T. Satyanarayana · G. Kunze
Editors

Yeast Biotechnology: Diversity and Applications

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Brief CVs of the Editors

After completing M.Sc. and Ph.D. at the University of Saugar (India), T. Satyanarayana had post-doctoral stints at the University of Bhopal and France. In 1988, he joined the Department of Microbiology, University of Delhi South Campus as Reader and became Professor in 1998. During this period, his research efforts have been focused on understanding the diversity of yeasts and thermophilic fungi and bacteria, their enzymes and potential applications, production of ectomycorrhizal fungal inoculum and heterotrophic carbon sequestration. He has published over 140 scientific papers and reviews and edited two books. He is fellow of the Association of Microbiologists of India, Mycological Society of India and Biotech Research Society of India, and a recipient of Dr. G.B. Manjrekar award of the Association of Microbiologists of India in 2004. He is in the editorial board of Bioresource Technology and Indian Journal of Microbiology.

Gotthard Kunze studied biology at the Ernst Moritz Arndt University in Greifswald. He got a post-doctoral fellowship and a position as scientific assistant at the Department of Biology of the university. In 1986 he joined as a research associate at the Institute of Genetics and Crop Plant Research (IPK) at Gatersleben. Since 1998 he is a visiting professor at the University Greifswald and since 1998 professor at the Technical University Anhalt at Köthen. During this period, he focused his research activities on yeast genetics (construction of new yeast host vector systems, heterologous gene expression, thermo- and osmoresistance in non-conventional yeasts and microbial yeast biosensors). Prof. Gotthard Kunze is the author of about 140 publications, editor of 2 books and teaches at the universities of Greifswald and Köthen.

Foreword

The objective of the book is to give a review of knowledge on the diversity and potential applications of yeasts to the researchers in this field and to the biotechnologists. This book is a collection of articles on yeasts, which will be very useful for those who desire to have an up-to-date volume. The researchers have attempted to communicate their significant observations and ideas to the scientific community. I believe that the book will expose students to new developments in the yeast research.

Yeast communities have been found in association with plants, animals and insects. Several species of yeasts have been isolated from specialized or extreme environments. Yeasts play a vital role in food chains, carbon, nitrogen and sulphur cycles. Yeasts are now being used to express foreign genes for producing human proteins of pharmaceutical interest. The products of modern yeast biotechnologies impinge on many commercially important sectors including food, beverages, chemicals, pharmaceuticals, industrial enzymes and agriculture. The vast majority of yeasts are beneficial to human life.

This book is divided into three parts, the first part, i.e., *'Diversity and Biology'* gives information about the various yeasts species. Diversity searches in the natural environment have resulted in the description of new yeast species at a rapid pace and the field is wide open to global exploration. The second part, i.e., *'Genetic and Molecular Insights'* aims at reviewing the use of this system as an experimental tool for conducting classical genetics. Yeasts have been recognized as a very important group of microorganisms on account of their extensive use in the fermentation industry and as a basic eukaryotic model cellular system. The latest developments in genomics and micro-array technology have allowed investigations of individual gene function by site-specific deletion method. The third part, i.e., *'Biotechnology Applications'* focuses on the hydrolysis of starchy and lignocellullosic substrates to sugars and their fermentation to ethanol, yeast enzymes and their potential applications. The survey is also given here of the production, the characteristics and the potential applications of currently well studied yeast extracellular polysaccharides. It emphasizes on the biological significance and their industrial applications.

I believe that the book would provide an overview of the recent developments in the domain of yeast research with some new ideas, which could serve as an inspiration and challenge for researchers in this field.

New Delhi
Dec. 24, 2007

Prof. Asis Datta
Former Vice-chancellor, JNU
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Preface

Yeasts are eukaryotic unicellular microfungi that are widely distributed in the natural environments. Although yeasts are not as ubiquitous as bacteria in the natural environments, they have been isolated from terrestrial, aquatic and atmospheric environments. Yeast communities have been found in association with plants, animals and insects. Several species of yeasts have also been isolated from specialized or extreme environments like those with low water potential (e.g. high sugar/salt concentrations), low temperature (e.g. yeasts isolated from Antarctica), and low oxygen availability (e.g. intestinal tracts of animals). Around 1500 species of yeasts belonging to over 100 genera have been described so far. It is estimated that only 1% of the extant yeasts on earth have been described till date. Therefore, global efforts are underway to recover new yeast species from a variety of normal and extreme environments.

Yeasts play an important role in food chains, and carbon, nitrogen and sulphur cycles. Yeasts can be genetically manipulated by hybridization, mutation, rare mating, cytoduction, spheroplast fusion, single chromosomal transfer and transformation using recombinant technology. Yeasts (e.g. *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*) are now being used to express foreign genes for producing human proteins of pharmaceutical interest. A landmark in biotechnology was reached in 1996 with the completion of the sequencing of the entire genome of *S. cerevisiae*. The genome sequencing of three more yeasts (*Schizosaccharomyces pombe*, *Candida albicans* and *Cryptococcus neoformans*) have recently been completed. *S. cerevisiae* has now become a central player in the development of an entirely new approach to biological research – systems biology. The systems biology was made possible because the yeast genome had been sequenced, micro-array analysis of yeast mRNA had been developed, databases of protein-protein interactions were available, rapid MS analysis of protein presence and abundance was possible, and computing facilities to process all of the information were available.

The products of modern yeast biotechnologies impinge on many commercially important sectors including food, beverages, chemicals, pharmaceuticals, industrial enzymes, agriculture (e.g. *S. cerevisiae* has the potential in stimulating cereal plant defences against fungal pathogens, yeasts like *Debaryomyces hansenii* can be used in the biocontrol of fungal fruit diseases, live yeasts like *S. cerevisiae* stabilize

rumen environment, provide dicarboxylic acids to stimulate rumen bacteria) and the environment (e.g. yeasts biosorb heavy metals and detoxify chemical pollutants). Although the vast majority of yeasts are beneficial to human life, some are opportunistically pathogenic towards humans (e.g. candidiosis caused by *Candida albicans*, yeast infections in immuno-compromised individuals and AIDS patients).

The book is aimed at bringing together the scattered knowledge that has accumulated in the last few decades on aspects such as diversity of yeasts in normal and extreme environments, their ecology and adaptations, developments in their taxonomy and systematics, physiology and biochemistry, molecular aspects, and their potential biotechnological applications.

We are grateful to all the authors for readily accepting our invitation and contributing chapters for the book. We wish to thank Springer for publishing the book, and Dr. Deepak Chand Sharma, Mr. Bijender Singh and Mr. Rakesh Kumar for extending help in preparing the book.

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Part I
Diversity and Biology

Chapter 1
Antarctic Yeasts: Biodiversity and Potential Applications

S. Shivaji and G.S. Prasad

Contents

Abstract This review is an attempt in cataloguing the diversity of yeasts in Antarctica, highlight their biotechnological potential and understand the basis of adaptation to low temperature. As of now several psychrophilic and psychrotolerant yeasts from Antarctic soils and marine waters have been characterized with respect to their growth characteristics, ecological distribution and taxonomic significance. Interestingly most of these species belonged to basidiomycetous yeasts which as a group are known for their ability to circumvent and survive under stress conditions. Simultaneously their possible role as work horses in the biotechnological industry was recognized due to their ability to produce novel enzymes and biomolecules such as agents for the breakdown of xenobiotics, and novel pharmaceutical chemicals. The high activity of psychrophilic enzymes at low and moderate temperatures offers potential economic benefits. As of now lipases from *Pseudozyma antarctica* have been extensively studied to understand their unique thermal stability at 90°C and also because of its use in the pharmaceutical, agriculture, food, cosmetics and chemical industry. A few of the other enzymes which have been studied include

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extracellular alpha-amylase and glucoamylase from the yeast *Pseudozyma antarctica* (*Candida antarctica*), an extra-cellular protease from *Cryptococcus humicola*, an aspartyl proteinase from *Cryptococcus humicola,* a novel extracellular subtilase from *Leucosporidium antarcticum,* and a xylanase from *Cryptococcus adeliensis*

The ability of these yeasts to adapt to the low temperature conditions has also led to investigations directed towards characterizations of cold stress proteins and heat shock proteins so as to understand the role of these stress protein with respect to adaptation. Antarctic yeasts have also been used as model system to study the inter-relationship among free radicals, antioxidants and UV-induced cell damage.

Keywords Biodiversity, yeast, Antarctica, enzymes, lipase, psychrophilic

1.1 Introduction

The continent of Antarctica which occupies an area of 14 million square kilometers, is a major cold habitat, of which about 99% is covered by ice and snow (Holdgate, 1977). Apart from being very cold, this continent is considered to be a very extreme habitat due to the fact that it is also the driest (Vincent, 1988; Claridge and Campbell, 1977; Campbell and Claridge, 2000), windiest and iciest of all known habitats of the world with high solar radiation at least during the summer season (Smith et al., 1992). Despite these extreme conditions, Antarctica is host to a number of life forms demonstrated by the presence of bacteria, yeasts, fungi, lichens, small invertebrates, many species of birds and mammals (Cameron et al., 1970; Vishniac and Mainzer, 1972; Vincent, 1988; Wynn-Williams, 1990). All these life forms have evolved special mechanisms to overcome the influence of low temperature, high salinity and high radiation.

The microorganisms that thrive in the extreme environment of Antarctica are cold loving and are referred to as psychrophiles. Psychrophilic (cold-loving) organisms differ from the psychrotolerant (cold-tolerant) organisms, on the basis of their cardinal temperatures. Psychrophilic yeasts have an optimum temperature for growth at about 15°C or lower, a maximum up to 25°C but are still capable of growing at 0°C or below (Morita, 1975; Arthur and Watson, 1976); in contrast the psychrotolerant microorganisms are those that are capable of growing at 5°C and below, regardless of whether the optimum temperature was about 15°C or more (van Uden, 1984; Vishniac, 1987). Psychrophiles are unable to grow above 20°C and are widely prevalent in permanently cold habitats, such as in polar regions, at high altitudes or in the deep sea. In contrast, the psychrotolerant which grow over a wider range of temperature and show better growth rates above 20°C are predominant in environments with periodic low temperatures. In Antarctica, psychrophilic and psychrotolerant microorganisms are believed to play key roles in the biodegradation of organic matter and the cycling of essential nutrients (Russell, 1990).

Psychrophilic bacteria, yeasts and other microorganisms define the lower limits of temperature for the survival of life forms. In this context the psychrophilic bacteria and yeasts of Antarctica could serve as excellent model systems to understand the molecular basis of survival at low temperatures. As yet, biological studies in Antarctica have mostly focused on the diversity of bacteria (Shivaji, 2005; Shivaji et al., 2005a; Prabagaran et al., 2006), their taxonomic position (Shivaji et al., 2004; Shivaji et al., 2005b, 2005c), their biotechnological potential (Cavicchioli et al., 2002) and as model systems to understand adaptation of microorganisms to the low temperature (Shivaji et al., 2007; Chintalapati et al., 2006, 2007; Kiran et al., 2004, 2005; Jagannadham et al., 1991, 2000; Chattopadhyay et al., 1997; Ray et al., 1994a, b, c). However, similar studies on yeasts are very limited. This review focuses primarily on the diversity and cataloging of yeasts from Antarctica and their biotechnological potential.

1.2 Diversity of Yeasts in Antarctica

Yeasts are a versatile group of eukaryotic microorganisms which are heterogeneous in their nutritional abilities and are capable of surviving in a range of habitats (Lachance and Starmer, 1988) such as in deep sea (Seiburth, 1979; Fell, 1976), moist and uneven surfaces including polluted waters (Hagler and Ahearn, 1987), on dry substrates and in the presence of high concentrations of salt and sugar (Ingram, 1958). Turkiewicz et al. (2003) suggested that yeasts may be better adapted to low temperatures than bacteria. Therefore, it is not surprising that yeasts belonging to genera such as *Bullera, Candida, Cryptococcus*, *Cystofilobasidium, Debaryomyces, Kondoa, Leucosporidium, Metschnikowia, Mrakia, Pseudozyma*, *Rhodotorula, Sakaguchia, Sporopachydermia, Sympodiomyces* and *Trichosporon* have been identified in various habitats of Antarctica.

1.2.1 Distribution of Yeasts in Antarctica

The entire Antarctic region is cold and therefore the distribution of yeasts in Antarctica if dependent only on temperature one should be able to see yeasts uniformly distributed. But this is not so. The most northern and southern sampling sites in Antarctica are separated by 10° of latitude. Despite this separation, psychrophiles appeared to be random in their distribution and did not increase with latitude (di Menna, 1960, 1966a). The possible reasons for not obtaining yeasts from some Antarctic samples could be due to the fact that the isolation methods were unsuitable, the incubation temperatures being too high or too low, the incubation time too short, or the medium is too acidic or because of too low osmotic pressure (di Menna, 1966a). Yeasts were usually found in substrates which are acidic rather than alkaline, but inspection of the results showed that high pH values were

not in themselves inhibitory. It was also observed that the yeasts found in Antarctic soils appeared to be dependent on plants. Babyeva and Golubev (1969) isolated more yeasts at 5°C than at higher temperature and showed that forty percent of their 63 isolates were "obligate psychrophiles", failing to grow above 20°C. Vishniac (1996) concluded that the biodiversity of yeasts and filamentous fungi in terrestrial Antarctic ecosystems increases with the availability of water and energy. Further it was also suggested that yeasts predominate in continental Antarctica compared to maritime and sub-Antarctic habitats (Vishniac, 1996).

1.2.2 Survival of Yeasts in Antarctica

Over the years attempts have been made to understand as to how psychrophilic yeasts survive at low temperatures (< 20°C) (Inniss, 1975; Larkin and Stokes, 1968).On the basis of melting points of major fatty acids present in yeasts, it was proposed that the psychrophilic yeasts would be able to grow at temperatures as low as −10°C. Further thermotolerance to temperatures > 20°C may be attributed to the capacity of these yeasts to synthesize heat shock proteins (hsp) and (or) trehalose accumulation as in *Mrakia frigida, Leucosporidium fellii* and *L. scottii* but not in *L. antarcticum* (Deegenaars and Watson, 1997, 1998). In fact based on these studies it was speculated that hsp 110 may play a role in stress tolerance in psychrophilic yeasts, similar to that of hsp 104 in mesophilic species.

1.2.3 Lipid Composition of the Membranes and Psychrophily

Several studies have clearly indicated that the ability to modulate membrane fluidity by regulating the synthesis of fatty acids is very crucial for low temperature adaptation (Shivaji et al., 2007; Chintalapati et al., 2005). As a thumb rule, low growth temperature increases the proportion of unsaturated fatty acids compared to the saturated fatty acids. This phenomenon applies to bacteria or yeasts (Shivaji et al., 2007; Chintalapati et al., 2005; Sato and Murata, 1980; Sato et al., 1979; Murata et al., 1992; Wada and Murata, 1990; Arthur and Watson, 1976) and in several species of psychrophilic yeasts the unsaturated fatty acids, constituted 50–90% of the total fatty acid composition as in species of *Mrakia, Candida, Torulopsi, Leucosporidium*, and *Cryptococcus* (Watson, 1987; Thomas-Hall and Watson, 2002). Sabri et al. (2001) showed that the inability of *Rhodotorula aurantiaca* to grow at temperatures close to 20°C was due to high accumulation of myristoyl-CoA (C_{14}-CoA), (28-fold higher than in cells cultivated at 0°C temperature). Silver et al. (1977), observed that the cessation of growth at temperatures above 20°C in the psychrophilic yeast *Leucosporidium stokesii* is due to the inability of the yeast to complete an event(s) associated with nuclear division

such as DNA synthesis and normal cell division cycle (Silver and Sinclair, 1979). Meyer et al. (1975) observed that the psychrophilic yeasts are more sensitive to freeze-thaw cycles compared to mesophilic yeasts.

1.2.4 Yeasts of the Genus Cryptococcus

The abundance of yeast in Antarctica varies depending on the habitat. In fact even in the same habitat, such as soil, the number varied from total absence to as many as 100,000 yeasts per gram of soil (di Menna, 1966a). *Cryptococcus* is the most predominant group of yeasts in the Antarctic. In this genus C. *laurentii* and *C. albidus* are more predominant compared to *C. luteolus* and *C. diffluens* (di Menna, 1966a). Several new species of *Cryptococcus* have been reported from various habitats in Antarctic such as *Cryptococcus friedmannii* from an Antarctic cryptoendolithic community (Vishniac, 1985a); *Cryptococcus vishniacii* (Vishniac and Hempfling, 1979a, b; Vishniac and Baharaeen, 1982), *Cryptococcus antarcticus* (Vishniac and Kurtzman, 1992; Vishniac and Onofri, 2003), *Cryptococcus albidosimilis* (Vishniac and Kurtzman, 1992), *Cryptococcus socialis* (Vishniac, 1985b), and *Cryptococcus consortionis* (Vishniac, 1985b) from Arctic soils; *Cryptococcus victoriae* (Montes et al., 1999), *Cryptococcus adeliensis, Cryptococcus albidus, C. laurentii* and *Candida oleophila* (Scorzetti et al., 2000; Pavlova et al., 2001) from mosses and lichens; *Cryptococcus nyarrowii* and *Cryptococcus statzelliae* from soil and snow samples (Thomas-Hall et al., 2002). Some strains of yeasts belonging to the same species appeared to be very different morphologically. Interestingly *Cryptococcus nyarrowii* was represented by two different coloured strains CBS 8804^T (pink colonies) and CBS 8805 (yellow colonies). Other yeast strains (CBS 8908, CBS 8915 and CBS 8920) such as *Cryptococcus victoriae*, *Cryptococcus watticus* sp. nov. (CBS 9496^T) were also isolated from samples collected from the Vestvold Hills, Davis Base (Guffogg et al., 2004).

Cryptococcus laurentii and *C. albidus* are considered as ubiquitous, and are reported by almost all investigators from Antarctica. This could be due to incorrect delineation of these species, as several tests used for identifying them are variable (Fell and Statzell-Tallman, 1998; Barnett et al., 2000; Takashima et al., 2003; Fonseca et al., 2000; Sugita et al., 2000). Sequence analysis of D1/D2 domain of the large subunit rRNA gene and the ITS region has resulted in description of several new species of *Cryptococcus* which were earlier thought to be either *C. albidus* or *C. laurentii,* based on phenotypic methods (Takashima et al., 2003; Middelhoven, 2005). It is also difficult to discriminate *Cryptococcus laurentii* from *C. cellulolyticus, C. flavus, C. humicola and C. hungaricus* based on physiological characters (Barnett et al., 2000). Sugita et al. (2000) reported genetic diversity in the ITS and D1/D2 regions among the clinical isolates of *C. laurentii* and 10 isolates examined in that study were found to belong to seven different species. Similarly, Fonseca et al. (2000) examined several strains of "*Cryptococcus albidus*", using sequence

analysis of the D1/D2 domain of large subunit rRNA gene and established eight new species.

According to Vincent (1988), the *Cryptococcus* yeasts recovered in Antarctic lakes were clearly the result of wash-in from adjacent soils. Moreover, polar soil yeasts, which occur in significant numbers, were found mostly in soil samples that also contain moss, lichen or microalgal material. Vishniac (1995) demonstrated that *Cryptococcus albidus,* a dominant soil organism, was capable of rapid growth when introduced into autoclaved soil, following which viability was retained for 2 months. It was suggested that sterilization altered the nutritional value of the soil in a manner similar to natural weathering factors. Consistent with this, the growth of indigenous soil yeasts would be a function of the frequency and intensity of disturbances of the soil.

1.2.5 Yeasts of Other Genera

Yeasts belonging to the genus *Candida* appear to be quite common in Antarctica but not as predominant as the *Cryptococcus* yeasts. Several strains of *Candida* spp. such as *Candida nivalis, Candida gelida* and *Candida frigida*, presently known as *Mrakia frigida* (di Menna, 1966b), *Candida humicola, Cadida famata, Candida ingeniosa* and *Candida auricullariae* (Ray et al., 1989) and *Candida oleophila.* (Pavlova et al., 2001) have been isolated from soil and moss. *Candida* (*Torulopsis*) *austromarina* (Fell and Hunter, 1974) has been reclassified as *Candida sake* on the basis of identity of the D1/D2 regions of rDNA. (Kurtzman and Robnett, 1998). All these yeasts were found to be psychrophilic. *Candida* isolates were also identified in various other habitats of Antarctica such as in water, associated with algae, penguin dung etc. (Goto et al., 1969). Other yeasts isolated from Antarctica include *Leucosporidium* (Fell et al., 1969), *Debaryomyces hansenii* (Biswas et al., unpublished results), *Rhodotorula rubra*, (Ray et al., 1989), *Rhodotorula minuta* (Pavlova et al., 2001), *Rhodotorula mucilaginosa* (Pavlova et al., 2001), *Bullera alba* (Ray et al., 1989), *Mrakia frigida* (Biswas et al., unpublished results) and *Mrakia psychrophila* closely related to *Mrakia frigida* (Xin and Zhou, 2007).

1.3 Antarctic Yeasts in Culture Collections

It is interesting to note that about 90% of the yeasts isolated from Antarctica are of basidiomycetous origin (Table 1.1).

The Centraalbureau voor Schimmecultures (CBS), Utrecht, Netherlands has 125 Antarctic yeast strains and the American Type Culture Collection (ATCC), USA has 18 Antarctic yeast cultures, including type strains of nine species of *Cryptococcus* (Table 1.1). Based on the sequence analysis of D1/D2 domain of 26S rRNA gene (Fell et al., 2000) and ITS regions (Scorzetti et al., 2002) these nine type strains

Table 1.1 Antarctic yeast strains available at The Centraalbureau voor Schimmecultures, Utrecht, the Netherlands

Accepted scientific name	CBS accession number	Habitat	Site of collection
Candida davisiana Guffogg et al.	CBS 9495	Soil	Antarctica, Davis base, Vestvold Hills, Moss Cirque
Candida parapsilosis group II	CBS 8548	-	Antarctica
Candida psychrophila (S. Goto et al.) S.A. Meyer & Yarrow	CBS 5956	Dung of penguin	Antarctica, Ross Island, Cape Royds
Candida sake (Saito & Oda) van Uden & H.R. Buckley	CBS 5957	Stream water	Antarctica, Lake Bonney
Cryptococcus adeliensis Scorzetti et al.	CBS 8351	Decayed algae	Antarctica, Dumont d'Urville base
Cryptococcus albidosimilis Vishniac & Kurtzman	CBS 7711	Soil	Antarctica, South Victoria Land, Wright Valley, Linnaeus Terrace
Cryptococcus albidus (Saito) C.E. Skinner et al. var. *albidus*	CBS 9809	Soil	Antarctica, Victoria Land, Edmonson Point
Cryptococcus antarcticus Vishniac & Kurtzman var. *antarcticus* Vishniac & Kurtzman	CBS 7687	Soil	Antarctica, University Valley
Cryptococcus antarcticus Vishniac & Kurtzman var *circumpolaris* Vishniac & Onofri	CBS 7689	Soil	Antarctica, University Valley
Cryptococcus consortionis Vishniac	CBS 7159	Soil	Antarctica, South Victoria Land, Linnaeus Terrace
Cryptococcus friedmannii Vishniac	CBS 7160	Soil	Antarctica, Ross Desert
Cryptococcus humicola (Daszewska) Golubev	CBS 5958	Water	Antarctica, Lake Vanda
Cryptococcus mycelialis Golubev, V.I & Golubev, N.V	CBS 7712	Soil	Antarctica, East Falkland Island
Cryptococcus nyarrowii Thomas-Hall & Watson	CBS 8805	Soil and lichen	Antarctica, Lichen Valley, Vestfold Hills, Davis base
Cryptococcus nyarrowii Thomas-Hall & Watson	CBS 8804	Bird	Antarctic, Lichen Valley, Vestfold Hills, Davis base
Cryptococcus socialis Vishniac	CBS 7158	Soil	Antarctica, South Victoria Land, Linnaeus Terrace
Cryptococcus victoriae Montes et al.	CBS 8685	Soil	Antarctica, Victoria Land
Cryptococcus vishniacii Vishniac & Hempfling var. *vishniacii*	CBS 6808	Soil	Antarctica, Mount Baldr

(continued)

Table 1.1 (continued)

Accepted scientific name	CBS accession number	Habitat	Site of collection
Cryptococcus watticus Guffogg et al.	CBS 9496	Soil	Antarctic, Davis base, Vestfold Hills, Watts Lake
Cystofilobasidium bisporidii (Fell et al.) Oberwinkler & Bandoni	CBS 6346	Sea water	Antarctic Ocean
Cystofilobasidium capitatum (Fell et al.) Oberwinkler & Bandoni	CBS 6358	Zooplankton	Antarctic Ocean
Cystofilobasidium infirmominiatum (Fell et al.) Hamamoto et al	CBS 6350	Zooplankton	Antarctic Ocean
Kondoa malvinella (Fell & Hunter) Y. Yamada et al.	CBS 6082	Sea water	Antarctica
Leucosporidium antarcticum Fell et al.	CBS 5942	Sea water	Antarctica, Weddell Sea off Joinville Island
Leucosporidium scottii Fell et al.	CBS 5930	Sea water	Antarctica
Metschnikowia australis (Fell & Hunter) Mendonça-Hagler et al.	CBS 5847	Sea water	Antarctic Ocean
Metschnikowia koreensis Hong et al.	CBS 9068	-	Antarctica
Mrakia frigida (Fell et al.) Y. Yamada & Komagata	CBS 5266	Soil	Antarctica, Scott Base
Rhodosporidium sphaerocarpum S.Y. Newell & Fell	CBS 5939	Sea water	Antarctica, Marguerite Bay
Rhodotorula minuta (Saito) F.C. Harrison var. minuta	CBS 9810	Soil	Antarctica, Victoria Land, Edmonson Point
Rhodotorula sp. F.C. Harrison	CBS 8940	Water	Antarctica, Chelnok lake
Sakaguchia dacryoidea (Fell et al.) Y. Yamada et al.	CBS 6353	Sea water	Antarctic Ocean
Sporopachydermia lactativora Rodrigues de Miranda	CBS 5771	Sea water	Antarctic Ocean
Sympodiomyces parvus Fell and Statzell-Tallman	CBS 6147	Sea water	Antarctic Ocean
Trichosporon pullulans (Lindner) Diddens & Lodder	CBS 5108	Soil	Antarctica

were identified as being synonyms to *Cryptococcus vishniacii* var. *vishniacii.* The Microbial Type Culture Collection and Gene Bank (MTCC) in India has 25 Antarctic yeasts in its collection, isolated from the Schirmacher Oasis region of Antarctica (Ray et al., 1989).

1.4 Are Antarctic Yeasts Endemic?

The larger question in microbial ecology is whether microbes are endemic? The continent of Antarctica due to its remoteness and isolation from the remaining landmass of the earth for millions of years should be amongst the first places to look for endemic organisms and also to examine the evolutionary processes that can give rise to microbial speciation. *Cryptococcus antarcticus* and *C. vishniacii* occur in Antarctica and as of now are unknown outside Antarctica (Vishniac, 1999). But this may not be sufficient evidence in support of endemism since many other yeasts are widely distributed. *Candida antarctica* (reclassified as *Pseudozyma antarctica*), was first isolated from Antarctica (Goto et al., 1969); but later it was identified from Japanese natural samples and from flowers in India (Saluja and Prasad, unpublished observations). Similarly, *Cryptococcus victoriae*, first reported from Antarctica (Montes et al., 1999) is also found in flower and soil samples in India (Saluja and Prasad, unpublished observations). The yeast genus *Leucosporidium* originally isolated from Antarctica was later isolated from temperate climates (Summerbell, 1983). However, the species of the genus *Mrakia* seems to be confined to cold habitats. Besides, Antarctica it has been reported from other cold habitats such as European Alps (Margesin et al., 2005), Hokkaido, Japan (Nakagawa et al., 2004), glacial and subglacial waters of northwest Patagonia, Argentina (Brizzio et al., 2007), Western Siberia (Poliakova et al., 2001) and Tinto river in southwestern Spain (Lopez-Archilla et al., 2004). A new species *Mrakia curviuscula* was isolated from forest substrates collected in the central part of European Russia (Bab'eva et al., 2002). It appears that organisms are extremely versatile in their adaptive capabilities and therefore would break the shackles of endemism and attain an ubiquitous distribution.

1.5 Biotechnological Potential of Antarctic Yeasts

1.5.1 Enzymes from Antarctic Yeasts

Bioprospecting for biomolecules such as enzymes, pigments, polyunsaturated fatty acids etc. from psychrophilic yeasts has gained momentum with the realization that these yeasts due to their unique ability to survive and grow at low temperatures

Table 1.2 Enzymes produced by Antarctic yeasts

Enzyme	Yeast	Reference
Proteinase	*Cryptococcus friedmannii*	Vishniac, 1985
Serine proteinase	*Leucosporidium antarctium*	Turkiewicz et al., 2003
Aspartyl proteinase	*Candida humicola*	Ray et al., 1992
Xylanase	*Cryptococcus adeliensis*	Gomes et al., 2000; Petrescu et al., 2000
Xylanase	*Cryptococcus albidosimilis* (*Cryptococcus albidus* TAE85)	Amoresano et al., 2000
Lipase	*Leucosporidium antarcticum*	Turkiewicz et al., 2003
Lipases A and B	*Pseudozyma antarctica* (*Candida antarctica*)	
α-Glucosidase	*Leucosporidium antarcticum*	Turkiewicz et al., 2003
α-Amylase	*Candida antarctica*	De Mot and Verachtert, 1987
Glucosamylase	*Candida antarctica*	De Mot and Verachtert, 1987
Acid phosphatase	*Leucosporidium antarcticum*	Turkiewicz et al., 2003
Alkaline phosphatase	*Leucosporidium antarcticum*	Turkiewicz et al., 2003
Beta-fructofuranosidase	*Leucosporidium antarcticum*	Turkiewicz et al., 2003

would be producing enzymes which are cold active and also other biomolecules so as to facilitate their survival at low temperatures. Cold active enzymes may provide interesting clues that would add to our understanding of the relationship between structure, stability and activity of enzymes at low temperatures (Gerday et al., 1997). Most biological systems show 2–3 times reduced reaction rate when the temperature is decreased by 10°C. Enzymes from psychrophilic microorganisms are thought to have evolved a more flexible structure when compared to their mesophilic and thermophilic counterparts. This character probably originates from weakening of intramolecular interactions and is supposed to be responsible for the increased catalytic efficiency and the low thermal stability of psychrophilic enzymes in general (Feller and Gerday, 1997). Several different types of enzymes have been characterized from psychrophilic yeasts (Table 1.2).

1.5.2 Lipases

Two lipases from *Pseudozyma antarctica* (*Candida antarctica*) namely CAL-A and CAL-B have been patented and used for various processes such as preparation of optically active amines, acid ethyl esters, triglycerides, alkyl ester derivatives of restaurant grease (Hsu et al., 2003), hydrolysis of fats, hydrolysis of water insoluble esters of fats, hydrolysis of a mixture of (chloromethyldimethylsilyl)-2-propenyl acetate (Rubio et al., 2001), synthesis of polyesters etc. which are useful to the detergent, food, pharmaceutical and other industries (UNEP report on Antarctic bioprospecting, 2004). Thus both these lipases have extensive applications (de Maria et al., 2005) and CAL-A is considered as the most thermostable lipase known, being able to work efficiently even at above 90°C

(Anderson et al., 1998; Kirk et al., 2002). The biotechnological applications of *Candida antarctica* lipase has been reviewed (de Maria et al., 2005) and detail properties related to the catalytic properties (Passicos et al., 2004) substrate specificity (Raza et al., 2001; Larios et al., 2004; Arsan and Parkin, 2000) and thermostability have been studied (Anderson et al., 1998; Kirk and Christensen, 2002). The immobilized form of CAL-B is thermostable even under non-aqueous conditions (Arroyo et al., 1998; Koops et al., 1999). Recently, DNA shuffling was used to create chimeric CAL-B with improved activity toward the hydrolysis of diethyl 3-(3′,4′-dichlorophenyl) glutarate (DDG) (Suen et al., 2004). Three variants of the *Candida antarctica* CAL-B lipase have been constructed and it was found that the variant containing the T103G mutation, that introduces the consensus sequence G-X-S-X-G found in most other known lipases, showed increased thermostability but retained only half the specific activity of the native enzyme (Patkar et al., 1998).

1.5.3 Xylanases

Antarctic yeast *Cryptococcus adeliensis* produces a cold-adapted xylanase (Scorzetii et al., 2000; Gomes et al., 2000). In addition to xylanase, this strain also showed activities of endoglucanase, β-mannanase, β-xylosidase, β-glucosidase, and α-L-arabinofuranosidase enzymes. The authors observed that the broad pH and temperature ranges suggest that the xylanase of *C. adeliensis* exists in multiple forms, but could not determine the isoenzymic composition of the crude xylanase. They predicted that the very low thermal stability of the *C. adeliensis* xylanase is most probably the result of increased protein flexibility. Petrescu et al. (2000) studied a xylanase of *Cryptococcus adeliensis which* shared 84% identity with its mesophilic counterpart from *Cryptococcus albidus,* but was less thermostable than its mesophilic homologue. The cold-adapted xylanase displayed a lower activation energy and a higher catalytic efficiency in the range of 0–20°C. These observations suggested a less compact, more flexible molecular structure. Molecular modeling indicated that the adaptation to cold consists of discrete changes in the three-dimensional structure that may lead to a less compact hydrophobic packing, to the loss of one salt bridge, and destabilization of the helices. The structural characterization of the xylanase from the psychrophilic antarctic yeast *Cryptococcus albidosimilis* (*C. albidus* TAE85), showed that it is a glycoprotein made up of 338 amino acids (Amoresano et al., 2000) and has both the N- and O-linked glycans and suggested that the glycosylation system in cold-adapted organisms might have similarities as well as differences with respect to mesophilic and thermophilic yeasts. Xylanases may be suitable for applications such as digestion of industrial or sewage wastes and decomposition of agricultural residues at low or ambient temperatures. The yeast *C. adeliensis*, with its ability to produce xylanase and other enzymes, may find application as a probiotic inclusion and a therapeutic agent in food.

1.5.4 Proteases and Other Enzymes

Ray et al. (1992) examined the extracellular protease from a psychrotolerant dimorphic yeast *Candida humicola,* isolated from Antarctic soil. Secretion of the enzyme was greater during exponential growth and low temperatures than during growth at higher temperatures. The enzyme was active from 0 to 45°C, with optimum activity at 37°C. Turkiewicz et al. (2003) reported an extracellular serine proteinase, lap2, from the psychrophilic antarctic yeast *Leucosporidium antarcticum* 171. This enzyme was a glycoprotein, and was most active at temperatures between 20 to 30°C with an optimum at 25°C. Partial activity of the enzyme was retained at zero (20 to 25% activity) and subzero temperatures (18% activity at −10°C). The proteinase lap2 is the first psychrophilic subtilase in this family.

An α-amylase and a glucoamylase were purified to homogeneity from the culture fluid of β-cyclodextrin-grown *Candida antarctica* CBS 6678 (De Mot and Verachtert, 1987). α-Amylase was active on cyclodextrins, whereas debranching activity was demonstrated for glucoamylase. List of enzymes reported from Antarctic yeast species is given in Table 1.2.

1.6 Other Applications of Antarctic Yeasts

Candida sp. which was isolated from the upper layer of Lake Vanda in the McMurdo Dry Valleys, Antarctica was used for removing nitrogen and nitrate from water samples at low temperature (5°C) (Katayama-Hirayama, et al., 2003). Some yeasts from the Antarctic have been used as model systems to study the effects of UV-radiation (Tsimako et al., 2002) and these attempts could form the basis of future studies to establish inter-relationship among free radicals, antioxidants and UV-induced cell damage. In a recent paper, Libkind et al. (2006) suggested that in Patagonian freshwater yeasts there is an apparent relationship between the ability to produce photoprotective compounds, their tolerance to UV exposure and their success in colonizing habitats highly exposed to UV. Similar mechanism may be in operation in the yeast strains isolated from Antarctica.

1.7 Conclusions

Culture collections are important repositories of microbial biodiversity and are essential for the long-term availability of authentic cultures. They also serve as key sources of taxonomic expertise and are needed for the long-term preservation of strains and organisms for biotechnological research. Unfortunately, many researchers do not understand and appreciate the importance of depositing their cultures in known culture collections, as a result some cultures isolated from exotic locations are lost forever, once the researcher retires or changes his field of research. For this

reason, of the several hundreds of yeast cultures isolated from Antarctica, very limited numbers are available from the culture collections in the World. Extensive research into the biodiversity of Antarctic yeasts from various habitats of Antarctica is essential to establish yeast species richness, to identify various strategies by which they adapt to low temperatures and to unravel the molecular basis of their adaptation to the extreme conditions in Antarctica like low temperature, low water activity and low nutrient availability.

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Chapter 2
Basidiomycetous Yeasts: Current Status

Devendra K. Choudhary and Bhavdish N. Johri

Contents

Abstract The approach to yeast identification has significantly changed in just a few decades due to rapid increase in basic biological knowledge, increased interest in the practical applications and biodiversity of this important microbial group, and enormous technological advances especially in the sphere of molecular tools. While some conventional methods are still tenable, many molecular techniques have been developed that allow for strain classification at all taxonomic levels. However, the oldest tool of microbiology, the microscope, is still a fundamental accessory for studies involving yeast biology, biodiversity and taxonomy.

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The basidiomycetous yeasts, are currently recognized, in three classes of the Basidiomycota: Ustilaginomycetes, Urediniomycetes and Hymenomycetes. These yeasts have considerable economic, agricultural and medical importance and estimates suggest that the number of known yeasts represents only about 1 to 5% of the species that exist in nature. There is an increased interest in exploration of these species for economic exploitation and there is a need to understand their biodiversity and ecological roles.

Identification and phylogenetic placement of the basidiomycetous yeasts is not always easy, partly because of their polyphyletic nature. The unifying characteristic of these fungi is a predominant unicellular growth phase. Separation of yeasts into the three classes of fungi is based on septal morphology, cell wall composition and rDNA analysis. Generic diagnosis is based on sexual and vegetative biology, in addition to physiological tests such as growth on inositol or D-glucuronic acid and formation of extracellular starch-like compounds. Species are usually differentiated by physiological attributes, particularly the utilization of carbon and nitrogen sources, and by measurement of DNA reassociations between closely related species. Currently approximately 50 genera and 250 species of basidiomycetous yeasts are known. Molecular methods used in their identification include, species-specific PCR primers, analysis of RFLPs PFGE, randomly amplified polymorphic DNA (RAPD) and single-stranded conformational polymorphisms (SSCP). Significant advances in basidiomycete systematics have resulted from sequence analysis of the large and small subunits of rDNA.

Basidiomycetous yeast species are associated with living plants, *viz*., *Sporobolomyces* and *Phaffia.* Several species have been found to play a prominent role in biocontrol of plant disease whereas others have application in agro based industry. For example, *Phaffia rhodozyma* produces a pigment astaxanthin that has considerable market in aquaculture industry. On the other hand several species produce polysaccharases and can store lipids in amounts reaching upto 65% of their biomass. Some species of *Cryptococcus, Rhodotorula* and *Trichosporon* can degrade varied aromatic compounds and thus are a candidate in bioremediation. On the negative side is the pathogenic *Filobasidiella neoformans* that poses medical problem since both the varieties of this basidiomycetous yeast infect the lungs which can result in pneumococcal-type pneumonia. Lipophilic *Malassezia* spp. are associated with skin surfaces but can cause serious pulmonary and other infections.

Diversity searches in the natural environment have resulted in description of new species within the basidiomycetous yeasts at a rapid pace and the field is wide open to global exploration.

Keywords Basidiomycetous yeasts, systematics, molecular methods, biocontrol, Astaxanthin, polysaccharases

2.1 Introduction and Definition

Yeasts are described as unicellular fungi and are generally characterized by the absence of coenocytic hyphae. They are represented usually by small cells which reproduce by budding or by the formation of a cross wall, followed by fission. During budding, the cell wall of the mother cell inflates and blows out to form a 'bud', which is subsequently released as a daughter cell. However, several yeast exhibit formation of hyphae or pseudohyphae, made up of chains of elongated bud.

The term 'yeast' has no taxonomic standing of its own *per se* and represents a growth form in several groups of unrelated fungi. Some fungi are dimorphic (two life stages) and exhibit a 'yeast' stage that shifts to mycelial growth under certain cultural conditions. Yeasts include fungi with sexual forms (basidiomycetous yeast and ascomycetous yeasts) and asexual forms. Basidiomycetous yeasts can be differentiated from the ascomycetous yeasts based on urease test which is positive in the former group.

The basidiomycetes are divided into subclasses: Hymenomycetes (mushroom-forming basidiomycetes), Urediniomycetes (rusts), and the Ustilaginomycetes (smuts). Some basidiomycetous members grow in culture with budding cells.

In this chapter, the diversity, taxonomy and systematics, and importance of basidiomycetous yeast are stressed. Beginning as a loose group of asporogenous forms, basidiomycetous yeasts now stand on firm footing by way of diversity, ecology and phylogenetic consideration.

2.2 Diversity and Ecology

The unicellular nature of yeasts makes them better suited for deep liquid substrates or moist and uneven surfaces. Therefore, yeasts grow typically in moist environments where there is an abundant supply of simple, soluble nutrients such as sugars and amino acids. This explains why they are common on leaf and fruit surfaces, on roots and in various types of food. Exceptions are those that degrade polymers, such as starch and cellulose. Basidiomycetous yeasts are widely distributed on a variety of substrates such as angiosperm wood and bark, weathered and dead inflorescence and mushrooms (McLaughlin et al., 2004).

Yeasts are found in widely different aquatic and terrestrial sources, as also in certain restricted habitats (Table 2.1). They are also found associated with the body of certain animals where they act as intestinal commensals (Lachance and Starmer, 1998). The type of nutrients reaching the soil determines the yeast microflora although some forms are permanent residents in soil, *viz., Cryptococcus, Rhodotorula* species, and *Sporobolomyces* species (Spencer and Spencer, 1997; Lachance and Starmer, 1998).

In a survey of basidiomycetous yeast, *Fellomyces fuzhouensis* was considered as potentially pathogenic; this species exclusively reproduces by conidiogenesis (Gabriel

Table 2.1 Distribution of major genera of basidiomycetous yeasts in various habitats

Habitat	Genera
Plants	*Sporobolomyces, Rodotorula, Cryptococcus* sp.
Tree exudates	*Phaffia rhodozyma*
Insect	*Cryptococcus* sp.
Soil	*Cryptococcus neoformans, Rhodotorula, Sporobolomyces* sp.
Water body	*Cryptococcus, Rhodotorula, Trichosporon* sp., *Rhodosporodium*
Animal	*Cryptococcus, Rhodotorula, Trichosporon* sp.
Atmosphere	*Cryptococcus neoformans, Rhodotorula, Sporobolomyces*

et al., 2000). *Sporobolomyces roseus* Kluyver & van Neil is a common ballistoconidium-forming yeast species which occurs in several different habitats, but most frequently in the phyllosphere (Nakase, 2000).

The distribution of yeast species in various water sources varies quite widely from a few cells ml^{-1} in unpolluted water to more than a million ml^{-1} in effluents. In polluted water the number of yeast increases proportionally with the degree of pollution (Lachance and Starmer, 1998). Some, such as red yeasts have been used as indices of pollution. Fresh water yeasts as well as those found in sea include those from other ecosystems, which have been washed into these water sources (Vincent, 1988). Aquatic species of basidiomycetous forms generally include species of *Cryptococcus, Rhodotorula and Sporobolomyces* (Hagler and Ahearn, 1987).

The diversity of ballistoconidium – forming yeasts has been studied extensively in phyllosphere in north-east China. As many as 250 yeast strains were isolated from 39 leaf samples of various plants collected from Changbai Mountain, Jilin Province, north-east China; of these six strains were classified into one group by conventional and chemotaxonomic characterization. Two separate groups, representing two novel *Bensingtonia* species *viz., Bensingtonia changbaiensis* sp. nov. (type strain CB 346^T = AS 2.2310^T = CBS 9497) and *Bensingtonia sorbi* sp. nov. (type strain, CB 288^T = AS 2.2303^T = CBS = 9498^T), were recognized based on 26S rDNA D1/D2 domain, ITS region and 18S rDNA sequence analyses (Wang et al., 2003).

The *cactus*-yeast-*Drosophila* system has been used as an example (Starmer et al., 1991) to show the role of dispersal as a factor that is highly specific since it may involve insects that feed only on certain Cactus species (Lachance, 2003). The yeasts present in certain habitats provide sufficient benefits such as food enrichment and detoxification to the vector. This ensures further dispersal of the yeast community to a new environment. Furthermore, the compatibility of yeast species within a niche influences the species found in that habitat (Lachance and Starmer, 1998).

Yeast habitats are often rich in simple organic carbon, sometimes very high in moisture, acidic or occasionally alkaline. This diversity in habitat-types confirms that yeasts are able to grow over a broad range of growth conditions. These features enable one to predict their distribution; however, new yeast species isolated from

the varying habitats are formed due to selection pressures exerted by the environment. The observed similarities and differences in yeasts found in a particular environment play a vital role in observing the evolution as it progresses. Thus, ecology of yeasts is believed to involve the effects of the physical environment on the yeast cells and the interaction of the yeast species with other microorganisms (Spencer and Spencer, 1997).

2.3 Isolation and Maintenance

2.3.1 Isolation of Basidiomycetous Yeasts

Yeasts, like other heterotrophic living organisms require carbon, nitrogen, phosphorus, trace elements and growth factors as sources of nutrition. Yeasts rarely occur in the absence of either molds or bacteria and since they do not occur naturally as pure cultures it is important to analyse the components that are crucial. Hence, selective techniques are often used for the recovery of yeasts, using media that permit the yeast to grow while suppressing molds and bacteria (Yarrow, 1998).

For isolation purposes, the direct streak plating technique is recommended. The preferred medium is yeast-malt (YM) agar which is acidified to pH 3.7 using either hydrochloric acid or phosphoric acid. Acidification is preferred over the incorporation of antibiotics and fungistatic agents (Lachance and Starmer, 1998). Fungistatic agents are used with caution as some of these compounds may also inhibit certain yeasts (Yarrow, 1998). Cultures are usually incubated between 20°C and 25°C since most basidiomycetous yeasts are mesophilic. Optimum temperatures for growth are higher for some yeasts and lower for others. The psychrophilic taxa require temperatures of between 4°C and 15°C as their optimum temperatures. Higher temperatures, in the range of 30–37°C, are often required for yeasts that are strictly associated with warm-blooded animals (Yarrow, 1998).

Recovery of yeasts present in low numbers requires use of enrichment media and conditions that favour their growth over other microorganisms. Usually a sample is inoculated into a liquid medium with a pH of 3.7 to 3.8. Air can be excluded from the culture to discourage the development of moulds although this method leads to the appearance of fermentative strains excluding the aerobes. Sterile pharmaceutical paraffin can be poured on the surface of the media to a depth of 1 cm to exclude air (Yarrow, 1998).

The use and ability of some yeasts to either transform toxic compounds to valuable compounds or to detoxify these compounds has been well documented (Copley, 1998; Fetzner, 1998).

The carbon sources usually utilized by microorganisms contain glucose or other carbohydrates and are used as carbon and energy source. Microbes with the ability

to utilize non-carbohydrate carbon sources such as alkanes, branched alkanes (Demain et al., 1998), low molecular weight aromatics and cyclic alkanes are important because these compounds are environmental pollutants (Van Beilen et al., 1998, 2003). In addition, the degradation of cyclic alkanes by microbes is important in nature and in technological applications such as wastewater, waste gas treatment, bioremediation and biocatalysis (Sikkema et al., 1995). In particular, cyclohexane is becoming increasingly important as an industrial solvent because it is relatively nontoxic compared to benzene, a known carcinogen, used previously as an industrial solvent (Uribe et al., 1990; Sikkema et al., 1995).

Monoterpenes are branched chain C_{10} hydrocarbons widely distributed in nature. The most widespread terpene in the world is limonene, which is formed by over 300 plants (Colocousi et al., 1996; van der Werf and de Bont, 1998; King and Dickinson, 2000, 2003). The biotransformation of limonene by microorganisms with the potential production of more valuable natural flavour compounds, has been reported in bacteria and basidiomycetous yeasts (van der Werf et al., 1999). This degradation pathway was determined by biochemical studies (van der Werf et al., 1999). Some yeast strains, all belonging to the alkane-utilizing yeasts, can hydroxylate monoterpenes (Van Rensburg et al., 1997), but ascomycetous yeast strains in general do not utilize monoterpenes as sole carbon source. The only report on the isolation of yeasts able to grow on monoterpenes has been on basidiomycetous yeasts (Thanh et al., 2004).

2.3.2 Maintenance of Cultures

The best medium for maintaining yeast cultures requires the addition of glucose to the media as sole source of carbon. This is preferred since the risk of changes in growth and fermentative pattern, due to the selection of mutants is minimized (Scheda and Yarrow, 1966). However, an unstable strain can change its properties within a few days due to the selection pressure when grown on media containing malt extract. Consequently, YM agar slopes as well as yeast-glucose-peptone or malt agar are used to maintain yeast flora (Yarrow, 1998).

Numerous yeast strains are stored at temperatures between 4°C and 12°C by sub-culturing at intervals of six to eight months. The frequency of subculturing differs among yeasts, with some such as *Arxiozyma* and *Malassezia* requiring subculturing every month because they are more sensitive to prolonged storage. The teleomorphic members of the ascomycetous and basidiomycetous yeasts are known to lose the ability to sporulate on successive cultivation on laboratory media. The extent to which yeasts lose their ability to sporulate differs among yeasts and may range from a few weeks to several years (Yarrow, 1998). As a result, it is best to preserve important strains using techniques such as lyophilization (Kirsop and Kurtzman, 1988), L-drying (Mikata and Banno, 1989) and freezing in either liquid nitrogen or a mechanical freezer at temperatures between −60°C and −135°C (Yarrow, 1998). The method currently being mostly used is freezing in liquid

nitrogen (cryopreservation), which uses a cryoprotectant such as glycerol to ensure high rates of survival as well as genetic stability.

2.4 Cytological Characteristics

Basidiomycetous yeasts are characterized by electron dense, layered cell walls (Simmons and Ahearn, 1987) and septal morphology. Septal morphology has been used as a primary phylogenetic character for discrimination within the basidiomycetous yeasts (Moore, 1998). For example, species belonging to urediniomycetous forms have septa with simple pores (Fig. 2.1) in which the cell wall is attenuated towards the central pore during the hyphal state. Multiple pores have however been reported in *Kriegeria eriophori* instead of single pore (McLaughlin et al., 1995). Mannose is excessively present in the cell wall, glucose to some extent; fucose and rhamnose are rarely present and xylose is entirely absent (Prillinger et al., 1991). Starch-like compounds and inositol are absent in urediniomycetous yeasts.

Ustilaginomycetous yeasts are characterized by 'micropore-like' septa (Fig. 2.2) which possesses an inflated margin. The occurrence of inflated margin can vary in species. The micropore septa do not have tapering cell walls and lacks a true pore (Bauer et al., 1997). The level of glucose is high; galactose and mannose are present to a certain extent whereas xylose is absent (Prillinger et al., 1990).

Hymenomycetous yeasts possess dolipore septum (Fig. 2.3) wherein cell walls contain glucose, mannose and xylose (Roeijmans et al., 1998). Inositol is usually assimilated and starch-like compounds are produced by a majority of them.

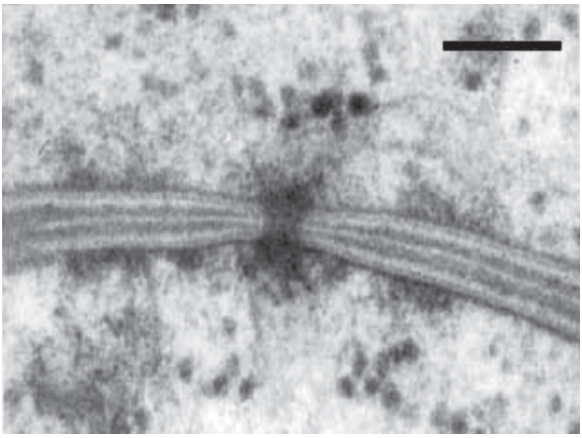

Fig. 2.1 Septal pore type in Urediniomycetes. From Bauer, R. et al. 1997. *Can. J. Bot.* **75**: 1273–1314. Copyright NRC Research Press. Reproduced with permission

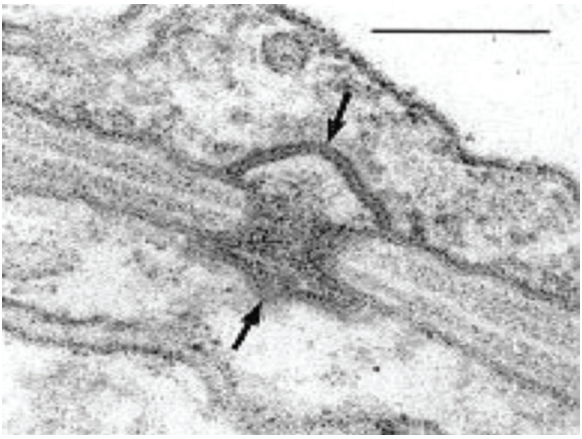

Fig. 2.2 Transmission electron micrograph showing a typical septal pore apparatus of the Ustilaginomycetes (*Entyloma callitrichis*) with two membrane caps (arrows). Scale bar = 0.1 μm. From Bauer, R. et al. 1997. *Can. J. Bot.* **75**: 1273–1314. Copyright NRC Research Press. Reproduced with permission

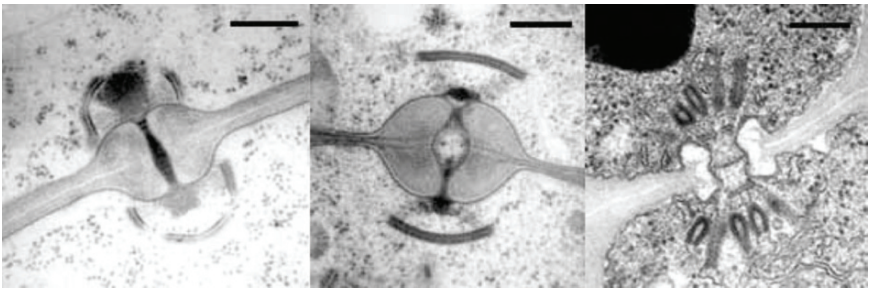

Fig. 2.3 Septal pore types in the Hymenomycetes. Left: Dolipore with perforated parenthesomes of *Schizophyllum commune* (Bar = 0.25 μm) Center: Dolipore with continuous parenthesomes of *Tulasnella* sp. (Bar = 0.25 μm). Right: Dolipore with cup-shaped parenthesomes of *Tremella* sp. (Bar = 0.2 μm) From Bauer, R. et al. 1997. *Can. J. Bot.* **75**: 1273–1314. Copyright NRC Research Press.Reproduced with permission

Sampaio et al. (2002) described two new genera *Bullerobasidium* and *Papiliotrema* with three new species, *B. oberjochense, P. bandonii* and *B. murrhardtense* by integrated analysis of morphological, ultrastructural, physiological and molecular data. They placed these new genera and species into the order Tremellales since they compared with those of closely related taxa.

Two general types of teleomorphs are found among the basidiomycetous yeast (Boekhout et al., 1993). In the first, teliospores are formed and germinate to produce a basidium that bears basidiospores. The second type of sexual cycle has no

teliospores. Basidia develop on hyphae or yeast cells and give rise to basidiospores in a manner similar to jelly fungi (Kurtzman and Fell, 1998). When these sexual structures are not present, basidiomycetous yeasts are morphologically indistinguishable from ascomycetous yeasts, except that morphology of the bud scars can sometimes be different.

More useful diagnostic characteristics of the basidiomycetous yeasts are the presence of clamp connections, the red stained colonies with diazonium blue B (colonies of ascomycetes remain unstained) (van der Walt and Hopsu-Hava, 1976), + ve urease reaction and a high G + C content. TEM studies have demonstrated that the inner walls of the basidiomycetous yeasts are typically lamellar, in contrast to the uniform inner layer of ascomycetes (Kreger-van Rij and Veenhuis, 1971). TEM is also useful to demonstrate dolipore (complex barrel-shaped structures formed in the septa, which are covered on both sides by a membrane called parenthosome) in basidiomycetous yeast. The presences of carotenoids and ballistoconidia (forcibly ejected vegetative cells) in some taxa have been used as a criterion for assignment to a genus.

Ultrastructural features correlate well with chemosynthetic characteristics and are regarded as reliable systematic criteria in higher taxonomic rank. However, at lower level, rRNA sequence analysis shows that *Rhodotorula, Sporobolomyces*, and *Cryptococcus* are polyphyletic, confirming that commonly used phenotypic characteristics are insufficient for defining anamorphic genera (Nakase et al., 1991).

Prillinger and coworkers (1990, 1991) and Dorfler (1990) investigated carbohydrate composition of the purified cell wall in yeast states of Basidiomycetes. They found that xylose, rhamnose, fucose and galactose as well as the ratio of glucose to mannose were important in differentiation of higher taxa. Employing GLC-analysis of purified cell wall hydrolysates in basidiomycetous yeast, Prillinger et al. (1991) identified three different carbohydrate profiles, Glucose-Mannose-Galactose (*Ustilago*-type that exhibited high amount of glucose and balanced amount of mannose and galactose), Mannose-Galactose-Fucose (*Microbotryum*-type, that showed greater amount of mannose compared to that of glucose and balanced amount of galactose and fucose) and Glucose-Mannose-Xylose (*Tremella*-type wherein higher amount of glucose and balanced amount of mannose and xylose was observed); a fourth type in the systematics of basidiomycetous yeast, was the so-called *Dacrymyces*-type that exhibited presence of xylose and balanced amounts of glucose and mannose in the cell walls, e.g. *Dacrymyces* and *Calocera.*

2.5 Taxonomy of the Basidiomycetous Yeasts

The principles of yeast taxonomy encompass identification, naming and placing organisms in their proper evolutionary framework. Historically, basidiomycetous yeasts have been placed into three taxonomic classes namely the Hymenomycetes, Urediniomycetes and Ustilaginomycetes.

2.5.1 Natural Classification System

Natural classification systems are based on evolution, and have been the preferred method for systematics. This system addresses the species concept, and also shows the phylogeny or sequence of events that are involved in evolution. The species concept recognizes that different species have different phenotypic characters by which they can be recognized (van der Walt, 2000).

2.5.2 Biological Species Concept

Modern biologists have arrived at the biological species concept that is characterized by four distinct criteria. These are, a reproductive unit, an ecological unit, a genetic unit and, an evolutionary entity. This was further enhanced by another dimension of the species through the introduction of the so-called phylogenetic species concept that focused on the interpretation of the ribosomal nucleotide sequence analysis thereby excluding the phenotypic, genetic or ecological criteria. The two concepts namely the biological species concept and phylogenetic species concept have little in common. The biological species concept is not easily applied in practical systematics resulting in having to adhere to the type-based species and the extensive use of phenotypic differentiation (Kurtzman, 1987; Boekhout and Kurtzman, 1996). By definition, the biological species concept excludes the asexual (anamorphic) yeast species. Barnett and co-authors (2000) have listed 93 characters for identification purposes concentrating on just phenotypic characteristics.

To overcome the noted drawbacks from the two preceding concepts, yeast taxonomists had to consider analysis of the nuclear genome that had already been extensively used in prokaryotic systems. The base composition of the nuclear genome was believed to reflect the ancestral descent at molecular level reducing reliance on phenotypic characterization (van der Walt, 2000).

2.5.3 Conventional Identification

The criteria and tests for identification of basidiomycetous yeasts involve firstly, observation of culture characteristics, which include colour, shape and texture of the colonies. The production of extra-cellular polysaccharides is also observed by the resulting mucoid growth. There are distinctive colours such as yellow, orange and red those are peculiar to certain genera of basidiomycetous yeasts, viz., *Phaffia*, *Rhodosporidium* and *Sporidiobolus*. However, the colour produced by a majority of yeasts ranges from whitish through cream to buff (Yarrow, 1998). This is followed by observation of asexual structures, which include shape and size of the vegetative cells. The mode of conidia formation is important and provides information, which aids in the identification of a strain. Budding starts by forming a small outgrowth

at some point on the surface of the cell without the cell changing in size. The increase in size is seen in a newly formed bud, which eventually separates from the parent cell (mother). Holoblastic budding results from outgrowth of the entire cell wall of the parent cell, the bud separates from the narrow base leaving a scar through which no further budding occurs. This type of budding is characteristic of the Saccharomycetales and their anamorphic states while enteroblastic is characteristic of basidiomycetous yeasts and results in formation of a collaret due to recurrent formation and abscission of a succession of buds (Yarrow, 1998).

The vegetative cells have different shapes that include globose, subglobose, ellipsoidal, ovoidal, cylindrical, botuliform, elongate, apiculate, lunate and triangular. The shape may reflect the type of reproduction and in some cases it is peculiar to particular genera or species viz., the bottle-shaped cells of *Malassezia* (Yarrow, 1998).

Sexual structures are investigated with respect to arrangement, cell wall ornamentations, number, shape and size of basidiospores (Fig. 2.4). Other types of spores formed that aid identification include endospores which are vegetative cells, formed within discrete cells and hyphae. They cannot be stained selectively. However, they can also be observed in old cultures on YM agar. Asexual endospores are observed in strains of the genera *Cryptococcus, Cystofilobasidium,* and *Trichosporon* whereas they are uncommon in other genera.

2.5.4 Species Differentiation Based on ITS and D1/D2 Regions

van der Aa Kühle and Jespersen (2003) investigated phylogenetic analysis of closely related species by amplifying the region spanning two intergenic transcribed spacers (ITS1 & ITS2) and the 5.8S ribosomal subunit. This region is located between the 18S and the 28S rRNA genes in yeasts. The ITS region is subdivided into ITS 1 region which separates the conserved 18S and the 5.8S rRNA genes (Frutos et al., 2004). The ITS 2 region is found between 5.8S and 28S rRNA genes. The ITS 1 and 2 have been shown to play a role in primary rRNA processing (Musters et al., 1990).

D1/D2 domain: This region refers to the variable domain of the large subunit (26S) ribosomal DNA or the complete small subunit and is approximately 600 bases in size. Conspecific strains are separated by less than 1% nucleotide substitution whereas biological species are separated by greater than 1% nucleotide substitution. Kurtzman and Robnett (1998) have shown that most yeast species can be identified from sequence divergence in this region, which represents a partial sequence of the 26S rDNA (Kurtzman and Robnett, 1998; Fell et al., 2000).

The sequencing of the D1/D2 domain has been extensively used to identify yeasts (Phaff et al., 1999; Hong et al., 2001; Scorzetti et al., 2002). According to Frutos et al. (2004) it is accepted universally as the main tool for yeast taxonomy. Databases of the D1/D2 sequences are now available for all currently recognized ascomycetous and basidiomycetous yeasts (Kurtzman and Robnett, 1995, 1997, 1998; Guffogg et al., 2004). This extensive available database makes the task of species identification much easier (Kurtzman, 2001; Starmer et al., 2001; Wesselink

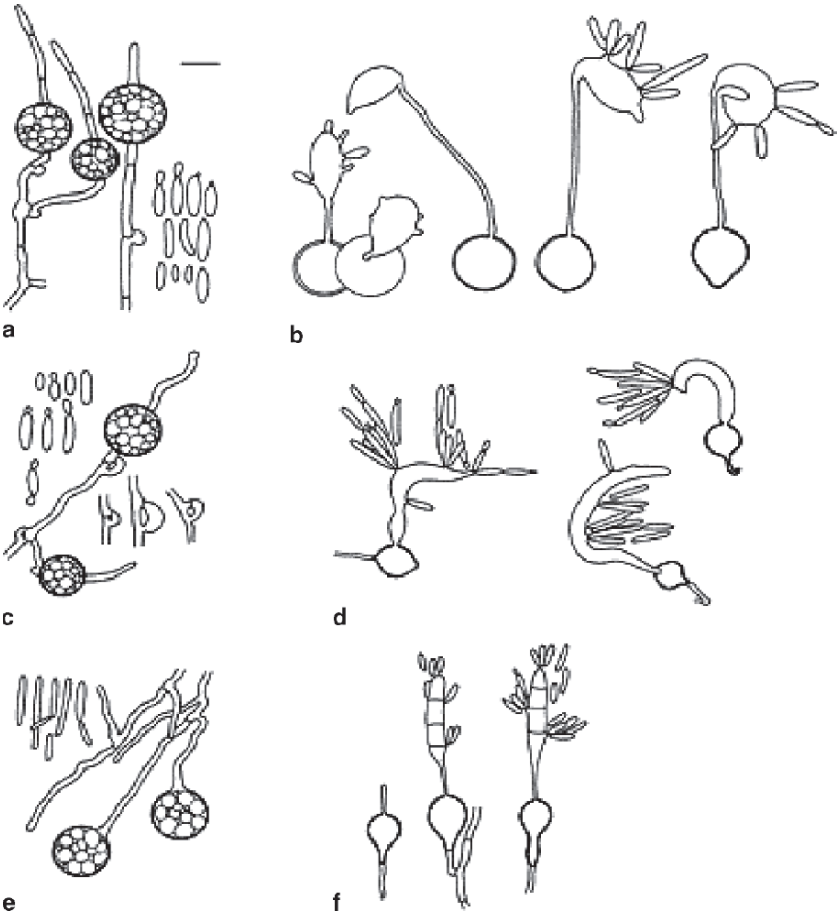

Fig. 2.4 Line drawings of different developmental stages of basidiomycetous yeast cells (after 4–6 days on MYP agar), mycelium and teliospores (after 1–2 weeks on PDA) (**a**, **c**, **e**) are shown; germinated teliospores, basidia and sessile basidiospores (**b**, **d**, **f**) are shown. From Sampaio, J.P. et al. 2004. *Int. J. Syst. Evol. Microbiol.* **54**: 1401–1407. Copyright IUMS. Reproduced with permission

et al., 2002) and serve as reliable and practical criteria for identification of most known yeasts (Abliz et al., 2004). It has been extensively used to characterize most of the basidiomycetous species at strain level.

2.6 Systematics of Basidiomycetous Yeasts

Basidiomycetous yeasts have been divided into different lineages based on the analysis of different region of rDNA viz., four main clusters according to 26S rDNA (Sporidiales, Tremellales, Filobasidiales and related taxa and, the

Ustilaginales; Fell et al., 1995); and three main lineages according to 18S rDNA or the D1/D2 region of the large subunit rDNA (Ustilaginomycetes, Urediniomycetes and Hymenomycetes; Fell et al., 2000).

Not only nuclear DNA (n DNA) approach but also another approach wherein characterization of genes present in extrachromosomal organelles provides a pivotal way to infer phylogeny of closely related species, viz., the rapid evolution of the mitochondrial genome, its lack of recombination and its maternal inheritance, makes it an attractive marker for inferring the phylogeny of closely related species (Manceau et al., 1999). Phylogenetic relationships of *Rhodotorula* spp. were studied by employing partial sequences of mitochondrial cytochrome b gene (Biswas et al., 2001). Based on *mt cyt* b gene, the basidiomycetous yeasts are distributed into two main clusters: one containing Tremellales, Filobasidiales and their anamorphs, and the other, Ustilaginales, Sporidiales and their anamorphs.

Fell et al. (2000) examined the phylogenetic diversity of yeasts among the Ustilaginomycetes, Urediniomycetes, and Hymenomycetes. The results obtained confirmed some accepted concepts, *viz.,* that yeasts are a heterogeneous group of organisms and that many genera are representative of artificial assemblages, e.g. the genus *Cryptococcus* occurs in the following Hymenomycetes clade: Tremellales, Trichosporonales, Filobasidiales and Cystofilobasidiales. Similarly species of *Rhodotorula* occur in *Microbotryum, Sporidiobolus* and *Erythrobasidium* clades of the Urediniomycetes and Microstromatales clade of the Ustilaginomycetes.

Middelhoven et al. (2004) isolated three novel species, *Trichosporon vadense* sp. nov. (type strain CBS 8901^T), *Trihcosporon smithiae* sp. nov. (type strain CBS 8370^T) and *Trichosporon gamsii* sp. nov. (type strain CBS 8245^T), from soil and novel species *T. scarabaeorum* sp. nov. (type strain CBS 5601^T) and *T. dehoogi* sp. nov. (sp. nov. CBS 8686^T) from insect of unknown origin, respectively. These new species were quite different from other related species based on phylogenetic position and physiological characteristics.

A novel species of *Cryptococcus* was recovered from salt farm on the Taean Peninsula in Korea, i.e. *C. taeanensis*. The isolate exhibited typical physiology of the genus *C. vuillemin*, but was quite distinct from previously described species in the genus based on its large subunit rRNA D1/D2 domain sequence (Shin et al., 2005).

2.6.1 Yeast Species of the Hymenomycetes

Swann and Taylor (1995a, b, c) recommended two subclasses among the Hymenomycetes based on sequence analysis of small subunit rDNA, (a) the Hymenomycetidae, containing the non-yeast-like macrofungi, mushrooms and puffballs; and (b) the Tremellomycetidae. These authors analyzed D1/D2 region and kept them in four major clades (Fig. 2.5) of the Tremellomycetidae: the Tremellales, the Trichosporonales, the Filobasidiales and the Cystofilobasidiales. The hymenomycetous yeast genus, *Cryptococcus*, is polyphyletic and occurs in all four clades whereas remaining genera occur in single clades: a. the Tremellales – *Bullera,*

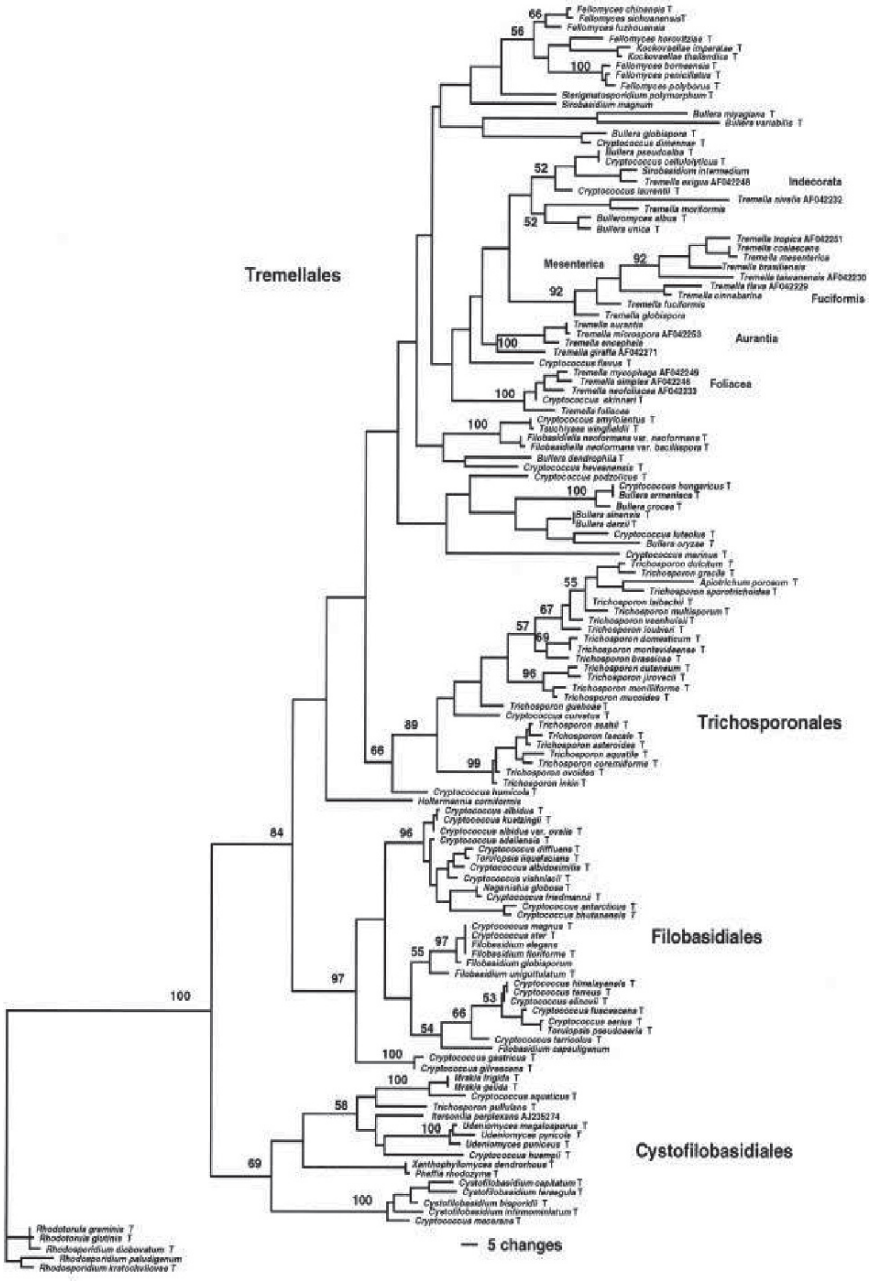

Fig. 2.5 Hymenomycetous yeasts: phylogenetic analysis of the D1/D2 region of the large-subunit rDNA (one of 100 equally parsimonious trees). From Fell et al. 2000. *IJSEM* **50**: 1351–1371. Copyright IUMS. Reproduced with permission

Bulleromyces, Fellomyces, Filobasidiella, Kockovaella and *Tsuchiyaea*; b. the Trichosporonales – all species of *Trichosporon* with the exception of *Trichosporon pullulans* which occurs in the cystofilobasidiales; c. the Filobasidiales – *Filobasidium*; and, d. Cystofilobasidiales – *Cystofilobasidium, Mrakia, Phaffia, Urediniomyces* and *Xanthophyllomyces.*

2.6.2 Yeast Species of the Urediniomycetes

The group urediniomycetes constitutes four major clades (Fig. 2.6) with the genera *Microbotryum, Sporidiobolus, Agaricostilbum* and *Erythrobasidium*. Three other genera once occupied two or more clades. *Bensingtonia* occurs in *Microbotryum* and *Agaricostilbum* clades; *Rhodotorula* in *Microbotryum, Sporidiobolus,* and *Erythrobasidium* and *Sporobolomyces* occur in all four clades. Genera found in single clade were:

a) Microbotryum clade – *Leucosporidium*
b) Sporidiobolus clade – *Rhodosporidium* and *Sporidiobolus*
c) Agaricostilbum clade – *Kondoa, Kurtzmanomyces* and *Sterigmatomyces*
d) Erythrobasidium clade – *Erythrobasidium, Sakaguchia* and *Occultifur*

2.6.3 Distribution of Yeasts among the Ustilaginomycetes

A majority of yeast species examined within the Ustilaginomycete consist of the D1/D2 sequences; originally generally isolated from plants, they were distributed into several clades (Fig. 2.7): *Pseudozyme* spp. and *Rhodotorula acheniorum* are in the subclass Ustilaginomycetidae, whereas *Rhodotorula phylloplana* and *Sympodiomycopsis paphiopedili* are in the subclass Exobasidiomycetidae. *Tilletia* is phylogenetically associated with the Tilletiales.

2.7 Basidiomycetous Yeast – Life Cycle

The life cycle of basidiomycetous yeast normally alternates between diplophase and haplophase. Both ploidies can exist as stable cultures. In heterothallic strains, haploid cells are of two mating types, a and α. Mating of a and α cells results in a/α diploids that are unable to mate but can undergo meiosis. The eight haploid (basidiospores) products resulting from meiosis of a diploid cell are contained within the wall of the mother cell (the basidium). Digestion of the basidium and separation of the basidiospores by micromanipulation yield the eight haploid meiotic products.

Fig. 2.6 Urediniomycetous yeasts, representing four clades (*Microbotryum*, *Sporidiobolus*, *Erythrobasidium* and *Agaricostilbum*): phylogenetic analysis of the D1/D2 region of the large-subunit rDNA (one of 100 equally parsimonious trees). From Fell et al. 2000. *IJSEM* **50**: 1351–1371. Copyright IUMS. Reproduced with permission

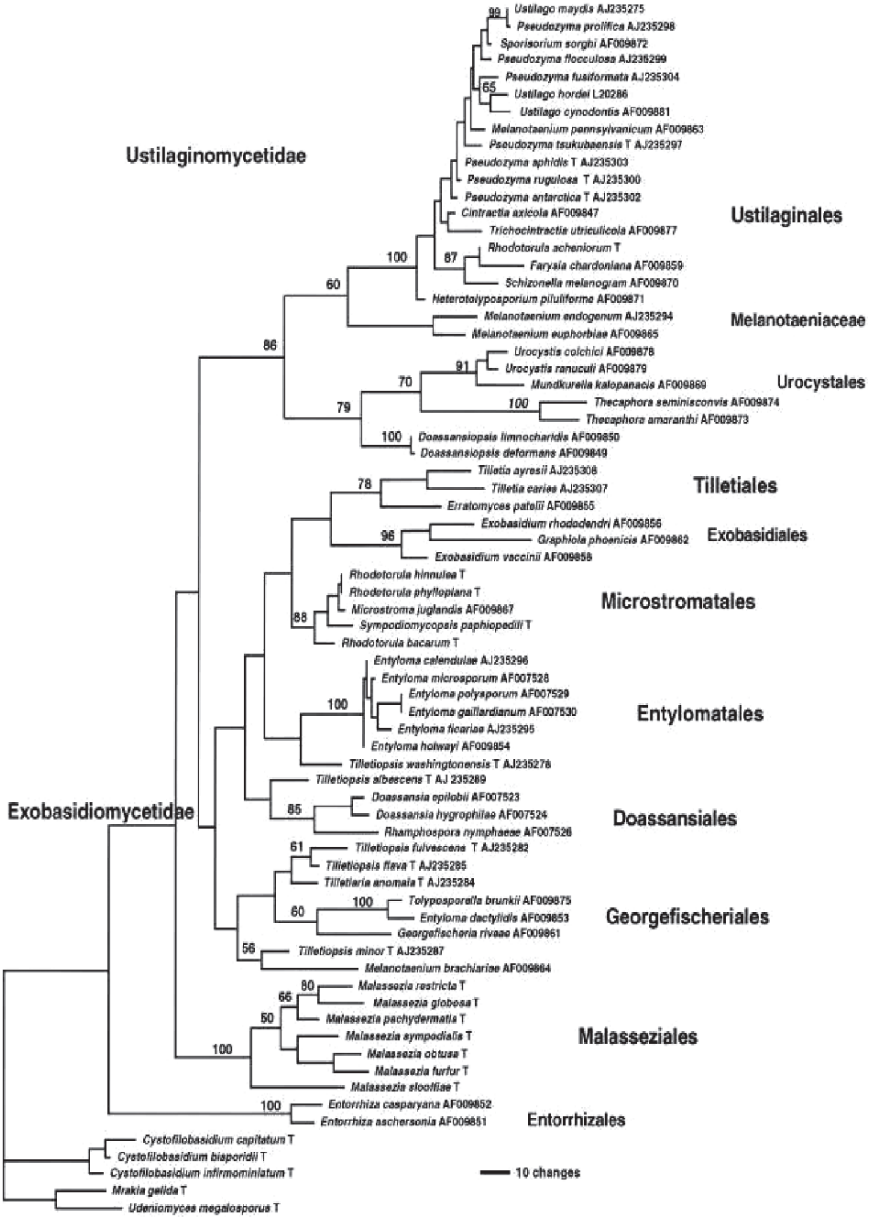

Fig. 2.7 Ustilaginomycetous fungi and associated yeasts: phylogenetic analysis of the D1/D2 region of the large-subunit rDNA (one of 100 equally parsimonious trees. From Fell et al. 2000. *IJSEM* **50**: 1351–1371. Copyright IUMS. Reproduced with permission

Analysis of the segregation patterns of different heterozygous markers among the four spores constitutes tetrad analysis and reveals the linkage between two genes (or between a gene and its centromere) (Mortimer and Schild, 1981).

On the whole, genetic distance in yeast appears to be remarkably proportional to physical distance, with a global average of 3 kb/cM. A large variety of protocols for genetic manipulation in yeast are available (Guthrie and Fink, 1991; Johnston, 1994). Yeast has a generation time of ca 80 min and mass production of cells is easy. Simple procedures for the isolation of high molecular weight DNA, rDNA, mRNA, and tRNA are at hand. It is possible to isolate intact nuclei or cell organelles such as intact mitochondria (maintaining respiratory competence). High efficiency transformation of yeast cells is achieved, for example, by the lithium acetate procedure (Ito et al., 1983) or by electroporation. A large variety of vectors have been designed to introduce and to maintain or express recombinant DNA in yeast cells (e.g. Guthrie and Fink, 1991; Johnston, 1994). Furthermore, a large number of yeast strains carry auxotrophic markers, drug resistance markers or defined mutations. Culture collections are maintained, for example, at the Yeast Genetic Stock Center (YGSC) and the American Type Culture Collection (ATCC). Mutant strains with defined gene deletions together with clones carrying the corresponding gene cassettes have emerged from the EUROFAN and TRANSATLANTIC projects. Ordered cosmid libraries using different vectors were constructed during the yeast-sequencing project (e.g. Thierry et al., 1995; Riles et al., 1993; Stucka and Feldmann, 1994).

2.8 Basidiomycetous Yeasts as Model System

2.8.1 Cryptococcus neoformans

Studies of non-vertebrate hosts during the recent past decade have yielded deeper insights into the molecular mechanisms of microbial pathogenesis and host defense. Apparently similar bacterial virulence factors are involved in pathogenesis in hosts that are evolutionarily far apart, *viz*., plants, nematodes, and mammals (Aballay and Ausubel, 2002). *Caenorhabditis elegans,* a nematode has proved particularly important host for study of bacterial pathogenesis.

Steenbergen et al. (2001) reported the use of the free-living amoeba *Acanthamoeba castellanii* as a model for study of survival strategies used by the human opportunistic fungal pathogen *Cryptococcus neoformans* after ingestion by macrophages. These workers observed that *C. neoformans* was phagocytosed by *A. castellanii*, and that once inside, the yeast replicated, eventually killing the amoeba. The process appeared remarkably similar to that in mammalian macrophages (Levitz, 2001); an acapsular strain of *C. neoformans* did not survive when incubated with *A. castellanii* and a phospholipase mutant exhibited decreased replication rate in amoeba, processes similar to those in macrophages.

These observations suggest that cryptococcal characteristics that contribute to mammalian virulence also promote fungal survival in free-living amoebae. In view of the successful use of *C. elegans* and *A. castellanii* to study bacterial and fungal pathogenesis, especially among immunocompromised patients (Marty and Mylonakis, 2002), such observations are of special relevance.

Study of the pathogenic mechanisms of *C. neoformans* has been enhanced substantially by the development of transformation protocols, homologous recombination for genetic manipulations, and reproducible animal models (Hua et al., 2000). The most important *C. neoformans* virulence factors identified so far include the polysaccharide capsule (Perfect et al., 1998), laccase (an enzyme essential for melanin production) (Jacobson, 2000), and at least two signal transduction cascades (Waugh et al., 2002). These observations suggest that the developmental aspects of *C. neoformans* pathogenesis in humans can be modeled using simple invertebrate nematode *C. elegans* as an experimental host.

2.8.2 Fellomyces fuzhouensis

A new, potentially pathogenic yeast which reproduces by conidiogenesis, can also act as model system

In conformity with other 'long neck yeasts', *Fellomyces* is characterized by formation of conidia, a unique vegetative structure in so far as yeasts are concerned. While, majority of yeast species reproduce vegetatively by budding, fission or by 'bud-fission', conidiogenesis, was discovered in the genus *Sterigmatomyces;* this involves the development of a sterigma, a tube-like, slender projection, that bears a single, spherical or ovoidal conidium (Fell, 1966). In the genus *Sterigmatomyces*, conidia separate from sterigmata by cleavage at the midpoint, whereas in the genus *Fellomyces* they separate at the distal ends of sterigmata (Hamamoto et al., 1998).

Compared to the budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe*, *Fellomyces fuzhouensis* is rich in membranous organelles, especially long cisternae of the endoplasmic reticulum and large mitochondria. The density of cristae of mitochondria is related to the physiological activity (growth) of each cellular compartment, i.e. conidiogenous cell, sterigma and conidium. The multilamellar cell wall in conidiogenous, ageing cells is reminiscent of the cell wall of the Basidiomycetes (Kreger-van Rij and Veenhuis, 1971) and shows that this organism was correctly classified as a basidiomycetous yeast (Fell et al., 2000).

Based on the use of ultrastructural methodologies, the genus *Fellomyces* has been separated from *Sterigmatomyces* on the basis of breaking-off of the empty sterigma from the conidiogenous cell; this is in addition to differences in biochemical characterization and chemical composition of the wall. The long, empty sterigma visualised in *F. fuzhouensis* is due to the breakage that occurs in the narrowest part of the sterigma at the conidium base; this area also shows presence of an actin ring.

Conidia could easily be detached when cultured in liquid media on a shaker but, on solid media, subsequent cell generations remain connected in a network.

2.8.3 Isolation and Characterization of Carotenoid Hyperproducing Mutants of Yeast

The carotenoid pigment astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is an important component in feeds of aquacultural animals. It is produced as a secondary metabolite by the yeast *Phaffia rhodozyma*, and the isolation of rare mutants that produce increased quantities is limited by the lack of genetic selections. As a model system for enriching mutants increased in production of secondary metabolites, quantitative flow cytometry/cell sorting (FCCS) have been used to isolate astaxanthin hyperproducing mutants of this yeast (An et al., 1991).

2.9 Importance of Basidiomycetous Yeasts

Besides being a diverse group of fungi, basidiomycetous yeasts have considerable industrial and medical importance. Among these, *Cryptococcus* (Tremellales) is medically the most important genus. *Cryptococcus neoformans* is ubiquitous in the environment and serves as a model organism for fungal pathogenesis, and an opportunistic human pathogen of global importance.

This yeast encodes unique genes that contribute towards its unusual virulence properties; comparison of two phenotypically distinct strains reveals variation in gene content, in addition to sequence polymorphism between the genome. The genus *Cryptococcus* has 34 species of diverse relationships.

Virulence in *C. neoformans* is mediated predominantly by a polysaccharide capsule that surrounds the cell wall and has multiple effects on the host immune system. Capsule provides a physical barrier that interferes with the normal phagocytosis and clearance by the immune system. Capsule components inhibit the production of proinflammatory cytokines, deplete complement components, and reduce leukocyte migration to sites of inflammation (Buchanan and Murphy, 1998). The capsule also constitutes the major diagnostic feature of cryptococcosis, because its components can be detected in the bloodstream and can be visualized with light microscopy by using India ink staining. The capsule excludes the ink particles and forms characteristic halos, whose diameters are often several times that of the cell. The elaborate structure of the capsule can be appreciated by electron microscopy (Fig. 2.8).

Genus *Trichosporon* is characterized by the production of arthroconidia and ballistoconidia and its species are able to assimilate many C compounds and degrade urea (Gueho et al., 1994a,b). The clinical manifestation caused by

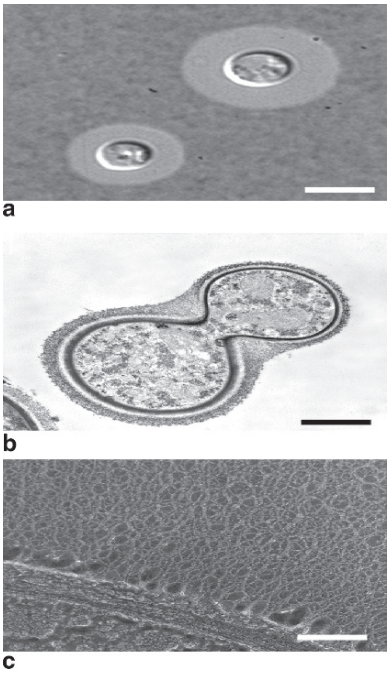

Fig. 2.8 Views of the cryptococcal capsule. (**a**) Differential interference contrast micrograph of cells that were mixed with India ink after induction of capsule formation by growth in low-iron medium. Scale bar, 3 μm. (**b**) Thin-section micrograph of a budding cell fixed in the presence of ruthenium red dye. Scale bar, 1 μm. (**c**) Quick-freeze, deep-etch image of the edge of a cell, with an arc of cell wall separating the cell interior (lower left) from the abundant capsule fibers emanating upwards. Scale bar, 0.15 μm. Pierini and Doering 2001. *Mol. Microbiol.* **41:** 105–115. Copyright Blackwell Publishing. Reproduced with permission

Trichosporon spp. vary widely from mild superficial infections such as 'white piedra' to localized or disseminated infections in patients with hematological malignancies (Herbrechet et al., 1993; Tashiro et al., 1995). Six species, *T. asahii, T. inkin, T. mucoides, T. cutaneum., T. ovoides* and *T. asteroids* are of clinical significance. *Rhodotorula, Sporobolomyces* and *Tilletiopsis* are anamorphic genera with pathogenic species assigned to the order Ustilaginales. *Rhodotorula* is characterized by pink colonies and budding cells with narrow bud scars. The teleomorphs of the type species, *R. mucilaginosa,* produce dominantly ballistoconidia but also produces teliospores in culture after mating with compatible strains. Kurtzman and Fell (1998) recovered 34 species of *Rhodotorula*, of which only three, *R. glutinis, R.mucilaginosa,* and *R. minuta* were found to be pathogenic (Gyaurgieva et al., 1996; Nucci et al., 1995). These three species can be distinguished from each other by the assimilation or lack of nitrate and raffinose. *Sporobolomyces salmonicolor*, the anamorph of *S. johnsonii*, also develops salmon pink colonies but they are

differentiated from *Rhodotorula* spp. by the production of ballistoconidia on large sterigmata. This agent has been reported as a cause of infection in AIDS patient (Plazas et al., 1994).

Phaffia rhodozyma has the ability to secrete β-carotene and astaxanthin; 'white-haze' symptoms occur on apple as a result of colonization by ballistoconidium forming fungi (Hui et al., 2005). It occurs in mild form in the field and subsequently it becomes problematic after ultra-low oxygen storage and, is considered as a post harvest disorder. *Rhodosporidium toruloides* (anamorph, *R. glutinis*) is a common phylloplane epiphyte which has biocontrol potential (Buck and Andrews, 1999).

Buzzuni and Martini (2002) recovered 155 strains of basidiomycetous yeasts from extreme environments to assess their extracellular enzymatic activity profiles. Approximately one-third basidiomycetes exhibited caseinolytic activity; this character was expressed almost exclusively by isolates of *Cryptococcus* sp. and *Pseudozyma* sp. In particular, 83% of *Pseudozyma antarctica* strains were caseinolytic and only half of caseinolytic basidiomycetes were able to hydrolyze gelatine whereas a few strains were chitinolytic.

Thirty-seven basidiomycetous yeasts belonging to 30 different species within seven genera were grown on media containing L-cysteine or L-methionine as sole nitrogen sources with the objective of evaluating production of volatile organic sulfur compounds (VOSC). The headspace of yeast cultures was analyzed by the solid-phase microextraction (SPME) sampling method, and volatile compounds were quantified and identified by GC-MS techniques. Ten strains assimilating L-methionine produced the following VOSCs: 3-(methylthio)-1-propanol, methanethiol, S-methyl thioacetate, dimethyl disulfide, dimethyl trisulfide, allyl methyl sulphide and 4,5-dihydro-3(2H)-thiophenone. The production level was usually small (<1 mg l^{-1}) except for 3-(methylthio)-1-propanol (40 and 400 mg l^{-1}). Higher alcohols (isobutyl alcohol, isoamyl alcohol and active amyl alcohol) and esters (ethyl acetate, ethyl propionate, n-propyl acetate, isobutyl acetate, n-propyl propionate, n-butyl acetate, isoamyl acetate, amyl acetate, isoamyl propionate, amyl propionate and 2-phenylmethyl acetate) were also sporadically produced. This is the first report of production of VOSCs by basidiomycetous yeasts. Consequently, basidiomycetous yeasts may be considered as an interesting new group of microbial production of VOSCs for the flavour industry (Buzzuni et al., 2005).

A cold-active polygalacturonase (PG) has been characterized from the extracellular fraction of *Cystofilobasidium capitatum* strain PPY-1. The purified PG from strain PPY-1 has a molecular mass of about 44 kDa, and exhibits high activity at 0°C, with optimum of 45°C. Although the Km value for polygalacturonate as a substrate at 45°C is 11.2 mg ml^{-}, it decreases gradually with decreasing temperature; it was 0.66 mg ml^{-} at 0°C. Moreover, the cleavage pattern of this enzyme is endo-type. These findings suggest that this PG from strain PPY-1 is a novel type of cold-active endo-PG that is able to degrade pectin compounds at low temperatures (Nakagawa et al., 2005).

2.10 Future Perspectives

Since the release of the yeast genome DNA sequence, there has been an expected change in the technology of yeast research as well as a rather surprising change in its goals. Most of the new understanding of individual yeast gene functions has come from comparative genomics and relatively little from the high-throughput genomic technologies. The latter have, however, fueled the changes in goals, from a focus on individual genes and their interactions to a focus on the system-level transactions that make the robustly functioning organisms as pioneer find in the nature.

The future of genome-scale technologies is, indeed, very promising. It is not clear whether the slow rate at which new annotations are verified is caused by problems in data analysis and representation, or by a more simple lack of focus on the need for such verification. Some methods, now in early stages of development, will no doubt help: among these are methods based on natural variation (examples include Brem et al., 2002; Steinmetz et al., 2002; Fay et al., 2004), methods that are not limited to nonessential genes (e.g. synthetic lethality with conditional alleles) (Tong et al., 2004) or titratable promoter alleles (Mnaimneh et al., 2004), methods that study the locations and movements of intracellular molecules (Ghaemmaghami et al., 2003; Huh et al., 2003), and methods that use more biological information from other species (for example, Harbison et al., 2004). Fortunately, in the post–genome-sequence era, it is much easier to acquire this kind of information on a comprehensive scale, and this appears to be the path forward. Another challenge of this nature is to understand the basis on which selection acts on the ensemble of genes, proteins, networks, and systems to produce organisms capable of surviving in new environments.

Finally, there remains the eternal issue of verification. One expects that the need for tests of hypotheses generated by genome-scale experiments and quantitative models will persist for a very long time. As has always been the case, every model (and the data used to generate it) must be tested, and to be tested, it must be specified in full and available to the public. The yeast community has an excellent record in this regard, one that we believe is a major reason that yeast continues to be the very model of a model organism.

Keeping in mind that fungi are significant and increasingly cause morbidity and mortality in immunocompromised patients and that the opportunistic infections they cause are severe and life threatening, rapid diagnosis and efficient therapeutic measures are essential. Of course, collaboration between the clinicians and the laboratory is absolutely necessary. Additionally, the precise diagnosis is based on the morphologic identification of the tissue forms in biopsy material or pus by microscopy and culture. The final diagnosis of the agent is achieved only after culture of material is obtained from the lesions. Detection of specific antibodies and specific antigens and/or metabolites in body fluids or tissues may be of great value in the future, especially molecular diagnostic techniques.

In conclusion, the interaction of *C. elegans* with the yeast *C. neoformans* involves a number of genes that are also important during the host pathogen interaction during mammalian infection. Identification of new *C. neoformans* virulence factors using this model may lead to new targets for antifungal therapies as well as a deeper understanding of the host–fungus interaction.

A new taxon in the genus *Sporidiobolus* has been described as *Sporidiobolus metaroseus* sp. nov. (type strain CBS 7683^T) (Valerio et al., 2008). Margesin et al. (2007) reported three novel psychrophilic species of the genus *Rhodotorula viz., R. psychrophila* sp. nov. (type strain PB19^T=CBS 10440^T=DSM 18768^T), *R. psychrophenolica* sp. nov. (type strain AG21^T=CBS 10438^T=DSM 18767^T) and *R. glacialis* sp. nov. (type strain A19^T=CBS 10436^T=DSM 18766^T). In addition, a novel species *viz., Rhodotorula subericola* has been isolated from bark of *Quercus suber* (cork oak) (Belloch et al., 2007). Besides, a novel species *Rhodotorula pacifica*, was isolated from sediments collected on the deep-sea floor in the northwest Pacific Ocean (Nagahama et al., 2006). Shin et al. (2006) described two novel basidiomycetous yeasts species *viz., Cryptococcus mujuensis* sp. nov. (type strain KCTC 17231^T=CBS 10308^T) and *Cryptococcus cuniculi* sp. nov. (type strain KCTC 17232^T=CBS 10309^T). In addition, Wang et al. (2006) reported two novel ustilaginomycetous anamorphic yeast species *viz., Pseudozyma hubeiensis* and *Pseudozyma shanxiensis*, recovered from wilting leaves of various plants in China. Pohl et al. (2006) reported a novel anamorphic basidiomycetous yeast, *Cryptococcus anemochoreius* sp. nov. (type strain CBS 10258^T).

In addition, a novel anamorphic genus, *Farysizyma*, is created in the *Ustilaginales* to accomodate three novel epiphytic basidiomycetous yeast species *viz., F. itapuensis, F. setubalensis* and *F. taiwaniana,* based on nucleotide sequences of the D1/D2 domains of the 26S rRNA gene and the ITS region (Inacio et al., 2008). Statzell-Tallman et al. (2008) reported a novel teleomorphic yeast species, *Kwoniella mangroviensis,* from mangrove habitats in the Florida Everglades and Bahamas. Two new *Cryptococcus* species *viz., C. bestiolae* and *C. dejecticola*, were isolated from litchi fruit borer *Conopomorpha sinensis* Bradley (Thanh et al., 2006). *Pseudozyma jejuensis* sp. nov., a novel cutinolytic ustilaginomycetous yeast species (Seo et al., 2007), isolated from orange leaves on Jeju island in South Korea, and two new taxa, *Malassezia caprae* sp. nov. (type strain MA 383=CBS 10434), isolated mainly from goat, and *M. equina* sp. nov. (type strain MA146=CBS 9969), isolated from horses (Cabanes et al. (2007), using analysis of the of the D1/D2 regions of the 26S rRNA gene and the ITS1+2 regions. Cafarchia et al. (2008) described genetic variants of *Malassezia pachydermatis*, isolated from canine skin. In addition, Paulina et al. (2008) compared the *Malassezia* microbiota from six healthy body locations and two psoriatic lesions, and evaluated its stability over time using multiplex real-time PCR. *Malassezia restricta* was the most abundant species in the majority of samples, and high amounts of *Malassezia globosa* were also reported.

Eigenheer et al. (2007) conducted a proteomic analysis of secreted and cell wall-bound proteins with an acapsular strain of *C. neoformans*, to study the extracellular proteome of the human fungal pathogen *C. neoformans*. Different isolates of *C. neoformans* express ectophosphatase activity, which is not influenced by capsule

size or serotype, suggesting that ectoenzyme expression can contribute to the pathogenesis of *C. neoformans* (Collopy-Junior et al., 2006). Extracellular phospholipase B (PLB) is a virulence determinant of *C. neoformans* and *C. gattii*, that causes cryptococcosis (Wu et al., 2007). van Staden et al. (2007) investigaated phytase activity in ten *Cryptococcus* strains, of which the *Cryptococcus laurentii* ABO 510 strain showed the highest level of activity. Dunlap et al. (2007) determined the effect of cold adaptation on the physicochemical properties of *C. flavescens* that may be responsible for its improved desiccation tolerance. A combination of *C. laurentii* with indole-3-acetic acid (IAA) at 0.1 mg ml^{-1} was more effective in suppressing blue and gray mold diseases (*Penicillium expansum* and *Botrytis cinerea*) on pea fruit than application of *C. laurentii* alone (Yu and Zheng, 2007). In addition, *Pseudozyma antarctica* is one of the best producer of the glycolipid biosurfactants known as mannosylerythritol lipids (MELs), which show not only excellent surface-active properties but also versatile biochemical actions (Morita et al., 2007).

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Chapter 3
Hansenula polymorpha (Pichia angusta): Biology and Applications

Gotthard Kunze, Hyun Ah Kang, and Gerd Gellissen

Contents

Abstract *Hansenula polymorpha* (*Pichia angusta*) belongs to a limited number of methylotrophic yeast species. It is able to assimilate nitrate and can grow on a range of carbon sources. Furthermore, *H. polymorpha* is a thermo-tolerant microorganism with some strains growing at temperatures up to 50°C and more. These unusual characteristics render *H. polymorpha* attractive as a model organism to study the development and functions of peroxisomes and the biochemistry of nitrate assimilation. *H. polymorpha* provides an established platform for heterologous gene expression and is distinguished by an impressive track record as producer of recombinant proteins that include commercially available pharmaceuticals like hepatitis B vaccine, insulin and the IFNα-2a.

Keywords Methylotrophic yeast, nitrate, thermotolerant, peroxisomes, recombinant proteins

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3.1 History, Phylogenetic Position, Basic Genetics and Biochemistry of *H. polymorpha*

A limited number of yeast species are able to utilize methanol as a sole energy and carbon source. The range of methylotrophic yeasts includes *Candida boidinii, Pichia methanolica, Pichia pastoris* and *Hansenula polymorpha*, all of them provide attractive platforms for heterologous gene expression (Gellissen, 2000). Especially *H. polymorpha* has found successful application in industrial production of heterologous proteins as detailed later (Gellissen, 2002; Guengerich et al., 2004). In basic research, it is used as a model organism for peroxisomal function and biogenesis as well as nitrate assimilation (Gellissen and Veenhuis, 2001; van der Klei and Veenhuis, 2002; Siverio, 2002; Kang et al., 2002). The presence of a nitrate assimilation pathway is a feature not shared by the other methylotrophs. Since *H. polymorpha* is the most thermo-tolerant representative of this group, it might also be better suited as source and for the production of proteins considered for crystallographic studies. The first methylotrophic yeast described was *Kloeckera* sp. No 2201, later re-identified as *Candida boidinii* (Ogata et al., 1969). Subsequently the other species, including *H. polymorpha*, have been identified as methanol-assimilating yeasts (Hazeu et al., 1972).

Three basic *H. polymorpha* strains with unclear relationships and of independent origins have been used in basic research and in the production of recombinant proteins, which exhibit different features. The strain CBS4732 (CCY38-22-2; ATCC34438, NRRL-Y-5445) was initially isolated from irrigated soil near a distillery in Pernambuco, Brazil (Morais and Maia, 1959); DL-1 (NRRL-Y-7560; ATCC26012) was also isolated from soil by Levine and Cooney (1973); and NCYC495 (CBS1976; ATAA14754, NRLL-Y-1798) was obtained from spoiled concentrated orange juice in Florida, which was initially designated as *Hansenula angusta* (Wickerham, 1951). The strains CBS4732 and NCYY495 can be mated, while the strain DL-1 cannot be mated with the other two (K. Lahtchev, personal communication).

The genus *Hansenula* H. et P. Sydow includes ascosporogenic yeast species exhibiting spherical, spheroidal, ellipsoidal, oblong, cylindrical, or elongated cells. One to four ascospores are formed. Ascigenic cells are diploid arising from conjugation of haploid cells. The genus is predominantly heterothallic. *H. polymorpha* is probably homothallic exhibiting an easy inter-conversion between the haploid and diploid state (Teunisson et al., 1960; Middelhoven, 2002). After performing DNA/DNA reassociation studies, it was proposed to merge both genera and transfer *Hansenula* species with hat-shaped ascospores to *Pichia* Hansen emend Kurtzman (Kurtzman, 1984), although *Hansenula* spp. can grow on nitrate and *Pichia* spp. cannot. Kurtzman and Robnett (1998) provided a phylogenetic tree in which nitrate-positive and nitrate-negative *Pichia* are clustered, demonstrating the unreliability of nitrate assimilation for prediction of kinship. The leading taxonomy monographs follow this proposal, re-naming *H. polymorpha* as *Pichia angusta* (Kurtzman and Fell, 1998; Barnett et al. 2000). However, the merging of the genera

is still criticized by some taxonomists, and there are arguments for maintaining the established and popular name *Hansenula polymorpha* (Middelhoven, 2002; Sudbery, 2003) (Fig. 3.1).

Some strains of *H. polymorpha* can tolerate temperatures of 49°C and higher (Teunisson et al., 1960; Reinders et al., 1999). Cells grown under conditions of elevated temperature accumulate trehalose as thermo-protective compound. It was shown that trehalose synthesis is not required for growth under these conditions, but for acquisition of thermo-tolerance (Reinders et al., 1999). The synthetic steps for trehalose synthesis have been detailed for *H. polymorpha* and *TPS1,* the key enzyme gene of this pathway, has been isolated and characterized (Romano, 1998; Reinders et al., 1999). Transcripts of this gene encoding trehalose-6-phosphate synthase were found to be very abundant in cells grown at elevated temperature, but to be present in high quantities when grown even at normal temperature (Reinders et al., 1999). The *TPS1*-derived promoter provides an attractive element to drive constitutive heterologous gene expression which can be further boosted at temperatures above 42°C (Amuel et al., 2000; Suckow and Gellissen, 2002).

All methylotrophic yeasts share an identical methanol utilization pathway (Tani, 1984; Yurimoto et al., 2002; Kang and Gellissen, 2005) (Fig. 3.2).

Growth on methanol is accompanied by a massive proliferation of peroxisomes in which the initial enzymatic steps of this pathway take place (Gellissen and Veenhuis, 2001; van der Klei and Veenhuis, 2002; Yurimoto et al., 2002). During growth on methanol key enzymes of the methanol metabolism are present in high amounts. An especially high abundance can be observed for MOX, FMD, and DHAS (Gellissen et al., 1992a). Their presence is regulated at the transcriptional level of the respective genes. In the related species, *C. boidinii*, *P. methanolica*, and *P. pastoris*, this gene expression strictly depends on the presence of methanol or methanol derivatives (Gellissen, 2000), whereas in *H. polymorpha* strong expression is elicited by appropriate levels of glycerol or under conditions of glucose starvation (Kang and Gellissen, 2005). This expression profile has been confirmed by micro-array analysis of transcripts isolated from cells grown under the respective

Eukaryota **(Superkingdom)**
Fungi **(Kingdom)**
Ascomycota **(Phylum)**
Saccharomycotina **(Subphylum)**
Saccharomycetes **(Class)**
Saccharomycetales **(Order)**
Saccharomycetaceae **(Family)**
Pichia **(Genus)**
Pichia angusta **(Species)**

Fig. 3.1 Taxonomy of *P. angusta* (synonym: *H. polymorpha*) (Kurtzman and Fell, 1998; Barnett et al. 2000)

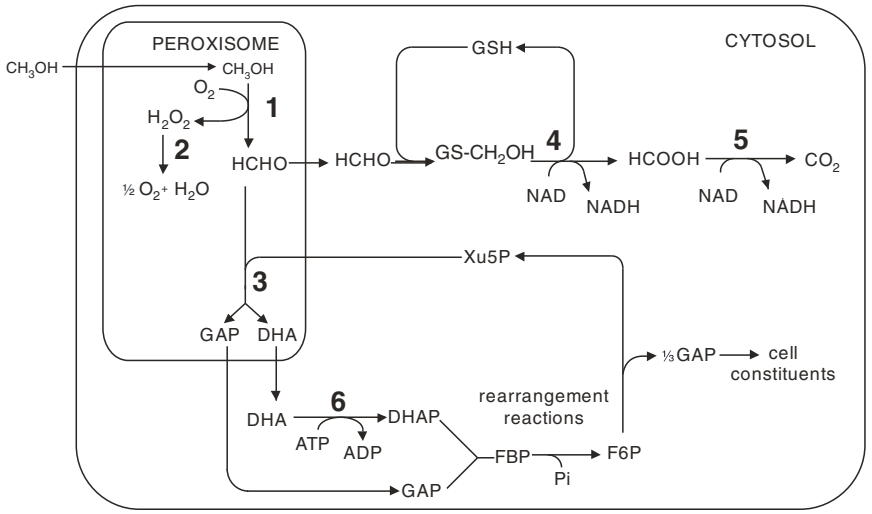

Fig. 3.2 The methanol utilization pathway and its compartmentalization in methylotrophic yeasts (modified after Kang and Gellissen 2005). (1) Methanol is oxidized by alcohol oxidase to generate formaldehyde and hydrogen peroxide. (2) The toxic hydrogen peroxide is decomposed by catalase to water and oxygen. (3,4) Within a dissimilatory pathway the formaldehyde is oxidized by two subsequent dehydrogenase reactions to carbon dioxide, catalyzed by a formaldehyde dehydrogenase (FLD) and a formate dehydrogenase (FMD or FDH). (5) For assimilation the formaldehyde reacts with xylulose-5-phosphate (Xu_5P) by the action of dihydroxyacetone synthase (DHAS) to generate the C_3 compounds glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone (DHA). (6) DHA is phosphorylated by dihydroxyacetone kinase (DHAK) to dihydroxyacetone phosphate (DHAP). (7) GAP and DHAP yield in an aldolase reaction fructose 1,6-biphosphate (FBP). (8) In further steps of the pentose phosphate cycle fructose-5-phosphate and xylulose-5-phosphate are finally generated

conditions (H.A. Kang, personal communication). Expression of methanol utilization pathway genes is thus subject to de-repression mechanism in absence of glucose in *H. polymorpha*, but to a methanol-dependent induction mechanism in all other methylotrophs conferred by inherent properties of the respective promoters. Again these promoter elements constitute strong components for the control of heterologous gene expression that can be regulated by carbon source addition. In *H. polymorpha* the elements derived from the *MOX* and the *FMD* genes are particularly attractive in providing the unique possibility of eliciting high promoter activity without methanol addition to a culture medium.

Identified and characterized genes of the *H. polymorpha* methanol utilization pathway are boxed and are shown in the pathway position of the encoded enzymes. The genes are *MOX* (Ledeboer et al. 1985), *DAS* (Janowicz et al., 1985), *CAT* (Didion and Roggenkamp 1992), *DAK* (Tikhomirova et al., 1988), *FLD1* (Baerends et al., 2002), and *FMD* (Hollenberg and Janowicz, 1988).

3.2 Strains

From the three *H. polymorpha* parental strains, NCYC495, CBS4732, and DL-1 (see previous section), some 50 other strains have been derived. DL-1 strains are not employed in classical genetic analyses. The inability of the DL-1 strain to copulate makes this strain inconvenient for classical genetic manipulation exploiting meiotic segregation. However, the relatively high frequency of homologous recombination in the DL-1 strain enables application of several molecular genetic techniques developed in *S. cerevisiae* to the DL-1 strain. The DL-1 strain has certain advantages in that it has a higher growth rate and adapts more quickly to culture media than the other parental strains; additionally DL-1 strains have a higher frequency of homologous recombination than other strains (Kang et al., 2002; Lahtchev, 2002). Several host strains suitable for heterologous protein expression, including auxotrophic mutants, protease-deficient strains, and *mox*-negative strains, have been constructed in the DL-1 strain mostly using gene disruption techniques. A pop-out cassette using *HpURA3* as a selection marker has been constructed to recover the auxotrophic marker for the subsequent gene disruption or for subsequent transformation with expression vectors (Kang et al., 2002). A similar range of auxotrophic mutants are derived from the CBS4732 background, mostly using chemical mutagenesis methods and mating techniques (Lahtchev, 2002).

Most classical genetic techniques have been performed using NCYC495, which shows mating and sporulation (Lahtchev, 2002). Unlike the other two parental strains, NCYC495 does not grow well on methanol-containing media and therefore does not have the strong methanol pathway-derived promoters available to the other strains for gene expression. Instead, NCYC495 has other interesting applications, including its employment for the study of nitrate assimilation mentioned before (Siverio, 2002). Cells from CBS4732 grow well on methanol and show strong mating and sporulation (Lahtchev, 2002). Both CBS4732 and DL-1 and their derivatives are employed in the production of recombinant products (Kang et al., 2001a; Gellissen, 2000, 2002; Müller et al., 2002; Park et al., 2004, see also the forthcoming section of this chapter). In contrast to DL-1 strains, some sub-strains of CBS4732

Table 3.1 Comparison of selected gene sequences from *H. polymorpha* strains CBS4732 and DL-1

Gene name	Amino acid identity (%)	Nucleic acid identity (%)	Accession no.*	Reference
CST13	96.7%	95.8%	AF454544	Kim et al., 2002
CPY	98.0%	95.9%	U67174	KRIBB
GSH2	96.0%	94.5%	AF435121	KRIBB
MNN9	96.3%	95.5%	AF264786	Kim et al., 2001
PMI40	97.9%	94.9%	AF454544	Kim et al., 2002
PMR1	98.5%	95.2%	U92083	Kang et al., 1998a, b
YPT1	99.5%	97.2%	AF454544	Kim et al., 2002
Average	97.6%	96.6%		

*The sequences of genes isolated from the DL-1 strain were obtained from GenBank and compared with those from the CBS4732 strain (Ramezani-Rad et al., 2003).

are not easily applied to recombinatory methods, perhaps due to their high mitotic stability (Suckow and Gellissen, 2002). For a selection of *H. polymorpha* strains and for protocols specific to parental strains, see Degelmann et al. (2002) (Table 3.1).

3.3 *N*-Linked Glycosylation in *H. polymorpha*

Initial processing of *N*-linked glycans on glycoproteins occurs in the endoplasmic reticulum (ER) of eukaryotes and results in the core oligosaccharide, $Man_8GlcNAc_2$. Further maturation of oligosaccharides in the Golgi apparatus is quite variable among yeast species (Gemmill and Trimble, 1999). Yeasts elongate the core oligosaccharide mostly by addition of mannose, leading to the formation of core-sized structures ($Man <_{15} GlcNAc_2$) as well as hypermannose structures ($Man_{50-200}GlcNAc_2$) with extended poly-α-1,6 outer mannose chains, which are decorated with various carbohydrate side chains in a species-specific manner. In *S. cerevisiae*, the linear backbone of the outer chain is often composed of 50 or more mannoses, highly branched by addition of α-1,2-linked mannoses and terminally capped with α-1,3-linked mannoses, generating heavily hypermannosylated glycoproteins. α-1,3-linked terminal mannose are considered to cause allergenic reactions. The outer chain also contains several mannosylphosphate residues, providing the oligosaccharide with a negative net charge (Jigami and Odani, 1999; Kim et al., 2004). Compared to *S. cerevisiae*, the mannose outer chains of *N*-linked oligosaccharides generally appear to be much shorter in *H. polymorpha* (Kang et al., 1998b), although extensive hyperglycosylation has also been reported in a few cases of recombinant glycoproteins produced in this yeast (Müller et al., 1998). A recent study on the structure of the oligosaccharides derived from the recombinant *Aspergillus niger* glucose oxidase (GOD) and the cell wall mannoproteins derived from *H. polymorpha* has revealed that most oligosaccharide species attached to the recombinant GOD have core-sized structures ($Man_{8-12}GlcNAc_2$) without terminal α-1,3-linked mannose residues (Kim et al., 2004). Therefore, the outer chain processing in the *N*-linked glycosylation pathway in *H. polymorpha* appears to be similar to that in *P. pastoris*, with the addition of shorter outer chains to the core and no terminal α-1,3-linked mannose addition (Montesino et al., 1998; Bretthauer and Castellano, 1999).

Differences between yeast and mammalian N-glycosylation are a major limitation for yeasts to be used in the production of recombinant glycoproteins for therapeutic use. Glycoproteins derived from yeast expression systems contain *N*-glycans of the high mannose type, in humans both *N*-glycans of a high mannose type and of a terminally sialylated complex type exist. Attempts have been made to genetically modify glycosylation processes in *S. cerevisiae* (Chiba et al., 1998) and *P. pastoris* (Callewaert et al., 2001) to trim the yeast *N*-glycans of the high mannose type to the human glycans of the $(Man)_5$-$(GlcNAc_2)$ intermediate type. A more advanced achievement has been made to genetically re-engineer the glycosylation pathway of *P. pastoris* to produce the complex human *N*-glycan *N*-acetylglucosamine$_2$-mannose$_3$-*N*-acetylglucosamine ($GlcNAc_2Man_3GlcNAc_2$) (Hamilton

et al., 2003). Recently, EPO could be produced in an authentic form with the addition of sialylated complex *N*-glycans (Hamilton et al., 2006). In *H. polymorpha* genes of N-glycosylation have been characterized and successful approaches have been taken to replace such genes by those from other fungal organisms to re-engineer strains adding humanized N-glycans of the intermediate mannose type (Kim et al., 2006). Co-expression of secretory pathway genes has resulted in a reduced extent of over-glycosylation (co-expression of a *S. cerevisiae*-derived *CMK2* gene) in case of a secreted recombinant phytase or in the presence of N-glycan structures corresponding in size to core-glycosylation (co-expression of a *H. polymorpha* –derived *CNE1* gene) in case of secreted IFNγ (Steinborn et al., 2006).

3.4 Characteristics of the *H. polymorpha* Genome

The entire genome of strain CBS4732 (CCY38-22-2; ATCC34438; NRRL-Y-5445) has been characterized (Ramezani-Rad et al., 2003), for the other biotechnologically applied strain DL-1 (NRRL-Y-7560; ATCC26012) data on karyotyping are available (Kang and Gellissen, 2005). Pulsed-field gel electrophoresis of *H. polymorpha* chromosomes revealed that both strains have 6 chromosomes, ranging from 0.9 to 1.9 Mbp, but the electrophoretic patterns of their chromosomes are quite different. The sequence identity of the open reading frame for the selected genes ranges between 94.5–97.2%, with an average value of 96.6%. The sequence differences are observed to be much more magnificent at the 5′- and 3′-untranslated regions, which might be involved in controlling gene expression. This implies that the two strains are closely related but have distinct genetic and physiological characteristics (Ramezani-Rad et al., 2003).

For sequencing of *H. polymorpha* of strain CBS4732, a BAC library with approximately 17x coverage was constructed in a pBACe3.6 vector (Osoegawa et al., 1998, 1999). Sequencing resulted in the characterization of 8.733 million base pairs assembled into 48 contigs. The derived sequence covers over 90% of the estimated total genome content of 9.5 Mbp located on 6 chromosomes which range in size between 0.9 and 2.2 Mbp. Out of the sequenced 8.73 Mb 5848 ORFs have been extracted for proteins longer than 80 amino acids. 389 ORFs smaller than 100 amino acids were identified. 4771 ORFs have homologues to known proteins (81.6%). Calculation of the gene density and protein length, taking into account the gene numbers, showed an average gene density of 1 gene/1.5 kb and an average protein length of 440 amino acids. 91 introns have been identified by homology to known proteins and confirmed by using GeneWise (Birney and Durbin, 2000). 80 tRNAs were identified, corresponding to all 20 amino acids. From approximately 50 rRNA clusters 7 clusters have been fully sequenced. All clusters are completely identical and have a precise length of 5033 bp (Table 3.2).

The main functional categories and their distribution in the gene set are manually predicted for energy, 4%; cellular communication, signal transduction mechanism, 3%; protein synthesis, 6%; cell rescue, defense and virulence, 4%; cellular

Table 3.2 *H. polymorpha* CBS4732 genome statistics

Contigs: 48	48
Total length of contigs:	8,733,442 bp
Average contig length:	182 kb
No. of extracted ORFs:	5,848
No. of ORFs < 100 aa:	389
Average gene density:	1 gene/1.5 kb
Average gene size (start-stop):	1.3 kb (1,320 nt)
Average protein length:	440 aa

Table 3.3 Functional categorization of genes

Functional category	No. ORFs	%
Metabolism	1114	19
Energy	231	4
Cell growth, Cell division and DNA synthesis	518	9
Transcription	767	13
Protein synthesis	323	6
Protein destination	1014	17
Transport facilitation	423	7
Cellular transport and transport mechanisms	518	9
Control of cellular organization	417	7
Cellular communication/signal transduction	170	3
Cell rescue, defense, and virulence	260	4
Cell fate	282	5
Regulation of/interaction with cellular environment	184	3

transport and transport mechanisms, 9%; cell cycle and DNA processing, 9%; protein fate (folding, modification, destination) 17%; transcription, 13% and metabolism, 19% (Ramezani-Rad et al., 2003) (Table 3.3).

Based on the sequence data a genome micro-array has been established (Oh et al., 2004; Park et al., 2007) that is currently applied to transcript profiling of *H. polymorpha*, among others to carbon source-dependent gene expression as pointed out before.

3.5 The *H. polymorpha*-Based Expression Platform

Generation of recombinant *H. polymorpha* strains of a CBS4732 background typically employs vectors that are mitotically stable integrated into the genome of the host (Gellissen and Hollenberg, 1997). The traditional vectors applied to transformation are provided as circular plasmids. Despite the presence of HARS or S. *cerevisiae*–derived ARS sequences they are integrated and not of an episomal fate. These plasmids may integrate into the host DNA over a number of generations, resulting in strains with as many as 100 integrated plasmids present in tandem repeats (Gellissen, 2000; Kang et al., 2002). Obviously the circular plasmids are not randomly integrated

as initially postulated but recombine with genomic sequences represented on the vector. This was shown with a particular vector harboring a *FMD* promoter/*HBsAg* fusion where recombination within the *FMD* gene was observed (Dahlems U, personal communication). It seems most likely that homologous recombination also takes place with vectors equipped with *MOX*, *TPS1* and other promoter elements. A high number of integrated copies are not always a prerequisite for high-level expression, especially in the case of secretory protein production. In a particular case, four copies of a HARS vector were sufficient to obtain efficient production of a *Schwanniomyces occidentalis*-derived glucoamylase in *H. polymorpha* CBS (Gellissen et al., 1992b). Other examples are *H. polymorpha* DL-1-based production strains for human urinary-type plasminogen activator (u-PA) and human serum albumin (HSA). In these cases, a single- or two-copy integration of the expression vector resulted in the maximal levels of recombinant u-PA or HSA secreted into culture supernatants (Kang et al., 2002). Targeted integration into sites others than the highly expressed methanol pathway genes require much longer target gene overlaps than those used in *S. cerevisiae* (Gonzalez et al., 1999; van Dijk et al., 2001). Plasmids harboring one of a set of several cloned sub-telomeric ARS sequences derived from the DL-1 strain have been described. These vectors homologously integrate into a genomic counterpart and result in recombinant strains harbouring single or multiple tandemly repeated copies at the respective sub-telomeric genomic locus (Sohn et al., 1999; Kim et al., 2003). A set of vectors has been constructed to target the heterologous DNA to the rDNA locus of *H. polymorpha*. *Arxula adeninivorans* and *H. polymorpha*-derived rDNA targeting elements have been defined for optimal integration and expression characteristics when present on wide-range expression/integration vectors. They are included in the wide-range yeast (CoMed™) system described elsewhere in the book (Steinborn et al., 2005, 2006).

After transformation, cells are plated on selective media according to the requirements of the selection marker gene contained on the plasmid. Macroscopic colonies typically appear after 4–5 days of incubation at 37°C. Colonies are then transferred within a ‘passaging step’ to liquid selective medium and incubated under vigorous shaking for 24 h to 48 h at 37°C. An aliquot of the dense culture is then used to inoculate fresh selective medium, and the incubation is repeated. After 3–8 subsequently applied passaging steps, the initially episomal plasmid is integrated into the genome.

For heterologous gene expression in *H. polymorpha* a range of homologous and heterologous promoters of extraordinary strength is available. *MOX* and *FMD* promoters are derived from genes of the methanol degradation pathway that can be carbon source-regulated as described before. The *TPS1* promoter, derived from the trehalose 6-phosphate synthase gene of *H. polymorpha*, is constitutive with regard to different carbon sources and can be influenced by different temperatures (Amuel et al., 2000). In combination with a high copy numbers of the integrated plasmid these strong promoters can provide very high expression rates of the heterologous gene in selected strains. For secreted phytase, product levels of up to 13.5 g l^{-1} have been obtained. This extremely high productivity was elicited by the *FMD* promoter applying conditions of glucose starvation to culturing (Mayer et al., 1999). In addition

to homologous promoters elements derived from alternative yeasts can be employed. A particularly attractive component is the strong constitutive *A. adeninivorans*-derived *TEF1* promoter (Steinborn et al., 2006). Other available but less frequently applied regulative promoters are derived from inducible genes encoding enzymes involved in nitrate assimilation (e.g. *YNT1*, *YNI1*, *YNR1*, which can be induced by nitrate and repressed by ammonium - Avila et al., 1998), or the enzyme acid phosphatase (the *PHO1* promoter - Phongdara et al., 1998), and the *FLD1* promoter, yet another element derived from a methanol utilization pathway gene (Baerends et al., 2002). Examples of constitutive promoters are *ACT* (Kang et al., 2001b), *GAP* (Heo et al., 2003), and *PMA1* (Cox et al., 2000). The *PMA1* promoter even competes with the outstanding *MOX* promoter in terms of high expression levels; *PMA1* provides an attractive element for the co-expression of genes on industrial scale (Cox et al., 2000).

Signal sequences may be fused to the target open reading frame (ORF) for direct release of synthesized proteins directly into the media, or into a pre-selected cell compartment, such as the peroxisome, the vacuole, or for targeting to the cell surface. For ER-mediated secretion a range of pre-leader sequences such as that derived from the *PHO1* gene encoding a repressible acid phosphatase (Phongdara et al., 1998) and a *S. occidentalis*-derived *GAM1* sequence (van Djik et al., 2000; Weydemann et al., 1995) are available. Most commonly, the *S. cerevisiae*-derived the MFα1 pre-pro- leader is employed as the preferred component (Brake et al., 1984; Gellissen, 2000) Available targeting signals include PTS1 and PTS2 for addressing the peroxisome (van Djik et al., 2000); glycosylphosphatidylinositol (GPI)-anchoring motifs derived from the GPI-anchored cell surface proteins, such as *HpSED1*, *HpGAS1*, *HpTIP1*, and *HpCWP1*, have been exploited to develop a cell surface display system in *H. polymorpha*. When the recombinant glucose oxidase (GOD) was produced as a fusion protein to these anchoring motifs, most enzyme activity was detected on the cell surface (Kim et al., 2002).

Expression and integration vectors in *H. polymorpha* are composed of prokaryotic and yeast DNA incorporating elements described before or combinations thereof (Gellissen and Hollenberg, 1997). Vectors are either supplied as circular plasmid or linearized and targeted to a specific genomic locus. Possible targets for homologous integration include the *MOX*/*TRP* locus (Agaphonov et al., 1995), an ARS sequence (Agaphonov et al., 1999; Sohn et al., 1996), the *URA3* gene (Brito et al., 1999), the *LEU2* gene (Agaphonov et al., 1999), the *GAP* promoter region (Heo et al., 2003), or the rDNA cluster (Klabunde et al., 2002, 2003). In the novel wide-range yeast vector (CoMed™) vector system individual modules consisting of expression cassettes equipped with attractive promoters of choice, selection markers, rDNA targeting sequences or ARS sequences can be combined (Steinborn et al., 2006) (Fig. 3.3).

For detailed information of various traditional vectors the reader is referred to previous publications (Suckow and Gellissen, 2002; Kang et al., 2002; Guengerich et al., 2004). Plasmids that have been successfully developed for industrial use of CBS4732-based strains include pFPMT121 for *FMD* promoter-controlled production of phytase (Mayer et al., 1999; Suckow and Gellissen, 2002) and a derivative of pMPT121 for *MOX* promoter-controlled production of the anti-coagulant hirudin (Avgerinos et al., 2001; Suckow and Gellissen, 2002).

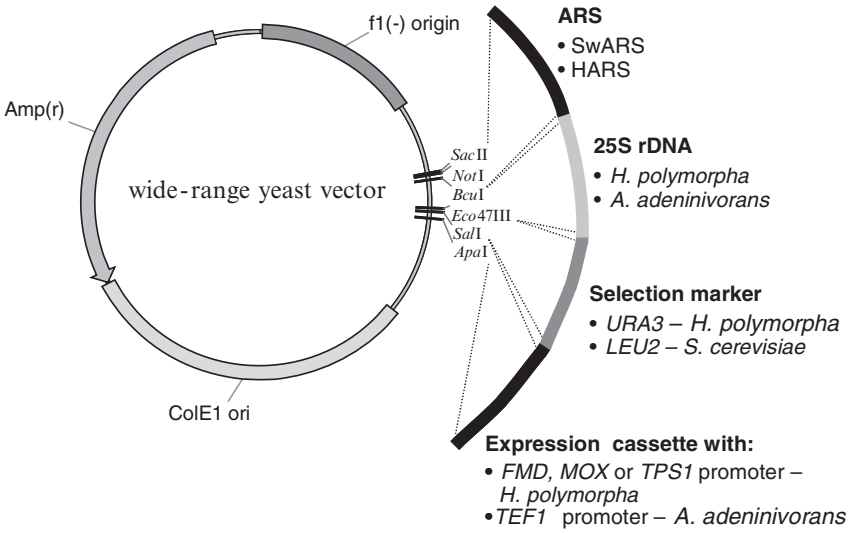

Fig. 3.3 General design of the wide-range yeast vector (CoMed™) with elements to address *H. polymorpha.* The vector contains all *E. coli* elements for propagation in the *E. coli* system and a MCS for integration of ARS, rDNA, selection marker and expression cassette modules. For this purpose, ARS fragments are flanked by *Sac*II and *Bcu*I restriction sites, rDNA regions by *Bcu*I and *Eco*47III restriction sites, selection markers by *Eco*47III and *Sal*I restriction sites and promoter elements by *Sal*I and *Apa*I restriction sites

3.6 Product and Process Examples

We now briefly summarize a few industrially relevant examples of *H. polymorpha*-based processes, all of them leaning on CBS4732-derived strains employing *MOX* or *FMD* promoters for expression control of the foreign gene. More detailed description of fermentation and purification procedures can be found in recent articles (Jenzelewski, 2002; Hellwig et al., 2005). The design of a fermentation procedure greatly depends on characteristics of the host cell, the intended routing of the recombinant gene product, and most importantly on the promoter elements used. The commonly used culture media are based on simple synthetic components. They contain trace metal ions and adequate nitrogen sources, which are required for efficient gene expression and cell yield, but no proteins. The total fermentation time varies between 60 and 150 hours. Due to the inherent versatile characteristics of the two methanol pathway-derived promoters fermentation modes vary, for the most part in the supplemented carbon source: glycerol, methanol, glucose, and combinations thereof may be selected.

In processes for secretory heterologous proteins usually a ‘one-carbon source’ mode is employed supplementing the culture medium with glycerol only. A hirudin production process may serve as an example for this fermentation mode. In this process a strain was employed that harbours 40 copies of an expression cassette for an *MFα1* prepro-sequence/ hirudin fusion gene under control of the *MOX* promoter (Weydemann et al., 1995; Avgerinos et al., 2001;

Bartelsen et al., 2002). Hirudin production was promoted by reducing initial glycerol concentration and maintaining it on a suitable level by a pO_2-controlled addition of the carbon source. The fermentation is started with 3% (w/v) glycerol at the beginning of fermentation. After consumption of the carbon source after 25 hours the pO_2-controlled feeding mode is initiated resulting in a glycerol concentration between 0.05 and 0.3% (w/v) (derepression of the *MOX* promoter). The fermentation run is terminated after 36 hours of derepression (total fermentation time of 72 hours). Then the broth is harvested and the secreted product is purified from the supernatant by a sequence of ultrafiltration, ion exchange, and gel filtration steps.

In case of HBsAg production, a 'two carbon source' fermentation mode was employed (Brocke et al., 2005). The producer strain harbours high copy numbers of an expression cassette with the coding sequence for the small surface antigen (S-antigen) under control of methanol pathway promoters. The selected strain is fermented on a 50 l scale. The product-containing cells are generated via a two fermenter cascade, consisting of a 5 l seed inoculating the 50 l main fermenter. The initial steps of fermentation closely follow those described for the production of hirudin. Cultivation is started with a glycerol feed in a fed-batch mode, to be followed by subsequent semi-continuous glycerol feeding controlled by the dissolved oxygen level in the culture broth. This de-repression phase is then followed by a batchwise feeding with methanol in the final fermentation mode. The product concentration increases to amounts in the multigram range. It consists of a lipoprotein particle in which the recombinant HBsAg is inserted into host-derived membranes. Addition of methanol also serves for the proliferation of organelles and consequently for the synthesis and proliferation of membranes. Methanol is thus needed in this case to provide a high-yield and balanced co-production of both components of the particle. For downstream processing the harvested cells are disrupted and the particles are purified in a multi-step procedure that includes adsorption of a debris-free extract to a matrix and the subsequent application of a sequence of ion exchange, ultra-filtration, gel filtration, and ultra-centrifugation steps (Schaefer et al., 2001, 2002; Brocke et al., 2005).

For the production of phytase *H. polymorpha* has been used in a particularly efficient process (Mayer et al., 1999; Papendieck et al., 2002), a prerequisite for an economically competitive production of a technical enzyme. A strain was generated in which the phytase sequence is under control of the *FMD* promoter. A fermentation procedure was developed to achieve high levels of enzyme production. The active status of the *FMD*-promoter was maintained by glucose starvation (fermentation with minimal levels of continuously fed glucose). Strains were found to produce the recombinant phytase at levels ranging up to 13.5 g l^{-1} (Mayer et al., 1999). The secreted product is purified through a series of steps, including flocculation centrifugation, dead-end filtration, and a final ultra-filtration yielding a high-quality, highly concentrated product at a recovery rate up to 92% (Tables 3.4 and 3.5).

Table 3.4 *H. polymorpha*-based products (selection)

	Product	Status	Brand name	Reference
Pharmaceutical	HBsAg (*adr*)	Launched	HepaVax Gene	Schaefer et al., 2002
	HbsAg (*adw*)	Launched		Schaefer et al., 2002
	insulin	Launched	AgB	
	IFNα -2a	Process transfer	Wosulin	Müller et al., 2001
	HSA	Pilot scale completed		Heo et al., 2003
	EGF	Lab scale completed		Heo et al., 2002
Food additive	Hexose oxidase	Launched	Grindamyl-Surebake	Cook and Thygesen, 2003
Feed additive	Phytase	Registration		Mayer et al., 1999
Enzymes	Levansucrase	Lab scale completed		Park et al., 2004

Table 3.5 Selection of *H. polymorpha* host strains

Strain	Genotype	Phenotype	Source
Parental strain			
DL-1	wild-type (NRRL-Y-7560,ATCC26012)		Levine and Cooney, 1973
DL-1-L	*leu2*	Leu^-	Sohn et al., 1996
uDL10	*leu2 ura3*	Leu^-Ura^-	KRIBB
DL1Δ-A	*leu2 Δade2*	Leu^-Ade^-	CRC
uDLB11	*leu2 ura3 Δpep4::lacZ*	$Leu^-Ura^-Pep4^-$	KRIBB
uDLB12	*leu2 ura3 Δprc1::lacZ*	$Leu^-Ura^-Prc1^-$	KRIBB
uDLB13	*leu2 ura3 Δkex1::lacZ*	$Leu^-Ura^-Kex1^-$	KRIBB
DLT2	*leu2 Δmox-trp3::ScLEU2*	Mox^-Trp^-	CRC
DL1-LΔM	*leu2 Δmox*	Leu^-Mox^-	CRC)
Parental strain			
NCYC495	wild-type (CBS1976, ATAA14754, NRRL-Y-1798, VKM-Y-1397)		Wickerham, 1951
L1	*leu1-1**	Leu^-	Gleeson et al., 1986
A11	*ade11-1*	Ade^-	Parpinello et al., 1998
M6	*met6-1*	Met^-	Parpinello et al., 1998
Nitrate assimilation-related strains			
NAG1995	Δ*ynr1::URA3*, *leu1-1**	Ynr1 Leu^-	Avila et al., 1995
NAG1996	Δ*yni1::URA3*, *leu1-1**	Yni1 Leu^-	Brito et al., 1996
NAG997	Δ*ynt1::URA3*, *leu1-1**	Ynt1 Leu^-	Pérez et al., 1997
NAG998	Δ*yna1::URA3*, *leu1-1**	Yna1 Leu^-	Avila et al., 1998

(continued)

Table 3.5 (continued)

Strain	Genotype	Phenotype	source
Parental strain			
CBS4732	wild-type (CCY38-22-2, ATCC34438, NRRL-Y-5445)		Morais and Maia, 1959
LR9/RB11/ MedHp1	*ura3-1*		Roggenkamp et al., 1986; Weydemann et al., 1995
RB11/MedHp2	*ura3 leu1-1*		Kang and Gellissen, 2005
RB14/MedHp3	*ura3 leu1-1, ade*		
A16	*leu2* *trp3 mox*	Leu$^-$ Trp$^-$Mox$^-$	Veale et al., 1992
1B	*ade2-88 leu2-2*	Ade$^-$Leu$^-$	Bogdanova et al., 1998
1-HP065	*ade2-88 ura2-1 met 4-220*	Ade$^-$Leu$^-$Met$^-$	Mannazzu et al., 1997
14C	*leu2-2 cat1-14*	Leu$^-$Cat$^-$	Lahtchev, 2002
5C-HP156	*ade2-88*	Ade$^-$	Lahtchev, 2002
8V	*leu2*	Leu$^-$	Agaphonov et al., 1995

* *leu1-1* and *leu2* correspond to the same gene.

3.7 Future Directions and Conclusions

Despite the most favorable characteristics of the *H. polymorpha*-based platform for application in heterologous gene expression, problems and limitations can be encountered in particular strain and product developments as is similarly and more frequently observed in other yeast systems. These limitations include overglycosylation (Agaphonov et al., 2001), retention within the ER (Agaphonov et al., 2002), poor secretion, impaired processing (Müller et al., 2002; Gellissen et al., 2002) and proteolytic degradation (Suckow and Gellissen, 2002). A possible strategy to overcome these limitations is to identify genes and gene products that may upon disruption or co-expression positively influence the performance of respective strains. As such, the *S. cerevisiae*-derived *KEX2* gene provided a greatly improved processing of a IFN-2a pre-pro-sequence in *H. polymorpha* in which production of predominantly N-terminally extended molecules had been observed before (Müller et al., 2001; Gellissen et al., 2002). In other examples co-expression of a *S. cerevisiae*-derived *CMK2* or the *H. polymorpha CNE1* (calnexin) gene has led to an improved secretion and a reduction in overglycosylation of a secreted enzyme and a cytokine as described before.

The tools of functional genomics established in the recent past will further the identification of genes hat can potentially ease limitations and drawback of

heterologous protein production in a given case. However, there is obviously no single yeast platform that is optimal for all proteins. It is, therefore, advisable to assess several yeast platforms in parallel for their capability to produce a particular protein in desired amounts and quality. This approach is now greatly facilitated by the newly developed wide-range yeast vector system (CoMed™) (Steinborn et al., 2006). The range of established yeast expression platforms are transformed simultaneously by an individual single vector, thereby enabling an assessment in parallel of such strains for criteria like efficient secretion or authentic processing and modification.

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Chapter 4
Debaryomyces hansenii: An Osmotolerant and Halotolerant Yeast

Monika Aggarwal and Alok K. Mondal

Contents

Abstract The yeast *Debaryomyces hansenii* which was isolated from saline environments such as sea water, concentrated brines, salty food, is one of the most halotolerant species. It can grow in media containing as high as 4 M NaCl, while the growth of *Saccharomyces cerevisiae* is limited in media with more than 1.7 M NaCl. This species is very important for food industry as it is used for surface ripening of cheese and meat products. In the recent past, there is growing interest in understanding the molecular mechanisms of high halotolerance exhibited by *D. hansenii*. Availability of genome sequence of *D. hansenii* has opened up new vistas in this direction.

Keywords *Debaryomyces hansenii*, halotolerance, saline environment, halophily, osmotolerance

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4.1 Introduction

Debaryomyces hansenii is an osmotolerant, halotolerant, xerotolerant and a cryotolerant marine yeast. It was originally isolated from hyper-saline environ ments such as seawater (Norkrans, 1966) and concentrated brines (Onishi, 1963). It is the yeast most frequently found in traditional cheese and sausages, with a recognized contribution to special flavors in these products (Seiler and Busse, 1990; Saldanha-da-Gama et al., 1997; Mortensen et al., 2005; Mounier et al., 2005). In this respect, *D. hansenii* is different from other yeast species, the prevalence of which is cheese dependent (Fleet, 1990). It is also common in dairies as it is able to grow in the presence of salt at low temperature and to metabolize lactic and citric acids. Besides this it can be isolated from other sources such as meat, wine, beer, sakemoto, rennet, tobacco, salmon, fruit and soil (Barnett et al., 2000; Davenport, 1980) as well as from high-sugar products (a_w as low as 0.62) (Tilbury, 1980; Butinar et al., 2005). In the salterns on the Atlantic coast in Namibia and in the Great Salt Lake brine *D. hansenii* has been isolated (Butinar et al., 2005). This shows that *D. hansenii* can be found in many habitats with low water activity. The presence of this species of yeast in such environments is due to its high osmotolerance. It can tolerate salinity levels up to 4 M NaCl, whereas *Saccharomyces cerevisiae* growth is inhibited when salinity reaches above 1.7 M NaCl. It is capable of accumulating sodium ion at high concentrations without being intoxicated, even in the presence of low concentrations of potassium (Prista et al., 1997). Moreover, in environments where the concentration of potassium is low, as is frequently found in nature, sodium significantly improves its growth and protects it from additional stress factors such as high temperature and extreme pH (Gonzalez-Hernandez et al., 2004; Prista et al., 2005). For this characteristic, *D. hansenii* is being designated as the halophilic yeast. In the recent past, *Debaryomyces hansenii* has become a model of osmotolerance of eukaryotic microorganisms. Its high cryo- and osmotolerance is highly advantageous from biotechnological point of view as it allows quasi-non-sterile production under high osmolarity conditions that should reduce production cost dramatically. This species of yeast has enormous biotechnological potential in agro-food sector. Although *D. hansenii* is considered as non-pathogenic, one case of bone infection (Wong et al., 1982) associated with this yeast was reported and several clinical samples have been identified as *D. hansenii*. Wagner et al (2005) have reported a case of invasive infection due to *D. hansenii* and *Scopulariopsis brevicaulis* in a stem cell transplant patient receiving liposomal Amphotericin B and Caspofungin for suspected Aspergillosis. Further, association of this species with psoriasis, patient with angina, infected nails, infected hands and wounds has been shown (van Uden and Fell, 1968). Mattsson et al. (1999) identified feral pigeons as carriers of *D. hansenii.*

The peculiar behavior of this yeast together with its ubiquity in salty environments highlights that *D. hansenii* is a genetically and biochemically interesting

yeast with considerable biotechnological promise. This chapter introduces the phylogeny, general physiology, metabolism, genome organization and molecular biology of *D. hansenii* before discussing the current biotechnological applications.

4.2 Phylogeny

Debaryomyces hansenii is an ascomycetous yeast. Sequence of the 18S ribosomal DNA unambiguously placed *D. hansenii* within the hemiascomycete subdivision (Wilmotte et al., 1993). The hierarchical details of this species of yeast include: Kingdom, Fungi; Phylum, Ascomycota; Class, Saccharomycetes; Order, Saccharomycetales; Family, Saccharomycetaceae and Genus, Debaryomyces. Cai et al. (1996) examined the complete 18S rRNA gene (18S rDNA) sequences of three *Debaryomyces* species (*D. hansenii*, *D. udenii* and *D. castellii*) and *Candida guilliermondii* (anamorph of *Pichia* (*Yamadazyma*) *guilliermondii*). They suggested that the *Debaryomyces* species and *C. guillermondii* were closely related. However, due to the lack of additional 18S rDNA sequence data from other *Debaryomyces* species, their phylogenetic relationships remained unclear. Based on partial sequence analysis of D1/D2 regions of 26S rRNA gene, Kurtzman and Robnett (1998) showed that *Debaryomyces* species is not monophyletic. They also suggested that *Debaryomyces* species separate into four clades that are represented by *D. hansenii*, *D. polymorphous*, *D. melissophilus* and *D. etchellsii*, and stated that basal branches of these four clades were weakly supported and that additional data were needed before generic boundaries could be confidently drawn. ITS sequence analysis by Martorell et al. (2005) also suggested that genus *Debaryomyces* is polyphyletic confirming the earlier observations.

The species *D. hansenii* comprises of two varieties, *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*, these two show 56% DNA re-association. Kurtzman and Robnett (1998) found one base substitution and one base deletion between two varieties of *D. hansenii* in the D1/d2 domain. These two varieties differ by one base substitution in ITS2 region, however no substitution was found in ITS1 sequence (Martorell et al., 2005). Some yeast strains that were earlier considered as *D. hansenii,* based on phenotypic characters, were shown to belong to a new species *Debaryomyces prosopidis* (Phaff et al., 1998).

The two varieties exhibit marked differences in the growth temperatures. The maximum growth temperature of *D. hansenii* var. *fabryi* is 36–39°C, whereas *D. hansenii* var. *hansenii* can grow in temperatures up to 35°C. However, the var. *fabryi* is not very often found and is poorly characterized. Optimum growth temperature of *D. hansenii* is between 20 and 25°C, and the growth, however, has been reported between 5°C and 10°C and even below 0°C (Davenport, 1980). At 10°C, this yeast is capable of growth at pH 4.0–6.0 in water activities (a_w) up to 0.99 (van den Tempel and Jacobsen, 2000).

The techniques that have been used to discriminate between the two varieties includes hybridization to species specific sequences, pulsed-field gel electrophoresis

(PFGE), restriction fragment length polymorphism (RFLP) and random amplification of polymorphic DNA (RAPD) and Fourier-Transform Infrared Micro-spectroscopy. Corredor et al. (2000) have shown that two RAPD products F01pro and M18pro are useful for the identification and typing of *D. hansenii* strains. Both the probes hybridized specifically to the *D. hansenii* var. *hansenii* strains only. Unique probes corresponding to actin *ACT1*, glycerol-3-phosphate dehydrogenase *GPD1* and beta-glucosidase *LAC4* encoding genes recognized chromosomal bands from var. *hansenii* specifically and not from var. *fabryi* thus, strongly suggesting that strains of this variety actually represent a different taxon, as suggested by a number of previous authors (Nakase and Suzuki, 1985; Prillinger et al., 1999). Further, by using the technique of PFGE they have shown that the two strains differ markedly at the level of genomic organization (Corredor et al., 2003). Similar discrimination between the two varieties of *D. hansenii*, by using a combination of mitochondrial (mt) DNA RFLP and RAPD analysis, by using appropriate primers have been shown by Romano et al. (1996). *Debaryomyces* species can be identified both quickly and correctly by direct sequence comparison of the ribosomal 5.8S-ITS region. The two varieties of *D. hansenii* can be distinguished by following the techniques of ITS-PCR and mt-DNA RFLP (Petersen et al., 2001). Both the methods have been shown to be useful for subspecies typing and investigation of the microbial succession between strains of *D. hansenii* during the ripening process of surface ripened cheeses. In contrast to Corredor et al. (2003), results from the studies of Petersen and Jespersen (2004) showed that two varieties of *D. hansenii* couldn't be divided into separate groups. Wenning et al. (2002) have shown Fourier-transform infrared (FT-IR) micro-spectroscopy as a useful method for the identification of *D. hansenii* strains. The mean strain identification rate was 91% for *D. hansenii* and 92% for *S. cerevisiae*. Strains of *D. hansenii* can also be distinguished from other yeast species by using a chromogenic substrate salmon-Gluc and X-Gal (de-Siloniz et al., 2000). The taxonomic classification of the two varieties of *D. hansenii* seems likely to change in the near future as further analytical methods are developed and applied.

4.3 Sexual Reproduction

D. hansenii is haploid yeast that reproduces vegetatively by multilateral budding. It is a homothallic yeast (Dujon et al., 2004; Fabre et al., 2004). Homothallism is defined as a state that is caused by the gene conversion between the *MAT* locus and two *MAT*-like loci during cellular division of haploid cells (Herskowitz et al., 1992; Haber, 1998). *D. hansenii* has a single *MAT*-like locus that seems to be a mosaic of *MAT***a** and *MAT*α genes, which is in accordance with homothallic life style of this species. This is in contrast to what exists in other species, where *MAT* loci have either 'a' or 'alpha' type information. Sequence analysis revealed that although *D. hansenii* possesses all maturation factors and receptors of both **a** and α pheromones, but no gene encoding **a** factor precursor have been found. Further, no gene encoding

HO endo-nuclease homologue has been found, thus, indicating the absence of mating-type switching. Establishment of mechanism responsible for the homothallism in *D. hansenii* should, thus, provide precious information. The species has an essentially haplo-diplontic life cycle (Fabre et al., 2004). However, according to Forrest et al. (1987) the species has a haplontic life cycle.

Sexual reproduction occurs very rarely. According to van der Walt et al. (1977) somatogamous autogamy is the main agency of diploidization, and that the species is largely inbreeding. However, sexual reproduction via heterogamous conjugation had also been suggested (Forrest et al., 1987). Conjugation leads to a short diplophase that is followed by meiosis and ascospore formation. Ascus formation involves fusion between a mother cell and a bud while still attached to each other, via short protuberances developed between the cross wall between them. Nuclear fusion takes place in the channel that connects the two cells. Meiosis occurs in the mother cell. Generally, only one lobe of the meiotic nucleus is surrounded by a prospore wall and it becomes the nucleus of a spore (Kreger van Rij and Veenhuis, 1975). The rest of the nucleus disappears. The spores are spheroidal with a warty wall. The wartiness is not always distinct under that light microspore. With the scanning electron microscope, warts appear as small blunt protuberances or as small ridges (Kurtzman et al., 1975). Usually one, seldom two spores are formed per ascus. A unique wale type of surface ornamentation of ascospores in the two strains of *D. hansenii* was observed by Banno and Mikata (1985). The spores germinate by swelling in the ascus and forming one or more buds. The view of sexual reproduction by heterogamous conjugation was further supported by Forrest et al. (1987). Isogamous conjugation also occurs (Nakase et al., 1998).

The dimorphism from yeast-to-mycelial forms has been reported in *D. hansenii* (Cruz et al., 2000). Hyphal growth is induced when the organism is cultured continuously in a xylose containing media made from hemi-cellulosic acid hydrolysates of barley bran. This dimorphism has been ascribed to be dependent on both the dilution rates of the culture medium as well as the dissolved O_2 concentration. Further, the yeast-to-mycelia transition is induced by adding selected amounts of acid-soluble lignin (BBH) in media with xylose or glucose.

4.4 General Physiology

D. hansenii is a highly halotolerant yeast. Unlike *S. cerevisiae*, which is a 'sodium excluder', *D. hansenii* is the 'sodium includer' yeast (Prista et al., 2005). It can grow in a media containing as high as 4 M NaCl, while the growth of *S. cerevisiae* is limited in a media containing 1.7 M NaCl. In this species of yeast, NaCl and KCl has similar effect thus, indicating that NaCl created only osmotic effect and specific toxicity of sodium is not involved as has been shown in *S. cerevisiae* (Prista et al., 1997). Further, growth was even stimulated by 0.5 M NaCl. However, LiCl has a specific inhibitory effect, although relatively weaker than in *S. cerevisiae*. It can grow in a media containing as high as 0.6 M LiCl (our unpublished observation).

In the absence of Na^+ and presence of K^+ ions at a low concentration (as low as 50 mM), growth has been observed for both the species. However, under these conditions, growth of *S. cerevisiae* was completely inhibited by 0.6 M NaCl (Camacho et al., 1981). On the other hand, growth of *D. hansenii* was stimulated by NaCl at concentrations up to 1 M NaCl. Moreover, under these conditions, the value for the specific growth rate and the final biomass was close to the values obtained in normal growth medium. This difference in tolerance to Na^+ ions, however, is not due to the difference in the efficiency of transporters involved in sodium efflux (Prista et al., 1997, 1998). These results highlights that in *D. hansenii* LiCl is far more toxic than NaCl and, hence, different resistance mechanisms are involved.

Presence of salt appeared to stimulate the growth of *D. hansenii* at higher temperature that normally did not support its growth. Moreover, the stress effect of extreme pH had been shown to be relieved by presence of 0.25 M NaCl (Almagro et al., 2000). These studies further highlight the salt loving or halophilic nature of this species of yeast. Since *D. hansenii* is a highly halo- and osmotolerant yeast, it is quite plausible to think that genetic material from this species may confer tolerance to osmotic effect and salt stress to heterologous host. *S. cerevisiae* harboring genetic material from *D. hansenii* and thus, conferring a different phenotype like growth at high salt concentration or under alkaline conditions has been demonstrated (Almagro et al., 2001; Prista et al., 2002).

According to Norkans (1968), *D. hansenii* is capable of both respiration and fermentation. However, Gancedo and Serrano (1989) described it as exclusively respiratory. Further studies carried out by Neves et al. (1997) supported the observation made by Norkans. Recently, it has been shown that this species is 'almost' Crabtree negative yeast but clearly Pasteur negative yeast, probably due to its low fermentative capacity which is mainly because of its lower phosphofructokinase activity (Sanchez et al., 2006). Crabtree effect relates to the decrease in the production of ethanol in the presence of oxygen and Pasteur effect relates to the decrease of glucose consumption in the presence of oxygen.

Cyanide-resistant respiration (CRR), a widespread metabolic pathway among yeasts, is very common in Crabtree-negative yeasts (incapable of aerobic fermentation) and in non-fermentative yeasts. It is conferred by a salicylhydroxamic acid (SHAM)-sensitive alternative oxidase that transfers electrons from ubiquinol to oxygen, bypassing the cytochrome chain. Although the involvement of this pathway in the fine adjustment of energy provision to the cell has been proposed, its physiological role remains obscure (Veiga et al., 2003a, 2003b, 2003c). Under aerobic conditions, a respiratory pathway alternative to the cytochrome chain (i.e. CRR) is triggered by stress conditions (such as starvation under aerobic conditions, decreasing pH or incubation of the culture in a narrow temperature range below the maximum temperature for growth) in *D. hansenii*. Besides this, in *D. hansenii* the activity of mitochondrial alternative oxidase is also triggered by the presence of 1.5–2.0 M NaCl. Therefore, such a relationship between stress situations and CRR must be taken into account in studies on the performance of spoilage yeasts in the food processing environments where several forms of stress are common.

4.5 Genomic Exploration

Whether the gene content of the genome can be correlated to the adaptive properties of species with different lifestyles? Could the mechanism of eukaryotic genome evolution be answered by comparative genomics? Although complicated by the multiplicity of events that have taken place throughout the history of individual lineages, the comparison of sequences of several unexplored yeast that covers an evolutionary range comparable to the entire phylum of chordates provides with a unique opportunity to investigate how genes involved in adaptation have been shaped by evolution.

The whole genome of *D. hansenii* has recently been sequenced and annotated by the consortium Genolevures and is now available at http://cbi.labri.fr/Genolevures/. It is compared with the complete genome sequence of three species widely spread over the hemiascomycete phylum: *Candida glabrata*, the second most prominent causative agent of human fungal infection (candidiasis) and phylogenetically related to *S. cerevisiae*; *Kluyveromyces lactis*, a milk loving yeast and *Yarrowia lipolytica*, an alkane using yeast that shares a number of common properties with the filamentous yeast in the Genolevures project. Analysis of the genome sequences revealed the role of processes such as tandem gene repeat formation, segmental duplication, massive genome duplication and extensive gene loss in the evolution of these species (Dujon et al., 2004).

4.5.1 Overview of the Genome

The genome of *D. hansenii* var *hansenii* type strain CBS767 is made of seven chromosomes ranging in size from 1.25 Mb to 2.33 Mb (Dujon et al., 2004). The total size of the genome is 12.2 Mb excluding ribosomal DNA (rDNA). It has the highest coding capacity among yeasts explored in the Genolevures project, amounting to 79.2% of the genome. The putative number of coding sequences (CDs) is 6906. Three distinct intra-chromosomal rDNA repeat loci are present in *D. hansenii*, whereas, single, seven and two loci are present in *S. cerevisiae*, *Y. lipolytica* and *C. glabrata* respectively. Variability also exists for the 5S rRNA gene copies. With a complement of 205 tRNA gene types corresponding to 43 types of tRNA, it has the largest number of potentially co-transcribed tRNA gene pairs. As compared to *S. cerevisiae*, *C. glabrata* and *K. lactis* which possess 42-tDNA set, *D. hansenii* has a 43-tDNA set. tDNA pairs are made of two distinct tRNA genes in the same orientation and separated from each other by a short distance (distance being shorter than the minimal 5′′ sequence required for transcription, thus, suggesting a common transcription of the two genes). Unlike the other three species of yeast, *D. hansenii* possesses eight identical copies of tDNA-lys that are present in tandem and are separated by intergenic distances sufficient for independent transcription. Like *C. albicans*, it uses an alternative genetic yeast code in which the CUG codon (leucine) is used as a serine codon (Tekaia et al., 2000; Sugita and Nakase, 1999).

D. hansenii is also the yeast with the most redundant genome, with an overall redundancy of 49.2%. Gene redundancy here is defined as the presence of genes representing conserved gene families that were present in organisms' last ancestor as well as gene families that have emerged or disappeared since specification. The global degree of genome redundancy, estimated from the number of paralogous gene copies per protein family indicates that this species of yeast has the most duplicated genome (901 sets) than *S. cerevisiae*, *C. glabrata, Y. lipolytica* and *K. lactis*. Among 97 newly identified members of paralogous gene families, 23 genes have putative role in transport, thus, indicating an important over-representation of this type of activities in *D. hansenii* as compared to *S. cerevisiae*. Similar over-representation exists for a large number of orthologues within a functional class however most striking is with the orthologues involved in 'transport facilitation'. The largest excess is for the 'allantoin and allantoate transporters' class. Besides this, multi-genic families encoding multi-drug resistance proteins and hexose transporters are more expanded in *D. hansenii* than in the other four yeasts. Tandem gene duplications are 5 to 10 times more frequent in this yeast. Tandem paralogues identified in *D. hansenii* are not strictly identical in sequence, indicating possible functional specialization and hence, limiting their destruction by popout. Few duplicated blocks (5 blocks, 3 in tandem) are present (Lepingle et al., 2000). Acquisition of new genes by horizontal gene transfer, a phenomenon rare in hemiascomycetes, has also been detected in *D. hansenii* (Dujon et al., 2004). Transposable elements are found in all eukaryotic organisms. In fungi, they participate to a large extent to genome plasticity through transposition and homologous recombination. Transposable elements are also present in *D. hansenii* and are more related to those found in higher eukaryotes (Lepingle et al., 2000).

4.5.2 Transporters

Trans-membrane transporter proteins make up to 10% of the coding genes in the hemiascomycete phylum (Hertogh et al., 2006). These may be considered as metabolic checkpoints for complex anabolic or catabolic pathways. These proteins have been classified according to the transport classification system (TC) (Saier, 2000). This classification system allocates five digits to each phylogenetic cluster of transporters in the order: class, subclass, families or super families, subfamilies and finally the fifth digit (clusters) that identifies the substrate or the range of substrates transported. Comparative analysis of the sequences encoding these proteins has allowed the identification of 'species-specific' transporter subfamilies that have either emerged or lost in the hemiascomycete phylum and to distinguish them from 'ubiquitous' subfamilies of transporters. It allows identifying the 'homoplasic' subfamilies of transporters that are conserved transiently in the different hemiascomycete species. A few examples of subfamilies in *D. hansenii* are: anion: cation symporter subfamily that takes up anionic vitamins in symport with protons or other cations (27 transporters including 9 members of the 'allantoate clusters'),

sugar porters (48 transporters, 21 members are gained and their substrates are undetermined), drug: proton antiporter-1 subfamily that pumps out a variety of hydrophobic drugs (24 transporters in *D. hansenii* as compared to 12 and 8 in *S. cerevisiae* and *K. lactis*) and large amino acid-polyamine-organocation family that transports a variety of amino acid in yeasts (24 members compared to 14–18 in the other species). Further, the species that exhibit the highest number of unique Hemiascomycete transporters is *D. hansenii* (9 specific transporters) compared to 5 in *Y. lipolytica* and one each in *S. cerevisiae*, *K. lactis* and *C. glabrata*. Mitochondrial carriers although are present in *Y. lipolytica* but are absent in *D. hansenii* and afterwards. This shows that the emergence of *Y. lipolytica* and *D. hansenii* is accompanied by a drastic gain of transporters compared to those during emergence of the other three species and thus, establishes the involvement of transporters in the evolution mechanism of speciation. The molecular mechanism of emergences or losses of these transporters genes during evolution are not understood.

Besides this, cell employs coordinated functions of different transporters for cation influx and efflux. In natural environments, sodium belongs to the abundant and potassium to the scarce ions. High internal concentrations of Na^+ (or its analogue Li^+) are generally toxic for cells. On the other hand, K^+ is required for many physiological functions such as regulation of cell volume and intracellular pH, protein synthesis, enzyme activation and this cation is accumulated in cells at a fairly high concentration. To maintain an optimum cytoplasmic concentration of potassium and a stable high intracellular K^+/Na^+ ratio, cells employ three distinct strategies: strict discrimination among alkali metal cations at the level of influx (higher affinity of transporters for potassium than for sodium), efficient efflux of toxic cations from cells, and selective sequestration (compartmentation) of cations in organelles (Sychrova, 2004).

In *S. cerevisiae*, sodium ions have been shown to enter the cell through the potassium transport system(s) encoded by the *TRK* genes (*TRK1* and *TRK2*) (Borst-Pauwels, 1981; Gaber, 1992; Rodriguez-Navarro and Ramos, 1984). However, specific sodium uptake system has not been identified as yet. Similarly in *D. hansenii*, nothing has been published in relation to the genes encoding the proteins mediating influx of sodium and lithium ions. The existence of genes orthologous to the *TRK* in *D. hansenii* has been reported (Prista et al., 2005).

Norkrans and Kylin (1969) explored the capacity of this species of yeast to tolerate high concentrations of NaCl in the growth medium. They have shown that *D. hansenii* when grown in presence of NaCl, accumulates high concentration of Na^+ inside the cell. But interestingly, when placed in presence of K^+ or Rb^+, it rapidly extrudes Na^+ to maintain high concentrations of K^+ inside the cell. The uptake of Rb^+ ions was found to be stimulated by the presence of NaCl in the medium (Prista et al., 1997). These results were further supported by the studies done by Thome-Oritz et al. (1998). The authors have postulated the existence of (i) an ATPase functioning as a proton pump and thus, generating a membrane potential difference that would drive K^+ ions through K^+ ion uniporter (ii) a K^+/H^+ exchange system (iii) a cation/cation exchange system. Analysis of the genome data also indicated the existence of homologues of Pma1p and Vma2p in *D. hansenii* (Prista et al., 2005). Similar

proteins in *S. cerevisiae* have been shown to generate a transmembrane potential and a pH gradient by massive proton efflux (Thome-Oritz et al., 1998; Gonzalez-Hernandez et al., 2004).

Ion transport systems mediating the efflux of sodium ions either out of the cell (Na^+ -ATPases and Na^+/H^+ antiporters) or their sequestration into the vacuoles have been identified. In *S. cerevisiae,* Na^+ -ATPases are encoded by *ENA1*/*PMR2* (*ENA* for efflux of natrium), the first unit of tandem array of four to five genes, depending on the strain, that encode the homologous P-type ATPases (Garciadeblas et al., 1993; Wieland et al., 1995). The expression of *ENA1* is modulated by osmotic stress and high pH values at the level of transcription. Under low salt concentrations (0.3 M), induction is mediated by HOG-MAPK kinase pathway, whereas under high salt conditions (0.8 M) by calcineurin which antagonizes the negative regulator, cAMP-dependent protein kinase (Marquez and Serrano, 1996; Hirata et al., 1995). In addition, it is also regulated post translationally by calcium-calmodulin via a calcineurin-independent mechanism (Wieland et al., 1995). *ENA1* encoded Na^+ -ATPases functions at alkaline pH values. In contrast, *NHA1* encoded Na^+/H^+ antiporters mediates Na^+ and K^+ efflux through the plasma membrane, required for alkali cation tolerance at acidic pH (Banuleos et al., 1998). Bedsides this, Na^+/H^+ antiporters are also involved in the regulation of intracellular pH. Thus, both systems have a complementary action to maintain the intracellular steady-state concentration of K^+ and Na^+. The two genes (*DhENA1* and *DhENA2*) encoding *ENA* homologues, which are involved in Na^+ extrusion, have been isolated and characterized (Almagro et al., 2001). *DhENA1* is expressed under conditions of high Na^+ concentrations. However, a high pH value along with the high Na^+ concentration is required for the *DhENA2* expression. It is suggested that these Ena proteins do not determine the Na^+ extrusion, as in *S. cerevisiae*, but plays an important role in maintaining the balanced levels of intracellular cations. These proteins are, thus, involved in ionic homeostasis of the cell.

The gene (*DhNHA1*) encoding *NHA1* homologue has been isolated and characterized from *D. hansenii* (Velkova and Sychrova, 2006). It shares the highest degree of identity with the antiporters of two other osmotolerant yeast species, *C. albicans* (*CNH1*) (Kinclova et al., 2001a) and *P. sorbitophila* (Banuelos et al., 2002). Thus, like the Na^+/H^+ antiporters from these yeast species, *DhNHA1* also exhibits broad substrate specificity. These antiporters might also play role in cell volume, intracellular pH and maintenance of cell cation homeostasis. On the other hand antiporters from *Zygosaccharomyces rouxii* (Kinclova et al., 2001b) and *Schizosaccharomyces pombe* (Jia et al., 1992; Kinclova et al., 2002) are able to transport only Na^+ and Li^+. Thus, depending upon the substrate specificity, yeast Na^+/H^+ antiporters can be divided into two subfamilies with different functions in yeast physiology. The gene encoding *DhNHX*, i.e. the homologue of *NHX1* gene of *S. cerevisiae* has been annotated in *D. hansenii* (Prista et al., 2005). *NHX1* functions to sequester the sodium ions into the cell vacuole. These results suggests that the ability of *D. hansenii* to adapt to high salt concentrations is not due a particular Na^+ ion extrusion system, but is due to the mechanism leading to the intrinsic resistance to the toxic effect of cations (Gonzalez-Hernandez et al., 2004).

4.6 Salt Toxicity and Defense Responses

The genetic analysis of salt tolerance in yeast has disclosed several adoptive mechanisms that cell has developed to counteract the stress due to changes in the osmolarity of the surrounding medium. The most important ones among these are the reactions crucial for maintaining ion homeostasis and cellular targets of salt toxicity. In the following sections these aspects are dealt with in detail.

4.6.1 Metabolism

When exposed to high osmolar medium, yeast cells respond by synthesizing and accumulating compatible solutes, called as osmolytes, inside the cell to counterbalance the external osmotic pressure (Mager and Varela, 1993). Glycerol accumulates as a major osmolyte in *S. cerevisiae*. It is synthesized in the cytosol via reduction of dihydroxyacetone phosphate in two steps that are catalyzed, respectively, by cytosolic NADH-dependent glycerol-3-phosphate dehydrogenase and a glycerol-3-phosphatase (Albertyn et al., 1994). The first enzyme in this pathway is encoded by two genes, the osmoresponsive *GPD1* (Larsson et al., 1993) and *GPD2* (Eriksson et al., 1995). The second enzyme is again encoded by two genes, the constitutively expressed *GPP1* and the osmotically induced *GPP2* (Norbeck et al., 1996). Increased accumulation of glycerol during osmotic stress is due to the enhanced activity of *GPD1*, thereby, resulting in the enhanced production and also because of the enhanced retention inside the cell, achieved by regulating the activity of *FPS1* (Olz et al., 1993; Blomberg and Adler, 1989; van Aelst et al., 1993). Fps1 behaves as a glycerol channel, responsible for glycerol leakage, that would remain closed in hyper-osmotic environments keeping glycerol inside the cells (Oliviera et al., 2003). Both the genes, i.e. *GPD1* and *FPS1* are regulated by HOG pathway (Albertyn et al., 1994). Although in *D. hansenii* glycerol accumulates as a dominant polyol during log phase like *S. cerevisiae* and arabinitol as dominant solute in the stationary-phase cells (Gustafsson and Norkans, 1976; Adler and Gustafsson, 1980; Nobre and da Costa, 1985), the information regarding the glycerol response in this yeast is still scarce. Besides this, trehalose, glutamic acid, erythritol, mannitol and alanine could also be accumulated in the cell in response to the osmotic stress. Recently, the gene encoding glycerol-3-phosphate dehydrogenase (*DhGPD1*) has been isolated from *D. hansenii* (Thome, 2004). A key role for this enzyme in glycerol production in *D. hansenii* has been demonstrated (Adler et al., 1985). The enzyme has been purified and characterized in vitro (Nilsson and Adler, 1990). Increased transcriptional activation of *DhGPD*, in presence of high salt stress, has been demonstrated (Lucas et al., 1990; Thome and Trench, 1999). Further, the ability to synthesize glycerol under osmotic stress does not differ much between *D. hansenii* and *S. cerevisiae* but the former is capable of retaining more glycerol than the other (Edgley and Brown 1983;

Brown, 1978). This is because of the presence of both an osmoregulatory-active glycerol transport system (Adler et al., 1985; Lucas et al., 1990) and by its ability to regulate the permeability of the plasma membrane (Oliviera et al., 2003). The first osmoregulatory-active glycerol transport system in *D. hansenii*, that accumulates glycerol up to 150-fold in the presence of 1 M NaCl, was described by Lucas et al. (1990). On the basis of their studies, these authors suggested the involvement of sodium-glycerol symporter in *D. hansenii*.

It is quite plausible that in halophilic organisms, the expression of genes encoding the enzymes of the central metabolic pathways could be modulated in order to increase transcription and enzyme synthesis, to overcome the negative effects of high salt concentration on enzyme activity. In this regard, Guerrero et al. (2005) studied the expression level of NADP-glutamate dehydrogenase involved in glutamate biosynthesis and ammonium assimilation in *D. hansenii*. They have shown that the expression of *DhGDH1* increases several fold in the presence of NaCl, whereas reverse is true for *DhGLN1*. Furthermore, the salt regulated expression of these two genes is by the action of a particular mechanism specific to *D. hansenii*, as its heterologous expression is not modulated by NaCl.

4.6.2 Signal Transduction Pathways

In eukaryotes, mitogen activated protein kinase (MAPK) cascades constitute very important signal transduction pathways that are conduits of various extracellular stimuli arising out of fluctuations in the environment or cell-cell communications. These cascades control various aspects of cellular physiology such as cell proliferation, differentiation and adaptive response to changes in the extracellular environment. A typical MAPK cascade is composed of three conserved families of protein kinases; the MAPK (also known as ERK, i.e. extracellular signal regulated kinase); a MAPK activator: MAPK kinase (MAPKK, MEK, MKK, extracellular signal regulated kinase kinase); and a MEK activator: MAPK kinase kinase (MAPKKK, MEK kinase [MEKK], extracellular signal regulated kinase kinase kinase) (Robinson and Cobb, 1997; Banuett, 1998). These kinases act in succession to form a cascade that ultimately modulates the activity of both cytoplasmic and nuclear targets by phosphorylation (Martinez-Pastor et al., 1996; Raitt et al., 2000). MAPK pathways seem to be essentially conserved from lower eukaryotes such as yeast to higher organisms, though their numbers and functional complexities vary. In *S. cerevisiae,* five MAPK modules have been identified so far, they regulate: mating, filamentation, sporulation, high-osmolarity responses and cell wall remodeling (Gustin et al., 1998; reviewed by Herskowitz et al., 1995; Madhani and Fink, 1998). Among these pathways, one is required for spore wall assembly and normally not present in growing cells, whereas other four are present in growing cells.

Very little is known about the signal transduction pathways in the marine yeast, *D. hansenii*, except the high osmolarity glycerol response pathway (HOG pathway) that mediates cellular adaptation process under high osmolar conditions. In *S. cerevisiae*

HOG pathway is specifically stimulated by osmotic shock (Brewester et al., 1993). This pathway receives signal from two upstream branches. One branch is the two component signaling system comprising Sln1, Ypd1 and Ssk1 (Maeda et al., 1994; Posas et al., 1996). Ssk1p activates the functionally redundant MAPKKKs Ssk2p/Ssk22p. A second branch of HOG1 is activated by the osmosensor Sho1p (Maeda et al., 1995), which signals to MAPKKK Ste11p (Posas and Saito, 1997). These MAPKKKs independently activate the MAPKK Pbs2p, which in turn dually phosphorylates and activates the MAPK Hog1p. Besides its role as a MAPKK, Pbs2p also functions as a scaffold protein in HOG pathway. Thus, Pbs2p plays a very complex role as it receives signals from both the upstream branches and allows the HOG pathway to operate over a wide range of sensitivity. Analysis of the genome sequence revealed that most of the components of HOG pathway are present in *D. hansenii*. Recently homologues of *HOG* and *PBS2*, has been isolated and characterized from *D. hansenii* (Bansal and Mondal, 2000; Bansal et al., 2001). Although *DHOG1* complemented the *hog1* mutation, the *PBS2* homologue (*DPBS2*) could partially complement the osmo-sensitivity of *pbs2* mutation in *S. cerevisiae* (Bansal et al., 2001). This partial complementation could be abrogated by replacing the C-terminal region of Dpbs2 with the homologous region of Pbs2p (Sharma and Mondal, 2005). This was due to an increase in nuclear translocation of Hog1p upon osmo-stress. Therefore, the C-terminal region of Pbs2p has an important role in nuclear translocation of Hog1p. Moreover, *DPBS2* was found to complement the polymyxin B sensitivity phenotype but had no perceptible effect on the calcofluor resistant phenotype of the *pbs2* mutation. Another interesting feature of Dpbs2p is the presence of a MAPK docking motif that appears to be essential for its function (Sharma and Mondal, 2006).

In *S. cerevisiae*, the activity of the HOG pathway is tightly controlled. Exposure to hyper osmolarity leads to rapid but transient activation of Hog1p by phosphorylation. Phosphorylated Hog1p translocated to the nucleus for a brief period to elicit the transcriptional responses necessary for osmoadaptation (Ferrigno et al., 1998; Reiser et al., 1999). Deactivation and subsequent re-entry of the Hog1p in the cytoplasm precedes the resumption of cellular growth. The prolonged activation of this pathway is detrimental for cell growth in *S. cerevisiae*. Under moderate stress conditions (0.7 M NaCl) the activation of HOG pathway in *D. hansenii* is quite similar to that in *S. cerevisiae*. However, the dynamics of the HOG pathway activation in *D. hansenii* differs considerably under severe osmotic stress. Sustained activation of Dhog1p has been observed in *D. hansenii* in the presence of 2.0 M NaCl. Interestingly, except for a brief period in the nucleus, the activated Dhog1p mostly remained in the cytoplasm (Sharma et al., 2005). These studies thus indicated important cytoplasmic role of phosphorylated Dhog1p in *D. hansenii*. Identification of the cytoplasmic targets of Dhog1p could provide us important clues for understanding the high osmotolerance exhibited by this yeast. HOG pathway is the only stress activated protein kinase pathway in yeast. The types of stress conditions that activate HOG pathway differ among yeast species. In *D. hansenii* this pathway is activated by osmostress, oxidative stress and UV stress. However, the heat stress did not activate this pathway. In this regard *D. hansenii* is quite similar to *Candida*

albicans (Sharma et al., 2005). UV induced activation of this pathway could be mainly as a reaction to oxidative stress due to formation of free radical.

4.6.3 Halotolerance Genes

Halotolerance genes are defined as the genes that have the capability to improve salt tolerance. Using *S. cerevisiae* as a model system, a few genes that are importance for halotolerance in yeast have been identified. Most prominent among them are *HAL1, HAL2, HAL3, HAL4* and *HAL5. HAL1* (Gaxiola et al., 1992; Rios et al., 1997) and *HAL3* (Ferrando et al., 1995) modulate intracellular sodium and potassium concentrations and encode components of the regulatory machinery for ion homeostasis. Their effects are mediated by the *ENA1*/*PMR2* gene. *HAL4* and *HAL5* encoded protein kinases are also required for ion homeostasis however their action is mediated through the coordinate regulation of potassium ion transporters of yeast cells, *TRK1* and *TRK2* (Mulet et al., 1999). On the other hand, *HAL2* encodes an enzyme, 3′(2′)5′-bisphosphate nucleotidase that catalyzes a side reaction essential for sulfate assimilation in yeast (Glaser et al., 1993). These enzymes are highly sensitive to sodium and lithium (Murguia et al., 1995) and thus, perform a crucial rate-limiting metabolic step during salt stress in yeast (Murguia et al., 1996). Genome sequence revealed that except *HAL1*, all these genes are present in *D. hansenii*.

Recently, a *HAL2* homologue, *DHAL2*, has been isolated and characterized from *D. hansenii* (Aggarwal et al., 2005). Biochemical as well as in vivo studies has revealed that Dhal2p has much higher intrinsic salt tolerance than that of the other homologues. It is quite plausible that in halotolerant organisms 'specific targets of salt toxicity' have been evolved to be more robust and less amenable to salt inhibition. It could tolerate much higher concentration of Na^+ and Li^+ (IC_{50} = 180 mM and 2.4 mM respectively) than most of the known homologues. With respect to sodium sensitivity, Dhal2p is comparable to *SAL2* of *A. thaliana*, which exhibited maximum salt tolerance (IC_{50} for Na^+ and Li^+ = 200 mM and 10 mM respectively). However, in case of lithium, Dhal2p seems to be more sensitive. Hal2p belongs to a larger super-family of phosphatases, Mg^{2+} -dependent Li^+ sensitive phosphomonoesterases. A number of *HAL2* homologues from plant, mammal and yeast have been characterized. Although they require Mg^{2+} for their activity, higher Mg^{2+} concentration has inhibitory effect on these enzymes. In this regard, *D. hansenii* homologue is quite different (Aggarwal et al., 2005).

Another interesting and distinguishing feature of Dhal2p is that, two distinct isoforms of this enzyme existed in *D. hansenii* (Aggarwal and Mondal, 2006). One of them is a constitutively expressed cytosolic form. Like the other known homologues, this form of Dhal2p is a typical PAPase. Whereas the other isoform of Dhal2p is membrane bound. It is accumulated in the endoplasmic reticulum only in response to high salt stress conditions and is a PIPase, i.e. enzyme acting on both inositol-1,4-bisphosphate and PAP. In this respect, the membrane-bound form was like a dually active BPntase prevalent in multi-cellular organisms (Lopez-Coronado et al., 1999). These types of enzymes have been suggested to play an important role

in inositol signaling. Modulation of Na^+ efflux system by the expression of *SAL1* in *S. cerevisiae* (Quintero et al., 1996), increase in sensitivity to abscisic acid and to damage by drought, low temperature or salt stress by mutation in *SAL1*/*FRY1* in *Arabidopsis thaliana* (Xiong et al., 2001) are few examples to quote. Studies done by Aggarwal and Mondal (2006) have further shown that the *S. cerevisiae* cells expressing both the cytosolic and membrane bound isoforms exhibited a higher level of salt tolerance than those expressing only the cytosolic form of Dhal2p. These studies thus, clearly suggested that the membrane-bound form of Dhal2p, unique to *D. hansenii*, played an important role under high salt stress conditions. Moreover, such a modulation of sub-cellular localization and substrate specificity of Dhal2p could be an important adaptive mechanism for growth of *D. hansenii* under high salt stress conditions. These studies, furthermore, highlight the importance of *D. hansenii* as a model to study halotolerance.

4.7 Biotechnological Applications

Studies done in recent past have shown that *D. hansenii* has enormous biotechnological potential in agro-food industry. It is commonly found in cheeses and sausages (Seiler and Busse, 1990; Saldanha-da-Gama et al., 1997) and in fact, plays an important in cheese making. The characteristic properties of the genus *Debaryomyces* that endows it with such a potential has recently been reviewed by Breuer and Harms (2006). It includes its ability to adhere to a solid surface (a prerequisite for initiation of growth to occur) (Mortensen et al., 2005), its salt tolerance, capacity to grow at low temperature and low water activity (a_w), ability to produce proteolytic and lipolytic enzymes that can metabolize milk proteins and fat (Besancon et al., 1992; Davenport, 1980; Fleet and Mian, 1987; Roostita and Fleet, 1996) and ability to inhibit the germination of undesired microorganisms in cheese brines (Deiana et al., 1984; Fatichenti et al., 1983). Furthermore, in comparison to other cheese ripening yeast, *D. hansenii* produces volatile sulphur compounds (Arfi et al., 2002). Similarly the involvement of *Debaryomyces* in meat fermentation has been known for a long time. Besides producing volatile compounds, the yeasts also influence the sensory properties of the meat (Dura et al., 2004a, 2004b). Recently few enzymes like prolyl amino peptidase, arginyl amino peptidase (Bolumar et al., 2003a, 2003b) and glutaminase (Dura et al., 2004c) of *D. hansenii* has been isolated and characterized. However, much less is known about the possible involvement and their role in ripening of a fermented sausage and meat fermentation.

One of attractive biotechnological potential of *D. hansenii* is its use in the production of xylitol from D-xylose or wood hydrolysate (Girio et al., 1996, 2000; Parajo et al., 1997). Xylitol, as a sweetener, is an important raw material in food industry for the production of sugar-free confection and food (Cruz et al., 2000). Conversion of xylose to xylitol by *D. hansenii* has been studied quite extensively. *D. hansenii* could use both pentoses and hexoses individually as well as in mixture. The presence of hexoses particularly glucose did not inhibit the consumption of pentoses such as xylose (Nobre et al., 1999). This interesting property needs further

investigation, as it will be useful for developing strains of other yeast species to utilize sugars in a mixture simultaneously. The initial steps for the metabolism of xylose in *D. hansenii* involve a NADPH-dependent xylose reductase and a NAD^+ linked xylitol dehydrogenase (Converti and Dominguez, 2001; Girio et al., 1994). Since these two enzymes utilize different co-actor, the availability of oxygen during the fermentation has tremendous influence in the overall yield of xylitol from xylose. Although production of xylitol by *D. hansenii* awaits commercial exploitation at present, insights into the regulation of pentose metabolism will definitely help towards achieving this objective. Besides xylitol, *D. hansenii* can be used to synthesize a range of other useful product. It produces xylitol as well as arabinitol from pentose sugar. D-Arabinitol is produced in the stationary phase simultaneously with the excretion of riboflavin in the batch cultivation. The importance of this yeast species as cell factories for producing chemicals like pyruvic acid, D-arabinitol has recently been highlighted (Breuer and Harms, 2006). Some strains of *D. hansenii* exhibit anti-fungal activity. Effectiveness of these strains as a biocontrol agent of fruit spoilage fungi has also been demonstrated (Fleet, 1992; Droby et al., 1989; Ramirez-Orozco et al., 2001).

4.8 Future Perspectives

Although *D. hansenii* had been known in the literature for a long time, the surge of scientific interest in this organism is very recent. This is evident from the increase in the number of publications on this organism in the past ten years. A cursory glance through the published literature, certainly suggests that this is an interesting yeast, with enormous potential for both fundamental research and applied research. The availability of the whole genome sequence of *D. hansenii* offers new possibility for an integrated approach to unravel the extremophilic nature of this organism. Comparative genomics could be very useful. Development of molecular genetic tools is an urgent requirement. This will facilitate the development of better strains for commercial exploitation. It will also help to garner the detailed information about the signal transduction pathways, genetic network and regulation, an important step for understanding and exploitation of the halotolerance/halophily of this organism.

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Chapter 5
Candida famata (Debaryomyces hansenii)

Andriy A. Sibirny and Andriy Y. Voronovsky

Contents

Abstract *Debaryomyces hansenii* (teleomorph of asporogenous strains known as *Candida famata*) belongs to the group of so named 'flavinogenic yeasts' capable of riboflavin oversynthesis during starvation for iron. Some strains of *C. famata* belong to the most flavinogenic organisms known (accumulate 20 mg of riboflavin in 1 ml of the medium) and were used for industrial production of riboflavin in USA for long time. Many strains of *D. hansenii* are characterized by high salt tolerance and are used for ageing of cheeses whereas some others are able to convert xylose to xylitol, anti-caries sweetener. Transformation system has been developed for *D. hansenii*. It includes collection of host recipient strains, vectors with complementation and dominant markers and several transformation protocols based on protoplasting and electroporation. Besides, methods of multicopy gene insertion and insertional mutagenesis have been developed and several strong constitutive and regulatable promoters have been cloned. All structural genes of riboflavin synthesis and some regulatory genes involved in this process have been

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identified. Genome of *D. hansenii* has been sequenced in the frame of French National program 'Genolevure' and is opened for public access.

Keywords Riboflavin, *D. hansenii*, *C. famata*, flavinogenic, transformation, insertional mutagenesis

5.1 Introduction

Candida famata (teleomorph: *Debaryomyces hansenii*) is osmotolerant yeast able to grow in the presence of high concentrations of NaCl. The yeast tolerates 4 M of the salt, whereas growth of *Saccharomyces cerevisiae* is completely inhibited by 1.7 M NaCl (Onishi, 1963; Prista et al., 1997). Both *C. famata* and *D. hansenii* strains are able to overproduce riboflavin (vitamin B_2) in iron-deficient media (Gadd and Edwards, 1986; Shavlovsky and Logvinenko, 1988). Some *C. famata* mutants are the most flavinogenic organisms known (Heefner et al., 1988, 1992, 1993; Stahmann et al., 2000). Strains of *D. hansenii* and *C. famata* are found in habitats with high salinity levels, such as sea water, brines, salted food (cheeses, sausages) (Norkrans, 1966; Seiler and Busse, 1990; Lépingle et al., 2000). Ability of *D. hansenii* to grow in the presence of high NaCl concentrations resulted in a designation of the species as halotolerant (or, according to some authors: halophilic) yeast. Some *D. hansenii* strains are considered as potential producers of xylitol (Parajo et al., 1996; Roseiro et al., 1991). Osmotolerance of *D. hansenii* is advantageous for some biotechnological applications; it allows quasi-non-sterile production and high product/educt concentrations, conditions which should reduce production costs (Breuer and Harms, 2006). *D. hansenii* (*C. famata*) belongs to the monophyletic clade containing organisms that translate CTG as serine instead of leucine (Fitzpatrick et al., 2006). This chapter mainly is focused on the flavinogenic strains of the anamorph *C. famata*.

Complete sequence of *D. hansenii* genome has been published (http://cbi.labri.fr/Genolevures/elt/DEHA) and is available for public use (Dujon et al., 2004). It opens new opportunities for study and elucidation of molecular mechanisms of halotolerance and riboflavin overproduction in *D. hansenii* (*C. famata*) and using this yeast in basic and applied research.

Recently, the review on *D. hansenii* has been appeared (Breuer and Harms, 2006). This useful review contains the comprehensive data on many aspects of physiology, biochemistry, genetics and potential biotechnological applications of *D. hansenii*. However, in spite of the fact that the only industrial biotechnological application of *D. hansenii* to date is the use of the mutant strain of anamorph *C. famata* for riboflavin production (at Archer Daniels Midland Co. in USA), authors did not pay attention on this important aspect. They also did not mention on development of transformation system for *C. famata* and cloning structural and regulatory genes involved in riboflavin synthesis. Our review aims to fill these gaps.

5.2 History of Research, Phylogeny and Physiology

D. hansenii is studied actively as osmotolerant yeast since 1960-ies (Onishi, 1963; Norkrans, 1966, 1968) and, nowadays, obvious progress has been achieved in studying mechanisms underlying high salt tolerance of the yeast (Prista et al., 2005; Velkova and Sychrova, 2006). First articles on capability of *C. famata* strains to overproduce riboflavin were published at 1940-ies – 1950-ies (Tanner et al., 1945; Goodwin and McEvoy, 1959). Since then efficient approaches for improvement of *C. famata* riboflavin production were developed and very active overproducers were isolated (Heefner et al., 1988, 1992, 1993). A transformation system based on the *C. famata* strain L20105 (*leu2*) deficient in β-isopropylmalate dehydrogenase as a recipient and vectors containing the *S. cerevisiae LEU2* gene as a selective marker was developed for *C. famata* (Voronovsky et al., 2002; Abbas et al., 2006). Genomic fragments of the anamorph containing genes participating in the synthesis of riboflavin were cloned and sequenced (Dmytruk et al., 2004; Voronovsky et al., 2004). Recently, a method of insertional mutagenesis was applied successfully for *C. famata* and genes involved in positive regulation of riboflavin synthesis were identified (Dmytruk et al., 2006). Complete sequence of *D. hansenii* genome was published in July 2004 (http://cbi.labri.fr/Genolevures/elt/DEHA). All these achievements in the field of molecular biology of *D. hansenii* (*C. famata*) open new opportunities for study and clarification of metabolic peculiarities (halotolerance, riboflavin overproduction) of this yeast.

The species *D. hansenii* (anamorph: *C. famata*) is a haploid yeast that reproduces vegetatively by multilateral budding. Most *D. hansenii* strains mate very rarely and diploidize transiently by somatogamous autogamy to form asci containing generally a single spore (Kreger and Veenhuis, 1975; van der Walt et al., 1977). *D. hansenii* (*C. famata*) belongs to the monophyletic clade containing organisms that translate CTG as serine instead of leucine (Fitzpatrick et al., 2006). The species *D. hansenii* (Zopf) Lodder & Kreger comprises two varieties: *D. hansenii* (Zopf) Lodder & Kreger var. *hansenii* (anamorph: *C. famata* (Harrison) S.A. Meyer & Yarrow var. *famata*) and *D. hansenii* var. *fabryi* (Ota) Nakase & M. Suzuki (anamorph: *C. famata* (Harrison) S.A. Meyer & Yarrow var. *flareri* (Ciferri & Redaelli) Nakase & M. Suzuki) (Nakase and Suzuki, 1985; Nakase et al., 1998). The varieties of *D. hansenii* differ in the electrophoretic mobility of their glucose-6-phosphate dehydrogenases. They also differ in maximum growth temperatures: var. *hansenii* can grow at temperatures up to 35°C while var. *fabryi* is able to grow up to 39°C (Nakase and Suzuki, 1985). The varieties also differ in sequences of their 26S rDNA genes (Kurtzman and Robnett, 1997, 1998). All these differences were used for discrimination of the two varieties of *D. hansenii*. In addition, the pulse-field gel electrophoresis (PFGE) was used to discriminate between the two varieties (Corredor et al., 2003; Petersen and Jespersen, 2004). However, different results were obtained by the two groups of authors: Corredor et al. (2003) stated a marked chromosomal polymorphism in *D. hansenii* strains. This suggested that strains belonging to *D. hansenii* var. *fabryi* represent a different taxon from

D. hansenii var. *hansenii*. But, according to the other authors (Petersen and Jespersen, 2004), PFGE analysis did not result in a division of the two varieties to separate groups. So, further change and improvement of the taxonomic classification of *D. hansenii* varieties will be possible after development of new analytical methods.

D. hansenii (*C. famata*) has some remarkable physiological properties. The yeast can grow at concentrations of NaCl up to 2.5 M and tolerate 4 M of the salt (Prista et al., 1997; Lépingle et al., 2000). *D. hansenii* assimilates a broad spectrum of carbon substrates: glucose, galactose, sucrose, maltose, cellobiose, trehalose, raffinose, xylose, arabinose (Nakase et al., 1998). The optimal temperature for the yeast is 25–28°C, but growth at 5–10°C was also reported (Davenport, 1980). *D. hansenii* utilizes *n*-alkanes (Yadav and Loper, 1999). It has a poor growth in the absence of oxygen, therefore fermentation of glucose, galactose, sucrose, maltose, trehalose and raffinose by *D. hansenii* is weak (Nakase et al., 1998). The yeast does not utilize nitrate, but it can assimilate nitrite (Nakase et al., 1998). *D. hansenii* (*C. famata*) overproduces riboflavin in iron-deficient media (Gadd and Edwards, 1986; Shavlovsky and Logvinenko, 1988). Some of developed *C. famata* strains are the most flavinogenic organisms known (Heefner et al., 1988, 1992, 1993; Stahmann et al., 2000).

D. hansenii has a mitochondrial alternative oxidase (in addition to the cytochrome *c* oxidase) that is triggered by 1.5–2 M NaCl and can act as the terminal oxidase for an electron transfer chain that branches from the core pathway at the ubiquinone pool level. The alternative oxidase is insensitive to cyanide and antimycin A. The cyanide-resistant respiration pathway reduces the amount of energy from the cytochrome *c* pathway by diverting some of the electron flux to the alternative oxidase (Veiga et al., 2003a, b).

Generally the yeast *D. hansenii* (*C. famata*) is considered as non-pathogenic. There are many harmless strains of *D. hansenii* and *C. famata* involved in ripening of cheeses and sausages (Seiler and Busse, 1990; Saldanha-da-Gama et al., 1997). The industrial strain of *C. famata* involved in the production of riboflavin is also known (Stahmann et al., 2000). On the other hand, clinical isolates of *D. hansenii* and *C. famata* are not rare (Nishikawa et al., 1996). Hence, the possible pathogenicity of the yeast has to be investigated in more detail.

5.3 Mechanisms for the Halotolerance of the Yeast

A few peculiar characteristics provide the remarkable halotolerance of *D. hansenii* (*C. famata*); particularly, capability to keep glycerol at high concentrations inside the cells and less sensitivity of metabolism of the yeast to intracellular sodium concentrations are considered the most important among the characteristics.

The osmoregulation in *D. hansenii* has been studying for many years by many groups. Correlation between the intracellular polyol (glycerol and arabinitol) concentration and the salinity of the medium was established (Larsson et al., 1990).

^{13}C NMR studies showed that glycerol is the predominant end product when *D. hansenii* metabolizes glucose in the presence of 8% NaCl (Jovall et al., 1990). There was shown a key role of the enzyme glycerol-3-phosphate dehydrogenase (GPD) in the production of glycerol in *D. hansenii* (Adler et al., 1985). The enzyme was purified and characterized *in vitro* (Nilsson and Adler, 1990). The specific activity of *D. hansenii* GPD was doubled when the salinity of the medium increased to 1.4 M, but this stimulation was weaker than that observed in *S. cerevisiae* (Andre et al., 1991). The *GPD1* gene of *D. hansenii* (*DhGPD1*) was isolated and a high degree of homology with *GPD1* of *S. cerevisiae* was found (Thome, 2004). Immunoblot analysis and Northern blotting confirmed the previous observation on the role of GPD in osmoregulation (Thome and Trench, 1999). Recently, the significance of cell wall for the induction of *DhGPD1* gene and consequently for osmotic regulation in *D. hansenii* was shown (Thome, 2007).

It was established that enzymes leading to pyruvate, in particular glyceraldehyde-3-P dehydrogenase, were inhibited under the salt-induced stress in *D. hansenii* (Neves et al., 1997). It can explain the increased diversion of glycolytic flux towards the glycerol pathway in the yeast.

Glutamate in *D. hansenii* protects GPD against the toxic effect of salt (Nilsson and Adler, 1990). The increase of glutamate dehydrogenase activity in the yeast in the presence of salt was reported. Glutamate is suggested as the intracellular counter ion under high ionic strength (Alba-Lois et al., 2004).

Studies directed on identification of protein targets for Na^+ toxicity were performed. The *HAL2* gene was identified in *S. cerevisiae* as an important target of salt toxicity (Glaser et al., 1993). The overexpression of the gene significantly increased salt tolerance. The product of *HAL2* is a nucleotidase that hydrolyses 3′-phosphoadenosine-5′-phosphate (PAP) to AMP, recycling adenosine. A gene homologous to *HAL2* was identified in *D. hansenii* (Prista et al., 2005). Preliminary results suggested that, *in vitro*, Hal2p from *D. hansenii* was more salt-resistant than the corresponding protein from *S. cerevisiae* (Prista et al., 2005).

The only production of glycerol is not sufficient to provide high osmotolerance; the osmolyte has to be kept inside the cells at high concentrations. The osmoregulatory-active glycerol transport system was described for *D. hansenii* (Lucas et al., 1990). This transport system accumulates glycerol significantly (up to 150-fold) in the presence of 1M NaCl. Correlation between the maximum ratios of glycerol accumulation and the concentration of extracellular NaCl led to the hypothesis about involvement of sodium-glycerol symporter in the system. The data also suggested that potassium may be a co-substrate instead of sodium when the sodium concentration is low. Yeasts able to grow in the presence of 3M NaCl were able to accumulate glycerol in an energy-dependent process (Lages et al., 1999). There was shown for the range of yeasts (including *D. hansenii*) that accumulation of glycerol was reinforced by the presence of 1M NaCl (Lages et al., 1999).

The capability to maintain a high intracellular concentration of glycerol does not depend only on active transport of glycerol. It also depends on the permeability of the plasma membrane: the low permeability prevents glycerol leakage.

The permeability coefficient (ε) for glycerol diffusion in *D. hansenii* is lower compare to that in *S. cerevisiae* (Lages et al., 1999; Larsson et al., 1990; Prista et al., 2005). Searching in the Genolevures database for glycerol permeases (like the Fps1p of *S. cerevisiae*) or putative MIP channels for glycerol did not result in finding the corresponding *D. hansenii* homologues. This supports the view that *D. hansenii* is better equipped to retain than to release glycerol, opposite to *S. cerevisiae* (Prista et al., 2005).

The process of sodium efflux has been studied actively in *D. hansenii* since the sodium extrusion is a main way of defence for other yeasts during growth in the presence of salt. Particularly, two genes specifically involved in salt extrusion were cloned and characterized in *D. hansenii*. The genes code for Na^+-ATPases (Almagro et al., 2001). They were designated *DhENA1* and *DhENA2*. Northern analysis demonstrated the elevated expression of *DhENA1* gene in the presence of high NaCl concentrations. The gene *DhENA2* for its increased expression required high pH in addition to high sodium. The genes complemented NaCl-sensitivity of the *S. cerevisiae* mutant lacking the sodium efflux systems. Resulting transformants acquired sodium tolerance and ability to extrude the cation (Almagro et al., 2001). But the recovered tolerance was far from the tolerance level of *D. hansenii*. Thus, sodium extrusion alone is not sufficient to explain the high salt tolerance of *D. hansenii*.

Search in the Genolevures database found a range of *D. hansenii* genes that may play a role in Na^+ and K^+ efflux. The gene *DhNHX* coding for a putative protein mediating the transport of Na^+ into the vacuole was also found (Prista et al., 2005). Real significance of the genes in regulation of cation content and halotolerance in *D. hansenii* has to be tested by experimental studies.

5.4 Strains and Media

The wild-type *C. famata* strain VKM Y-9 (Russian Collection of Microorganisms, Poushchino, Russia) and its derivative, the leucine auxotroph *C. famata* L20105 (*leu2*, NRRL Y-30292) were used in our studies (A. Sibirny and A. Voronovsky; Institute of Cell Biology NAS of Ukraine, Lviv, Ukraine). Both strains overproduce riboflavin in iron-deficient media. For complete genome sequencing, the strain *D. hansenii* var. *hansenii* CBS767 was used. The sequenced strain also overproduces riboflavin in iron-deficient media (Voronovsky et al., 2004).

The *C. famata* strains were grown at 28°C in YPD (1% yeast extract, 1.5% peptone, 2% glucose) or minimal media, YNB (0.67% Difco yeast nitrogen base without amino acids, containing 2% glucose), or modified Burkholder medium (Voronovsky et al., 2002; Shavlovsky et al., 1978). Leucine at 40 mg $\times$ L^{-1} was added to minimal media for cultivation of the *leu2* mutant L20105. Iron-deficient media contained about 0.18 μM of iron. Iron was removed from the medium with 8-hydroxyquinoline as described earlier (Cowart et al., 1980). Iron supplemented media contained 7.2μM iron added as ammonium ferrous sulfate hexahydrate.

5.5 Genetic Data and Techniques

5.5.1 Transformation System for the Flavinogenic Yeast C. famata

5.5.1.1 Identification of *C. famata* Recipient Strains Deficient in *LEU2* gene

For development of *C. famata* transformation system, the collection of *C. famata* leucine auxotrophic mutants was isolated. Cells of wild-type strain VKM Y-9 were UV-irradiated and plated on the modified Burkholder medium containing L-leucine. 16 leucine auxotrophic mutants (Leu^-) of *C. famata* were isolated after testing 45,180 colonies. Identification of *leu2* mutants was carried out by spheroplast transformation of five randomly picked Leu^- mutants by plasmids harboring the *S. cerevisiae LEU2* gene coding for β-isopropylmalate dehydrogenase. Three Leu^- strains were transformed by the plasmids to Leu^+ colonies. These mutants were designated L203, L2012 and L20105. All three mutants were stable with a reversion frequency to prototrophy of less than 10^{-7}. Thus, there was concluded that the mutants L203, L2012 and L20105 have a deficiency in gene homologous to the *S. cerevisiae LEU2* gene encoding β-isopropylmalate dehydrogenase. The strain L20105 was used in further studies as a recipient in transformations using the *S. cerevisiae LEU2* gene as selective marker (Voronovsky et al., 2002; Abbas et al., 2006).

5.5.1.2 Cloning of *C. famata* Autonomously Replicating Sequences (ARS) and Obtaining Replicative Plasmids Providing Effective Transformation of This Yeast

Plasmids carrying heterologous autonomously replicating sequences (ARS) could not provide the high transformation frequency for *C. famata*. Efficient transformation is important factor for the host-vector system that is developed for gene cloning. A strategy of *C. famata* ARS cloning consisted in the isolation of this sequence from the yeast genome library constructed on a vector that does not contain any yeast plasmid replicator. The recombinant plasmid p19L2 (Voronovsky et al., 2002) was used for the library construction. The plasmid contains the *S. cerevisiae LEU2* gene as selectable marker. This plasmid transformed the *C. famata* mutant L20105 inefficiently. The gene library of *C. famata* wild-type strain VKM Y-9 was constructed on the basis of plasmid p19L2. The plasmid DNA of resulting library transformed the strain L20105 to leucine prototrophy with frequency six times higher than that with p19L2. Thus, the increased transformation frequency suggested the presence of ARS elements among inserts of the constructed gene library. For ARS cloning, plasmid DNA was isolated from *C. famata* transformants obtained after transformation with the gene library. First, total DNA was isolated from approximately 40,000 such transformants. This DNA was used for transformation of *E. coli* DH5α with following isolation of the plasmid DNA from 16 randomly picked separate bacterial clones. Electrophoretic analysis showed that the vectors can be divided into five groups by their sizes. One plasmid was picked from each group for further studies.

Restriction analysis of the plasmids was carried out and the results were compared to the restriction digest of the vector p19L2. The approximate length of the inserts was identified (from 0.25 to 6 kb). The frequency of spheroplast transformation by plasmids containing the inserts appeared to be, on average, 13 times higher than that obtained using the vector p19L2. Corresponding plasmid DNA was rescued through *E. coli* retransformation from yeast transformants obtained by insert-containing plasmids but not from transformants L20105/p19L2. High transformation frequencies and extrachromosomal status of insert-containing vectors suggested that the cloned fragments possess with the ARS function. These plasmids were named pCfARS1, pCfARS6, pCfARS11, pCfARS7 and pCfARS16. An ARS fragment of the smallest size of plasmid pCfARS16 (the CfARS16 insert) was sequenced (accession number AF435949 in GenBank database). The CfARS16 fragment had features characteristic for ARS elements. Namely, it had a region rich in adenine plus thymine (A + T) (more than 81%) and contained a 10-of-11-bp match to the *S. cerevisiae* ARS Consensus Sequence, ACS: (A/T)TTTAT(A/G) TTT(A/T) (Rowley et al., 1994; Voronovsky et al., 2002).

5.5.1.3 Optimization of Transformation Methods for *C. famata*

5.5.1.3.1 Spheroplast Transformation

C. famata spheroplast transformations were performed by the method described earlier for *P. pastoris* (Sreekrishna and Kropp, 1996) with some modifications. During spheroplast transformation, sucrose was used as an osmotic stabilizer instead of sorbitol, as there was found that sorbitol strongly reduces *C. famata* spheroplast viability. Important factor influencing the spheroplast transformation frequency appeared to be the purity of the enzyme used for cell wall hydrolysis. Lyticase (Sigma, 'crude') resulted in transformation frequency for the strain L20105 with an average of 1.44×10^3 transformants per μg DNA. Lyticase (Sigma, 'partially purified') resulted in more than 40 times higher transformation frequency (6.3×10^4 transformants per μg DNA). The other important factor influencing *C. famata* spheroplast transformation efficiency is the quality and/or supplier of PEG. We found that the best stock for transformation was PEG 3,350 (Merck, Germany) or PEG 3000 (Ferak, Germany). The use of PEG 3,350 Carbowax (Fisher, USA) was found to provide transformation frequencies 10 times lower than those of the PEGs mentioned above (Voronovsky et al., 2002; Abbas et al., 2006).

5.5.1.3.2 Electrotransformation

The *C. famata* strain L20105 (*leu2*) and plasmids pCfARS6 or pCfARS16 were used for optimization of the electrotransformation parameters for this yeast. Our protocol was based on two electrotransformation procedures previously described for *S. cerevisiae* (Becker and Guarente, 1991) and *H. polymorpha* (Faber et al., 1994). Electroporation was carried out with the Electro Cell Manipulator 600

(ECM600) from BTX, USA, using 2-mm cuvettes. The following important factors for electrotransformation efficiency were examined: significance of DTT treatment of cells, electric field strength, the cell culture age, and pulse duration. There was found out that the pretreatment of *C. famata* cells with DTT is essential for transformation. Omission of this step resulted in the total absence of transformants. Optimal field strength for electrotransformation of *C. famata* strain L20105 was determined. For this, electroporations of the strain under different field strengths were carried out. There was identified that the field strength of 11.5 kV cm^{-1} provided the highest transformation frequencies: 0.8–1 × 10^5 transformants μg^{-1} DNA. It is known that cells from exponential growth phase are the most suitable for highly efficient electrotransformation of yeasts (Becker and Guarente, 1991; Faber et al., 1994). Cells of strain L20105 from different stages of exponential growth were tested for their suitability for electrotransformation. Prior to electrotransformation, the cells were concentrated by centrifugation and resuspended in 1M sucrose (used as electroporation buffer) to achieve equal cell densities (approx. 4 × 10^9 cells ml^{-1}). The cells (0.2 ml per sample) were then transformed with 0.3 μg of plasmid. There was determined that the most efficient transformation frequency was achieved with cells of OD_{540} = 6 (2.7 × 10^7 cells ml^{-1}). The optimal pulse duration for electrotransformation of strain L20105 was also established. This parameter is determined by the timing resistance. For many yeast species, including strains of *S. cerevisiae*, *H. polymorpha*, *Pichia methanolica* and *Schizosaccharomyces pombe*, the optimal value of pulse duration during electroporation appeared to be 4–5 ms (Becker and Guarente, 1991; Faber et al., 1994). This pulse length was provided by the timing resistance of ~129 Ω. Therefore, the pulse duration of ~4.5 ms (129 Ω) was used during the determination of optimal field strength for electroporation of *C. famata* L20105. After that, the effect of different pulse lengths on electroporation of strain L20105 was investigated. There was established that the optimal pulse duration for *C. famata* appeared to be ~4.5 ms (129 Ω).

Thus, efficient transformation system for the flavinogenic yeast *C. famata* was developed. It is based on a mutant strain of *C. famata* L20105, which is defective in a gene homologous to the *S. cerevisiae LEU2* gene encoding β-isopropylmalate dehydrogenase, and on vectors containing the *S. cerevisiae LEU2* gene as a selective marker. For DNA transfer, two methods were optimized and used: spheroplast transformation and electroporation. The transformation system was successfully used for cloning of *C. famata* genes for riboflavin synthesis (see Sect. 5.5.3).

5.5.2 Insertional Mutagenesis of C. famata

5.5.2.1 Insertion Cassette, Transformation Frequency and Integration Events

Identification of regulatory mutations by functional complementation usually is difficult to fulfil due to similarity in the growth patterns of wild-type strains and derived mutants. Therefore, other methods for generation of mutations with subsequent

rapid identification of defective elements have to be applied in the cases. Insertional mutagenesis belongs to techniques providing significant improvements in the procedure for cloning and identification of regulatory genes. The method has been successfully used to tag genes in many fungi including different yeast species (Tilburn et al., 1990; Schiestl and Petes, 1991; Kuspa and Loomis, 1992; Kang and Metzenberg, 1993; Granado et al., 1997; Van Dijk et al., 2001).

Development of the method for insertional mutagenesis of *C. famata* was started with the construction of insertion cassette. The integrative plasmid pL2 was constructed as the cassette (Dmytruk et al., 2006). This plasmid consists of *S. cerevisiae LEU2* gene inserted into the bacterial vector pUC19. A range of restriction sites were reduced in the plasmid; this allowed using many different endonucleases for the recovery of genomic fragments. The unique site *Sal*I was used for linearization of the plasmid.

The *C. famata* strain L20105 (*leu2*) was transformed by the linearised DNA of plasmid pL2. Addition of the restriction enzyme *Sal*I to transformation mixtures increased transformation frequencies only slightly (up to 1.5 times). Stability of resulting pL2 transformants was tested. Twelve randomly selected Leu^+ transformants were picked up and cultivated by turns in minimal and non-selective rich media. In all cases transformants remained Leu^+ prototrophs, indicating stable integration of the vector pL2 into the *C. famata* genome. Random integration at different sites of linearized plasmid pL2 into genome of the recipient strain was shown by analysis of the *C. famata* transformants using Southern hybridization. The analysis also revealed that in 95% of the transformants a single molecule of the cassette was present in genome (Dmytruk et al., 2006).

5.5.2.2 Generation and Identification of *C. famata* Regulatory Mutants Unable to Overproduce Riboflavin

The *C. famata leu2* strain was transformed by the *Sal*I-linearised pL2 plasmid. Obtained approx. 3000 Leu^+ transformants were replica plated onto the ferrozine-containing (iron deplete) medium. Three mutants affected in riboflavin overproduction on the medium were selected. These mutants 101R, 4R and 1R were stable and used for further analysis. Study of riboflavin production by the isolated mutants in liquid medium showed that they are unable to overproduce riboflavin either in iron replete or iron deplete media. Therefore there was hypothesized that the mutants 101R, 1R and 4R have mutations in one or more regulatory genes required for riboflavin production.

In order to identify the sites of insertion of cassette pL2, the genomic DNA from selected mutants affected in riboflavin overproduction was isolated. Restriction enzymes, which do not cleave pL2, were used to isolate of the integrated plasmid together with flanking genomic sequences. Flanking fragments together with the vector were isolated from the mutants 101R, 1R and 4R by transformation with the self-ligated genomic DNA, digested with *Bam*HI, *Sac*I and *Hind*III, respectively, into *E. coli*. All rescued plasmids were amplified and sequenced.

Resulting sequences revealed that, in selected strains 101R, 4R and 1R, the insertional cassette disrupted the gene *RIB1* (GTP cyclohydrolase II) and the orthologs of *S. cerevisiae* genes *MET2* (homoserine O-acetyltransferase) and *SEF1* (putative transcription factor), respectively.

5.5.2.3 Confirmation of Gene Disruption

Confirmation that the observed phenotype is a result of insertion cassette integration, but not the secondary mutation occurring elsewhere in the genome, is an essential part of the study. For this purpose, the insertion mutants 101R, 4R and 1R were transformed with plasmids carrying the respective genes (*RIB1*, *MET2* and *SEF1*) isolated from wild-type strains. In the case of 101R mutant, the *C. famata RIB1* gene that was cloned and sequenced earlier was used (Dmytruk et al., 2004; Voronovsky et al., 2004). In two other cases (4R and 1R), the corresponding genes of *D. hansenii* CBS767 strain were used (homologs of *S. cerevisiae MET2* and *SEF1*; the sequences were taken from the Genolevures database). As the isolated insertion regulatory mutants did not contain auxotrophic selective markers, a dominant marker was used to select the transformants. There was found that growth of *C. famata* is inhibited by phleomycin (2.0 mg l^{-1}) in the rich YPD medium. The *Staphylococcus aureus ble* (*Sable*) gene confers resistance to bleomycin, phleomycin and zeocin. *C. famata* (as was mentioned in Sect. 5.5.2) uses an alternative genetic code in which the CUG codon (leucine) codes for serine. Fortunately, the *ble* gene does not contain CUG codons within its open reading frame (Semon et al., 1987). Therefore this gene was used as a dominant selection marker for transformant selection.

Plasmids that bear *CfRIB1*, *DhMET2* or *DhSEF1* genes were constructed on the base of the vector containing *Sable* gene driven with the *C. famata TEF1* promoter (Dmytruk et al., 2006). These plasmids were used for transformations of the corresponding 101R, 4R and 1R strains. Phleomycin resistant colonies were selected. Transformants were stabilized and verified by PCR using specific primers for the *Sable* gene. The genes *CfRIB1*, *DhMET2* and *DhSEF1* complemented corresponding mutations and the obtained transformants acquired the ability to overproduce riboflavin in the iron depleted medium. The functional complementation of the *C. famata* strains 101R, 4R and 1R by the genes *CfRIB1*, *DhMET2* or *DhSEF1*, is a sufficient evidence to assert that the observed phenotype of isolated mutants is a result of disruption of the genes *RIB1*, *MET2* and *SEF1*.

Some speculations can be put forward to explain the inability of mutants 101R, 4R and 1R to overproduce riboflavin. In the mutant 101R, integration of the cassette pL2 resulted in disruption of the *RIB1* gene (the first gene of the riboflavin synthesis pathway), cutting off 33 amino acids from the N-terminus. However the insertion did not result in riboflavin auxotrophy of the 101R mutant. The insertion created a hybrid ORF, in which the start cogon ATG originates from the bacterial part of the pL2 vector. Blast analysis showed that the catalytic domain of the GTP cyclohydrolase II is located in the C-terminus. Thus, insertion of the cassette led to

the separation of iron-regulated promoter from the ORF of *RIB1* gene, and the formation of a truncated version of Rib1p. Such alteration apparently led to the synthesis of small amounts of shortened Rib1p and resulted in the inability of the 101R strain to overproduce riboflavin in iron depleted media.

Analysis of the 4R mutant led to the identification of the *MET2* gene, which is involved in some way in the regulation of riboflavin synthesis and iron acquisition in *C. famata*. Disruption of the gene did not result in methionine auxotrophy. This implies that two alternative pathways for the formation of cystathionine via o-succinyl-L-homoserine and o-acetyl-L-homoserine exist in *C. famata*, as has been described for the yeast *Candida albicans* (http://www.genome.ad.jp/dbget-bin/get_pathway?org_name = cal&mapno = 00271) Contrary to *C. albicans*, in *S. cerevisiae* formation of cystathionine from homoserine occurs exclusively via o-acetyl-L-homoserine (Langin et al., 1986). The finding of *MET2* paralog (45% homology) in the *D. hansenii* genome could also be a possible explanation for the absence of methionine auxotrophy in the 4R mutant. The addition of methionine to the iron deplete medium partially restored the ability to oversynthesize riboflavin in the 4R mutant. Additionally, the 4R strain had two-fold increased intracellular concentration of iron during cultivation in iron deplete/replete media. This increased concentration of iron apparently caused the inability of the 4R mutant to overproduce riboflavin in the iron deplete medium. It can suggest that methionine shortage somehow activates iron transport into the cells of the 4R strain (*met2* mutant).

Disruption of the *SEF1* gene, encoding a potential transcription factor, was identified after analysis of the 1R mutant. Data concerning Sef1p functions are poor. The *Kluyveromyces lactis SEF1* and its *S. cerevisiae* homologue are able to suppress the mutation in Rpm2p, which is a protein subunit of yeast mitochondrial RNase P, an enzyme responsible for the 5′ maturation of mitochondrial tRNAs. DNA sequence analysis of the *K. lactis SEF1* gene revealed that it contained the Zn(2)-Cys(6) binuclear cluster motif found in a growing number of yeast transcription factors (Groom et al., 1998). Apparently *SEF1* also acts as a regulatory gene in riboflavin synthesis in *C. famata*. However, the mechanisms of such regulation remain to be elucidated (Dmytruk et al., 2006).

5.5.3 *Identified* C. famata *Genes Coding for Enzymes of Riboflavin Synthesis*

For cloning of *C. famata* genes involved in riboflavin synthesis, the riboflavin-auxotrophic mutants from the strain L20105 (*leu2*) were isolated. Their biochemical identification was carried out by identification of accumulated intermediates of riboflavin synthesis in the cultural medium of the mutants (Dmytruk et al., 2004). As a result, mutants deficient in GTP cyclohydrolase II (*rib1*), specific reductase (*rib2*), 6,7-dimethyl-8-ribityllumazine synthase (*rib5*), 3,4-dihydroxy-2-butanone-4-phosphate synthase (*rib6*) and riboflavin synthase (*rib7*) were isolated. All of them also carried the *leu2* mutation. The mutants were used as recipients for

transformation with the genomic DNA library of *C. famata* VKM Y-9 to clone corresponding genes by complementation of growth defects on the medium without leucine and riboflavin. The DNA fragments containing genes *RIB1, RIB2, RIB5, RIB6* and *RIB7* were isolated as a result of the cloning. Plasmids with these genes successfully complemented riboflavin auxotrophies of corresponding mutants of another flavinogenic yeast species, *Pichia guilliermondii* (Dmytruk et al., 2004; Abbas et al., 2006).

Cloned DNA fragments containing *C. famata RIB* genes were sequenced (Voronovsky et al., 2004).

The 1,763 bp *C. famata* DNA fragment (EMBL Accession No AJ810169) of plasmid pCR1Xb (Dmytruk et al., 2004) contains 1,029 bp open reading frame (ORF) encoding a protein of 343 amino acids with calculated molecular mass of 38.1 kDa. The derived amino acid sequence shows extensive homology to the GTP cyclohydrolase II sequences of yeasts, other fungi and prokaryotes. Data on comparisons of the amino acid sequences together with results on the functional complementation of *C. famata* and *P. guilliermondii rib1* mutations by the plasmid pCR1Xb are sufficient to indicate that the 1,029 bp ORF of 1,763 bp *C. famata* DNA fragment from the plasmid encodes the riboflavin synthesis enzyme GTP cyclohydrolase II (*RIB1* gene).

The 1,501 bp *C. famata* DNA fragment (EMBL Accession No. AJ810170) of plasmid pCR2-1 (Dmytruk et al., 2004) contains 786 bp ORF encoding a protein of 262 amino acids with calculated molecular mass of 29.1 kDa. The derived amino acid sequence shows homology to sequences of corresponding reductases of yeasts, other fungi and prokaryotes. Data on comparisons of the amino acid sequences together with results on the functional complementation of *C. famata* and *P. guilliermondii rib2* mutations by the plasmid pCR2-1 suggest that the 786 bp ORF of 1,501 bp *C. famata* DNA fragment encodes the riboflavin synthesis enzyme HTP reductase (*RIB2* gene).

The analysis of 1,465 bp *C. famata* DNA fragment (EMBL Accession No AJ810173) of plasmid pPR5 (Dmytruk et al., 2004) found a presence of 615 bp ORF (845–1,459; see the DNA fragment AJ810173 of EMBL Bank) containing the putative intron of 123 bp (888–1,010). The intron holds the 5′ (GTAAGT, 888–893) and 3′ (TAG, 1,008–1,010) splice sites and also the branch site (TACTAAC, 996–1,002) (Bon et al., 2003). The ORF without the intron is 495 bp and encodes a protein of 164 amino acids with calculated molecular mass of 18.2 kDa. The derived amino acid sequence shows extensive homology to the dimethylribityllumazine synthase sequences of yeast, other fungi and prokaryotes. Data on comparisons of the amino acid sequences, together with results on the functional complementation of *C. famata* and *P. guilliermondii rib5* mutations by the plasmid pPR5, demonstrate that the ORF of *C. famata* DNA fragment from the plasmid encodes the riboflavin synthesis enzyme dimethylribityllumazine synthase (*RIB5* gene).

The 1,333 bp *C. famata* DNA fragment (EMBL Accession No. AJ810171) of plasmid pF (Dmytruk et al., 2004) contains a 612 bp ORF encoding a protein of 204 amino acids with calculated molecular mass of 22.7 kDa. The derived amino acid sequence indicates extensive homology to the dihydroxybutanone phosphate

synthase sequences of yeasts, other fungi and prokaryotes. Data on comparisons of the amino acid sequences, together with results on the functional complementation of *C. famata* and *P. guilliermondii rib6* mutations by the plasmid pF, prove that the 612 bp ORF of 1,333 bp *C. famata* DNA fragment from the plasmid encodes the riboflavin synthesis enzyme dihydroxybutanone phosphate synthase (*RIB6* gene).

The 1,515 bp *C. famata* DNA fragment (EMBL Accession No. AJ810172) of plasmid pCR7 (Dmytruk et al., 2004) contains a 711 bp ORF encoding a protein of 237 amino acids with calculated molecular mass of 26.3 kDa. The derived amino acid sequence shows extensive homology to the riboflavin synthase sequences of yeasts, other fungi and prokaryotes. Data on comparisons of the amino acid sequences together with results on the functional complementation of *C. famata* and *P. guilliermondii rib7* mutations by the plasmid pCR7 are sufficient to assert that the 711 bp ORF of 1,515 bp *C. famata* DNA fragment from the plasmid encodes the riboflavin synthesis enzyme riboflavin synthase (*RIB7* gene).

Attempts to clone the *C. famata RIB3* gene encoding DRAP deaminase from the genomic DNA library by functional complementation of growth defect of corresponding auxotrophic mutant of *P. guilliermondii* were unsuccessful (available *rib3* mutants of *C. famata* were not applicable during these attempts because of their instability) (Dmytruk et al., 2004). Therefore, the gene was isolated as a homolog of corresponding yeast genes from genomic DNA of *D. hansenii* CBS767 after publication of complete genomic sequence of the strain. The homolog of yeast DRAP deaminase genes was isolated by PCR as a 2,569 bp DNA fragment containing the 1,821 bp ORF. The fragment was incorporated into the plasmid p19PR3 (Voronovsky et al., 2004). The isolated gene complemented riboflavin auxotrophy of *P. guilliermondii* strain *rib3* deficient in DRAP deaminase. Thus, high homology to DRAP deaminase genes of yeasts, other fungi and prokaryotes and capability of complementation of *P. guilliermondii rib3* mutation are sufficient to assert that the 1,821 bp ORF of 2,569 bp *D. hansenii* DNA fragment encodes the riboflavin synthesis enzyme DRAP deaminase (*RIB3* gene).

Thus, the *C. famata* (*D. hansenii*) genes for riboflavin synthesis encoding GTP cyclohydrolase II (*RIB1*), HTP reductase (*RIB2*), 6,7-dimethyl-8-ribityllumazine synthase (*rib5*), 3,4-dihydroxy-2-butanone-4-phosphate synthase (*rib6*), riboflavin synthase (*rib7*) and DRAP deaminase (*RIB3*) were cloned and identified. In addition, the involvement of *C. famata* (*D. hansenii*) genes homologous to *S. cerevisiae MET2* (homoserine O-acetyltransferase) and *SEF1* (putative transcription factor) in positive regulation of riboflavin synthesis was shown (see Sects. 5.5.2.2 and 5.5.2.3).

5.5.4 Development of a Promoter Assay System in C. famata

A system for analysis of promoter activities was developed for *C. famata,* based on the *K. lactis LAC4* gene encoding β-galactosidase as a reporter gene and *C. famata* mutant *lac4* unable for lactose utilization as a recipient strain (Ishchuk et al., 2008). The *E. coli* β-galactosidase gene *lacZ* could not be used in the system because of

difference in codon usage between the bacterium and *C. famata*. The *C. famata* mutant *lac4* was transformed with the plasmid containing analyzable promoters fused with the promoterless *LAC4* gene. Resulting transformants (unlike the mutant *lac4*) were able to utilize lactose as a sole carbon source. The promoter strength was estimated on the basis of β-galactosidase activity assayed in the transformants. Different promoters of *C. famata* and *D. hansenii* (a teleomorph of *C. famata*) were analyzed using this approach. The results showed an adequacy of the *K. lactis LAC4* gene for evaluation of promoter strength in *C. famata*.

C. famata and *D. hansenii* promoters *CfTEF1* (*C. famata* translation elongation factor 1A), *CfPGI1* (*C. famata* phosphoglucoisomerase), *DhPGK1* (phosphoglycerate kinase of *D. hansenii*), *DhPGI1* (*D. hansenii* phosphoglucoisomerase), *DhPHO5* (*D. hansenii* acid phosphatase) and *DhTPI1* (*D. hansenii* triosephosphate isomerase) were cloned by PCR using the genome database 'Genolevures' for *D.hansenii* (http://cbi.labri.fr/Genolevures/index.php). The *CfTEF1* and *CfPGI1* promoters were isolated by the inverse PCR. The primers were designed for isolation of central region of the *D. hansenii TEF1* and *PGI1* ORFs. Corresponding fragments of expected size were amplified using these primers with total DNA of *C. famata* VKM Y-9 as a template. These primers were used to design inverse primers complementary to corresponding *C. famata* genome regions. In such a way, the sequences of *C. famata* VKM Y-9 genomic fragments containing promoters and initial part of both *TEF1* and *PGI1* ORFs were isolated. Sequence comparison of the *TEF1* fragments of strains *C. famata* VKM Y-9 and *D. hansenii* CBS 767 showed 100% identity of initial ORF fragments but just partial homology for promoters. Sequence comparison of the *PGI1* genomic fragments of strains *C. famata* VKM Y-9 and *D. hansenii* CBS 767 showed again a very high level of identity (91%) for coding regions and much lower homology for analyzed promoter region.

Activities of promoters of the genes *CfTEF1*, *CfPGI1*, *DhPGK1*, *DhPGI1*, *DhPHO5* and *DhTPI1* were tested in *C. famata* using the *K. lactis LAC4* reporter gene. It is known that the promoter of yeast *TEF1* (translation elongation factor 1α) gene as well as promoters of the glycolytic genes *PGK1* (phosphoglycerate kinase), *PGI1* (phosphoglucoisomerase) and *TPI1* (triosephosphate isomerase) are strong and constitutive under many different conditions. The promoter for *PHO5* (acid phosphatase) gene is regulable and induced by limitation of phosphate. Plasmids bearing the mentioned above promoters fused with the *K. lactis LAC4* gene were introduced into the *C. famata lac4* mutant. Quantitative liquid β-galactosidase assays performed in the obtained transformants showed very low activities for the promoters *DhPGI1*, *DhPHO5* and *DhTPI1* in *C. famata* cells in spite of a high degree of relatedness between the *C. famata* and *D. hansenii* strains. Only one of the tested *D. hansenii* promoters, *DhPGK1*, showed relatively high β-galactosidase activity, which was comparable with that of the *CfPGI1* promoter. The promoters *DhPGK1* and *CfPGI1* displayed 3- and almost 4-times lower activities of β-galactosidase, respectively, compared to those expressed from the promoter *CfTEF1*. Thus, the promoter *CfTEF1* was found to be a strongest one among tested (Ishchuk et al., 2008).

The developed *LAC4* reporter system can be used as a potent tool for understanding the regulation of riboflavin synthesis, halotolerance and other processes in *C. famata* and *D. hansenii*.

5.6 Applied Aspects

This section describes current uses of *D. hansenii* (*C. famata*) in production of riboflavin, cheesemaking, meat fermentation, involvement of *D. hansenii* lytic enzymes in food industry (particularly in wine-making), the potential of the yeast in production of xylitol and some other chemicals.

5.6.1 Production of Riboflavin

The ability of *C. famata* (also known as *Candida flareri, Torulopsis candida*) to overproduce riboflavin in iron-deficient media is known for 60 years (Tanner et al., 1945). This species possesses the highest flavinogenic potential among other yeasts capable to overproduce riboflavin under iron limitation. Wild-type strains can accumulate in the medium near 600 μg ml^{-1} (Levine et al., 1949) whereas other flavinogenic yeasts accumulate 5–300 μg ml^{-1} and normally yeasts accumulate not more than 1–2 μg of riboflavin per ml (Sibirny et al., 2006).

Specially selected mutant of *C. famata* dep8, along with mutants of other microorganisms (bacterium *Bacillus subtilis,* mycelial fungus *Ahbya gossypii*) are used for industrial production of riboflavin (Stahmann et al., 2000). The *C. famata* riboflavin overproducing strain dep8 was isolated by classic multistep selection procedure (Heefner et al., 1988, 1992, 1993). On the first step of selection, cells of the wild-type strain *C. famata* NRRL Y245 mutagenized with nitrozoguanidine and adenine-deficient mutant was isolated which accumulated red pigment at high concentration of exogenous adenine. This suggested that mutant has impaired regulation of purine synthesis *de novo* (it is known that riboflavin is synthesized from purine precursor, GTP) (Shavlovsky and Logvinenko, 1988). Independently, the mutant was isolated from the wild-type strain which was resistant to riboflavin synthesis inhibition exerted by 5′-AMP. On the next stage of selection, mutants from first steps were hybridized by protoplast fusion. One of hybrid, named as GA18, showed 14 times increased riboflavin productivity relative to wild-type strain. After mutagenesis of GA18, several yellow colonies were picked up. The most stable of them, strain GA18Y8-6#2 was again mutagenized and plated on the medium with toxic glucose analog, 2-deoxyglucose. One most flavinogenic 2-deoxyglucose-resistant strain was again mutagenized and plated on the medium with toxic purine analog, 4-aminopyrazolo-(3,4-d)pyrimidine. The most flavinogenic strain, designated as ATCC 20755 was isolated after such selection step. From it, the new 2-deoxyglucose mutants were isolated, which served for several next rounds of selection for yellow

colonies on the medium with high riboflavin content and poor for nutrients. The best flavinogenic mutant, designated as dep8, was used for isolation of the mutants resistant to tubericidin, another purine structural analog. Several new mutants were isolated, however, they did not differ significantly from strain dep8 by their flavinogenic potential (Heefner et al., 1988, 1992, 1993; Sibirny et al., 2006).

Isolated strain accumulated during large-scale fermentation (volume of fermenter, 450 l, duration of fermentation, 200 h) around 20,000 μg riboflavin in 1 ml of the medium, which is comparable to riboflavin titers reached by other riboflavin overproducers (*B. subtilis, A. gossypii*) (Stahmann et al., 2000). The strain dep8, however, is not very stable and can revert to quite non-flavinogenic revertants (A. Sibirny, non-published observation). It is important to identify reverting locus/loci and the nature of evolved mutations. Depending on results of such studies, the strategy for strain stabilization can be developed. For fulfillment this work, methods of molecular genetics developed for *C. famata* can be used. One may assume that identified recently regulatory gene of positive control *SEF1* (Dmytruk et al., 2006) is important for riboflavin oversynthesis. If successful, the stable *C. famata* riboflavin overproducers can be superior to bacterial and fungal competitors. Indeed, the process based on the use of *B. subtilis* is sensitive to phagolysis whereas riboflavin overproduction by *A. gossypii* starts only after finishing the growth and during lysis of mycelium (Stahmann et al., 2000). The process based on *C. famata* is free from these drawbacks as yeasts are resistant to phagolysis and riboflavin synthesis occurs during growth and propagation of the producer. Further improvement of strains of industrial producer *C. famata* dep8 can be reached using developed methods for genetic manipulation of this species (Voronovsky et al., 2002; Dmytruk et al., 2006; Abbas et al., 2006). For example, structural genes of riboflavin synthesis can be amplified, GTP cyclohydrolase can be engineered to decrease or to block totally feedback inhibition exerted by FAD, genes of purine biosynthesis pathway can be overexpressed.

5.6.2 Cheese Production

D. hansenii (*C. famata*) is an essential species of microflora of many dairy products including cheeses. It is the common yeast species found in all types of cheese. *D. hansenii* is also present in brines of semi-hard and hard cheeses (Fleet, 1990). The prevalence of this yeast in cheeses and brines is a result of its peculiar properties: salt tolerance, ability to produce proteolytic and lipolytic enzymes that can metabolize milk proteins and fat, capacity to grow at low temperatures and low water activities (Fleet and Mian, 1987; Roostita and Fleet, 1996; Wyder and Puhan, 1999; Prista et al., 2005). In addition, there was shown a capability of *D. hansenii* to inhibit the germination in cheese brines of undesired microorganisms (such as *Clostridium butyricum* and *C. tyrobutyricum*) by out-competing them for nutrients and producing antimicrobial metabolites (Deiana et al., 1984; Fatichenti et al., 1983; Breuer and Harms, 2006). *D. hansenii* also modifies the cheese microenvironment

and thus supports some desired bacteria and/or fungi and protect cheeses from undesired carbohydrate fermentations (van den Tempel and Jacobsen, 2000; Breuer and Harms, 2006). The capacity of *D. hansenii* to assimilate lactate, citrate, lactose and galactose in addition to glucose is very important for cheese making: it places the yeast among favorable components of starter cultures for cheese production (Fatichenti et al., 1983; Welthagen and Viljoen, 1998).

D. hansenii is capable of synthesis of volatile acids and cheesy flavor compounds, namely methyl ketones with fruity, rose, cheesy, moldy, wine odors, and 2-phenylethanol (the faded-rose odor) (Arfi et al., 2002; Leclercq-Perlat et al., 2004). The capacity of *D. hansenii* and *K. marxianus* to govern the sensory properties of cheeses has been exploited in starter cultures containing these two species (Seiler and Busse, 1990). Like many cheese-ripening yeasts, *D. hansenii* can synthesize *S*-methylthioacetate, the most prevalent volatile sulphur compound in cheese. The yeast can also produce methional. This compound was found in some kinds of cheeses, for instance, in Cheddar and Camembert. Methional contributes to development of a strong Cheddar flavor (Ferreira and Viljoen, 2003). There was also found that *D. hansenii* capable to produce substantial amounts of another volatile sulphur compound: methylthiopropanal (Arfi et al., 2002).

D. hansenii possesses the peptidase activity. A peptidase with activity to β-casein-derived peptides, which significantly influenced proteolysis in cheese, was found in the yeast. There was also reported on capability of *D. hansenii* isolated from cheese to digest both α- and β-casein (Klein et al., 2002; Kumura et al., 2002; Leclercq-Perlat et al., 2000).

D. hansenii can produce pigments (Hansen et al., 2001; van den Tempel and Jacobsen, 2000). The production by this yeast of reddish-brown pigments involved in the brown surface discoloration of Portuguese ewes' cheese was reported (Carreira et al., 1998). The tyrosinase activity in *D. hansenii* was shown. It initiates oxidation of tyrosine to melanine (Nichol et al., 1996). The pigment production during ascospore formation that resulted in pigmented ascospores and brown color of cultures was also described (Nakase et al., 1998).

D. hansenii and some other yeasts involved in cheese ageing provide developing an appropriate bacterial surface flora by supplying with growth factors for the bacteria, such as vitamins and amino acids. Simultaneously, the yeasts, including *D. hansenii*, produce aroma components, lipolytic and proteolytic enzymes that govern the process of ripening (El Soda, 1986; Fleet and Mian, 1987; Guerzoni et al., 1993; Lépingle et al., 2000; Petersen and Jespersen, 2004). *D. hansenii* can grow both on the surface and inside of processed cheeses. The yeast was found in the curd or in the cheese interior, depending on the kind of cheese and composition of the involved starter culture (Deiana et al., 1984; Fatichenti et al., 1983; Ferreira and Viljoen, 2003; Fleet, 1990; Roostita and Fleet, 1996; van den Tempel and Jacobsen, 2000; Welthagen and Viljoen, 1998).

Thus, *D. hansenii* plays important and diverse roles in cheese production. No doubt, additional studies of the yeast and the process are needed to provide knowledge-based improvements of production efficiency and cheese quality.

5.6.3 Meat Fermentation

D. hansenii is the most common yeast species found in different sausages and minced beef (Dalton et al., 1984). There was shown that *Debaryomyces* could generate ammonia and several volatile compounds, alter the free amino acid contents of dry-cured sausages. Addition of *Debaryomyces* sp. as a starter culture generally modified the flavour profile of the sausages (Dura et al., 2004b). Effect of *Debaryomyces* spp. on production of volatile compounds during the ripening of dry-cured sausages by inhibiting the generation of lipid oxidation products and promoting the generation of ethyl esters, processes that contribute to the development of typical sausage aroma was also reported (Flores et al., 2004). A decrease of pH to 4.5 during the growth of *Debaryomyces* under dry-cured sausage processing conditions increased the yield of alcohols and aldehydes, while the transition from exponential to stationary growth phase diminished alcohol and aldehyde production but increased acid generation (Dura et al., 2004b). Similar results were reported for the modification of flavour profiles of dry-cured ham (Martin et al., 2004). Thus, representatives of *Debaryomyces* spp. govern the sensory properties of the meat by producing volatile compounds.

A few enzymes of *D. hansenii* or *Debaryomyces* spp. involved in meat fermentation were described. Activities of prolyl aminopeptidase, arginyl aminopeptidase and glutaminase during the ripening of dry-cured sausages were reported (Bolumar et al., 2003a, b; Dura et al., 2004a). Nitrate, nitrite, glucose and ascorbic acid (curing agents), which inhibit growth of the pathogen *Clostridium botulinum* and are responsible for the pink colour associated with cured meats and for particular meat flavours, had no significant effect at levels typically used in meat processing on the activity of glutaminase (Dura et al., 2004a).

Yeasts play not only a positive role in meat processing. There is a range of reports on identification of yeasts (including *D. hansenii*) in the surface slime of sausages (Lodder 1970; Breuer and Harms, 2006). Thus, the yeasts are involved in spoilage of sausages.

Current knowledge on the effect of *D. hansenii* on meat fermentation is far not sufficient. Further studies are needed for elucidation of the effect and use of this yeast in the meat fermentation.

5.6.4 Lytic Enzymes

Some lytic enzymes of *D. hansenii* are involved in wine production. Particularly, β-glucosidases from *Debaryomyces* can liberate monoterpenols from β-D-glucopyranoside, β-D-xylopyranoside, α-L-arabinofuranoside and α-L-rhamnopyranoside. This property is useful for a possible enzyme treatment as part of the processing of terpenol-containing juices, leading to an increase of flavor compounds in the wine (Yanai and Sato, 1999). Some non-conventional yeasts, including *Debaryomyces*,

exhibit significantly higher levels of β-glucosidase activity in the absence of glucose than *S. cerevisiae*, indicating that they have a stronger role in flavor development during wine production than baker's yeast (Charoenchai et al., 1997). The production of extracellular glucose-tolerant and thermophilic β-glucosidases in wine making by 48 yeast strains belonging to the genera *Candida, Debaryomyces, Kluyveromyces* and *Pichia* was explored (Saha and Bothast, 1996). The β-glucosidases of *Debaryomyces* strains had very low activity, but they were able to hydrolyse cellobiose without inhibition of the enzymes by glucose. Moreover, the enzymes had a high optimal temperature: 65°C. The intracellular β-glucosidase from *D. hansenii* was purified and analysed (Yanai and Sato, 1999). It was tested in the fermentation of Muscat juices. This enzyme has a significant tolerance to high concentrations of glucose in grape juices, in contrast to commercially available β-glucosidases of mycelial fungi, which are sensitive to glucose (Yanai and Sato, 1999).

D. hansenii along with other non-*Saccharomyces* wine yeasts possesses with esterase, one of the main enzymes involved in wine production (Besancon et al., 1995; Esteve-Zarsoso et al., 1998). The enzyme was isolated from *D. hansenii* and partially characterized (Besancon et al., 1995). The enzyme hydrolysed tributyrin and ethyl butyrate with maximal activity, methyl and ethyl esters of short fatty acids (C_2–C_5) rapidly and esters of longer-chain fatty acids (C_6–C_{14}) moderately quickly. Aliphatic and aromatic acetate esters were also hydrolysed by the esterase.

D. hansenii has an activity of superoxide dismutase (SOD), a metalloenzyme catalysing the dismutation of superoxide radicals. The yeast can be a source of the enzyme. SOD has important applications in medicine and food industry, including anti-inflammation, immune-response modulation, malignant tumor regression, radiation and chemotherapy protection, premenstrual syndrome, arthritis, and anti-ageing treatments, during the use of hyperbaric chambers, and against oxidative stress in general (Garcia-Gonzalez and Ochoa, 1999; Orozco et al., 1998). The SOD gene of *D. hansenii* was cloned and sequenced (Hernandez-Saavedra and Romero-Geraldo, 2001). Potentially, SOD production using *D. hansenii* is very competitive compared to current methods, due to ability of the yeast to grow in media with a wide range of cultivation parameters (Ochoa et al., 1995).

5.6.5 Production of Xylitol

D. hansenii is one of the best xylitol-producing yeasts (Parajo et al., 1997; Roseiro et al., 1991). The capability of the yeast to produce xylitol from D-xylose is studied actively for many years (Roseiro et al., 1991; Girio et al., 1994; Parajo et al., 1997). However, profitable industrial technology for production of xylitol using this yeast is still not developed.

Xylitol is used in the food industry as a sweetener and anti-caries tool. It is employed in the manufacture of sugar-free food for diabetics (Cruz et al., 2000a). Xylitol is formed as a metabolic intermediate of D-xylose fermentation, where D-xylose is converted into xylitol by xylose reductase (XR). The enzyme has broad

substrate specificity for aldehydes and aldol sugars. The overproduction of xylitol by *D. hansenii* was shown to be a result of combination of high NADPH-dependent XR activity and low xylitol dehydrogenase (XDH) activity (Converti and Dominguez, 2001). *D. hansenii* can convert xylose to xylitol as effective as other yeast representatives of xylitol producers: *C. guilliermondii*, *C. boidinii* and *C. parapsilosis* (Barbosa et al., 1988; Furlan et al., 1991). The level of oxygen supply is very important for conversion of D-xylose to xylitol by *D. hansenii*. The conversion cannot be performed under strictly anaerobic conditions, because of very low activity of XR in the absence of oxygen. It prevents assimilation of D-xylose by the yeast (Dominguez, 1998; Roseiro et al., 1991). On the other hand, under strictly aerobic conditions the reaction products are used for biomass production resulting in very little or absence of xylitol. High oxygen levels inhibit XR more than XDH, and thus affect xylitol production in *D. hansenii* (Girio et al., 1994). Therefore, the accumulation of xylitol is increased by limited aeration (Parajo et al., 1997; Converti and Dominguez, 2001). *D. hansenii* cultivated on oxygen-excess chemostat produced neither ethanol nor xylitol over the entire range of dilution rates tested (Nobre et al., 2002). On oxygen-limited chemostat the metabolism of the yeast changed substantially and, due to oxidative phosphorylation limitation, the cell biomass decreased and xylitol became the major extracellular product.

There are a few publications describing the capability of *D. hansenii* to grow in sugar mixtures. Particularly, according to the one article, growth on pentoses was slower than growth on hexoses, but the values obtained for biomass yields were very similar with the two types of sugars. Furthermore, when mixtures of two sugars were used, a preference for one carbon source did not inhibit consumption of the other (Nobre et al., 1999). It is important during fermentation of hydrolysates of hemicelluloses, which often consist of sugar mixtures. The other study showed a dependence of the growth of *D. hansenii* on the type of carbohydrate supplied (Tavares et al., 2000). According to the latter article, D-glucose inhibited or retarded the utilization of D-xylose, and the yeast preferentially assimilated the sugars in a substrate mixture in the order D-glucose, D-mannose, D-xylose.

Dilute-acid hydrolysis of hemicellulose results in a range of byproducts (inhibitors), such as furfural and hydroxymethylfurfural, acetic acid, lignin degradation products, phenols. These compounds limit fermentation of resulting hydrolysates. Submission of the hydrolysates to detoxification treatments minimized inhibitory effects. Particularly, activated charcoal adsorption removed most of the lignin-derived compounds; evaporation was able to reduce the acetic acid concentration below the inhibition threshold. The combination of the treatments gave improvement of fermentation of the D-xylose solutions into xylitol (Converti et al., 1999). During growth of *D. hansenii* on undetoxified dilute-acid hydrolysate of brewery's spent grain, the yeast showed high biomass yields and productivity (Carvalheiro et al., 2004). Detoxification of the substrate with activated charcoal did not increase biomass yield and improved the volumetric productivity just slightly. Thus, detoxification was not useful in the study (Carvalheiro et al., 2004). The obtained results also suggest that *D. hansenii* (or some strains at least) during growth in some lignocellulosic hydrolysates has a resistance to fermentation inhibitors.

Optimization of xylitol production processes is very important for achievement of maximal productivity and yield of the final product. The importance of high cell concentrations for efficient xylitol production was shown. For instance, a low xylitol productivity (0.088 g $(l \times h)^{-1}$) and product yield (0.57 g g^{-1}) were reached when the starting cell concentration below 16 g biomass l^{-1} and D-xylose concentration above 70 g xylose l^{-1} were taken for fermentation. But, when initial cell concentration was increased to 50 g biomass l^{-1} at the same D-xylose concentration (>70 g xylose l^{-1}), the xylitol productivity amounted to 0.50 g $(l \times h)^{-1}$ and the yield was 0.73 g g^{-1} (Parajo et al., 1996). The high xylitol productivity (2.53 g $(l \times h)^{-1}$) was achieved in continuous fermentation of *D. hansenii* with maintaining the high cell concentration (with cell recycling at a dilution rate of 0.284 h^{-1}) (Cruz et al., 2000b). In addition to cell concentration, the substrate concentration is an important factor as well. High xylitol productivities (0.481–0.694 g $(l \times h)^{-1}$) and product yields (0.74–0.83 g g^{-1}) were reached during fermentation of *D. hansenii* at xylose concentrations between 90 and 200 g l^{-1} (Converti et al., 2002). The further increase of D-xylose concentrations (over 200 g l^{-1}) resulted in substrate inhibition. A study of effects of starting xylose concentration and oxygen mass flow rate on xylitol production from rice straw hydrolysate by *D. hansenii* found the optimal D-xylose concentration (71 g l^{-1}) and oxygen mass flow rate (4.1 mg O_2 s^{-1}) required to reach the maximal productivity (0.53 g $(l \times h)^{-1}$), product yield (0.71 g g^{-1}) and final xylitol concentration (42.2 g l^{-1}) (De Faveri et al., 2004).

Data presented on xylitol production by *D. hansenii* show that this process has been intensively studied. However, both the yeast and the process have to be further improved to provide a profitable industrial technology for production of the alditol.

In addition to xylitol, *D. hansenii* can synthesize other biotechnologically relevant products. Particularly, the *C. famata* strain is capable to produce D-arabinitol from D-glucose (Ahmed et al., 1999). The efficient conversion of glucose to arabinitol by *D. hansenii* (*C. famata*) can be used as a first step for production of D-xylitol from D-glucose (Mayer et al., 2002). Thiamine auxotrophs of *D. hansenii* accumulate pyruvic acid under thiamine limitation. Thus, potentially, the yeast can be used for production of the acid. *Debaryomyces* has a biotechnological advantage because it is capable to use inorganic ammonium as a sole nitrogen source (Yanai et al., 1994). Pyruvic acid is widely used in chemical, pharmaceutical and agrochemical industries. The acid is produced mainly by chemical method. But, the biotechnological production of pyruvic acid using the yeast *Candida glabrata* is also known (Li et al., 2001). The yield of pyruvic acid has to be improved significantly for *D. hansenii* to provide the industrially profitable process using the yeast.

5.7 Future Perspectives

C. famata (D. hansenii) possesses several interesting peculiarities, among which the highest productivity of riboflavin synthesis among all natural yeast isolates is apparently the most outstanding feature. Due to sophisticated scheme of classic

selection, very active industrial producer was obtained which accumulate more than 20 mg riboflavin per ml of the cultural broth. However, to compete with modern industrial riboflavin producers *B. subtilis* and *A. gossypii*, isolated on the base of molecular genetics methods, available industrial producer *C. famata* dep8 has to be improved using developed modern approaches of metabolic engineering. Strategies for strain improvement can include the amplification of structural genes of riboflavin synthesis (all genes or those limiting the process), protein engineering of GTP cyclohydrolase for decrease or total alleviation of feedback inhibition of this enzyme by FAD, increase in supplying the riboflavin synthesis with purine precursor, which can be achieved by overexpression the pathway of purine synthesis *de novo* and reactions from IMP to GMP as well as by impairment of allosteric inhibition of glycine phosphoribosylaminotransferase, the first enzyme of purine nucleotide biosynthesis pathway. Additionally, genetic stability of current producer has to be elevated. Riboflavin overproducers can be used as parental strains for construction of the producers of flavin nucleotides, FMN and FAD. As in *C. famata* riboflavin synthesis is not repressed by flavins, this organism can easier permit producers of flavin nucleotide relative to other riboflavin producers. However, question of toxicity of excess of flavin nucleotide content in the cell remains this possibility the open question.

Modern approaches of metabolic engineering and natural ability of *D. hansenii* to produce pentitols (xylitol and arabitol) opens possibility to construct efficient strains which will be able for efficient conversion of cheap glucose to xylitol or arabitol (with subsequent conversion of the last compound to xylitol using bacterial isomerase). As xylitol market is large and increasing, the corresponding work is highly desirable.

Much attention has been paid to natural tolerance of *D. hansenii* to high salt concentration. This feature promises potential benefit for many biotechnological processes as this yeast can be cultivated in semi-sterile conditions (Breuer and Harms, 2006). However, molecular mechanisms of high salt tolerance are far from elucidation and have to be studied in more details. Further development deserves ability of this species to produce some enzymes and toxins, to participate in cheese ripening and other processes.

To achieve these goals, researchers need to improve molecular genetic tools for studying this species. It is especially important to develop methods which provide homologous recombination, precise gene integration and knock out for *C. famata (D. hansenii)*.

5.8 Conclusions

C. famata (D. hansenii) represents the group of osmotolerant yeast strains capable of growing at high salt concentration (up to 4 mM NaCl). This yeast is natural overproducer of riboflavin, capable of xylitol, arabitol and industrially important enzyme production. Strains of this species are involved in ripening of cheeses and

in meat fermentation. Industrial riboflavin production based on *C. famata* strain dep8 exists. During last several years, much progress was achieved in molecular genetics study of *C. famata* (*D. hansenii*) due to complete sequencing genome of the type strain CBS767 and development of transformation system and methods of insertional mutagenesis for the strain VKM Y-9. These achievements are the pre-requisite for further development of existing process of riboflavin production as well as for creation the strains capable of production flavin nucleotides, xylitol from glucose and some industrial enzymes.

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Chapter 6
Pichia guilliermondii

Andriy A. Sibirny and Yuriy R. Boretsky

Contents

Abstract *Pichia guilliermondii* (asporogenous strains of this species are designated as *Candida guilliermondii*) is the model organism of a group so named "flavinogenic yeasts" capable of riboflavin oversynthesis during starvation for iron. Besides, some strains of this species efficiently convert xylose to xylitol, an anti-caries sweetener. However, there are also pathogenic *C. guilliermondii* strains. This species has been used for studying enzymology of riboflavin synthesis due to overproduction of participating enzymes and intermediates under iron-limiting conditions as well as for identification of genes of negative and positive action involved in such a regulation. Besides, *P. guilliermondii* was used for identification and studying the properties of the systems for active transport of riboflavin in the cell (riboflavin permease) and out of the cell (riboflavin "excretase"). The genetic line of *P. guilliermondii* with high fertility has been selected and the methods of classic genetics (hybridization and analysis of meiotic segregation) have been developed. More recently, tools for molecular genetic studies of *P. guilliermondii* have been developed which include collection of host strains, vectors with recessive

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and dominant markers, several transformation protocols including that for gene knock out. Recently, the genome of this yeast species was sequenced and become publicly available (http://www.broad.mit.edu).

Keywords Flavinogenic yeasts, riboflavin, iron-limiting condition, riboflavin, permease, *Pichia guilliermondii*

6.1 Introduction

Pichia guilliermondii (asporogenous strains of this species are designated as *Candida guilliermondii*) belongs to the group so named "flavinogenic yeasts" capable of riboflavin oversynthesis during starvation for iron (Tanner et al., 1945). Other yeast species which can be referred as "flavinogenic" are: *Candida famata* (other names: *Candida flareri, Torulopsis candida*, teleomorph is known as *Debaryomyces hansenii*), *Schwanniomyces occidentalis* and *Candida albicans* (Shavlovsky and Logvinenko, 1988a; Knight et al. 2002). *P. guilliermondii* is often considered as the model organism for the group of flavinogenic yeasts as only for this flavinogenic yeast species methods both of classic and molecular genetics have been developed (Sibirnyi et al., 1977b; Boretsky et al., 1999). Besides, some strains of this species efficiently convert xylose to xylitol, anti-caries sweetener (Canettieri et al., 2001). However, there are also pathogenic *C. guilliermondii* strains (Krcmery and Barnes, 2002). *P. guilliermondii* has been used for studying enzymology of riboflavin synthesis due to overproduction of participating enzymes and intermediates under iron-limiting conditions as well as for identification of genes of negative and positive action involved in such a regulation. Besides, *P. guilliermondii* was used for identification and studying the properties of the systems for active transport of riboflavin into the cell (riboflavin permease) and out of the cell (riboflavin "excretase"). The genetic line of *P. guilliermondii* with high fertility has been selected and the methods of classic genetics (hybridization and analysis of meiotic segregation) have been developed (Sibirnyi et al., 1977b, c). More recently, tools for molecular genetic studies of *P. guilliermondii* have been developed which include collection of host strains, vectors with recessive markers and several transformation protocols including that for gene knock-out. Since 2005 genome of this yeast species was sequenced and become publicly available (http://www.broad.mit.edu).

6.2 Classification of *Pichia guilliermondii,* Physiology and Research History

Pichia guilliermondii Wickerham represents a collection of sporogenous strains which formerly were classified as the asporogenous species *Candida guilliermondii* (Cast.) Langeron a. Guerra (Wickerham and Burton, 1954; Wickerham, 1966). This

means that each strain of *C. guilliermondii* which is able to hybridize with any strain of *P. guilliermondii* must be transferred to the latter species, for example, even the type strain C. *guilliermondii* ATCC 9058 must now be considered as *P. guilliermondii* (Sibirny et al., 1977b). It is not known at the moment, how many strains really belong to species *Candida guilliermondii*, i.e. are unable to hybridize.

According to web site of Broad Institute, *Candida guilliermondii* (teleomorph *Pichia guilliermondii*) is a rarely observed pathogen, accounting for a few percent of all candidemias (see: *http://www.broad.mit.edu/annotation/genome/candida_guilliermondii/Info.html#t0*). However, for cancer patients, *C. guilliermondii* is the primary cause of fungemia. *C. guilliermondii* is more distantly related to *C. albicans* than *C. tropicalis*, but is more closely related than *C. lusitaniae*. *C. guilliermondii* shares in common with *C. albicans*, *C. tropicalis*, and *C. lusitaniae* the fact that CTG encodes serine rather than leucine, which is a marked distinction from other more distantly related hemiascomycetes including *Ashbya gossypii*, *Saccharomyces cerevisiae*, and *Candida glabrata*. Statement that "*C. guilliermondii* is haploid and has retained a complete sexual cycle including meiosis and sporulation" is contradictory to common view that genus *Candida* includes only asporogenous species.

Earlier, the genetics of *P. guilliermondii* was studied almost exclusively in the former USSR (mostly in Lviv and Kiev, Ukraine), and the former GDR (in Greifswald). The interest to genetic studies of this species was based primarily on its ability to utilize hydrocarbons as sole carbon and energy source (Shchelokova et al., 1974) and production of single-cell protein from hydrocarbons. Formerly, the industrial strain used for that purpose was erroneously identified in the USSR as *Candida guilliermondii,* however, it had been later re-identified as *Candida maltosa* (Bykov et al., 1987). The second reason is the potential of *P. guilliermondii* to convert xylose to xylitol, an anti-caries sweetener (Sene et al., 2001; Carvalho et al., 2002; Rodrigues et al., 2006). This yeast species appears to be the most effective organism for bioconversion of xylose into xylitol and is able to utilize even hemicellulosic hydrolysates obtained by acid hydrolysis (Canettieri et al., 2001). Another reason causing an interest to this yeast species is potential pathogenic properties of some clinical isolates classified as a C*andida guilliermondii* (Krcmery and Barnes, 2002; de Vos et al., 2005). It should be stressed that no cases of candidiasis caused by laboratory strains of *P. guilliermondii* were noted during decades of work with this species, at our institute. Moreover, all patented *P. guilliermondii* strains were properly checked before patenting, and no one of them was found to be pathogenic to laboratory animals. Thus, it can be stated that type strains of *P. guilliermondii* belong to GRAS organisms (Generally Recognized As Safe) and can be used as a model strains for different purposes.

An ability of *P. guilliermondii* strains to overproduce riboflavin (vitamin B_2) during growth in iron-deficient media (Tanner et al., 1945) attracts an especial interest (Shavlovsky and Logvinenko, 1988b). 146 strains of *P. guilliermondii* out of 147 analyzed were able to excrete a yellow pigment, identified as riboflavin, during cultivation in iron-deficient medium (Shavlovsky et al., 1978). Thus, the ability to overproduce riboflavin in the cultural liquid during cultivation in iron-deficient media, could be a species characteristic of *P. guilliermondii*. Such ability is known

for several yeast species that belong to different genera *(Candida famata (Debaryomyces hansenii), Debaryomyces subglobosus, Schwanniomyces occidentalis, P. guilliermondii*) (Tanner et al., 1945; Shavlovsky and Logvinenko, 1988b). Although this phenomenon was first described in 1945, neither the physiological role, nor the mechanisms of iron-dependent regulation of flavinogenesis are known. *P. guilliermondii* can be considered as the model organism for this group, due to development of the methods for classic and molecular genetics of this species. Unlike *D. hansenii* and other representatives of this group, *P. guilliermondii,* being heterothallic species, exists in both haploid and diploid states and can be induced to mate and sporulate. Genetic lines producing large amounts of spores have been isolated and used to study the regulation of riboflavin biosynthesis in this yeast species (Sibirny et al., 1977b). More recently, nucleotide sequence of the genome of this yeast species become publicly available at http://www.broad.mit.edu. Cells of *P. guilliermondii* are heterogeneous, mostly elongate in shape (approx. 2 × 10 μ), sometimes forming a pseudomycelium. The natural habitat of the species is diverse. A study of 140 strains of *P. guilliermondii* isolated from natural habitats showed that the most frequent source of their isolation is oil-containing soil (123 strains); others were isolated from plant leaves, lake water, and cow paunch (Zharova et al., 1980).

All known strains do not utilize lactose, starch, and inositol, whereas they differ in their ability to utilize D-ribose, D-arabinose, D-cellobiose, D-melibiose, salicin, L-rhamnose, L-sorbose, and dulcitol. All known strains utilize hydrocarbons (natural mixtures or n-hexadecane) as sole source of carbon and energy (Kreger van Rij, 1970). Growth on other respiratory substrates, such as ethanol, glycerol, succinate, or citrate, is satisfactory. *P. guilliermondii* is a typical representative of aerobic yeasts, and cannot grow under strictly anaerobic conditions. The standard growth temperature for *P. guilliermondii* is 30°C. The upper limit is near 42°C. Standard media for yeast cultivation can be used for laboratory cultivation of *P. guilliermondii* (YEPD or YEPS in which glucose is substituted by sucrose, are used as "complete" media). As minimal medium, modified Burkholder medium is used (Burkholder, 1943; Shavlovsky et al., 1978). Other standard media can also be used in work with *P. guilliermondii* (Sibirny et al., 1977b).

6.3 Biosynthesis of Riboflavin and Its Regulation

6.3.1 Elucidation of Riboflavin Biosynthetic Pathway

As mentioned before, *P. guilliermondii* was the only genetically studied eukaryote able to regulate riboflavin biosynthesis in response to iron concentration in the medium. This organism was used for identification of structural genes of flavinogenesis as well as for studying mutations impairing a regulation of this biosynthetic pathway.

Using strains of opposite mating types of *P. guilliermondii* genetic lines, 114 riboflavin-deficient mutants were isolated after UV irradiation. Biochemical study based on analysis of accumulated riboflavin intermediates (and their derivatives)

divided them into four biochemical groups. Complementation analysis of 106 mutants revealed 7 complementation classes *(RIB1* to *R1B7).* Later one of them (*RIB4*) was omitted as a double (*rib1 rib5*) mutant. The strains of the biochemical group, accumulating no specific products, corresponded to complementation group I (*rib1*). They lost activity of GTP cyclohydrolase II which catalyses first reaction of riboflavin biosynthesis. *S*trains accumulating blue fluorescenting compounds (apparently derivatives of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinedione 5′-phosphate and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione) were combined into biochemical group 2 which comprises two complementation classes *RIB2* and *RIB3*. Strains able to grow in the medium supplemented with 3,4-dihydroxybutanone (this compound directly react with 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione yielding 6,7-dimethyl-8-ribityllumazine – immediate precursor of riboflavin) were combined into biochemical group 3 which also comprises two complementation classes, namely *RIB5* and *RIB6*. And finally, mutants accumulating 6,7-dimethyl-8-ribityllumazine were combined into group 4 which corresponds to complementation class *RIB7*. They were shown to be defective in a gene specifying riboflavin synthase that catalyses last step of riboflavin biosynthesis (Shavlovsky et al., 1979). Biochemical and genetic studies of *P. guilliermondii* riboflavin auxotrophs showed complete identity of known steps of riboflavin biogenesis with those in *S. cerevisiae*. Later, detailed enzymological analysis of *P. guilliermondii* riboflavin auxotrophs was carried out. Such analysis, by the way, helped to identify new enzymes catalyzing, unstudied steps of riboflavin biosynthesis (Logvinenko et al., 1985). Also, well characterized *P. guilliermondii* riboflavin auxotrophs were used to facilitate identification of genes coding for enzymes of riboflavin biosynthesis in *Candida famata* (Voronovsky et al., 2002; Voronovsky et al., 2004). The formation of 6,7-dimethyl-8-ribityllumazine was studied using extracts of *rib5* and *rib6 P. guilliermondii* mutants with impaired synthesis of proteins P1 and P2, respectively. It was shown that synthesis of 6,7-dimethyl-8-ribityllumazine took place in extracts of *rib5* mutant (active P1 protein) in the presence of 2,4-dihydroxy-5-amino-6-ribitylaminopyrimidine and the compound formed from ribulose-5-phosphate by extracts of *rib6* mutant (active P2 protein). No lumazine was formed in extracts of *rib6* mutant from pyrimidine substrate and ribose-5-phosphate preincubated pre-incubated with extracts of *rib5* mutant. It was decided that P1 protein (the product of RIB5 gene) participates in the biosynthesis of 6,7-dimethyl-8-ribityllumazine from 2,4-dihydroxy-5-amino-6-ribitylaminopyrimidine and an aliphatic intermediate, which is formed from ribulose-5-phosphate, under the action of P2 protein (the product of RIB6 gene). Thus, it was shown that ribulose-5-phosphate is a second precursor of riboflavin (Logvinenko et al., 1987). Later the aliphatic precursor formed from ribulose-5-phosphate was determined as 3,4-dihydroxy-2-butanone-4-phosphate (Volk and Bacher, 1988, 1990).

At present reactions of this biochemical pathway is studied in details (Bacher, 1991). The initial step in the biosynthetic pathway is the opening of the imidazole ring of GTP catalyzed by the enzyme, GTP cyclohydrolase II (Shavlovsky et al., 1980). The product of this enzyme has been reported to be 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate. This intermediate is converted

to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione by a sequence of side chain reduction, ring deamination, and dephosphorylation. The hypothetical enzyme involved in the dephosphorylation of 5-amino-6-ribitylamino 5′-phosphate is still unknown. The conversion of 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione to 6,7-dimethyl-8-ribityllumazine by the enzyme, 6,7-dimethyl-8-ribityllumazine synthase, requires a second substrate, 3,4-dihydroxy-2-butanone 4-phosphate, which is obtained from ribulose 5-phosphate by the catalytic action of 3,4-dihydroxy-2-butanone-4-phosphate synthase. Finally, 6,7-dimethyl-8-ribityllumazine is converted to riboflavin by a dismutation reaction catalyzed by riboflavin synthase (Bacher, 1991; Fischer and Bacher, 2005). Thus, one mole of GTP and two moles of ribulose 5-phosphate are required to biosynthetically generate one mole of riboflavin. A scheme of riboflavin biosynthesis is given in Fig. 6.1.

Finally riboflavin is converted into flavin mononucleotide and then into flavin adenine dinucleotide (FMN and FAD correspondingly), which are universally required for indispensable redox reactions in all cellular organisms. The first reaction is catalyzed by riboflavin kinases. The second reaction is catalysed by FAD synthetases (FMN adenylyltransferase). UTP, GTP, ADP and CTP, besides ATP, can be used as a phosphate donors by the kinase purified from *P. guilliermondii* (Kashchenko et al., 1978). In contrast only ATP was utilized by the enzyme purified from *Bacillus subtilis*. This enzyme preparation also differs from other flavokinases by specificity to reduced form of flavins. It catalysed formation of FMN-H2 and FAD-H2 from reduced riboflavin and ATP, but not the synthesis of FAD from FMN and ATP (Kearney et al., 1979). One can suggests that in general eukaryotes possess two different enzyme catalyzing conversion of riboflavin to FAD whereas bacteria often possess a bifunctional enzyme which exhibit both flavokinase and FMN adenylyltransferase activities (Hagihara et al., 1995; Mack et al., 1998; Santos et al., 2000).

6.3.2 Regulation of Riboflavin Biosynthetic Pathway

Several groups of the mutants affected in the regulation of riboflavin biosynthesis by iron ions were isolated in *P. guilliermondii*, which differed genetically and physiologically. Original approaches were used for isolation of derepressed mutants. One of them is based on selection of the mutants resistant to the riboflavin structural analog, 7-methyl-8-trifluoromethyl-10-(1′-D-ribityl)isoalloxazine. Since wild-type strains of *P. guilliermondii* are resistant to riboflavin analogs due to their slow penetration into the cell, mutants with multiple sensitivity to antibiotics and antimetabolites (Sibirny et al., 1977a, b) were used as initial strains for selection (Shavlovsky et al., 1980b).

Besides, it was found that high concentrations of sulphate or phosphate anions induce increasing sensitivity to antibiotics and antimetabolites (including riboflavin analogs) of the wild-type strains of *P. guilliermondii* and of other yeast species (Sibirny and Shavlovsky, 1981); it was possible to isolate mutants resistant to riboflavin analogs directly from wild-type strains on plates containing high sulphate

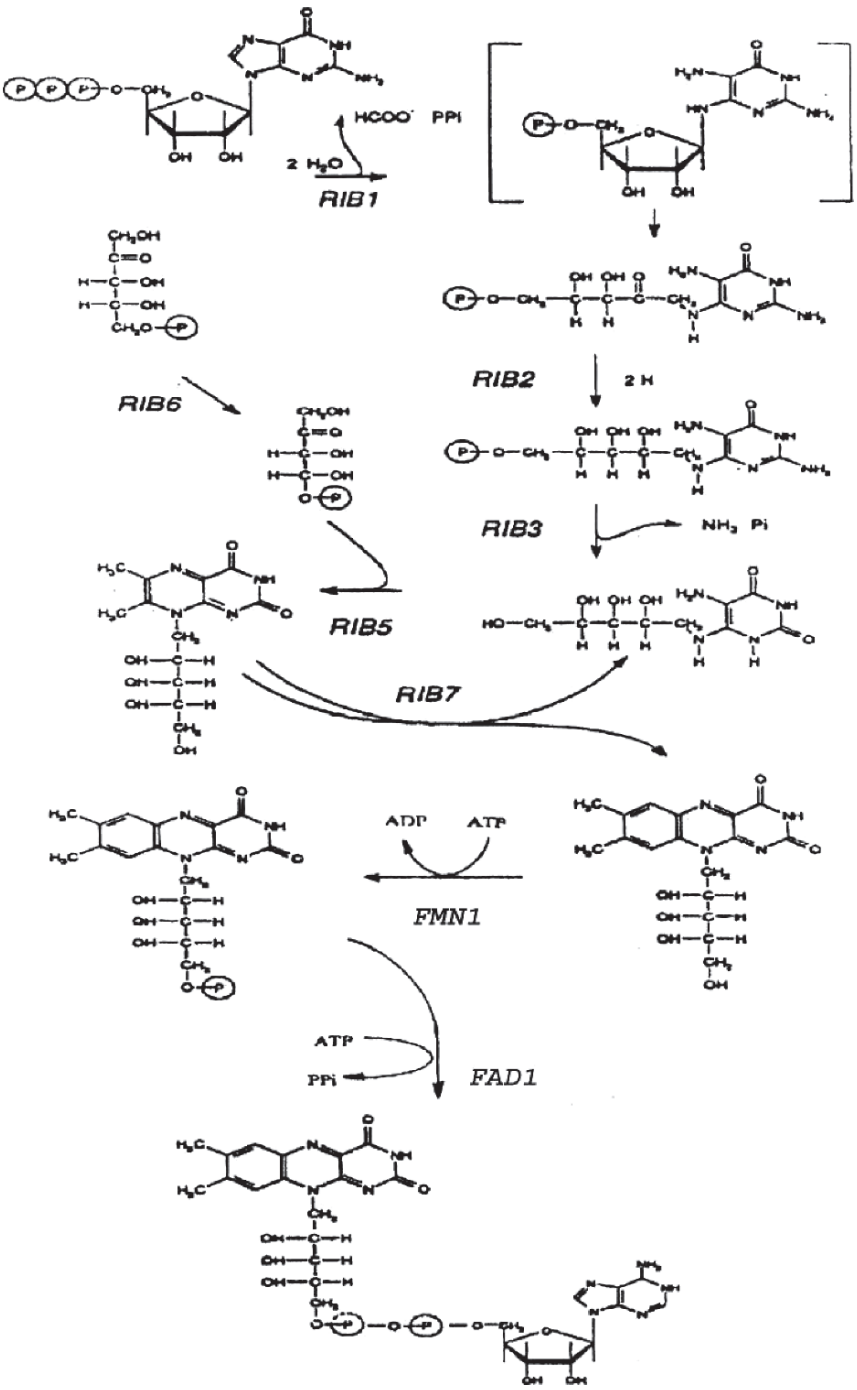

Fig. 6.1 Riboflavin biosynthesis

concentrations (Shavlovsky et al., 1985a). Additionally, derepressed mutants were isolated from previously selected temperature sensitive *rib1* mutants as pseudorevertants able to grow in iron-rich media at the elevated (restrictive for its *rib1* parental strains) temperature (Shavlovsky et al., 1985a). A third approach for isolation of riboflavin-derepressed mutants used leaky *rib2* auxotrophs as initial strains. Activity of the second enzyme of flavinogenesis in the leaky *rib2* strain was so low that they grew without exogenous riboflavin only in iron-deficient media, where other enzymes were derepressed. Selection of leaky *rib2* mutants capable of growing in iron-rich medium without riboflavin allowed the isolation of regulatory mutations enhancing riboflavin production in *P. guilliermondii* (Shavlovsky et al., 1982).

As a result of the use of three above-mentioned methods, riboflavin-overproducing mutants forming two groups of complementation (designated *rib80* and *rib81*) were isolated. Selected mutants over-synthesized riboflavin and exhibited significantly enhanced rate of radioactive iron uptake. Both groups of mutations appeared to be recessive, monogenic and possessed nuclear localization. (Shavlovsky et al., 1982, 1985a, b). Phenotype of *rib80* mutants depends on carbon source in the medium. They did not overproduce riboflavin and did not hyper accumulate iron when glucose in the medium is substituted by dulcitol, glycerol or succinate (Shavlovsky et al., 1990). It was speculated that the corresponding *RIB80* and *RIB81* genes together encode a heterooligomeric regulatory protein complex of negative action (Shavlovsky et al., 1982, 1988a).

Later, in 1991, *P. guilliermondii rib1* riboflavin deficient mutant able to grow in media containing 10 times lower concentration of riboflavin (20 mg l^{-1}) was selected. Cells of this strain actively reduced riboflavin, methylene blue, 2,3,5-triphenyltetrazoliumchloride, they also possessed high activity of ferrireductase and hyperaccumulated iron in the cells. This mutation (designated *hit1* – high iron transport) was shown to cause riboflavin overproduction when introduced in *P. guilliermondii* riboflavin prototrophic strains (Stenchuk et al., 1991; Fedorovich et al., 1999).

In addition, selection of *P. guilliermondii* mutants defective in regulation of RF biosynthesis and iron homeostasis representing 6 new groups of complementation (designated *red1-red6*) was reported recently (Stenchuk and Kapustiak, 2003).

It should be noted that all mutations mentioned above possess pleiotropic effect on *P. guilliermondii* metabolism. All of them cause defects in regulation of iron acquisition and lead to oxidative stress, in addition to riboflavin overproduction (Shavlovskii et al., 1992; Fedorovich et al., 1999; Protchenko et al., 2000).

It was shown that expression of *RIB1* and *RIB7* genes coding for the first and the last enzymes of RF biosynthesis is significantly enhanced in both *red6* and *rib81* mutants. Results of Northern blotting demonstrated that cells of the wild-type strain exhibited low levels of *RIB1* and *RIB7* genes expression when the medium is supplemented with 3.6 μM iron. Decrease of iron concentration in the medium caused at least 10-fold increase in mRNA levels both of the genes analyzed. Obtained results suggested that an increase in RF production by *P. guilliermondii* cells caused by iron starvation or regulatory mutations correlates with elevated level of mRNAs of key enzymes involved in this biosynthetic pathway. Thus, it can be supposed that regulation of riboflavin biosynthesis by iron in *P. guilliermondii* occurs mainly at the transcriptional level (Boretsky et al., 2005).

The *P. guilliermondii* regulatory mutants *rib83* and *rib84,* unable to overproduce riboflavin in iron-deficient medium, were also isolated (Shavlovsky et al., 1989). They were selected as mutants that have lost the ability to excrete riboflavin in an iron-deficient medium. It has been suggested that the corresponding genes encode regulatory proteins of the positive control involved in expression of structural genes of riboflavin synthesis. Double *rib80 rib83* and *rib81 rib83* mutants were unable to overproduce riboflavin, which suggests on epistasis of the *RIB83* gene over the genes *RIB80* and *RIB81*. Notably, cells of *rib83* mutants grown under iron deficiency conditions possess decreased iron transport when compared to the wild-type strain (Stenchuk et al., 2001). One may suggest that mutation *rib83* blocks high affinity iron transport in *P. guilliermondii.* In favor of this assumption, growth rate of *rib83* mutants is significantly decreased in iron deficient media as compared to the wild-type strain. Possibly, mutations *rib83* inactivates an unknown transcription factor required for expression of genes involved in riboflavin biosynthesis and high affinity iron transport in *P. guilliermondii.* It could be speculated that products of genes of positive and negative type of action form a cascade system for proper transmission of the regulatory signals and provide coordinated regulation of iron acquisition and riboflavin biosynthesis that is essential for *P. guilliermondii* viability.

Unfortunately, no regulatory genes of riboflavin synthesis were isolated so far, therefore molecular mechanisms of iron-dependent regulation of riboflavin synthesis remain to be quite speculative. The difficulties in regulatory gene cloning arose from the absence of differences in growth patterns between wild-type strains and regulatory mutants in most of tested media. To overcome this problem, methods of insertional mutagenesis can be useful. Recent communication from this lab on successful development of method for insertional mutagenesis in another flavinogenic yeast, *C. famata*, and identification of several regulatory genes of positive action, involved in riboflavin synthesis (Dmytruk et al., 2006) makes cloning of regulatory genes of riboflavin synthesis in *P. guilliermondii* the immediate task for the future research.

6.4 Riboflavin Transport

Most of the wild-type strains and the riboflavin-deficient mutants of *P. guilliermondii* are incapable of riboflavin-mediated transport from the medium (Sibirny et al., 1977d). It should be noted that riboflavin-deficient mutants of *P. guilliermondii* required for optimal growth addition to the medium up to 200 mg l^{-1} of riboflavin. This concentration is close to saturated solutions of this vitamin (near 400 mg l^{-1} in neutral water solution).

Using riboflavin auxotrophs, the secondary auxotrophs growing in media containing only 2.0 mg l^{-1} of riboflavin were selected. They were shown to possess multi drug sensitivity. However, selected mutants were unable to accumulate significant amounts of the vitamin in the cells due to uptake of this vitamin from the medium (Sibirny et al., 1977a).

After an additional step of mutagenesis, *P. guilliermondii* mutants able to grow in media containing less than 0.3 mg l^{-1} of riboflavin were selected. On media

containing 100–200 mg l^{-1} of riboflavin they accumulated significant amounts of intracellular riboflavin turning color of the colonies to yellow. This observation suggested on existence of the system for active transport of the vitamin in such mutants.

Two independent riboflavin-deficient mutants MS1–3 and RA68–2 which grew at very low concentration of exogenous vitamin and possessed riboflavin transport systems with different properties designated as RF-permease I and RF-permease II, respectively, have been isolated (Sibirny et al., 1977d; Shavlovsky and Sibirny, 1985).

Properties of RF-permease I were studied using strain MS1–3 and riboflavin prototrophs, isolated from it. Riboflavin uptake via RF-permease I was saturable and displayed an apparent *Km* of 0.17 mM and velocity 0.27 nmoles/(min × mg of dry weight cell). The pH optimum was at 5.8. Transport system was very specific as did not catalyzed transport of several structural analogs of riboflavin and FMN and FAD were not transported by this system at all. Activity of RF-permease I rises 30–50 fold in 4 hours after substitution of glucose by sucrose, maltose or other α-glucosides in the medium. It was found out the coordinate regulation of RF permease I and α-glucosidase and identified genes of negative and positive control involved in such regulation (Sibirny et al., 1979; Sibirny and Shavlovsky, 1984a). Genetic analysis revealed three unlinked regulatory genes designated *RFP80, RFP81* (of negative action (recessive mutations *rfp80, rfp81*), and *RFP82,* a gene of positive action (dominant mutations $RFP82^c$) that are involved in regulation of riboflavin transport. The recessive mutations *rfp82* were isolated as meiotic segregants from an intragenic recombination between two $RFP82^c$ – alleles or as mutants resistant to riboflavin analogues in a medium with the riboflavin permease inducer sucrose. Interallelic complementation was found within the *RFP80* and *RFP82* loci, and the corresponding maps were constructed (Sibirny and Shavlovsky, 1984a). The epistasis-hypostasis test showed that the gene *RFP82* acts after the gene *RFP80.* A model for the action of regulatory products of the identified genes, which form a cascade system in expression of riboflavin permease and a-glucosidase, was presented (Sibirny and Shavlovsky, 1984a).

It was shown that cells of MS1–3 strain grown on sucrose are able to uptake riboflavin even without exogenous energy source in the reaction mixture, however, inhibitors of energy metabolism (uncouplers of oxidative phosphorylation, inhibitors of ATPase and others) strongly inhibited riboflavin uptake. These data suggest on existence of system of active transport of riboflavin, which differs from that described in *S. cerevisiae* (Perl et al., 1976; Reihl and Stolz, 2005). Ability to transport exogenous riboflavin appeared to be a semidominant feature since colonies of diploid hybrids (between strain MS1-2 and the wild-type strain) exhibited slightly yellow color after long incubation on solid media containing 200 mg l^{-1} of riboflavin. Concentration of intracellular riboflavin in MS1-3 strain could reach up to 6 g l^{-1}. Significant part of the vitamin was accumulated in vacuoles as crystals. It was shown that riboflavin transport by this strain is significantly reduced by glucose, sorbose but not by fructose, galactose and mannose when added to the reaction mixture.

After additional steps of UV mutagenesis the mutant 9i, derivative of the strain MS1-3, was selected. This mutant was able to grow in media containing 0.005 mg l^{-1}

of riboflavin. Riboflavin uptake by this strain displayed an apparent *Km* of 0.031 mM and maximal velocity was 1.0 nmoles/ (min × mg of dry weight cell). This strain has been successfully used to develop a method for microbiological assay of riboflavin and for new method for measurement of riboflavin kinase activity (Sibirny and Shavlovsky, 1984b; Kashchenko et al., 1991).

In contrast to RF-permease I, RF-permease II activity (strain *RA68–2*) did not depend on type of carbon source (sucrose or glucose) in the media. Both sucrose and glucose did not effect RF-permease II activity when added to the reaction mixture (Shavlovsky and Sibirny, 1985; Sibirny, 1986). Velocity of riboflavin transport via RF-permease II is 10 times lower as compared to the first transport system.

The mechanism of evolution of these transport systems, which are cryptic in the wild-type strains, is not known. In addition to riboflavin permeases, P. *guilliermondii* possesses a riboflavin-excreting system which is present also in the wild-type strains (Shavlovsky et al., 1977; Sibirny et al., 1978). Riboflavin permease II and riboflavin excretase are apparently synthesized constitutively (Sibirny et al., 1977d, 1978). Mutants defective in riboflavin excretase were isolated. If to combine genetic defect in riboflavin excretase and overexpression of riboflavin synthesis, resulted strain accumulated in cells huge amounts of riboflavin which exceeded normal amount of this vitamin in yeast cells up to 1000 times. Cells of such mutants can be used as rich source of vitamin in the content of animal feed (Sibirny and Shavlovsky, 1984b; Sibirny, 1986).

6.5 Genetic Techniques

6.5.1 Life Cycle

All known natural or collection strains of *P. guilliermondii* are heterothallic (Wickerham and Burton, 1954; Kreger van-Rij, 1970; Shchelokova et al., 1974; Zharova et al., 1977). The mating types were designated mat^+ and mat^- (Sibirny et al., 1977b). Homothallic natural or collection strains are not described, though some of meiotic segregants of the hybrids obtained by protoplast fusion manifest a homothallic phenotype. Such a phenotype was designated as pseudohomothallic ($mat^{+,-}$) as these strains segregate mat^+ and mat^- clones during cultivation in complete synthetic medium. The pseudohomothallic phenotype was completely eliminated in the meiotic pedigree of $mat^{+,-}$ x mat^+ or $mat^{+,-}$ x mat^- hybrids (Sibirny, 1986). It was hypothesized that such pseudohomothallic ($mat^{+,-}$) strains are aneuploids.

6.5.2 Sexual Crosses

Cell conjugation between prototrophic strains of opposite mating types was observed on wort agar (Zharova et al., 1977). Study of sexual hybridization between auxotrophic mutants showed that most efficient matings occurred in solid media

with sodium acetate or tomato juice (Sibirny et al., 1977b). Hybridization was not observed in complete media. Thus, optimal conditions for crossing of *P. guilliermondii* appeared to be poor starvation media. Later, such a conclusion found support during the investigation of hybridization conditions for other yeast species. It was shown that yeast species found in natural habitats as haploids (haplonts) efficiently cross in poor media, whereas diplontic species (found in nature as diploids) hybridize predominantly in rich media (Naumov et al., 1980, 1981).

The following procedure of sexual hybridization is generally used for *P. guilliermondii* (Sibirny et al., 1977b). Auxotrophic strains of opposite mating types with complementary nutritional requirements are grown as a streak on complete YEPD or YEPS media. The strains are crossed on plates with acetate medium (sodium acetate, 1%; potassium chloride, 0.5%), incubated for 2–3 days, and then are replica-plated onto minimal medium. The prototrophic hybrids are formed at the contact sites of the streak cross.

6.5.3 Protoplast Fusion

Hybridization can also be obtained by protoplast fusion (Sibirny et al., 1982; Klinner and Böttcher (1984). In this case, hybrids were obtained between auxotrophs belonging to opposite or to the same mating type. The maximal frequency of hybridization by protoplast fusion was near 2×10^{-2} (Sibirny et al., 1982).

In addition to intraspecific *P. guilliermondii* hybrids, protoplast fusion was used for isolation interspecific hybrids with *Pichia kudriavzevii* and *Hansenula polymorpha* (Sibirny et al., 1982; Kashchenko et al., 1987).

Sexual or protoplast fusion hybrids of *P. guilliermondii* appeared to be very stable mononuclear diploids during growth on synthetic or complete media, and did not sporulate under such conditions (Zharova et al., 1980; Klinner and Böttcher, 1984; Sibirny, 1986). It is interesting to note that ploidy of the protoplast fusion hybrids never exceeded the diploid level (Klinner and Böttcher, 1984; Büttner et al., 1985). It was suggested that protoplast fusion hybrids appeared to be aneuploids in many cases.

Hybrids of *P. guilliermondii* were able to sporulate on acetate media. Most hybrids produced only one to two spores after 5–6 days of incubation at room temperature, but some pairs of strains gave diploids, which produced up to 40% asci with spores (Sibirny et al., 1977b). Incubation at 30°C depressed the sporulation. Five other media tested did not induce sporulation (Sibirny, 1986).

6.5.4 Protocol for Isolation and Fusion of Protoplasts

Cells are cultivated in sugar-mineral medium containing yeast extract (0.5%), 0.05 M potassium phosphate buffer, pH 6.0, and growth factors for auxotrophs (40 μg ml^{-1}). Cultivation is run for 16–24 h to middle exponential growth phase (cell mass

0.7–l.0 mg dry weight ml^{-1}). Cells are sedimented by centrifugation and washed twice with water.

Cells (50–100 mg dry weight ml^{-1}) are incubated in the following mixture: 0.05 M Tris-HCl, pH 7.0; 0.4 M $CaCl_2$; 0.01 M dithiothreitol; Zymolyase 20000 (2 mg ml^{-1}; Seikagaku Corp.) or β-Glucuronidase (10–15 mg ml^{-1}; Sigma) for 30– 40 min at 37°C with periodic mixing. Control of protoplasting is monitored using phase contrast microscopy. Yield of protoplasts usually reached 100%.

Protoplasts are separated from incubation medium by centrifugation at 2000 rpm for 5 min at 4°C, twice washed with cold (4°C) 0.4 M $CaCl_2$, and then are resuspended in the same solution at 7.5 mg dry weight ml^{-1}.

Suspensions of a pair of auxotrophic mutants containing 50×10^6 of protoplasts ml^{-1} are mixed in a 1:1 ratio, sedimented by centrifugation, and resuspended in the medium inducing protoplast fusion (25% polyethylene glycol 6000, containing 0.1 M $CaCl_2$, in 0.05 M Tris-HCl buffer, pH 8.6). Final concentration of the protoplasts of both strains is 50×10^6 ml^{-1} mixture. The obtained suspension is incubated for 20 min at 30°C with periodic mixing.

The protoplast mixture is spread onto a surface of an agar medium containing an osmotic stabilizer (1 M sucrose or 1 M sorbitol). Any additional pouring of top-overlaid mild agar is not necessary for *P. guilliermondii.*

6.5.5 Analysis of Meiotic Segregants

Asci of hybrids contain as a rule two spores, sometimes one-spored asci appeared, while three- or four-spored asci were seldom found. Asci have oval or elongate forms, while spores are characterized by round or hat-shaped forms (Sibirny et al., 1977b). Preferential production of two-spored asci is the result of degeneration of several nuclei formed during meiosis (Zharova et al., 1977, 1980).

Meiotic segregants of sexual hybrids, which produced abundant amounts of spores frequently, gave diploids characterized by low spore frequency. A genetic line, i.e. haploid strains hybrids of which produced a large amount of spores, was selected by inbreeding; using sister crosses between several consecutive pedigrees of meiotic segregants (Sibirnyi et al., 1977c). The strains denied from this genetic line easily crossed and sporulated, producing up to 60% of two-spored asci. Unfortunately, strains which appeared to be capable of producing four-spored asci were not isolated. Thus, tetrad analysis is impossible for *P. guilliermondii.* Electron microscopic studies showed that, during meiosis, diploid cells formed three or four nuclei but only part of them were surrounded by a spore envelope and formed spores. Apparently the other nuclei degenerated (Zharova et al., 1977, 1980).

Several methods of elimination of vegetative diploid cells for random spore analysis were developed. The method, based on selective killing of diploid vegetative cells by elevated temperature, (55°C and 60°C), was unsuccessful. More appropriate appeared to be the method based on eliminating vegetative cells by vaseline oil or killing diploid cells by ethanol or diethyl ester. The most suitable method,

which used 20% ethanol to eliminate non-sporulated hybrid cells for random spore analysis, is given below. Survival of spores of *P. guilliermondii* genetic line is equal to 86% (Sibirny et al., 1977b, c; Sibirny, 1986).

6.5.6 Protocol for Random Spore Analysis

A sporulating diploid cell suspension (2–2.5 mg dry weight ml^{-1}) is incubated at 30°C for 2 h in helicase (β-Glucuronidase) solution (10–15 mg ml^{-1}) for digestion of asci envelopes. Then the suspension is gently homogenized in a glass homogenizer to separate the spores, diluted to a cell concentration of approx. 0.6 mg dry weight ml^{-1}, and 1/5 part (by volume) of ethanol is added. The suspension is incubated with permanent shaking for 10–12 min and, after dilution with 20% ethanol, is spread onto YEPD medium.

P. guilliermondii does not contain its own plasmids. Therefore, plasmids based on a *Saccharomyces cerevisiae* vector were used for *P. guilliermondii* transformation at the beginning (Kunze et al., 1985; Zakal'skii et al., 1990; Logvinenko et al., 1993). Efficiency of transformation was low (10–20 transformants mg^{-1} of plasmid DNA), suggesting that autonomously replicating sequences (ARS-elements) derived from *S. cerevisiae* are not functional in *P. guilliermondii* cells.

6.6 Transformation

P. guilliermondii genes *RIB1* and *RIB7* coding respectively for GTP cyclohydrolase II and riboflavinsyntase were cloned by functional complementation of corresponding *E. coli* riboflavin deficient strains (Zakal'skii et al., 1990; Logvinenko et al., 1993). Later nucleotide sequence both of the genes was determined and was found to encode polypeptides sharing significant homologies with the corresponding enzymes in other organisms (Liauta-Teglivets et al., 1995; Boretsky, unpublished data). It was observed that efficiency of transformation of *P. guilliermondii rib1* mutants with plasmids containing the *RIB1* gene was about 150 transformants mg^{-1} of plasmid DNA. In contrast efficiency of transformation of *P. guilliermondii rib7* mutants with plasmids containing the *RIB7* gene was only 10–20 transformants mg^{-1} of plasmid DNA. Analysis of nucleotide sequences of the cloned *RIB1* gene revealed A + T rich sequence containing two stretches of homology to yeast ARS consensus sequence. Cloning of the 3′-located sequence into plasmid carrying the *RIB7* gene increased efficiency of transformation of *P. guilliermondii rib7* mutants by two orders of magnitude and provide autonomous replication of constructed plasmid. Thus, *P. guilliermondii* ARS-elements was identified and had been used to construct *P. guilliermondii/ E. coli* shuttle vectors (Boretsky et al., 1999).

Using constructed plasmids a several protocols of yeast transformation were tested and properly modified in order to increase transformation efficiency of *P. guilliermondii*. At present, lithium acetate transformation protocol provided

efficiency up to 1000 transformants μg^{-1} of DNA of a replicative plasmid whereas spheroplast transformation or electroporation both routinely give 3–5 × 10^4 transformants μg^{-1} of DNA of a replicative plasmid. Protocols for these procedures are given below.

A modified protocol reported by Ito is currently used to treat the *P. guilliermondii* cells with lithium acetate (Ito et al., 1983). Yeast cells were grown overnight aerobically in YPD medium to an optical density of $OD_{600} \leq 0.5$ and pelleted at 3,000 g for 10 min. Usually we use 100–150 ml of the medium. The cells were washed with water, pelleted and re-suspended in 10 ml of 0.1 M lithium acetate, TE buffer pH 7.5 (LiAc/TE buffer). After incubation (1 hour at 30°C) they were pelleted and re-suspended in fresh LiAc/TE buffer to a final concentration of 5 × 10^9 cells ml^{-1}. Aliquots of 50 µl were dispensed into 1.5 ml tubes. Plasmid DNA (1–10 µg in 1–10 µl of TE buffer) and 250 µl of 50% PEG in LiAc/TE buffer were added and mixed vigorously. After incubation at 30°C for 30 min, the cells were heat-shocked (15 min, 42°C), chilled in ice for 1 min, pelleted, re-suspended in 1 ml YPD and incubated at 30°C for 1 h. Finally the cells were pelleted again, re-suspended in 150 µl of 1 M sucrose, plated on a selective medium and incubated at 30°C for 3–6 days.

Spheroplast transformation was done using a modified protocol for *Pichia pastoris* transformation (Cregg et al., 1985). Yeast cells were grown aerobically overnight in YPD medium to an optical density of $OD_{600} \leq 0.3$ and pelleted at 3000 g for 10 min. Usually we use 200–350 ml of the medium. The cells were pelleted at 3000 g for 10 min washed with water, pelleted and re-suspended in 10 ml of 1 M sucrose, 50 mM dithiothreitol, 25 mM EDTA, pH 8.0. After 15 min incubation at room temperature cells were pelleted again, washed 2 times with 1 M sucrose and re-suspended in 1 M sucrose, 25 mM EDTA, 100 mM sodium citrate pH 5.8. Zymolyase (or lyticase) was added and cells were incubated for 10–40 min (30°C, slow agitation) in order to get approximately 5–10% of spheroplasted cells. More intensive treatment with an enzyme increases percentage of spheroplasts, but significantly reduces efficiency of transformation. Thus, quantity of an enzyme used for the treatment of cells should be justified before. Cells are pelleted at 2,000x g for 5 min, washed twice with 1 M sucrose, once with 1M sucrose, 10 mM $CaCl_2$ and re-suspended in the last solution to a final density 2 × 10^8 cells ml^{-1}. To re-suspend the pelleted cells and spheroplasts slowly and repeatedly draw them into a pipette and release them into the tube was done. 100 µl aliquots of the cell suspension were dispensed into 1.5 ml tubes and 0.1–1 µg of transforming DNA together with 5 µg of single strand carrier DNA were added. After 20 min of incubation at 25°C 1 ml of solution of 20% PEG 3350, 10 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$ was added and immediately (gently, avoid vortexing) mixed. The mixture was incubated for additional 15 min at 25°C. Spheroplasts and cells were pelleted (1000x g for 10 min), re-suspended in 1 M sucrose, 10 mM $CaCl_2$ and incubated for 30 min at 25°C. Aliquots of the suspension were plated on selective medium containing 1 M sucrose and incubated for 3–6 days at 30°C.

For yeast electroporation, a modified protocol of (Becker and Guarente, 1991) was used. Yeast cells were grown aerobically overnight in a rich medium (YPD) to an optical density of $OD_{600} \leq 0.5$, chilled on ice and pelleted at 3000x g for 10 min.

All subsequent manipulations should be done at 2–4°C. The cells were washed with 0.1 M Li acetate, 10 mM Tris-HCl, 1 mM EDTA (pH 7.5), twice with ice-cold water, and twice with 1 M sucrose. They were then re-suspended in 1 M sucrose to a final concentration of 5×10^9 cells ml^{-1}. Aliquots of 200 µl were dispensed into 1.5 ml tubes. Plasmid DNA (0.05–0.50 µg in 1–2 µl of TE buffer) was added and mixed gently. The mixture was transferred into prechilled 1 mm electroporation cuvettes. Electroporation was performed as follows: resistance – 200 Ω; capacitance – 25 µF; voltage – 1.8 kV. The cells were washed out from the cuvettes with 1 ml of YPD medium, incubated for 1 hour at 30°C, pelleted and re-suspended in 1 M sucrose, plated on selective medium and incubated at 30°C for 3–5 days.

6.7 Plasmid Rescue

All constructed *P. guilliermondii/ E. coli* shuttle vectors and plasmids are rather unstable in the yeast cells. Even after 5–8 divisions under selective pressure only 5–30% of cells of transformants contain a plasmid bearing selective marker. Never the less plasmid DNA can be rescued from *P. guilliermondii* transformants. Fresh colony of a transformant was inoculated in 3 ml of YPD medium and grown at 30°C for approximately 15 hours. Cells were pelleted, washed with water, and resuspended in 0.3 ml of water. 0.6 ml of acetone was added. After 10 min incubation cells were pelleted, re-suspended in 1 ml of acetone and incubated for 15 min at 37°C. Washed cells were pelleted at 5000g for 7 min, dried and re-suspended in 0.2 ml of 50 mM Tris-HCl buffer pH 8.0, 50 mM EDTA, 0.5% SDS, 100 mM NaCl. The cell suspension was incubated for 30 min in ice-water bath with occasional shaking. Cells were removed by centrifugation; 0.02 ml of 3 M potassium acetate pH 5.0 were added to supernatant and mixed. To precipitate DNA two volumes of prechilled ethanol were added and mixture was incubated at 0°C for 1 h. DNA was pelleted by centrifugation washed with 70% ethanol, dissolved in 20 µl of TE buffer and used for transformation of an *E. coli* strain.

Calculations based on comparison of *E. coli* transformation efficiencies suggested that about 0.001–0.01 µg of the plasmid DNA could be purified from *P. guilliermondii* transformants using procedure described above.

6.8 Construction of Knockout Strains

The widely used *URA3* marker appeared to be the most convenient and useful selectable markers for *P. guilliermondii* transformation, since no antibiotic resistance genes are reported for this yeast species. We isolated *P. guilliermondii ura3* auxotrophs using a positive selection procedure with 5-fluoroorotic acid and used them as recipients for transformation experiments (Boretsky unpublished). To identify whether the *URA3* gene was impaired in FOA-resistant strains we constructed a pAGU3 plasmid carrying the PgARS and *S. cerevisiae URA3* gene and

used it for complementation experiments. The constructed plasmid complemented uracil deficiency in well characterized *S. cerevisiae* YPH499 and *E. coli pyrF* strains. Despite the presence of PgARS, the efficiency of transformation of the *P. guilliermondii ura3* auxotrophs was very low: 40–50 transformants per 1 μg of DNA. A reason for that could be ambiguity of CUG codon in *P. guilliermondii* like it was reported for many *Candida* species (Sugiyama et al., 1995). To avoid this problem we substituted the single CUG codon encoding a conservative leucine residue (L45) in the *URA3* gene by another leucine codon, CUC. The efficiency of the transformation of *P. guilliermondii ura3* auxotrophs with the newly constructed pAGU34 plasmid that carries the modified gene was 100 fold increased when compared to the pAGU3 plasmid. This result can suggest that the CTG codon codes for serine (like in *Candida* species) and not leucine in *P. guilliermondii* cells (Boretsky unpublished).

Before the *P. guilliermondii* genome sequenced, only 2 genes, *RIB1* and *RIB7*, were cloned and sequenced. The *P. guilliermondii RIB1* gene coding for GTP cyclohydrolase II has been shown to be partially overlapped with an ARS element which decreases the probability of homologous recombination in this yeast species (Piniaga et al., 2002). Thus, the only target available for an initial knock-out experiment was the RIB7 gene coding for riboflavin synthase.

Three plasmids designated p72, p724D and p724R were constructed in which *RIB7* structural gene was replaced by the modified *S. cerevisiae URA3* gene as described above. Plasmid p72 was digested with *Bam*HI and *Pae*I restriction endonucleases generating the *rib7::URA 3–2,3* deletion cassette. Plasmids p724D and p724R were digested with *Bam*HI restriction endonuclease generating the *rib7::URA3–2,8D* and *rib7::URA3–2,8R* deletion cassettes, respectively.

The appropriate *ura3* mutant of *P. guilliermondii* was transformed with these cassettes using the spheroplasting procedure, Li-Ac method and electroporation. To get a larger number of transformants, the electroporation procedure was used in the initial experiments. Efficiency of transformation was approximately 1000 transformants per 1 μg of DNA for all cassettes. However, no stable riboflavin deficient recombinant strains were selected among approximately 400, 280 and 200 transformants obtained with cassettes *rib7::URA3–2,3 rib7::URA3–2,8D* and *rib7::URA3–2,8R*, respectively, after the electroporation procedure. Similar results were obtained when the spheroplasting procedure was applied for transformation – no stable riboflavin deficient strains were selected among 116 transformants checked. In contrast, approximately 12% of stable riboflavin auxotrophs were selected among uracil prototrophs when the recipient strain was transformed with *rib7::URA3–2,8D* cassette using the lithium acetate procedure. Efficiency of transformation was approximately 200 transformants per 1 μg of DNA. The number of riboflavin auxotrophs was decreased to 3% when using deletion cassette *rib7::URA3–2,8R* that has an opposite orientation of the *URA3* gene. No riboflavin auxotrophs were selected with the cassette *rib7::URA3–2,3* that harbors a shortened terminator sequence of the *RIB7* gene (Fig. 6.2a).

In order to confirm deletion of the *RIB7* gene, riboflavin auxotrophs obtained with *rib7::URA3–2,8D* cassettes were further checked. For that purpose, two

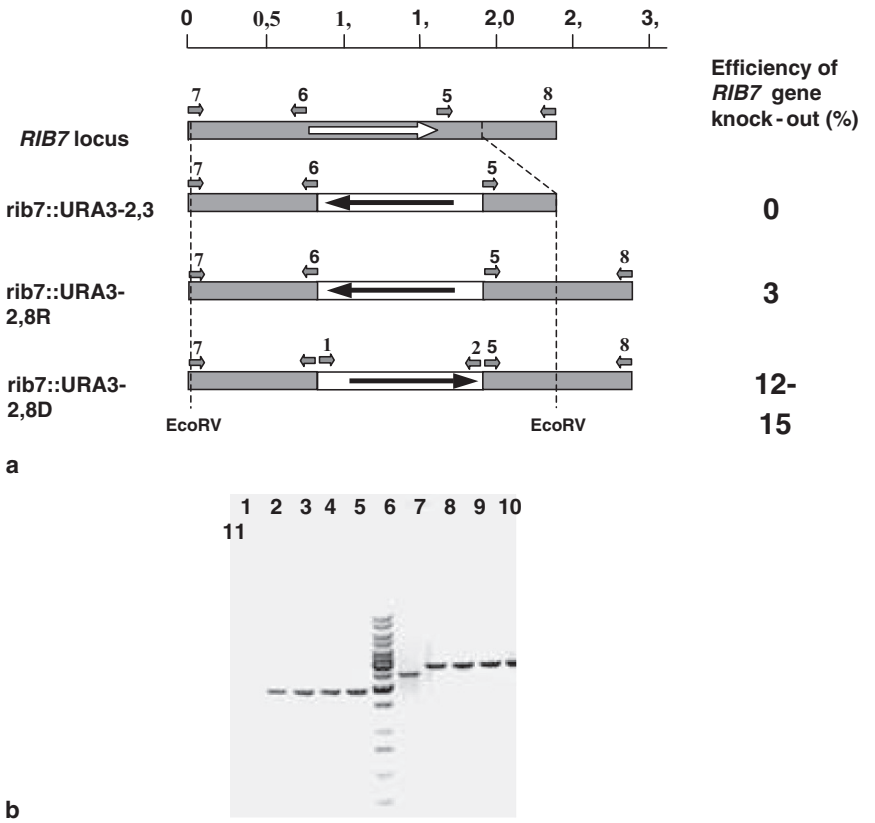

Fig. 6.2 Deletion of the *RIB7* gene in *P. guilliermondii*. (**a**) Scheme of cassettes used to delete *RIB7* gene. ⇒- *RIB7* structural gene of *P. guilliermondii.* ➡- *URA3* structural gene of *S. cerevisiae.* (**b**) Checking of knock-out recombinant strains by means of PCR Lane 1 – DNA-signals obtained with recipient strain (primer 7 and primer 2; Table 2); lanes 2, 3, 4, 5 – DNA-signals obtained with recombinant strains *Δrib7–1*, *Δrib7–2*, *Δrib7–3*, *Δrib7–4* correspondingly, (primer 7 and primer 2); lane 6–1 kb DNA ladder "Fermentas" (from the top: 10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3.5 kb, 3 kb, 2.5 kb, 2 kb, 1.5 kb, 1 kb, 0.75 kb, 0.5 kb, 0.25 kb); lane 7 – DNA-signals obtained with recipient strain (primer 7 and primer 8); lanes 8, 9, 10, 11 – DNA-signals obtained with recombinant strains *Δrib7–1*, *Δrib7–2*, *Δrib7–3*, *Δrib7–4* correspondingly (primer 7 and primer 8)

PCR amplifications were performed using the total DNA of transformants as a template. 1.9 kb DNA fragments were obtained with primers 7 and 2 when total DNA of selected recombinant strains was used as a template whereas no signal was generated in the case of recipient strain. Another set of primers (namely 7 and 8) also gave expected results: 2.4 kb and 2.8 kb signals in the cases of the recipient and recombinant strains respectively (Fig. 6.2b). Taken together, the obtained results suggest that in these transformants the *rib7::URA3–2,8D* deletion cassettes integrated into genome by homologous recombination which lead to a knock-out of the *RIB7* structural gene. To further prove the feasibility of this approach, we

constructed a deletion cassette *rib1::URA3–10* (that contains 1.6 kb and 1.4 kb promoter and terminator sequences of the *RIB1* gene respectively) and transformed it into the same recipient strain using the lithium acetate procedure. Efficiency of transformation was approximately 10000 transformants per 1 μg of DNA. Only 2 of 2000 transformants checked exhibited riboflavin deficiency. Deletion of *RIB1* gene was confirmed by means of PCR using total DNA of these transformants and a set of appropriate primers. The decreased efficiency of *RIB1* knock-out could be a suggestion that the ARS element adjacent to the GTP cyclohydrolase structural gene reduces the efficiency of homologous recombination. Thus, two genes were deleted successfully in *P. guilliermondii* (Boretsky unpublished). The developed transformation system can be used for identification of genes involved in the regulation of riboflavin biosynthesis and for other studies that require functional analysis of *P. guilliermondii* genome.

6.9 Conclusions

In contrast to other yeast species that overproduce riboflavin under conditions of iron deprivation, *P. guilliermondii* exists in both haploid and diploid forms, can be easily stimulated to mate and sporulate. Genetic line producing large amounts of spores was selected. More recently, tools for molecular genetic studies of *P. guilliermondii* have been developed which include collection of host strains, vectors and several transformation protocols including that for gene knock-out. Genome of *P. guilliermondii* has been sequenced recently and is publicly available. Thus, *P. guilliermondii* seems to be an attractive model for studying the specific iron-dependent regulation of cellular metabolisms, more specifically, control of riboflavin biosynthesis. Besides, some strains of this species efficiently convert xylose to xylitol, anti-caries sweetener. Production both of riboflavin and xylitol are of great industrial interest. But regulation of these metabolic pathways by *P. guilliermondii* has to be studied in details. At present, even the most active mutants of *P. guilliermondii* produce much less riboflavin than the other industrial producers, based on yeast *C. famata*, bacterium *B. subtilis* and fungus *Ashbya gossypii*. Selection of the more active *P. guilliermondii* riboflavin producers is possible, but will demand a strong increase in specific activity of GTP cyclohydrolase, which apparently limits flavinogenesis and the enhancement of this process by the purine precursor, GTP. The mutants of *P. guilliermondii* capable of active riboflavin transport efficiently accumulate this vitamin from diluted solutions inside the cells. It is possible to use such mutants for concentration of riboflavin from cultural liquids of weak producers. System of energy-dependent riboflavin excretion of riboflavin, which differs from that of riboflavin permease has been found in *P. guilliermondii.* Mutants defective in riboflavin excretase have been isolated which accumulate huge amounts of riboflavin inside the cells. It is suggested that obtaining the riboflavin-enriched yeast biomass as a source of riboflavin would be a much cheaper procedure than other possible methods for concentration of this vitamin from diluted solutions.

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Chapter 7
Assimilation of Unusual Carbon Compounds

Wouter J. Middelhoven

Contents

Abstract Yeast taxa traditionally are distinguished by growth tests on several sugars and organic acids. During the last decades it became apparent that many yeast species assimilate a much greater variety of naturally occurring carbon compounds as sole source of carbon and energy. These abilities are indicative of a greater role of yeasts in the carbon cycle than previously assumed. Especially in acidic soils and other habitats, yeasts may play a role in the degradation of carbon compounds. Such compounds include purines like uric acid and adenine, aliphatic amines, diamines and hydroxyamines, phenolics and other benzene compounds and polysaccharides. Assimilation of purines and amines is a feature of many ascomycetes and basidiomycetes. However, benzene compounds are degraded by only a few ascomycetous yeasts (e.g. the *Stephanoascus/*

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Blastobotrys clade and black yeastlike fungi) but by many basidiomycetes, e.g. Filobasidiales, Trichosporonales, red yeasts producing ballistoconidia and related species, but not by Tremellales. Assimilation of polysaccharides is wide-spread among basidiomycetes.

Growth tests on these compounds separate *Trichosporon* species that otherwise are hardly distinguishable. Yeasts able to degrade phenolics can be applied for cresol removal from polluted soil and styrene removal from air by biofilters containing black yeast. Yeasts growing on polysaccharides may be a valuable source of hydrolytic enzymes that can be applied in food technology. Biodegradative abilities of yeasts inhabiting aerial plant surfaces and the fate of these yeasts during anaerobiosis and lactic acid fermentation are also dealt with.

Keywords Adenine, amines, benzene compounds, methanol, phenolics, polysaccharides, purines

7.1 Introduction

Yeast taxa traditionally are distinguished by their ability to produce ascospores, by cell wall structure and by morphology, e.g. the presence of budding cells, splitting cells and hyphae. These characteristics suffice for distinction of yeast genera, but at the species level physiological growth tests are required. These include ability to ferment sugars into ethanol and carbon dioxide and ability to utilize a great variety of naturally occurring organic compounds as sole sources of carbon and energy, and several nitrogenous compounds as sole nitrogen source. Additional distinguishing characteristics are maximum growth temperature, osmotolerance and vitamin requirement.

The organic compounds used in traditional growth tests include monosaccharides, disaccharides, trisaccharides, inulin and soluble starch, polyols and several organic acids. These growth tests are necessary for species description and identification, but do not give a complete picture of the degradative abilities shown by some yeast species. An example is methanol assimilation that until 1969 was generally believed not to be shown by any yeast species. Other studies revealed assimilation of alkanes, purines, C2 compounds, amines, diamines, hydroxyamines, benzene compounds and polysaccharides. In the following sections attention will be paid to assimilation of these unusual carbon compounds.

Yeasts in nature are found in many habitats, e.g. soil, or are associated with insects, mushrooms and animals, Men included, some being opportunistic pathogens. Another habitat rich in yeast species is the aerial surface of plants. The degradative abilities of these yeasts and their fate during anaerobiosis and lactic acid fermentation will also be dealt with.

7.2 Assimilation of C1 And C2 Compounds, Purines, Aliphatic Amines and Alkanes

7.2.1 C1 Compounds

Until 1969 when Ogata et al. (1969) isolated *Candida boidinii* from an enrichment culture on methanol it was generally believed that methanol could not support growth of any yeast species. Screening of the CBS yeast collection (Hazeu et al., 1972) showed that this character is exclusively shown by some ascomycetous yeast species. Sequencing of 26S rDNA nuclear bases pointed to close phylogenetic relationship of these *Candida* and *Pichia* species (Kurtzman and Robnett, 1998). Levine and Cooney (1973) isolated *Hansenula anomala*, later renamed *Pichia angusta*, a thermotolerant methylotrophic yeast. Methylotrophic yeasts metabolize methanol by an alcohol oxidase (Sahm and Wagner, 1973) that together with catalase is located in so-called peroxisomes (van Dijken et al., 1975). Detailed studies on the formation of these cell organelles were carried out by the group of Veenhuis in Groningen. For review see van der Klei and Veenhuis (2002). Production of single cell protein from methanol is feasible. It must be kept in mind that methylotrophic bacteria may give higher cell yields as they generate more energy from this substrate, due to activity of a specific methanol dehydrogenase rather than an alcohol oxidase. Methylotrophic yeasts respiring on methanol show a relatively high substrate constant for molecular oxygen, viz. 0.5-1.3 mg O_2 per litre rather than < 0.15 mg O_2 per litre when ethanol is respired (Middelhoven et al., 1976a). This implies that the maximum specific growth rate of yeast cultures on methanol can be attained only at oxygen concentrations near half air saturation. Some strains of *Candida boidinii* prefer nitrate to ammonium when growing in a methanol ammonium nitrate medium (Middelhoven et al., 1976a, b). These strains excrete substantial amounts of ammonia when growing in methanol potassium nitrate medium under oxygen-limited conditions, but not in well-aerated cultures or anaerobically (Middelhoven et al., 1976b). This nitrate reduction appears not to be linked to energy generation.

Different alcohol oxidases have been detected in methylotrophic yeasts (Szamecs et al., 2005). A detailed study of alcohol oxidase was presented by Ozimek et al. (2005). An alternative pathway of methanol catabolism starts with an alcohol dehydrogenase (Sakay et al., 1995). Methylformate is an intermediate and this pathway is particularly active at high concentrations of methanol and formaldehyde. Possibly it plays a part in detoxification of the latter.

Hexamethylenetetramine (urotropine) is a formaldehyde derivate. It is spontaneously formed in aqueous solutions of formaldehyde and ammonia. The compound is stable but subject to hydrolysis under acidic conditions. It does not support growth of yeasts when administered as carbon source, but 46 out of 60 yeast species tested readily assimilated urotropine as sole nitrogen source (Middelhoven and van Doesburg, 2007). These species include basidiomycetes and ascomycetes, methylotrophs as well as non-methylotrophs. Urotropine is not known to occur in nature, but spontaneous formation of small amounts in the cell from ammonia and formaldehyde is feasible.

7.2.2 Purines

Enrichment cultures on uric acid as sole carbon source, inoculated with soil, yielded an ascomycetous yeast identified as *Candida famata* (anamorph of *Debaryomyces hansenii*) and two basidiomycetes belonging to the genus *Trichosporon* (Middelhoven et al., 1983). In spite of its complex chemical structure, uric acid from a physiological viewpoint is a C2 compound as it is metabolized via glyoxylate, both in the ascomycete and in the basidiomycetous yeasts studied. From glyoxylate energy is generated and cell constituents are produced. The pathway of urate catabolism begins with urate oxidase by which allantoin is formed, that in three successive hydrolytic steps is converted into glyoxylate and two moles of urea (Middelhoven et al., 1983). Urate oxidase is located in peroxisomes (Middelhoven et al., 1983; Veenhuis et al., 1985). Several soil samples yielded yeast strains able to grow at the expense of uric acid (Middelhoven et al., 1985). These strains belong to some ascomycetous genera, e.g. *Candida, Stephanoascus* and *Arxula* and to basidiomycetous genera like *Cryptococcus* and *Trichosporon*. All the ascomycetous strains assimilated n-hexadecane as well. Yeastlike endosymbionts of the brown planthopper recycle uric acid stored in the insect's tissues (Sasaki et al., 1996).

Enrichment cultures on adenine as sole carbon source yielded some strains of *Stephanoascus ciferrii* (Middelhoven et al., 1985) and of a novel species, initially named *Trichosporon adeninovorans* (Middelhoven et al., 1984), later renamed as *Arxula adeninivorans* (van der Walt et al., 1990). The latter species assimilated adenine, uric acid, several aliphatic amines and amino acids (Middelhoven et al., 1991). In addition to these nitrogenous carbon compounds, several benzene compounds supported growth of *A. adeninivorans* (Middelhoven et al., 1991). The group of Kunze in Gatersleben detected many excreted hydrolytic enzymes in cultures of this industrially promising yeast species. A detailed genetic study was done by the group of Bode and Samsonova in Greifswald. These topics will be dealt with in other chapters of this book.

7.2.3 C2 Compounds

Enrichment of yeasts growing on glycollate as sole carbon source was unsuccessful. Similarly, enrichments from soil on allantoin were also unsuccessful, in spite of allantoin being the first intermediate of urate catabolism. Allantoin is an excellent nitrogen source for many yeasts, *Saccharomyces cerevisiae* included (Middelhoven, 1977; Middelhoven and Arkesteyn, 1981). Inoculation with a rotten mushroom resulted in isolation of *Cryptococcus allantoinivorans* sp. nov. (Middelhoven, 2005). This species belongs to the *Cryptococcus laurentii* complex. *C. allantoinivorans* was the only species of this group growing on allantoin. The other eight species studied assimilated one or more C2 compounds like ethanol, ethylamine, ethanolamine

and glycine. The pattern of C2 compound utilization shown by the type strains appeared to be species-specific (Middelhoven, 2005).

7.2.4 Amines

It has been known for a long time that amines support growth of yeasts when administered as sole nitrogen source. Van der Walt (1962) introduced a growth test on ethylamine as a diagnostic tool. Van Dijken and Bos (1981) showed that 60% of 461 yeast species utilized at least one primary amine as sole nitrogen source, but they failed to demonstrate assimilation as sole carbon source. Middelhoven et al. (1983, 1984, 1985) showed that several primary amines, diamines and hydroxyamines were assimilated as sole carbon source by many ascomycetous and basidiomycetous yeast species. The formula of Difco Yeast Nitrogen Base had to be amended for attaining complete substrate utilization. For this purpose, ammonium sulphate was omitted and the phosphate buffer concentration was increased tenfold.

Amines are oxidized to the corresponding aldehydes by amine oxidases (for review see Large, 1986) that are located in peroxisomes (Zwart et al., 1980, 1983). Yeasts produce different amine oxidases: methylamine oxidase is most active with short-chain primary amines, ethanolamine included (Zwart and Harder, 1983). Long-chain amines, benzylamine and isobutylamine are oxidized by benzylamine oxidase that may attack putrescine as well (Green et al., 1982). This type of benzylamine oxidase was detected in *Candida utilis* and *Pichia pastoris*. *Kluyveromyces* spp. and *Candida boidinii* produced a benzylamine oxidase that did not attack putrescine (Haywood and Large, 1981; Heath and Large, 1984). All of these amine oxidases showed pH optima of 7.0–7.5. The benzylamine oxidase of *Kluyveromyces marxianus* has been completely characterised and compared to other copper amine oxidases by Corpillo et al. (2003).

Middelhoven et al. (1986) grew five yeast species on amines as sole carbon source and screened cell-free extracts for amine oxidase activity, using primary amines, hydroxyamines and diamines of different chain lengths, isobutylamine and benzylamine as enzyme substrates. In cells of *Trichosporon cutaneum* (later renamed *T. domesticum*), grown on ethylamine or butylamine, maximum activities towards primary amines was found with propylamine and butylamine at pH 7.0, towards hydroxyamines at chain lengths C_4-C_6 (pH 7.0) and towards diamines at C_8-C_9 (pH 8.0). Cell-free extracts of *T. domesticum* grown on putrescine displayed little activity on primary and hydroxyamines, but attacked putrescine and cadaverine at pH 8.0. In putrescine-grown cells of the other basidiomycete studied, viz. *Cryptococcus laurentii*, putrescine was the preferred substrate, but significant activity on monoamines (C_3-C_4) and hydroxyamines (C_3-C_6) was also present, isobutylamine and benzylamine not being oxidized, all tested at pH 8.0. Putrescine-grown cells of three ascomycetes, viz. *Candida famata*, *Arxula adeninivorans* and *Stephanoascus ciferrii* were most active on diamines C_7-C_9 (pH 8.0) and hydroxyamines C_4-C_6. Of the monoamines tested, optimum chain lengths were C_4-C_{12} for *C. famata,* C_3-C_5

for *A. adeninivorans* and C_4-C_5 for *S. ciferrii*. The results presented by Middelhoven et al. (1986) show a greater variability of yeast amine oxidases than earlier studies. This appears from a higher pH optimum and a preference for long-chain diamines.

7.2.5 Hydrocarbons

Long-chain alkanes (C_{12}-C_{16}) are assimilated by many yeast species as sole carbon source (Markovetz and Kallio, 1964; Scheda and Bos, 1966; Hug and Fiechter, 1972). Bos and de Bruyn (1973) proposed growth tests on alkanes as a diagnostic tool. Unfortunately, growth may be very slow and is dependent on the methods used. Moreover, not all yeast species have been screened for this character. Middelhoven (2001) stated that the slant culture method of Markovetz and Kallio (1964) gives better results than growth tests in liquid media. Alkane-utilizing yeast cultures excrete emulsifiers that facilitate uptake of these hydrophobic liquids (Zinjada and Pant, 2002).

7.3 Phenolic and Other Benzene Compounds

7.3.1 General

Aromatic compounds received little attention by yeast taxonomists. This is regrettable as the benzene ring is a widely distributed unit of chemical structure in the biosphere. Some reports on phenol degradation appeared already in the late fifties. Zimmermann (1958) reported degradation of phenol by suspensions of some basidiomycetous yeast cells. Di Menna (1959) showed assimilation of lignin-related compounds like ferulic and vanillic acids by basidiomycetous yeasts isolated from the phyllosphere of pasture plants. To avoid growth inhibition due to toxicity of these compounds, she administered these at a low concentration, viz. 100 mg per litre mineral salts medium. Middelhoven (1993) applied the slant culture method, described by Middelhoven et al. (1991, 1992b, 2004) to demonstrate growth at the expense of toxic compounds. Sampaio (1999) carried out growth tests on aromatic compounds in liquid media, at 1 gram per litre. A comparison of these methods shows that more positive growth responses are seen with the slant culture method than in liquid medium. Sampaio (1994) isolated many basidiomycetous yeast strains from enrichment cultures on lignin-related benzene compounds, inoculated with plant leaves or soil. A comparison with type cultures learned that members of the *Trichosporon* and *Filobasidium* clades assimilated many benzene compounds, but members of the *Tremellales* and of the *Cystofilobasidium* clade generally did not. Middelhoven et al. (1992b) isolated yeasts from soil samples previously polluted

with some benzene compounds. Several basidiomycetes predominated, but *Schizoblastosporion starkeyi-henricii* was also among the isolates. Soil supplied with phloroglucinol yielded *Cryptococcus* spp., later reidentified as *Trichosporon porosum* (Stautz) (Middelhoven et al., 2001).

Middelhoven (1993) screened a yeast culture collection, isolates of Middelhoven et al. (1992b) included, representing about 20% of all species known at that time, for growth on phenol and 3-hydroxybenzoate. Fifteen ascomycetous yeast species and thirteen basidiomycetous yeast species and yeastlike fungi responded positively and were selected for further study. They were tested for growth on 84 benzene compounds, of which 63 supported growth of at least one yeast species. The black ascomycetous yeastlike fungus *Exophiala jeanselmei* assimilated 54 of these compounds. About 30 of these were assimilated by *Trichosporon* spp., *Rhodotorula* spp, *Leucosporidium scottii* and *Cryptococcus elinovii* and about 20 by *Arxula adininivorans* and the closely related *Stephanoascus ciferrii*. On the other hand, none of the *Saccharomyces* spp., *Kluyveromyces* spp. and *Pichia* spp. tested grew on any of these compounds. *Candida tropicalis* and *Debaryomyces hansenii* assimilated only phenol and dihydroxybenzenes.

Compounds not assimilated by any yeast strain tested could be classed in 3 groups. Compounds with more than one carbon side chain, such as xylenols and toluic acids, a branched side chain or with an amino group attached to the ring, such as anthranilic acid and 4-aminobenzoic acid. Repeated attempts to enrich yeasts on anthranilic acid were unsuccessful. This is contrary to the paper of Anderson and Dagley (1981) who described the catabolic pathway in *Trichosporon cutaneum*. Other compounds not assimilated by any yeast strain tested were 2-hydroxycinnamic acid and its internal lactone coumarin, umbelliferone, esculetin, 3,4,5-trimethoxycinnamic acid, benzaldehyde and para-quinone. Failure to grow on these compounds may be due to steric hindrance or toxicity.

7.3.2 Catabolism

Aerobic degradation of benzene compounds is achieved by ring fission. This may occur between two adjacent hydroxyl groups, e.g. in catechol, hydroxyhydroquinone, protocatechuate (3,4-dihydroxybenzoate) and homoprotocatechuate (3,4-dihydroxyphenylacetate), or next to a hydroxyl group in para (1,4) position, e.g. in gentisate (2,5-dihydroxybenzoate) or homogentisate (2,5-dihydroxyphenylacetate). Ring fission is catalyzed by dioxygenases. Ring fission substrates are formed by monooxygenases that introduce hydroxyl groups with concomitant oxidation of NAD(P)H. These pathways are common in aerobic microorganisms. Biochemical studies on yeasts were carried out by the groups of Halina Neujahr (Stockholm, Sweden) and S. Dagley (St. Paul, Minnesota). For review, see Middelhoven (1993) who studied the occurrence of cleavage enzymes in various phylogenetic groups. The catechol branch of the 3-oxoadipate pathway and its hydroxyhydroquinone variant were involved in phenol and resorcinol catabolism in ascomycetes as well

as in basidiomycetes. However, these two groups of yeasts showed characteristic differences in hydroxybenzoate catabolism. In the yeastlike fungus *Exophiala jeanselmei* and in basidiomycetes of the genera *Cryptococcus, Leucosporidium* and *Rhodotorula* the protocatechuate branch of the 3-oxoadipate pathway was induced by growth on 3- and 4-hydroxybenzoic acids. In three *Trichosporon* species and in all ascomycetous yeasts tested, 4-hydroxybenzoate was catabolized via protocatechuate and hydroxyhydroquinone. These yeasts were unable to cleave protocatechuate. 3-Hydroxybenzoic and 3-hydroxycinnamic acids were assimilated in ascomycetous yeasts via the gentisate pathway (Middelhoven et al., 1992a), but in basidiomycetes via protocatechuate.

Tannins are important plant constituents. Condensed tannins are covalently bound benzene compounds. Yeasts are unable to degrade these (Bhat et al., 1998). Hydrolysable tannins consist of glucose moieties esterified with substituted benzoic acids, e.g. gallic acid (3,4,5-trihydroxybenzoic acid). Some yeasts produce tannase that hydrolyses the ester bond (Bhat et al., 1998). Tannase of a *Candida* sp. has been purified by Aoki et al. (1976). Gallic acid supports growth of some *Trichosporon* sp., *Rhodotorula* sp. and *Leucosporidium scottii* (Middelhoven, 1993). Some *Trichosporon* spp. assimilate hydrolysable tannic acid (Middelhoven, 2004).

Cresol removal from polluted soil was speeded up by inoculation with *Rhodotorula* sp. cells (Middelhoven et al., 1992b), later described as *Rhodotorula cresolica* sp. nov. (Middelhoven and Spaaij, 1997). It was demonstrated that this yeast utilized ortho-cresol in competition with the soil microflora. Cox et al. (1996) used biofilters to remove styrene vapour from air. The black yeastlike fungus *Exophiala jeanselmei* is the active agent in these filters. Cinnamic acid is converted into styrene by growing cultures and cellfree extracts of *Cryptococcus elinovii*. (Middelhoven and Sollewijn Gelpke, 1995).

7.4 Polysaccharides

Growth tests on soluble starch and inulin are traditionally used for distinction of yeast species. Some yeasts are able to ferment these polysaccharides. Other polysaccharides did not receive much attention. Some hydrolytic enzymes excreted by yeasts that convert these polymers into low-molecular fragments are or were commercially available, e.g. xylanase from *Aureobasidium pullulans* (Leathers, 1989) and *Cryptococcus albidus* (Biely and Vrsanska, 1988). Most of the commercially available polysaccharide hydrolases are produced by filamentous fungi. A thermolabile xylanase is produced by *Cryptococcus adeliae* (Gomes et al., 2000).

In the taxonomic growth tests soluble starch is used. Some yeast species are also able to assimilate raw, unmodified starch. De Mot et al. (1984a) did a comparative study on starch degradation by ascomycetous species. Of 73 species tested, *Endomycopsis, Lipomyces, Pichia* and *Schwanniomyces* spp. were the most active. Another comparative study on starch assimilation was carried out by McCormack

and Barnett (1986). A thermostable α-amylase digesting raw starch is produced by a *Cryptococcus* sp. (Fuji et al., 1996) and by *Lipomyces starkeyi* (Punpeng et al., 1992). Wanderley et al. (2004) studied the α-amylase of *Cryptococcus flavus*. Debranching enzymes that hydrolyze 1,6 glucosidic bonds in amylopectin were produced by many yeast species. Highest activities were found with *Endomycopsis, Lipomyces, Filobasidium, Leucosporidium* and *Trichosporon* spp. grown on pullulan (poly-α-1,6-maltotriose) (de Mot et al., 1984b). Dextran (α-1,6-glucan) is another polymer of D-glucose, produced by *Leuconostoc mesenteroides* and some other bacteria. *Lipomyces starkeyi* produces an endodextranase (Webb and Spencer-Martins, 1983; Koenig and Day, 1988), but *Lipomyces lipofer* an exodextranase (Ramos and Spencer-Martins, 1983). A β-mannanase is produced by *Trichosporon cutaneum* CBS 5790 (Oda and Tonomura, 1996), later renamed *T. laibachii* of which it is the type strain. Kremnicky et al. (1996) screened many yeast species for mannanase production and found *Aureobasidium pullulans* and *Stephanoascus ciferrii* to be the best producers.

Pectic substances are complex structural polysaccharides of plant origin that contain a large proportion of partially methyl-esterified galacturonic acid residues linked by α-1,4-glycosidic linkages. These D-galacturonic acid residues present in the backbone of the pectin chain are interrupted by L-rhamnose units, to which arabinose and galactose residues can be attached. Roelofsen (1953) observed that yeasts belonging to the genera *Candida, Pichia, Saccharomyces* and *Zygosaccharomyces* attacked cell wall pectin. Many of these strains were isolated from fermenting cocoa. Salt-tolerant yeasts are also pectolytic and play a part in the softening of cucumbers pickled in brine (Bell and Etchells, 1956). Assimilation of pectic substances by yeasts during the dew-retting process of flax was reported by Wieringa (1956). A red yeast, initially named *Rhodotorula lini*, was responsible for this process. Frederiksen (1956) named it *Rhodotorula macerans.* Its present name is *Cryptococcus macerans*.

A review of pectolytic yeast species was given by Blanco et al. (1999). Endopolygalacturonase is the chief enzyme excreted into the growth medium (Luh and Phaff, 1951, 1953). In most species it is produced constitutively, but is subject to glucose repression. Several yeast species such as *Kluyveromyces* and *Saccharomyces* spp. are unable to grow on pectin or galacturonic acid (Schwan et al., 1997; Blanco et al., 1999). Hence, these yeasts must be involved in colonisation of plants and fruits. Tropical fruits are a habitat of many pectolytic yeasts (da Silva et al., 2005). Commercially available pectinases are produced by *Aspergillus niger*, but yeast enzymes could offer an alternative. Pectinases are used for softening of baby foods and for clarification of fruit juices. Large-scale production of endopolygalacturonase by *K. marxianus* has been reported by Almeida et al. (2004). It is also produced by *Debaryomyces hansenii* (da Silva et al., 2005), but *Stephanoascus smithiae* excreted an exopolygalacturonase. Pectin methylesterase was not secreted. Pectin lyase was produced by some strains of *K. wickerhamii*, *S. smithiae* and *Pichia anomala* (da Silva et al., 2005).

Middelhoven (2005) isolated yeast strains from a habitat rich in polysaccharides, viz. rotten wood. Several ascomycetous and basidiomycetous yeast species were

recovered and were tested for growth on polysaccharides. The ascomycetes assimilated none or a few, but the basidiomycetes tested assimilated soluble starch, pullulan, dextran, xylan, polygalacturonate, galactomannan and tannic acid or at least some of these. *Cryptococcus podzolicus* and *Trichosporon porosum* were the most active species. Previously, (Middelhoven, 2004) proposed growth tests on these polysaccharides for distinction of saprotrophic *Trichosporon* species. Pathogenic *Trichosporon* species generally do not assimilate these polysaccharides (Middelhoven, 2003). None of the yeasts tested was able to assimilate carboxymethyl cellulose, colloidal chitin, arabinogalactan and gum xanthan.

Some yeast species display hydrolytic activity towards polysaccharides but are unable to grow at the expense of the monomer. Gainvors et al. (1994) detected pectin-degrading enzymes in wine yeasts, but only one of 32 tested strains excreted these enzymes.

7.4.1 Immunology

Yeasts excrete heat-stable immunogenic polysaccharides that can be detected by enzyme-linked immunosorbent assays, ELISA (Middelhoven and Notermans, 1988; Middelhoven et al., 1998; Middelhoven and Notermans, 1993). The antigens of most ascomycetous yeast species were almost species-specific, but those of basidiomycetes gave cross reactions with many yeast species, ascomycetes included. This ELISA method permits specific detection of ascomycetous yeasts, even after their death. However, the preparation of specific antibodies is laborious. Hence, the technique became obsolete and was replaced with rDNA nuclear base sequencing and methods derived thereof. Nevertheless, the striking difference in behaviour of ascomycetes and basidiomycetes is interesting from a scientific viewpoint.

7.5 Yeasts Inhabiting Plants and Silage

7.5.1 Phyllosphere

The aerial surface of plants is covered by a thin layer of microorganisms. Ruinen (1956, 1961) introduced the term “phyllosphere”. This term is still used, though some authors prefer phylloplane to designate this intriguing habitat. The phyllosphere microflora of tropical foliage consists of bacteria, free-living nitrogen-fixing of the genus *Beyerinckia* included, and many yeasts (Ruinen, 1963, 1965), mainly *Cryptococcus* spp. and red basidiomycetes that produce ballistoconidia. These yeasts are able to decompose the plant’s cuticle, thus promoting leakage of nutrients from which the microflora benefits (Ruinen, 1965). Many of these yeasts produce, and even excrete, large amounts of lipids when grown in an unbalanced growth medium

containing an excess of sugars (Ruinen and Deinema, 1964; Stodola et al., 1967). Ballistoconidiogenous yeasts and their relatives were also detected on fresh maize foliage grown in a temperate climate Middelhoven and van Baalen, 1988). Sugar cane foliage is also inhabited by basidiomycetous yeasts (de Azaredo et al., 1998), of which *Cryptococcus albidus, C. laurentii* and *Rhodotorula mucilaginosa* were the most numerous; the ascomycete *Debaryomyces hansenii* was also present.

Plants growing in an arid climate, however, were inhabited by other yeasts as appeared from a study of 24 different plant species (Middelhoven, 1997). Ascomycetes predominated and about half of the isolates (22) were identified as *Debaryomyces hansenii*. Other ascomycetes were black yeastlike fungi like *Hormonema dematioides* (4 strains), *Hortaea werneckii* (1 strain) and 2 strains of a novel species *Hormonema schizolunatum* (Middelhoven and de Hoog, 1997). Basidiomycetes were represented by *Cryptococcus albidus* and *C. laurentii* (both by 4 strains) and by 5 red *Rhodotorula* spp., tentatively identified as *R. glutinis* and *R. mucilaginosa*. In addition to these true yeasts two yeastlike fungi of basidiomycetous affiliation were isolated, viz. an unidentified *Pseudozyma* sp. and a strain of *Cerinosterus cyanescens* that lacked the characteristic diffusible blue pigment (Middelhoven et al., 2000c).

Phyllosphere yeasts may protect the plant against infection by phytopathogenic fungi (Ruinen, 1961, 1963; Fokkema et al., 1979). Many of these yeasts attach to hyphae and conidia of these fungi (Allen et al., 2004). Phyllosphere yeasts and yeastlike fungi produce antibacterial compounds (McCormack et al., 1994). A monograph on the phyllosphere appeared some years ago (Lindow et al., 2002).

7.5.2 Biodegradation

Several phyllosphere yeast strains were screened for growth on plant constituents (Middelhoven, 1997). All of them readily assimilated lipid compounds, either hydrolytically or oxidatively or both. All strains grew on olive oil (*Hormonema* spp. excepted) and lecithin, indicating that these species may hydrolyze the cuticle. Assimilation of n-hexadecane was shown by all strains of *D. hansenii, R. glutinis, C. cyanescens and Pseudozyma* sp. This may be indicative of an oxidative attack of the cuticle that consists of long-chain aliphatic compounds.

The black yeastlike fungi, *Cryptococcus* sp. and *R. glutinis* assimilated xylan, pectin and starch. Disaccharides common in plants, like sucrose, maltose and cellobiose were assimilated by almost all strains tested.

Most strains assimilated protein, i.e. native casein, and several individual amino acids, lysine and methionine excepted. DNA and deoxyribose were no suitable carbon sources, but DNA and nucleic acid bases were assimilated as sole nitrogen sources, DNA and cytosine by all strains tested, the other bases giving variable results. RNA was assimilated as sole carbon and nitrogen source by almost all strains tested.

Phenolic compounds are important plant constituents. *D. hansenii* strains grew on phenol and hydroquinone, but not on more complex compounds. Some of these,

e.g. cinnamic acid and its hydroxy and methoxy derivatives, gallic and tannic acids supported growth of some *Rhodotorula* strains and of some yeastlike fungi. For more details, see Middelhoven (1997). The general conclusion is that the yeast flora of the phyllosphere benefits from many plant constituents. The latter are set free by mechanical damage and by leakage of the cuticle and cell wall that may be provoked by lipolytic activity of the yeast cells.

7.5.3 Maize Silage

The aerobic phyllosphere yeast flora drastically changes when plant material is compressed and stored under anaerobic conditions, as happens during ensiling. In maize silage the aerobic yeasts had vanished after two days, before lactic acid fermentation was complete, and were replaced with fermentative yeast species (Middelhoven and van Baalen, 1988). *Candida lambica, C. milleri* and *C. holmii* (anamorph of *Saccharomyces exiguus*) predominated this yeast flora. These species, along with *C. krusei* and *S. dairenensis*, had previously been demonstrated as dominant yeast species in an analysis of 13 different maize silages (Middelhoven and Franzen, 1986).

Another maize silage yielded a novel species, viz. *S. bulderi* (Middelhoven et al., 2000a), related to *S. exiguus*. It is able to ferment gluconolactone by a novel pathway for alcoholic fermentation (van Dijken et al., 2002) in which a NADP-dependent glucose dehydrogenase and enzymes of the pentose phosphate pathway participate.

Numbers of *C. lambica, C. milleri* and *C. holmii* increased during ensiling to approximately 10^7 per gram after 5 days, but gradually decreased to 10^4 after 4 months. Obviously, conditions in silage are adverse, also for these acid-tolerant yeasts. These are the principal agents responsible for aerobic deterioration of maize silage as appears from their increase to about 10^9 per gram after 4 days, at the expense of acetic and lactic acids. Some yeast species, e.g. *C. famata, Geotrichum candidum* and *Hansenula (Pichia) anomala* occurred in lower numbers. In addition to lactic acid and ethanol, these species assimilated minor products of bacterial sugar fermentation such as acetoin and butane-2,3-diol (Middelhoven and van Baalen, 1988).

7.5.4 Other Ensiled Crops

Whole-crop maize that was ensiled at 25°C or 30°C, rather than at 20°C as usual was inhabited by the same fermentative yeast species, but these were accompanied with ascomycetous non-fermentative fungi, i.e. *Exophiala jeanselmei* and *Verticillium psalliotae*, by the non-fermentative basidiomycete *Rhodotorula mucilaginosa* and the weakly fermentative ascomycete *Arxula adeninivorans* (Middelhoven et al., 1990). Other ensiled crops were inhabited by the same yeasts as present in maize silage at 20°C. These crops were beetroot foliage, grass, lucern, Jerusalem artichoke

foliage, green chicory foliage and hemp foliage. However, ensiled crops containing mustard oils were inhabited by other yeast species, i.e. *Rhodotorula minuta, Stephanoascus ciferrii, Candida famata* and *Trichosporon cutaneum* (later reidentified as *T. gracile*). These species were isolated from ensiled turnip foliage, rocket and leek.

7.6 Phylogeny and Distinction of Taxa

7.6.1 Phylogeny

In some cases a physiological character is only shown by a group of related species. A well-known example is growth on methanol that is a character of related ascomycetous species and not of other yeasts (Kurtzman and Robnett, 1998). Yeast species able to utilize adenine as sole carbon source are also found in only one clade. Phylogenetic analysis based on nuclear ribosomal base sequencing revealed close phylogenetic relationship of *Stephanoascus ciferrii* and *Arxula adeninivorans* (Kurtzman and Robnett, 1998). Several *Blastobotrys* species belong to the same clade of about 12 species. Middelhoven and Kurtzman (2003) showed that nearly all these species assimilated adenine as sole carbon source, a character not shown by other yeasts studied. In addition to adenine, these 12 species assimilated n-hexadecane, uric acid, isobutanol, L-leucine, L-isoleucine and putrescine. Some of these characteristics were also shown by other ascomycetes, but the 12 species of the *Stephanoascus/Blastobotrys* clade showed these all together, suggesting a relation between phylogeny and physiology.

Middelhoven and Kurtzman (2003) stated that yeast species of which respiratory deficient mutants are known do cluster in four clades according to the tree proposed by Kurtzman and Robnett, 1998). However, in a more recent phylogenetic analysis (Kurtzman and Robnett, 2003) these species were scattered over the tree. This is no surprise as several different mutations can lead to respiratory deficiency.

7.6.2 Trichosporon

The imperfect basidiomycetous genus *Trichosporon* is characterized by assimilation of many unusual carbon compounds. Growth tests on uric acid, ethylamine, L-4-hydroxyproline, tyramine and L-phenylalanine as sole source of carbon and nitrogen, and on quinate, 4-ethylphenol, 2,3-dihydroxybenzoate and orcinol as sole carbon source were used for distinction of pathogenic and suspected pathogenic species, able to grow at 37°C (Middelhoven, 2003) and an identification key based on these tests was provided. A key to saprotrophic *Trichosporon* species was provided by Middelhoven (2000b, 2004). In addition to the growth tests that distinguish

pathogenic species, some plant constituents such as xylan, polygalacturonate, galactomannan, tannic acid, phloroglucinol and orcinol were used as well.

Species of the genus *Trichosporon* cluster in four clades (Middelhoven et al., 2004), viz. the Ovoides, Cutaneum, Porosum and Gracile clades. The Ovoides clade contains most of the pathogenic species able to grow at 37°C and on uric acid, but not on polygalacturonate, quinate, phloroglucinol, melibiose and raffinose. The other three clades are variable for these characters, except the Porosum clade of which nearly all members assimilate polygalacturonate, quinate, phloroglucinol, melibiose and raffinose. The Gracile clade is negative for phloroglucinol and erythritol and variable for the other characteristics. Assimilation of dextran and tannic acid is exclusively shown by some species of the Porosum clade (Middelhoven, 2004).

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Chapter 8
Ecology and Biodiversity of Yeasts with Potential Value in Biotechnology

T. Deak

Contents

Abstract In the latest edition of the standard treatise of yeasts, in 1998, 700 species were described. Since then, the number of recognized yeast species has doubled, with a steep increase particularly in the number of the basidiomycetous yeasts. Of all these yeast species, only about a dozen is used at industrial scale, and some 70–80 species have been shown at laboratory scale to possess potential value in biotechnology; their ratio is, in the best case, 5–10%. If it is accepted, that according to a modest estimate, the known yeast species represent only 5% of the total number which may inhabit the Earth, then there is ample room to search for new species with novel potential to exploit. Where could these yeasts be discovered?

In recent years we are witnessing great progress in exploring the diverse ecological niches of yeasts, and revealing the great diversity of species living in the various habitats. Still, compared to the profusing metabolic capability of bacteria living in the soil, surprisingly less is known about the soil yeasts. Much remains to be learned on yeasts associated with insects, invertebrates and fishes in the deep ocean, inhabiting tropical forests, or striving in extreme environments. It could reasonably be expected, that among the numerous species to be discovered in specific and unusual habitats, many will be found to possess enzymes, carry out metabolic routes and show physiological properties which hold out promises to be valuable for biotechnological applications. This chapter will examine these potential values from the point of view of ecology and biodiversity of yeasts.

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8.1 Introduction

Yeasts have been used for making bread, beer and wine since ancient ages. Their role in fermentation was recognized by Pasteur, and the first pure cultures (starters) of brewer's and wine yeast were obtained by Hansen and Müller-Thurgau, respectively, at the end of the 19th century. Since then the application of yeast starters has become a standard practice in the industrial fermentation not only for food and beverages but also for a broad variety of other products made by yeasts or from yeast cells.

The traditional fermentation processes are carried out by a single species of yeasts, *Saccharomyces cerevisiae*, hence for many, its name is synonymous with yeasts, and it is thought that all yeasts are fermentative. Contrary to general belief, there have been described more than thousand species of yeasts, about half of them not being able to ferment; nevertheless many of these have gained a significant role in biotechnology.

Improvement of starter cultures relies on classical genetic techniques such as hybridization and mutagenesis followed by selection. Recently, it has become possible to tailor production strains for purpose by methods of recombinant gene technology (genetic engineering). Public concern and legal regulation may raise difficulties in commercial application of genetically modified organisms and their products. However, there is an alternative way of finding novel strains with better producing properties, and it is to search for them among the existing organisms.

In recent years we are witnessing an increasing awareness of the importance of the biodiversity in nature, and of conserving and sustainable utilizing it. Beyond plants and animals, great progress has been made in exploring the diverse ecological niches of microorganisms, among them yeasts. Much remains to be learned about the great diversity of species and the profusing metabolic capabilities of yeasts striving in extreme environments. It could be reasonably expected that among the numerous species living in specific and unusual habitats – many of them are to be discovered – several strains would be found possessing enzymes, carrying out metabolic routes and showing physiological attributes which hold out promises to be valuable for biotechnological exploitation.

This chapter will examine these potential values from the point of view of ecology and biodiversity of yeasts. Some recent overviews of the subject are Demain et al. (1998), Walker (1998), Buzzini and Vaughan-Martini (2006).

8.2 Biodiversity of Yeasts

Biodiversity of yeasts can be characterized from taxonomic and ecological point of view. Taxonomically, yeasts form an artificial group of fungi comprising mostly unicellular organisms reproducing vegetatively by budding (blastoconidia).

However, several yeasts can develop true hyphae similar to those of moulds, whereas other species may form pseudohyphae from elongated cells remaining attached together after budding. Instead of budding, some yeasts propagate by arthroconidia arising from cell division or splitting of hyphae. Sexual reproduction is known in less than half of yeast species; this may result in the generation of ascospores or basidiospores, with or without preceding conjugation. In all, yeasts represent a phylogenetically diverse group of fungi, that can be classified either to Ascomycetes (e.g. *Saccharomyces, Candida*) or Basidiomycetes (e.g. *Filobasidiella, Rhodotorula*); moreover the small genus of *Schizosaccharomyces* and few other species belong to neither, formerly was regarded a separate group called Archiascomycetes.

The number of recognized yeast genera and species are increasing steadily. In a little more than 50 years, between the appearance of the 1st edition and the 5th edition of the taxonomic monograph of yeast, the number of genera increased from 26 to 133, and the number of species described from 164 to more than one thousand

Table 8.1 Yeast species of current and potential use in biotechnology

Species	Application
Candida milleri	Sourdough
C. shehatae	Bioethanol
C. sake	Biocontrol
C. oleophila	Biocontrol
C. maltosa	SCP on hydrocarbons
Debaryomyces hansenii	Cheese, sausage ripening, proteases
D. (Schwanniomyces) occidentalis	Amylase
Eremothecium ashbyi	Riboflavin
Geotrichum candidum	Cheese ripening
Hanseniaspora uvarum	Wine fermentation
Kluyveromyces marxianus	Milk fermentation, SCP from whey
K. lactis	Milk fermentation, SCP from whey
Pachysolen tannophilus	Bioethanol
Phaffia rhodozyma	Astaxanthin
Pichia angusta (Hansenula polymorpha)	Bioethanol
P. anomala	Biocontrol
P. jadinii (C. utilis)	Feedstock
P. pastoris	Heterologous proteins
P. stipitis	Bioethanol
Pseudozyma flocculosa	Biocontrol
Rhodotorula glutinis	Carotene
Schizosaccharomyces pombe	Cider fermentation
Saccharomyces cerevisiae	Brewer's, baker's, wine yeast, bioethanol, invertase, heterologous proteins
S. exiguus	Sourdough
S. boulardii (S. cerevisiae)	Probiotics
Saccharomycopsis fibuligera	Amylase
Torulaspora delbrueckii	Sourdough
Zygosaccharomyces rouxii	Soy sauce

Data from Abbas (2006); Buzzini and Vaughan-Martini (2006); Walker (1998); Demain et al. (1998)

(Lodder, 1970; Kurtzman, Fell and Boekhout, 2006), at the time of writing, the 5th edition has not appeared, and the exact figure is not known). However, the real number of existing yeast species may well exceed that of already described ones. Prudent attempts only try to estimate the existing number of fungal species as 1.5 million of which 72000 (4.8%) described (Hawksworth, 2001; Hammond, 1995). If yeasts make up about 1.0–1.5% of all known fungi, then the number of their existing species would approximately fall between 15 to 24 thousand. Of all these yeast species, only about a dozen is currently used at industrial level, and some 70 to 80 species have been tested at laboratory scale showing potential value in biotechnology application (Table 8.1). The questions arise: how much more could be exploited, and where could these yeasts be discovered? Part of the answer lies in exploring the ecology of yeasts.

8.3 Ecology of Yeasts

Yeasts live in community or biocoenosis with other organisms, which is the biotic component making up an ecosystem together with its abiotic components. The abiotic (physical and chemical) components of the ecosystem is frequently referred to as the environment. The physicochemical attributes of the environment act on the organisms as intrinsic and extrinsic ecological factors, and define the habitat in which they could exist. The properties of organisms (implicit ecological factors) as opposed to those factors provided by the environment would determine how the organisms could strive, survive or die in a given habitat (Boddy and Wimpenny, 1992). The biotic component also includes the sources and vectors contributing to the colonization of habitats, as well as the interactions between the members of the community, which are sometimes the most influencing ecological factors (e.g. synergistic or antagonistic) of an ecosystem.

Ecosystems differ in kind and size. The soil, the sea, a forest, or an animal body are natural ecosystems, an arable land, an orchard or a cow in stable are under the impact of human influence, whereas foods can be entirely artificial – nevertheless, they can be considered for ecosystems, and from microbiological point of view, they are certainly those. The extension of an ecosystem may be as large as the ocean, or as small as a leaf of a plant or a morsel of soil, but they provide equally habitats for microorganisms under their characteristic impact of ecological factors.

Ecological factors exert limits on microbial biodiversity. Microorganisms exist everywhere on Earth when physical or chemical conditions permit. One of the most surprising outcomes of the recent exploration of microbial biodiversity has been the recognition of the wide range of physiological conditions under which microbes flourish. Microorganisms have been discovered in niches not conceived to be habitable. Some thrive at temperatures close to 100°C in hot springs and at temperatures above the boiling point of water in submarine hot vents. Others are found in the ice of both Poles, some live in saturated salt brines or at pH extremes lower than 1 or

Table 8.2 Examples of extremophiles in natural habitats and foods

Environmental parameter	Microbial type	Natural habitat	Foods
Temperature			
Low < 10°C	Psychrophile	*Psychrobacter*	Some *Pseudomonas*
High > 50°C	Thermophile	*Synechococcus*	*Bacillus, Clostridium*
> 80°C	Hyperthermophile	*Pyrobolus*	None
pH			
Low ≤ 1	Acidophile	*Thiobacillus*	*Acetobacter*
High ≥ 9	Alkalophile	*Natronbacterium*	*Metschnikowia*
Low a_w < 0.85	Xerotolerant	*Penicillium*	*Debaryomyces*
< 0.65	Xerophile	*Xeromyces*	*Zygosaccharomyces*
Oxygen			
Cannot tolerate	Obligate anaerobe	*Methanococcus*	*Clostridium*
Tolerate low O_2	Microaerophile	*Spirillum*	*Campylobacter*
Neutral	Facultative	*Enterobacter*	*Saccharomyces*
Require O_2	Obligate aerobe	*Macrococcus*	*Rhodotorula*
Radioactivity	Radioduric	*Deinococcus*	None
High hydrostatic pressure	Barophilic	*Shewanella*	None

higher than 12, still others bear high hydrostatic pressure or high radioactivity. These various forms are collectively called as extremophiles. Among them several new species have been found, moreover, new classes and phyla of bacteria have been recognized, most of them placed in the recently recognized third domain of life, the Archaea (Woese and Fox, 1977; Woese et al.,1990; Staley et al., 1997; Hugenholtz et al., 1998). Extreme physiological properties are, however, not limited to prokaryotes, the same peculiar characteristics are found among eukaryotes, as well (Roberts, 1998; Rotschild and Mancinelli, 2001; Moreira and López-Garcia, 2002). Moreover, extreme living conditions are not restricted to natural habitats, similar conditions are provided by some preserved foods, and the microorganisms striving them can be also considered extremophiles. For example, most of the major food spoilage yeasts could be termed such, they are extremely osmophilic (e.g. *Zygosaccharomyces rouxii*), halotolerant (*Candida etchellsii*), ethanol-tolerant (*Saccharomyces cerevisiae*), resistant to weak-acid preservatives (*Zygosaccharomyces bailii*) or others (Table 8.2) (Stratford, 2006; Raspor and Zupan, 2006).

8.4 Diversity of Yeast Used in Industrial Fermentation

The term ‘industrial fermentation’ is meant in the present context all kinds of processes from the traditional alcoholic fermentation of beer and wine, to the aerobic propagation of baker’s yeast, and to novel products of biotechnology made with so-called non-conventional yeasts, other than strains of *Saccharomyces*, including not only industrial, but also agricultural, environmental and medical applications and utilizations.

In this brief overview, the enormously broad and vast field of actual and potential use of yeasts cannot be covered exhaustively. Some examples will only be given to illustrate current and future trends in five major fields of exploitation: 1. Food and beverage fermentations, 2. Products of cell mass and cell constituents, 3. Bioethanol, 4. Pharmaceutical and bioactive substances, and 5. Other uses. In discussing the improvement of production strains, special attention will be paid to the potential exploitation of yeasts hidden in the biodiversity as contrasted to improvement by genetic modification.

8.4.1 Food and Beverages

The traditional use of yeasts for the production of bread, beer and wine has been comprehensively reviewed (Dequin et al., 2003; Pretorius, 2000; Bonjean and Guillaume, 2003; Dufour et al., 2003). Since the beginning of the 20th century, the application of yeast starters has become a standard practice in the industrial fermentation of these products.

In brewing, where the malt is fermented practically by the starter alone, the pivotal role of *Saccharomyces cerevisiae* as pitching yeast is unquestioned. Efforts have been directed only to improve the performance of this starter, as will be discussed below. Use of other starters at the previous stage of steeping and malting have been considered only recently. Sometimes the barley is heavily contaminated by mycotoxin producing fusaria. It has been shown that *Geotrichum candidum* starter culture can be used for the protection of barley, and its presence also increases the enzymatic potential of malt (Linko et al., 1998; Foszczynska et al., 2004).

The situation is different in the fermentation of must and in the leavening of bread, where the pure starter, when used, does not remain alone but a mixed association with other yeasts develops, often together with lactic acid bacteria and accompanied by other bacteria and molds. In enology, it has been long debated to what degree the autochthonous yeasts may contribute in the fermentation of aroma and 'bouqet' of the wine. Some even question the use of starter strain and prefer the indigenous yeasts to maintain the specific character of the 'terroir'. Experiments on lab scale and by mini-vinification have been conducted showing the potential role of yeasts other than *Saccharomyces cerevisiae* in wine making. Among these, *Hanseniaspora guilliermondii* and its anamorph *Kloeckera apiculata*, *Pichia fermentans, Candida stellata* and others have been suggested as novel adjuncts in simultaneous or sequential mixtures with *Saccharomyces cerevisiae* (Clemente-Jimenez et al., 2005; Moreira et al., 2005).

Starters are intensively used in the leavening of bread and various other baked goods. The baker's yeasts also belong to the species of *S. cerevisiae*, being special strains of it. Lactobacilli play also an important role in sourdough, and are often associated with yeasts. In addition to *Saccharomyces cerevisiae*, which can be added as baking yeasts, at least 25 different yeast species has been described from sourdough (Meroth et al., 2003), among them *Candida milleri, C. glabrata,*

C. krusei may become dominant, however, further studies are necessary to determine their importance in the fermentation and to select appropriate species for use as starter culture (Vogel, 1997). Currently, *Saccharomyces exiguus* and *Candida humilis* have been considered in commercial sourdough preparations (Hammes et al., 2005; de Wuyst and Neysens, 2005).

Over the years, the use of yeasts for the production of food and beverages has been broadened to include dairy, meat and bakery products, spirits and alcoholic beverages other than wine (Fröhlich-Wyder, 2003; Samelis and Sofos, 2003; Hammes et al., 2005). The role of yeasts in the fermentation of some of these products has been well known for long, e.g. in kefyr and sourdough. In most cases, however, yeasts were considered in these products as spoilage organisms, or, in the best case, as innocuous, allochthonous members of the microbiota. Studies disclosing the rich biodiversity of yeasts in many of these products have also revealed, that certain yeasts may play a beneficial role in fermentation and ripening.

In the fermentation of dairy products and some kind of meat products starters are used as well. In these, the dominant microorganisms are lactic acid bacteria, however, yeasts and other microbes may join them contributing in the development of flavour and texture. In kefyr grains, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* and *Torulaspora delbrueckii* live in strong symbiotic association with lactic acid bacteria (Narvhus and Gadaga, 2003). Although lactic acid starters are used primarily for the fermentation of cheeses, adventitious yeasts always participate in their ripening and maturation (Ferreira and Viljoen, 2003; Das et al., 2005; Leroy et al., 2006). In addition to *Saccharomyces cerevisiae*, due to their proteolytic and lipolytic activity, *Debaryomyces. hansenii* and *Yarrowia lipolytica* are regarded as good candidates for ripening agents in soft cheeses (van den Tempel and Jakobsen, 2000; Guerzoni et al., 2001; Suzzi et al., 2001; Ferreira and Viljoen, 2003), and *Geotrichum candidum* in the production of Camembert cheese (Molimard et al., 1994; Boutrou and Guéguen 2005). Of these, *Debaryomyces hansenii* has already been commercialized as potential adjunct culture (Durá et al., 2004; Flores et al., 2004). Less is known about the involvement of yeasts in the ripening of sausages. *Debaryomyces hansenii* or other lipolytic yeasts may be considered as commercial starter cultures (Olesen and Stahnke, 2000).

In the fermentation of the dairy and meat products, yeasts are only second to lactic acid bacteria, whereas in the alcoholic fermentation of various beverages other than wine, yeasts play a significant role. In many of them, e.g. cider, sake, tequila, rum and others, beyond *S. cerevisiae*, other yeasts can be dominant in developing characteristic flavor and aroma.

In recent years, the rich and varied microbiota participating in various other food and beverage fermentations has been the subject of detailed studies, and it has emerged that some species may be applied as adjuncts to improve the quality of product. Several yeast species are noted among the potent candidates. In pickled cucumbers, the mixed fermentation of *Saccharomyces rosei* (now *Torulaspora delbrueckii*) with lactic acid bacteria has been considered (Passos et al., 1997). The fermentation of coffee, cocoa, cider, olives and a number of various indigenous traditional products have recently been the subject of intensive studies, which shed

light of the complex microbial interactions and most important species (Jespersen et al., 2005). Among them there are several yeast species with the potential to be developed into a starter culture (Schwan and Wheals, 2003; Coton et al., 2006). The participation and role of yeasts of mixed fermentation, such as soy sauce, oriental products, coffee and cacao awaits further exploratory studies. Of these fermentations, with the participation of mixed microbial associations, it is perhaps the production of soy sauce the microbiology of which is best known. The process is controlled by the starters of the koji mold *Aspergillus oryzae* or *A. sojae* and the moromi yeast, *Zygosaccharomyces rouxii* (Hanya and Nakadai, 2003). The 'soy yeast', *Zygosaccharomyces rouxii*, is undoubtedly one of the main producer of aroma compounds, however, less is known about the contribution of some 20 to 25 other yeast species isolated from various stages of soy sauce production. The microbiota of indigenous (traditional, oriental) fermentations is so variable that no definite picture can be drawn on the yeasts (and other microorganisms) present in these products. Preparation of most indigenous fermented products is still a traditional art at small scale rather than a controlled process. At least 20 to 30 yeast species may participate to some degree in the development of the characteristic quality of these naturally fermented foods (Narvhus and Gadaga, 2003; Sanni and Lönner, 1993), while in the fermentation of several commodities it is the lactic acid and other bacteria and/or molds which play the determining role.

8.4.2 Yeast Cell Mass and Commodity Products

In addition to the main fermented foods and beverages, the second major group of commodities include those made from yeast cell mass or cell-derived products. Among these are pressed baker's yeast and active dried yeast, food and feed yeasts, yeasts autolysates and extracts, as well as cell components such as enzymes, vitamins, carotenoids, lipids, steroids, polysaccharides, glucans, nucleotides, flavours and many others. Several of these are important ingredients and adjuncts in the production of food and beverages, whereas others find application in chemical, pharmaceutical, cosmetic and other industries. It would be far beyond the space of this chapter to give an overview of all these, and reference is made to comprehensive reviews appeared previously and more recently (Reed and Nagodawithana, 1991; Halasz and Lasztity, 1991; Abbas, 2006).

Pichia jadinii, better known in asexual form as *Candida utilis*, is the most widely used species for the production of cell mass for animal feed. It grows abundantly on molasses, and to a certain degree also on agricultural and industrial wastes (wood hydrolysate, sulfite liquor). It has been an ongoing effort of research to find or develop a yeast species or strain being able directly utilizing lignocellulosic materials, for the bioconversion of renewable agricultural products and residues to feedstock and/or industrial fuel. Although a few yeast species has the metabolic capability to hydrolyse starch (e.g. *Debaryomyces occidentalis, Saccharomycopsis fibuligera*), and to utilize cellobiose and xylose after the partial

hydrolysis of woody materials (e.g. *Candida shehatae, Pichia stipitis*), the economically feasible solution has not yet achieved, even with genetically engineered strains (see below) (Jeffries and Kurtzman, 1994; Leathers, 2003).

A large number of yeast species has been recognized for their ability to utilize hydrocarbons as sole carbon and energy sources (e.g. *Candida maltosa, C. tropicalis, Yarrowia lipolytica* and many others; (Tanaka and Fukui, 1989; Fickers et al., 2005). In the 1970es, large industrial plants were set up to produce single cell protein on this source. After the explosion of oil prices, this technology became unprofitable and ceased. Nowadays, hydrocarbon utilizing yeasts can be used for the degradation of oil spills and remediation of the environment. A large group of yeasts, however, is capable of utilizing methanol, which could serve for an inexpensive source of producing single cell proteins from the anaerobic decomposition of agricultural wastes.

Baker's yeast is a main product as well as a source of many derived products together with spent brewer's yeast. However, beyond *Saccharomyces cerevisiae*, increasing lists of other yeast species are being exploited in producing and manufacturing these commodities. Whey is a major waste in the dairy industry, and lactose utilizing yeasts, such as *Kluyveromyces marxianus*, can be used for the production of protein-rich cell mass as well as valuable bioingredients, oligonucleotides, flavor enhancers (Belem and Lee, 1998). Further examples are: *Candida utilis* for feed, *Kluyveromyces lactis* for aromas and lactase, *Rhodotorula glutinis, Sporobolomyces pararoseus, Phaffia rhodozyma* for carotenoids and colorants, *Rhodotorula glutinis* also for lipids, *Debaryomyces (Schwanniomyces) occidentalis* for amylase, *Eremothecium ashbyi* for riboflavin, *Yarrowia lipolytica* for citric acid and lipase, *Sporidiobolus salmonicolor* for flavor compounds (Dufossé et al., 2002).

Pectinolytic enzymes are important in the food industry for improving juice extraction and clarification. Instead of addition of pectinases, *Saccharomyces cerevisiae* wine strains can be transformed to constitutively overexpress its own endopolygalacturonase gene (Fernández-González et al., 2005). Production of pectinases is not uncommon among yeasts; 7% of species belonging to six genera isolated from tropical fruits secreted pectinolytic enzymes (da Silva et al., 2005).

8.4.3 Bioethanol

Considering the exploitation of yeasts beyond the field of food and beverages, the most important biotechnological application is the production of bioethanol for gasoline additive or even substitute. In some countries, particularly Brazil, and also in the USA and Canada, considerable amount of ethanol is fermented from cane juice or other sugar-rich agricultural raw materials (Wheals et al., 1999). *Saccharomyces cerevisiae* is used for this purpose, and current interest is directed to the improvement of fermentation technology and to the utilization of cheap agro-industrial by-products or wastes. In this regard, the conversion to ethanol of

lignocellulosic hydrolysates with yeasts, such as *Pichia stipitis, Candida shehatae* or *Pachysolen tannophilus*, which can ferment cellobiose and xylose, is of primary concern. The ethanol yield is far less than in the case of *Saccharomyces cerevisiae,* and approaches have been made to the genetic transformation of *Saccharomyces cerevisiae* with genes for xylose fermentation (Kuyper et al., 2005). A multitransformant strain containing not less than four foreign genes was engineered capable of directly degrading cellulose (van Rensburg et al., 1998). Wild type of a methylotrophic yeast, *Pichia angusta* (*Hansenula polymorpha*) is able to ferment cellobiose and xylose to ethanol (Ryabova et al., 2003). *Kluyveromyces marxianus* can be used to ferment inulin and produce ethanol from many plant feedstock. Recently, a strain of *Kluyveromyces marxianus* has been used for bioethanol production also from cheese whey (Kargi and Ozmihci, 2006).

8.4.4 Pharmaceutical and Bioactive Products

As a further biotechnological extension, yeasts can be utilized for the production of compounds of pharmaceutical value. Few of these can be obtained from natural strains of *S. cerevisiae* or non-conventional yeasts, and more by genetically modified (GM) strains expressing heterologous proteins. Since the beginning of the 1980s a number of vaccines, antigens, hormones and other biotherapeutic compounds have been cloned into yeasts and expressed at laboratory scale, and some of these (e.g. insulin, interferon, hepatitis A antigen) have reached commercial production as well. GM yeasts will be discussed below in more details. In biotherapeutic respect, the potential use of yeast as probiotics should be mentioned. Compared with the widely accepted probiotic activity of lactic acid bacteria and bifidobacteria, yeasts are less recognized although some strains of *Saccharomyces cerevisiae* referred to as *'Saccharomyces boulardii'* have been used to control gastrointestinal disorders (McFarland et al., 1993). More recently, it has been shown that viable and dead cells, in particular cell wall preparates (glucomannans) can be applied to bound and remove mycotoxins from the intestine of poultries and also from juices (Bejaoui, 2004; Yiannikouris et al., 2004; Basmacioglu et al., 2005), moreover, a new yeast species, *Trichosporon mycotoxinovorans*, was described with the ability to degrade mycotoxins (Molnar et al., 2004).

8.4.5 Other Uses of Yeasts

Miscellaneous further, potential applications of yeasts relate to both foods and other biotechnological products and processes. Yeasts of certain capability of biodegradation have been considered for bioremediation and action in environmental protection. Hydrocarbon assimilating yeasts may be useful for the degradation of oil spills, yeast cells as biosorbent can be used for the removal of heavy metals and

radioactive isotopes, and stains of *Trichosporon cutaneum* and the yeast-like *Aureobasidium pullulans* able to degrade phenols and other aromatic compounds can be used for their removal from industrial effluents. Olive oil manufacture results in large quantities of black wastewaters due to phenolic compounds which could be decolorized by depolymerization of the phenolics by *Geotrichum candidum* (Ayed et al., 2005). Another case is the reduction of the pesticide, glyphosphate residues in wheat flour during proofing of yeasted dough (*Saccharomyces cerevisiae*) as demonstrated by Low et al. (2005).

An area attracting growing interest is the application of yeasts for biocontrol. Some yeast species, in particular *Pichia guilliermondii, P. anomala*, and *Debaryomyces hansenii* inhibit the growth of certain moulds attacking fruits and grains. The possible use of antagonistic yeasts to control post-harvest diseases and production of mycotoxins has been reviewed (Wisniewski and Wilson, 1992; Druvefors and Schnürer, 2005; Suzzi et al., 2005).

8.5 Improvement of Yeast Strains Used in Production

Since the creation of the first pure cultures of yeast, intensive research has been carried out leading to production of industrial strains with improved properties, made first by selection and hybridization, later by protoplast fusion and cytoduction, and from the 1980s on, by genetic engineering. These studies have been excellently reviewed by Hammond (1995); Dequin (2001); Pretorius and Westhuizen (1991); Schuller and Casal (2005).

In the field of traditional food and beverage fermentations, only a few will be mentioned of the broad purposes of improvement for technologically important properties. Among these were in brewing: carbohydrate utilization, fermentation of dextrins, flocculation and filtration, reduction of H_2S and diacetyl production, osmotolerance (high gravity wort); in baking: fast dough raising, organic acid resistance, rehydration tolerance; in wine making: ethanol tolerance, fermentation capacity, absence of off-flavours. These targets have been achieved with some success by the application of classical genetic techniques such as mutagenesis and hybridization followed by selection, and more recently protoplast fusion and cytoduction. However, a major limitation of these classical genetic techniques has been in general the difficulty of adding or removing one feature without altering gross performance. In particular, the stable genetic constitution of polyploid/aneuploid industrial strains, lack of mating type characteristics, and poor sporulation all restricted the possibilities of broad strain improvement. The potential of recombinant gene technology (genetic engineering) has provided more possibilities, and holds out much promises of specific modifications.

The principal aims of genetic modification is the transformation of host cell by introduction of foreign genes. It is beyond the scope of this chapter to go into details of the techniques for transformation and cloning (only few of the extensive list of reference manuals: Broach et al. (1991); Evans (1996); Jones et al. (1992);

Pringle et al. (1997). Briefly, the major steps are: 1. identifying the target gene and obtaining the DNA fragment from a genomic cDNA library or by PCR amplification; 2. creating a suitable plasmid vector; 3. joining the DNA fragment to the vector DNA generating a recombinant DNA molecule; 4. inserting the recombinant into host cell; 5. screening transformed cells and selecting the target gene using appropriate marker system.

Yeasts are excellent hosts for the production of recombinant proteins, offering ease of genetic manipulation, and cultivation to high cell density with a fast growth rate. Moreover, yeasts are able to perform complex eucaryotic-type posttranslational modification and produce proteins similar to mammalian origin. *S. cerevisiae*, the genetically best characterized organisms, is the host used most frequently for transformation. However, the *S. cerevisiae* transformation system has some limitations in that the proteins are often overglycosylated and may contain a terminal group suspected to be allergenic; the yield of recombinant proteins is relatively low, and the narrow substrate specificity of the species limits fermentation design. Some of the non-conventional yeasts, such as *Pichia pastoris, Pichia angusta (Hansenula polymorpha*) and others, may be more advantageous host, although the number of cloned genes, the availability of molecular genetic tools, and the understanding of metabolic regulation are limited compared with *Saccharomyces cerevisiae* (Cereghino and Cregg, 2000).

The primary approaches have been directed to the genetic improvement of the production characteristics of *Saccharomyces cerevisiae* starter strains used in brewing, wine making and baking. Table 8.3 gives some examples of these. Note, that in several cases the genetic modification is achieved by self-cloning, i.e. the GM strain does not contain foreign gene from organisms other than *Saccharomyces cerevisiae*.

Developing of transgenic strains has been extended to the broader field of biotechnology, in particular for the production of bioethanol and pharmaceuticals. Sequential introduction of multiple genetic alterations into a single host genome is now not exceptional. Examples are the total biosynthesis of the steroid hydrocortisone involving as many as 13-engineered genes (Szczebara et al., 2003), and a celluluse fermenting yeast containing genes from four different organisms (van Rensburg et al., 1998).

In this regard it is worth mentioning, that screening among yeast isolates from natural sources revealed rich sources of cellulose decomposing strains, several of which turned out to be novel species (Buzzini and Martini, 2002; Nakase et al., 1994; Carreiro et al., 2004). A potential producing strains may well be found among these isolates.

Genetic modification of microorganisms and, in particular, crop plants, have been the subject of big controversy and being debated heavily both in scientific circles and by the general public. In these days, the great developments and achievements already made in the field should be taken seriously, and the issues arising from technological, environmental, economic, social, ethical and political point of views should be discussed critically and rationally (Pretorius, 2000; Schuller and Casal, 2005; Verstrepen et al., 2006). Concerns about GMOs and GM

Table 8.3 Genetically modified *Saccharomyces cerevisiae* starters for brewing, baking and wine making

Improvement	Proteins, genes	Sources
Wine yeast		
Clarification, no haze	Pectate lyase *pelA*	*Erwinia chrysanthemi*
Endopolygalacturonase	*PGU1*	*S. cerevisiae*
Flocculation	Flocculin *FLO1*	*S. cerevisiae*
Flor formation	Adhesin *FLO11*	*S. cerevisiae*
Stress tolerance	Trehalose *TPS1,2*	*S. cerevisiae*
Ethanol tolerance	Sterols *SUT1*	*S. cerevisiae*
Glycerol overproduction	Glycerol-P-dehydr. *GPD1*	*S. cerevisiae*
Resveratrol production	β-Glucosidase *bglN*	*C. molischiana*
Malolactic fermentation	Permease, *mae1* malic enzyme *mleS*	*Schizo. pombe* *Lactococcus lactis*
Brewer's yeast		
Dextrin fermentation	Glucoamylase *STA2* Amyloglucosidase *AMG*	*S. cerev.* var. *diastaticus* *Aspergillus awamori*
Flocculation	Glucanase EG1	*Trichoderma reesii*
Diacetyl elimination	Acetoacetate decarboxylase *ALDC*	*Enterobacter aerogenes*
Reduced H_2S production	Sulfuhydrase *MET25*	*S. cerevisiae*
Acetate esters production	Acetyltransferase *ATF1*	*S. cerevisiae*
Antibacterial property	Pediocin *pedA* Leucocin *lcaB*	*Pediococcus cerevisiae* *Leuconostoc carnosum*
Baker's yeast		
Melibiose utilization	α-Galactosidase *MEL1*	*S. bayanus*
Maltose utilization	Stronger promoter *ADH*	*S. cerevisiae*
Cryoresistance	Aquaporin AQY1	*Sch. pombe*
Osmotolerance	Glycerol synthesis GPD1	*S. cerevisiae*

Data from: Randez-Gil et al. (1999); Dequin (2001); Schuller and Casal (2005); Hammond (1995); Panadero et al. (2005); Pretorius (2000); Pretorius et al. (2003); Verstrepen et al. (2006); Gonzalez-Candelas et al. (1995).

products are beyond the scope of this review. Regarding microorganisms only, it should be realized, however, that vaccines, drugs, enzymes produced by genetically engineered strains have been on the market for years, and are not just beneficial but also indispensible. Several of them are produced by GM yeasts, such as interferons, somatostatin, insulin, chymosin and others.

A different issue is, however, when not the purified product but the organisms itself containing foreign genes is included in the consumables or foods. Baker's yeast with high maltase activity, brewer's yeast with glucoamylase for dextrin hydrolysis, and a sake yeast with enhanced ethyl caproate flavor (Akada, 2002) have got approval by respective authorities, however, have not been commercialized because the lack of public acceptance refrained industry from putting to use (Moseley, 1999). Recently, the appearance on the US market of recombinant wine yeast capable of malolactic fermentation may sign a breakthrough in this respect (Cummins, 2005).

Examples for the expression of heterologous genes in *S. cerevisiae* and other yeast hosts are listed in Tables 8.4 and 8.5.

Table 8.4 Genetic modification of *Saccharomyces cerevisiae* expressing foreign genes

Foreign gene	Donor species	Result
β-Galactosidase	*Kluyveromyces lactis*	Lactose utilization
L-Galactose dehydrogenase	*Arabidopsis thailana*	Ascorbic acid (vitamin C)
α-Amylase^{+}	*Lipomyces kononenkoae*	
Glucoamylase	*S'copsis fibuligera*	Starch fermentation
Xylose isomerase	*Piromyces* sp. fungus	Xylose fermentation
α-Glucuronidase	*Aureobasidium pullulans*	Xylan degradation
Cellobiase	*Endomyces fibuliger*	Cellulose degradation
$^{+}$Endo-β-glucanase	*Butyrivibrio fibrisolvens*	
$^{+}$Cellobiohydrolase	*Phaanerochaete chrysosporium*	
$^{+}$Cellodextrinase	*Ruminococcus flavefaciens*	
Pectate lyase	*Fusarium solani*	Pectin hydrolysis
Eight foreign genes and disruption of five host genes	Mammalian	Hydrocortison

Data from Rubio-Teixera et al. (2000); Sauer et al. (2004); Knox et al. (2004); Kuyper et al. (2005); de Wet et al. (2006); van Rensburg et al. (1998); Szczebara et al. 2003.

Table 8.5 Examples of the production of foreign proteins in non-conventional yeasts

Yeast	Protein	Year of publication
Schizosaccharomyces pombe	Invertase from *S. cerevisiae*	1985
	α-amylase from *D. occidentalis*	1989
	Glucoamylase from *S. diastaticus*	1986
Pichia pastoris	β-galactosidase	1987
	Hepatitis B antigen	1987
	Bovine lysozyme	1989
	Human epidermal growth factor	1990
Pichia angusta	β-lactamase	1988
(*Hansenula polymorpha*)	Glucoamylase	1991
	Human serum albumin	1990
Kluyveromyces lactis	Prochymosin	1990
	Human serum albumin	1991
	α-Amylase from *D. occidentalis*	1989
Yarrowia lipolytica	Porcine α-interferon	1990
	Bovine prochymosin	1988
	Human proinsulin	1993
Zygosaccharomyces bailii	Lactate dehydrogenase	2004

Data from Romanos et al. (1992); Madzak et al. (2004)

8.6 Conclusions

The use of selected strains of *Saccharomyces cerevisiae* has provided tremendous advantages in traditional fermentation and novel biotechnology industries. The methods of conventional breeding, hybridization and selection, though have already resulted in numerous innovations and improvement in the properties of traditional starters, are nevertheless somewhat limited in their capacity. The application of

molecular techniques and recombinant gene technologies, as further possible ways for the development of novel starters will have to receive serious consideration in the future. Introduction of foreign genes into baking, brewing and wine yeasts, and to a number of non-conventional yeast species, has resulted in many improved strains genetically modified at laboratory scale. Only few of them have got legal approval but the lack of public acceptance refrained industry from commercial application. Hence, the exploration of the rich and yet only partially known biodiversity of natural ecosystems, among them indigenous fermentations, is a promising and challenging way for the quest of novel potential starters and adjuncts in the production not only of food and beverages, but also across various biotechnology sectors from bioenergy and pharmaceuticals to bioremediation and environmental protection. Yeasts have been and will continue being important contributors to benefit our life.

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Chapter 9
Yeasts Diversity in Fermented Foods and Beverages

Jyoti Prakash Tamang and Graham H. Fleet

Contents

Abstract People across the world have learnt to culture and use the essential microorganisms for production of fermented foods and alcoholic beverages. A fermented food is produced either spontaneously or by adding mixed/pure starter culture(s). Yeasts are among the essential functional microorganisms encountered in many fermented foods, and are commercially used in production of baker's yeast, breads, wine, beer, cheese, etc. In Asia, moulds are predominant followed by amylolytic and alcohol-producing yeasts in the fermentation processes, whereas in Africa, Europe, Australia and America, fermented products are prepared exclusively using bacteria or bacteria-yeasts mixed cultures. This chapter would focus on the varieties of fermented foods and alcoholic beverages produced by yeasts, their microbiology and role in food fermentation, widely used commercial starters (pilot production, molecular aspects), production technology of some common commercial fermented foods and alcoholic beverages, toxicity and food safety using yeasts cultures and socio-economy.

Keywords Amylolytic yeasts, fermented foods, alcoholic beverages, food safety, yeasts, food fermentation

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9.1 Introduction

A fermented food or beverage is defined as an edible product prepared from the raw or cooked materials of plant or animal origins by microorganisms either naturally or by adding mixed or pure culture(s) (Campbell-Platt, 1994; Holzapfel, 2002). The essential objective of food fermentation is to carry over supplies from the time of plenty to those of deficit (Tamang, 2000). Traditionally people knew how to culture the beneficial microorganisms, mostly lactic acid bacteria, yeasts and filamentous moulds, for production of foods for consumption. What was the scientific explanation and identity of these microorganisms were unknown to them.

Microorganisms are present in or on the ingredients, utensils, environment, and are selected through adaptation to the substrate for fermentation (Hesseltine, 1983; Tamang, 1998). 'Rice-soybean-fish-alcoholic beverage' diet is the characteristic food culture of the East and South East Asia, whereas 'wheat-milk and milk products-meat-wine' is the basic diet in the Western part of the world. In East Asia rice is a staple food whereas in West Asia wheat or barley is a staple food. Milk products similar to West and alcoholic beverages similar to East are encountered in food habits of the people of the Himalayas (Tamang, 2005). In Asia, moulds are predominant microorganisms in the fermentation processes, whereas in Africa, Europe and America, fermented foods are prepared exclusively using bacteria or bacteria-yeasts mixed cultures; moulds seem to be little or never used (Tamang, 1998). However, in the Indian sub-continent, mostly due to wide variation in agro-climatic conditions and diverse form of dietary culture, all major groups of micro-organism (bacteria-yeasts-moulds) are associated with fermented foods showing the transition of food culture (Tamang and Holzapfel, 1999).

Yeasts play vital roles in production of many traditional fermented foods and beverages across the world (Aidoo et al., 2006) signifying the food culture of the regions and the community. Functional yeasts genera associated with fermented foods and beverages are mostly *Brettanomyces* (its perfect stage, *Dekkera*)*, Candida, Cryptococcus, Debaryomyces, Galactomyces, Geotrichum, Hansenula, Hanseniaspora* (its asexual counterpart *Kloeckera*), *Hyphopichia, Kluyveromyces, Metschnikowia, Pichia, Rhodotorula, Saccharomyces, Saccharomycodes, Saccharomycopsis, Schizosaccharomyces, Torulopsis, Trichosporon, Yarrowia* and *Zygosaccharomyces* (Kurtzman and Fell, 1998; Pretorius, 2000; Nout and Aidoo, 2002; Tsuyoshi et al., 2005). Table 9.1 shows the common fermented foods and beverages, mostly prepared by yeasts, or in combination with bacteria and moulds. This chapter deals with the role of yeasts in production of traditional fermented foods and beverages mostly concerning their diversity and identity.

9.2 Fermented Meat Products

Fermented meat products are divided into two broad groups: those made from whole meat pieces or slices, such as dried meat, cured hams and jerky, and those made by chopping or comminuting the meat, usually called sausages and salami-type

Table 9.1 Foods and beverages fermented by yeasts

Food	Substrate	Nature and use	Microorganisms	Country
Fermented legume products				
Bhallae	Black gram	Deep-fried patties; snack	LAB, yeasts	India
Dhokla	Bengal gram	Spongy cake; snack	LAB, yeasts	India
Maseura	Black lentil	Dry, ball-like, brittle; condiment	LAB, Yeasts	India, Nepal
Papad	Black gram	Circular wafers; spicy condiment, snack	LAB, yeasts	India
Vadai	Black gram	Deep fried patties; snack	LAB, yeasts	India
Wari	Black gram	Dry, ball-like hollow, brittle; condiment	LAB, yeasts	India
Fermented cereal products				
Dosa	Rice (finely grounded), black gram	Spongy, pan cake; staple food	LAB, yeasts	India
Enjera	Tef flour, wheat	Sour, leavened, pancake-like bread; staple food	LAB, yeasts	Ethiopia
Idli	Rice (coarsely grounded), black gram	Steamed, spongy pancake; breakfast	LAB, yeasts	India
Jalebi	Wheat flour	Crispy, deep fried pretzels; confection snack	Yeasts, LAB	India, Nepal, Bangladesh
Kenkey	Maize	Acidic, maize dumpling, steamed; staple food	LAB, yeasts	Ghana
Kisra	Sorghum	Thin pancake bread; staple food	LAB, yeasts	Sudan
Masa	Rice/millet or sorghum	Shallow fried millet or sorghum flour cake; snack food	LAB, yeasts	Nigeria
Nan	Wheat flour	Leavened flat baked bread; staple food	Yeasts, LAB	India, Pakistan, Afghanistan
Puto	Rice	Rice cake; breakfast and snack food	LAB, yeasts	Philippine, China
Rabadi	Barley, maize, wheat, pearl millet	Cereal flour with buttermilk; staple food	LAB, yeasts	India
Selroti	Rice-wheat flour-milk	Pretzel-like, deep fried; bread	Yeasts, LAB	India, Nepal, Bhutan
Sourdough	Wheat flour	Leavened baked bread; staple food	Yeasts, LAB	America

(continued)

Table 9.1 (continued)

Food	Substrate	Nature and use	Microorganisms	Country
Fermented milk products				
Cheese	Animal milk	Fermented dairy product; side-dish	LAB, yeasts	World-wide
Chhu or *sheden*	Cow/Yak milk	Soft, strong flavoured; curry	LAB, yeasts	India, Nepal, Bhutan, China (Tibet)
Chhurpi	Cow/Yak milk	Soft, cheese-like; curry, pickle	LAB, yeasts	Nepal, India, Bhutan
Dahi	Cow milk	Curd; savory	LAB, yeasts	India
Mohi	Cow milk	Butter-milk	LAB, yeasts	India
Kefir	Goat, sheep, or cow milk	Acidic, mildly alcoholic, effervescent milk	LAB, yeasts	East Europe, middle east countries, north America
Kishk	Sheep milk, wheat	Milk-wheat mixture; dried balls	LAB, yeasts	Greece, Turkey, Egypt, Libya, Iran
Koumiss	Horse, donkey or camel milk	Acid/alcoholic milk	LAB, yeasts	North European conutries, Russia, Mongolia, China
Fermented meat products				
Cured hams	Beef/pork	Whole meat product, side-dish	LAB, micrococci, Yeasts	World-wide
Kargyong	Beef/yak/pork	Sausages; curry	LAB, micrococci, Yeasts	India, Bhutan, China (Tibet)
Kheuri	Beef/yak/pork	Sausages; curry	LAB, micrococci, Yeasts	India, Bhutan, China (Tibet)
Salami	Beef/pork	Meat product, side-dish	LAB, micrococci, Yeasts	World-wide
Sausages	Beef/pork	Sausages, side-dish	LAB, micrococci, Yeasts	World-wide
Fermented/dry fish products				
Gnuchi	River fish	Smoked, sun-dried; curry	LAB, micrococci, *Bacillus*, yeasts	India
Hentak	Fish	Dried fish mixed aroid plants, fermented; curry	LAB, yeasts	India
Ngari	Fish	Fermented fish product; curry	LAB, yeasts	India
Sidra	Fish	Dried fish; curry	LAB, micrococci, yeasts	India, Nepal
Sukako maacha	River fish	Smoked, sun-dried; curry	LAB, micrococci, *Bacillus*, yeasts	India, Nepal

Sukuti	Fish	Dried fish; curry	LAB, micrococci, yeasts	India, Nepal
Tungtap	Fish	Fermented; pickle	LAB, yeasts	India
Mixed-amylolytic starter cultures				
Appon dobai	Rice, wild herbs	Starter culture	Filamentous moulds, Yeasts, LAB	India
Bubod	Rice, wild herbs	Dry, ball-like starter culture	Filamentous moulds, Yeasts, LAB	The Philippines
Chiu-yueh	Rice, wild herbs	Starter culture to ferment alcoholic beverages	Filamentous moulds, Yeasts, LAB	China, Singapore
Hamei	Rice, wild herbs	Starter culture to ferment alcoholic beverages	Filamentous moulds, Yeasts, LAB	India
Longpang	Rice, wild herbs	Starter culture to ferment alcoholic beverages	Filamentous moulds, Yeasts, LAB	Thailand
Marcha	Rice, wild herbs, spices	Dry, ball-like starter culture	Filamentous moulds, Yeasts, LAB	Nepal, India
Men	Rice, wild herbs, spices	Dry, ball-like starter culture	Filamentous moulds, Yeasts	Vietnam
Nuruk	Rice, wild herbs	Starter culture to ferment alcoholic beverages	Filamentous moulds, Yeasts, LAB	Korea
Phab	Wheat, wild herbs	Starter culture to ferment alcoholic beverages	Filamentous moulds, Yeasts, LAB	China (Tibet), Bhutan
Ragi	Rice, wild herbs	Dry, ball-like starter culture	Filamentous moulds, Yeasts, LAB	Indonesia
Alcoholic beverages				
Aara	Cereals	Clear distilled liquor; alcoholic drink	Yeasts, LAB	India
Appo	Finger millet	Mild-alcoholic, beverage	Filamentous moulds, Yeasts, LAB	India
Atingba	Rice	Alcoholic, sweet-sour paste beverage	Yeasts, LAB	India
Bhaati jaanr	Rice	Mild-alcoholic, sweet-sour, food beverage; paste	Filamentous moulds, Yeasts, LAB	India, Nepal, Bhutan

(continued)

Table 9.1 (continued)

Food	Substrate	Nature and use	Microorganisms	Country
Beer	Barley, hubs	Mild alcoholic beverage	Yeasts	World-wide
Chyang/ Chee	Finger millet	Mild-alcoholic, slightly sweet-acidic; beverage	Filamentous moulds, Yeasts, LAB	Bhutan, China (Tibet)
Cider	Apple	Clear alcoholic drink	Yeasts	France, Spain, Ireland, Slovenia
Gahoon ko jaanr	Wheat	Mild-alcoholic, slightly acidic, beverage	Filamentous moulds, Yeasts, LAB	India, Nepal, Bhutan
Jou	Rice	Alcoholic beverage	Yeasts, LAB	India
Kodo ko jaanr	Finger millet	Mild-alcoholic, slightly sweet-acidic; beverage	Filamentous moulds, Yeasts, LAB	India, Nepal
Makai ko jaanr	Maize	Alcoholic, sweet-sour, food beverage; paste	Filamentous moulds, Yeasts, LAB	India, Nepal, Bhutan
Ruou nep	Rice	Alcoholic drink	Moulds, Yeasts	Vietnam
Oh	Rice-millet	Soft, beverage	Yeasts, LAB	India
Pona	Rice	Alcoholic, sweet-sour, beverage	Yeasts, LAB	India
Raksi/Arak	Cereals	Clear distilled liquor; alcoholic drink	Filamentous moulds, Yeasts, LAB	India, Nepal, Bhutan
Simal tarul ko jaanr	Cassava tuber	Mild-alcoholic, sweet-sour, food beverage; paste	Filamentous moulds, Yeasts, LAB	India, Nepal
Wine	Grapes	Clear alcoholic drink	Yeasts	World-wide
Zutho	Rice	Alcoholic beverage	Yeasts, LAB	India
Non-alcoholic beverages				
Kombucha or tea fungus	Tea liquor	Non-alcoholic drink	LAB, Yeasts	China, Japan, Indonesia, Russia

Note: LAB, lactic acid bacteria

products (Campbell-Platt and Cook, 1995). Meat processing is the combination of chemical curing, microbial fermentation and drying which together give stable, safe, ready-to-eat products (Bacus, 1984). Microorganisms involved in the production of fermented meats are lactic acid bacteria, staphylococci, micrococci, moulds and yeasts (Cook, 1995).

Although bacteria are considered to have the dominant role in meat fermentation, the contribution of yeasts nevertheless is significant (Romano et al., 2006). There are two stages during processing where yeasts growth and activity are considered relevant. They exhibit limited growth along with bacterium during the initial stage of fermentation (e.g. as in the production of salami-type sausages), and their populations remain high and active during subsequent storage and maturation of the product (e.g. as for salami and dry-cured hams).

The ingredients for sausage production include chopped or dried meat (beef, pork or sheep), sodium chloride, sodium nitrite/nitrate, sucrose (obtained) and a range of species according to the product (Bacus, 1986). Initial populations of yeasts are generally low (less than 10^3–10^4 cfu/g), and originate from the ingredients and processing equipment. Yeasts are naturally present on the hides of animals and readily contaminate fresh meat during slaughtering (Dillon and Board, 1991). Studies in several countries have now shown that yeast grow during the initial stages of fermentation, along with bacteria, growing final populations of 10^6–10^7 cfu/g. The total populations usually remain at these levels or decrease slightly during the subsequent stages of maturation and storage, which may last for several months (Samelis et al., 1993; Abunyewa et al., 2000; Coppola et al., 2000; Encinas et al., 2000; Gardini et al., 2001; Ferreira et al., 2006). Similarly total yeast populations of 10^6–10^8 cfu/g are frequently found during the storage or ripening of dry-cured hams (Nú ez et al., 1996; Saldanha-da Gama et al., 1997). Numerous factors can alter the growth of yeast during the production of fermented sausages and cured hams and include smoking, addition of spices, salt concentration, relative humidity and temperature. Encinas et al. (2000) found difference in yeast counts between smoked and non-smoked sausages. Inhibitory effects of garlic powder on yeast growth during sausage production have been reported (Ghamnnoum, 1990; Olesen and Stahnke, 2000).

A diversity of yeast species has been isolated from fermented sausages and cured hams produced in different countries with little exception (Metaxopoulos et al., 1996; Martin et al., 2006). *Debaryomyces hansenii* and its anomorph *Candida famata* are the most frequently isolated and quantitatively the most significant yeasts in the production of fermented sausages and cured hams. Various other species in the genera *Candida, Cryptococcus, Pichia, Rhodotorula, Trichosporon, Metschnikowia* and *Yarrowia* have been associated with these products, with *Y. lipolytica, R. mucilaginosa, C. zeylanoides* and *M. pulcherrima* deserving special mention. Recently, both culture and culture-independent-DNA analyses have confirmed the predominance of *D. hansenii* in sausage fermentations (Rantsiou et al., 2005). *D. hansenii* is reported from a traditional South Italian processed sausages along with bacteria (Baruzzi et al., 2006). Moreover, molecular analyses have demonstrated substantive strain heterogenecity in the strains of *D. hansenii* and *Y. lipoltica* isolated from these products (Gardini et al., 2001).

It is generally considered that yeasts make a positive contribution to colour and flavour developments in fermented sausages and cured-hams (Mauriello et al., 2004), but the precise mechanisms are not understood. Significant metabolic properties in this context include their oxygen scavenging ability for production of extracellular proteases and lipases, utilization of organic acids such as lactic acid, and production of flavour volatiles such as alcohols, acids, esters and various carboxyls (Romano et al., 2006). *Debaryomyces hansenii* is known for its ability to hydrolyze meat proteins (Martin et al., 2003; Flores et al., 2004), while *Y. lipolytica* has strong lipase and proteolytic activities (Gardini et al., 2001). However, there is significant strain variation among these properties. Both *D. hansenii* and *Y. lipolytica* have been proposed as candidates for development of novel starter cultures for production of sausage and ham (Gardini et al., 2001; Martin et al., 2006) where they could enhance, flavour, texture and colour applied and continue to decrease processing times.

There are few reports regarding the contribution of yeasts to biogenic amines production in fermented foods. In *Debaryomyces* and *Candida* isolated from fermented meats, a histidine decarboxylase activity was found, which was higher than that observed for lactic acid bacteria and staphylococci (Suzzi and Gardini, 2003).

Tamang and Rai (unpublished data) found the populations of yeasts in kargyong and kheuri, ethnic fermented sausages of Sikkim, Bhutan and Tibet at the level of 10^5 cfu/g along with predominant lactic acid bacteria.

9.3 Fermented Milk Products

Fermented milk products are generally classified as (i) low acid products (e.g. cultured buttermilk and cultured cream); (ii) medium acid products (e.g. yogurt and cheese); (iii) high acid products (e.g. Bulgarian sour milk); and (iv) acid alcohol products (e.g. kefir, koumiss) (Kosikowski, 1977; Oberman and Libudzisz, 1998). In terms of microbiology, the production of these commodities is usually associated with the growth and metabolic activities of lactic acid bacteria during the initial fermentation of milk. The contribution of yeasts to the manufacture of these products is a relatively recent observation that is reviewed by Fleet (1990), Jakobsen and Narvhus (1996) and Frohlich-Wyder (2003). It is now widely demonstrated that yeasts make a significant and positive contribution to the maturation process of many types of cheese and may grow in association with lactic acid bacteria in the fermentation of products such as kefir and koumis (Stanley, 1998).

Low populations of yeasts (less than 10^3 cfu/ml) occur in raw or pasteurized milks (Fleet and Mian, 1987; Deak and Beuchat, 1995). Milk is an excellent substrate for their growth and in the absence of competing bacteria; they quickly grow to populations as high as 10^7–10^8 cfu/ml (Roostita and Fleet, 1996a). However, they exhibit little growth in milk inoculated with high levels of lactic acid bacteria. Consequently, they are not significant in the milk fermentation stages of cheese or

yogurt production (Viljoen, 2006). Surveys of cheeses in the retail markets, globally, consistently show the presence of high populations of yeasts (10^6–10^9 cfu/g), especially in soft-brined cheese and mould ripened soft to semi-soft varieties such as Brie, Camembert and blue veined cheeses (Roostita and Fleet, 1996b). Detailed investigations of the microbial ecology of cheese production have revealed that yeasts are major component of the microflora of cheese maturation. The coagulated curd from milk fermentation contains little yeast. However, soon after the curd is brined, yeasts quickly grow to 10^6–10^9 cfu/g, and generally remain at these levels throughout subsequent maturation. They are distributed throughout the entire curd, but populations on the outer curd are usually 10–100-fold higher than those of the inner curd (Devoyod, 1990; Addis et al., 2001). These yeasts originate as natural contaminants of the processing environment, principally coming from the brine (salt), surface of equipment, the air and workers.

A significant diversity of yeast species has been isolated from cheeses and includes species of *Candida, Debaryomyces*, *Kluyveromyces*, *Saccharomyces*, *Pichia*, *Torulospora*, *Yarrowia*, *Rhodotorula*, *Cryptococcus, Galactomyces* and *Trichosporon* (Frohlich-Wyder, 2003; Vasdinyei and Deak, 2003; Romano et al., 2006). However, the most prevalent species are *D. hansenii*, *Y. lipolitica, S. cerevisiae, K. marxianus* and *G. geotrichum* (Addis et al., 2001; Romano et al., 2006). *D. hansenii* is notable for its tolerance to salt and occurrence in cheese brines, and exhibits dominant growth in cheese throughout the maturation process. DNA-based molecular typing and phenotypic analyses have demonstrated significant strain heterogenecity within cheese isolates of the species (Suzzi et al., 2000; Hansen and Jakobsen, 2001; Petersen et al., 2002).

The function of yeasts during cheese maturation and their contribution to cheese quality requires further investigation. However, they are considered to impart on cheese flavour and texture through proteolytic and lipolytic activities, (as *D. hansenii* and *Y. lipolytica*), fermentation of residual lactose (e.g. *K. marxianus*), and utilization of organic acids such as lactic and citric acids (Fleet, 1990). Moreover, they may stimulate or inhibit the growth of bacteria and filamentous fungi that contribute to maturation of some cheeses (Viljoen, 2006). Generally, their activities during maturation are considered positive, creating commercial interest in using selected strains of *D. hansenii*, *S. cerevisiae* and *Y. lipolytica* as novel starter cultures in cheese manufacture (Frohlich-Wyder, 2003; Romano et al., 2006).

Kefir is an acidic mildly alcoholic beverage prepared by fermenting the milk of goats, sheep or cows with kefir grains obtained from the previous batch of kefir. It is popular in Eastern and Coastal European as well as in Scandinavia and is considered as a health promoting, probiotic beverage (Oberman and Libudzisz, 1998; Frohlich-Wyder, 2003). Milk fermentation is conducted at 18–22°C for about 20–24 h, after which newly formed kefir grains are removed and the liquid cooked for consumption. Microbiologically, the fermentation is a symbiotic interaction between lactic acid bacteria, yeasts and acetic acid bacteria. During fermentation and subsequent refrigeration of the product, the yeasts grows to about 10^5–10^8 cfu/ml. Species of *K. marxianus* and *S. cerevisiae* generally dominate but less frequently isolated species include *Torulaspora delbrueckii*, *S. unisporus* and *S. exiguus* (Beshkova

et al., 2002; Frohlich-Wyder, 2003). The yeasts contribute characteristic gassiness (CO_2), ethanol and other flavours to the product (García Fontán et al., 2006).

Koumiss is an effervescent acid or alcoholic fermented milky white-greyish liquid made primarily from mare milk (Kosikowski, 1977). The primary fermenting microorganisms in koumiss are *Lb. bulgaricus*, yeasts *Candida kefyr,* and *Torulopsis* spp. (Kosikowski, 1977; Tamime, 1981).

Laban rayeb (laban) is a traditional Lebanese fermented milk product, which has slightly acid taste with aroma resembling that of buttermilk (Morcos et al., 1973; Oberman, 1985). The predominating organisms are *Lactococcus lactis* subsp. *lactis, Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus* (Chammas et al., 2006) and lactose fermenting yeasts (Vedamuthu, 1982).

Misti dahi (sweetened dahi, mishti doi, lal dahi or payodhi) is a sweetened fermented milk product from the eastern part of India (Ray and Srinivasan, 1972). Gupta et al. (2000) optimized the production of misti dahi from buffalo milk using starter combinations comprising (i) *Streptococcus salivarius* subsp. *thermophilus*, *Lb. acidophilus* and *Lb. delbrueckii* subsp. *bulgaricus*; and (ii) *Lb. acidophilus*, *Lactococcus lactis* subsp. *lactis* and *S. cerevisiae*.

The people of the Eastern Himalayan regions of India, Nepal, Bhutan and Tibet in China, prepare a variety of ethnic fermented milk products for long centuries (Tamang, 2005). Some of these milk products are dahi, mohi, gheu, chhurpi, chhu**,** somar and philu (Dewan and Tamang, 2007). Species of lactic acid bacteria are the dominant microorganisms in these ethnic fermented milk products with high population levels up to 10^8 cfu/g, followed by yeasts with a load of 10^7 cfu/g (Tamang et al., 2000; Dewan and Tamang, 2007). Prevalence of yeasts in fermented cow-milk and yak-milk products of the Sikkim Himalayas was 60% and 45.5%, respectively (Dewan, 2002). *Saccharomycopsis crataegensis* and *Candida castellii* are reported from chhu, an indigenous fermented cheese-like product of Sikkim (Dewan and Tamang, 2006). Presence of high number of yeasts (10^6 cfu/g) indicates some role during spontaneous fermentation of chhu. Yeasts bring about desirable fermentation changes in fermented milk products (Westall and Filtenborg, 1998). Lactic acid bacteria and *Candida kefyr* constitute a part of microflora in amasi, Zimbabwean fermented cow-milk product (Gadaga et al., 2001).

9.4 Fermented Cereal Products

The production of bread is an ancient biotechnological process that is based on the fermentation of a wheat flour-dough, but flours from other cereals such as rye, are also used. It has evolved into a modern industrialized process, the details of which are well-reviewed (Jenson, 1998; Hammes and Ganzle, 1998; Decock and Cappelle, 2005). Sourdough bread is acidic-tasting aerated bread, made from rye, wheat or mixed flours of Europe and South America (Campbell-Platt, 1987). Sourdough fermentation is a mixture of flour, water and salt and if kept at room temperature, it will undergo a natural fermentation that involves the growth of

indigenous yeasts and LAB (Brandt, 2007). Essentially, bread dough is a mixture of flour, water and salt and if kept at room temperature, it will undergo a natural fermentation that involves the growth of indigenous yeasts and lactic acid bacteria. The microbial ecology of these fermentations has been well studied, leading to the recognition that *S. cerevisiae* is the principal yeast of most bread fermentations (Jenson, 1998; Hammes et al., 2005). Today, selected strains of *S. cerevisiae* (baker's yeast) are extensively produced at commercial products for bread production. Typically, the bread manufacturer buys baker's yeast as a dried, compressed or liquid product and adds it to the dough mixture to give populations of 10^8–10^9 cfu/g. The dough mixture is then incubated at 30–35°C for varying times (depending on the process) for fermentation. The substrates for fermentation are mainly maltose and glucose that are released by the breakdown of wheat starch by endogenous amylases of the flour. Yeast fermentation serves several functions. Gas (CO_2) production causes expansion and leavening of the dough, ultimately affecting bread texture, density and volume. Some of the CO_2 dissolved to form carbonic acid. Ethanol produced by the yeast, yeast enzymes that affect cereal proteins and carbonic acid, influence the rheological properties of the dough again impacting on final bread texture and structure. A vat assay of secondary metabolites (alcohols, acids, esters, aldehydes, ketones, etc.) produced by the yeast and any bacterium associated with the fermentation, constitute distinctive flavour to the bread. Most of these metabolites are volatile and are lost during baking, but they also undergo complex reactions with other dough components during baking to affect flavours (Jenson, 1998; Hammes and Ganzle, 1998; Decock and Cappelle, 2005). There has been extensive selection development and genetic improvement of specific strains of *S. cerevisiae* for the bread processing industries (Rande-Gil et al., 1999; Dequin, 2001; Bonjean and Guillaume, 2003). The principle requirements of the strains are rapid fermentation and CO_2 production from maltose and glucose, and generation of good bread flavours. However, strains used for fermenting sweet doughs that certain added sucrose need to be more osmotolerant and strains used for the production of frozen dough must be freeze-thaw tolerant. In addition, the various strains of baker's yeasts need to have properties that enable them to be produced efficiently and economically on a large commercial scale and to have good storage and stability properties as dried yeasts, either compressed yeasts or yeast slurries. Strains to be processed as dried yeasts need to be more tolerant of heat stresses than compressed yeasts. Thus, there is significant physiological, biochemical and genetic diversity in strains of *S. cerevisiae* used for bread production (Jenson, 1998; Rondez-Gil, 1999). In some cases, species other than *S. cerevisiae* could offer better functionality in some criteria. For example, *Torulaspora delbrueckii* and *Kluyveromyces thermotolerans* are more freeze tolerant than *S. cerevisiae* and could be used to prepare frozen dough breads (Jenson, 1998; Alves-Araújo et al., 2004).

Many types of bread, especially in European countries, are still produced by traditional processes where no commercial strains of baker's yeast (*S. cerevisiae*) are added. Indigenous yeasts and lactic acid bacteria conduct dough fermentation, and the resultant products are generally called sourdough breads because they have

higher contents of lactic acid and acetic acid due to the bacterial growth (Hammes and Ganzle, 1998; Hammes et al., 2005; De Vuyst and Neysens, 2005; Rehman et al., 2006). San Francisco sourdough bread falls into this category. Various studies have been conducted in recent years to understand the microbial ecology of this fermentation. While indigenous *S. cerevisiae* is still prominent in many of these fermentations, the presence and growth of other yeast species are significant and these include *Saccharomyces exiguus, Candida milleri, Candida humilis, Candida krusei* (*Issatchenkia orientalis*), *Pichia anomola, Pichia membranifaciens* and *Yarrowia lipolytica* (Gobbetti, 1998; Corsetti et al., 2001; Paramithiotis et al., 2000; Gullo et al., 2002; Foschino et al., 2004; Veinocchi et al., 2004). These yeasts have evolved to grow in temperature with the lactic acid bacteria of these dough, including *Lactobacillus sanfranciscensis* (unique to these ecosystems), *Lactobacillus plantarum*, and various other species *of Lactobacillus, Pediococcus* and *Leuconostoc* (Hammes et al., 2005; de Vuyst and Neysens, 2005). Commercial starter cultures of these yeast-bacterial combinations are now available (Decock and Cappelle, 2005). Finished bread products are not immune to spoilage by yeasts if not properly stored. If slightly moist, they can develop a fermentative odour and flavour due to growth of *S. cerevisiae*. Growth of *Pichia burtonii* produces visible, white or chalky discoloration (Legan and Voysey, 1991).

Consumption of rice as a staple food in Asia has resulted in a traditional cereal fermentation with moulds and yeasts (Haard et al., 1999). Varieties of traditional non-alcoholic cereal-based fermented foods are mostly prepared and consumed in Africa as staple foods (Nout, 2001; Blandino et al., 2003). Fermented cereal-based gruels are generally used as naturally fortified weaning foods for young children in Africa (Efiuvwevwere and Akona, 1995; Tou et al., 2007).

Enjera (Injera) is thin soft bread, with numerous eyes, or gas holes, baked in Ethiopia from the cereal tef (*Eragrotis tef*) and eaten at nearly every meal with meat, vegetable or legume stew, with each person eating two or three per day. Stewart and Getachew (1962) isolated fungi including yeasts *Pullaria, Aspergillus, Penicillium, Rhodotorula, Hermodendrum, Candida guilliermondii* and a number of unidentified bacteria from samples of *enjera* batter. Yeasts appeared in all stages of *enjera* fermentation but disappeared later due to decrease in pH of the product (Gashe, 1985).

Idli is an acid-leavened and steamed cake made by bacterial fermentation of a thick batter made from coarsely ground rice and dehulled black gram. Idli cakes are soft, moist and spongy, have desirable sour flavour, and is eaten as breakfast in South India. Dosa batter is very similar to idli batter, except that both the rice and black gram are finely grounded. The batter is thinner than that of idli and is fried as than, crisp pancake and eaten directly in South India. Though, lactic acid bacteria are predominant microflora in idli and dosa fermentation, yeasts have also been reported from the product. Soni and Sandhu (1989, 1990, 1991) reported the principal yeasts in idli and dosa as *S. cerevisiae, D. hansenii, H. anomala, T. candida* and *Trichosporon beigelii*. Addition of yeasts in idli and dosa fermentation contributes to leavening and flavour development and results in enhanced contents of thiamine and riboflavin. However, the presence of yeasts can interfere with acidification of

the batter since the yeasts utilize a portion of the fermentable sugars that otherwise would be used for production of lactic acid supplementation of the batter ingredients (Venkatasubbaiah et al., 1985; Steinkraus, 1996).

Jalebi is a traditional Indian crispy sweet, deep fried pretzel made from wheat flour and eaten as confection snack food (Chitale, 2000). The batter is prepared by mixing wheat flour with curd and then fried in oil and the fried jalebi are taken out from the pan and soak in sugar syrup immediately for 4–5 h (Batra, 1986). LAB along with yeasts *Saccharomyces bayanus, S. cerevisiae* and *Hansenula anomala* have been reported from jalebi (Batra and Millner, 1974; Soni and Sandhu, 1990).

Rabadi is a fermented cereal-based food prepared by mixing flour of wheat, barley, pearl millet or maize with buttermilk of North-West India (Gupta et al., 1992a). Single as well as mixed culture fermentation of pearl millet with yeast (*S. cerevisiae* or *S. diastaticus*) and LAB (*Lb. brevis* or *Lb. fermentum*) was developed for utilization of pearl millet by fermentation (Khetarpaul and Chauhan, 1990a). Fermentation of pearl millet with pure cultures of yeast and lactobacilli has been found effective method for improving its nutritive value: increased bioavailability of minerals (Khetarpaul and Chauhan, 1989); improved starch and protein digestibility (Khetarpaul and Chauhan, 1990a); increased total soluble sugar, reducing and non-reducing sugar content with decrease in starch (Khetarpaul and Chauhan, 1990b); elimination of anti-nutrients (Khetarpaul and Chauhan, 1991a); and brought an improvement in biological utilization (Khetarpaul and Chauhan, 1991b; Gupta et al., 1992b).

Kenkey is a popular fermented maize product of Ghana that is acidic, dumpling like, wrapped in leaves or maize cob sheaths, and usually steamed, eaten as a staple food with soup (Amoa and Muller, 1976). *Leuconostoc mesenteroides, Pediococcus acidilactici* and *Lactobacillus fermentum*, and yeast *Geotrichum candidum* were reported from kenkey (Christian, 1970; Halm et al., 1993).

Kisra is a thin pancake like fermented bread made from whole sorghum flour and it is the staple food of Sudan served regularly for at least in one of the three meals of the day (Elkhalifa, 2000). Mohammed et al. (1991) reported species of LAB and yeasts *Candida intermedia*, *Debaryomyces hansenii*, and few moulds in samples of kisra.

Masa is a popular shallow fried fermented product which is obtained through fermentation of rice, millet or sorghum and is widely produced in Northern Nigeria (Efiuvwevwere, and Ezeama, 1996). A wide range of microorganisms was isolated during the early stage of masa fermentation between 0–6 h with *Bacillus* spp., *Lactobacillus* spp., *Saccharomyces* spp., *Enterobacter* spp., *Aspergillus* spp., *Penicillium* spp., and *Rhizopus* spp., however, after 8 h, all disappear except *Saccharomyces* spp. with few *Bacillus* spp. which become dominant in the fermentation (Efiuvwevwere and Ezeama, 1996).

Puto is a fermented rice cake consumed as a breakfast and snack food in the Philippines and is generally served with grated coconut; it is closely related to Indian *idli* except that it contains no legume (Sanchez, 1996). During puto fermentation, the yeasts and microaerophilic bacteria increased in number with time where the predominant organism was always *Leuc. mesenteroides*, followed by *S. faecalis* and then *S. cerevisiae* (Cooke et al., 1987; Rosario, 1987). It was found out that the

yeast along with *Leuc. mesenteroides* played an important role in leavening the batter for puto (Tongananta and Orillo, 1996).

Selroti is a popular fermented rice-based ring shaped, spongy, pretzel-like, deep-fried food item commonly consumed by the Nepalis of Sikkim and the Darjeeling hills in India, Nepal and Bhutan (Tamang, 2005). Selroti is mostly prepared at home (75.6%) comparable to market purchase (Tamang et al., 2007a). The microbial population of selroti batters revealed that LAB present in viable numbers above 10^8 cfu/g, followed by yeasts around 10^5 cfu/g, respectively, which included *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*, *Debaryomyces hansenii, Pichia burtonii* and *Zygosaccharomyces rouxii* (Hannah, 2007). It was found that selroti batters produced using a mixture of pure culture strains of *Leuc. mesenteroides* BS1:B1 and *S. cerevisiae* BA1:Y2, selected on the superior technological property, at 28°C for 4h had organoleptically scored the highest acceptability among the consumers (Hannah, 2007).

9.5 Alcoholic Beverages

Yeasts are intimately involved with the production all alcoholic beverages. This association depends on the ability of some yeast species to rapidly and efficiently ferment sugar into ethanol and also their ability to tolerate ethanol concentration of 15–20% v/v. Alcoholic beverages represent a vast diversity of products ranging from table wines, fortified wines, sparkling wines, beer, sake, cider and other fruit wines, distilled alcoholic products to many traditional fermented beverages (Fleet,1998; Lea and Piggott, 2003).

Wines generally refer to those products originating from the alcoholic fermentation of grape juice. The international wine industry is extensive and produces many different types and styles of wines depending on the cultivar of the grape, the geographical origin of the grape and wine making process. Until 50–75 years ago, most wines were produced by so called spontaneous or natural alcoholic fermentation of the grape juice by indigenous yeast flora. These yeasts originated from the surface of the grape berry, the surface of winery equipment that come in contact with the juice during crushing, pressing, pumping and fermentation, and the air (Raspor et al., 2006). Many years of research have identified various strains of *S. cerevisiae* and *S. bayanus* as the principal yeasts of wine fermentation. Today many wine makers purchase commercially prepared dried preparation of these yeasts for inoculation into grape juice and initiation of the alcoholic fermentation (Fleet,1998; Pretorius, 2000) However, it needs to be understood that indigenous yeasts are always present in the juice and usually grow in cooperation and competition with any inoculated strain (Fleet, 2003).

Very few yeasts 10^4–10^3 cfu/g are found on immature grape berries but they increase to 10^4–10^6 cfu/g of the berries mature to harvest. Unripe grapes harbour a predominance of *Rhodoturula, Sporobolomyces, Cryptococcus* and *Candida* species, along with the yeasts-like fungus *Aureobasidium pullulans*. Most of these

species also occur on mature grapes but at this stage, species of *Hanseniaspora* (anamorph *Kloeckera*) and *Metschnikowia* predominate. Damaged grapes with increased availability of fermentable carbohydrate have increased population of *Hanseniaspora* and *Metschnikowia* species as well as the other yeasts, including *Saccharomyces* and *Zygosaccharomyces*. *Saccharomyces* yeasts are rarely isolated from healthy mature grapes berries by plate culture methods but they occur at low populations detectable by enrichment culture procedures.

Freshly extracted grape juice harbours a yeast population of 10^3–10^5 cfu/ml, comprised mostly of *Hanseniaspora/Kloeckera* species, but species of *Candida, Metschnikowia, Pichia, Kluyveromyces* and *Rhodotorula* also occur. The juice will also contain low populations of indigenous species of *Saccharomyces*, depending on the extent of their occurrence on grapes and equipment used to process the juice. Fermentation is initiated by the growth of various species of the non-Saccharomyces yeasts (e.g. *Hanseniaspora uvarum, Kloeckera apiculata, Candida stellata, Candida colliculosa, Metschnikowia pulcherrima, Kluyveromyces thermotolerans*) as well as *Saccharomyces* yeast is generally limited to the first 2–4 days of fermentation, after which they die off (Moreira et al., 2005). They achieve maximum populations of 10^6–10^7 cfu/ml before death, thereby imparting on the metabolic behaviour of the fermentation and products released into the wine are, consequently, wine flavour and quality. Their death is attributed to an inability to tolerate the increasing concentrations of ethanol which is largely produced by the *Saccharomyces* species. After 4 days or so, the fermentation is continued and completed by the *Saccharomyces* species especially strains of *S. cerevisiae*, *S. bayanus* and in some cases *S. paradoxus*. Molecular fingerprinting of isolates from the wine fermentation has isolated further ecological complexity and diversity, in the most species are represented by more than one strain. For example, many genetically distinct strains of *S. cerevisiae* have been isolated from the one fermentation. Thus, wine fermentations reflect not only an ecological succession of different yeast species, but also successional growth of strains within a species (Fleet, 2003). Apart from ecological diversity, the species and strains of wine fermentations also reflect significant metabolic or biochemical diversity. Different species and even different strains within a species can produce substantially different profiles of metabolic end products such as organic acids, higher alcohols, esters and sulphur volatiles (Romano et al., 2003). Apart from contributing to the alcoholic fermentation, yeasts can also spoil the wine, especially during the stages of bulk storage and maturation in the cellar and after packaging. Ethanol-tolerant fermentative species such as *Zygosaccharomyces bailii, Saccharomycodes ludwigii* and *Dekkera* (*Brettanomyces*) *bruxellensis* are particularly notable and lead to a variety of spoilage off-flavour. Wine exposed to air quickly develops a surface flora of weakly fermentative or oxidative yeasts in the genera *Candida, Pichia* and *Hansenula,* with *Pichia membranifaciens* being significant (Sponholz, 1993; du Toit and Pretorius, 2000; Loureiro and Malfeito-Ferriera, 2003).

Beer is the fermented extract of malted cereal grains principally barley. It has an ethanol content of 2–8%, and a distinctive flavour which originates from constituents of the malt extracts of hops, and products of yeast metabolism. Barley is germinated

and kilned in a process called malting. The malted barley is extracted with water under carefully controlled conditions (mashing) to give the extract, termed wort, which contains fermentable carbohydrates and other nutrients for yeast metabolism. The wort is boiled with hops, clarified and then fermented. After fermentation, the beer is clarified, conditioned or matured if necessary and then packaged (Hammond, 1993; Fleet, 1998; Dufour et al., 2003). The boiled wort is essentially sterile and brewers conduct the fermentation by inoculating (pitching) it with pure cultures of yeasts. Generally brewers maintain and propagate their own stocks of yeasts principally, species and strains of *Saccharomyces*. Current taxonomy has these strains in the species, *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*, although former literature describes brewing yeasts within the species *Saccharomyces carlsbergensis* and *Saccharomyces uvarum*. Strains within these species have been merged into either *S. cerevisiae* or *S. pastorianus* (Kurtzman, 2003; Kurtzman and Robnett, 2003).

There is great diversity in the styles of beer produced internationally and indeed within the one brewery (e.g. ale, lager, stout, pilsner, etc.) and this depends on the raw materials, mashing, fermentation and conditioning processes and the strain of yeast. Ecologically, the fermentation should be relatively homogeneous being conducted by the strain of yeast that is inoculated (10^6 cfu/ml) into the boiled wort. However, there is significant metabolic, physiological and genetic diversity in the strains used, and these determine the final flavour and quality of the beer and the efficiency of the process (Dufour et al., 2003). Some key technological properties used by brewers in selecting strains for particular beers include: profile of aroma volatiles produced (as higher alcohols, esters, carbonyl compounds, sulphur volatiles); rate and extent of sugar (glucose, maltose, maltotriose fermentation; fermentation at lower (7–15°C) or higher (25–30°C) temperatures; flocculation and sedimentation characteristics; oxygen requirement; ethanol and osmotolerance-especially in relation to performance in high gravity brewing. Most brewers recycle their yeasts in order to improve efficiency and decrease wastes. After fermentation, the yeast cells are harvested, acid washed to decrease bacterial contamination, stored at 4–5°C and then used to inoculate the next batch of wort. Generally, brewers yeasts can be recycled about 7–8 times, before their performance decreases. This process can lead to increase in the proportion of mutant strains (as respiratory deficient petite mutants) and killer strains within the total yeast population and comprise beer quality (Russel and Stewart, 1995; Dufour et al., 2003).

Growth of any yeast other than the desired strains during fermentation and maturation or any yeast after packaging will lead to off-flavours and turbidity, essentially the beer is spoiled. Many fermented species within the genera *Saccharomyces, Kluyveromyces, Torulaspora, Zygosaccharomyces* and *Dekkera* (*Brettanomyces*) are capable of causing such spoilage. Exposure of beer to air will encourage the growth of oxidative species in the genera of *Pichia*, *Debaryomyces, Candida* and *Hansenula*. Species of Dekkera produce high levels of acetic acid and esters, *Pichia* and *Hansenula* species give excessive ester production while others can give phenolic off-flavours and ferment residual dextrans in the beer (Fleet, 1998; Dufour et al., 2003; Romano et al., 2006). Good manufacturing and hygiene practices are needed to prevent these outcomes.

Some beers, such as those produced in African countries and the limbic beers of Belgium are still produced by traditional processes where mixtures of wild yeasts and bacteria conduct the fermentations. In these cases, the yeasts represent various mixtures of *Saccharomyces* and *Dekkera* spp. (Jespersen, 2003; Viljoen, 2006).

Sparkling wines and fortified wines are all produced from a base wine that is fermented from grape juice with yeasts in a similar way to the table wines just described (Fleet, 1998, 2001). With sparkling wines, the base wine is subjected to a secondary fermentation which traditionally is conducted in the bottle to retain the dissolved carbon dioxide (fizz or sparkle) produced during this fermentation. About 2% of fermentable sugar is added to the base wine, along with a selected strain of *S. cerevisiae*. The mix is bottled and sealed, and fermented for 6 months to 2 years. The yeast grows, ferments the sugar, sediments out and autolyses to contribute unique flavour and other physical properties to the wine. Finally, the yeast is removed from the wine in a specialized process called disgorgement (Howe, 2003). Special strains of *S. cerevisiae* are required for the secondary fermentation. Criteria for these strains include: fermentation under conditions in the bottle- high ethanol concentration (8–12%), low pH (as low as 3.0), low nutrient availability, and increasing pressure of carbon dioxide (upto 600 kpa); ability to flocculate and sediment to facilitate yeast cell removal from the wine; undergo autolysis and give good flavours (Fleet, 2001).

Fortified wines include products such as sherry and port that have ethanol concentrations of 15–22%. This higher ethanol concentration is achieved by addition of ethanol (usually derived from the distillation of wine products) at certain stages during the process (Reader and Dominiquez, 2003). Generally, the higher ethanol concentration suppresses further yeast growth so that they no longer have a role in the process. However, in the case of 'finos', sherries, the wine is fortified in wooden casks by a staged, maturation process called the 'solera' system. This process encourages the formation of a surface film or velum of yeast growth (flor) at the air-wine interface. Essentially, the velum is a thick, wrinkled layer of yeast biomass that largely consists of a unique mixture of hydrophobic strains of *S. cerevisiae*, but strains of *Torulaspora delbureckii, Dakkera bruxellensis, Candida* spp. and *Zygosaccharomyces* spp. may be found. The oxidative metabolism of the velum yeasts decreases the concentrations of wine acid, glycerol and alcohol and substantially increases the concentration of acetaldehyde, thereby contributing unique flavours to these sherry products (Esteve-Zarzoso et al., 2001, 2004).

Using the principles of wine and beer production, almost any fruit juice or cereal extract can be fermented by yeasts to yield an alcoholic product. The raw material must give a supply of fermentable carbohydrates (glucose, fructose, sucrose) and the initial pH should be sufficiently low (as pH 3.0–4.0) to restrict the growth of bacteria. If the pH is not sufficiently low, then the extract will need to be heat processed to reduce the bacterial population, and starter cultures of yeast then inoculated (e.g. as for beer production).

Among the fruit wines, cider produced from apple juice has received most study (Beech, 1993). Like grape wines, it is produced by traditional indigenous fermentation or by inoculation with selected strains of *S. cerevisiae* or *S. bayanus*. In either

case, there is a successional development of yeast species and strains throughout the process. Initially, of *Hanseniaspora uvarum/Kloeckera apiculata* contribute to the fermentation along with species of *Metschnikowia, Candida* and *Pichia*. These give way to a predominance of *S. cerevisiae* and *S. bayanus* that complete the fermentation and demonstrate significant strain diversity (Naumov et al., 2001; Morrissey et al., 2004). Later during maturation *Dekkera, Zygosaccharomyces*, *Saccharomycodes* and *Hanseniaspora* species may develop, either enriching the product flavour or contributing to spoilage (Valles et al., 2007).

Distilled liquor or 'wines' produced from the fermentation of rice extracts are popular in East and South East Asia. Saké production in Japan is well known (Fleet, 1998). Initially, the rice is fermented in a solid substrate process with amylolytic and proteolytic filamentous fungi (*Aspergillus*, *Rhizopus* spp.) to produce a mix (Koji) that is rich in fermentable sugar and starch degrading enzymes. The koji is then mixed with steamed rice, water and a traditional or selected yeast starter culture for alcoholic fermentation. After fermentation, the liquid material is separated from the solids to give the wine. Unique strains of *S. cerevisiae* have evolved to conduct those fermentations generating products with high ethanol content (12–20%), attractive flavour and aroma and odour (Kodama,1993; Dung et al., 2005, 2006).

Distilled products (spirits) form a large part of the market for alcoholic beverages. Although a diverse range of spirit product is available, a general scheme for their production can be presented as (i) selection of the raw material (ii) processing of the raw material to give a fermentable extract (iii) alcoholic fermentation by yeast, principally by strains of *S. cerevisiae* (iv) distillation of the fermented material to give the distillate product and (v) post-distillation processing (Watson, 1993; Bluhm, 1995). Whisky is a distillate from fermented cereals such as malted barley, rum is made by distilling fermented sugar cane or molasses, brandies are prepared from distillates of fermented fruit juices, such as grapes. In addition, there is a large range of flavoured spirits, such as gin, vodka, etc. They are generally produced by alcoholic fermentation of extracts from cereals or their agricultural commodities and then distilled. Specific flavours are added to the distillate to give particular products (Bluhm, 1995).

Specific distillers strains of *S. cerevisiae* are generally purchased from yeast-producing companies and inoculated into the raw material extract of 10^6–10^7 cfu/ml to initiate and conduct the fermentation. However, they must compete with the growth of any other yeasts and bacteria that may be naturally present in the extract. The key criteria for strain selection include efficient production of high concentrations of ethanol, and production of a desired profile of flavours volatile that carry over into the distillate (Watson, 1993). In some cases, traditional processes are still conducted, where indigenous yeasts make a significant contribution to the fermentation and product flavour. In tequila production from fermenting agave juice, species of *Kloeckera africana*, *Candida magnolia* and *Candida krusei* contribute to the fermentation in addition to *S. cerevisiae* (Lachance, 1995) Rum production from molasses fermentation may involve contributions from *Schizosaccharomyces pombe*, as well as *S. cerevisiae* (Fahrasame and Ganow Parfact, 1998). Brazilian rum, cachucha, is distilled from fermented cane juice where the successional

growth of several yeast species occur. A diversity of different strains of *S. cerevisiae* eventually dominates the fermentation after initial growth of other species within the genera *Pichia*, *Candida* and *Kluyveromyces*. In some case, *Schizosaccharomyces pombe* was dominant yeast (Pataro et al., 2000; Schwan et al., 2001).

9.5.1 Mixed Starter Cultures

In Asia, three types of inocula as starters are commercially produced to convert starchy materials to sugars and subsequently to alcohol and organic acids (Hesseltine et al., 1988; Fleet, 1998; Thapa, 2001):

i. In koji, pure cultures of *Aspergillus oryzae* and *Aspergillus sojae* are used in combination. At the same time, they produce amylases that convert starch to fermentable sugars, which are then used for the second stage yeast fermentation to make miso and shoyu, while proteases are formed to break down the soybean protein.
ii. In second-type, whole-wheat flour with its associated flora is moistened and made into large compact cakes, which are incubated to select certain desirable organisms. The cakes, after a period of incubation, are used to inoculate large masses of starchy material, which is then fermented to produce alcohol. Cakes contain yeasts and filamentous moulds. This inoculum is used in the so-called kao-liang process for making alcohol.
iii. The third type of starter is a mixed culture of yeast, filamentous moulds and bacteria. This starter is in the form of flattened or round balls of various sizes, compact in texture, and dry. The starter is inoculated with some previous starter. This mixed flora is allowed to develop for a short time, then dried, and used to make either alcohol or fermented foods from starchy materials. The starters have a variety of names such as marcha in India, Nepal and Bhutan, ragi in Indonesia, bubod in the Philippines, chiu-chu in China and Taiwan, loogpang in Thailand, nuruk in Korea, men in Vietnam (Tamang et al., 1996; Thapa, 2001; Dung et al., 2007), which are used as starters for a number of fermentations based on rice and cassava or other cereals in Asia.

Marcha or murcha is the mixed dough inocula prepared as a dry, round to flattened; creamy white to dusty white, solid ball like starter which is used to produce sweet-sour alcoholic drinks, commonly called jaanr in the Himalayan regions of India, Nepal, Bhutan and Tibet in China (Shrestha et al., 2002; Tamang, 2005). During production of marcha, mainly soaked glutinous rice are crushed, mixed with wild herbs and spices, made into paste by adding water, starter (marcha) from previous batch is mixed, shaped into balls, wrapped in fern leaves, covered with jute sags, left to ferment for 1–3 days and sun-dried. Marcha is a Nepali word. Bhutia calls it phab, and the Lepcha calls it buth (Tamang et al., 1996). Microorganisms of marcha included filamentous moulds *Mucor circinelloides*, *Mucor hiemalis, Rhizopus chinensis* and *Rhizopus stolonifer* variety *lyococcus*; yeasts *Saccharomycopsis fibuligera*,

Saccharomycopsis capsularis, Pichia anomala, Pichia burtonii, Saccharomyces cerevisiae, *Saccharomyces bayanus* and *Candida glabrata*, and lactic acid bacteria *Pediococcus pentosaceus, Lb. bifermentans* (Tamang and Sarkar, 1995; Thapa, 2001; Tsuyoshi et al., 2005).

Based on phylogenetic, morphological and physiological characterization, yeast strains isolated from marcha were first classified into four groups (Group I, II, III, IV), and were identified as *Saccharomyces bayanus* (Group I), *Candida glabrata* (Group II), *Pichia anomala* (Group III), *Saccharomycopsis fibuligera*, *Saccharomycopsis capsularis,* and *Pichia burtonii* (Group IV) (Tsuyoshi et al., 2005). Among them, the Group I, II, and III strains produced ethanol. The isolates of Group IV had high amylolytic activity. Because all marcha samples tested contained both starch degraders and ethanol producers, it was hypothesized that all groups of yeasts (Group I, II, III, and IV) contribute to starch-based alcohol fermentation. *Rhizopus* spp. and amylolytic yeasts (mostly *Saccharomycopsis fibuligera*) degrade starch and produce glucose, and alcohol-producing yeasts (species of *Saccharomyces* and *Pichia*) rapidly grow on the resultant glucose to produce ethanol (Thapa, 2001). Marcha making technology reflects the traditional method of sub-culturing desirable inocula from previous batch to new culture using rice as base substrates. This technique preserves the microbial diversity essential for beverages production. Marcha retains its potency *in situ* for over a year or more. In Manipur, a similar mixed starter is called hamei (Tamang *et a*l., 2007b).

Ragi is a starter like marcha, used in Indonesia where rice is used as a substrate (Saono et al., 1974). Went and Prinsen-Geerligs (1896) found *Monilia javanicus* (*Pichia anomala*) and *Saccharomyces cerevisiae* as principal yeasts in ragi. The dominant yeasts species present in ragi are *Candida parapsilosis, C. melinii, C. lactosa, Hansenula subpelliculosa, H. anomala* and *H. malanga* (Dwidjoseputro and Wolf, 1970; Ardhana and Fleet, 1989). Studies of Saono and Basuki (1978) revealed that ragi and its fermented products such as tape keté la, tapé ketan hitam, oncom hitam and oncom mérah from various places in West Java contained *Candida* spp as dominant yeasts, and *Mucor* spp and *Rhizopus* spp as dominant among moulds.

Bubod is used as a starter in the Philippines (Tanimura et al., 1978). Kozaki and Uchimura (1990) reported the presence of *Mucor circinelloides*, *M. grisecyanus*, *Rhizopus cohnii*, *Saccharomyces cerevisiae* and *Saccharomycopsis fibuligera* in bubod. Hesseltine and Kurtzman (1990) reported that *Saccharomycopsis fibuligera* was dominant in bubod.

Nuruk is the starter for preparing Korean alcoholic drink yakju and takju, prepared from rice or wheat (Steinkraus, 1996). Generally, nuruk is prepared by natural inoculation of molds, bacteria, and yeasts; however, it can be prepared by inoculation with *Aspergillus usamii*. Kim (1968) isolated *Aspergillus oryzae* (10^7 cfu/g), *A. niger* (10^7 cfu/g), *Rhizopus* sp (10^6 cfu/g), anaerobic bacteria (10^7 cfu/g), aerobic bacteria (10^6 cfu/g) and yeasts (10^5 cfu/g) from nuruk.

Chiu-yueh or peh-yueh is the Chinese starter for lao-chao, fermented rice product of China. It is a gray-white ball containing yeasts and fungi grown on rice flour, which is closely related to ragi. Wei and Jong (1983) isolated yeasts and moulds

from chiu-yüeh and tested the ability of these microorganisms to convert steamed glutinous rice into a good quality lao-chao.

Loogpang is the starter commonly used in Thailand to prepare alcoholic drink and vinegar (Vachanavinich et al., 1994). Dhamcharee (1982) showed that the yeasts present were *Saccharomycopsis*, *Hansenula*, and *Saccharomyces* along with molds in loogpang. Sukhumavasi et al. (1975) isolated a strain of *Endomycopsis* (*Saccharomycopsis*) *fibuligera* from loogpang with high glucoamylase activity. Uchimura et al. (1991) reported the presence of *Saccharomycopsis fibuligera* and *Pediococcus* sp. in loogpang.

9.5.2 Asian Alcoholic Beverages

Alcoholic foods and beverages, in which amylolytic moulds and yeasts accomplish starch hydrolysis and fermentation, range from very primitive (Steinkraus, 1996). The main yeasts, which ferment saccharified rice starch to alcohol, are *Endomycopsis* (*Saccharomycopsis*) *burtonii*, *Saccharomycopsis fibuligera*, *Saccharomyces cerevisiae* and *Candida lactosa*; *Saccharomycopsis fibuligera* also produces amylolytic enzymes (Reiser and Gasperik, 1995; Yip et al., 1997; Brimer et al., 1998; Dung et al., 2005). Other yeasts genera *Hansenula, Pichia* and *Torulopsis* have also been isolated from rice wine. Esters, fusel oils, acids and other compounds which contribute to flavour are also produced (Nout and Aidoo, 2002). Some of common indigenous alcoholic beverages prepared by a mixed starters are kodo ko jaanr, bhaati jaanr, tapé, etc. *Saccharomycopsis fibuligera* which is one of the common yeasts in Asian starter cultures have amylolytic as well as some ethanol producing capacity (Limtong et al., 2002).

Kodo ko jaanr is the most common fermented mild-alcoholic beverage prepared from dry seeds of finger millet (*Eleusine coracana*), by using marcha in Sikkim, the Darjeeling hills and North East hills in India, Nepal, Bhutan and Tibet in China (Tamang, 2005). Kodo ko jaanr contributes to the mineral intake in daily diet of the local people (Thapa, 2001). Because of high calorie, ailing persons and post-natal women consume the extract of kodo ko jaanr to regain the strength. Population of yeasts and lactic acid bacteria was detected at the level of 10^7 cfu/g and 10^5 cfu/g, respectively. Yeasts consisted of *Pichia anomala*, *Saccharomyces cerevisiae*, *Candida glabrata*, *Saccharomycopsis fibuligera*, and lactic acid bacteria consisted of *Pediococcus pentosaceus* and *Lactobacillus bifermentans* in kodo ko jaanr samples. Microorganisms necessary for fermentation of finger millets into kodo ko jaanr are supplemented by marcha (Thapa and Tamang, 2004).

Thapa and Tamang (2006) studied the microbiological and physico-chemical changes during fermentation of kodo ko jaanr and found that population of yeasts increased significantly ($P < 0.05$) from 10^5 cfu/g to 10^7 cfu/g within second day. Maximum activities of saccharification and liquefaction of millets were observed on second day of fermentation. It was revealed that *Saccharomycopsis fibuligera* and *Rhizopus* spp. play the dominant role in saccharification process of finger millet in kodo ko jaanr fermentation.

On the basis of amylolytic activity 4 strains of *Rhizopus* spp., 2 strains of *Mucor* spp., 5 strains of *Saccharomycopsis fibuligera*, 4 strains of *Pichia anomala*, 4 strains of *Saccharomyces cerevisae* and 3 strains of *Candida glabrata* were selected for liquefying and saccharifying activities (Thapa and Tamang, 2006). None of the lactic acid bacteria showed amylolytic activity. Saccharifying activities were mostly shown by *Rhizopus* spp. and *Saccharomycopsis fibuligera* whereas liquefying activities were shown by *Saccharomycopsis fibuligera* and *Saccharomyces cerevisiae*. *Saccharomycopsis fibuligera* played the main roles in amylase production whereas *Rhizopus* seemed to supplement the saccharification (Wei and Jong, 1983; Uchimura et al., 1990). The result indicated that *Saccharomycopsis fibuligera* and *Rhizopus* spp. play the important role in saccharification process of kodo ko jaanr fermentation breaking starch of substrates into glucose for ethanol production (Thapa and Tamang, 2006). *Mucor* spp., *Pichia anomala* and *Candida glabrata, Saccharomyces cerevisiae* may supplement the saccharification.

Bhaati jaanr is an inexpensive high calorie mild-alcoholic beverage prepared from the steamed glutinous rice, consumed as a staple food beverage in the Eastern Himalayan regions of Nepal, India and Bhutan. It was revealed that *Saccharomycopsis fibuligera* and *Rhizopus* spp. play the important roles in saccharification process of rice in bhaati jaanr fermentation (Tamang and Thapa, 2006).

Tapé is a sweet-sour paste with an alcoholic flavour, prepared from glutinous rice or cassava or other cereals by using starter ragi in Indonesia (Ko, 1972). A combination of *Aspergillus rouxii* and *Endomycopsis* (*Saccharomycopsis*) *burtonii* reduced total solids by 50% in 192 h at 30°C, which raised the crude protein in tapé ketan by 16.5% on a dry basis (Cronk et al., 1979). Suprianto et al. (1989) reported that *Saccharomycopsis fibuligera* produced mainly α-amylase and *Rhizopus* sp. produced glucoamylase in tapé fermentation. They also found that liquefaction was not caused by amylases of *Saccharomycopsis* even though it produced high activity of α-amylase.

Tapai is the Malaysian fermented food-beverage produced by adding pulverized ragi or jui-piang. It is consumed as a desert but in East Malaysia it is the rice wine with lighter colour and less sweetness (Merican and Yeoh, 1989). *Candida* spp, *Saccharomycopsis fibuligera*, *Amylomyces rouxii*, *Mucor circinelloides*, *M. javanicus*, *Hansenula* spp, *Rhizopus oryzae*, and *R. chinensis* have been found in tapai (Wang and Hesseltine, 1970; Ko, 1972). Merican and Norrijah (1985) showed that the organisms necessary to produce a good tapai pulut consist of a mixture of *Amylomyces rouxii, Saccharomycopsis fibuligera* and *Hansenula anomala*, and for a good quality tapai ubi, the essential microorganisms are *Amylomyces rouxii,* and *Saccharomycopsis fibuligera*.

Lao-chao is a popular Chinese fermented food with sweet taste and fruity aroma, made from rice by using chiu-yueh or peh-yueh as starters (Wang and Hesseltine, 1970). It is served as a dessert and is a traditional diet for new mothers who believe that it helps them regain their strength. Wei and Jong (1983) reported the presence of *Rhizopus*, *Amylomyces*, *Torulopsis*, and *Hansenula* in lao-chao. Pure culture fermentation method of lao-chao was developed by Wang and Hesseltine (1970) and showed that good fermented rice was made when *Rhizopus chinensis* NRRL 3671, and *Saccharomycopsis* sp. NRRL Y7067, used as inocula instead of a commercial starter.

Yakju is the Korean alcoholic beverages, made from rice by using nuruk. The lower or diluted concentration of yakju is known as takju (Steinkraus, 1996). Microbial studies of yakju revealed the presence of yeasts, *Bacillus* spp. and *Lactobacillus* sp. and *Leuconostoc* spp. (Shin and Cho, 1970; Kim, 1970; Lee and Rhee, 1970). Kim and Lee (1970) reported that *Saccharomyces cerevisae* is the most important organism in alcohol production while *Hansenula* spp. play an important role in flavour development.

Basi is a traditional alcoholic beverage of the Philippines made by fermenting boiled, freshly extracted sugarcane juice with a mixture of yeast, bacteria and moulds or with organisms found in 'samac' (*Macharanga tanarius*) leaves, bark, or fruit (Tanimura et al., 1978). Kozaki (1976) reported that the dominant organisms in basi are *Saccharomyces*, *Saccharomycopsis* and lactic acid bacteria.

Ruou nep is the Vietnamese fermented rice wine prepared from rice using a traditional mixed starters called men (Aidoo et al., 2006). Defined granulated starters containing *A. rouxii* and *S. arvensis* in men make high-quality Vietnamese rice wine (Dung et al., 2005).

Zutho is an ethnic rice beer prepared and consumed by the Nagas in Nagaland in India. Teramoto et al. (2002) reported *S. cerevisiae* as main yeast in zutho fermentation.

9.6 Other Commodities

Yeasts have a key role in the production of a diverse range of other products that are commercially significant globally, or have significance as local, traditional products.

Cocoa beans are the raw material of chocolate manufacture, and require fermentation as one of the first stages in the chocolate production chain (Schwan and Wheals, 2004). The beans are contained in pods of the tree, *Theobroma cocao*, mostly cultivated in the tropical equatorial regions of the world. After harvesting, the beans are removed from the pods and placed as large masses in wooden boxes, on trays or as heaps covered with plantain leaves. They undergo a spontaneous, indigenous fermentation from the growth of naturally associated yeasts, bacteria and fungi. Numerous studies in different countries have described the microbial ecology of this fermentation (Schwan and Wheals, 2003). Yeasts have a prominent role in the fermentation, exhibiting a successional development through *Hanseniaspora uvarum* (*Kloeckera apiculata*), *Hanseniaspora quilliermundii* (*Kloeckera apis*), *Saccharomyces cerevisiae*, *Pichia membranifaciens* and other *Pichia* species, *Issatchenkia orientalis* (*Candida krusei*), various Candida species and *Kluyveromyces* species (Ardhana and Fleet, 2003; Jespersen et al., 2005).

During fermentation, the yeasts and other organisms degrade the previous pulp surrounding the seeds, ferment pulp sugars and create ethanolic acidic conditions that kiln the beans. Thereafter, the beans undergo endogenous biochemical reactions that develop the chemical precursors to chocolate flavour. Products generated

by yeast metabolism also diffuse into the beans to impart on their flavour (Schwan and Wheals, 2003, 2004).

Coffee beans are harvested from trees of the green coffee. Subsequently, they are processed by either wet or dry methods to remove pulp and mucilaginous materials that surround the seeds (Schwan and Wheals, 2003). Various species of yeasts and bacteria grow throughout these processes, producing an array of pectinolytic, hemicellulolytic and other enzymes that facilitate pulp and mucilage degradation. Other metabolic reactions may contribute either positively or negatively to coffee flavours and character, but precise linkages of these reactions to specific organisms are not clear. A diversity of yeast species has been isolated from coffee bean fermentations, including various species of *Candida*, *Saccharomyces*, *Kluyveromyces, Saccharomycopsis, Hanseniaspora, Pichia* and *Arxula*, but further studies are required to describe definitive associations and functionality with respect to the process (Silva et al., 2000; Masoud et al., 2004)

Soy sauce is another global product of major economic significance. The raw materials, soybeans and wheat, are initially fermented with the filamentous fungus *Aspergillus oryzae* or *Aslergillus sojoe,* in a solid substrate mode. During this stage, starch, proteins and other macromolecules in the raw materials are partially degraded to fermented sugars, amino acids and other nutrients necessary for subsequent fermentation by yeasts and lactic acid bacteria (Hanya and Nakadai, 2003). This material is then mixed with brine (16–20% NaCl) for the next stage of fermentation which generally takes several months. Lactic acid bacteria (*Tetragenococcus halophilus*) initiate the fermentation, which is then dominated by the growth of osmotolerant yeasts, *Zygosaccharomyces rouxii* followed by *Candida versatilis* or *Candida etchellsii*. These yeasts evolve naturally or may be added as starter cultures. *Zygosaccharomyces rouxii* conducts an alcoholic fermentation while the *Candida* spp. contribute characteristic flavours from the production of phenolic compounds. The yeasts also accumulate and produce significant amounts of glycerol and other polyols in response to the high salt environment. At the completion of fermentation, the product is clarified and packaged.

9.7 Conclusions

Yeasts food fermentation is practiced in nearly all the countries, along with bacterial and fungal fermentation, or in combination with them. It is concluded that in fermentation of any substrate, *Saccharomyces* ferments sugar, produces secondary metabolites; inhibits growth of mycotoxin-producing moulds and has several enzymatic activities such as lipolytic, proteolytic, pectinolytic, glycosidasic and urease activities. *Debaryomyces* contributes in sugar fermentation, increases pH of the substrates, and produces growth factors for bacteria. *Hanseniaspora* and *Candida* also contribute in sugar fermentation, production of secondary metabolites, and enzymatic activities. *Yarrowia lipolytica* also plays role in sugar fermentation, lipolytic, proteolytic and urease activities and reduction of fat rancidity in the product.

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Chapter 10
Utilization of Yeasts in Biological Control Programs

R.S. Pimenta, P.B. Morais, C.A. Rosa, and A. Corrêa Jr.

Contents

Abstract In an agricultural environment, the native flora is replaced by a commercial crop and consequently the native microbiota also undergoes changes and, no seldom, species with antagonistic action against pathogens are eliminated. The lack of natural competitors may result in an outburst of diseases or herbivores that will feed upon the growing crop. Several strategies such as: chemical control, pathogen resistant cultivars and biological control may be used to avoid economical loses in the crop. Biological control protocols are based on the assumption that in an undisturbed environment outbursts of diseases are seldom due to the presence of naturally occurring antagonists and therefore, the introduction/augmentation of antagonism in a disturbed environment will control the disease. A successful agent for biological control has to hold several characteristics such: antagonism against pathogens, well know biology, specificity, be ease to produce and apply, be safe to the environment. Yeast may present all of those characteristics and are used in several biological control protocols. We will discuss in this chapter the basic concepts of biological control, the use of yeasts as biological control agents and describe the commercial products that use yeasts for biological control.

Keywords Biological control, environment, competitors, antagonists, antagonism

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10.1 Introduction

The concept of biological control correlates well with the sustainable agriculture strategies because both exploit the natural biological cycles in search of crop production with reduced environmental impact (Spadaro and Gullino, 2004). Ecophysiological aspects of pathogen, host and their interaction with the biological control agent (BCA) are the fundamental information for the development of biological control strategies of plant disease since the core of this technology is the manipulation of the ecological interaction among the host, pathogen and BCA towards the decrease of pathogen damage. Population control in a natural habitat acts by different mechanisms such as environmental resistance and biological antagonism. Although abiotic environmental control is hard to achieve, it can effectively diminish pathogen damage in a crop. Among the techniques used for environmental control there are changes in temperature (green-house, solarization), pH, (soil correction), salinity, radiation incidence, humidity, as well as decrease in the availability of nutrients in the substrate and introduction of toxic compounds. Biotic control of pathogens can be achieved by the introduction or augmentation of existent antagonists in the ecological niche of the pathogens. Biotic antagonism limits growth by competition for space or food, parasitism, predation, inhibition of growth through the production of toxic substances and by producing environmental modification. These factors influence microbial activities and play very important roles in determining the spatial and temporal dynamics of microbial populations. Usually, the biotic activities modify the environment and these modifications change the community structure.

Microbial ecology examines the diversity and activity of microorganisms in Earth's biosphere (Xu, 2006). In a broad view, microbial ecologists organize and group microorganisms in specific metabolic categories related to its energy source and generation. Yeasts are ubiquitous unicellular fungi widespread in nature and colonize terrestrial, aerial and aquatic environments and also plant and animals surfaces, where the successful colonization is intimately related to their physiological adaptability to a highly variable environment (Rodrigues et al., 2006). Distribution of yeasts in nature is partially determined by nutritional characteristics of substrate, biogeographical characteristics and dispersal agents (Lachance et al., 2003; Pimenta, 2001).

All those natural characteristics should be taken in consideration when applying those organisms in a program of disease control and management. Yeasts are particularly interesting microorganisms in a Biological Control programs because they are relatively easy to produce and maintain and have several characteristics that can be manipulated in order to improve its use and efficiency.

Yeasts do not occur randomly throughout the biosphere, and each yeast community may be defined by its habitat (Lachance and Starmer, 1998). Yeast species may be defined as generalists or specialists, depending on their habitat occupation and physiological profiles. Generalist yeast has the ability to utilize diverse carbon compounds and due to this they can survive and grow in different environments. Specialist yeasts have a simple physiologic profile and obtain energy solely from

few carbon compounds and this limitation restricts their habitat amplitude. However, a simple physiologic profile allows faster growth. In contrast, the complex physiology, found in generalist yeasts allows a great range of food supply, but that leads to slow colonization (Morais et al., 1995; Abranches et al., 2000; Rosa and Péter, 2006). Depending on the metabolism type of a BCA yeast, a different strategy for applying a biological control program should be devised. Generalist yeasts are easier to maintain, and because they use several different media for growth they are generally more suitable for industrial production. However, because they are found in various environments, specificity towards a particular pathogen is not frequent. Such metabolic pattern is desired for a BCA to be used against opportunistic or non-specialist saprophytic pathogens. Specialist yeasts, on the other hand, are restricted to fewer environments and are more prone to exploit such environments quickly and with less inter-specific competition. These BCAs are more suitable for the development of specific pathogen control programs or to be applied preventively. However, they are usually more difficult to produce and use for biotechnological applications.

The transmission of infectious diseases is an inherently ecological process involving interactions among at least two, and often many species (Keesing et al., 2006). In an agricultural condition, a commercial crop replaces the native microorganisms, consequently the native microbiota is modified or completely changed. As a result, no seldom species with antagonistic action against pathogens are eliminated. The lack of natural competitors may result in an outburst of diseases or herbivores that will feed upon the growing crop. The knowledge of such environment and biotic changes are capital for the development of biological control strategies. In situations where populations of related host species grow sympatrically but isolated from other populations of both hosts, cross-species disease transmission can have great influences on disease dynamics and patterns of pathogen persistence (Carlsson-Granér, 2006).

Recently, there has been renewed interest in the potential effects of diversity on disease risk, partly because of the interest in identifying and evaluating utilitarian functions of biodiversity. Various empirical and modeling investigations have suggested that increased species diversity could reduce disease risk due to genetic variability. However, in particular situations, some studies propose an increase in disease risk caused by varying numbers of vectors and hosts (Keesing et al., 2006).

The usual approach for solving an agronomic problem is to focus in the disease and study the interaction of pathogen and host alone. Such view could inhibit the development of alternative control strategies that could be more efficient and environmentally safe. Designing and using a biological control protocol is an exercise of manipulating ecological conditions in a commercial environment. Unfortunately, the knowledge of ecological interactions in such environments is scarce. Therefore, is necessary an effort to understand the ecology of natural environments, the changes caused by agronomic use and apply this knowledge in developing strategies to decrease disease pressure in a crop. The efficient use of a biological control strategy is a challenge that should be pursued by the conjoint effort of the ecologist and the agronomist.

An important characteristic in commercial crops is the search for efficiency in production. Efficiency is usually measured by the volume of production. The factors that may decrease production such as diseases and pests are normally neglected and view as secondary. Strategies based of increased efficiency have generated high productive crops, but having special nutritional and environmental demands. Usually such crops do not cope well with environmental challenges such as diseases and pests. To overcome this problem, farmers compensate such inadequacy with the use of chemical soil correction and pesticides. The use of genetically improved cloned seeds surely has increased the volume of production by cultivated area for many crops, but at the same time, generated problems related to low genetic variation. The losses due to microbiological food deterioration may reach up to 5 to 20% of the crop yield in developed countries. In tropical areas the losses may be even higher, reaching up to 50% in countries where modern techniques for food storage and transport are not implemented (Eckert and Ogawa, 1985; Chand-Goyal and Spotts, 1997; Varma and Dubey, 2001; Janisiewicz and Korsten, 2002).

The classical way to approach disease control is based on exploiting aspects of the disease triangle theory. The disease triangle summarizes disease as an interaction among host, pathogen and the environment (Fig. 10.1).

Biological control protocols add a biotic factor to the equation, that will interact with the pathogen directly (antagonistic effect), with the host by changing its resistance characteristics (induced resistance) or through environmental changes (nutrient depletion, pH change, etc.) (Jeger and Spence, 2001).

Different plant substrates can be attacked by different pathogens, and the type of plant species can influence the BCA efficacy. Consequently, fruits, seeds, leaves, and flowers can be targets for pathogens and biocontrol agents. Therefore, the attack and protection can happen at different times of cultivation, from planting to post harvesting. Normally fruits have high sugar concentration and low pH. Leaves surfaces are poor in complex nutrients. However, stems, roots and seeds can offer a great and diverse supply of nutrients. With this, the biological controller needs to have a physiological profile compatible with host resources and also the ability to colonize the substrate.

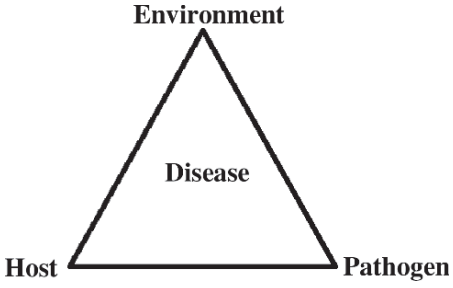

Fig. 10.1 Graphic representation of disease triangle

Since the 1960's the storage times of fruits have been increasing considerably and post-harvest diseases are today a major cause of economical losses. Actually, there are over a hundred thousand types of plant diseases and about eight thousand species of pathogenic fungi described, but only 100 fungal species are responsible for the majority of post harvest ones. All plants can be infected by one or more fungal species and some fungi can infect several plant species. Plant disease is the damage of cells or tissues, and result in development of symptoms by the pathogenic agent or environmental condition. Disease involves morphological or physiological modifications but also alterations in integrity or behavior. These modifications can result in partial damage or death of the plant or of its parts (Agrios, 1997; Tripathi and Dubey, 2004).

The post harvest storage time of fruits increased mainly due to new technologies for temperature and humidity control and the use of fungicides. Fungicides have been efficient in decreasing losses by deterioration of food, but also generated the increase of public health concerns and environmental problems mainly due to the carcinogenic and/or teratogenic proprieties of the compounds, and by their cumulative toxicity (Janisiewicz and Korsten, 2002). Therefore, the development of new environmentally friendly and healthy technologies is necessary. Among the possibilities, biological control of post-harvest diseases is particularly suitable. Generally, biological control is harmless to the public and if applied correctly will not cause any environmental damage.

Among the microorganisms that can be utilized as biocontrol agents, yeast has several characteristics such as ability for fast colonization and survival on the fruit surface for a long period of time and in different environmental conditions, that make them a good candidate for the development of biological control protocols (Droby et al., 1999, 2003). Different yeasts species are being utilized as biocontrol agents, for example: Candida oleophila and Pichia membranifaciens is commercially used for the control of Botrytis cinerea post harvest rot in apples (Jijakli and Lepoivre, 1998); Debaryomyces hansenii against Penicillium digitatum on the decay of grapefruit; Pichia guillermondii against Botrytis, Rhizopus, Penicillium and Alternaria on decaying tomato fruits; Cryptococcus laurentii, Cr. flavus and Cr. albidus for control of Mucor in pears; Candida sake against B. cinerea and Rhizopus nigricans on apple decay (Masih et al., 2000).

In this chapter we will discuss yeast ecology applied to biological control of postharvest disease of fruits and seeds, including the inhibition of aflatoxin production and possible probiotic proprieties of yeasts. We will bring to discussion different strategies of disease control, the methods for evaluating biological control efficiency and the perspectives for research and development in the field.

10.2 Postharvest Diseases

Postharvest diseases are those that may start in the field and develop during the cropping, transportation, packing and storage. The concept of disease in vegetables is not only applied to the plants or parts of plants that are affected by pathogenic

agents in the field, but also to deterioration of vegetable products, such as grains, roots, fruits, wood, during transportation, storage or in consumption phase. Usually, the most tender and succulent products, such as fruits, have the greatest susceptibility to the attack of pathogenic fungi because they have a high water and nutrient content. Fruits and flowers attacked by postharvest disease usually cause direct losses to the producer and to the seller, due to loss of quality or quantity of these products and are particularly pernicious because the loss occurs after the investment in the production, transport and storage is made (Agrios, 1997; Leggott and Shephard, 2001; Mercier and Jiménes, 2004).

A relatively small number of fungi and bacteria cause these diseases. Postharvest diseases are normally caused by primary parasites, like Rhizophus, Penicillium and Erwinia, that actively attack living tissues of vegetables, provoking degradatives lesions that usually serve as entrance to secondary infection. Lesions produced by mechanical injury during harvesting, transportation, storage and commercialisation of the fruits are also an entrance door for the pathogen. Facultative or accidental pathogens are saprophytic organisms and normally attack soft organs used as nutrient reserves. These pathogens are considered primitive parasites since they exhibit great severity and low specificity (Obagwu and Korsten, 2003; Stange et al., 2002). Spreading of the pathogen occurs normally from fruit to fruit within the storage area (Agrios, 1997; Huang et al., 2000).

Traditionally, the postharvest diseases are controlled by fungicides. However, the appearance of resistant varieties of pathogens as well as the difficulties of the implementation of protocols that guarantee the safe use, has made more and more problematic the application of these substances. The main problems involved in fungicide utilization are related to environmental pollution and public health concerns especially due to its carcinogenic and/or teratogenic properties (Harman et al., 1996; Masih et al., 2001; Janisiewicz and Korsten, 2002; Mercier and Jimenes, 2004).

10.3 Biological Control

Biological control is a natural phenomenon that occurs widely in the environment. It consists in growth control of a population or community by one or more antagonistic organisms. This control is established by a reciprocated influence of pathogen, host and environment and it potentially happens with all organisms and species, since all types of living forms have a pathogen. Biological control is spread on Earth and many species belonging to different taxa may take part on the maintenance of ecological equilibrium. However, anthropic environments such as plantations or cities have a different and normally less diverse community of organisms. According to Keesing et al. (2006), the lack of diversity may lead to ecological imbalance that may allow some species to increase in number and niche area while other populations are eliminated or reduced, increasing the possibility of appearance of epidemic disease.

The idea that some species can reduce the populations of others considered noxious is very old. Probably, the Chinese were the first to use a biological control strategy. They used a predacious ant Oecophylla smaragdna for the control of a herbivorous Lepidoptera in citrus crops, as early as the century III A. C. (Parra et al., 2002; Santos and Del-Claro, 2002). However, the first well documented case of success of classic biological control was the introduction of Rodolia cardinalis from Australia to California in 1,888 to control the white greenfly, Icerya purchasi. After two years of its introduction, C. cardinalis totally controlled the insect (van den Bosch et al., 1982). Biocontrol initiatives were first used to control insects, acaroids and weeds, but afterwards, its use became wider and other invertebrate, phytopathogens and also some vertebrates are now considered targets. Nowadays, biological control is used in several agronomical problems, among them the control of postharvest diseases.

The growing interest in the consumption of foods free of fungicides has led producers worldwide to demand new alternatives of control of diseases (Spadaro and Gullino, 2004). In this context, biological control of pathogens grows as an alternative, especially the biological control of postharvest diseases, and it represents a promising alternative to total or partial substitution of chemical pesticides (Janisiewicz, 1991; Harman et al., 1996; Chand-Goyal and Spotts, 1997).

Biological control is the use of several ecological interactions between pathogen and a BCA to decrease economical loss. The ecological interactions employed in the reduction of plagues and pathogens are: competition, parasitism, production of antibiotics, induction of resistance in the host and predation, being common the presence of more than one type of interaction (Bernard, 1999; Schoeman et al., 1999; Bapat and Shah, 2000; Qin et al., 2003, 2004).

Competition is an interaction among populations resulting in decrease in the number of individuals and may be classified in:

- Competition by mutual inhibition - when two populations inhibit actively each other.
- Competition for resource - in which each population affects negatively the other, in an indirect way, in the dispute for a limited resource, as space or nutrient.
- Antibiosis - in which a population is inhibited and the other is not affected, being usually mediated by antibiotics.
- Parasitism and predation are associations in which a population affects negatively another through a direct attack, depending on the other population as food or habitat.

Despite the ecological mechanism of disease control, most of the biological control protocols using microorganisms are developed similarly.

The classical biological control development starts with the isolation of naturally occurring strains of microorganisms associated with the target vegetable to obtain one or more antagonistic species in Nature. This step is followed by experiments to select the antagonist, the multiplication of the antagonist in laboratory and multiple inoculations in field using different and usually high concentrations of the antagonist. Even though this strategy is widely used, the isolation of antagonists in nature is

time consuming and not ensures commercial applicability. Frequently, the isolated microorganism after several cycles of multiplication in laboratory, loss its efficacy and environmental fitness, diminishing its use in the field. To increase the time of use of a particular BAC, several strategies have been developed, as a result of our better understanding of microorganism ecology and physiology. The integrated biological control is an example of such improvement. Integrated biological is an association of classical biological control with a GRAS (Generally Regarded As Safe) substance.

Among others, sodium bicarbonate, sodium carbonate, ethanol, ascorbic acid, acetic acid, lactic, benzoic, sorbic are considered as GRAS and used in integrated biological control protocols (Kang et al., 2003; Gamagae et al., 2003; Irtwange, 2006). This method, differing from the classical biological control, has presented similar results to chemical control. Most of the GRAS substances if used solely, presents some sort of disease control. Sodium bicarbonate has been used in orange disinfection since 1920 (Obagawu and Korsten, 2003). However, the use of this substance in high concentrations can produce burn-like lesions, depending on the type of treated fruit. The use of sodium carbonate and bicarbonate alone has controlled partially the citrus pathogens P. expansum, P. digitatum and P. italicum. However, its use is preventive and not curative, because sodium bicarbonate seems to act as fungistatic and it probably produces some poisonous effect in the spores (Palou et al., 2002; Gamagae et al., 2003; Yaoa et al., 2004). Therefore the use of sodium bicarbonate alone has not been capable to reduce in an effective way the incidence and severity of the lesions and it is not indicated as main strategy of control of citrus fruits pathogens (Smilanick et al., 1999). However, when sodium bicarbonate is applied in consortium with a BCA, the efficiency of control increases significantly.

10.4 Yeasts for Postharvest Control of Pathogens in Fruits

According to Cook et al. (1996), microorganisms are a vast resource still little explored for the control of plagues and phytopathogens. Among them, yeasts are particularly suitable for use as BCAs due to their ability of fast colonization of the vegetable surface, maintaining viability for long periods of time under different environmental conditions (Cartwright and Spurr, 1998; Droby et al., 1999, 2003; Janisiewicz et al., 2001). Yeasts and bacteria can prevent deterioration of foods during the stockpiling through competition with the pathogen for space and food (Wilson and Wisniewski, 1989; Roberts, 1990; Wisniewski et al., 1991; Avis and Belanger, 2002).

Pathogen control by yeasts, in pre- or postharvest diseases have been demonstrated extensively (Paulitz and Bélanger, 2001; Irtwange, 2006; Punja and Utkhede, 2003). Generally most of the antagonistic yeasts are obtained from the epiphytic microbiota associated with flowers and fruits and display a range of activities such as competition for nutrients and space, production of antagonistic

substances and predation (Goyal and Spotts, 1996; Piano et al., 1997). Young (1987) documented antagonistic interactions mediated by the production of soluble proteic molecules by yeasts that provoke disruption of cell membrane and cell wall. Starmer et al. (1987) concluded that the production of killer toxins is a strategy to eliminate competitor strains of the same or different species. The term predacious yeast was introduced by Lachance and Pang (1997) to describe certain yeast species that produces small feeding appendages or haustoria that penetrate and kill other yeast cells. Parasitism and degradation of hyphae of the pathogenic fungi by antagonistic yeasts have been linked to the adherence of the yeast cells to fungal hyphae and to high production of glucanase derived from different carbon sources (Odum, 1988; Chand-Goyal and Spotts, 1997; Lewis and Larkin, 1998). Among the antagonistic relationships among yeasts and other microorganisms, the production of antibiotic substances is not common. On the other hand, competition for resources is often observed. For example competition for L-proline, observed between the yeasts Candida membranifaciens and Cryptococcus laurentii, and Penicillium expansum can be responsible for the reduction of infection of apples by this pathogen (Blum et al., 2004).

Jijakli and Lepoivre (1998) showed that Candida oleophila that is found in fruits, and Pichia membranifaciens that is usually isolated from a great variety of habitats, specially fermented substrates, actively eliminated Botrytis cinerea from apples. According to Masih et al. (2000) Debaryomyces hansenii, an ubiquitous yeast can act against Penicillium digitatum in decaying grapefruit. Experiments showed that the ubiquitous yeast Pichia guilliermondii is effective against Botrytis, Rhizopus, Penicillium and Alternaria in decaying tomato fruits. The yeasts Cryptococcus laurentii, Cr. flavus and Cr. albidus are usually found in foliar surfaces and could potentially are used for control of Mucor in pears. Also, Candida sake, a yeast commonly isolated from fermentations, soil and water seems to act against B. cinerea and Rhizopus nigricans, which are the main causes of postharvest disease on apple.

Several yeasts used as BCAs have shown more effective results in control of phytopathogens when inoculated simultaneously with sodium bicarbonate that is a recognized as GRAS by the US FDA (United States Food and Drug Administration). Saccharomycopsis schoenii, a predacious yeast, was tested as a biological control agent against Penicillium italicum, P. expansum, and three strains of P. digitatum causing post harvest decay in oranges. In an integrated biological control test, treatment with S. crataegensis associated with sodium bicarbonate, resulted in no decay 96 h after the treatment (Pimenta, 2004).

10.5 Yeasts for Aflatoxin Inhibition in Food

Peanuts, coffee, corn and others substrates are often invaded by Aspergillus flavus and/or other mycotoxigenic fungi causing damage. Biodeterioration of seeds and grains in the field and during storage limits the stockpiling and reduces the nutritional

value of those foods (Reddy and Shetty, 1992; Prado et al., 1999; Sarimehmetoglu et al., 2004; Bittencourt et al., 2005; Erdogan, 2004).

One of the most important effects of postharvest diseases of seeds and grains is mycotoxin accumulation. Mycotoxins are almost certainly the main non-infectious dietary risk factor associated with food. Mycotoxins are secondary metabolites of molds that exert toxic effects on animal and humans. The toxic effect of mycotoxin on animal and human health is referred to as mycotoxicosis, the severity of which depends on the toxicity of the mycotoxin, the extent of exposure, age and nutritional status of the individual and possible synergistic effects of other chemicals to which the individual is exposed. Acute mycotoxicosis can cause serious and some times fatal diseases (Peraica et al., 1999). The toxic effects are mainly localized in liver as manifested by hepatic necrosis, bile duct proliferation, icterus and hemorrhage. Chronic toxicity in birds is characterized by loss of weight, decline in feed efficiency, drop in egg production and increased susceptibility to infections. The incidence of hepatocellular tumors, particularly in ducklings, is considered to be one of the serious consequences of aflatoxicoses (Krogh, 2004). Ergotism and the poisoning due to consumption of mushrooms are the most largely known examples of mycotoxicosis. The magnitude of this problem is exemplified by the consumption of moldy grains in Russia during the Second World War, when a sudden appearance of lesions in the skin, hemorrhage, bankruptcy of liverwort led to countless deaths of soldiers and animals. Similar symptoms were observed in thousands of birds dead poisoned by toxins present in peanut feed in the middle of the 60 decade (Christensen and Kaufmann, 1965; Agrios, 1997; Rastogi et al., 2004; Rasooli and Abyaneh, 2004; Keller et al., 2005).

Different mycotoxicosis result in serious diseases and can lead to death. Filamentous fungi produce these toxins mainly in stocked seeds and processed foods. Usually, the infection of the seeds occurs in the field or during the initial phases of the storage (Prado and Oliveira, 1996; Shephard and Leggott, 2000; Batista et al., 2003; Blesa et al., 2003). The contamination of foods with aflatoxins is more frequent in tropical and subtropical areas, where the climate favors the development of toxigenic fungi (Sabino et al., 1986, 1989; Whitaker, 2003). The disease control for molds in grains, vegetables and other plant products depends on certain precautions and conditions that can be applied before and during cropping and also during the storage (Prado et al., 1995; Prado and Oliveira, 1996; Widstrom et al., 2003). The grains should be protected from mechanical damages, in order to reduce the access of the mold to the internal tissue of the vegetable. Some strategies of reduction of toxins in foods have reached positive results, such as pasteurization, fermentation, addition of substances and filtration, among others. However, the demand for products “in nature” and the fact that many of these procedures result in undesirable alterations in the foods, such as, loss of nutritious substances and organoleptic alterations, led to several studies aiming to establish new control procedures (Leggott and Shephard, 2001; Dorner et al., 2003).

Pimenta (2004) showed that the concomitant inoculation of Saccharomycopsis schoenii in peanuts infected with Aspergillus flavus resulted in 73.5% decrease in the accumulation of aflatoxin in the grain. The same study observed that the amount of aflatoxin produced varied according to the concentration of mold cells. Previous

studies had already pointed the importance of the concentration of spores of A. flavus for toxin production (Odamtten et al., 1987; Sharma et al., 1980; Chulze et al., 1999). Usually aflatoxigenic molds produce more toxins when the initial inoculum is about 10^3 spores/ml. Measures to reduce or increase this optimal spore concentration would also reduce toxin production. On the other hand, control measures that would reduce populations to near 10^3 spores/ml would probably amplify toxin production. Another important aspect of control is that the inoculation of an antagonistic filamentous fungi or yeast, added to a toxigenic population lower than 10^3 spores/ml would also increase the number of cells and consequently lead to an increase in toxin production. The studies have shown that that inoculation of BCAs must use concentrations up to 10^6 cells/ml and that any procedure leading to reduction of the toxigenic population must reduce the initial population to levels below 10^1 cells/ml (Pimenta, 2004).

Another potential use of yeasts against toxigenic pathogens is as probiotics. Probiotic microbes are defined as those which upon ingestion in adequate amounts confer health benefits to the host by improving its intestinal microbial balance (Buzzini and Vaughan-Martini, 2006). When ingested as viable cells by the individual intoxicated with aflatoxin, the yeasts reach the gastrointestinal tract and lead to a return to the equilibrium of the microbiota and improve the complexing of toxins or their inducing agents. The use of live cells of Saccharomyces cerevisiae was capable of minimizes the histotoxicity of aflatoxins in mice (Baptista, 2001; Baptista et al., 2005; Pennacchia et al., 2006).

10.6 Control Strategies

The best strategy for the establishment of a biological control program consists in the utilization of ecological interactions that already exist in nature, including interactions between the crop and microorganisms. It is necessary therefore to search microbial species occurring in the geographic region or associated with the target plant. The yeast or other biocontrol microorganism should be obtained from the phylloplane (leaf surfaces), fruit surfaces, inner plant structures (endophytic microorganisms) or from soil. These protocols avoid the risk associated with introduction of exotic organisms and increase the chance of selecting an organism already suitable to survive and grow in the environmental conditions present in the application area.

The application of the biocontrol agent by inoculation should be made accordingly to the cultural traits of the crop to be protected. In situations of postharvest disease control, the inoculation with a massive population of the biocontroller should be made after harvesting but before storage to prevent pathogens from infecting fruit wounds immediately after harvesting. Wounds are normally the preferred infection route for most of the postharvest pathogens. Once the pathogen gains the interior of the fruit or grain it is virtually unreachable for chemical or biological control. Therefore, the protective inoculation should be made before any chance of wounding or fungal infection. Avoiding wounds is difficult in most cases,

since the act of harvesting fruits could produce injuries. In some cases a preharvest application should be considered (Janisiewicz and Korsten, 2002).

The association of biological control with physical control methods has also had success. An example of this combination is a prestorage treatment to control disease and plagues associated with modified methods for the ripening of the vegetable products. Reduction of humidity, use of heat or cold, modification of the atmosphere, irradiation treatments are among the most employed physical methods for diminishing storage loss (Leverentz et al., 2000; Pimenta, 2004). Low temperatures are recognized as very efficient to increase storage time because they delay physiological processes associated to the ripening and also reduces fungal growth. Humidity reduction preserves commodities by reducing pathogen metabolism but it is an expensive method compared to other methodologies. Treatments using heat generation (hot air at 38°C for 4 days) eliminate insects and phytopathogenic microorganisms. However, the greater limitations to the use of heat refer to the lack of residual protection against recontamination by opportunistic parasites and to injury pit can provokes in the host together with acceleration of the ripening process of several fruit species. Modified atmosphere inhibits the normal aerial growth of the mycelia and greatly prevents sporulation of pathogens. These alterations can be done by a reduction in O_2 and increase in CO_2 concentration, which reduce the ethylene synthesis delaying the ripening of the fruits. Ozone addition also reduces microbial proliferation. Ozone does not deposit a persistent residue on the product, and it is accepted by many organic growers' organizations (Palou et al., 2003; Moraes et al., 2006).

Induction of systemic resistance is a mechanism that operates through the activation of multiple defense proteins. Some BCA can interact with the host tissue particularly with wounds, increasing scarring processes and stimulating host tissue to produce enzymes and other substances that prevent pathogen growth, such as α-1,3-glucanase, chitinase, peroxidase, salicylic acid, terpenoids, and others (Punja and Utkhede, 2003; Spadaro and Gullino, 2004; Liu et al., 2005). An efficient BCA should present some characteristics, like fast growth, genetic stability, efficacy at low concentrations against a wide range of pathogens on various plant products, survival in adverse environmental conditions, growth on cheap substrates, lack of pathogenicity for the host plant and absence of production of metabolites potentially toxic to humans, resistance to the most frequently used pesticides and compatibility with other chemical and physical treatments (Spadaro and Gullino, 2004).

The time of endurance of BCA on substrate is indispensable. Once this period is determined, it will point to the viability and need for reinoculation protocols, although in most cases, reinoculation is not accessible or viable (Pimenta, 2004).

10.7 Conclusions/Tendencies

The transition from chemical to biological control is now evident and a combination of economic, political, and environmental factors has probably contributed to this change. There is increasing concern about the environmental effects and safety

of chemical pesticides and fungicides all over the world. Regulatory agencies have reacted to public pressure and introduced comprehensive legislation to reduce pesticide use. Biological control of postharvest diseases has been one of the most extensively studied alternatives and appears to be a viable technology. Several commercial products are already available and others will be available in the near future. During the past ten years, over 80 biocontrol products have been marketed worldwide and this reflects a growing demand for biocontrol strategies in crop production and storage (Chand-Goyal and Spotts, 1997; Irtwange, 2006).

The yeast Candida oleophila (strain I182) and two strains of a bacteria (Pseudomonas syringae) are registered as Aspire (Ecogen, Inc., Langhorne, PA), BioSave-100, and BioSave-110 (EcoScience Corp., Worchester, MA), respectively, for the control of postharvest diseases of citrus and apple fruits. The integrated control using biological agents associated with one or more physical and chemical (GRAS) treatments such as heat treatment, controlled and modified atmospheres, sodium bicarbonate, calcium chloride, and foodgrade preservatives will probably provide adequate control levels comparable to those achieved by traditional chemical fungicides.

This is an effective method for control of postharvest diseases that seems to be safe and possesses negligible risk to human health and the environment. The biological control of plant diseases using saprophytic, naturally occurring microorganisms which do not produce antifungal compounds, do not grow at human body temperature, and are consistent in controlling the target disease is a safe way to reduce postharvest losses (Chand-Goyal and Spots, 1997). The biological control is an important method for the postharvest control, considered not alone but as part of a multivariate strategy to be applied for reduction of pollution and intoxication. Phytopathogens are associated with plantations since the appearance of agriculture and they will endure along with the cultivation of vegetables by men. The major idea of a biological control program is the return to an equilibrium between species in modified environments (crops), and the reduction in use of toxic compounds in food reached satisfactory standards of food safety (Leverentz et al., 2000; Vivekananthan et al., 2004).

Recently, food safety is one of the most important restrictions to international trade and creates non tariff barriers between countries. The United States and European Union are the most important markets for fruits, and so the most rigorous consumers for safe foods (Skogstad, 2001). Regulators agencies in the United States, Canada, and Europe tend to be favorable towards biological pesticides and they encourage companies to register these products. For example, the costs of registration of biofungicides are lower than for chemical pesticides, and in many cases, registration is unnecessary. Biological products automatically enter a fast-track review process that speeds up registration. This initiative will probably be followed by other countries (Paulitz and Bélanger, 2001). Scientists, growers and consumers alike must accept the fact that BCAs are usually not as effective as pesticides, however, the benefits to the environment and public health compensate a less appealing appearance. The success of biological control greatly depends on influencing the consumer to prefer inner quality to outward appearance (Spadaro and Gullino, 2004).

The use of yeasts in postharvest biocontrol formulations apparently presents advantages over other organisms. Yeasts are easy to cultivate, fast growing and readily found in a variety of substrates and conditions. The use of genetic engineering and other tools of DNA manipulation may, in the near future, increase the use of yeasts to protect fruits and other foods but also to add desirable flavors, higher nutrient content and probiotic properties to foods.

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Chapter 11
Opportunistic Pathogenic Yeasts

Uma Banerjee

Contents

Abstract Advances in medical research, made during the last few decades, have improved the prophylactic, diagnostic and therapeutic capabilities for variety of infections/diseases. However, many of the prophylactic and therapeutic procedures have been seen in many instances to exact a price of host-vulnerability to an expanding group of opportunistic pathogens and yeasts are one of the important members in it. Fortunately amongst the vast majority of yeasts present in nature only few are considered to have the capability to cause infections when certain opportunities predisposes and these are termed as 'opportunistic pathogenic yeasts.' However, the term 'pathogenic' is quite tricky, as it depends of various factors of the host, the 'bug' and the environment to manifest the clinical infection. The borderline is expanding. In the present century with unprecedented increase in number of immune-compromised host in various disciplines of health care settings, where any yeast, which has the capability to grow at 37°C (normal body temperature of human), can be pathogenic and cause infection in particular situation.

Spectrum of infective yeasts varies in different geographical region and mainly depends upon nature of immune suppression of the patients and prevailing yeast in the environment. Opportunistic yeast pathogen mostly reported are *Candida* spp. (*albicans*, *tropicalis*, *krusei, parapsilosis, kefyr, glabrata, dubliensis, rugosa* and others), *Cryptococcus neoformans* (var *grubii*,var *neoformans* and var *gattii*), *Trichosporon* spp. and occasionally others like *Geotrichum* spp, *Pichia* spp. etc.

Among these, *C. albicans* has been regarded as the most common agent of invasive yeast infection.

The population of patients at risk has expanded to include those with a broad list of medical conditions, such as solid-organ and hematopoietic stem cell transplantation (HSCT), cancer, receipt of immunosuppressive therapy, HIV/AIDS, premature

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birth, advanced age, and major surgery. Furthermore, the etiology of these infections has changed. In the 1980s, yeasts (particularly *Candida albicans*) were the most common causative agents of invasive mycoses. However, presently non *albicans* species of *Candida* (NAC) account for >50% of infections. In addition, infections caused by other yeasts, such as *Trichosporon* species, have been reported. This chapter intends to high light important predisposing factors responsible for increase incidence of opportunistic yeast infection, its clinical significance, diagnostic approach for early detection of pathogenic yeast, guide line of therapy and epidemiology of important opportunistic yeast pathogen specially that of *Candida* and *Cryptococcus*.

Keywords Opportunistic yeast pathogen, candidosis, cryptococcosis, *Candida*, *Cryptococcus*, immune suppression

11.1 Introduction

Advances in medical research, made during the last few decades, have improved the prophylactic, diagnostic and therapeutic capabilities for a variety of infections/diseases. However, many of the prophylactic and therapeutic procedures have been seen in many instances to exact a price of host-vulnerability to an expanding group of opportunistic pathogens and yeasts are important members in it. Yeasts being ubiquitous in origin can be easily driven by environmental change linked to demography and the speed at which development is impacting human activities like road building, agricultural changes, population movements etc. Fortunately amongst the vast majority of yeasts present in nature, only few are known pathogens which can cause infection in human and animal. However the scenario has changed during last two decades. Many types of yeasts which were not known to cause infection earlier (termed as non pathogenic yeast), now shown to have the capability to cause infections when certain opportunities predisposes and these are termed as ‘opportunistic pathogenic yeasts’ (OPY). In last two to three decades OPY are increasingly reported from clinical manifestation of various disease conditions especially in immune-compromised patients (ICP).

The yeasts responsible for opportunistic infection (OI) differ in characteristics from that of conventional communicable pathogen. These are mainly low or non virulent, though the borderline is expanding. However the term ‘pathogenic’ is quite tricky as it depends on various factors of the host, the ‘bug’ and the environment to manifest the clinical infection. In recent years the definition of ‘pathogen’ is changing and the concept is evolving purely on the basis of ‘host-parasite’ interaction in particular situation. (Casadevall and Pirofski, 2002). Hence, these could be, non pathogenic in an individuals with intact immune system (*Candida albicans*) or known pathogen presenting in a different way than usual in immune-competent individuals (*Cryptococcus neoformans*) (Banerjee, 2005). Presently, with an unprecedented increase in number of immune-compromised patients (ICP) in

various disciplines of the health care system, particularly the current pandemic of HIV/AIDS, OPY have assumed great significance, where any yeast, which has the capability to grow at 37°C (normal body temperature of human), can be pathogenic and cause infection in a particular situation.

The severity of infection caused by these OPY depends mainly on the type and state of immune-suppression of the host, nature and bolus of infective yeast, the route of its entry and final site of lodgment. Many of these OPY have particular tissue tropism (e.g. *C. neoformans*) which is reflected in the characteristic diseases they produce. The clinical manifestation ranges from benign and localized lesion (either transient or chronic) to disseminated and sometimes fatal infection.

Spectrum of infective yeasts varies in different geographical regions and mainly depends upon the nature of immune suppression of the host and prevailing yeast in the environment (Advani et al., 1996; Musial et al., 1988; Banerjee et al., 1992, 1997; Handa et al., 1996; Rastogi, et al., 1999; Goswami et al., 2000; Jagarlamudi et al., 2000; Lattiff et al., 2004; Marques et al., 2000).

Opportunistic pathogenic yeast mostly reported are Candida spp (*albicans, tropicalis, krusei, parapsilosis, kefyr, glabrata, dubliensis, rugosa* and others) (Odds, 1988), *Cryptococcus neoformans* (var *grubii,* var *neoformans* and var gattii) (Casadevall and Perfect, 1998) Trichosporon spp. (Rippon, 1988; Ramos et al., 2004) and occasionally others like Geotrichum spp, Pichia spp. etc. While in present HIV/AIDS era, azole resistance *C. albicans* and non *albicans* Candida (NAC) has been highlighted. (Musial et al., 1988; Lattiff et al., 2004).

This chapter intends to highlight important predisposing factors responsible for increased incidence of OPY, its clinical significance, diagnostic approach for early detection, guidelines for therapy and epidemiology of important opportunistic yeasts like *Candida* and *Cryptococcus*. Emphasis will be provided in the background of Indian scenario.

Important opportunistic infections and pathogenic yeast associated with lesion:

11.2 Candidosis

Candidosis is one of the common endogenous opportunistic yeast infections. Candidosis arise in subjects who are predisposed like extremes of age, illness, debility or local reduction of host resistance to an overgrowth of their own yeast flora. Incidences of all types of candidosis, and especially deep-seated infections, have risen throughout the eras of antibiotic and immunosuppressive chemotherapy. Nosocomial candidosis is a distinct entity (Burnie et al., 1985; Pfaller, 1996). Of the causative agents, most common and virulent species is *C albicans*, (Odds, 1988) though other non-albicans Candida (NAC) species are increasingly being reported from all groups of patients. Incidence and prevalence of it varies in different geographical area. (Burnie et al., 1985; Wingard, 1995; Nguyen et al., 1996; Colombo et al., 1999 Krcmery and Barnes, 2002; Gutierrez, 2002; Almirante, 2006). In the HIV/AIDS era, reports of

fluconazole resistance *C. albicans* have come out and this creates lot of therapeutic problem. (Maenza et al., 1997; Yang et al., 2003) *C. albicans* causes mostly superficial mucocutaneous infection (Odds, 1988) though systemic infection is not uncommon (Sood et al., 1998; Pfaller et al., 2000; Leleu et al., 2002; Tortorano et al., 2004). Candidosis is significant in immunodeficiency in that it is commonest fungal infection found in neutropenia, (Kralovicova et al., 1997; Jagarlamudi, et al., 2000), cancer (Viscoli et al., 1999), transplant (Fotedar and Banerjee, 1996) and HIV/AIDS (Kumarasamy et al., 1995; Aggarwal et al., 1997; Lattiff et al., 2004). Extensive oesophageal candidosis is an AIDS defining condition.

Oropharyngeal candidosis (OPC) is at the top of the list of opportunistic infections in HIV disease reported worldwide before the era of highly active anti retroviral therapy (HEART), same is also true for Indian HIV positive patients (Lattiff et al., 2004; Banerjee, 2005). Our three studies on OPC in HIV/AIDS since 1992, with increase number of patients in each series, show still prevalent isolate as *C. albicans*, though emergence of non *albicans* Candida species, in concordance with experience of other investigators, has also been reported (Mirdha et al., 1993; Rastogi et al., 1999; Solomon and Ganesh, 2002; Lattiff et al., 2004; Banerjee, 2005).

Though oral candidosis, unless is very extensive, is not diagnostic of AIDS, it is of prognostic value as its presence indicates progression of the immunodeficiency. Even in immune-competent individuals certain other conditions predisposes for occurrence of mucocutaneous candidosis. As for instance, diabetes mellitus where patients have increased risk of developing vulvovaginal candidosis (VVC) (Reed, 1992; Peer et al., 1993; Goswami et al., 2000; Goswami et al., 2006). In persons with systemic infections, Candida species are now the fourth most commonly isolated pathogens from blood cultures (Pfaller et al., 2000).Yeast fungemia, especially that with Candida, occurs frequently in patients with indwelling catheters (Strinden et al., 1985; Sood et al., 1998; Fatkenheuer et al., 2003) and can result in endocarditis (Nguyen et al., 1996; Beynon et al., 2006) or pyelonephritis (Hall, 1980; Seidenfeld et al., 1982.), artificial heart valves, or other prosthetic devices (Kojic and Darouiche, 2004). Urinary tract infection (UTI) by different Candida species is a common clinical entity (Mirdha et al., 1998(b)). In advanced countries with the introduction and wide spread use of fluconazole overall incidence of candidemia caused by *C. albicans* has been decreased but it has increased the occurrence of NAC like *C. glabrata*. (Fidel et al., 1999). A retrospective study between 2001 to 2003 at AIIMS revealed that incidence of cultured proved candidemia increased from 7% (2001) to 10% (2002) to 10.3% (2003) with increase rate of isolation of NAC like *C. tropicalis*, *C. parapsilosis* and *C. glabrata* (Fig. 11.1).

Candida infection of the eye results from injury to cornea (keratitis) or can involve the retina (endopthalmitis) as one of the manifestation of candidosis spread by hematogenous dissemination (Chignell, 1992).

Candida species is readily isolated on most laboratory media, of which the most commonly used and economical media is Sabouraud dextrose agar (SDA). Since common Candida species isolated from clinical specimen are not inhibited by antibiotics or cycloheximide (except *C. glabrata*, some strain of *C. krusei* and

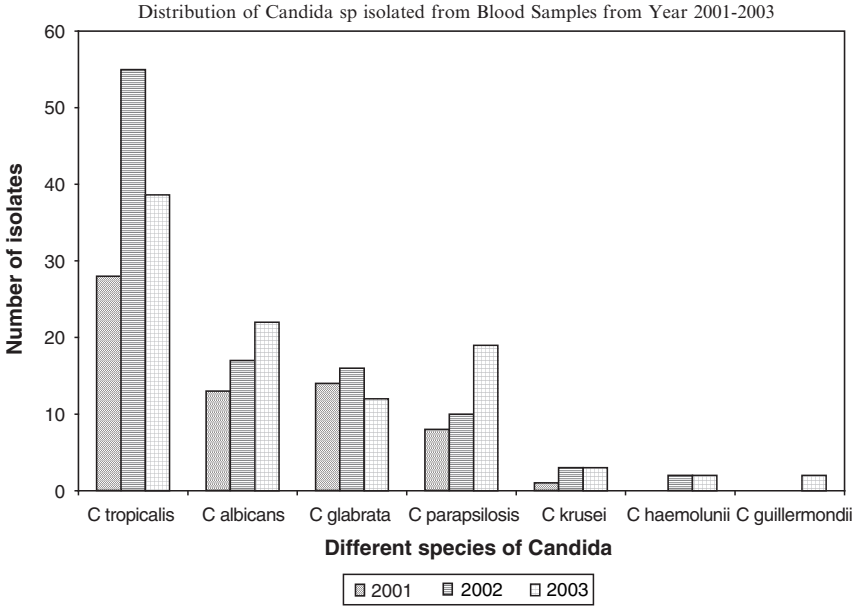


Fig. 11.1 Depicting rate of isolation of NAC like *C. tropicalis*, *C. parapsilosis* and *C. glabrata* from blood in the year 2001, 2002 and 2003

C. parapsilosis, which are cycloheximide sensitive), the use of media containing these antimicrobials are very helpful in isolation of Candida, specially from specimens which are not sterile (e.g. skin, sputum and urine), though there is difference in opinion of its use as yeast isolation media (Barnett et al., 1983). Most pathogenic strains grow well in 37°C as also at room temp (22°C–25°C).

Triphenyl terazolium chloride (TTZ) medium is a useful indicator screening medium which can identify Candida up to species level. *C. albicans* is unable to reduce tetrazolium dyes, so its colony appear creamish white, while other Candida species give colonies with various degrees of pink or red coloration. *C. tropicalis* consistently produce maroon color colony on it (Fig. 11.2).

Young colonies are white with soft consistency; the surface and margins of the colonies are smooth, although rough surfaced strains have been described. Old colonies frequently show a fringe of submerged mycelium which appears as feathery outgrowth deep in the agar.

In a wet mount, masses of budding cells and fragments of mycelium, often with budding cells attached indicative of presence of yeast like organism. Gram stain of smears shows Gram-positive budding yeasts with both pseudo-mycelium and true mycelium (Fig. 11.3).

The traditional specific test for rapid identification of *C. albicans* is the ‘germ tube’ test, in which *C. albicans* alone produces hyphal outgrowth from blastospore when incubated at 37°C in serum for 2–3 h. Germ tube test is a rapid screening test

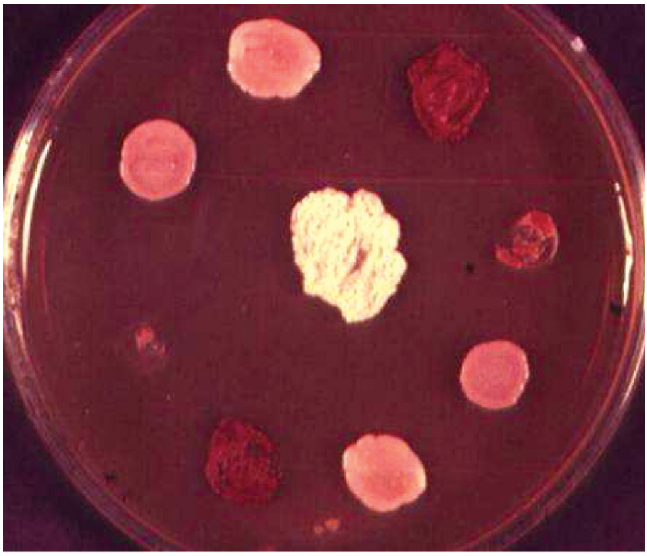
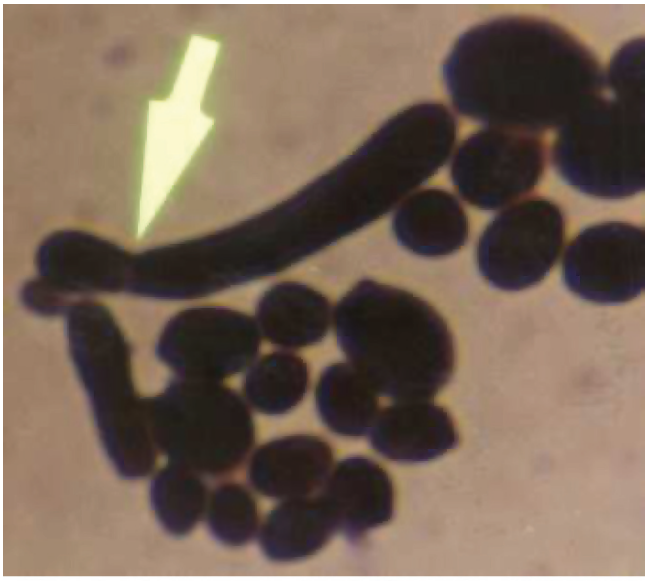


Fig. 11.2 TTZ medium with growth of different Candida species

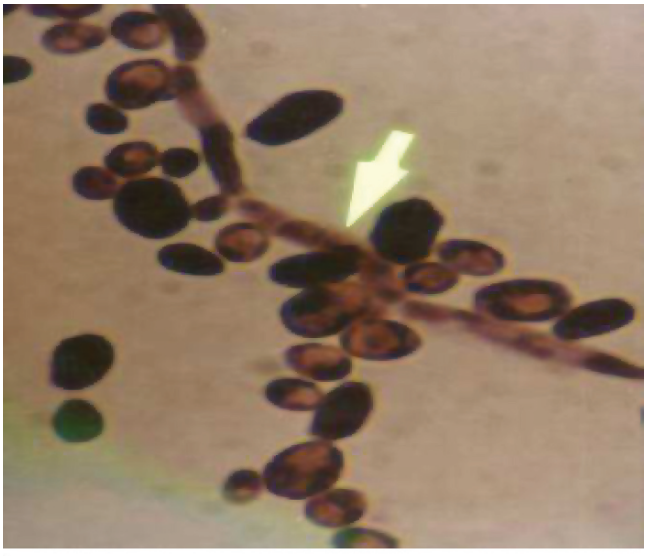
and occasional germ tube negative *C. albicans* has been reported (Odds, 1988). Morphological identification needs to be confirmed by chlamydospore formation. Chlamydospore, a resistance, resting stage of yeast, formation is a unique property specific to *C. albicans*. Corn meal agar (CMA) is a good media for testing this property. In a nutritionally deficient medium like CMA, *C. albicans* produces chlamydospores. This culture-based test is relatively rapid, cheap and easy to use. The chlamydospores are large circular structures with refractile thick walls, formed only in vitro (Fig. 11.4).

Inclusion of detergents (such as Tween 20) in the medium enhances the production of chlamydospores. The optimal temperature is 25°C–30°C, and low density of inoculum produces a better result. Ideal time of observation of CMA test is 48 h though after 24 h some strain can produce chlamydospores. CMA induces not only chlamydospores in *C. albicans* but also induce pseudo mycelium formation in isolates capable of this property. The use of CMA is therefore important not only to confirm identification of *C. albicans* but also for morphological examination of other species (Odds, 1988).

Biochemical characteristics of yeast by fermentation and assimilation of different sugars are also used to identify particular species but these are time consuming and some times produce variable results. Commercially made packages for identification of pathogenic Candida species have greatly enhanced the speed and ease in routine clinical laboratories. Of various systems, API 20 C is popular. Most of the kit rely mainly on physiological properties of the yeast. However many of the evaluator of these kits have stressed yeast morphology testing, which is unique in each species, should remain a significant part of any yeast identification and that identi-



a



b

Fig. 11.3 **(a)** Gram positive budding globular yeast cells characteristics of *C. albicans*. **(b)** Gram positive budding elongated yeast cell. Inter and intranodal bunch of blastospores (budding yeast cell) is characteristics of *C. tropicalis* Pseudo-mycelium (hyphae) designates elongated cells formed from blastospores (budding cells) which elongate, but do not break off from the mother cell. These filaments are very fragile and may break apart easily during smear preparation. On the other hand, true mycelium is formed by the elongation and branching of a germ-tube produced by the mother cell. Septae are formed along the length of the mycelium

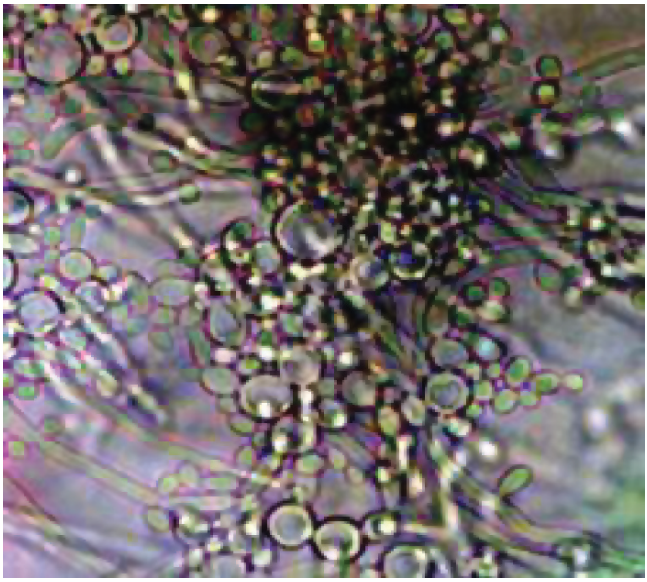


Fig. 11.4 Chlamydospores of *C. albicans* in CMA

fications based entirely on physiological properties can not be assumed to be correct (Odds, 1988).

Diagnosis:Specimens depend on the site of lesion. Usual specimens for investigation of candidosis are:

- Swabs/scraping from lesion/nail clippings
- Oesophageal brushing
- Blood for culture (hemoculture)
- Urine (suprapubic, especially in children)
- Other body fluids, where suspected
- CSF only in very rare cases
- Biopsy of tissue

Since Candida is a common and harmless commensal of mucous membrane and digestive tract of normal individual, demonstration of a few cells in a smear or isolation from specimens such as sputum, swabs from mucosal surfaces etc, have little significance. However, the presence of a large number of yeasts in a fresh specimen, in absence of any other known pathogenic organism, as well as repeated isolation of the same organism from the same site may have some diagnostic significance.

In the blood, urine (suprapubic or collected with sterile precautions), CSF and sample from closed inflammatory foci, the presence of Candida, whatever the species and the number of cells, is of pathogenic significance. It is wise, however,

to request repeat specimens, wherever possible, to rule out possible contamination from the skin when the specimen was obtained.

Quality control needs to be maintained at each step, starting from collection to the processing of samples in the laboratory till the final identification.

For example, to differentiate from colonization to the actual pathogenic role of particular yeast, multiple samples, specifically from the site that is normally not sterile, is advisable. For definitive diagnosis of OI, repeated demonstration and/or isolation of the same yeast from the same site of lesion or same yeast from multiple sites (depending on clinical presentation) are essential.

11.3 Cryptococcosis

Cryptococcosis, encountered world wide, is a serious, often fatal infection caused by the opportunistic yeast pathogen *Cryptococcus neoformans*, the only pathogenic species of the genus Cryptococcus. *Cryptococcus neoformans* is a cosmopolitan, free-living, saprophytic encapsulated yeast. It can survive in a variety of environmental niches. Source of infection is exogenous, mainly soil contaminated with bird dropping particularly pigeon droppings. Various large trees, especially *Eucalyptus camaldulensis*, another important source of this infection. Occasionally it has been isolated from healthy individuals

Depending on the structure of its polysaccharide capsule, *C. neoformans* has been typed in 5 serotypes (A, B, C, D and AD), and it exists in two varieties, var *neoformans* and var *gatti*. Recently a new variety has been suggested (*grubii*) for the serotype A. C *neoformans* is primarily a pulmonary pathogen, and infection generally begins through respiratory route with primary pulmonary invasion. In immune-competent individuals, it mainly remains as in apparent sub-clinical infection. In immune-suppressed patients, it spreads and occasionally becomes disseminated. *C. neoformans* has a predilection for the central nervous system (CNS). For many years, it was considered to be a rare disease; however, now it is recognized to be quite common, probably due to the fact that it is diagnosed more frequently.

Cryptococcosis is mostly chronic infection commonly seen in immunocompromised as well as immunocompetent patients, and sometimes also in association with other infections (Rippon, 1988; Kwon-Chung and Bennett. 1992; Mirdha et al., 1998). Chronic meningitis is the commonest presenting symptom (Diamond, 1990). It has gained increasing medical importance in recent years, particularly in the milieu of HIV/AIDS and it is predicted that it will be ‘Mycosis in future’ (Drouhet, 1997). Extra-pulmonary cryptococcosis is now regarded in an AIDS surveillance case definition (US Public Health Service, 1995). Cryptococcosis was first diagnosed in India in 1941 (Banerjee, et al., 2001(c))**.** It has since made its presence felt, more so in the AIDS era. Cases are diagnosed every year, though the actual annual number of cases in India varies (Banerjee et al., 2001(a), 2001(b) and Banerjee, 2005). Figure 11.5 shows the number of cases of cryptococcosis in AIIMS since 1985 as well as the cumulative index, in which the levels of annual occurrence

are clearly seen. Though occasionally diagnosed, increase in the number of cryptococcosis cases has been observed in AIIMS since 1993 (Banerjee et al., 1994), some times with spurt of infection (Banerjee et al., 1995). In three successive studies, expanding over a decade, we have observed that patients with cryptococcosis not only may present with various unusual clinical manifestation (Banerjee et al., 2001(c)) but also cryptococcosis can occur in substantial percentage (more than 40%) of individuals in whom there is no apparent immune-suppression (Banerjee et al., 2001(c); Banerjee 2005).

In this situation, two possibilities emerge; first, immune-suppression thresholds and parameters are not yet fully understood, and secondly, exact mechanisms of pathogenesis of cryptococcosis are not yet fully explained (Casadevall and Perfect, 1998). Related to these is the possibility of a strain variation occurring in the Indian clinical isolates (Banerjee et al., 2001(c)).

Concentrating on the factor of strain variation, in recent studies, we have found that unique strains of *C. neoformans* are circulating in the environment as different clusters (Jain et al., 2005). Some of these strains are widely distributed in nature. (Banerjee et al., 2005). Genetic analysis of sequential isolates from hospitalized patients with prolonged illness revealed microevolution taking place in particular strain (Jain et al., 2005). Further research is needed to ascertain association of microevolution of the infective strain with chronicity of disease as seen in cryptococcosis. Furthermore in vitro phase variation detected in both variety of clinical isolates (var *neoformans* and var *gattii*) as phenotypic switching (Fig. 11.6). In vivo experiments on switch variety corroborated the association with virulence

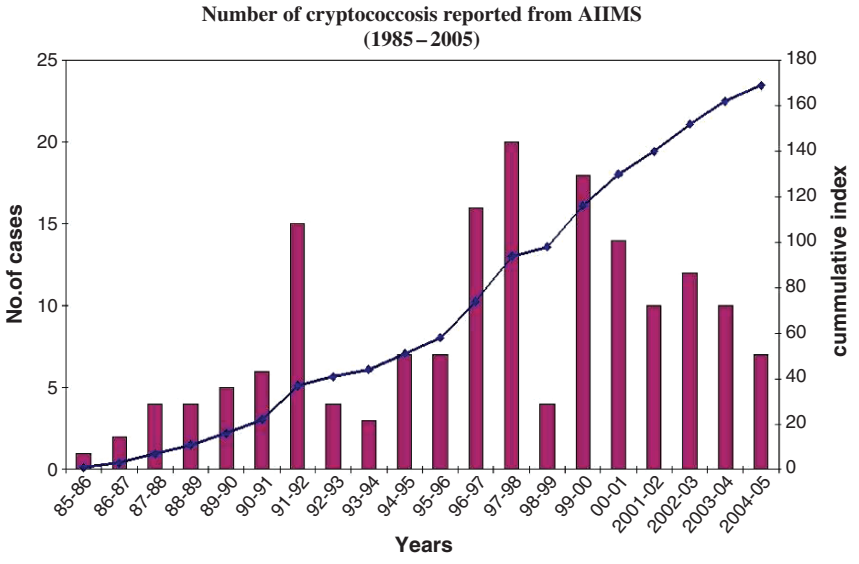


Fig. 11.5 Year wise distribution and cumulative index of laboratory proved Cryptococcosis at AIIMS between 1985–2005

and promotion of dissemination of infection (Jain et al., 2006). Well characterised polysaccharide capsule has been demonstrated in *C. neoformans* (Fig. 11.7) and shown to be associated with its virulence. It is composed of long, unbranched polymers of α-1,3-mannan with monosacharide branches of xylose and glucoronic acid. Capsule synthesis is mediated by a specific α GTP binding (G) protein encoded by the GPA1 gene in *C. neoformans* (Alspaugh et al., 1997). It is hypothesized that capsule protects the yeast from desiccation or reduce its ability to ingest and destroyed by soil amebae when in enviornment and prevents phagocytosis in side host's body, but there is no direct correlation between size of the capsule and virulence of the strains (Casadevall and Perfect, 1998).

Out of 416 clinical isolates studied in our laboratory, various thickness of large, medium and narrow capsulated strains have been demonstrated (Fig. 11.8) in clinical specimen by India Ink/nigrosin mount from both immnosuppressed and aparently immune competent patients.

Microcapsulated/acapsular strain considered as non pathogenic. Though rare, we have for the first time isolated micro-capsulated dwarf strain (Fig. 11.9) from endocardities following mitral valve prosthesis which has experimentally proved to be pathogenic strain (Banerjee et al., 1997).

Surprisingly we have found difference in cell size/capsule ratio in melanin positive (Mel+) and negative albino strain (Mel−) (Mandal et al., 2005) (Fig. 11.10a and b).

C. neoformans is relatively unique in its possession of an enzyme system (laccases) that allows it to metabolize a variety of catachol (such as dopa, dopamine, norephedrine and ephedrine) to a pigment melanin. (Polachek, 1991). Melanin

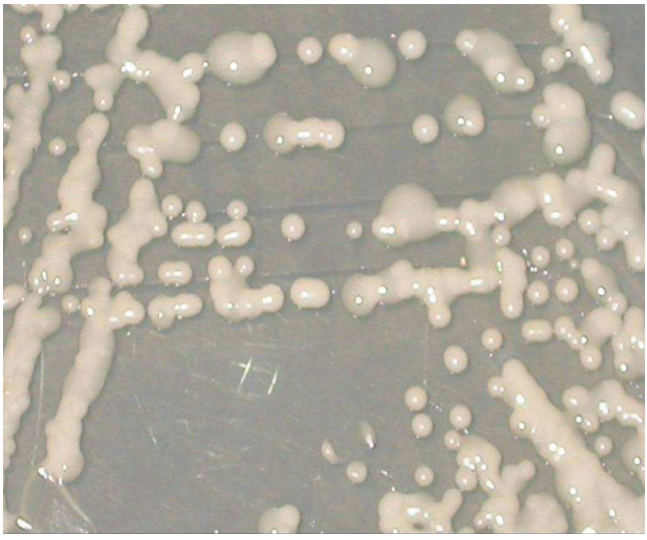


Fig. 11.6 Phenotypic switching of *C. neoformans* colony on SDA from smooth to mucoid

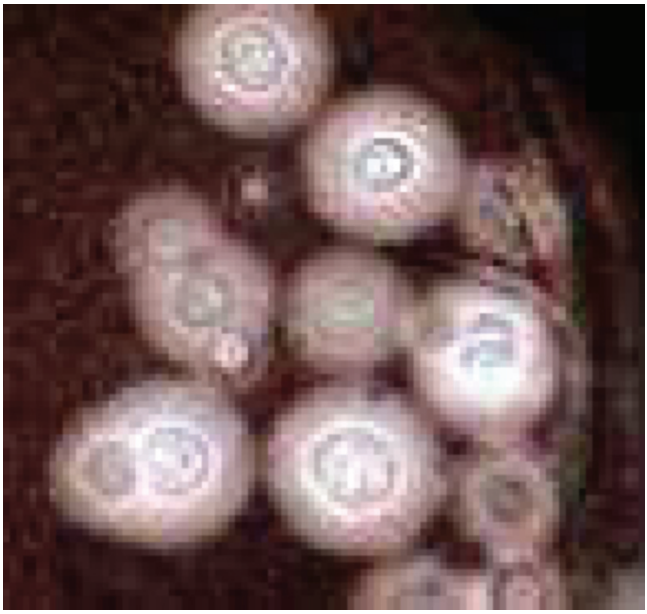


Fig. 11.7 India Ink mount of CSF showing encapsulated budding yeasts of *C. neoformans* in different stage of development

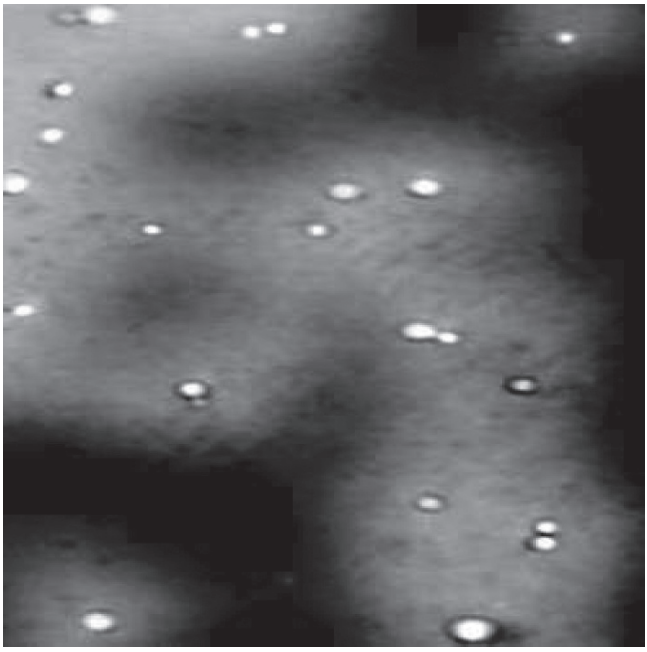


Fig. 11.8 India ink mount of acapsulated, dwarf strain of *C. neoformans* in microthrombus in a patient with cryptococcal endocarditis

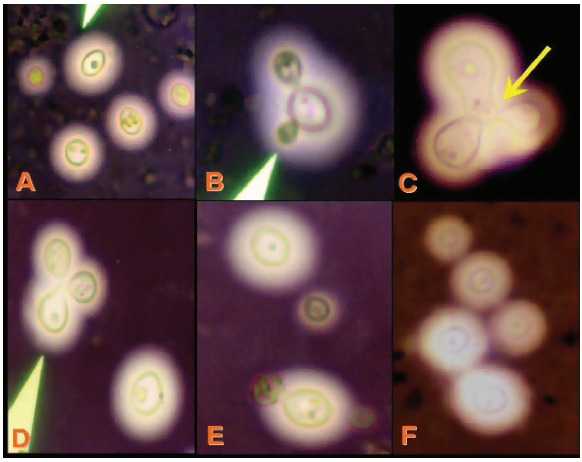


Fig. 11.9 India ink mount of CSF from different patients showing different dimension of capsules along with various cellular architecture. (**A**) and (**D**) depicts oval cells of *C. neoformans* var *gattii.* (**C**) denotes severe alteration of cellular architecture in a isolate from severely immunosuppressed host. (**E**) commonly seen in immune competent patients. (**B**) and (**F**) unusual form

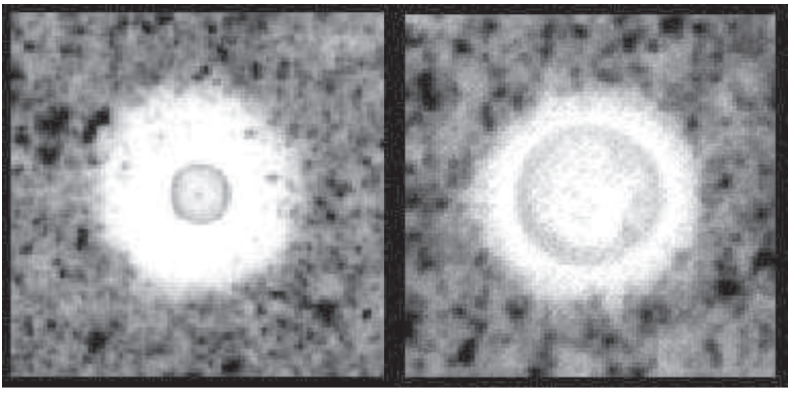


Fig. 11.10 Difference in cell size and capsule ratio in Melanin positive and Melanin negative isolates of *C. neoformans*

appears to be concentrated in the inner aspects of the cell wall of the yeast and acts as an antioxidant which protect the yeast against solar radiation and environmental oxidants as also in vivo protection against oxidative damage by professional phagocytes (Casadevall and Perfect, 1998).

Like capsule, melanin production is one of the virulent facator (Mel+) attributed to pathogenic strain of *C. neoformans* and melanin negative (Mel–) strains are considered as non-pathogenic. This property of *C. neoformans* has been used as a

diagnostic test for definitive identification of pathogenic Cryptococcus. Our experience revealed that melanin variable pathogenic strains are not infrequent (Fig. 11.11).

Optimum incubation time and temperature need to be provided to detect a stable melanin negative/variable strain. Recently we also demonstrated the pathogenic role of some melanine negative (Mel−) albino strain as an infective agent, causing serious meningitis in both HIV positive and negative patients either alone or in association with virulent melanine positive strain (Mandal et al., 2005). It is apparent that a single strain may represent a heterogeneous population of cell (Fig. 11.12) in which specific phenotypic clones can be selected. It has been further substantiated that Mel− strains had lacasse gene but due to some unknown reason failed to express it (Mandal et al., 2005). Cell size of Mel- strains was smaller compared to its counter part Mel+ strain but capsule size was significantly larger (Fig. 11.10), which further support its pathogenic potential. It is suggested that whenever unsual strains isolated from clinical specmen, detail investigations need to be conducted before dismisial of particular isolate as non pathogenic.

Identification of serotype/variety status of isolates of *Cryptococcus* not specifically relevant to diagnosis of disease but has significance not only in epidemiology, pathogenesis and clinical presentations but also on the therapeutic outcome. Serotype A, *var grubii*, is the most common serotype worldwide (Kwon-Chung and Bennett, 1984;Casadevall and Perfect, 1998), same as in India though 2% of our 45 isolate tested was untypable (Banerjee et al., 2004). In contrast to the conventional belief that var *gattii* (serotype B) does not infect HIV-positive patients and not present in India, there are few reports, both from north and south India, about the

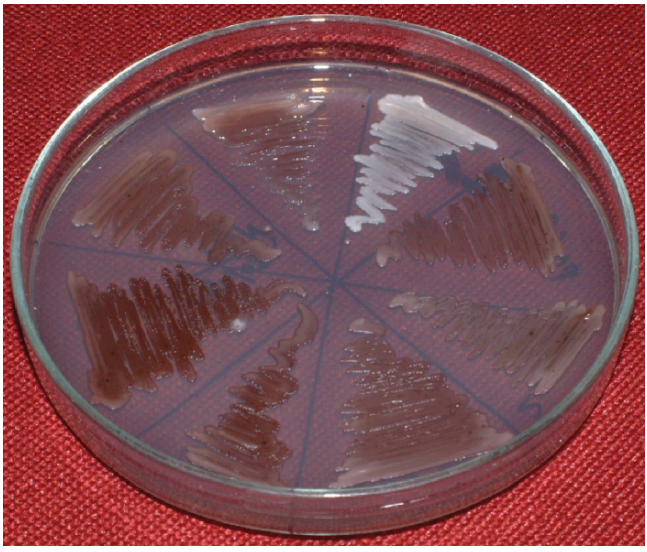


Fig. 11.11 Caffic acid agar showing melanin variable clinical isolates of *C. neoformans* along with non melanin producer yeast *C. albicans* (white colony) as control

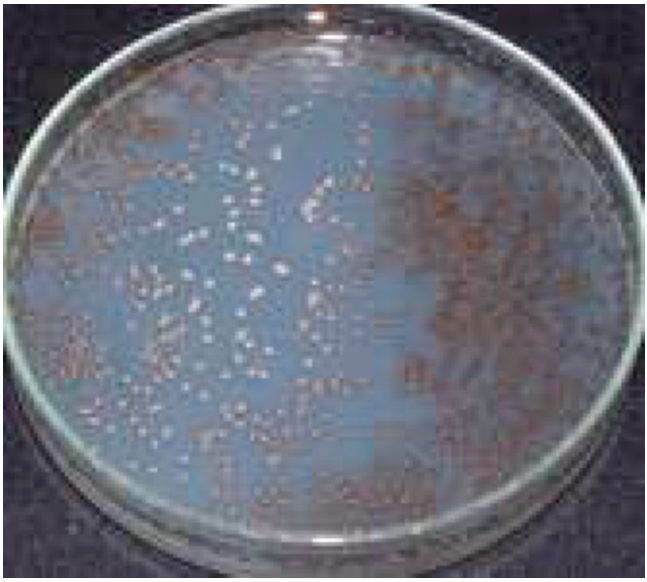


Fig. 11.12 A single CSF isolate on L Dopa agar showing heterogeneous population (strong and weak melanin positive and albino (white) population of *C. neoformans* cells

isolation of it in HIV infected patients (Banerjee et al., 2001a, 2004). This indicates the widespread reservoir of this agent in India, which is evidenced by a significant epidemiological study from the north (Chakrabarty et al., 1997). Identification of this variety of the isolate is particularly important, as it is quite often isolated from immunocompetent patients. This variety is relatively refractory to the treatment and prolonged course and /or increased dosage of antifungal may be necessary for therapy. Besides, sequelae of cryptococcosis is much more in *gattii* variety.

In vitro antimicrobial test is one of the integral part of a diagnostic set up in clinical microbiology. In contrast to the wide application of the in vitro antibacterial susceptibility test, development and adoption of in vitro antifungal test is still evolving. This test system is essential for therapeutic guidance in OPY in HIV disease, particularly in the case of therapy with fluconazole, widely used triazole, which is being used for prophylaxis of OPC and life long maintenance therapy in patients who have been treated for chronic meningitis with amphotericin B. There are reports of appearance of growing population of fluconazole resistant *C. albicans* in HIV disease, 6% per cent of our isolates from OPC in HIV positive patient showed in vitro resistant against fluconazole (Lattiff et al., 2004). On the other hand, 16% isolates of *Cryptococcus*, though not overly resistant, have higher inhibitory concentration (MIC) against fluconazole (Datta et al., 2003), many of these patients did hot have prior exposure to the drug. Reason of it unclear.

Recently transposon has been detected for the first time in some of our clinical isolates (Jain et al., 2005). Its role in acquisition of drug resistance need to be explored. Situation though is not alarming at this point, intensive research in these areas along with surveillance programs is, therefore, a prime necessity to forecast the advent of a resistant population.

Clinical Manifestation of Cryptococcosis: The clinical presentations of human cryptococcosis often varied and because of the variety of signs and symptoms, clinical evidence of infection without laboratory diagnosis can be difficult at times in both high and low risk patients. When disseminated from its primary location lung, it can lodge in any organ of the body and damage it, but brain becomes the organ with unique and still unexplained propensity for becoming a body site for clinical disease (Casadevall and Perfect, 1998). Therefore, most of the available data describe either lung or CNS manifestation of the infection.

CNS cryptococcosis: Onset of clinical symptoms often insidious, initially may linger for weeks or months as fever of unknown origin before classical manifestation of chronic meningitis sets in. Sever headache with minimal or no neck rigidity is one of the important feature of CNS cryptococcosis. Drowsiness and alteration in sensorium, with advancement of disease process. Cerebral cryptococcal granuloma is quite common.

Pulmonary cryptococcosis: Often presents with asymptomatic subclinical infection, usually detected during investigation to exclude other diseases. It is mainly diagnosed by extensive laboratory investigations and radiology.

Diagnosis: Specimen should be collected according to the symptom of the patient and suspected site of localization of infection.

Most common specimen is CSF; others are other body fluids, sputum, skin scrapping, biopsy tissues, prostatic fluid (in case of relapse) and blood for culture where indicated. If the initial demonstration/isolation is from an extra pulmonary site, attempts should be made to examine CSF irrespective of clinical presentation, to rule out asymptomatic meningeal involvement.

Diagnosis by conventional methods of direct demonstration of encapsulated budding yeast in the clinical specimen followed by successful culture. Large volume specimen increases the chances of microscopic demonstration and recovery of the infective yeast. Blood culture is helpful in diagnosis especially in the disseminated condition. However once cryptococcemia develops, fatality is almost certain even with specific antifungal therapy (Banerjee et al., 2004).

Most *C. neoformans* strains can be identified by morphological and biochemical characteristics. In its identification, certain features, such as encapsulation, growth at 37°C and production of melanin will presumptively identify the yeast as *C. neoformans.* Stain like India Ink and its various modifications – nigrosin is very useful, rapid and inexpensive diagnostic test for demonstration of *C. neoformans* in clinical specimen. It is a negative stain which demonstrates encapsulated budding yeast cells in various stages of development (Figs. 11.7 and 11.9). Its sensitivity varies depending on yeast load in a particular specimen. In CSF it can be observed when yeast concentration ranged between 10^3 to 10^4 CFU/ml. Sensitivity can be improved by centrifuging CSF (i.e. 500 rpm for 10 min) and using pellet for

staining. *C. neoformans* can be stained by Gram stain as gram variable budding yeast. Yeasts have been identified from various body sites and tissues with histological stain ranging from nonspecific Papanicolaou, hematoxylin and eosin, and acridine orange preparation to more specific fungal stains such as Calcofluor, which stain fungal chitin, or Gomori methenamine silver stain. Mucicarmine, periodic–Schiff, and alcian blue have been used to demonstrate capsular material in surrounding yeast in tissue. Immuno histochemistry not only can pinpoint specific localized site of lodgment of the yeast but also can identify specific soluble polysaccharide in the tissue even in absence of yeast cell.

In clinical specimens, yeast cell of *C. neoformans* are mostly globose in shape, although some may be oval to lemon shaped and var *gattii* cells may be actually be elliptical (Fig. 11.9). *C. neoformans* does not produce hyphae or pseudohyphae.

Biochemically *C. neoformans* is quite inert and does not ferment sugars. It can assimilate inositol but not nitrate. In its carbon assimilation profile, it will utilize galactose, maltose, galactitol and sucrose. However, it will not assimilate lactose or melibiose and its growth is strain variable with erythritol (Casadevall and Perfect, 1998).

There are a series of commercially available micro method systems employing modified conventional biochemical tests (API 20C, API 32C, Vitek Yeast Biochemical Card etc) which are being used for identification of *C. neoformans* in clinical laboratory in advanced countries but these tests, though reduce turn around time, are expensive. On the other hand conventional techniques though good and cost effective may not always offer the expected discriminatory power for early detection of infection and strain identification. It mainly depends upon the stage of the disease, suitable collection of appropriate specimen in adequate amount and proper processing in the laboratory.

Since the last decade there have been rapid development of different molecular techniques (Vilgalys and Hester, 1990; Huffnagle and Gander,1993; Mitchell et al., 1994; Hoper et al., 1993), which can rapidly distinguish *C. neoformans* from other yeast with in mixed sample or in tissue with 100% sensitivity and specificity. It seems some of these methods can be adopted directly in clinical specimen but besides being expensive, at this point of time it is uncertain when these molecular strategies will prove to supplement, complement or completely replace conventional method of diagnosis. (Casadevall and Perfect, 1998).

C. neoformans is not fastidious yeast and can grow on standard bacterial and fungal culture media. Sabouraud Dextrose Agar (SDA) mostly used in diagnostic service laboratory. Colonies may appear within 48–72 h of incubation, but may take a longer time depending on the fungal load and strain of *C. neoformans. C. neoformans* grows well at 25°C but incubation should be both at 25°C and 30°C to 37°C for clinical specimens. This range of temperature may actually speed up isolation, since some strains of var *gattii* may require prolonged incubation (5 to 7 days) and may not easily grow at higher temperature (35°C to 37°C). Thermo tolerance study conducted on 131 clinical isolates in our laboratory, 62% showed better growth in 30°C. Most isolates of *C. neoformans* are inhibited by cycloheximide under 25 μg/ml. Occasionally, nutritionally aberrant strains are reported, though specific

nutritional requirement of these strains remains unclear. It emphasizes that for some strains different media may be required for primary isolation. For instance, direct India Ink positive CSF/ histologically positive tissue which failed to grow, ideally may need to be cultured on several different media before yeast viability or non viability can be definitely determined. However in clinical practice quite often it is difficult to obtain large volume specimen, particularly from children and critically ill patients, for this purpose. Most of these culture negative cases are probably due to too few yeast at the site of infection to be detected. We have some success in this situation, by incubating a part of the sample at 25°C and reculture it after 24–48 h.

Canavanine-glycine-bromothymol blue (CGB) agar can be used to differentiate var *neoformans* from var *gattii.* Var *gattii* is resistant to canavanine and there will be good growth. It metabolizes glycine with production of ammonia which turns the medium blue. Var *neoformans* neither grows nor change the colour of the medium. Inoculum size standardization is very important in this test, and suitable positive and negative control need to included in each batch of test.

In general, colonies of *C. neoformans* are opaque, smooth or mucoid, soft in texture and creamy in colour which may turn tan or brown on prolonged incubation. There is variability among strains in their ability to appear mucoid. Mucilaginous character of colony is directly proportional to size of capsule around the yeast. *C. neoformans* can exhibit various morphological different forms of colony character especially after prolonged incubation. (Banerjee, 2005) Upon repeated subcultures (for instance, for maintenance of isolates) the colonies tend to become dry as the capsule size decreases.

For isolation of *C. neoformans* from heavily contaminated specimen like sputum in clinical practice or environmental sample, niger seed agar (Staib's medium) is an excellent selective medium. *C. neoformans* appears as brown colony due to its ability to break down caffic acid to melanin. Caffic acid agar is also available commercially and worked well in our hand. This medium can be used as a supplement as a primary culture medium for sputum, skin scrapping and urine in patients with HIV disease which increase the sensitivity for detection of *C. neoformans*, since these patients are frequently colonized with other yeasts (Staib et al.,1987; Denning et al.,1990). Comparative analysis of efficacy of bird seed with sunflower seed agar we have found that early appearance of colony and pigmentation is better in sunflower seed agar medium.

Phenotypic change of phase variation, from smooth to mucoid colony and vice versa has been observed, (Fig. 11.6) as phenotypic switch, in both var *neoformans* and var *gattii* (Jain et al., 2005) variety. The genetics of the switching mechanisms for certain phenotypes in the morphological changes of colonies and their relevance to the pathobiology of the yeast await further research.

Serology is an important adjunct of the indirect evidence of infection. Cryptococcal capsular polysaccharide antigen detection by latex agglutination is the test of choice with very high sensitivity and specificity. Antibody detection has prognostic rather than diagnostic value. Antibody becomes positive as the patient

recovers and antigen titer drops. However, it is doubtful that AIDS patients ever recover sufficiently so as to produce detectable antibodies.

A clinical service laboratory needs to be equipped with battery of tests including anti fungal susceptibility and ready for timely detection and identification of a particular unusual form of yeast, which may be pathogenic, for effective patient management.

11.4 Conclusions

Any yeast can be a pathogen in a particular situation and cause infection in an ICP. It is also to be borne in mind that being eukaryotic in nature, yeast infections, compared to other microbial infections like bacteria, virus etc, takes time to clinically manifest. Absence of pathogenomic signs and symptoms suggestive of classical manifestations of particular yeast infection, particularly the deep seated ones in ICP, often makes it difficult to recognize it, especially in the early stage of infection when yeast burden in the system is low, consequently delaying specific intervention. Confident diagnosis relies heavily on experience along with a combination of clinical, radiological, microbiological, histopathological and serological evidences. Though continuous addition of newer methods takes place, introduction of sophisticated equipment and techniques in day-to-day working of the laboratory invariably increases the cost of laboratory investigations. Therefore, at the primary level, the clinical mycologist has to fall back upon simple established standard procedures. No one single procedure may help in proper diagnosis, since each has its own limitations. Therefore, all these procedures (WHO, 2001) should be incorporated in a standard mycological laboratory as far as practicable.

OPY that can be diagnosed with reasonable accuracy by physical examination (oral candidosis) or by inexpensive laboratory techniques (*C neoformans* by India ink), may be documented more frequently than OPY requiring more cumbersome procedures of collection of specimen (oesophageal candidosis, VVC, endocarditis, endopthalmitis, prostatitis etc.) (Banerjee, 2005). Determining the spectrum of OPY and the changing pattern over the years, in a given region requires adequate surveillance and good local diagnostic services. However for definitive diagnosis of etiological role of these ‘opportunistic’ yeast pathogens strict criteria for ‘documented’, ‘possible’ and ‘probable’ yeast infection should be followed with adequate quality control. Communication with reference laboratory helps in confirmation of local diagnosis.

In spite of best effort to correlate a particular yeast as a pathogen, in nature constantly ‘new’ strains evolve or ‘old’ one change their character (phase variation, acquire resistance against antifungal etc.). Awareness of the local situation, documentation, proper preservation of isolates and timely communication of newly acquired data helps in understanding of pathobiology of the yeast and regional epidemiology. Endogenous infection as in Candidosis is difficult to control/or

eradicate. Early specific diagnosis followed by specific intervention in time can save the life of many patients. Nosocomial infection however can be reduced with good hospital infection control program. Situation is different in cryptococcosis. Since all Cryptococcus in the world are being maintained in environment, their capacity to evolve, acquire new genes and search new hosts is facilitated by proximity of host to environment. It can strike at an opportune time and periodically transmit to human being. Early detection and intervention can prevent the infection to spread to CNS and complication there after.

Follow up and monitoring of the patients infected with OPY is necessary especially in ICP, as not only relapse rate is quite high even after initial successful therapeutic outcome, some times spectrum of infective yeast changes during the course of therapy. (Foteder and Banerjee, 1996). Scenario of present status of OPY is expected to change in highly active antiretroviral therapy (HAART) era. Besides, presently good number of newer antifungals are available. Judicious use of these will help better patient management. Improvement, upgradation and networking of an effective data preservation system, specially that of the laboratory documented cases of uncommon yeast causing infection is essential. A high level of alertness is needed at both clinical and laboratory level and routine surveillance studies need to be undertaken.

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Chapter 12
Interaction Between Yeasts and Zinc

Raffaele De Nicola and Graeme Walker

Contents

Abstract Zinc is an essential trace element in biological systems. For example, it acts as a cellular membrane stabiliser, plays a critical role in gene expression and genome modification and activates nearly 300 enzymes, including alcohol dehydrogenase. The present chapter will be focused on the influence of zinc on cell physiology of industrial yeast strains of *Saccharomyces cerevisiae*, with special regard to the uptake and subsequent utilisation of this metal. Zinc uptake by yeast is metabolism-dependent, with most of the available zinc translocated very quickly into the vacuole. At cell division, zinc is distributed from mother to daughter cells and this effectively lowers the individual cellular zinc concentration, which may become zinc depleted at the onset of the fermentation. Zinc influences yeast fermentative performance and examples will be provided relating to brewing and wine fermentations. Industrial yeasts are subjected to several stresses that may impair fermentation performance. Such stresses may also impact on yeast cell zinc homeostasis. This chapter will discuss the practical implications for the correct management of zinc bioavailability for yeast-based biotechnologies aimed at improving yeast growth, viability, fermentation performance and resistance to environmental stresses.

Keywords Zinc, gene expression, industrial yeast, vacuole, fermentative performance, homeostasis

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12.1 Introduction

Yeasts require mineral nutrients for cellular growth and metabolism (Jones and Greenfield, 1984). Bulk metals, such as potassium and magnesium, are required at millimolar concentrations (mg l^{-1}), whereas trace elements, such as calcium, zinc, manganese, iron and copper are required at micromolar concentrations (μg l^{-1}). Other ions may be toxic, even at very low concentrations (μg l^{-1}) and include: Pb, Cd, Cr, Hg, Ni, and Al. The utilisation of such ions by yeast cells is often influenced by many factors including metal ion deficiency or excess, chelating/absorbing material in the media and the presence of other ions acting as antagonists (Jones and Gadd, 1990). Metals play structural roles in cellular organelles, proteins and phospholipids and influence cell-cell interactions in the phenomenon of flocculation. Metals are also required for uptake of other nutrients (e.g. Mg-ATPase), gene expression, cell division, growth, fermentation and in energy maintenance. At defined concentrations, some metals may help cells to cope with environmental stresses (Walker, 2004). Table 12.1 summarises the principle functions of essential metal ions in yeast physiology.

Regarding fermentation, zinc is well known as an essential ion for alcohol dehydrogenase (ADH) (Magonet et al., 1992), which facilitates the conversion of acetaldehyde into ethanol at the end of fermentation. In addition, intracellular magnesium may stimulate the activity of pyruvate decarboxylase in brewing strains of *Saccharomyces cerevisiae* (Smith, 2001). If present at high concentration, calcium ions may be detrimental and inhibit both growth and fermentation, due to the antagonism of essential magnesium-dependent reactions (Walker et al., 1996). Yeast cells actively extrude calcium, a metal playing a key role in the phenomenon of flocculation by stabilising bridges between lectin proteins of the cell wall and carbohydrates receptors on another cell, thus facilitating adhesion between adjacent cells (Miki et al., 1982).

In brewing, such a capacity to form flocs facilitates the separation between cells from immature (green) beer at the end of fermentation with cold temperatures

Table 12.1 Functions of essential metal ions in yeast physiology (adapted from Walker, 2004)

Metal ion	Optimal concentration in growth medium*	Main cellular function
Macroelements:		
K	2–4 mM	Osmoregulation, enzyme activity
Mg	2–4 mM	Enzyme activity, cell division
Microelements:		
Ca	<μM	Second messenger, yeast flocculation
Fe	1–3 μM	Haem—proteins, cytochromes
Zn	4–8 μM	Enzyme activity, protein structure
Mn	2–4 μM	Enzyme cofactor
Cu	1.5 μM	Redox pigments

* Values depending on strain, culture conditions and presence of chelators in the media.

promoting this phenomenon (Briggs et al., 2004). It is an intriguing possibility that the rate of calcium ion extrusion could increase to promote the flocculation.

Yeast intracellular ionic composition is different from that of the external medium as is the case for most microorganisms. As a consequence, yeast cells have evolved a system of transporters in order to exploit environmental changes and survive when nutrients are limited or when they are in excess. The metabolic status of cells and the prevailing growth conditions will determine the cellular capacity of yeasts to take up metal ions. Other factors such as low temperature, metabolic inhibitors and absence of energy-yielding substrates are known to negatively influence this uptake. Metal ion uptake into the cell occurs in two stages: biosorption and absorption/translocation. The former is also referred to as the metabolic-independent phase while the latter as the metabolic-dependent phase (Table 12.2).

After an initial passive cell wall binding, the transport of divalent cations is dependent on the activity of the plasma membrane H±-ATPase (Jones and Gadd, 1990). The membrane potential influences ion transport, including metals. For example, the cell may extrude K^+ to increase polarisation of the plasma membrane in order to facilitate ion uptake. This has been documented in the lager yeast *S. carlsbergensis*, where uptake of Mn^{2+}, Mg^{2+} and Zn^{2+} was accompanied by K^+ efflux (Okorokov et al., 1983; Jones and Gadd, 1990). The transport of an ion across a membrane is mediated by specific sets of transporter proteins. Various metal ion transport systems are known and occasionally, several uptake systems with different affinities have been described for the same metal ion. The main genes responsible for encoding these metal ion transporters have been recently identified and they are listed in Table 12.3.

Once the ions have crossed the plasma membrane, many mechanisms work together to ensure that cellular ion homeostasis is maintained, through highly regulated processes of uptake, storage and secretion. The understanding of these mechanisms is fundamental for industrial fermentation design for processes aimed at maximising biomass (e.g. dried yeast starter cultures for winemaking and baker's yeast production) and for production of fermentation compounds such as beer, wine or fuel ethanol. More precisely, the object of these homeostatic mechanisms is two-fold: to prevent accumulation of the metals in the freely reactive form (metal detoxification pathways) and to ensure proper delivery of the ion to target metalloproteins (metal utilisation pathways). Some ions may be sequestered in the nucleus, or in the cytoplasm through polyphosphates or calmodulin, a protein serving as the major intracellular

Table 12.2 Two-stage metal uptake systems in yeast cells (adapted from Mowll and Gadd, 1983)

Phenomenon	Description	Energy requirement	Cell localisation
Biosorption	Unspecific binding of the ions to the cell wall. Cells may be dead.	No	Cell wall
Absorption	Transport is aided by a proton-pumping ATPase	Yes	Plasma membrane (then translocation to cytosol)

Table 12.3 Main genes encoding transporters for metal uptake in yeast cells

Element	Genes	Comments
Zn	*ZRT1/2, ZRT3*	High and low affinity system. Also vacuolar transport
Fe	*FRE1/2/3/4/5/6/7 FET3, FTR1*	Fet4p is a metal transport also for Zn^{2+}, Mn^{2+} and Cu^{2+}
Mn	*SMF1/2/3*	Also for iron. Only high affinity system
Cu	*CTR1, 2, 3*	High and low affinity system
Mg	*ALR1/2, MRS2*	Membrane and mitochondrial transporters
Ca	*PMR1, PMC1*	Golgi and vacuole Ca^{2+}-ATPase

Information from Van Ho et al., 2002; Walker, 2004.

receptor for calcium and which mediates many effects of this ion (Cyert, 2001). Metallothioneins, cysteine-rich proteins of low molecular weight, are also known to bind various metals (especially copper) by means of their cysteine group, minimising their toxicity. Metallochaperones are soluble proteins that ensure the safe transfer of ions (e.g. copper and probably iron) to intracellular sites where they are required (O'Halloran and Culotta, 2000). Some ions may be compartmentalised in organelles for later utilisation. Other ions, such as zinc, magnesium and manganese can be stored in the vacuole. In some cases, ions may remain free in the cytoplasm at very low concentrations to become available for metabolic functions. In industry, the capacity to accumulate and store metals in the vacuole or other compartments can be used to pre-condition yeast cells with metal ions prior to inoculation.

This chapter focuses on yeast interactions with zinc and discusses implications of this for yeast-based industrial processes.

12.2 Critical Review

Zinc is a transition and group II element with atomic number 30 and an atomic weight 65.37 Daltons. Under physiological conditions zinc is very stable and exists in the divalent state. It is not redox active, since neither the potential oxidised form Zn^{3+}, nor the potential reduced form Zn^{+} occurs in cells. Zinc is able to form bonds with many molecules including sulphur from cysteine, nitrogen from histidine and oxygen from glutamate, aspartate and water (Berg and Shi, 1996). The characteristics of zinc's coordination sphere enables a variety of complex geometries to be formed and to allow zinc to participate in enzymatic oxido-reduction reactions in coordination with organic cofactors (Vallee and Auld, 1992).

In biological systems, zinc may be present in the bound form or as cellular free zinc depending on the type (neurons, blood cells, yeasts, etc.) and status of the cell. Its cellular concentration usually ranges from femtomolar to millimolar but this former level may occasionally drop in the range of picomolar to nanomolar concentrations (Outten and O'Halloran, 2001).

In biological systems, zinc is an essential trace element, estimated to be required for almost 3% of the yeast proteome function (Eide, 1998). Its abundance as a transition metal is second only to that of iron (Vallee, 1988). It is now known to be an integral component of a large number of proteins and enzymes, being indispensable for the function of nearly 300 of them (Vallee and Auld, 1990): e.g. acid and alkaline phosphatase, aldolases, Cu, Zn-superoxide dismutase and alcohol dehydrogenase (Leskovac et al., 2002). Zinc also plays a purely structural role in few enzymes (Berg and Shi, 1996). Zinc participates in a wide variety of metabolic processes including carbohydrate, lipid, protein and nucleic acid synthesis or degradation. Zinc ions can form bridges between lipid molecules reducing the capacity of the phosphate groups bound to zinc to take up water. These bonds make the membrane surface more hydrophobic and rigid (Binder et al., 2001), thus influencing membrane fluidity (Garcia et al., 2005). Excess zinc can influence respiration at the level of mitochondrial aconitase activity (Rhodes and Klug, 1993; Costello et al., 1997). Zinc is also the main constituent of the zinc finger proteins that bind specific DNA sequences, playing a role in gene expression (Rebar and Miller, 2004). This property is mainly due to the lack of redox activity of zinc which stabilises DNA and RNA molecules and prevents radical reaction resulting in nucleic acid damage (Berg and Shi, 1996). Zinc is also critically placed to control apoptosis, by suppressing major pathways leading to this phenomenon. Apoptosis is an active energy dependent process of programmed cell death, closely linked with oxidative stress. Zinc acts as a cytoprotectant and minimises oxidative damage by stabilising both lipids and proteins, increasing glutathione, the main cellular antioxidant, and by suppressing the activation of caspase-3, a protease that cleaves to substrates causing critical morphological changes (Truong-Tran et al., 2001).

Zinc plays a central role in the enzyme function in yeast cells, especially alcohol dehydrogenase (ADH). Zinc is also required for endoplasmic reticulum function in *S. cerevisiae* (Ellis et al., 2004). The average zinc cellular content in baker's yeast cells has been defined to be 0.12 g kg^{-1} dry weight (Jones and Greenfield, 1984), this value being strain and growth condition dependent. Zinc deficiency depresses yeast growth with cells tending to swell and to form clusters (Obata et al., 1996). Zinc depletion in *S. cerevisiae* also results in a decrease of the activity of phospholipid synthesis and subsequent alteration of phospholipid composition (Iwanyshyn et al., 2004).

Zinc is a hydrophilic, highly charged ion, which cannot cross biological membranes by passive diffusion and so must be translocated by transporter proteins (Guerinot and Eide, 1999). In *S. cerevisiae*, zinc uptake and homeostasis are controlled both at transcriptional and post-translational levels. The transcriptional system operates at moderate intracellular zinc levels, between 0.01 and 0.07 nmol Zn/million cells, and the post-translational level operates when intracellular zinc levels are above 0.07 nmol Zn/million cells (Zhao et al., 1998; Gitan et al., 1998). At the transcriptional level**,** three or more uptake systems are known to control zinc uptake. One system has a high affinity (K_d = 10 nmol l^{-1}) for extracellular zinc and is active in zinc limited cells (Zhao and Eide, 1996a). A second system has a lower affinity (K_d = 100 nmol l^{-1}) for extracellular zinc, and is active in zinc-replete cells

(Zhao and Eide, 1996b). These systems are very specific for zinc and are not involved in uptake of other metals. One transport protein works for each of the two systems: Zrt1 for the high affinity system and Zrt2 for the low affinity system. These proteins are localised in the plasma membrane and share 44% of identity in the amino acid sequence and 67% of similarity (Eide, 2003). A third system is also known to take part to zinc uptake: the transport protein Fet4. Fet4 is not only zinc specific but is also involved in iron and copper uptake (Waters and Eide, 2002). This protein, together with Zrt1 and Zrt2, belongs to the ZIP family of metal ion transporters. *ZRT1* and *ZRT2* are the genes encoding for Zrt1 and Zrt2, respectively and *FET4* for the transporter Fet4. The *ZAP1* gene, through its encoded activator protein Zap1, strictly controls the functioning of these genes and its own functioning, thanks to a mechanism of auto-regulation. Any controlled gene has one or more zinc responsive elements (ZRE) in their promoters. Zap1 binds to ZRE to maximise the expression of the target genes. Zinc levels play an important role in this mechanism. For example, under severe zinc deficiency, Zap1 is produced at high levels and the affinity for the *ZRT1* is kept very high. The affinity for *ZRT2* is reduced, maybe because other proteins bind with the promoter of this gene, which consequently becomes unavailable for Zap1 (Eide, 2003). This results in up-regulation of *ZRT1* and down-regulation of *ZRT2* expression.

At the post-translational level, zinc uptake is controlled by a mechanism of degradation of the protein Zrt1. At high zinc concentrations, Zrt1 is transferred to the vacuole and inactivated through a mechanism of endocytosis and proteolysis (Gitan et al., 1998). A summary of the zinc transporters is reported in Table 12.4.

Their localisation is depicted in Fig. 12.1 Once zinc is taken up into the cell, this metal is utilised for metabolic functions in the cytoplasm and in several organelles including mitochondria and endoplasmic reticulum. If zinc exceeds the requirements needed by the yeast cells, several mechanisms may be activated in order to store zinc until it may be required. The yeast vacuole is known to accumulate several divalent cations, such as Zn^{2+} (White and Gadd, 1987), Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Sr^{2+}, Ni^{2+} and the monovalent cations K^{+}, Li^{+}, and Cs^{+} (Okorokov et al., 1985; Ramsay and Gadd, 1997). Even zinc transport into the vacuole is controlled at the

Table 12.4 Summary of main transporters involved in zinc homeostasis

Transporter protein	Location	Transporter family	Zinc cell status
Zrt1	Plasma membrane	ZIP	Depleted
Zrt2	Plasma membrane	ZIP	Replete
Fet4	Plasma membrane	ZIP	Normal
Zrt3	Vacuole	ZIP	From replete to depleted
Zrc1	Vacuole	CDF	From depleted to replete
Cot1	Vacuole	CDF	From depleted to replete
Msc2	Endoplasmic reticulum	CDF	Normal
Zip7	Golgi apparatus	ZIP	Depleted

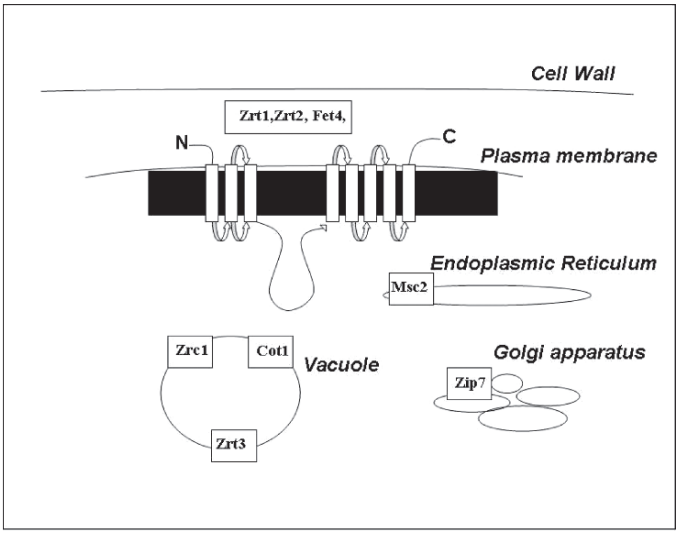

Fig. 12.1 Localisation of the main zinc transporters in the yeast cell. Information from Li and Kaplan (1998), Miyabe et al. (2001), MacDiarmid, et al. (2000, 2002), Waters and Eide (2002), Eide (2003), Ellis et al. (2004), Huang, et al. (2005) and relates to *S. cerevisiae*. See text for further information

transcriptional level. Three transporters are known to be localised in the vacuole: Zrc1 (Miyabe et al., 2001), Cot1 (Li and Kaplan, 1998) and Zrt3 (MacDiarmid et al., 2000, 2002). While the role of Zrc1 and Cot1 is to retain zinc brought in by endocytosis, the role of Zrt3 is to mobilise zinc stores out of the vacuole when zinc replete cells are exposed to zinc limiting conditions. A fourth transporter Msc2, already known to influence zinc homeostasis (Li and Kaplan, 2001), has been recently localised in the endoplasmic reticulum where it is involved in supplying zinc to this compartment (Ellis et al., 2004). The transporter Zip7, associated with the Golgi apparatus, has been found to have a role in zinc homeostasis, transporting zinc from the Golgi apparatus to the cytoplasm when yeast cells are grown in zinc depleted medium (Huang et al., 2005). When zinc starved cells are suddenly exposed to high zinc concentrations, a "zinc-shock" occurs (MacDiarmid et al., 2003). Zinc rapidly crosses the plasma membrane to accumulate in the cytoplasm first and then into the vacuole.

As a consequence, the transcription of genes encoding for the plasma membrane transporters is down regulated and the Zrt1 protein is inactivated by endocytosis. This system prevents excessive zinc uptake. At the same time, the genes encoding for the vacuolar transporters are up regulated. Studies with mutants have given very interesting information on the way zinc transport mechanisms act during zinc shock.

In *S. cerevisiae*, other systems have been described to play a role in zinc homeostasis. For example, Devirgiliis et al. (2004) have used specific fluorophores to detect zinc localisation and have been able to observe very small vesicles in the cytoplasmic periphery they named yeast *zincosomes*. These vesicles appear very

rapidly when zinc depleted cells are transferred to a medium containing micromolar zinc concentrations and disappear very slowly when the same cells are placed in zinc-depleted medium. They may play a role as transporters of zinc ions to the sites quickly requiring this metal and to the vacuole to storage. In *S. cerevisiae* there is no evidence of metallothioneins acting in zinc sequestration (Palmiter, 1998) although in *Schizosaccharomyces pombe* the gene *zym1*, encoding for one metallothionein, may play role in zinc tolerance and zinc storage (Borrelly et al., 2002).

The high capacity of *S. cerevisiae* to sequester heavy metals may be employed in bioremediation to clean up polluted industrial sites (Volesky and May-Phillips, 1995). A more comprehensive study has been recently published by Mapolelo et al. (2005) who studied the heavy metal uptake properties of various strains of *S. cerevisiae* in dam water, stream water, treated wastewater and industrial effluents. This potential of yeasts as tools for decontamination, including zinc, would be another novel application for this most useful microorganism.

12.3 Analysis

In industrial fermentation media, several divalent cations such as zinc, magnesium, manganese, calcium are known to play important roles in yeast cell physiology and in dictating the progress of fermentation. Zinc media concentrations in the range 0.25–0.50 ppm have been reported to be optimal for cell growth and 1–2 ppm for glycolysis (Jones and Greenfield, 1984; De Nicola, 2006). The concentration of this element is variable in different industrial media such as wine must, cane molasses or brewer's wort. Part of zinc may also not be available (bio-available) to yeast cells because it is bound to some compounds present in the media, for example, hop acids. Sometimes zinc may be present as zinc oxide, depending on the pH and the level of oxidation of the medium (Pourbaix, 1963). Zinc in this form is not taken up by yeast cells.

In the wine-making industry, for example, zinc concentration in grape wine must be between 0.04 and 7.8 ppm and the average is 0.90 ppm (Cabanis and Flanzy, 1998). Usually in winemaking such zinc levels are satisfactory for the progress of fermentation. Average zinc concentration in beet molasses is 40 ppm while in sugar cane molasses is 13 ppm (Curtin, 1973) with optimal concentration for ethanol production in the range 1–5 ppm. In brewing, the mineral content in malt is usually 2–3% of the dry weight depending on the agronomic conditions and the trace element content of soil (zinc, manganese, iron, calcium and copper). During malt wort preparation, a minor amount of metals is extracted, for example, less than 5% of zinc, iron and strontium (Jacobsen and Lie, 1979; Jacobsen et al., 1982). Jacobsen and Lie (1977) have analysed the degree of extraction of various elements and found an interesting correlation between zinc concentration in wort and the peptide and amino acid level, concluding that the cysteine groups may be active in the process of zinc sequestration. Other compounds are also known to take part to the process of ion chelation: phenols (Fe), α-amylase (Ca) and phytic acid (Ca, Zn and Fe). The level of these compounds in wort may depend on the technical processes used during its

preparation. Zinc levels usually decrease during the wort mashing, lautering and boiling (Daveloose, 1987; Jacobsen and Lie, 1977; Jacobsen et al., 1982; Kreder, 1999). For example, a concentrated mash has a decreased protein level and consequently a lower level of zinc-binding compounds. During lautering or filtering of the mash, some ions may be washed out. In this respect, zinc ions become complexed in precipitated trub during the wort boiling and cooling and may become unavailable to yeast cells. Zinc binding with trub is loose. Kreder (1999) has demonstrated that keeping part of the trub into the wort during fermentation is beneficial, because zinc ions are slowly released into the medium. Since the trub has other nutritional and physical qualities, it is also needed for proper yeast growth and fermentation performance. As a result, zinc levels in malt wort may be below 0.1 ppm.

Zinc deficiencies during fermentation may cause serious problems in terms of diminished yeast cell growth and reduced ethanol production. Generally speaking, when zinc levels fall below around 0.1 ppm, then fermentation problems may be encountered (Helin and Slaughter, 1977; Jacobsen and Volden, 1981; Bromberg et al., 1997) and this may lead to slow and incomplete fermentations, which are termed "sluggish" (Jacobsen et al., 1982).

The excess of zinc in brewer's wort is rare and synergistic effects with manganese have been reported (Helin and Slaughter, 1977; Jones and Greenfield, 1984). It is clear that it is very difficult to determine the limits of zinc tolerance since this may depend on the interaction with other elements. Generally speaking, in the range of 5–50 ppm, several divalent cations influence the uptake of other divalent cations (Helin and Slaughter, 1977). High gravity wort may help yeast cells to tolerate extremely high zinc concentrations up to 1310 ppm. This effect has been demonstrated in some lager yeast strains that keep the same attenuation time and maintain a relatively good viability (about 50%) compared to the viability they would have in normal gravity worts. This may depend on the different tolerance to zinc of various strains as well as the greater buffering capacity of high gravity wort (Rees and Stewart, 1998). In relation to other ions, zinc is known to have the following interaction properties (Jones and Greenfield, 1984): prevention of Cu^{2+} and Cd^{2+} toxicity, enhancement of the beneficial growth properties of Ca^{2+} and Mn^{2+}, Cu^{2+} level stimulation of Zn^{2+} absorption, stimulation of growth in communion with Mn^{2+} and Cu^{2+}.

Glucose, as a source of energy, strongly stimulates zinc uptake. In yeast peptone dextrose medium (YPD) with various sugar concentrations (Hall, 2001), it was shown that industrial yeasts growing and fermenting in high sugar concentration (5%), accumulated higher levels of zinc compared to cells growing and respiring in low sugar concentration (0.1%). Most likely, high levels of glucose provided enhanced energy to drive accumulation of intracellular zinc. The stimulatory effect of glucose on zinc uptake was inhibited by the following metabolic inhibitors: antimycin A (Mowll and Gadd, 1983), potassium cyanide, 2,4-dinitrophenol (DNP), DCCD, diethylstilboestrol (DES) and 2-deoxyglucose (White and Gadd, 1987). Although these compounds showed a clear metabolic dependent mechanism of zinc uptake, unfortunately they may interfere with various metabolic pathways or may have non specific effects. Therefore, they do not prove any specific mechanism implicated in zinc uptake. In *Candida utilis,* zinc uptake was strongly impaired in starved cells by the presence of

the protein synthesis inhibitor cycloheximide (Failla et al., 1976). This demonstrated that *de novo* proteins were involved in zinc uptake.

Zinc uptake exhibits Michaelis-Menten kinetics. A variety of dissociation constants have been found for yeasts (Borst-Pauwels, 1981). This variation has been found even within the same yeast species and may be attributed to different techniques used to measure zinc or to the presence in the medium of buffers or complexing anions. More precisely for *S. cerevisiae*, two systems have been found: one high affinity system with K_d = 10 nmol, active in zinc-limited cells (Zhao and Eide, 1996a) and one low affinity system with K_d = 100 nmol, active in zinc-replete cells (Zhao and Eide, 1996b).

Growth medium pH may affect zinc uptake suggesting some sort of proton symport mechanism. In *C. utilis*, zinc uptake rate decreased while pH increased from 4.8 to 8.2 (Failla et al., 1976; Failla and Weinberg, 1977). This may have also depended on the reduced bioavailability of zinc which may form complexes with polyphosphates, carbonates and hydroxides at pH values over 6.8 (Ross, 1994) or the formation of zinc oxide, which depends on the level of oxidation of the medium (Pourbaix, 1963). Uptake of heavy metals like cadmium is usually accompanied by release of K^+ (Norris and Kelly, 1977; Lichko et al., 1982; Mowll and Gadd, 1983). This phenomenon is not present in *S. cerevisiae* during zinc uptake, when zinc extra-cellular levels are at normal concentrations but it can be observed at very high zinc concentrations (Mowll and Gadd, 1983).

Figure 12.2 shows typical zinc uptake kinetics by a distiller's yeast strain (*S. cerevisiae*) cultivated in malt wort. Zinc is accumulated in most yeast strains within the first hour of growth with cells reaching the maximum mean zinc cell

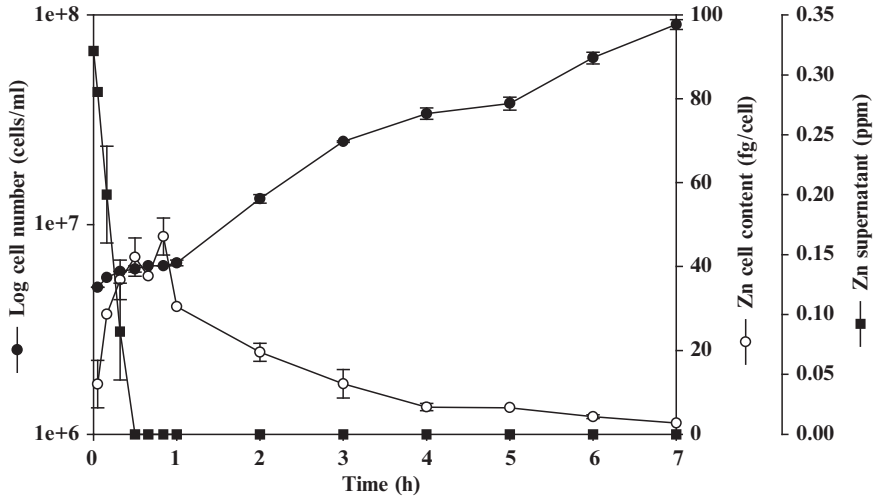

Fig. 12.2 Zinc uptake by a distiller's yeast strain of *S. cerevisiae* in industrial media. A distiller's yeast strain was cultivated in shake flasks in malt wort (zinc at 0.32 ppm), at 25°C for 24 h. Figure represents cell growth, zinc cell content and zinc supernatant concentrations in the first 7 h of growth (De Nicola, 2006)

content. After cell division, the average zinc gradually decreases (De Nicola, 2006). In the same study, zinc uptake was unaffected by calcium concentrations in the range 16–76 ppm, as well as various sources of zinc salts (Zn sulphate, Zn acetate and Zn chloride). In brewer's wort, zinc is occasionally present at sub-optimal concentrations for growth and fermentation. Zinc uptake studies by industrial yeast strains have been carried out by Mochaba et al. (1996) in small-scale 2 l fermenters and in 60 l fermenters, at 11°C, using malt wort with zinc levels up to 0.75 ppm. They found an immediate cellular zinc increase in the first hours after inoculation, followed by zinc level fluctuations in the remaining days of fermentation.

Zinc movement within the yeast cell occurs through various barriers. The first obstacle is represented by the cell wall. The cell wall constitutes 15–25% of the dry weight of the cell and it is essentially composed of a highly dynamic structure that is responsible for protecting the cell from rapid changes in external osmotic potential (Levin, 2005). Specific binding regions for metal ions are present on the cell wall which may change depending on the yeast strain or the media in use (Engl and Kunz, 1995). The zinc specific binding in the cell wall is the sulphydryl group of cysteine located in the mannoprotein fraction (Brady and Duncan, 1994; Mochaba et al., 1996). An energy dependent metabolism acting during zinc uptake has been reported in several studies. For example, White and Gadd (1987) determined the proportion of compartmented zinc within the cell: 56% in the soluble vacuolar fraction, 39% bound to insoluble components, probably the cell wall, and only 5% was found in the cytosol. An ATP-dependent zinc uptake system whose properties were consistent with a Zn^{2+}/H^{+} antiport was localised in isolated yeast vacuoles (White and Gadd, 1987). Lichko et al. (1982) have previously reported that intracellular K^{+} is concentrated in the vacuoles and that yeast cells may lose up to 30% of vacuolar K^{+} as they accumulate divalent cations. As previously mentioned, this phenomenon was only observed in *S. cerevisiae* when extra-cellular zinc levels were very high. In brewing experiments conducted in 60 l fermenters, Mochaba et al. (1996) have analysed yeast cellular zinc localisation throughout fermentation, finding high zinc concentrations in the mannoprotein and in the intracellular fractions. Further, at the end of the fermentation, zinc ions were translocated to the outer surface presumably to aid in maltotriose uptake. De Nicola (2006) showed that a low percentage of total cell associated zinc, in the range 5–30%, was bound to the cell wall.

In yeast cells killed by heat treatment, zinc uptake was minimal and probably reflected binding to the cell wall material. This association was loose and zinc was readily released back to the medium. It was concluded by De Nicola (2006) that biosorption by bond formation between zinc and the cell wall was not relevant during zinc uptake and an active translocation mechanism was likely. Free zinc was not visualised in the proximity of cell walls of yeast cells by using a specific zinc probe, probe Fura Zin-1 (MacDiarmid et al., 2003). Instead, zinc ions were quickly translocated into the yeast cell and stored into the vacuole (Fig. 12.3).

The yeast vacuole is a reservoir for several nutrients (Lichko et al., 1982) and vacuolar polyphosphate bodies, known as volutin granules, have already been shown to be associated with zinc (Jones and Gadd, 1990). Zinc is probably stored in this organelle to be mobilised under zinc-limiting conditions or to be directed to

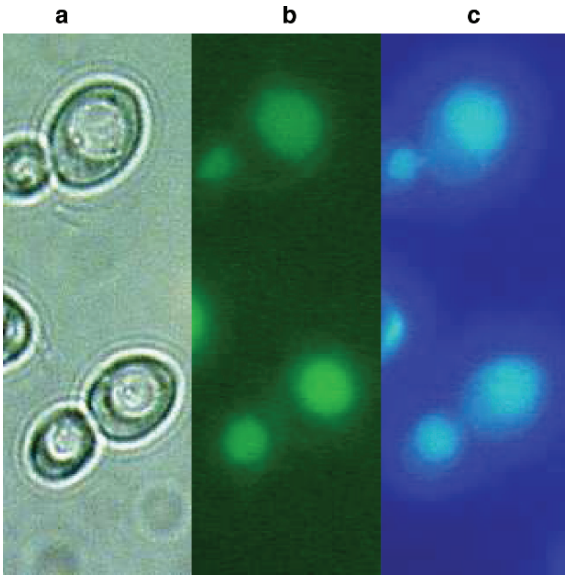

Fig. 12.3 Free zinc ions and vacuole localisation in yeast cells. Yeast cells of a brewing yeast strain were cultured in EMM3 medium (Zn at 10 ppm) and stained with both Fluo-Zn3 for zinc visualisation (**b**) and Cell-Tracker B for vacuole visualisation (**c**). Pictures were taken using a Leica microscope under bright field (**a**), using a green filter (**b**) and a blue filter (**c**) (De Nicola, 2006)

the daughter cell during budding and cell division (Eide, 2003). Zinc storage in the vacuole regulates the mechanisms of zinc distribution among the cellular population since zinc may be inherited by daughter cells during budding. The vacuole is actively divided between mother and daughter cells. This inheritance initiates early in the cell cycle and ends in G_2, just prior to nuclear migration (Weisman, 2003). A portion of the vacuole extends into the emerging bud enabling continued exchange of vacuole contents and therefore zinc between mother and daughter vacuoles. Such a mechanism could generate heterogeneities in yeast populations with respect to their zinc status especially under conditions where zinc uptake from the medium was faster than the rate of cell division. In alcohol production processes by fermentation, a major effort is made to keep yeast cells under appropriate physical, chemical and nutrient conditions in order to minimise cell growth and stimulate metabolic pathways leading to optimal alcohol production. In laboratory conditions with laboratory strains, however, growing cells produce alcohol 33 times faster than non-growing cells (Ingledew, 1999). Compromise efforts are made to keep yeasts under conditions that do not lead to low growth rates or to cell death. Control and management of micronutrient levels, such as zinc, are vital for the correct progress of fermentation and to encourage a predominantly fermentative, rather than respiratory, mode of metabolism in the yeast strains employed in alcohol production.

Optimal zinc concentrations reduce the attenuation time considerably compared with zinc-limited conditions (Skanks et al., 1997). Sometimes the specific fermentation rate changed only after several successive fermentations (Bromberg et al., 1997).

In the brewing industry, due to technological processes employed to prepare malt wort that may lead to lack of zinc in the medium, zinc levels may be determined, throughout the fermentation process and in the final beer. Zinc supplements may be employed when zinc levels are very low to avoid sluggish and incomplete fermentations. Moreover, although present, some of the zinc may be not bioavailable to the yeast cells.

In the literature, various levels of zinc have been proposed for optimal fermentation performance in brewing: between 0.1 and 0.15 ppm (Bromberg et al., 1997), 0.18 ppm (Helin and Slaughter, 1977) and between 0.05 and 0.30 ppm (Jacobsen et al., 1981). Precise zinc cellular requirements are obviously yeast strain dependent (Rees and Stewart, 1998) but the malt wort quality and the fermentation conditions also cause variations in zinc demand by yeast cells.

Taylor and Orton (1973) demonstrated zinc to be an inhibitor of flocculation only at very high concentrations, above 6500 ppm and pH 7.5, concluding that the role of zinc in flocculation is unimportant in brewing as these conditions are unrealistic. An experiment *in vitro* showed that in terms of flocculation, lager strains are not affected by the presence of zinc, while some ale strains flocculated when the zinc concentration reached the so-called saturation point at 2.6 ppm (Raspor et al., 1990). Above this level, de-flocculation occurred. This phenomenon may be explained by differences in the cell surface structures and may be employed to differentiate between ale and lager flocculating strains. In recent years, a study on yeast propagation demonstrated that only the addition of 0.30 ppm of zinc may have a consistent effect on flocculation (Wackerbauer et al., 2004). During fermentation, zinc may interact with other cations such as calcium, involved in the flocculation process, thus promoting this phenomenon. The hypothesis that zinc is involved in flocculation is also supported by zinc limitation studies in aerobic continuous culture (De Nicola, 2006). The gene *MUC1*, was found 50 and 30 times more up-regulated in zinc-limitation conditions compared to, respectively, carbon and nitrogen limitation albeit in a non-flocculent laboratory strain. This gene is involved in yeast flocculation (Guo et al., 2000) through the synthesis of the protein Flo11p, member of the second group of the Flo family proteins, uniformly localised around the cell surface of the haploid cells but only in few surface areas of diploid yeast cells. Zinc depleted cells were found to form clusters (Obata et al., 1996).

Positive influences of zinc on the synthesis of esters and higher alcohols as well as the decrease of acetaldehyde levels have been widely documented (Hodgson and Moir, 1990; Seaton et al., 1990; Skanks et al., 1997; Quilter et al., 2003). With regard to production of volatiles and higher alcohols by yeast, Skanks et al. (1997) have shown that elevated zinc concentrations increased the levels of higher alcohols and esters but reduced acetaldehyde levels. Lager yeast cells preconditioned with zinc produce distillates with higher alcohols (2-methyl-1-buthanol, 3-methyl-1-buthanol and isobutanol), esters and aldehydes and without iso-amyl acetate (Melville, 2003). Although addition of 0.5 ppm of zinc increased volatile organic compounds, it may also increase the concentration of medium chain fatty acids (MCFA) which can cause unwanted soapy, fatty and rancid taste (Villa et al., 1999) and reduce foam stability (Lange et al., 2004).

In the bioethanol (fuel alcohol) industry, zinc levels in beet molasses are much higher than in grape must or brewers wort and zinc deficiencies are rare. In the wine-making industry, as in the bioethanol industry, it is unusual to carry out analyses of zinc levels since the metal concentration in grape must is usually deemed satisfactory for the progress of the fermentation. In winemaking, research on zinc interaction with yeasts is mostly performed by companies involved in the commercialisation of yeast supplements. Commercial preparations, based on nutrients such as organic and inorganic nitrogen, fatty acids, sterols, vitamins and mineral salts, including zinc, are usually added during yeast rehydration and propagation to ensure that yeast cells are supplemented with satisfactory levels of nutrients prior to cell inoculation. These actions aim to guarantee that yeast cells are healthy and active from the early stages of the fermentation. Unfortunately, in wine making, most of the research has been focused only on agronomic studies of the vineyard soils or grape fertilizers based on zinc (Christensen, 1980; Christensen and Jensen, 1978) and there are very few studies available on the influence of zinc with wine yeast strains. In a wine making simulated experiment, zinc uptake patterns by a wine yeast strain showed high zinc uptake in the first two days of fermentation, followed by release or leakage back into the medium after 48 h (Fig. 12.4).

Most likely when ethanol levels were above 6% (Fig. 12.5), yeast cells continuously exposed to such a relatively high concentration may have had altered plasma membrane permeability, resulting in the loss of zinc ions. In this regard, (Learmonth and Gratton, 2002) reported that ethanol stress increased the membrane fluidity of the yeast cells. Yeast growth of the two wine strains was not dramatically affected by the zinc concentrations of this study. At the end of the fermentation, the yeast

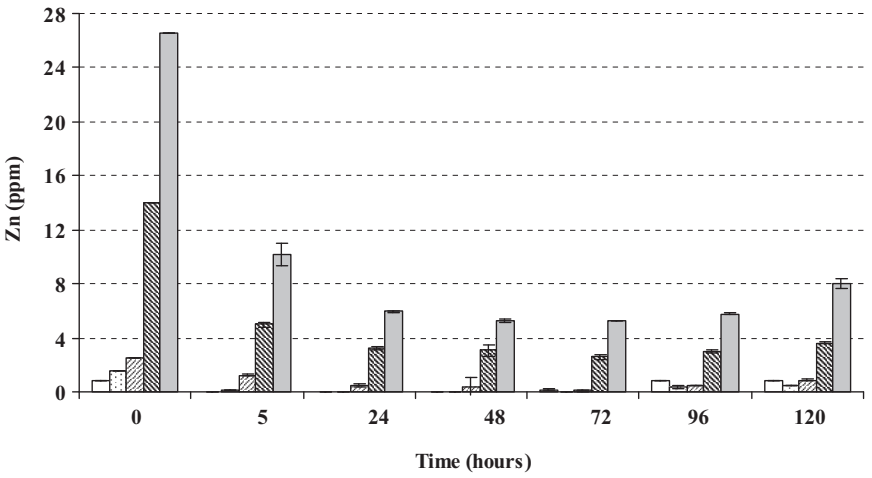

Fig. 12.4 Zinc uptake during grape juice fermentation by a wine yeast strain. Yeast cells of a wine strain were inoculated in 1 l Imhoff conical vessels, in grape juice with variable zinc levels. Fermentation was carried out at 25°C for 120 h (5 days). Zn residual levels in supernatants were analysed throughout fermentation by atomic absorbance spectrophotometry. Concentrations of zinc tested were as follows: 0.9 (□), 1.5 (□), 2.5 (▨), 14 (▩), 26.5 (▤) ppm

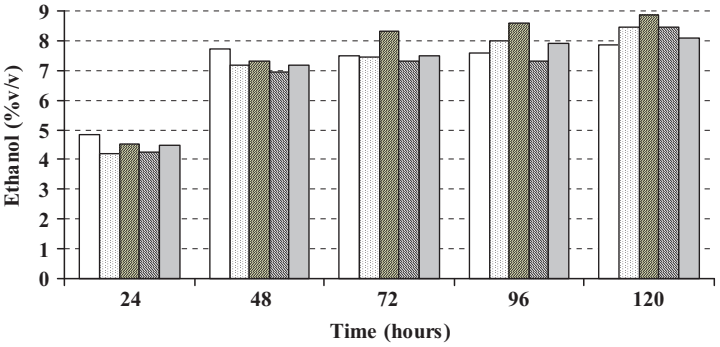

Fig. 12.5 Influence of zinc on ethanol production during wine fermentation. Conditions employed were as described in Figure 12.4. Fermentation performance of the wine strain was evaluated by analysing the ethanol produced daily. Concentrations of zinc tested were as follows: 0.9 (□), 1.5 (▨), 2.5 (■), 14 (■), 26.5 (▤) ppm

cone formed at the bottom of the fermenters was approximately of the same size in all the fermenters with the exception of 0.9 ppm of zinc, having a slower growth rate and lower final biomass. Zinc concentrations of 2.5 ppm accelerated the flocculation process of the yeast cells and gave a slightly higher final biomass. The viability was not affected by any of the zinc concentrations tested and at the end of the fermentation was 84%. Various zinc concentrations tested did not dramatically affect the fermentation rate. Higher ethanol production was determined at 2.5 ppm of zinc (Fig. 12.5). This result, together with a higher growth rate, appeared to indicate that this concentration had a general beneficial effect on this strain.

The physico-chemical properties of the environment can strongly influence the physiology of yeast cells, affecting both viability and vitality. In the fermentation alcohol industries, at any stage of the process, yeast cells can encounter a variety of stresses which can have a significant impact on cell growth and fermentation performance. Yeasts respond to stresses by changing their metabolic activities and by adapting their physiology in order to protect the cellular components from damage, to survive and to recover when optimal environmental conditions are re-established. The understanding and the correct management of the interactions between environmental stresses and yeasts is fundamental for brewers, wine-makers and distillers who want to optimise cell growth, viability and fermentation performance. In industrial processes, the stresses most commonly encountered are chemical (e.g. ethanol and other metabolite toxicity, oxidative, anaerobiosis, pH changes and acid wash treatments, nutrient limitation/starvation, metal ion toxicity/limitation), physical (e.g. osmostress, changes in temperatures, dehydration, rehydration, mechanical sheer, hydrostatic pressure) and biological (e.g. cell aging, genotypic changes or competition from other organisms) (Walker, 1998).

With regard to chemical and physical stresses encountered during industrial fermentation processes, magnesium is already known to protect cells from osmostress (D'Amore et al., 1988), ethanol (Birch and Walker, 2000), toxic metals (Blackwell

et al., 1997; Karamushka and Gadd, 1994) and the oxidant effect of free radicals (Szantay, 1995). As a consequence, Mg-enriched cells retain viability and vitality under stress. Magnesium ions may protect the integrity of cell plasma membrane during stress insults by stabilising its lipid bilayer and decreasing its membrane fluidity (Walker, 1999). Although an antioxidant role has been ascribed to zinc (Truong-Tran et al., 2001) and charge-neutralisation properties of membrane phospholipids have been assigned to this metal ion (Binder et al., 2001), to date, no information has been provided on how this metal may protect yeast cells from industrial stresses.

Experiments carried out with a brewing strain of *S. cerevisiae* under temperature, ethanol and combined temperature/ethanol stresses (Fig. 12.6), showed a significant release of zinc ions accompanied by loss of culture viability. After 1 and 5 h exposure, cells retained zinc and viability following heat or ethanol stress, although the combination of these insults had a synergistic dramatic effect on the viability of the cells which were all dead after only one hour from the beginning of the stress. After 24 h exposure, all cells died, although some residual zinc was associated with the dead cell mass probably due to tight cell wall biosorption (de Nicola, 2006).

The influence of ethanol on membrane permeability has already been observed by Learmonth and Gratton (2002). In that study, the authors used the probe Laurdan to determine the Generalised Polarization (GP) parameter, as an index of cellular

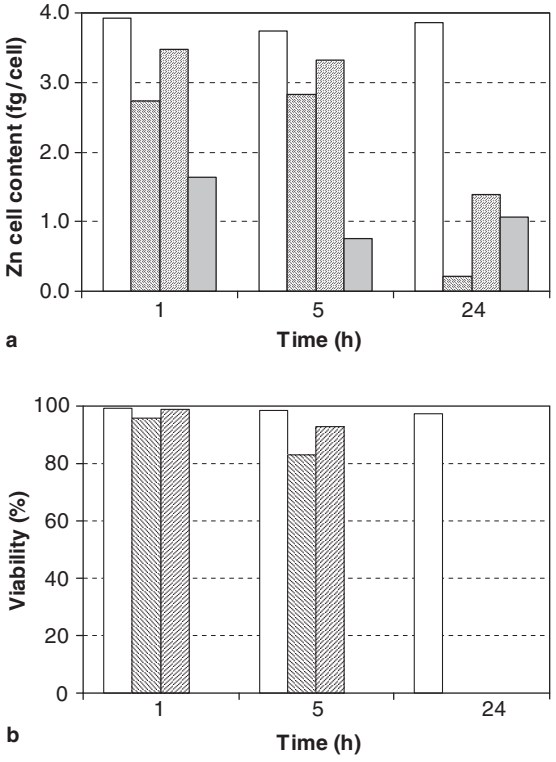

Fig. 12.6 Influence of temperature and ethanol stress on intracellular zinc in yeast. A lager brewing yeast strain was cultured in malt wort, resuspended in distilled water and stressed at 45 °C or in ethanol for 24 h. Zinc cell content (**a**) and cell viability (**b**) were analysed. Stress conditions tested were as follows: control in distilled water at 25 °C (□), temperature 45 °C (▩), ethanol 20% at 25 °C (v/v) (▩), temperature 45°C + ethanol 20% (v/v) (▩)

membrane fluidity. It is plausible that highest membrane fluidities at high ethanol concentrations influenced the permeability of the tonoplast, the vacuolar membrane. Since most cellular zinc is stored in the vacuole when accumulated in large amounts (Fig. 12.3 and MacDiarmid et al., 2003), changes in fluidity of the tonoplast may have determined zinc release. This theory is also supported by recent studies on the influence of zinc on phospholipid synthesis (Iwanyshyn et al., 2004; Han et al., 2005; Carman, 2005). Zinc depletion was found to highly affect the *DPP1* gene encoding DGPP phosphatase (Lyons et al., 2000) an enzyme associated with the vacuolar membrane and involved in the synthesis of phosphatidate and diacylglycerol. Lack of zinc reduced these two compounds and phosphatidylserine, increasing the production of phosphatidylinositol (Han et al., 2005). In general, the mechanisms by which zinc influences phospholipid synthesis appear to be complex and associated not only with the *DPP1* gene.

As for ethanol, temperature is known to influence the plasma membrane by increasing its fluidity (Learmonth and Gratton, 2002). The yeast plasma membrane has been recently suggested to be the primary cellular compartment controlling heat stress tolerance (Guyot et al., 2005). Interestingly, magnesium and zinc cell contents as well as ATP levels were affected with similar patterns, suggesting that the phenomenon of compound release was more general and not only specific for zinc (De Nicola, 2006). There is evidence that ethanol and temperature stresses influenced plasma membrane fluidity allowing a general release of metal ions. It is conceivable that a suitable level of zinc may stabilise the plasma membrane and help yeast cells to alleviate the effects of some environmental stresses.

Several genes have been determined to be up- and down regulated during zinc deficiency stress. For example, Lyons et al. (2000) have determined 15% of differentially expressed genes, 46 of which regulated by Zap1. Higgins et al. (2003) have shown that 76 genes were induced or repressed fivefold in a brewing yeast strain grown in conditions of zinc depletion with two genes, *YOR387c* and *YGL258w*, found to be induced 2 h after zinc starvation. Both studies were performed in batch cultures, with continuously changing environmental conditions. Experimental replication and acceptable levels of reproducibility are fundamental conditions in transcriptome analyses as shown by an inter-laboratory study of comparison between chemostat cultures with that in shake-flasks (Piper et al., 2002). Recent studies of zinc limitation in chemostat continuous culture have revealed a more robust set of genes involved during zinc deficiency. Chemostat culture allowed to control specific growth rate and prevented the occurrence of specific-growth-rate-related responses. The transcriptional regulation of this gene set was independent of the oxygen supply (De Nicola et al., 2007).

12.4 Future Perspectives and Conclusions

Zinc interaction with yeast, its transport and utilisation, during brewing and other industrial processes, are phenomena still not completely understood. To date, most of the efforts of the applied research have been concentrated towards the determination

of zinc in industrial media and specifically in malt wort and the discovery of new technologies to improve such availability. Most of the studies on zinc uptake kinetics were merely aimed at describing variation of zinc wort levels during fermentation and in determining the zinc concentration for the best fermentative performance.

With regard to zinc uptake, optimal zinc accumulation occurred using active and healthy cells, with high levels of available sugars as source of energy and at relatively high temperatures. Therefore, zinc supplementations to industrial media may be appropriate at the moment of inoculum additions and not during yeast slurry cold storage or acid-washing.

Since no major differences were found in terms of zinc uptake using different zinc salts, it is therefore advisable to use an economically convenient salt as a source of supplementary zinc, such as zinc sulphate. Although high zinc concentrations may delay fermentation rates, they may increase the synthesis of some esters and higher alcohols, altering taste and aroma of fermented alcoholic beverages. Further studies are required to elucidate the role of zinc in these pathways. The range of zinc levels in the media reported in the literature are merely indicative as conditions may change in relation to the yeast strain used, media composition and environmental characteristics. Industries employing yeasts in fermentation processes would be advised to perform tests aimed at verifying the optimal zinc concentration for their strains and processes employed. The practice to pre-enrich yeast cells with zinc may be implemented as demonstrated previously for magnesium (Walker and Smith, 1999). This may be very useful for industries adhering to food purity laws such as the German Reinheitsgebot which stipulates that the brewing of beer must only employ malted barley, hops, water and yeast. Experimental studies in yeast propagation vessels may give useful indications on how to optimise intracellular zinc accumulation. During the fermentation process, at any stage, brewers should control the viable status of their yeast cells and avoid any undue stresses. These may lead to diminished fermentation performance and loss of viability, as well as cellular zinc and magnesium. Yeast cells may concomitantly encounter more than a single stress and it would be useful to determine the degree of zinc release during a combination of more stresses at the same time, as shown for combined temperature/ethanol stresses in this paper. For example, in brewing, after a fermentation process and prior to pitching into a subsequent fermentation vessel, yeast cells can be kept in storage tanks at high cell density, starved of nutrients, exposed to high ethanol and low temperature, for variable periods of time. The stress caused by the combination of these factors can influence cell viability as well as zinc cellular homeostasis.

The practice to recycle yeast biomass for several fermentations (as well as in some fuel alcohol processes), should be carried out carefully. A fraction of the yeast cone is usually discarded. Heterogeneity in age and fermentation performance was found in cells from different yeast crop fractions (Powell et al., 2004) and mother cells may retain high levels of vacuolar zinc. The combination of these two conditions may cause the loss of a part of the yeast biomass representing an important reservoir of zinc. A specific set of over-expressed genes during fermentation process may be utilised as zinc-responsive molecular biomarker to determine zinc cellular

status. This approach may be used in those industries using yeasts, as a modern tool to determine the condition of zinc cellular deficiency and to gain deeper insight into metabolic responses to zinc bioavailability. Although some potential genes have already been identified, further research is required to confirm these findings, using approaches with higher levels of reproducibility, for example, in Zn-limited chemostat continuous culture. This has been the focus of our recent research efforts (De Nicola et al., 2007).

Further investigations are needed on the interaction of zinc with other metal ions such as Mg, Ca, Mn and Cu. For example, during industrial processes, zinc may exert a synergistic effect with Mg on plasma membrane stability. It is conceivable that an optimal level of intracellular zinc is required for plasma membrane stability during chemical and physical stresses. In addition, zinc may alleviate the inhibitory effects produced by ions such as Cu. Industries having problems with fermentation due to high residual levels of copper, may conceivably employ supplementary zinc as an efficient antagonist.

This study has highlighted the need to improve our fundamental understanding of yeast nutrition and cell physiology that will ultimately prove to be of practical benefit to yeast-based industries.

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Chapter 13
Glutathione Production in Yeast

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Contents

Abstract Glutathione, γ-glutamyl-cysteinyl-glycine, is the most abundant non-protein thiol found in almost all eukaryotic cells (and in some prokaryotes). The tripeptide, which is synthesized non-ribosomally by the consecutive action of two soluble enzymes, is needed for carrying out numerous functions in the cell, most important of which is the maintenance of the redox buffer. The cycle of glutathione biosynthesis and degradation forms part of the γ-glutamyl cycle in most organisms although the latter half of the pathway has not been demonstrated in yeasts. Our current understanding of how glutathione levels are controlled at different levels in the cell is described. Several different routes and processes have been attempted to increase commercial production of glutathione using both yeast and bacteria. In this article we discuss the history of glutathione production in

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yeast. The current bottlenecks for increased glutathione production are presented based on our current understanding of the regulation of glutathione homeostasis, and possible strategies for overcoming these limitations for further enhancing and improving glutathione production are discussed.

Keywords Glutathione, sulphur, yeast, biosynthesis, rate-limiting steps

13.1 Introduction

Glutathione (GSH), γ-L-glutamyl-L-cysteinyl-glycine, is the most abundant non-protein thiol present in almost all eukaryotes, with the exception of a few parasitic protozoans such as *Entamoeba histolytica* and *Giardia duodenalis* that lack mitochondria. Glutathione is also present in some bacteria such as the cyanobacteria and the proteobacteria, and a few gram-positive bacteria. It appears to be absent in the archaebacteria (Fahey and Sundquist, 1991).

Glutathione was first discovered in ethanolic extracts of yeast in 1888 by J. de Rey-Pailhade, in Montpellier, France, as a substance having the ability to hydrogenate sulphur (Pailhade, 1888; Meister, 1988). It was named as 'philothion' based on its reactivity towards sulphur. Many years later F.G. Hopkins crystallized the substance from yeast extracts and initially characterized it as a dipeptide of glutamic acid and cysteine (Hopkins, 1921). Based on this structure, Hopkins suggested the name 'glutathione'. However, subsequent work of Hunter and Eagles questioned the dipeptide structure (Hunter and Eagles, 1927), and Hopkins used improved purification techniques to establish the fact that glutathione was a tripeptide containing cysteine, glutamic acid and glycine (Hopkins, 1929). Pirie and Pinhey (1929) deduced the tripeptide sequence based on titration data and this was later confirmed by the work of Harington and Mead (Harington and Mead, 1935).

Glutathione plays several important roles in the cell. It functions as the major cellular redox buffer that is a consequence of its high concentrations in the cell (1–10 mM), its low redox potential (–240 mV), and its stability that results from the unusual γ-glutamyl-cysteine bond. Intracellular glutathione cycles between the reduced (GSH) and the oxidized forms (GSSG), thereby generating a redox couple that determines and regulates the redox status of the cell (Meister and Anderson, 1983; Schafer and Buettner, 2001; Ganguly et al., 2003; Ostergaard et al., 2004). In addition to its central role as the cellular redox buffer, glutathione plays several roles that includes the detoxification of metals and free radicals, in sulphur storage, in gene regulation and in signaling (Sies, 1999).

Glutathione is essential for the growth of eukaryotic cells. In yeasts, for example, absence of the first enzyme involved in glutathione biosynthesis leads to glutathione auxotrophy (Grant et al., 1996; Chaudhuri et al., 1997), while in mouse, knockout of the first enzyme involved in glutathione biosynthesis leads to embryonic lethality (Dalton et al., 2000). Apart from it's essentiality for growth, the levels of glutathione

and the ratios of the reduced to oxidized glutathione levels are crucial for the proper functioning of the cells especially under stress conditions. In humans, many diseases have now been correlated with decreased levels of glutathione (Wu et al., 2004; Reid and Jahoor, 2001).

13.2 Uses of Glutathione and Glutathione Analogues

One of the major uses of glutathione is in the replenishment of glutathione in glutathione-deficient disease states that are observed in cystic fibrosis, liver diseases, Parkinson's disease, Alzheimer's disease, surgical trauma, age-related disorders and HIV infection. Owing to the poor absorption of glutathione, many formulations, such as liposomal glutathione (Cooke and Drury, 2005), glutathione in association with either ascorbic acid, α-lipoic acid, or with selenium are being explored. Glutathione analogues and precursors are also being developed for circumventing the problem of poor absorption and transport. N-acetyl cysteine is an acetylated analogue of cysteine that can cross the cell membrane, to be rapidly deacetylated inside the cell, allowing the cysteine released to augment GSH synthesis. Other precursors include the compound L-2-oxothiazolidine-4-carboxylate which is a substrate for the 5-oxoprolinase that catalyze it conversion to s-carboxy cysteine, which after decarboxylation becomes incorporated into GSH. GSH deficiency can also be tackled by glutathione esters like GSH monoethyl esters, synthetic compounds prepared by linking the glycyl end of GSH into ester bond. Due to hydrophobicity these esters can cross the membrane easily and action of esterase will release the free GSH inside the cell. An important GSH precursors is γ-glutamylcysteine.

Several glutathione analogues are being developed as inhibitors of glutathione-S-transferases (GST) in anticancer drugs (Schultz et al., 1997; Rosen et al., 2004). The GSTs play a major role in resistance against chemotherapeutic treatment in tumors and the resistance is directly correlated with the over expression of GSTs.

Glutathione analogues are also being developed as inhibitors of glutathionylspermidine synthetase (GspS) an enzyme involved in the synthesis of trypanothione [*N* 1, *N* 8-bis- (glutathionyl)spermidine], the intramolecular thiol that functions in place of glutathione in *Trypanosoma* spp. and *Leishmania* spp (Amssoms et al., 2002a; Amssoms et al., 2002b). Phosphonates, phosphonamidates and phosphinates derived from glutathione like tripeptides are inhibitors of GspS. Diesters based on N-benzyloxycarbonyl-S-(2,4 dinitrophenyl) GSH containing linear or branched alcohols are also potent inhibitors of this enzyme. Due to their hydrophobicity these molecules can cross the membrane and are hydrolyzed in the cell to the active compounds (Daunes and D'Silva, 2002).

Other uses of glutathione are in various cosmetically acceptable components like emulsifiers, oily substances, and moisturizers primarily to enhance the whitening effect on skin, improving the effect of the cosmetic formulation. Several different

formulations and compositions including anti-aging composition for skin care and antisun products also seem apparent. Precursors of glutathione, γ-glutamylcysteine have potential use in the flavour industry.

In addition to their use and potential in the health and cosmetic industries, glutathione and its analogues also find extensive use as reagents and buffers. Several modified forms of glutathione can be used as chromatographic affinity ligands, binding reagents and enzyme inhibitors (Castro et al., 1993). Variously modified compounds have different propensities for binding human GSTs of different classes. Another example is biotinylated glutathione (BioGEE), a cell permeant, biotinylated glutathione analog for the *in vivo* detection of stress-induced glutathionylation of proteins.

Although most of the analogues are currently being synthesized chemically, glutathione and its precursors like γ-glutamyl cysteine are obtained through fermentation from yeasts.

13.3 Glutathione Biosynthesis

The biosynthesis of glutathione in the cell occurs through non-ribosomal synthesis of the tripeptide by the sequential action of two soluble enzymes in the cytosol. Both enzymatic reactions are ATP dependent ligations. The first step is the formation of the dipeptide γ-glutamylcysteine (γ-GC) from L-glutamic acid and L-cysteine by the enzyme γ-glutamylcysteine synthetase (Gsh1p/GshA) (also known as glutamate-cysteine ligase, EC 6.3.2.2, GSH1). The second step catalyzed by the enzyme Glutathione synthetase (Gsh2p/GshB) (EC 6.3.2.3, GSHII) involves the ligation of glycine to the C-terminal site of γ-GC to form GSH. Glutathione biosynthesis thus forms part of the γ-glutamyl cycle of glutathione biosynthesis and degradation (Fig. 13.1). *MET3,14, 16, 1, 5, 8, 10*:sulphate-reducing pathway; *MET15*:O-acetylhomoserine sulphydrylase; *MET6*: homocysteine methyl-transferase; *SAM1*, *SAM2*:S-adenosylmethionine synthase; *SAH1*:S-adenosylhomocysteine hydrolase; *STR4*:cytathionine β-synthase; *STR1*:cystathionine γ-lyase; *GSH1*: γ-glutamylcysteine synthetase; *GSH2*:GSH synthetase; *ECM38*: γ-glutamyl transpeptidase; *CGase*: cysteinylglycine dipeptidase; *STR2*:cystathionine γ-synthase; *STR3*:cystathionine b-lyase; APS:5'-adenylylsulphate; PAPS:3'–phospho-5'-adenylylsulphate Glutathione levels in the cell, in addition to being dependant on the levels and activities of the two enzymes that are dedicated for glutathione biosynthesis, are also dependant on the production of ATP and the levels of the substrates, cysteine, glutamate and glycine.

With these substrates and energy requirements, the biosynthetic pathway of glutathione in cell extends beyond the γ-glutamyl cycle to include the glycolytic pathway, the Krebs cycle and sulphur assimilation pathway (Fig. 13.2). These are the pathways which feed the key nutrients – glutamate, cysteine and glycine – and energy molecules into the γ-glutamyl cycle for glutathione biosynthesis.

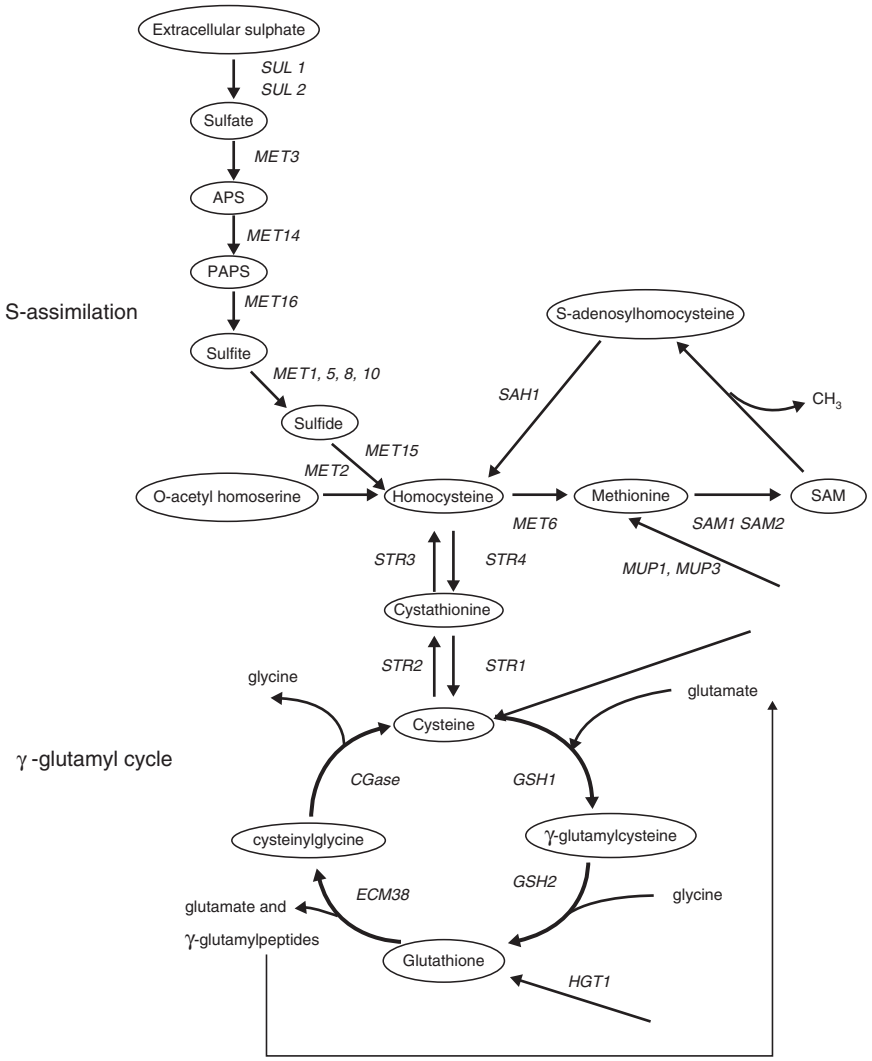

Fig. 13.1 Schematic representation of the sulphur assimilation pathway and γ-glutamyl cycle pathway in *S. cerevisiae*

13.4 Regulation of Glutathione Biosynthesis

Considering the physiological significance and the relative abundance of this tripetide in the cell, the entire process of glutathione biosynthesis and degradation is under tight regulation in the cell. Although yeasts can also transport glutathione from the extracellular medium, the major factor determining glutathione production in the cell is glutathione biosynthesis. Glutathione biosynthesis is regulated at different levels as indicated below:

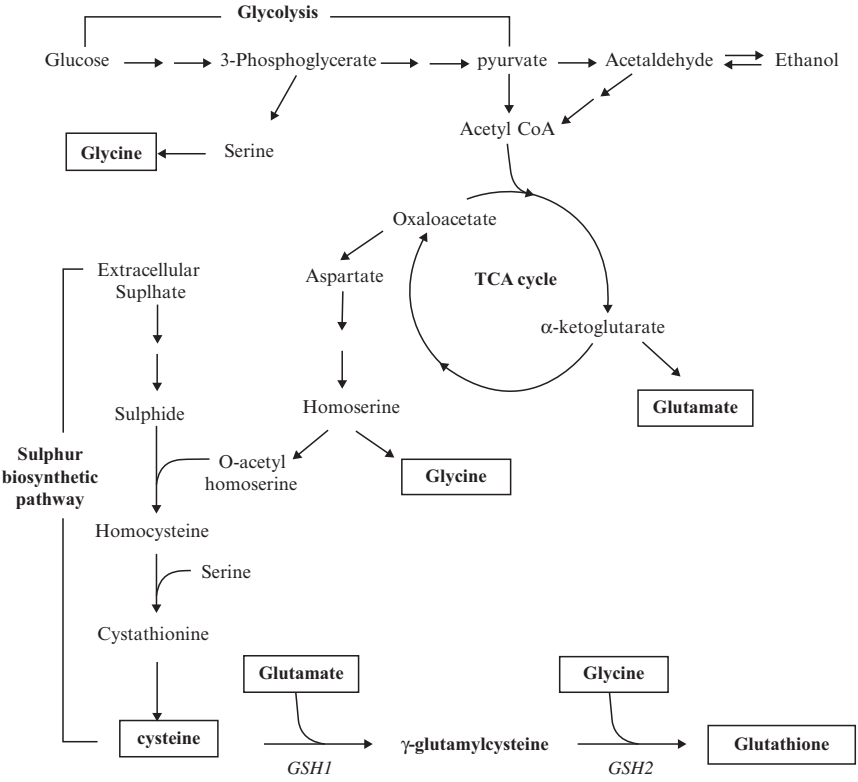

Fig. 13.2 Glutathione biosynthesis in *S. cerevisiae* and its interaction with other pathways

- Regulation of *GSH1* at the transcriptional level
- Regulation of the γ-glutamyl-cysteine synthase enzyme (Gsh1p) at the post-translational level
- Regulation by the substrate levels, in particular, cysteine.

13.4.1 Regulation of GSH1 at the Transcriptional Level

The *GSH1* gene of *Saccharomyces cerevisiae* that encodes γ-glutamylcysteine synthetase, the rate-limiting step of glutathione biosynthesis, is under transcriptional regulation.

The *GSH1* gene is upregulated under oxidative stress conditions mediated by H_2O_2 and/or chemicals with thiol reactivity (electrophiles such as CDNB). Interestingly, the induction of *GSH1* in response to peroxide stress is dependent on the presence of glutamate, glutamine, lysine, and to some extent glycine amino

acids in the medium, although the exact reason for the dependence on the amino acids is not clear. (Stephen and Jamieson, 1997; Dormer et al., 2000). Examination of the two oxidative stress transcription factors, Yap1p and Skn7p, for their involvement in the regulation of *GSH1* have revealed that of these two transcription factors, only the Yap1p (and not Skn7p) is required for the induction of the *GSH1* gene (Brombacher.K et al., 2006).

In addition to oxidative stress, sulphur limitation has been shown to induce the *GSH1* gene. Cadmium which has also been shown to regulate *GSH1*, and the regulation by cadmium has been shown to be dependant on the sulphur regulatory pathway (Dormer et al., 2002). Genome-wide studies with cadmium have revealed that cadmium also leads to an induction in the sulphur regulatory pathway owing to an increased requirement of glutathione under these conditions (Fauchon et al., 2002). The principal factor mediating regulation of sulphur assimilatory pathway is the bZIP protein, Met4p (Thomas et al., 1992). A functional Met4p is required for the activation of many sulphur assimilatory genes and has also been found to be required for the regulation of the *GSH1* gene as well. Extensive work on the regulation of sulphur assimilatory genes has established that a combination of three proteins Met4p, Met28p (or Met 31p/Met32p) and Cbf1p are required for activation of the sulphur assimilatory genes (reviewed by Thomas and Surdin-Kerjan, 1997). In the case of *GSH1* also, the Met4p, Met31p/Met32p and Cbf1p proteins have been found to be involved in the regulation of *GSH1*. The Cbf1p functions more as a repressor, and deletion of Cbf1p lead to a de-repression of the *GSH1* gene (Wheeler et al., 2003). Deletion of either of the transcription factors Yap1p and Met4p leads to an absence of induction of *GSH1*. However, while deletion of *yap1* in a *cbf1* background leads to loss of induction, deletion of *met4* in a *cbf1* background does not lead to loss of induction. Furthermore, Yap1p mediated activation of *GSH1* in response to cadmium toxicity is dependent on Met4p. These observations suggest that under cadmium stress conditions in the cell, Cbf1p binds to Met4p allowing Yap1p to bind to the Yap1p Response Element (YRE) sequence in the *GSH1* promoter thereby mediating *GSH1* activation (Wheeler et al., 2003).The *GSH1* gene thus appears to be regulated by Yap1p-dependant activation in response to the sulphur status that is monitored through a Met4p dependant mechanism.

The *GSH2* gene, which encodes the second enzyme in glutathione biosynthesis, glutathione synthetase (Gsh2p) is upregulated in response to oxidative stress in a Yap1p dependent manner, with its promoter having the YRE-sequence to which the Yap1p protein binds and induces the expression of the gene (Sugiyama et al., 2000).

13.4.2 Regulation of γ-Glutamyl-Cysteine Synthase (Gsh1p) at the Post-Translational Level

The second line of regulatory control on Gsh1p operates at the post-translational level by feed back inhibition of the enzyme by GSH. This feedback inhibition is non-allosteric in nature, since glutathione binds to the glutamate and cysteine binding

pockets, rather than to a separate site on the enzyme. Both the γ-glutamyl and sulphydryl groups of GSH are important for the feedback inhibition of Gsh1p. In vitro kinetic analysis have established the Ki value for GSH feedback inhibition as 2 mM, which is consistent with the physiological concentrations of glutathione (Richman and Meister, 1975). Most of these studies have been carried out with the mammalian enzymes. The mammalian glutamate-cysteine ligase enzyme is a heterodimer consisted of a heavy catalytic subunit (GCLC) and a lighter, modifier or regulatory subunit (GCLM). Chen et al. (2005) have demonstrated a linear increase in the γ-glutamylcysteine (γ-GC) synthesis following complementation of recombinant GCLC with increasing amounts of GCLM in mammalian cells. Thus, the association of GCLM with GCLC alters the kinetics of the reaction, lowering the K_m for Glutamate and ATP, increasing the turnover rate (k_{cat}), but not altering the K_m for cysteine.

The yeast *S. cerevisiae* Gsh1p, differs from the mammalian enzyme and resembles the *Trypanosoma* enzyme in lacking the regulatory subunit. Kinetic studies with the *Trypanosoma* enzyme have however revealed that the single subunit enzyme is also under feedback regulation(Lueder and Phillips., 1996). However in the absence of the GCLM subunit in *S. cerevisiae,* it remains to be seen how the different substrates affect the activity of the *S. cerevisiae* monomeric enzyme. The presence of a modifier subunit in other yeasts also remains to be demonstrated.

13.4.3 Regulation by Cysteine Availability, the Rate Limiting Nutrient in Glutathione Biosynthesis

Studies have revealed that among the different substrates, cysteine is limiting for glutathione biosynthesis (Alfafara et al., 1992). Thus the sulphur regulatory pathway becomes important not only from the point of view of the transcriptional regulation of *GSH1* but also in the transcriptional regulation of enzymes involved in the biosynthesis of cysteine, beginning from the assimilation of sulphate. A recent study on yeast sulphur assimilation pathway (Lafaye et al., 2005) that combined proteomic, genomic and metabolomic data revealed that the intracellular concentration of sulphur metabolites are below K_m values of their respective enzymes, indicating, that under standard conditions, enzymes of sulphur pathway remain unsaturated. Nevertheless, precursors of GSH biosynthesis, like methionine, homocysteine, sulphide, sulphate etc, when added, increased the flux of all sulphur metabolites including GSH, even without over-expressing any rate-limiting enzyme. On the other hand, under sulphur starving conditions, a decrease in intra-cellular sulphur metabolites concentration was observed in spite of up-regulation of sulphur assimilatory genes. When yeast was subjected to stress (like Cd^{2+} stress) a general rise in sulphur metabolite flux was observed leading to overproduction of GSH. This study indicated a correlation between flux of metabolites in the sulphur assimilatory pathway and GSH biosynthesis.

In a recent study in human cells it was found that when cysteine levels drop in the cell <15–500 μM, the Gsh1p/GCL activity increases. This increased capacity for glutathione synthesis (although the absolute rate of glutathione synthesis still decreases because of lack of substrates) facilitates conversion of cysteine in the form of glutathione. This increase in GCL activity is dependent on upregulation of expression of GCLC and a greater increase in the expression of GCLM than GCLC (a 1.5 times increase in GCLM/GCLC molar ratio). This increased expression of GCLM gives rise to increased holoenzyme formation, which in turn is associated with an increase in activity state or k_{cat}. The increase in GCLC and GCLM subunit levels are accompanied by parallel increases in the mRNA levels for GCLC and GCLM, indicating that regulation occurs at the transcriptional level. GCLC is not saturated with GCLM and this highlights the cell's ability to efficiently regulate enzymatic activity and maintain GSH homeostasis in the face of sulphur amino acid limitation, even when GSH levels are not depleted (Stipanuk et al., 2006).

In the event of high intracellular cysteine levels, the glutathione synthesis increases as a result of increased saturation of the enzyme thus contributing to the removal of excess cysteine and thereby preventing toxicity. Thus, short-term regulation of GSH production by GCL occurs mainly via the availability of cysteine, the limiting substrate and perhaps by feedback inhibition of GSH.

In another study it was observed that $K_{m\ (Glu)}$ for glutamate of GCLC ranges between 1.7 mM and 3.5 mM whereas the $K_{m\ (Glu)}$ of GCL holoenzyme ranges between 0.5 mM and 1.8 mM. Thus, it suggests that $K_{m\ (Glu)}$ for GCLC is substantially higher than that for GCL holoenzyme. Similarly, $K_{m\ (ATP)}$ differed by approximately six fold between GCLC and GCL holoenzyme with the $K_{m\ (ATP)}$ of GCLC being higher than GCL holoenzyme (Chen et al., 2005).

13.5 Evolution of Methods for Glutathione Production

The methods of glutathione production have undergone a gradual evolution ever since its days of discovery in yeast extracts. Interestingly, even today, yeast remains the current choice for commercial production of glutathione. Tracing the development of the methods of glutathione production provides a beautiful example of how our understanding of basic science has been exploited for commercial purposes.

In the early days of its discovery in yeast, and other living organisms, glutathione was produced by solvent extraction from these natural sources. Hopkins developed an elegant method for the preparation of the tripeptide from the biological extracts in the form of its cuprous mercaptide (Hopkins, 1929). These preparative methods provided sufficient amount of purified glutathione to meet the demands for laboratory studies. However the low intracellular content of glutathione in yeast, coupled with limited availability of raw materials, made it difficult to meet the commercial demands.

Deciphering of the chemical structure of glutathione as γ-glutamylcysteinylglycine paved the way for its chemical synthesis. In 1935, Harington and Mead used a

modified version of benzylcarbonato method to make synthetic glutathione. The method basically uses N-benzylcarbonato amino acid derivatives to block the reactive amino terminus and a α-COOH ester of glutamate residue to bring the γ-COOH group into reaction. Despite the process being commercialized in 1952, it was not favoured for industrial production because it was time consuming and complicated. Moreover, the end product was an optically inactive mixture of the D-and L-isomers. Hence the end product had to be optically resolved, to separate the physiologically active L-isoform of glutathione, making the entire process more expensive.

The next breakthrough in the commercial production of glutathione came with the discovery and understanding of the glutathione biosynthetic pathway in the liver cells (Bloch, 1949). With the characterization of the Gsh1p and Gsh2p enzymes in glutathione biosynthesis, enzymatic methods based upon use of immobilized microbial cells (both with *Escherichia coli* and *Saccharomyces cerevisiae*), and enzymes in bioreactor systems containing the three constituent amino acids and an ATP regeneration systems were developed for the efficient production of glutathione. In a recent review Li et al. (2004) have summarized the extensive studies using this approach in the last three decades. Despite the observations that this method gives very high yields of glutathione (upto 9 g/l, Miwa, 1976) this method could never be scaled up to the industrial scale. The obvious drawback being the high cost of production owing to the use of the amino acids and ATP in the reactor. The low activities of Gsh1p and Gsh2p also become limiting factor in glutathione biosynthesis by enzymatic methods.

In addition to the enzymatic methods, another option explored to meet the commercial demands of glutathione at a reasonable cost was fermentative production using sugar materials as substrates. Extensive studies have been done in this field in last few decades (reviewed in Li et al., 2004) and currently yeast fermentation is the commercialized method for glutathione production. Kyowa Hakko Kogyo Co., a Japanese industry, which holds a number of patents in glutathione production, has been manufacturing it by extraction from baker's yeast since 1970 (Sakato and Tanaka, 1992).

Yeast has been the choice for the commercial production for glutathione by fermentation because some of the yeast contain high intracellular contents of glutathione (about 0.1–1%dry cell weight). Furthermore, like bacteria, yeast are fast growing organisms, can be grown to high cell densities on cheap substrates like glucose sugar and are easy to handle on large scale; but unlike bacteria, components of yeast cells do not cause any endotoxic reactions in humans. In fact, the food yeasts, such as *Saccharomyces cerevisiae* and *Candida utilis* are the preferred microorganisms for glutathione production.

With the aim to design optimal fermentation conditions so as to get maximum glutathione yields, the effects of different factors- media components, environmental factors, yeast growth rate in culture and amino acid supplementation in media- on cell growth and glutathione production have been explored (Table 13.1). Despite the adoption of the favourable cultivation strategies the maximum glutathione yield achieved in a fermentor is around 4 g/l (Ishii and Miyajima, 1989), which is almost half the yield obtained using enzymatic method (Miwa, 1976). Hence there is much scope for

Table 13.1 Optimization of some parameters to enhance glutathione production by yeast

Yeast Strain	Parameter optimized	Scale	Cell biomass (g/L)	GSH yield (mg/L)	Reference
S. cerevisiae KY5711	Specific growth rate of cells by controlling substrate feed rate	5 L fermentor	-	-	Shimizu et al. (1991)
S. cerevisiae KY6186	Sugar feeding rate in fed batch culture	120 kL fermentor	-	2,360	Sakato and Tanaka (1992)
S. cerevisiae KY5711	Specific growth rate of cells by controlling substrate feed rate; and addition of cysteine	3 L fermentor	-	-	Alfafara et al. (1992)
S. cerevisiae S-8H	Media optimization (Glucose–2.5%, peptone–4.0%, KH_2PO_4-0.027%, L-cysteine–0.06%.	Flask	-	160.1	Udeh and Achremowicz (1997)
S. cerevisiae FF-8	Media optimization (Glucose–3.0%, yeast extract–3.0%, KH_2PO_4–0.06%, L-cysteine–0.08%, biotin–10 µg/cm[3)]	Flask (t = 96 h)	8.5	204	Cha et al. (2004)
S. cerevisiae T65	Optimization of amino acid composition (2 mM cysteine after 6 h; 10 mM glutamate, 18 mM glycine, 3.35 mM cysteine after 12 h of fermentation.	5 L fermentor (t = 24 h)	19.8	329.3	Wen et al. (2005)
S. cerevisiae T65	Optimization of sugar feeding rate and amino acid composition (2 mM cysteine after 6 h; 10 mM glutamate, 18 mM glycine, 3.35 mM cysteine after 24, 44 and 56 h of fermentation).	5 L fermentor (t = 57 h)	140	2190	Wen et al. (2006)
C. utilis WSH 02–08	Temperature optimization (30°C for optimum cell growth; 26°C for maximum GSH production).	7 L fermentor (t = 30 h)	15.4	385	Wei et al. (2003)
Yeast	Different parameters (Initial glucose concentration–12 g/L, pH–6.0, broth volume–60 ml/500 ml flask, etc).	Flask (t = 12 h)	8.78	119.4	Li et al. (1998)

a-cell biomass was estimated from the dry cell weight

improvement and herein we can make use of recombinant DNA technology and our knowledge at the molecular level about the glutathione metabolism in the cells.

In the last decade, recombinant microorganisms have been used for glutathione production (Li et al., 2004). Among different strategies, the glutathione biosynthetic enzymes Gsh1p and Gsh2p have been over expressed in both *S. cerevisiae* and *E. coli,* however there was limited increase in the glutathione levels in either case (Ohtake et al., 1988, 1989; Gushima et al., 1983). The tight regulation of *GSH1* coupled with its feedback inhibition by glutathione can be one of the reasons for this lack of increment. In addition the limited availability of the amino acids especially cysteine and degradation of glutathione could also result in low glutathione levels despite the increased copy number of the biosynthetic enzymes. Thus, our understanding of these aspects at the molecular level can provide clues to overcome these bottlenecks and also design new strategies for glutathione production in yeast.

13.6 Bottlenecks in Glutathione Production in Yeast

Glutathione production in yeast has been intensely investigated and improved upon over the last few decades, and there seems scope to see further improvements. However, the further development of methods and strategies for enhancing glutathione production requires an understanding of the current limitations and bottlenecks to glutathione overproduction. Based on our current understanding of the glutathione metabolic pathway in yeast, some of the major bottlenecks in relation to enhancing glutathione overproduction that need to be addressed, include the following:

- Limiting nutrients, and biomass
- Levels of glutathione biosynthetic enzymes, Gsh1p, Gsh2p
- Feedback regulation of Gsh1p
- Glutathione toxicity
- Glutathione degradation

The following sections describe how these different problems and bottlenecks have been addressed in the past to achieve glutathione overproduction, and how further strategies could be employed to address the issues.

13.6.1 Limiting Nutrients and Biomass

13.6.1.1 Tackling the Limiting Nutrient Problem by Increased Biosynthesis of the Nutrients or Increased Transport

As highlighted in the section on the glutathione biosynthetic pathway, the metabolite flux through the different metabolic cycles in cells have a direct influence on the glutathione biosynthesis. Studies on effects of amino acid on glutathione production have revealed that cysteine is a rate-limiting nutrient in glutathione biosynthesis

(Alfafara et al., 1992; Wen et al., 2004). The addition of glutamate, glycine, serine, methionine, and arginine to growth medium also increases glutathione content of the cells (Wen et al., 2004).

These studies clearly highlight the possibility that manipulation of cellular amino acid levels, cysteine in particular, can lead to increased glutathione content. Genetic manipulation of the biosynthetic pathways thus becomes an important strategy. The sulphur assimilation pathway leading upto cysteine biosynthesis is under a tight transcriptional regulation by the intracellular cysteine levels (Thomas and Surdin-Kerjan, 1997). Overproduction of some of the key enzymes in the pathway have thus been explored to enhance cysteine and glutathione biosynthesis in plants. Youssefian et al. (2001) showed that a threefold increased activity of O-acetlyserine(thiol)lyase (OASTL), a key enzyme of plant sulphur metabolism, that catalyses the formation of cysteine from sulphide and O-acetlyserine, led to a twofold increase in cellular cysteine levels. Though the increase in cellular glutathione levels was not very significant, over-expression of cysteine biosynthetic genes need to be explored in yeast cells to see how they impact on glutathione levels.

Previous work done on *S. cerevisiae* has shown that it has poor cysteine uptake ability as it lacks a cysteine specific transporter, and instead employs non-specific low affinity amino acid permeases for cysteine uptake (During-Olsen et al., 1999). Recently we have identified a high affinity transporter that also has a high specificity for cysteine (Kaur and Bachhawat, 2007) and an expression of this from a constitutive promoter can be done to impart better cysteine uptake abilities in the yeast strain.

13.6.1.2 Tackling the Limiting Nutrient Problem by Fermentative Approaches: Feeding Strategies, Preventing Ethanolic Fermentation for Increased ATP (energy) and Cell Mass

One of the most frequently encountered problem in yeast fermentation is its ability to perform simultaneous respiration and fermentation under fully aerobic conditions. This phenomenon termed as ‘crabtree effect’, results from inhibition of the respiratory enzymes in the yeast due to presence of excess of glucose. As a consequence of this repression, the excess of glucose is diverted into less energy producing ethanolic fermentation, resulting in reduced cell biomass even under aerobic conditions as well as decreased production of the desired product. In particular, the crab tree effect could be detrimental to the glutathione production because shutting down of the TCA cycle directly limits the availability of the glutamate and indirectly decreases the cysteine flux for the biosynthesis (Fig. 13.2). Though ethanol can also be assimilated and used for glutathione production, it has been observed that in contrast to glucose as a sugar source, the glutathione yield is low with ethanol as a sugar source (Shimizu et al., 1991; Liu et al., 1999b). Based on these observations, controlling a constant ethanol concentration to maintain a zero net production is one strategy to prevent the crabtree effect and increase cell biomass and glutathione content in cell.

Ethanolic fermentation in fermentor is favoured at high glucose concentration and in aerobic glucose limited culture at high specific growth rates. Hence, optimization of glucose feeding rate in the fermentor, which in turn also influences the cell growth rate and glutathione content, have been tried. In a study by Shimizu et al. (1991) designed a mathematic model to predict optimal profile of the specific growth rate in a yeast fed batch culture, based on the mass balance around the fed batch system and the relationship between the specific growth rate and specific production rate of glutathione. They suggested that initially the cell mass can be controlled at its maximum by controlling feed rate in the fermentor and then the specific growth rate can be decreased to achieve maximum glutathione production. By adopting this strategy, production glutathione was increased by 41% over the control. Similarly, Sakato and Tanaka (1992) have developed a feedforward/feedback control system and used the knowledge of on-line oxygen consumption and ethanol production to regulate the sugar feed rate, achieving a 40% increase in glutathione production. Alfafara et al. (1993) developed improved fuzzy control logic to monitor ethanol production in the fermenter and hence monitor specific cell growth to maximize the glutathione production.

Apart from process optimization, the exact nature of carbon, nitrogen source and other nutrients in the medium can also influence the cell biomass and the glutathione production. Accordingly different studies (summarized in Table 13.1) have aimed at optimization of the nature and percentage composition of the carbon, nitrogen, salt and amino acids to achieve highest glutathione production in culture.

In particular, stress has been laid on the addition of amino acid in the medium and has been shown to have positive effect on glutathione production. Alfafara et al. (1992) suggested a single shot strategy for addition of cysteine in the shake flask culture, there by obtaining a two fold increase in glutathione content compared to control with no amino acid supplement. In contrast, observing that inclusion of cysteine in media could have a detrimental effect on cell growth, Wen et al. (2004, 2005) have designed a two-step addition strategy for amino acid supplementation to medium to enhance glutathione biosynthesis. In fact they have observed a beneficial effect for cysteine, glycine and glutamate on glutathione production and found that addition of 2 mM cysteine after 2 hour of fermentation and the combined amino acids (10 mM glutamic acid, 18 mM glycine, and 3.35 mM cysteine) after 7 h led to a 72.2% higher glutathione content then the control. These concentrations were optimized using the orthogonal matrix method for parameter optimization and then further scaled up for fed batch culture condition.

In a recent report, (Wen et al., 2006) the high-density fed batch yeast culture obtained by feedback control of ethanol to 0.3%, has been combined with amino acid modulation to obtain a biomass of 140 g/L and glutathione content of 2190 mg/L after 57 h of fermentation. Although this is a significant improvement over the other process, this needs to be combined with the other factors such as use of the mutants strains and metabolic engineering strategies to achieve further increase in cellular glutathione content.

13.6.2 Limiting Levels of Glutathione Biosynthetic Enzymes, Gsh1p,Gsh2p

The rate-limiting step in a pathway is often the major bottleneck that needs to be addressed in any effort in metabolic engineering. Studies on prokaryotes and eukaryotes have revealed that the Gsh1p encoded by *GSH1* gene is the rate-limiting enzyme of GSH biosynthesis. As a consequence, many studies have been carried out to overproduce glutathione by over-expressing the two glutathione biosynthetic genes. Lang-Hinrichs and Stahl (1988) reported that using suitable over expression constructs of *GSH1* and *GSH2* they could achieve a sixfold increase in GSH production. However in most other studies, carried out in several labs, over-expression of either *GSH1* or *GSH2* (or both) could not lead to more than twofold increase in glutathione levels (Ohtake, 1988, 1989; Grant et al., 1997). Studies in our own lab have also shown that over-expression of both *GSH1* and *GSH2* from strong constitutive promoters led to only a twofold increase in GSH levels (Ganguli and Bachhawat, unpublished observations). Studies by Wheeler et al. (2003) on the regulation of the *GSH1* gene have revealed that the *GSH1* gene is transcriptionally regulated by glutathione and methionine via the Met4p and Yap1p transcription factor as described earlier. The Yap1 transcription factor up-regulates *GSH1* expression under oxidative and other stress conditions which leads to GSH depletion whereas methionine down regulates the *GSH1* transcription via ubiquitination of Met4p. On the other hand, upon GSH depletion the Met4p binds to the Cbf1p (repressor of *GSH1* transcription) thereby effectively inactivating Cbf1p and allowing Yap1p to activate *GSH1* transcription. In an effort to identify multicopy suppressors of *gsh2* , Wheeler et al. (2002) identified the *CDC34* gene. The over-expression of *CDC34* (which encodes ubiquitin ligase) led to the over-expression of *GSH1*, although the exact mechanism by which this occurs is still unknown. In addition, *cbf1Δ* strains have constitutively higher levels of *GSH1* (Wheeler et al. (2002). These finding can be used to construct a *GSH1* over-expression strain that may produce more GSH although, they need to be evaluated under fermentation conditions.

13.6.3 Feedback Regulation of the γ-Glutamylcysteine Synthase Enzyme, Gsh1p

Several mutant strategies have been described in the patent literature for the isolation of feedback resistant mutants of γ-glutamylcysteine synthase, Gsh1p, the rate-limiting step in glutathione biosynthesis which is under feedback regulation by GSH. These strategies are based on resistance to the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG), and in *E. coli* to methylglyoxal, sodium azide etc. While these mutants have led to higher glutathione yields, details about the enzyme itself have not confirmed if indeed these are feedback resistant.

The γ-glutamylcysteine synthase enzyme has not yet been crystallized from yeast (or from homologues of the yeast enzyme). γ-glutamylcysteine synthase has evolved across different organisms in three distinct lineages. Lineage I includes yeasts, mammals, fly, worm, fungi and parasites. The second lineage is the bacterial lineage that involves the gram-negative bacteria. The third lineage is the plant enzyme as well as several plant associated bacteria. The three lineages have little sequence similarity between them as seen from pair-wise alignments. The enzyme has been crystallized from the *E. coli* Gsh1p lineage and the plant (*Brassica*) Gsh1p lineages. A model of the *Trypanosoma brucei* enzyme (Lineage I, to which the yeast enzyme belongs) has been proposed based on its similarity to glutamine synthetases. In spite of the absence of the crystal structure of the yeast enzyme (or of any enzyme of Lineage I), the modeling studies and the presence of short sequence motifs common to all the lineages of this enzyme, might still permit one to identify possible regions that could be targeted for the isolation of feedback resistant mutants of the γ-glutamyl-cysteine synthase enzyme.

An alternative approach to the problem of feedback regulation would be to prevent GSH from accumulating within the cells by increasing the excretion GSH. Strategies to enhance secretion of glutathione are discussed in the next section on glutathione toxicity.

13.6.4 Glutathione Toxicity

Glutathione is an essential metabolite in yeast, and intracellular levels have been reported to lie between 1–10 mM. However, significantly increasing the glutathione levels can be toxic for cells. This was revealed from a recent study by Srikanth et al. (2005), where the yeast glutathione transporter was over-expressed from a constitutive promoter. It was found that the excess glutathione being transported inside was resulting in glutathione toxicity. The cells were unable to tolerate fivefold higher than normal levels of intracellular GSH. As the ratios of oxidized to reduced glutathione did not seem to be altered, it appeared that the excess intracellular GSH might be leading to indiscriminate glutathionylation of proteins and subsequent breakdown of metabolic processes. Thus, if intracellular levels of GSH have to exceed these levels one would have to address the problem of toxicity.

One of the best ways to overcome the problem of glutathione toxicity would be to prevent intracellular accumulation of GSH by allowing the intracellular GSH to be excreted outside the cell. Endogenously produced glutathione in the yeast cytosol has been shown to be secreted at low levels under normal growth conditions, which is then taken up again by the glutathione transporter. The endogenous glutathione thus cycles with the glutathione in the extracellular medium. Deletion of the glutathione transporter leads to threefold higher levels of glutathione in the extracellular medium (Perrone et al., 2005). A recent genome wide study has attempted to identify genes, which upon disruption would lead to increased secretion GSH secretion into the intracellular medium (Perrone et al., 2005). In this study the

authors were able to identify gene disruptants, falling under 10 different functional classes that have led to GSH over excretion (2–37 fold) in the medium during the growth at stationary phase. Although the exact mechanism of GSH excretion is still not understood, interestingly, many mutants exhibited altered plasma membrane permeability as the cultures reached early stationary phase. The highest secretion was seen in the vacuolar biogenesis *vps2* mutant that was defective in endosomal protein sorting.

A second way to increase secretion is by modulating the fermentation conditions. Low pH fermentation is one condition that has recently been found to lead to increased secretion. In *S. cerevisiae*, growth at pH 3.5 leads to significant secretion of glutathione while pH 6 leads to virtually little or no glutathione excretion. However some mutants such as *ino1* and *ino4* that affect the membrane compositions are more resistant to decreased secretion at higher pH (Perrone et al., 2005). Studies by Nie et al. (2005) with *Candida utilis* have also shown that at pH 1.5, significant leakage of glutathione occurred into the extracellular medium without any loss in cell viability. Fermentation conditions were subsequently optimized (pH 1.2, for 3 h) to allow for glutathione secretion without loss in viability. The presence of branched chain amino acids in the fermentation was also found to decreases the efflux of GSH into the medium. Low concentrations of surfactants during cell growth have also been used to achieve higher levels of glutathione in the extracellular medium without affecting the growth and viability of the cells significantly.

Glutathione secreted into the medium has been found to be predominantly in the reduced form (as is seen intracellularly) at a ratio of GSH:GSSG of 25–50:1. However, a few mutants were found to secrete higher levels of GSSG (even though the intracellular levels ratios of GSH:GSSG were not changed). These mutants include those mutants that are affected in the mitochondrial function. The highest GSH:GSSG ratio was however seen in *pmr1*strains (*PMR1* encodes a golgi-specific calcium ATPase), where the extracellular GSH:GSSG ratios were 2:1. High levels of GSSG were seen in cells bearing defects in mitochondrial functions.

13.6.5 Glutathione Degradation

The γ-glutamyl transpeptidase (γ-GT) enzyme, till recently has been the only enzyme known to be involved in GSH degradation. *S. cerevisiae* contains a single γ-GT enzyme that is located in the vacuole. Studies on the regulation of the gene encoding γ-GT have indicated that it is under nitrogen regulatory circuit, but also regulated to a small extent by sulphur limitation (Springael and Penninckx, 2003; Kumar et al., 2003a). This fact needs to be kept in mind during the fermentative production of glutathione. An alternative strategy would be to use yeasts naturally defective in this enzyme (Kumar et al., 2003b), or work with yeast strains in which this enzyme has been deleted. Although the exact effects of yield increases have not been evaluated in strains lacking this enzyme versus strains having a functional enzyme, the presence of γ-GT can lead to losses both during fermentation and downstream processing.

Although the yeast *S. cerevisiae* has only one γ-GT enzyme that is localized to the vacuole, most other yeasts have two genes encoding for γ-GT. However limited studies have been carried out on the nature of the two enzymes, but this fact needs to be addressed when using yeast for glutathione overproduction studies.

Recent studies from our lab have provided evidence of an alternative pathway for glutathione degradation. We have now identified the components of this pathway and have cloned the genes for this pathway that appear to be previously uncharacterized ORFs from the yeast genome (Ganguli et al., 2007). It appears, from database searches, that many types of yeast have this alternative pathway and although this is a regulated pathway, the interference of this pathway in affecting glutathione yields has not been evaluated.

13.7 Downstream Processing and Purification of Glutathione

Two methods are commonly used for the purification of glutathione from either intracellular extracts or from the fermentation broths.

In the first method, the copper precipitation method, cells are lysed with hot water (95°C) or sulphuric acid (95°C), the disrupted cells clarified by centrifugation and followed by precipitation of glutathione using copper oxide. Cuprous oxide is added to the extract with stirring to afford a copper salt of glutathione. The white cuprous glutathione which forms as a pellet, is washed with water and treated with hydrogen sulfide (H_2S) to liberate glutathione in the aqueous solution. The cuprous sulfide formed is removed by centrifugation and filtering. After expulsion of the excess H_2S with a stream of nitrogen, the filtrate is transferred to a crystallizing dish and evaporated to dryness. The dry crystalline residue is washed first with 70% and finally with absolute alcohol.

The second method uses ion exchange chromatography. In this method, either partially purified extracts or concentrated fermentation broths are loaded onto the ion exchange resins to purify the glutathione. Ion-exchange chromatography is also used for the purification of oxidized glutathione (GSSG), which accounts for approximately 10% of the total glutathione (Kazuhiro et al., 2002; Kazuhiro et al., 2003;).

13.8 Glutathione Production in Bacteria

Glutathione in prokaryotes is mainly found in gram-negative proteobacteria and the cyanobacteria (Fahey and Sundquist, 1991; Smirnova and Oktyabrsky, 2005). Although glutathione has a role in stress tolerance in these bacteria, it is not essential for their growth. Owing to the ease of growing bacteria and the short generation times, several attempts have been made to use bacteria, particularly *Escherichia coli*, as a source to produce glutathione. We briefly describe some of the different strategies used to obtain increased glutathione production in bacteria.

Initial strategies have included mutant approaches (such as obtaining glutathione over-producing strains of *E. coli*, methylglyoxal and 8-hydroxyquinoline resistance mutants) (Kimura and Murata, 1986), recombinant DNA approaches where the *GSHA* and *GSHB* (genes equivalent to *GSH1* and *GSH2* of eukaryotes) were expressed in *E. coli*, (fermentation optimization strategies were applied in each case), as well cell-free systems using immobilized and permeabilized bacterial cells (Gushima et al., 1983; Liao et al., 2006). Although the latter gave good yields, the need for an ATP regeneration system decreased its feasibility for large-scale production.

To overcome the problem of ATP regeneration in cell-free systems, glutathione production has also been investigated in the cyanobacteria, *Phormidium lapideum*. *Phormidium lapideum* was considered useful for coupling of an ATP regeneration system to glutathione synthesis because it has photophosphorylatic energy capture power that can be coupled to biosynthesis of GSH. This study produced 1.49/gm wet cell weight GSH using cyanobacterium *Phormidium lapideum* in the presence of precursor amino acids and ATP; and the ATP was efficiently regenerated from ADP using light as an external energy source. GSH was also produced under dark conditions using ATP regenerated from respiration (Sawa et al., 1986)

In addition to bacterial organisms like *E. coli* that synthesize glutathione, attempts have also been made with other bacteria such as *Lactococcus lactis* that do not naturally synthesize glutathione but are able to take it up from the growth medium (Li et al., 2003). In a recent study Li et al. (2005) achieved an extremely high intracellular concentration of GSH (up to 140 mM) in *L. lactis* upon over-expression of the *GshA* and *GshB* genes from *E. coli*. Although the exact reasons for the high level of glutathione achieved in this bacteria is not known, two possible explanations that have been provided are the lack of inherent γ-GT activity and the possible absence of feedback inhibition of GSH on *GshA* in *L lactis*.

13.9 Conclusions and Future Perspectives

We have come a long way since the discovery of glutathione in yeast in 1888. Despite the studies being taken on understanding the role and regulation of glutathione biosynthesis in other organisms and systems, yeasts continue to play a central role in understanding glutathione homeostasis. Thus, for example, newer aspects on the regulation of glutathione biosynthesis have emerged in the last few years, revealing the complex interdependence of transcription factors of both the sulphur assimilatory and the oxidative stress response pathways. At the protein level though, studies with the yeast biosynthetic enzymes have lagged behind. Thus while the structures of the γ-glutamyl-cysteine synthase enzyme have been recently described in plants and in *E. coli* (both of which have quite different sequence similarities to the yeast enzyme), structural information of the yeast enzyme is still unavailable. A greater understanding of this key step at both the transcriptional and protein level is important for understanding glutathione biosynthesis, homeostasis and in the overproduction of glutathione from these cells.

The γ-glutamyl cycle that defines glutathione metabolism and homeostasis has been unchanged ever since it was first proposed in 1970 (Orlowski and Meister, 1970). However, the recent identification, a few years ago, of a high affinity yeast glutathione transporter (Bourbouloux et al., 2000), and now an alternative pathway for glutathione degradation that involves several new genes (Ganguli et al., 2007), suggest that the γ-glutamyl-cycle as described is neither complete nor universal. The demonstration also of a novel cysteine specific permease (Kaur and Bachhawat, 2007) that would be important for both sulphur and glutathione homeostasis suggests that not all components of this pathway have yet been described. Furthermore, the demonstration, in a genome-wide study that deletions in 276 genes of the yeast *S. cerevisiae* led to 2–37 fold increase in glutathione excretion, and the fact that these genes fell into 10 different functional classes indicates that we are still at an early stage of understanding all facets of glutathione homeostasis. As our understanding of these processes increases, newer improved strategies are likely to be employed for increasing glutathione production in yeast. The involvement of such a large number of genes in different pathways also indicates the need for a more integrated approach to understanding of glutathione homeostasis. A need also exists for more quantitative models that would describe these processes.

Although much of our understanding of glutathione homeostasis at the molecular level is based on our understanding of *S. cerevisiae*, it is a strain of *C. utilis*, another food yeast, that is currently being exploited for commercial production of glutathione. However, the genome sequence of *C. utilis* is not available, nor are the tools for studying it adequate. Furthermore, differences in metabolic pathways (among other aspects) are likely to exist between these yeasts. Comparisons of the sulphur assimilatory pathways of *S. pombe*, *Aspergillus nidulans* and *S. cerevisiae*, for example, have revealed important differences, that may lead to consequent differences on glutathione production in these yeasts (Brzywczy et al., 2002).

Finally, in addition to glutathione, several glutathione analogues and precursors are also important commercially, as well as many other metabolites of related pathways. Most of the analogues are produced by the synthetic chemistry route, however, precursors such as γ-glutamyl cysteine, analogues such as S-lactoyl glutathione, and metabolites such as S-adenosine methionine that is in the closely linked methionine biosynthetic pathway, can be produced by yeasts. Thus the possibility of yeasts in fermentation, or as biocatalysts for the production of these analogues of glutathione like S-lactoyl glutathione (Liu et al., 1999a), S-adenosyl-L-methionine (Lin et al., 2004; Liu et al., 2004), in addition to glutathione itself, needs to be investigated with greater intensity.

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Chapter 14
The Fermentative and Aromatic Ability of *Kloeckera* and *Hanseniaspora* Yeasts

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Contents

Abstract Spontaneous alcoholic fermentation from grape, agave and others musts into an alcoholic beverage is usually characterized by the presence of several non-*Saccharomyces* yeasts. These genera yeasts are dominant in the early stages of the alcoholic fermentation. However the genera *Hanseniaspora* and *Kloeckera* may survive at a significant level during fermentation and can influence the chemical

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composition of the beverage. Several strains belonging to the species *Kloeckera apiculata* and *Hanseniaspora guilliermondii* have been extensively studied in relation to the formation of some metabolic compounds affecting the bouquet of the final product. Indeed some apiculate yeast showed positive oenological properties and their use in the alcoholic fermentations has been suggested to enhance the aroma and flavor profiles. The non-*Saccharomyces* yeasts have the capability to produce and secrete enzymes in the medium, such as β-glucosidases, which release monoterpenes derived from their glycosylated form. These compounds contribute to the higher fruit-like characteristic of final product. This chapter reviews metabolic activity of *Kloeckera* and *Hanseniaspora* yeasts in several aspects: fermentative capability, aromatic compounds production and transformation of aromatic precursor present in the must, also covers the molecular methods for identifying of the yeast.

Keywords Fermentation, bouquet, apiculate yeast, aroma and flavor, grape must

14.1 Introduction

Alcoholic beverage production in today's world is a complex process. There are infinite parameters that can be altered to produce alcoholic beverage with different flavor profiles. Spontaneous alcoholic fermentation occurs mainly by a succession of different yeast and bacteria population, which are affected by environmental factor. In fact yeasts, medium composition and culture conditions impact the alcoholic fermentation process and aromatic quality of final beverage.

Several authors have reported that the first fermentation stage is dominated by *non-Saccharomyces* apiculate yeast activity, mainly the *Kloeckera* and *Hanseniaspora* strains genus. The high substrate concentration tolerance (>200 g l^{-1}) of the *Kloeckera* and *Hanseniaspora* strains explains their dominance at the initial stages of fermentation. The growth of these yeasts is limited to the first days of fermentation. The progressive disappearance of the *non-Saccharomyces* strains is attributed to lower capability adaptation to gradual increase of ethanol concentration. This fact makes possible the growth of more tolerant *Saccharomyces* strains. Considerable physiological characteristics found in the *Saccharomyces* strains allow them to dominate alcoholic fermentation, the main one being high alcohol concentration tolerance.

The aroma-developing properties of *Kloeckera* and *Hanseniaspora* and their contribution to beverage bouquet determined by the survival period in fermentation make this species a very important object of study. Persistence of the *non-Saccharomyces* species depends on several factors, such as the temperature, pH, nutrient availability, *Saccharomyces* inoculum concentration, kind and concentration of antimicrobial compound, toxin killer sensibility, indigenous microorganism concentration present in fermentation juice and process technology.

This chapter focuses mainly on *Kloeckeras* and *Hanseniaspora* yeast strains studied in wine process. Metabolic, nutritional and aromatic aspects are reviewed.

In addition, fermentative and aromatic abilities are compared to other non-*Saccharomyces* and *Saccharomyces cerevisiae*, the universal yeast used in alcoholic fermentation.

14.2 Biodiversity and Ecology of the Yeasts Used in Fermented Alcoholic Beverages

Yeasts are found throughout nature all over the World. To this day, more than 700 yeast species have been classified in 100 genera (Kurtzman and Fell, 1998). However this number is only a tiny fraction of biodiversity. Hawksworth and Monchacca (1994) estimated that 62,000 genera and 669, 000 yeast species are yet to be described. Yeasts are not capable of moving and depend on vectors, such as wind, insects or man. Fermentative yeasts are found in 2 different habitats: raw material and factories where they processed. In wine-production, grape microflora varies according to the grape variety, climatic influences, viniculture practices etc. (Pretorius, 2000). *Kloeckera* and *Hanseniaspora* are the predominant species on the surface of the grape and represents 50–75% of the total yeast population (Fleet, 1993). The other yeast genera present are: *Candida, Brettanomyces, Cryptococcus, Kluyveromyces, Metschnikowia, Pichia* and *Rhodotorula.* In contrast, *S. cerevisiae* is scarce in vineyards, but abundant in grape juice and must-coated surfaces of winery equipment (Fleet and Heard, 1993). *S. cerevisiae* is preferred for initiating wine alcoholic fermentation due to their most efficient fermentative catabolism. However in the future, some winemakers might prefer using a mixture of indigenous yeast species and strains as starter cultures to increase aroma production.

14.2.1 Spontaneous Fermentation

The natural fermentation of grape must is usually started by low-alcohol-tolerant apiculate yeasts (*Kloeckera/Hanseniaspora*) dominating the initial stages of alcoholic fermentation in spontaneous and inoculated fermentation. These yeasts die off when ethanol concentration increases and are replaced by the strongly fermentative *S. cerevisiae* yeast (Heard and Fleet, 1986; Satora and Tuszynski, 2005). In non-inoculated fermentation, *Kloeckera* yeast is present up to 10^6 CFU ml^{-1}, being the dominant species and representing 50 to 75% of the total population. The main yeast, *S. cerevisiae*, is present, generally with very low population, less than 50 CFU ml^{-1} (Fleet and Heard, 1993). It has been reported that the growth and the survival of *K. apiculata* is not suppressed by the inoculated *S. cerevisiae* strain (Heard and Fleet, 1986). Nevertheless, another author suggests that *S. cerevisiae* produces compounds that are toxic to apiculate yeasts, other than ethanol and killer toxins (Perez-Nevado et al., 2006).

In addition, during various fermentation stages, it is possible to isolate other cultures belonging to other yeast genera, such as *Candida, Torulaspora, Kluyveromyces* and *Metschnikowia* (Fleet et al., 1984; Heard and Fleet, 1986; Pardo et al., 1989).

According to Pretorius et al. (1999), the intervention of only 15 genera of yeasts has been shown during the wine-making process: *Brettanomyces* (and their sexual Dekkera), *Candida, Cryptococcus, Debaryomyces, Hanseniaspora* and their equivalent asexual *Kloeckera, Kluyveromyces, Metschnikowia, Pichia, Rhodotorula, Saccharomyces, Saccharomycodes, Schizosaccharomyces* and *Zygosaccharomyces*. In the specific case of tequila, only one paper has been published, Lachance (1995), about yeast characterization in the manufacturing process. The author identified 10 genera: *Brettanomyces, Candida, Hanseniaspora, Kluyveromyces, Pichia, Saccharomyces, Saccharomycodes, Zygosaccharomyces, Torulaspora* and *Iwatchenkia,* eight of which are present in the wine production process.

14.2.2 Inoculated Fermentation

The introduction of a pure strain allows fermentation to be regulated and accelerated, thereby avoiding the action of certain indigenous populations and, consequently, the production of a specific aromatic compound. In winemaking, inoculation with a starter culture allows a high population of selected *S. cerevisiae* strain to assure its dominance.

The results are quick alcohol production, short fermentation, more predictable aromas and a decrease in the growth of non-*Saccharomyces* present in the must. The foregoing prevents the risk of stuck fermentation and the production of undesirable aromas. However, simpler and less interesting aroma production has been detected when fermentation is dominated by only one yeast type (Romano et al., 1992; Romano et al., 1997; Ciani and Maccarelli, 1998). Ciani et al. (2006), reported that the use of multistarter with apiculate yeast and *Saccharomyces*, showed analytical profiles of wines comparable to or better than those exhibited by pure culture of *S. cerevisiae*. Highest means for acceptability were also obtained by Pinot Noir musts fermented by *P. membranaefaciens* and Chardomay, fermented by *Kloeckera apiculata* (Mamede et al., 2005). This strategy revalues the role of non-*Saccharomyces* yeast and can increase the interest of starter cultures alone or cultures mixed with *S. cerevisiae.* However, our knowledge of the metabolic interactions between *S. cerevisiae* and non-*Saccharomyces* wine yeasts under winemaking conditions needs to be improved.

There are a few studies reporting the kinetic parameter of wine yeast in pure and mixed cultures. Moreira et al. (2005) reported that the specific growth rates in pure culture of *S. cerevisiae, H. uvarum* and *H. guilliermondii* are similar to those in mixed culture of, *H. guilliermondii-S. cerevisiae* and *H. uvarum-S. cerevisiae*. Nevertheless, in mixed cultures of the three yeasts, the specific growth rates of *S. cerevisiae* and *H. uvarum* decreased significantly, while the *H. guilliermondii* rate was not affected. Charoenchai et al. (1998); Ciani and Picciotti (1995) reported variations of specific growth rate of *S. cerevisiae* and *K. apiculata* cultivated on chemically defined grape juice and modified grape juice respectively. During

tequila production, the specific growth rates in pure culture of *Saccharomyces and Kloeckera* strains shown significant differences between genera: 0.373 ± 0.073 h^{-1} for *Saccharomyces* and 0.22 ± 0.1 h^{-1} for *Kloeckera* (Díaz-Montaño, 2004). Additionally, *Saccharomyces* specific growth rates in agave juice fermentation were similar to those reported in grape juice fermentation.

14.3 Molecular Techniques for the Analysis and the Identification of Yeasts

Traditionally, yeast identification has been made by conventional physiological techniques that are labor-intensive and can give ambiguous results (Kurtzman and Fell, 1998). Another disadvantage is their inability to discriminate among strains belonging to the same species. To avoid doubtful identification or misidentification, molecular techniques have been used by numerous authors to discriminate different wine yeast *Saccharomyces* and non-*Saccharomyces* (Capece et al., 2003, 2005). The sequence of the large subunit (26S) rDNA, especially region D1/D2, has been applied to study the phylogeny of different yeast groups and is an important tool in yeast identification (Balerias Couto et al., 2005). Recently, multigene sequence analysis was used, regarding their usefulness for reconstruction of phylogenetic relationships in the *Hanseniaspora–Kloeckera* species group (Cadez et al., 2006); Schültz and Gafner (1993) characterized strains of *Metschnikowia pulcherrima* and *Hanseniaspora uvarum* on the basis of their electrophoretic karyotypes. Esteve-Zarzoso et al. (1999) evaluated the use of restriction fragment length polymorphism (RFLP) of rDNA, amplified by polymerase chain reaction (PCR) to generate a database of restriction patterns for the routine identification of yeast species most frequently isolated from food. The same methods have been used to identify wine yeast species (Granchi et al., 1999; Torija et al., 2001). The molecular techniques employed to differentiate strains at intraspecific level include mtDNA restriction analysis (Comi et al., 2000), comparison of chromosomal DNA profiles (Cardinali et al., 1995), and analysis of random amplified polymorphic DNA by PCR (RAPD-PCR) (Quesada and Cenis, 1995; Cadez et al., 2003; Walczak et al.,2007). Recently (Flores et al., 2005) determined the variability and compared the genetic diversity obtained using amplified fragment length polymorphism (AFLP) markers in analyses of wine, tequila, mezcal, sotol and raicilla yeasts. This is the first report of molecular characterization of yeasts isolated from different traditional Mexican agave-distilled beverages, which shows high genetic differences with respect to wine strains.

14.4 Alcoholic Fermentative Process

Alcoholic fermentation consists of three main stages: transporting sugars to the interior of the cell, transforming sugars into pyruvate by means of glycolysis, and finally converting of acetaldehyde to ethanol.

14.4.1 Carbohydrate Transport

Transporting sugar is a key stage in alcoholic fermentation, as the internal concentration of sugars always remains low compared to the external concentration, (Gancedo and Serrano, 1989). Several authors (Mauricio and Salmon, 1992; Salmon et al.,1993) demonstrated in studies on winemaking that inhibiting sugar transport is the main factor restraining fermentative metabolism of wine yeast (particularly *S. cerevisiae*). Limiting fermentative metabolism in an industrial process can interrupt fermentation produce spontaneously, even when fermentable sugars are present in the must.

In *S. cerevisiae*, glucose and fructose are transported mainly by facilitated diffusion rather than active transport (Kruckeberg, 1996). The hexose transporter family (Hxt) consists of more than 20 proteins of high and low affinity (Bisson et al., 1993). Hexose transport through the plasmatic membrane in *S. cerevisiae* is known to be a control point in the metabolism of carbon compounds during fermentation (Elbing et al., 2004). However, for some non-conventional yeast, transporter kind can be different (Flores et al., 2000). *Candida utilis* transports sugar for proton symport, when the organism is grown to a low sugar concentration (van den Broek et al., 1997). Hofer and Nassar (1987) identified the hexose transporters in *Schizosaccharomyces pombe*, as H^+-symport. Scarce information exists in literature with regard to the transport mechanisms of monosaccharide through the membrane and its regulation in apiculate yeasts. With regard to the selectivity of sugar transport, it has been considered that the *S. cerevisiae* yeast is mainly glucosophilic. The residual fructose in wine is the result of the low capacity of fructose transport presented by *S. cerevisiae* yeast (Schültz and Gafner, 1995). The consumption of glucose and fructose in several strains of wine apiculate yeasts of the *Hanseniaspora guilliermondii*, *Hanseniaspora uvarum* (Ciani and Fatichenti, 1999) and *H. osmophila* genera (Granchi et al., 2002), were evaluated. The apiculate strains showed a wide viability in their preference with regard to the type of sugar. Most were glucosophilic (*H. osmophila/K. cortices* strains), although various were fructosophilic. Other yeasts consumed the two carbohydrates at the same speed (*Hansenispora uvarum/Kloeckera apiculata* and *H. guilliermondii*). These results show that the selective consumption of fructose is widely distributed along apiculate yeasts.

14.4.2 Carbohydrate Assimilation and Their Regulation

After the glucose is transported to the interior of the cell, it is phosphoriled (hexoquinases), to enter into the glycolysis cycle. The glycolysis final product is pyruvate that can be incorporated in two different metabolic ways: respiratory and fermentative. Both ways are regulated by the concentration of the substrate and dissolved oxygen in the medium, showing several phenomenons that are illustrated in Table 14.1.

Saccharomyces yeasts are Crabtree-positive, which means that glycolytic activity increases in the presence of high glucose concentration, diminishing the breathing

Table 14.1 Metabolic regulation: combined effect of sugar concentration and oxygen

Glucose (g l^{-1})	1–5	5–150	>150
Aerobiosis	Respiratory metabolism Pasteur Effect	Fermentative metabolism Crabtree Effect	Inhibition by substrate at respiratory and fermentative pathways
Anaerobiosis	Fermentative metabolism	Slow fermentative metabolism	

capacity in presence of oxygen, leading to an increment of the intracellular pyruvate concentration and ethanol formation (Käppeli, 1986). This phenomenon is due to the repressive action of glucose on the breathing enzymes. Albergaria et al. (2003) found that *Hanseniaspora guilliermondii* yeast was Crabtree-positive, in contrast to *Hanseniaspora uvarum*, that was Crabtree-negative, because aerobic alcoholic fermentation did not develop in batch cultures (Venturin et al., 1994) and chemostat cultures (Venturin et al., 1995a, b) in the presence of high concentrations of sugar. The different responses between Crabtree-positive and Crabtree-negative yeasts have been explained in terms of sugar consumption, glycolytic efficiency, anabolic limitation and enzymatic levels of pyruvate descarboxylase, alcohol dehydrogenase, pyruvate dehydrogenase, acetaldehyde dehydrogenase and acetyl CoA synthetase (**v**an Urk et al., 1988; Postma et al., 1989). Numerous studies have been performed on the balance of oxidative and fermentative metabolism in *S. cerevisiae* and *Candida utilis* (Verduyn, 1991, Verduyn et al., 1992; Käppeli, 1986). In Crabtree-positive yeasts (*S. cerevisiae*), the response to a pulse of glucose induces transcription mainly of the genes of the fermentative enzymes: pyruvate descarboxylase and alcohol dehydrogenase. On the other hand, Crabtree-negative yeasts (*C. utilis*) present low levels of fermentative enzymes and high activities of oxidative enzymes: pyruvate dehydrogenase, acetaldehyde dehydrogenase and the acetyl CoA synthetase, allowing mainly the generation of high concentrations of biomass, CO_2 and small quantities of ethanol. However, *H. uvarum* presents low acetyl CoA synthetase activity (0.05 U mg^{-1}) (Venturin et al., 1995a, b) with regard to other Crabtree-negative yeasts: *C. utilis* (0.50 U mg^{-1}) and *Kluyveromyces marxianus* (0.37 U mg^{-1}) (**v**an Urk et al., 1990).

The deficiency of acetyl CoA synthetase allows the accumulation of acetate and afterwards of ethanol in *H. uvarum*. Also, *H. uvarum* produces glycerol during the oxidative metabolism probably allowing the reoxidation of NADH generated during glycolysis (Venturin et al., 1995a). Likewise, the *H. guilliermondii* yeast (Crabtree-positive) presented yields similar to Crabtree-negative yeasts (Yx/s = 0.49 g g^{-1}) in a purely oxidative metabolism. Also under the same aerobic conditions, *H. guilliermondii* synthesized biomass, CO_2 and glycerol and in biosynthesized respiro-fermentative conditions, mainly ethanol, acetic acid, glycerol, CO_2 and malic acid. Venturin et al. (1995b) analyzed pyruvate descarboxylase activity in Crabtree-positive and Crabtree-negative yeasts in glucose limiting chemostat at different decreased flows. In the case of *S. cerevisiae* (Crabtree-positive), high levels

of pyruvate descarboxylase (0.67 U mg^{-1}) were present even in a low aerobic glucose-limited condition (Pronk et al., 1994). These activities were increased only two-fold under respiro-fermentative conditions (Weusthuis et al., 1994), in contrast to *H. uvarum*, in which single low activities of pyruvate descarboxylase could be detected in aerobic glucose limited cultures (0.20 U mg^{-1}). Likewise, *C. utilis* showed similar behavior (0.30 U mg^{-1}) (Weusthuis et al., 1994). These activities were increased in *H uvarum* (x 3.5) and in *C. utilis* (x7) when grown under oxygen limitation. This information suggests that the metabolism regulation of the Crabtree-negative yeasts *H. uvarum* and *C. utilis* can be controlled by the levels of oxygen (Weusthuis et al., 1994). Alcoholic fermentation occurs out only if oxygen is the limiting factor (Venturin, 1995b). On the other hand, (Steel et al., 2001) compared glucose catabolism for the pentose phosphate pathway in *S. cerevisiae* and *Kloeckera apiculata* yeasts, showing that *K. apiculata* catabolize smaller quantities of glucose through the pentose phosphate pathway than *S. cerevisiae*. The pentose phosphate or hexose monophosphate pathway is considered an alternative pathway in the degradation of glucose. This pathway allows the formation of two important products: NADPH and pentose phosphate. NADPH is used as a reducer in numerous reactions, mainly in lipid biosynthesis as well as other compounds whereas pentose phosphate (ribose 5-phosphate) is a precursor of nucleotides and nucleic acids. Likewise, it provides erythrose 4-phosphate for the synthesis of aromatic amino acids. Todd et al. (1995) reported considerable differences in the ribonucleic acid content between both strains, evidencing the different glucose catabolism requirements of pentose phosphate.

14.5 Factors Affecting Fermentation

Several factors impact fermentation rates and drive sluggish and stuck fermentation, but the important ones are: nutrient limitation, ethanol toxicity, toxicity for fatty and organic acids, the presence of killer factors, cation imbalance, temperatures carried to an extreme, pesticide and fungicide residues, microbial competition (Bisson, 1999).

14.5.1 Nutrients Limitation

The most studied conditions driving stuck and sluggish fermentation is the nutrient limitation (Bisson, 1999). Low fermentative capacities have been observed in *H. guilliermondii* (Albergaria et al., 2003) and *K. africana* (Díaz-Montaño, 2004) which are possibly due to a nutrient limitation.

The two macronutrients were frequently implied in the causes of stuck fermentation when present in small quantities are nitrogen and phosphate (Alexandre and Charpentier, 1998; Henschke and Jiranek, 1993). Micronutrients lacking vitamins

and minerals have been shown to limit fermentation speed (Bisson, 1999). The exhaustion of the thiamine leads to slow fermentation (Bataillon et al., 1996). High ethanol concentrations inhibit the translocation of amino acids and other nitrogen sources, so nitrogen must be available in the first stages of fermentation and stored inside the vacuole for later use (Boulton et al., 1996). Also, the addition of certain amino acids can increase the ability for quickly synthesizing degraded proteins as glucose transporters (Manginot et al., 1997). The phosphate limitation has been shown to impact biomass growth and yield. Phosphate is necessary to maintain cellular pools of Pi, ADP and ATP to drive glycolysis. Furthermore, mineral and cation deficiencies have been shown to impact fermentation rates (Blackwell et al., 1997). The minerals serve as cofactors in glycolysis. Limitations of some minerals such as Zn and Mg affect glucose catabolism while calcium limitation increases ethanol sensibility (Nabais et al., 1988).

14.5.2 Antimicrobial Compounds

Nutritional requirements of yeasts during the fermentation of grape juice can be influenced by the inhibitory substances present in the media. These compounds include killer toxins, chemical preservatives (especially sulfite) and agrochemicals containing heavy metals. Chemical preservatives can affect microbial activity causing an increment in the latency phase (Bisson, 1999). This behavior has been observed in pesticides containing copper (Tromp and De Klerk, 1988). Several pesticides have shown high antiseptic activity even with yeasts (Cabras et al., 1987). Recently, Cabras et al. (1999) studied the influence of six fungicides (azoxystrobin, cyprodinil, fludioxonil, mepanipyrim, pyrimethanil and tetraconazole), on *S. cerevisiae* and *K. apiculata* fermentative activity. The most of these pesticides improved the alcoholic production; this fact was especially observed with *K. apiculata*, which increased the alcoholic production from two- to three folds. Sulfur dioxide is used widely to suppress the growth of spoilage microorganisms in grape juice. The sulfite transport in wine yeasts is for simple diffusion (Walker, 1998), causing a decrease of intracellular pH. Even though *S. cerevisiae* is more tolerant to high concentrations of SO_2 than the non-*Saccharomyces* yeasts and bacteria, excessive doses of SO_2 can cause sluggish or stuck fermentation (Boulton et al.,1996). The susceptibility to SO_2 in non-*Saccharomyces* yeasts varies. For example, *K. apiculata* has been found to be susceptible to less than 5 mg l^{-1} free SO_2, but *Candida guilliermondii* and *Zygosaccharomyces* spp. were resistant to at least 10 times that concentration (Romano and Suzzi, 1993). Likewise, significant differences in resistance to sulphur dioxide was found in non-*Saccharomyces* grape and agave strains (Fiore et al., 2005).

Recently, the production by *S. cerevisiae* of fermentative metabolites potentially toxic for some non-*Saccharomyces* has been reported and not yet identified (Pérez-Nevado et al., 2006).

14.5.3 Toxin Killer

The killer activity was first reported in *S. cerevisiae* strains (Bevan and Makower, 1963). Since then, the characteristic killer has been detected in other yeast genera such as *Pichia* (Sawant et al., 1988), *Hansenula* (Polonelli et al., 1983), *Williopsis* (Walker et al., 1995) and *Kluyveromyces* (Young and Yagiu, 1978). Killer yeast strains produce an extracellular protein or glycoprotein (killer factor) that kills other sensitive yeasts. Neutral type yeasts are resistant to the killer factor but do not produce it. Killer-sensitive strains have also been discovered, these strains are immune to their own toxins but may be sensitive to other strains of toxins. The *Saccharomyces* yeasts produce species of protein killers: K_1, K_2, K_3 and K_{28}. The non-*Saccharomyces* yeasts generate species of protein killers: K_4 to K_{11}. The most of *Kloeckera* yeasts are neutral type and they are resistant to killer factor (Rodriguez et al., 2004; Sangorrín et al., 2001), an exception of toxin Kpkt of *K. phaffi* (Ciani and Fatichenti, 2001).

14.5.4 Temperature, pH, Oxygen and Culture Media Effects

The growth and the permanency of the non-*Saccharomyces* yeasts depend on fermentation conditions such as: temperature (Fleet and Heard, 1993), ethanol concentration (Kunkee, 1984), substrate concentration and pH (Charoenchai et al., 1998). Low temperature at the beginning of the fermentations results in prolonged survival of the non-Saccharomyces yeast. In contrast, *Saccharomyces* yeast populations dominated throughout the fermentation when the temperature of the cellar was maintained at a constant 16–18°C (Domizio et al., 2007). Several studies performed in wine (Erten et al., 2002) and cider (Bilbao et al., 1997) suggest that the growth of *K. apiculata* in the presence of *S. cerevisiae* is favored during fermentations performed below 20°C, allowing *K. apiculata* to prevail together with *S. cerevisiae* during fermentation (Bilbao et al., 1997). This situation can alter the chemical composition of wines, since the aromatic compounds depend mainly on the yeast (Mateo et al., 1991; Gil et al., 1996; Antonelli et al., 1999) and on fermentation temperature (Aragon et al., 1998).

Variation of medium pH between 3.0 and 4.0 did not significantly affect the growth rate or cell biomass of the non-*Saccharomyces* and *S. cerevisiae* strains (Charoenchai et al., 1998).

Culture media kind affects growth and fermentative capability of yeast. Recently, Arrizon et al. (2006) assessed different non-*Saccharomyces* and *S. cerevisiae* strains isolated of different origins cultivated on agave and grape must. Non-*Saccharomyces* grape strains did not ferment agave must in any conditions, whereas than non-*Saccharomyces* agave strains showed a moderate fermentative activity both in low sugar and high sugar concentration. On the contrary, non-*Saccharomyces* grape and agave strains were able to consume sugar and to produce ethanol in YPD medium, although to a lesser extent than *S. cerevisiae*.

During alcoholic fermentation, oxygen is a limiting factor for yeast growth. Visser et al. (1990) showed that *S. cerevisiae* is capable of rapid growth under strictly anaerobic conditions, whereas other yeasts, including the wine-related genera *Hanseniaspora, Kloeckera* and *Torulaspora,* grow poorly under the same conditions. The effect of oxygen on the survival of non-*Saccharomyces* yeasts during mixed culture fermentations of grape juice with *S. cerevisiae* has been reported. Oxygen clearly increased the survival time and decreased the death rate of *T. delbrueckii* and *K. thermotolerans* in mixed cultures, whereas it did not affect the growth and survival of *S. cerevisiae* (Hansen et al., 2001). It has also been shown that oxygen increases the time during which *Hanseniaspora valbyensis* coexists with *S. cerevisiae*, diminishing the mortality rate of *Hanseniaspora valbyensis* (Panon, 1997).

14.5.5 Ethanol Tolerance

Several studies have reported the role of the plasmatic membrane in the ethanol tolerance of *S. cerevisiae* (Beavan et al., 1982; D'Amore et al., 1990). High tolerance to ethanol thus correlates markedly with the level of fatty acid saturation and the fluidity of the membrane (Beavan et al., 1982; Alexandre et al., 1994). Sterols and unsaturated fatty acids also named survival factors cannot be synthesized under anaerobic conditions (Mauricio et al., 1997; Morrisey et al., 1999). Several authors have reported that the addition of fatty acids and ergosterol to the culture medium increases the ethanol yield and the ethanol tolerance of *S. cerevisiae* without requiring oxygen in the culture (Mauricio et al., 1997; Mishra and Prasad, 1989). *Hanseniaspora/Kloeckera* yeast is sensitive to ethanol concentrations of 5–6% v/v (Kunkee, 1984). Low temperatures increase ethanol tolerance (Fleet et al., 1989).

Pina et al. (2004) studied the kinetics of cell inactivation at high ethanol concentrations (>22.5% v/v) in different oxygen conditions with the addition of survival factors. These authors reported that the most abundant compounds found in the *S. cerevisiae*, *H. uvarum* and *H. guilliermondii* were: palmitic acid, oleic acid and ergosterol. However, the ergosterol/fatty acids ratio differs according to culture conditions and the yeast genus (Pina et al., 2004; Alexandre et al., 1994). *S. cerevisiae* aerobe cultures contain nearly 80% of unsaturated fatty acids, whereas in anaerobic ones, the phospholipids are typically enriched with the saturated fatty acids (Steels et al., 1994). *H. guilliermondii*, cultivated under aerobic conditions, produced high ergosterol and oleic acid contents, whereas anaerobiosis showed mainly palmitic acid and low ergoesterol concentration (Pina et al., 2004). The ergosterol and Tween 80 addition as sources of oleic acid in anaerobe cultures cultivated on high ethanol concentrations (> 22.5% v/v) has allowed an increase of cellular viability in *S. cerevisiae* and *H. guilliermondii* yeasts (Pina et al., 2004). Oleic acid and ergosterol play an equivalent role when they modulate ethanol tolerance in *H. guilliermondii*, although this response is not observed to all non-*Saccharomyces* yeasts. In the case of the most sensitive yeasts, *H. uvarum* and *T. delbrueckii*, the

presence of survival factors in anaerobic does not increase ethanol tolerance, even though the lipids have been incorporated into the membrane.

14.6 Aromatic Compounds

Alcoholic fermentation by yeast is associated with the production of a wide variety of fermentation products contributing to the flavor of drinks, as aromatic compounds or their precursors. There are nearly 400 volatile constituents, classified as: higher alcohols, fatty acid esters, bencenic compounds, lactones, terpens and certain particular metabolites (Cordonnier and Bayonove, 1986). Nevertheless, only the portion of these substances reaching the receiving organs is important from an aromatic point of view. The concentrations of most aromatic compounds in fermented alcoholic drinks are small, in the order of 10–50 ppm or fewer (Belitz and Grosch, 1988). To study them, it is necessary to perform extraction and concentration processes that allow their identification and later quantification (Mamede and Pastore, 2006).

14.6.1 Higher Alcohols

Higher alcohols are secondary metabolites of yeasts in alcoholic fermentation and they constitute the largest group of aromatic compounds in alcoholic drinks. Higher alcohols, also called fusel alcohol, have a strong pungent smell and taste of alcoholic drink (Rapp and Mandery, 1986). They are found in variable concentrations in wines, between 80 and 540 mg l^{-1}. When they are present in concentrations under 300 mg l^{-1}, they contribute to a desired complexity, but if they are above 400 mg l^{-1}, these compounds have a negative effect on the aroma. Higher alcohols are divided into two categories: aliphatic and aromatic. Aliphatic alcohols are the most significant in this group (Bertrand, 1986) and include propanol, isoamyl alcohol, isobutanol and isoamylic and amylic alcohols. Aromatic alcohol consists of 2-phenylethyl alcohol and tyrosol. It has been observed in wine that several factors influence the final concentration of higher alcohols in alcoholic beverages: vinicultural conditions and the use of different yeast strains during fermentation (Giudici et al.,1990). Also, the amino acid concentration in the medium affects the production of higher alcohols (Hernández-Orte et al., 2002). Likewise, ethanol concentration, fermentation temperature, must pH, aeration levels, solids levels, grape variety, maturity and skin contact time also affect the higher-alcohol concentration (Fleet and Heard, 1993).

Most aromatic compounds can be formed by yeast starting with exogenous amino acids for degradation, or starting with the assimilable sugars for biosynthesis of ketonic acids. Pyruvate decarboxylase converts the resulting keto acid into the corresponding branched-chain aldehyde, and the alcohol dehydrogenase catalyzes

the NADH-dependent reduction of this aldehyde to the corresponding fusel alcohol (Derrick and Large, 1993). Likewise, higher alcohols can be generated by the degradation of branched-chain amino acids following the Ehrlich pathway: leucine (isoamyl alcohol), valine (isobutanol), 2-amino-butyric acid (n-propanol), isoleucine (amyl alcohol) and phenylalanine (phenethyl alcohol) (Henschke and Jiranek, 1993). The yeast uses at least three aminotransferases, five descarboxylases and six deshydrogenases. Branched-chain amino acid uptake in *S. cerevisiae* is mediated by at least three transport proteins: the general amino acid permease Gap1p, the branched-chain amino acid permease Bap2p, and one or more unknown permeases (Didion et al., 1996).

14.6.1.1 n-Propanol

n-Propanol concentrations in wine are in the range of 14 to 17 ppm. The yeast genus (*S. cerevisiae, H. uvarum/Kloeckera* and *H. guilliermondii*) in pure and mixed culture (Rojas et al., 2003; Gil et al., 1996; Moreira et al., 2005) and the temperature (Erten, 2002) do not influence the concentration of this metabolite. However, differences have been observed in tequila; *Saccharomyces* yeasts generated greater quantity than *Kloeckeras* yeasts: 23 ± 9 ppm and 17.8 ± 5 ppm respectively (Díaz-Montaño, 2004). Similar results have been observed in wines (Romano et al., 2003).

14.6.1.2 Phenethyl Alcohol

The production of phenethyl alcohol using *Saccharomyces* yeasts is influenced by temperature. High concentrations of this metabolite have been detected at low temperatures (13°C). The production of phenethyl alcohol is also influenced by the yeast genus. Phenethyl alcohol is present in wine and tequila production in concentrations of 82.09 ± 0.97 (Rojas et al., 2003) and 22.4 ± 4.9 ppm (Díaz-Montaño, 2004) respectively. Other authors have reported a higher production in *H. guilliermondii* in mixed culture with *H. uvarum* (Moreira et al., 2005). Nevertheless, Gil et al. (1996) did not observe significant differences among the concentrations of this metabolite in pure and mixed cultures with *K. apiculata*, *H. uvarum* and several species of *Saccharomyces spp*.

14.6.1.3 Isobutanol

Isobutanol synthesis is strongly affected by the yeast genus. *S. cerevisiae* biosynthesizes isobutanol (Romano et al., 2003; Moreira et al., 2005; Aragon et al., 1998) in the range of 34.4–64.3 ppm in wine (Gil et al., 1996) and 20.9 ± 7.5 ppm in tequila (Díaz-Montaño, 2004). However, Rojas et al. (2003) observed that *H. guilliermondii* presented higher isobutanol production (57.61 ± 9.35 ppm)

than *S. cerevisiae* (16.43 ± 1.56 ppm), with a production of 32.34 ± 1.89 ppm in mixed culture. Other yeast genus, such as *H. uvarum and K. apiculate,* showed a low production (5–29 ppm) of this compound (Gil et al., 1996). On the another hand, *Kloeckera* yeast showed very low concentrations of isobutanol in the order of 7.7 ± 1.3 ppm in a tequila production process (Díaz-Montaño et al., 2004). Pinal et al. (1997) found that the isobutanol production by *S. cerevisiae* in agave fermentation is influenced mainly by yeast type and the carbon-nitrogen relationship.

14.6.1.4 Isoamyl and Amyl Alcohol

The *Saccharomyces* yeast shows a higher production of Isoamyl and amyl alcohol than the *Kloeckera/Hanseniaspora* yeasts in wines (Rojas et al., 2003; Gil et al., 1996; Romano et al., 1998, 2003) and in tequila (Díaz-Montaño, 2004). However, some authors have reported non significant differences in the production of this metabolite with *H. uvarum, H. guilliermondii* and *S. cerevisiae* in pure and mixed cultures (Moreira et al., 2005). The concentrations detected in wines with *Saccharomyces* are in the range of 164–282 ppm (Rojas et al., 2003; Gil et al., 1996), whereas the concentration of *H. guilliermondii* ranged of about 99.76 ± 8.38 ppm and 26.5 to 50.7 ppm for *K. apiculata* and *H. uvarum* yeasts. The concentrations obtained in tequila are lower in both genera: *Saccharomyces* yeasts show concentrations in the order of 64 ± 20 ppm and the *Kloeckera* yeast 18 ± 7 ppm (Díaz-Montaño, 2004). Isoamylic production in mixed culture of *Saccharomyces* and apiculate yeasts is lower than that of pure culture with *Saccharomyces* (167.53 ± 5.99 ppm) (Rojas et al., 2003); Erten (2002) found significant differences in the quantity of the isoamylic alcohols in the range of temperatures from 10 to 25°C in mixed culture of *Kloeckera* and *Saccharomyces* where concentration increased along with temperature increments. Other authors have reported that the production of this metabolite was not temperature-related (Aragon et al., 1998); Pinal et al. (1997) concluded that the production of isoamyl alcohols is influenced by the yeast, temperature and the carbon-nitrogen relationship.

14.6.2 Glycerol and Succinic Acid

Glycerol, together with ethanol, play a very important role in the fixation of aromas contributing to the viscosity of the wine (Navarre, 1992). Glycerol synthesis is a reaction coupled with glycolysis at the glyceraldehyde-3-phosphate level. Ethanol formation is only possible if the NAD^+ is regenerated. Regeneration of the enzymatic cofactor is occurs mainly in glycerol production. Also, the production of succinic acid and acetic acid reestablishes the NAD^+ to NADH. The *Kloeckera* and *Saccharomyces* yeasts produce glycerol in wine in the order of 1.36 to 4.44 g l^{-1} and 4.8 to 8.3 g l^{-1}, respectively (Ciani and Picciotti, 1995; Comi et al., 2001; Granchi et al., 2002; Brandolini et al., 2002). Likewise, *H. guilliermondii* produces glycerol

in similar ranges in S. cerevisiae (Rojas et al., 2003). With regard to succinic acid, Saccharomyces yeasts present concentrations from 0.45 to 0.71 g l^{-1} and 0.25 to 0.54 g l^{-1} with Kloeckera (Ciani and Picciotti, 1995).

14.6.3 Esters

Esters are formed by yeasts and bacteria during alcoholic fermentation (biological esterification) and very slowly in the course of wine aging (chemical esterification). Esters constitute one of most important groups of compounds that contribute largely to the desirable aromas of fermented beverages (Rapp and Mandery, 1986; Gil et al., 1996). The most significant esters are ethyl acetate (fruity, solvent-like), isoamyl acetate (pear-drops), isobutyl acetate (banana), ethyl caproate (apple) and 2-phenylethyl acetate (honey, fruity, flowery) (Rapp and Mandery, 1986). Volatile esters come from the reaction of saturated fatty acids with alcohol and sometimes with phenol.

Ethyl acetate is the most abundant of all esters, and when it is present in high concentrations, it produces off-flavors. Esters, especially ethyl acetate, are produced by *Kloeckera* yeasts, mainly (Romano, 2003; Rojas et al., 2001; Díaz-Montaño, 2004; Bilbao et al., 1997; Mamede et al., 2005; Zohre and Erten, 2002). Other authors report non significant differences in the production of ethyl acetate between pure and mixed cultures with *H. guilliermondii, H. uvarum* and *S. cerevisiae* (Moreira et al., 2005). However, in mixed cultures, the levels of ethyl acetate produced could contribute to the fruity notes and enhance the general complexity (Gil et al., 1996); Rojas et al. (2003) reported that *S. cerevisiae* increases the concentration of isoamyl acetate in mixed cultures with *H. guilliermondii*. Mamede and Pastore (2006) analyzed the volatile compounds on grape must fermentation by *K. apiculata*; ethyl propionate and propyl acetate were the compounds presents in highest concentration. However, Erten (2002) found significant differences in the quantity of ethyl acetate, ethyl butyrate, isoamyl acetate and ethyl hexanoate in the range of temperatures from 10 to 25°C in mixed cultures of *Kloeckera* and *Saccharomyces*. Low temperatures increase ethyl acetate concentration.

14.6.4 Carbonyl Compounds

The main carbonyl compound in wines is acetaldehyde, with a concentration of about 10–300 mg l^{-1}, and a sensory threshold value of 100 mg l^{-1} (Schreier et al., 1976). Wine yeasts produce this compound in very wide ranges, *S. cerevisiae* 0.5–286 ppm, *K. apiculata* 6–66 ppm and *H. guilliermondii* 10.5–28 ppm. The descriptors used for this compound at low concentrations are: apple-like, citrus-like and nutty. However, high concentrations confer an irritating scent to spicy. Acetaldehyde is one of the major metabolic intermediates, because it is the last precursor before the ethanol is formed. The end-product of glycolysis, pyruvate is converted to acetaldehyde

by pyruvate decarboxylase enzymes. Acetaldehyde is converted into ethanol by alcohol dehydrogenase enzymes (ADHI and ADHII) (Mesias et al., 1983). This step is crucial to maintaining the redox balance in the cell, NADH to NAD^+, required for glycolysis. Fermentation conditions and medium composition affect acetaldehyde production (Liu and Pilone, 2000). The use of sulfur dioxide results in an accumulation of this metabolite, and temperatures at 30°C inhibit the activity of the isoenzymes ADHI and ADHII (Romano et al., 1994). However other investigators report that highest acetaldehyde concentrations are produced in low temperatures (10°C) in mixed cultures of *Kloeckera* and *Saccharomyces* (Erten, 2002).

14.6.5 Volatile acids

Volatile acids constitute a large group of aromatic compounds synthesized by yeast. White wines have between 500–1000 mg l^{-1} of volatile acids, which break down into approximately 90% acetic acid and of about 10% fatty acids (Henschke and Jiranek, 1993). The acetic acid in *S. cerevisiae* is produced as an intermediate of the pyruvate dehydrogenase bypass, a pathway responsible for the conversion of pyruvate into acetyl-CoA through a series of reactions catalyzed by pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase. Acetaldehyde dehydrogenase forms acetate by oxidizing the acetaldehyde produced from pyruvate during fermentation (Pronk et al., 1994). Yeasts producing small quantities of acetic acid are characterized by presenting a high activity of the acetyl-CoA synthetase enzyme (Verduyn et al., 1990). This enzyme generates acetyl-CoA, starting with acetic acid, used in the synthesis of lipids.

Some investigators have reported that the production of acetic acid depends mainly on elevated temperatures (Erten, 2002) and yeast genus (mainly apiculate yeasts) (Bilbao et al., 1997; Díaz-Montaño, 2004; Romano et al., 2003). However, Rojas et al. (2003) reported similar concentrations of acetic acid with *H. guilliermondii* and *S. cerevisiae* in mixed and pure cultures, presenting concentrations higher than 900 ppm. Bilbao et al. (1997) observed differences in the concentrations with regard to temperature. The range of acetic acid concentrations in wines is in the order of 0.66–0.77 g l^{-1} with *Kloeckera* and 0.02–0.04 g l^{-1} with *S. cerevisiae* (Bilbao et al., 1997). In tequila production, *Kloeckera* synthesizes acetic acid in the range of 92.3 ± 18 ppm (Díaz-Montaño, 2004). These authors did not report acetic acid production by *S. cerevisiae* under these conditions.

14.6.6 Phenols

Volatile phenols can make a favorable contribution to the aroma of some wines; they can also contribute to off-flavor. Ethyl-phenols are known to produce a barnyard or stable smell if present in high concentrations. Vinylphenols produce a pharmaceutical

odor, particularly in white wines (Swiegers and Pretorius, 2005). These compounds are derived from ρ-hydroxycinnamic and ferulic acids under the action of yeasts and bacteria (du Toit and Pretorius, 2000). These phenolic acids can be decarboxylated into volatile phenols. They are usually decarboxylated into 4-vinyl derivatives and then reduced to 4-ethyl derivatives (Cavin et al., 1993). The *Brettanomyces/Dekkera* strains mostly produce 4-ethylphenol from ρ-coumaric acid (Chatonnet et al., 1992). The enzymes responsible for such decarboxylations are called phenolic acid decarboxylases (POF1 from *S. cerevisiae*) and several bacteria and fungi have been found to contain the genes coding them. The enzymes coded by these genes are not inhibited by other grape phenolics, and thus there is a high transformation of the vinylphenols into ethylphenols (Swiegers and Pretorius, 2005); Shinohara et al. (2000) analyzed the activity of wine yeasts to decarboxylated ferulic and ρ-coumaric acids in 74 strains of wild yeast (*S. cerevisiae*) and 23 strains of non-*Saccharomyces* yeast. The authors found that a majority of these yeasts were phenolic off-flavor producing strains.

The non-*Saccharomyces* yeasts belong to the genera: *Brettanomyces, Candida, Cryptococcus, Hansenula, Rhodotorula* and *Pichia* produced high or moderate phenolic off-flavors.

14.6.7 Monoterpenes

Part of the aromas of the wines is present under the form of heterosides and terpenics. These compounds are scentless and are called aroma precursors since they are susceptible to being transformed into volatile compounds participating in the aromas of alcoholic fermentation (Mateo and Jimenez, 2000; Swiegers and Pretorius, 2005). This transformation is made by hydrolysis through levurian enzymes: 1′ α-L-rhamnopiranosidase, *β*-glucosidase and 1′ α-L-arabinofuranosidase, located between the cellular wall and the plasmatic membrane in *S. cerevisiae* (Mateo and di Stefano, 1997). Enzymatic hydrolysis is carried out in two stages, in the first stage the α-L-rhamnosidase and the α-L-arabinofuranosidase or *β*-D-apiofuranosidase (depending on the structure of the aglycon moiety) cleave the 1,6-glycosidic. In the second stage, the monoterpens release mono-terpenyl *β*-D-glucosides by means of the action of *β*-glucosidase (Günata et al., 1990). These compounds are particularly abundant in the aromatic varieties of grapes such as: Muscat, Riesling and Gewürztraminer (Günata et al., 1990). The aroma of geraniol and nerol is described as rose-like, the linalool aroma as coriander, linalool oxides as camphorous, and nerol oxides as vegetative. Certain strains of *S. cerevisiae* possess *β*-glucosidase activity (Fia et al., 2005). However, their activity toward glycoside precursors seems to be very low (Hernandez et al., 2003), due to the inhibition of this enzyme for high substrate and ethanol concentrations (Mateo and di Stefano, 1997). The need to have microorganisms with *β*-glucosidases activities has stimulated the search in the non-*Saccharomyces* yeast group, such as: *Brettanomyces, Candida, Debaryomyces, Hanseniaspora* and *Pichia* (Fernandez et al., 2000; Garcia

et al., 2002; McMahon et al., 1999; Rodriguez et al., 2004). The results obtained have made evident that non-*Saccharomyces* yeasts produce mainly β-D-glucosidase (Ferreira et al., 2001; Manzanares et al., 2000; Rodriguez et al., 2004). The best β-glucosidase activity producers were all non-*Saccharomyces* yeasts belonging to: *Candida* (Rodriguez et al., 2004; Manzanares et al., 2000), *Hanseniaspora* (Manzanares et al., 2000), *K. apiculata* (Rodriguez et al., 2004; Ferreira et al., 2001), *Pichia anomalous* (Manzanares et al., 2000; Ferreira et al., 2001) and *Meshnikowia pulcherrima* (Ferreira et al., 2001). A significant increase in this enzyme's activity in the presence of oxygen has been observed (Rodriguez et al., 2004). On the contrary, (Strauss et al., 2001) studied the behavior of yeasts from the *Kloeckera, Candida, Debaryomyces, Rhodotorula, Pichia, Zygosaccharomyces, Hanseniaspora* and *Kluyveromyces* genera without detecting activity of β-glucosidase enzymes. Fia et al. (2005) used a new, rapid fluorimetric method to assay β-glucosidase activity. The authors found β-glucosidase activity in three *S. cerevisiae* strains, in one *Hanseniaspora valbyensts* strain and one *Brettanomyces anomalous* strain.

14.6.8 Ketones

Acetoin is formed in fermentation by the microbial activity of yeasts and bacteria (Romano and Suzzi, 1996). This compound is biosynthesized starting with pyruvic acid through the condensation of one molecule of pyruvate and another of active acetaldehyde which combines with thiamine pyrophosphate (acetaldehyde-TPP complex), both molecules form α-acetolactate. The diacetyl comes from the oxidative decarboxylation of α-acetolactate. Acetoin can be formed by the non oxidative decarboxylation of α-acetolactate acid or by the reduction of diacetyl (Romano and Suzzi, 1996). The main factor affecting acetoin production is the yeast type (Romano et al., 2003).

Numerous reports show that the production of this metabolite is characteristic of apiculate yeasts (Romano and Suzzi, 1996; Fleet and Heard, 1993; Romano et al., 2000, 2003; Mamede et al., 2005; Ciani et al., 2006). Romano and Suzzi (1996) studied 96 strains of *K. apiculata* and *Hanseniapora guilliermondii*, achieving that up to 60% of the *Kloeckera* yeasts produced between 100–200 ppm together with 60% of the *H. guilliermondii* yeasts. Romano et al. (1998, 2000) evaluated the stereoisomers of 2,3-butanediol and acetoin to differentiate *S. cerevisiae*, *K. apiculata* and other non-*Saccharomyces* wine strains (*C. stellata, M. pulcherrima* and *B. bruxellensis*). Significant differences were observed in the acetoin and 2,3-butanediol isomer concentration among the five species of yeasts, while no differences among strains of the same species were observed. The *S. cerevisiae* strain produces about 80% (R,R)-2,3-butanediol whereas *K. apiculata* produces 90% of (R,S)-2,3-butanediol, respectively. Other studies show that *S. ludwigii* yeast presents high concentrations of acetoin in the order of 100–200 ppm. Some strains of *S. ludwigii* can produce > 300 ppm.

Physical and nutritional factors affect acetoin production. Temperature increases the accumulation of this metabolite in the medium (Garcia et al., 1994) as well as high aeration levels (Cowland et al., 1966). Medium composition is important because more acetoin is generated in synthetic medium than in grape juice (Romano and Suzzi, 1992).

14.6.9 Sulfur Compounds

Sulfur-containing compounds have a profound effect on the flavor of wine, owing to their high volatility, reactivity and potency at very low thresholds. Generally, the aromatic contributions of these compounds are considered detrimental to wine quality (Mestres et al., 2000). This type of substance can impart aromas on cheese, cooked vegetables, onion, rubber, garlic, egg and rotten fish. The formation of sulfur compounds is affected by the organic (cysteine and methionine) and inorganic (SO_{4-}) S-containing substances and pesticides in the must, by the nutrient levels of grape musts and by yeast metabolism during fermentation (Swiegers and Pretorius, 2005). Very few reports are available in the literature about the production of sulfur compounds by non-Saccharomyces yeasts (Moreira et al., 2005). Romano et al. (1997) observed several *K. apiculata* and *H. guilliermondii* that produced less than 10 ppm of sulfur dioxide, and *K. apiculata* that produced higher amounts of hydrogen sulfite than *H. guilliermondii.* Moreira et al. (2005) found that heavy sulfur compounds were influenced by the yeast strain used. In general, pure cultures of *H. uvarum* led to the highest production of heavy sulfur compounds. The highest amounts of methionol, 3-methylthiopropionic acid and 2-methyl-tetrahydrothiophen-3-one were found in apiculate yeasts in pure culture and in mixed culture.

14.7 Analysis

In order to achieve alcoholic fermentation control, the complex microbial reaction in the process must be understood. Numerous researchers have studied the participation of *Saccharomyces* and non-*Saccharomyces* yeasts in several alcoholic drinks. Studies have been made on the genetic, metabolic, nutritional and aromatic aspects for distinguishing the fermentative and aromatic capacities of the yeast involved. Evolution of yeast populations and the individual evolution of the most important yeasts during spontaneous fermentation have also been studied under industrial conditions. In addition, yeast characterization by molecular, and physiological methods allow the identification of species and the polymorphism of this species. Currently, alcoholic fermentation control in the industry is performed by starter cultures of *S. cerevisiae,* although low aromatic compound production in alcoholic beverages has resulted from this strategy. Recently, the interest in using starter cultures with apiculate yeast and other non-*Saccharomyces* alone or in

mixed cultures with *S. cerevisiae* has increased. This method caused a positive effect on the aromatic compound production. It increased flavor composition for greater acceptability. It also improved sugar consumption in fermentation with *S. cerevisiae* (glucosophilic yeast) with selective fructose consumption. In addition, different specific growth rates were obtained in *H. guilliermondii, H. uvarum. S. cerevisiae* yeasts in pure and mixed cultures. This suggests that there are interactions between yeasts and culture medium types.

There is very little information on carbohydrate assimilation and its regulation in apiculate yeasts. Differences between non-*Saccharomyces* yeasts have been observed. *H. guilliermondii* behaves like Crabtree-positive yeast such as *S. cerevisiae*, *H. uvarum* and *C. utilis* are Crabtree-negative yeasts. There are other differences also; *Kloeckera* consumes lower quantities of sugar on the pentose-phosphate pathway than *S. cerevisiae*. The development of alcoholic fermentation by apiculate yeast not only depends on its genetic repertoire, certain external factors such as pH, temperature, the presence of inhibitor compounds, nutrient limitation and substrate concentration are involved, too. For years, the first cause of the lowest fermentative capacities by apiculate yeast has been high alcoholic sensibility, although early studies suggest that several factor are involved in these results, such as: high temperatures, low pH, strict anaerobic regime, toxic compounds other than ethanol (excessive SO_2, some pesticide and fungicide residues, the presence of the killer factor and organic and fatty acids) and nutrient limitation. Some studies have shown long periods of apiculate and *S. cerevisiae* yeasts coexisting at low temperatures. High ethanol production in pure culture of apiculate yeast was also reported. The apiculate yeast is characterized as a neutral type resistant to protein killers of *Saccharomyces* and non-*Saccharomyces* yeasts, with the exception of the Kpkt toxin of *K. phaffi*. In addition, they are not tolerant to high concentrations of SO_2.

In the aromatic area, the genotype influences the aromatic compound production. Most authors agree that *S. cerevisiae* strains produce higher amounts of amyl and isoamylic alcohols, n-propanol, 2-phenyl ethanol, acetaldehyde, isobutanol, diacetyl and phenol, whereas *Kloeckera* strains show higher production of acetic acid, monoterpens, acetoin and esters (mainly ethyl acetate). It also shows a significant variability of aromatic compounds between different yeast strains of the same species. Also, fermentation conditions, mainly temperature, oxygen and the medium culture composition, impact the aromatic compound formation and concentrations.

14.8 Perspectives

The use of multi-starter fermentation with non-*Saccharomyces* and *Saccharomyces* yeast could be an interesting alternative to improve alcoholic beverage quality. However, our knowledge of the metabolic interactions between *S. cerevisiae* and non-*Saccharomyces* needs to be improved.

The metabolic, genetics, nutritional and aromatic aspects of several species of apiculate yeasts need to be researched in order to know their fermentative capacities

and improve their yield with the manipulation of some external factors such as: pH, temperature, substrate concentration, oxygen quantities, carbon/nitrogen ratio, oligo-elements and vitamin concentrations. However, we need to detect and establish the physicochemical and biological factors affecting this genus and which do not allow it to survive until fermentation ends. In addition, studying the ethanol tolerance mechanism in *Hanseniaspora* yeasts is very important, as well as the development of efficient and quickest technologies allowing us to evaluate strain biodiversity in the *Hansenispora* genus.

More specific information is required concerning culture conditions affecting aromatic compound formation. Studies of the above points will contribute to enhancing knowledge of these genera, leading to efficient fermentation development with a high control of aromatic compound production.

14.9 Conclusions

The interest in *Hanseniaspora* and *Kloeckera* yeasts has increased throughout the years, as they begin to appear as starter cultures for world class alcoholic drink production.

Although apiculate yeasts do not display as high a fermentative capacity as *S. cerevisiae*, interest in this genus lies mainly in its ability to biosynthesize and/or release interesting aromatic compounds in cultures, thus enabling improvement of wine quality. Nevertheless, the increased knowledge of the nutrition and kinetic aspects of these strains provide us a basis for making this genus more competitive, from a fermentative standpoint.

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Chapter 15
Assimilatory Nitrate Reduction in *Hansenula polymorpha*

Beatrice Rossi and Enrico Berardi

Contents

Abstract In the last decade, the yeast *Hansenula polymorpha* (syn.: *Pichia angusta)* has become an excellent experimental model for genetic and molecular investigations of nitrate assimilation, a subject traditionally investigated in plants, filamentous fungi and bacteria. Among other advantages, *H. polymorpha* offers classical and molecular genetic tools, as well as the availability of genomic sequence data.

Assimilative nitrate metabolism in *H. polymorpha* has an enzymological layout that is similar to other fungal species, and undergoes nitrogen metabolite repression elicited by preferred nitrogen sources such as glutamine. Genes involved in nitrate assimilation are clustered and independently transcribed. The information that puzzles is the presence of two homologous, albeit different, transcriptional activators acting upon the nitrate cluster genes, as all other known fungal nitrate assimilatory pathways have only one activator of this family. Recent work enables a first outline of the interplay between these two activators to be depicted, and suggests that one of them plays a central role in chromatin remodelling within the cluster.

The information, which has recently emerged regarding complex post-translational down-regulatory mechanism acting upon the major nitrate transporter suggests that this protein plays a central role in the regulation of nitrate assimilation.

Nitrogen metabolite repression acting upon nitrate assimilative genes is also being investigated through the isolation and characterisation of *H. polymorpha Nmr* mutants. These studies have suggested that the repression mechanisms are mediated by several interacting factors in this organism, which are also believed to

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participate in nitrogen metabolite repression of other metabolic pathways. All these are involved in the utilisation of secondary nitrogen sources such as arginine, methylamine, urea and asparagine.

Keywords *Pichia angusta*, nitrate assimilation, gene cluster, transcriptional regulation, *trans*-activator, post-translational regulation, chromatin remodelling, nitrogen metabolite repression

15.1 Introduction

Assimilative nitrate reduction is the major pathway converting inorganic nitrogen to organic forms. It has been estimated that more than 2–10% megatons of organic nitrogen per year are produced by nitrate assimilating organisms, a variety of species including bacteria, fungi, algae and plants (Guerrero et al., 1981). According to Kay et al. (1990), as much as 25% of the energy of photosynthesis is consumed in driving nitrate assimilation.

For this reason, nitrate assimilation is crucially related to aspects of great relevance and impact for human life and biosphere equilibrium (e.g. crop yield improvement, concern over the use of nitrate fertilizers, costly in terms of production and in terms of ecological and toxicological impact).

The enzymological layout of assimilative nitrate metabolism (Fig. 15.1) is similar in all fungal species studied so far. It consists of a two-step reduction of nitrate to ammonia, catalysed by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR). NR catalyses the two-electron reduction of nitrate to nitrite; it is a complex multicentre enzyme that in eukaryotes uses NAD(P)H as electron donor

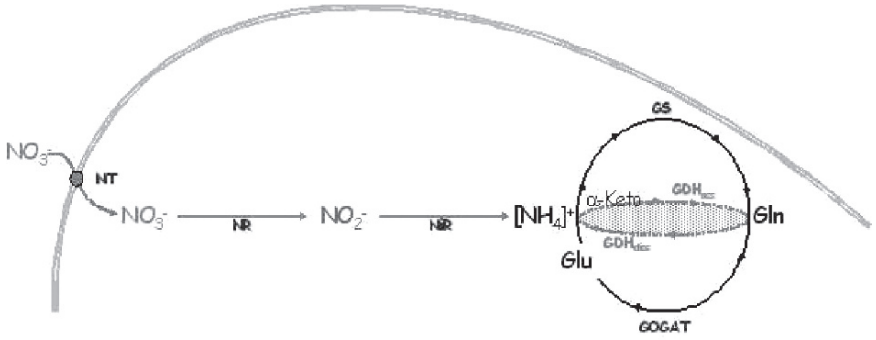

Fig. 15.1 Enzymological layout of assimilative nitrate metabolism in fungi. The two-step reduction of nitrate to ammonia is catalysed by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR). Glutamine Syntetase (GS) fixes ammonia to an α-ketoglutarate skeleton to form glutamine, which can undergo a second ammonia fixation (catalysed by GOGAT) to form Glutamate (Glu)

and contains FAD, ferrohaem and molybdopterin prosthetic groups. It is considered to be a limiting factor for growth, development and protein production in all nitrate-assimilating organisms. NiR catalyses the six-electron reduction of nitrite to ammonia; it is also a multicentre enzyme that in plants uses reduced ferredoxin as electron donor, whilst in bacteria and fungi uses NAD(P)H (Campbell and Kinghorn, 1990; and references therein).

Traditionally, genetic and molecular studies of nitrate assimilation have been done mostly in plants, filamentous fungi and bacteria (Wray and Kinghorn, 1989, and reviews therein). Although a large number of nitrate-assimilating yeasts exist (Barnett et al., 1984), only in the last fifteen years has a nitrate assimilating yeast become the object of molecular investigations, namely *Hansenula polymorpha* (syn.: *Pichia angusta)*. This ascomycete is an excellent experimental model, both for transport studies and for understanding regulatory mechanisms.

In most nitrate assimilating microbial species, nitrate is usually one of many possible nitrogenous compounds that can be utilised, and *H. polymorpha* is no exception. It is believed that this metabolic versatility is an adaptive strategy possibly evolved to confer species better adaptability in environments with variable substrate composition. As a consequence, complex regulatory circuits exist governing the assimilation of nitrogen compounds and the priority a given cell confers to each different nitrogen compound, in case two or more of these compounds occur simultaneously.

All biochemical and genetic studies related to nitrate in *H. polymorpha* descend from our laboratory in Ancona and from J. Siverio's group at La Laguna (Machin et al., 2004; Navarro et al., 2003, 2006; Pignocchi et al., 1998; Rossi et al., 2005; Serrani et al., 2001; Serrani and Berardi, 2005; Siverio, 2002 and references therein).

As for other microbial heterotrophs, also for *H. polymorpha,* nitrogen sources are conventionally classified as *primary* or *secondary* sources. The former are preferentially utilised and capable of sustaining high growth rates, as their incorporation in the living matter is energetically cheap (glutamine, glutamate, ammonia). The latter, which include nitrates and nitrites are only utilised when primary sources are absent (see Pignocchi et al., 1998). In the cell, the ability to switch on the metabolisms necessary for the utilisation of secondary nitrogen sources is rapidly developed, in response to the absence of *primary* sources and in the presence of a specific secondary source e.g. nitrate, that is regarded as *inducer* (Avila et al., 1995; Avila et al., 2002; Brito et al., 1996; Pignocchi et al., 1998; Serrani et al., 2001; Rossi et al., 2005).

A general picture of regulation of nitrogen source utilisation in fungi was sketched out as early as 1973 by Arst and Cove (1973) through imaginative studies on *Aspergillus* mutants (Fig. 15.2).

Any structural gene involved in the utilisation of a secondary nitrogen source was proposed to undergo dual control: (i) nitrogen metabolite repression, acting upon a trans-activating factor with general action (i.e. over all *Nmr*-sensitive genes), in a way that nitrogen depletion would release repression; (ii) induction, acting via factors that are specific for each secondary source utilisation (e.g. nitrate).

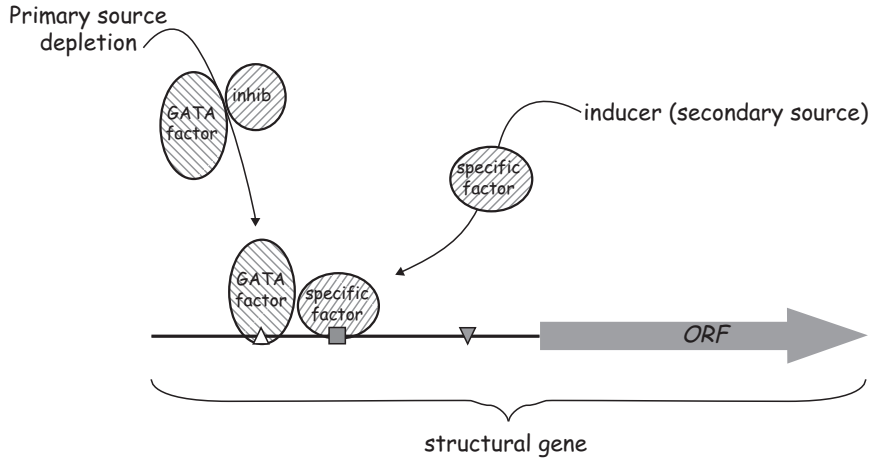

Fig. 15.2 General picture of regulation of nitrogen source utilisation in fungi

During the years further work (both with *Aspergillus* and *Neurospora)* confirmed and enriched this general picture, and new molecular players appeared, such as the specific inducer NirA, acting at different levels and enabling a much more complex picture to be sketched out (Bernreiter et al., 2007; Berger et al., 2006; Burger et al., 1991a, b; Caddick et al., 2006; Feng et al., 1996, 1998; Fu et al., 1995; Marzluf, 1997; Narendja et al., 2002; Strauss et al., 1998; Yuan et al., 1991).

15.2 Nitrate Assimilation in *H. polymorpha*

As said, nitrate assimilation in *H. polymorpha* is similar to that of other fungi, such as *Aspergillus nidulans* and *Neurospora crassa*. It requires *de novo* synthesis of one or more transporters (e.g. Ynt1p; Pérez et al., 1997), of NR (nitrate reductase i.e. NADPH: nitrate oxidoreductase, EC 1.6.6.3; Avila et al., 1995) and of NiR (nitrite reductase i.e. NADPH: nitrite oxidoreductase, EC 1.6.6.4; Brito et al., 1996). Once taken up, nitrate is firstly reduced to nitrite, then to ammonia, so as to enter cellular metabolic circuits (Fig. 15.3).

15.2.1 Nitrate Transport

As in other organisms (e.g. *Aspergillus nidulans; Chlamydomonas reinhardtii*; Unkles et al., 1991; Navarro et al., 2000; Galván et al., 1996), it has been suggested that *H. polymorpha* has more than one nitrate transporters (Machín et al., 2000; Machín et al., 2004), although only one transporter gene has been isolated so far

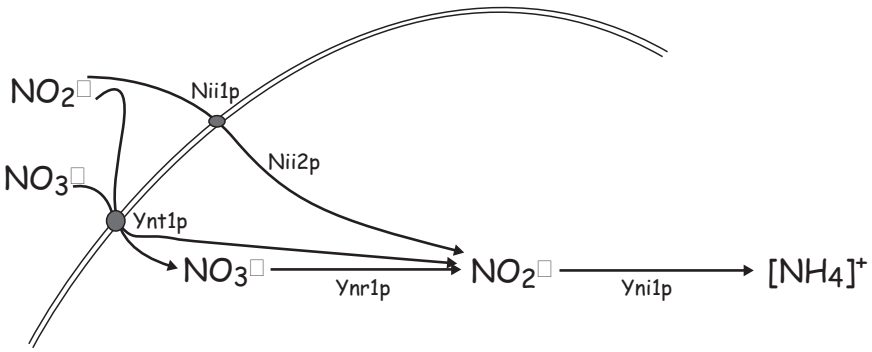

Fig. 15.3 Assimilative nitrate and nitrite reduction in *H. polymorpha*. Nitrate enters the cell *via* transporter Ynt1p, which is also involved in nitrite uptake. Nitrite is also taken up by specific transporter Nii1p. Nitrate reduction is catalysed by nitrate reductase Ynr1p. Nitrite reduction is catalysed by nitrite reductase Yni1p.)

*(YNT1,*Pérez et al., 1997). *YNT1* encodes a high affinity nitrate transporter (K_m 2–3 μM; V_{max} 0.5 nmol/NO_3/min/mg cell), believed to represent the most relevant one in quantitative terms (Machín et al., 2000). Ynt1p appears to be also a nitrite transporter (Machín et al., 2004) and belongs to group II of NNP family of nitrate transporters (nitrate nitrite porter; Forde, 2000), the same as *Asp. nidulans crnA* (*nrtA)* e *nrtB* (Unkles et al., 1991, 2001).

All group II members exhibits high sequence similarities and share the same membrane topology. In particular, a distinctive feature is a long hydrophilic sequence (90 aa) spanning between the sixth and seventh *trans*-membrane domain, and a C-terminal end shorter than that of other groups (*vide* Forde, 2000). Ynt1p plays a central role in the regulation of nitrate assimilation through a complex post-translational down-regulatory mechanism (Navarro et al., 2003, 2006). In the presence of primary nitrogen sources *Ynt1p-ubiquitin conjugates* are formed, associated to rapid vacuolar Ynt1p degradation. This fact brings about a quick decrease of nitrates transported into the cytoplasm, a reduction in the induction level of other genes involved in nitrate assimilation and, ultimately, a rapid modulation of nitrate uptake in response to nitrogen sources present in the medium. Navarro et al. have recently been shown that the central hydrophilic domain of Ynt1p harbours *PEST-like sequences,* deletion of which impedes the down-regulatory process of Ynt1p. In particular, site-directed mutagenesis showed that Lys-253 and Lys-270 within these domains are specifically involved in Ynt1p vacuole intake and degradation (Navarro et al., 2006). Interestingly, Ynt1p is degraded in response to glutamine, independently of glutamine repression, as shown by repression defective mutants (Navarro et al., 2006). Therefore, response to primary nitrogen sources seems to occur in two steps. A short term one, whereby Ynt1p down-regulation lowers nitrate intake by decreasing the number of transporter molecules; a long term one, whereby low intracellular nitrate levels would reduce the induction of nitrate assimilating gene expression (Navarro et al., 2006).

H. polymorpha is capable of utilising nitrite. This ion can enter the cell either via Ynt1p or via a different transporter. In our laboratory two complementation groups of mutants specifically impaired in nitrite assimilation *(nitrite$^-$ nitrate$^+$)* have been isolated, namely *Nii1* and *Nii2*. Experimental data suggests that *NII1* may represent a specific nitrite transporter, whereas complementation work led to the isolation of *NII2*. This gene is predicted to encode a membrane protein, homologue to Pho (Serrani and Berardi, 2005). Although Nii2p role remains unknown, by analogy to Pho, it can be speculated that it plays an accessory role in the maturation of the true transporter. It has been shown that nitrite specific system prevails at pH 4, whereas Ynt1p overcomes at pH 6. Furthermore, whereas Ynt1p is inactivated in the presence of glutamine, nitrite specific activator does not appear to be post-translationally regulated. Therefore, the latter is probably subject only to transcriptional regulation (Machín et al., 2004).

15.2.2 Nitrate Reduction

The first nitrate assimilatory step is the reduction of nitrate to nitrite, operated by nitrate reductase, the peptidic component of which is encoded by *YNR1* (Avila et al., 1995). YNR1p shows high similarity to those of plants and other fungi (Campbell, 1999; Okamoto et al., 1991; Johnstone et al., 1990; Unkles et al., 1992). *H. polymorpha* YNR1p requires various cofactors, including molybdopterinic group, haeme and FAD. Although NADPH is the preferred electron donor, also NADH can serve the purpose (Pignocchi et al., 1998).

Recently, it has been demonstrated in vitro that neither Ynr1p activity nor its stability are impaired by reduced nitrogen sources (ammonium, glutamine), suggesting that this enzyme is not regulated post-translationally (endogenous autoregulation; Navarro et al., 2003).

15.2.3 Nitrite Reduction

Nitrite is reduced to ammonium by nitrite reductase Yni1p, a 116.6 KDa protein encoded by *YNI1* (Brito et al., 1996). Yni1p shows 50% identity with NiR of other fungi. This is a single-copy gene, and no evidence suggests the presence of other isoforms (Siverio, 2002).

As other fungal and bacterial nitrite reductases, *H. polymorpha* Yni1p uses NAD(P)H as electron donors, whereas plants and algae employ ferredoxin (Siegel and Wilkerson, 1989). As typical for nitrite reductases, Yni1p has three prosthetic groups, namely 4Fe-4S center, a siro-haeme group, and a FAD molecule (Campbell and Kinghorn, 1990; Prodouz and Garrett, 1981).

15.3 Nitrate cluster in *H. polymorpha*

In *H. polymorpha,* genes encoding nitrate reductase (*YNR1*, Avila et al., 1995), nitrite reductase (*YNI1,*Brito et al., 1996; Garcìa-Lugo et al., 2000) and nitrate permease (*YNT1*, Pérez et al., 1997) are clustered and independently transcribed (Fig. 15.4; Ávila et al., 1995, 1998; Brito et al., 1996). This nitrate assimilation cluster also contains two regulatory genes (*YNA1,* Ávila et al., 1998; *YNA2,* Ávila et al., 2002) encoding two fungal zinc cluster proteins $Zn(II)_2Cys_6$ (cfr § 13.2.1), involved in nitrate induction mechanisms, as shown by the facts that their null mutants are unable to assimilate nitrate and to induce transcriptional activation of the three structural genes mentioned above.

Garcia-Lugo et al. (2000) showed that in the yeast *H. anomala,* at least two genes involved in nitrate utilisation are also clustered. In the *Aspergilli,* but not in other filamentous ascomycetes (Amaar and Moore, 1998; Johnstone et al., 1990; Kitamoto et al., 1995; Unkles et al., 1992), partial clustering of the nitrate utilisation genes has also been described. Clustering of nitrate assimilatory genes has been recently established in basidiomycetes (Jargeat et al., 2003). The alga *Chlamydomonas reinhardtii* also shows complete clustering of the nitrate assimilatory genes (Quesada et al., 1993, 1998). This phylogenetic distribution and the different gene order within the clusters may suggest that clustering of these genes has occurred independently more than once during evolution. In the *H. polymorpha* nitrate gene cluster the inter-ORF regions, are strikingly short, yielding a coding density as high as 92% (Siverio, 2002).

As mentioned, *YNA1* e *YNA2* encode two zinc cluster proteins, both involved in the induction process (Avila et al., 1998, 2002). Although they belong to the same protein family as the *Asp. nidulans* NirA and *N. crassa* NIT4 proteins (two well studied activators also specifically involved in nitrate assimilation; e. g., Bernreiter

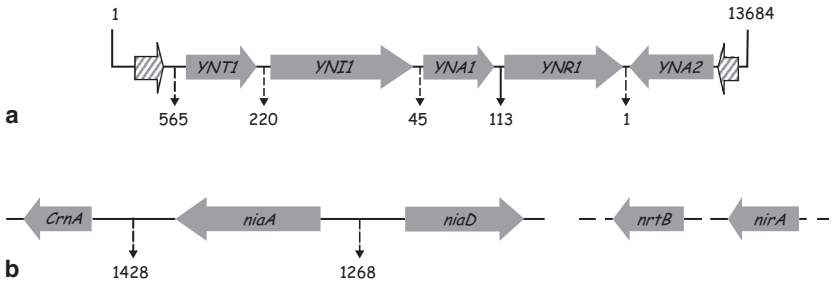

Fig. 15.4 *H. polymorpha* and *Asp. nidulans* nitrate utilization cluster (after Ávila et al.,1998, 2002). Coding regions involved in nitrate assimilation are in light blue (structural genes) or green (regulatory genes). The complete *H. polymorpha* nitrate cluster (A) has five nitrate assimilation genes. ORF and inter-ORF lengths are reported. YNT1, nitrate transporter; YNI1, nitrite reductase; YNA1, Zn(II)2Cys6 transcriptional activator; YNR1, nitrate reductase; YNA2, Zn(II)2Cys6 transcriptional activator; 1, ORF-1; 3, ORF-3. In *A. nidulans* (B) only three genes are clustered (crnA, nitrate transporter; niaA, nitrate reductase; niaD, nitrite reductase)

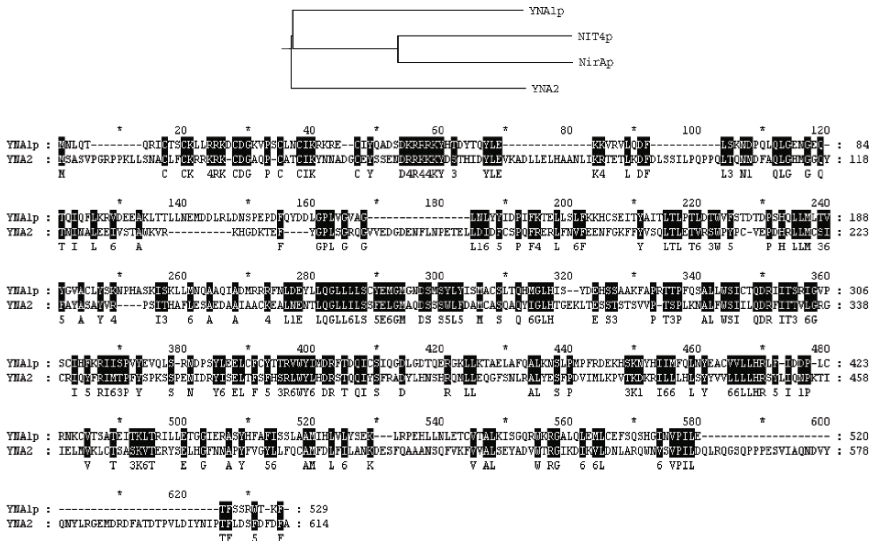

Fig. 15.5 Sequence relations of four nitrate specific trans-activators (above), and alignment of Yna1 and Yna2. Yna1p and Yna2p are from *H. polymorpha;* NIT4p is from *N. crassa*; NirAp is from *Asp. nidulans*

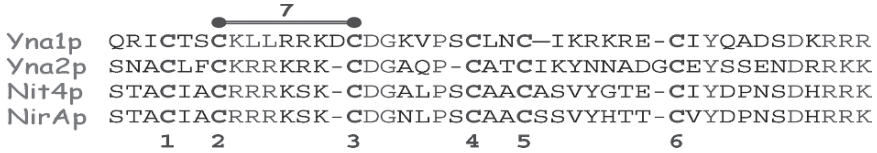

Fig. 15.6 Alignment of zinc cluster region of *trans*-acting factors Yna1 and Yna2 (*H. polymorpha*), Nit4p (*N. crassa)* and NirA (*Asp. nidulans).* This alignment shows the unique Cys-Cys spacer of Yna1, made of seven aminoacid, instead of six, as typical of all other member of this class of fungal transcriptional activators

et al., 2007; Berger et al., 2006; Burger et al., 1991a, b; Feng et al., 1996, 1998; Fu et al., 1995; Marzluf, 1997; Narendja et al., 2002; Strauss et al., 1998; Yuan et al., 1991) they share little similarity with these activators, except in the DNA binding domain (Fig. 15.5).

This similarity is no greater than that found with functionally unrelated proteins belonging to the same family. Yna1p shows a uniquely deviant Zinc cluster motif with seven amino acids, instead of six, between the second and the third cysteines (Avila et al., 1998).

A putative leucine-zipper follows the DNA binding domain of Yna2p. No such motif was found in Yna1p (Avila et al., 1998, 2002). Both, Yna1p and Yna2p are necessary for the expression of the nitrate assimilation genes *YNT1, YNI1* and *YNR1*. Whereas Yna1p appears to be required for *YNA2* induction, Yna2p is not needed for *YNA1* transcriptional activation (Avila et al., 2002). Recent work in our laboratory shows that, in the absence of Yna2p, *YNA1* expression is more sensitive to nitrogen catabolite repression than the wild type (Rossi, 2005) (Fig. 15.6).

15.4 Nitrate Assimilation Control in *H. polymorpha*

As yet, the amount of data available on nitrate assimilation control mechanisms in *H. polymorpha* does not allow to sketch out a model clearly indicating the complex interplay among different elements implicated in this process. However, combining various studies carried out both in Siverio's lab and by us, new important information has recently emerged, especially regarding the two clustered transcriptional activators, namely Yna1p and Yna2p.

As already described, the utilization of secondary nitrogen sources is only allowed in the absence of primary sources, combined to the presence of a secondary nitrogen sources, generally referred to as inducer (Avila et al., 1995; Brito et al., 1996; Pignocchi et al., 1998). Physiologic response elicited by primary sources and leading to repression of genes essential for the assimilation of a secondary sources is generally referred to as *nitrogen metabolite repression,* NMR (a denomination more frequently used in *Aspergillus* and *Neurospora,* and now in *H. polymorpha;* or as *nitrogen catabolic repression,* NCR (a denomination common in *S. cerevisiae;* Cooper, 1982; Cooper and Sumrada, 1983).

We have recently shown, in collaboration with Claudio Scazzocchio (Paris), the existence of a hierarchy in the repressing power of various nitrogen sources with respect to nitrate cluster genes in *H. polymorpha*. Glutamine shows the highest repressive power, followed by glutamate and ammonium. Proline was shown to be a *non-repressing, non-inducing source*, i.e. in regulatory terms, a *neutral* nitrogen source. Therefore, using proline as sole nitrogen source, experiments can be done in a physiological condition generally referred to as de-repression (Rossi, 2005).

Work in our laboratory showed that nitrate cluster structural genes *YNT1,* and *YNI1* and *YNR1* exhibit induction kinetics that are different to those observed for *YNA1* and *YNA2,* the cluster genes with positive regulatory functions. The former reaches high transcriptional levels only after 90–120 induction, while the latter strongly express after 10–20 min induction (Rossi, 2005).

In *H. polymorpha* rapamycin (an antifungal immunosuppressive macrolide; cfr § 13.2.2) blocks NMR, allowing *YNR1* expression even in the presence of glutamine or ammonium (Navarro et al., 2003). This fact suggests the involvement, in *H. polymorpha,* of TOR proteins (*T*arget *o*f *R*apamycin; see Cooper, 2002 and references therein) in post-translational regulation of nitrate assimilatory pathway, similarly to what is known in *S. cerevisiae* for other secondary nitrogen sources (Cooper, 2002 and references therein). This hypothesis is reinforced by the fact that *H. polymorpha* genome shows various ORFs encoding putative proteins which are highly similar to various *S. cerevisiae* factors participating in the TOR mechanism (J. Siverio, personal communication, Fig. 15.7).

Machín et al. (2004); Navarro et al. (2006) showed that, whereas nitrate reductase (Ynr1p) is not involved in the regulation of nitrate utilisation flux, nitrate transporter Ynt1p seems to play a key role in this game. As said, in addition to transcriptional regulation, Ynt1p is subject to post-translational regulation, and is rapidly inactivated in the presence of reduced nitrogen sources such as glutamine.

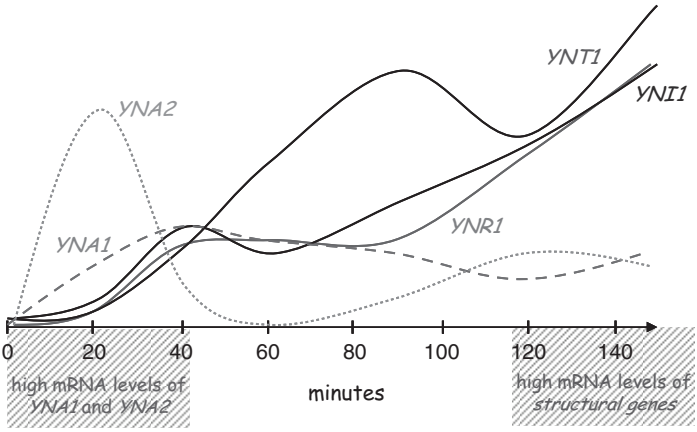

Fig. 15.7 Summary of typical mRNA induction dynamics of the nitrate assimilation genes in *H. polymorpha* after shift from proline to nitrate

A little puzzling is the presence of two homologous, albeit different, transcriptional activators acting upon the genes of the nitrate cluster, and both essential for their transcriptional activation (Yna1p e Yna2p; Brito et al., 1996; Ávila et al., 2002) – a pattern which differ from those characteristic of other fungal nitrate assimilatory pathways (*Aspergillus, Neurospora*, etc.). Work in our laboratories suggest that Yna1 positively influences also *YNA2*, whereas Yna2 does not act over *YNA1* expression, suggesting that auto-induction of *YNA1* is a possible inducing mechanism, with either Yna1p directly binding to *YNA1* promoter, or inducing a second gene, whose product could bind to *YNA1* promoter. Of course, other models are possible, including the involvement of an AreA-like GATA factor (hypothesised, but yet to be discovered) sufficient to induce *YNA1* (Fig. 15.8).

Our recent work has, however, showed that *YNA2* plays a central role in chromatin remodelling within *YNT1* and *YNI1* promoter regions, a task implicated by GATA factor AreA in *Asp. nidulans.*

It is largely recognised that chromatin remodelling plays an important role in eukaryotic transcriptional regulation. Disruption of nucleosomal structure accompanies the activation of many genes, and *H. polymorpha* nitrate gene cluster is no exception to that. In non-expressing conditions, *YNI1* and *YNT1* show highly structured chromatin organisation and well positioned nucleosomes. These structures undergo profound remodelling during gene activation (i.e. during nitrate growth; Rossi, 2005). Parallel analyses with *yna1 Δyna2 Δ*, showed that Yna2 is directly involved in those remodelling processes, whereas *YNA1* may only play an in direct role by activating *YNA2.*

Information on NMR mechanisms acting upon nitrate assimilative genes in *H. polymorpha* was obtained in our laboratory through the isolation and characterisation of Nmr mutants (Serrani et al., 2001; Rossi et al., 2005) (Fig. 15.9).

Study of mutants FM-32B3 (*nmr1-1*), FM-49A1 (*nmr2-1*) e FM-101B (*nmr4-1*) contributed to validate the idea that also in *H. polymorpha* repression mechanisms

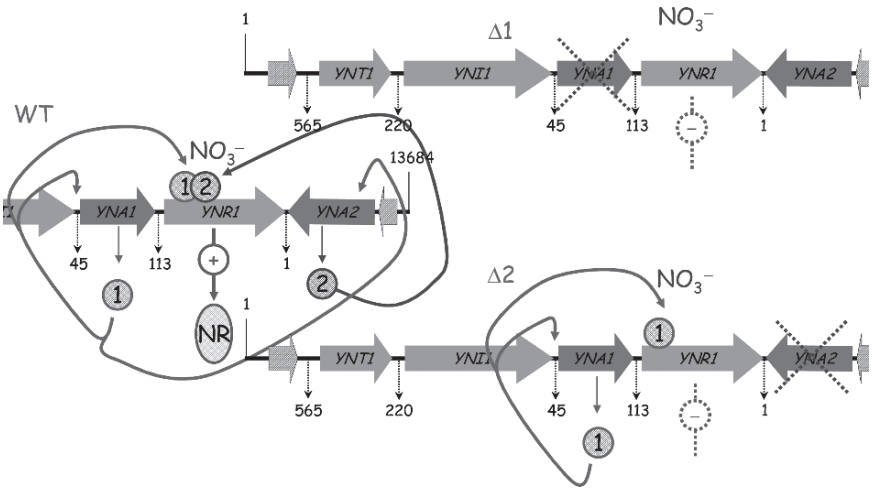

Fig. 15.8 Model showing possible role of Yna1 activator in the presence of nitrate (see text for details). Alternatively, a GATA factor may suffice to activate *YNA1*

are mediated by many interacting factors (Brito et al., 1996; Pignocchi et al., 1998; Serrani et al., 2001; Rossi et al., 2005). These factors are believed also to participate in nitrogen metabolite repression of other metabolic pathways, all involved in the utilisation of secondary nitrogen sources such as arginine, methylamine, urea and asparagine (Serrani et al., 2001; Rossi et al., 2005).

In particular, mutations in the *NMR1 locus* bring about de-repression of nitrate assimilation in the presence of glutamate, but not of glutamine suggesting that a glutamine-depending signalling circuit may coexist with a glutamate-depending one (Serrani et al., 2001). The presence of two regulatory circuits, a glutamate dependent one, and a glutamine dependent one, has been recently proposed also for *Asp. nidulans* (Margelis et al., 2001; Morozov et al., 2001; Mihlan et al., 2003) and constitutes a novelty.

The physiological and biochemical characterization of *nmr2-1* suggest that *NMR2,* as well as *NMR1,* is part of a negative regulatory response to reduced nitrogen compounds. However, *nmr2-1* causes a more general de-regulated phenotype, affecting nitrogen metabolite repression of NR in the presence of both, glutamine and glutamate and all other nitrogen source tested.

All together our data suggest that *NMR2* and *NMR1* are involved at different levels of the NMR regulative circuit. In particular, a reasonable hypothesis is that *NMR2* and *NMR1* have a role similar to the one of the negatively acting proteins *Asp. nidulans* NmrA, *N. crassa NMR1* or *S. cerevisiae* Ure2p, which inhibits AreA, NIT2 and Gln3p, respectively. Mutations in proteins involved in TOR signalling cascade acting on nitrogen metabolism could also be argued (Cooper, 2002 and references therein, Fig. 15.10).

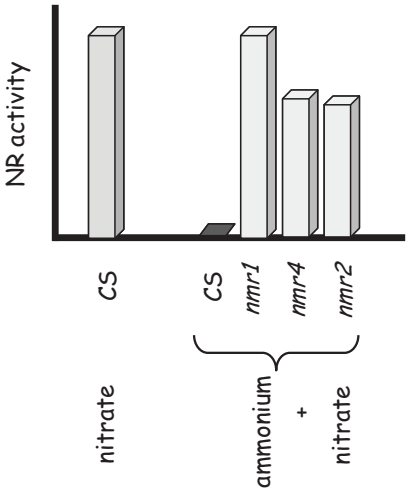

Fig. 15.9 Summary of *H. polymorpha nmr* mutants characterised so far (CS, control strain)

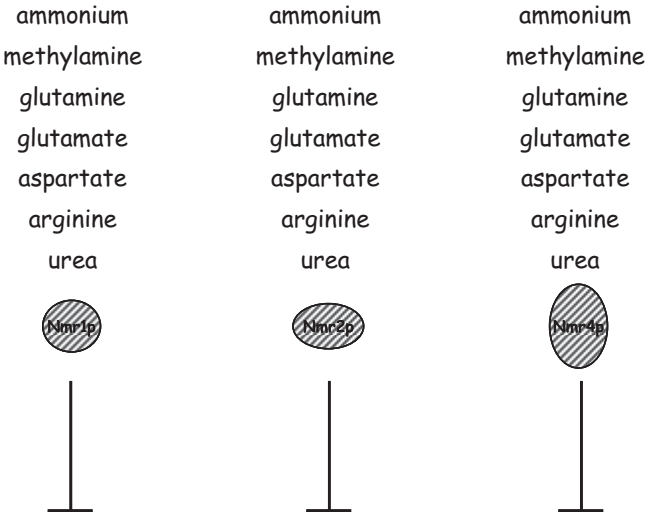

Fig. 15.10 Summary of Nmr1p, Nmr2p, Nmr4p roles. All three proteins are involved in *H. polymorpha* nitrogen metabolite repression of nitrate assimilation elicited by red, but not by green compound. Since Nmr2p is involved in Nmr exerted by all nitrogen compounds tested, it has been suggested that this protein may act downstream of Nmr1 and Nmr4p. Certain phenotypic traits of Nmr4 make it possible for this protein to act as an ammonium sensor. It has been suggested that a glutamine dependent circuit exists, separated from an ammonium dependent circuit

The characterisation of the *nmr4-1* mutant suggests that *NMR4* could act as a sensor also participating in ammonium assimilation. Further characterisation of this mutant, currently under way, may help to elucidate its role in nitrogen metabolite repression of *H. polymorpha*.

15.5 Conclusions

Interest in *H. polymorpha* has been steadily growing in recent years, both biologically and for commercial application. This yeast plays a leading role in peroxisome and heterologous protein expressions. As shown in this chapter, *H. polymorpha* is proving itself also an excellent model for nitrate assimilation studies. In spite of the small community involved in these studies, our understanding on *H. polymorpha* nitrate utilisation has deepened, adding significant molecular details to the previous portrait. In particular, recent exciting data concern transport studies, down-regulation of Ynt1p by ubiquitinylation processes and the role of Yna1p and Yna2p in the regulatory mechanisms controlling nitrate metabolism. As far as gene arrangement and functional organisation are concerned, it is noteworthy that *H. polymorpha* data, placed in relation to those of other well-established fungal models, have also a bearing in the evolutionary understanding of fungal nitrate assimilation. It is hoped that more research will soon enlarge this increasingly complex picture.

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Part II
Genetic and Molecular Insights

Chapter 16
Yeast Genetics and Biotechnological Applications

Saroj Mishra and Richa Baranwal

Contents

Abstract Yeast can be recognized as one of the very important groups of microorganisms on account of its extensive use in the fermentation industry and as a basic eukaryotic model cellular system. The yeast *Saccharomyces cerevisiae* has been extensively used to elucidate the genetics and regulation of several key functions in the cell such as cell mating, electron transport chain, protein trafficking, cell cycle events and others. Even before the genome sequence of the yeast was out, the structural organization and function of several of its genes was known. With the availability of the origin of replication from the 2 μm plasmid and the development of transformation system, it became the host of choice for expression of a number of important proteins. A large number of episomal and integrative shuttle vectors are available for expression of mammalian proteins. The latest developments in genomics and micro-array technology have allowed investigations of individual gene function by site-specific deletion method. The application of metabolic profiling has also assisted in understanding the cellular network operating in this yeast.

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This chapter is aimed at reviewing the use of this system as an experimental tool for conducting classical genetics. Various vector systems available, foreign genes expressed and the limitations as a host will be discussed. Finally, the use of various yeast enzymes in biotechnology sector will be reviewed.

Keywords Eukaryotic model, plasmid, genomics, micro-array technology, metabolic profiling

16.1 Introduction

Yeasts are single-celled, eukaryotic, saprophytic organisms which are more complex than bacteria. For typical budding yeast such as *Saccharomyces cerevisiae*, the size of the cell may be about 3–7 μm in width and 5–15 μm in length. This may increase to 15–20 μm during cell division. However, in case of fission yeast such as *Schizosaccharomyces pombe*, the cells can elongate to 18–20 μm at the time of cell division. In terms of structural organization, the yeasts are similar to other eukaryotic cells and are characterized by the presence of nucleus, mitochondria, large vacuoles etc. The DNA in *S. cerevisiae* is organized into 16 chromosomes (haploid number). The ease with which mutants can be isolated, characterized and mapped makes it an ideal system to carry out eukaryotic genetics. The pace with which the work can be carried out is as rapid as with the bacterial systems. Some of other properties that make it useful are its non-pathogenicity, ease of cultivation, rapid growth (the doubling time on glucose is 90 min and about 3.5–4 h on a non-fermentable carbon source), application of replica plating methods, mutant isolation and a well-defined haploid and a diploid life cycle. Various stable biochemical mutants can be isolated, many of them for essential functions, and expressed in the haploid state and complementation tests carried out in diploids to study the segregation of genes. Since the products of mitotic division are held together in a 4-spored ascus, the analysis of their phenotype gives a useful tool for following segregation of markers. Baker's yeast cells are also available commercially and provide a cheap source of cells.

Two significant findings have led to revolutionary advances in application of modern molecular biological techniques. These were the 'discovery' of the 2 μm plasmid, the origin of replication of which was used for construction of numerous stable plasmid vectors. The other was the development of a transformation system in *S. cerevisiae*. During early years, a large number of structural genes from this yeast were identified from plasmid libraries by complementation analysis in bacterium *Escherichia coli.* Many of the plasmids (Integrative YIp series) could enter into yeast chromosome by homologous recombination. External DNA containing partially homologous sequences can therefore be directed to specific locations in the yeast genome. Coupled with the high levels of gene conversions noticed in yeast, these have led to direct replacement of engineered sequences into their normal chromosomal locations. In fact, a library of mutants for each of the annotated genes

was constructed at Stanford University. These single gene knock-out libraries are available through commercial sources for use by scientists.

Very recently, transformations with single synthetic oligonucleotides have been reported in yeasts allowing manipulations of genes encoding proteins. This technique has been exploited for studying gene regulation, structure- function relations, chromosome structure etc. A number of mammalian proteins are also being expressed in yeast to study their functions. With the availability of the genome sequence of *S. cerevisiae,* a new way of doing science in this yeast has emerged.

16.2 Yeasts of Biotechnological Importance

A large number of genera have been found to be of industrial importance but the most commonly used yeast has been *S. cerevisiae*. Therefore, a reference to 'yeast' is associated with this genus and species. Yeasts have been used for preparation of wine, beer and bread for thousands of years. Due to a large body of information available on its life cycle, genetics, plasmid (the 2 μm plasmid), it was the first yeast to be used for production of heterologous proteins such as interferon (Hitzeman et al., 1981), hepatitis B surface antigen (Valenzuela et al., 1982). Many other yeasts such as *Arxula adeninivorans*, *Kluyveromyces lactis, Hansenula polymorpha, Pichia pastoris, S. pombe, Yarrowia lipolytica* have also been used successfully for production of heterologous proteins.

The choice of the alternate yeast systems is based on their ability to utilize cheap carbon sources. *Hansenula, Candida,* and *Pichia* have been used as hosts due to their ability to use methanol as a carbon source (Gleeson and Sudbery, 1988). C_1-compounds as sole source of carbon are utilized by *Candida boidinii* and some species of *Torulopsis* (Harder and Veenhuis, 1989; Sahm, 1977). These also use inorganic nitrogen source such as methylated amines increasing their versatility. Utilization of methanol by *Pichia* involves action by alcohol oxidase that converts methanol into formaldehyde. This is converted into carbon dioxide and water by consecutive activities of formaldehyde dehydrogenase and formate dehydrogenase. Hydrogen peroxide is also released as a by-product which is converted into water and oxygen by catalase. The promoter of methanol oxidase is inducible by methanol and repressed by glucose and ethanol which can serve to regulate induction of expression of foreign proteins. *P. pastoris* has been used for production of single cell protein (SCP) and heterologous proteins using the alcohol oxidase promoter. Some of these are described later.

Yeasts such as *Candida tropicalis*, species of *Torulopsis*, and *Y. lipolytica* have been selected based on their ability to utilize *n*-alkanes as a carbon source. Other hydrophobic substrates such as fatty acids and oils can also be utilized by *Y. lipolytica* (for review, see Fickers et al., 2005a) Some of these yeasts have been used for production of TCA cycle intermediates, vitamins and co-enzymes. The metabolism of *n*-alkanes is carried out by their conversion to fatty acids which are degraded to

CO_2 and H_2O by the β-oxidation pathway. The enzymes of this pathway are located in peroxisomes in these yeasts.

A more recently developed yeast for expression of foreign proteins is based on *Arxula adeninivorans*. In addition to being thermo- and halo-tolerant, it can grow on a number of carbon sources such as starch, tannic acid, *n*-alkanes and purines (for a recent review, see Steinborn et al., 2007a). Yeast such as *Brettanomyces naardenensis*, some species of *Pichia* and *Candida*, *Pachysolen tannophilus* are also capable of utilizing pentoses such as xylose for growth (Freer and Destory, 1983). As some such pentoses are a part of hemicellulose fraction in lignocellulosic complexes, there has been interest in use of these yeasts for biomass conversion. However, useful end products such as ethanol or acids are produced in low quantities by these yeast. Ethanol levels varying from 6.6–10.0 gl^{-1} have been obtained using *Candida shehate* and *Pichia etchellsii* (Kaur and Mishra,, unpublished data) and hence these have not been seriously considered for alcohol production. Since utilization of xylose requires additional enzyme (s), attempt has been made to express these enzymes in *S. cerevisiae* so that major fractions(hemicellulose/cellulose) of the lignocellulosics components can be utilized for alcohol production.

16.3 Genetic Material and Methods of Manipulation

The size of the yeast genome (about 15 Mbp) is about 4 times more than that of *E. coli* but unlike the latter, it is organized into 16 chromosomes. In the diploid cell, the number of chromosomes is 32. Several polyploids strains, the industrial strains, are extensively used but for general routine analysis of biochemical and genetic phenomenon, well defined yeast strains are used. Under light microscope, the nucleus is visible and is a distinguishing feature between bacterial and a yeast cell. Like most eukaryotes, the information is first processed into RNA's and then into proteins. A completely different mode of gene regulation is practiced in comparison to that seen in prokaryotes.

16.3.1 Chromosome Organization

Techniques such as pulse-field or orthogonal-field alternating gel electrophoresis have been used for the separation of the chromosomes. Each chromosome consists of a single double stranded DNA molecule (Fangman and Brewer, 1991) which is associated with histones H2, H3, and H4 to give rise to nucleosomes. Replication origins are located at an average distance of 40 kbp on the chromosome and are identical with autonomously replicating sequences (ARS). These can be identified by way of their imparting the property of autonomous replication to the plasmids harboring them. The size of the individual chromosome varies from 200–2200 kbp. Through the genome sequencing project, 6,183 open-reading frames (ORF's)

exceeding 100 amino acids were predicted out of which 5,800 were confirmed to correspond to protein-encoding genes (Sherman, 2006). The yeast genome is highly compact and 72% of DNA codes for genes. The average size of the gene is estimated at 1.45 kb, or 483 codons with a range from 40 to 4910 codons. A small proportion (3.8%) of ORF does contain introns which are predominantly in tRNA encoding genes. In spite of a large body of genetic information available for yeast, only 30% of predicted genes have been characterized experimentally. One of the most interesting features is the presence of retrotransposons, the movable DNA elements, which vary in number and position on the chromosomes in different strains of *S. cerevisiae*. The number is about 30 in common laboratory strains. The telomeres (TEL) are located at each end of the chromosome and are required for the stability of the chromosomes. These elements have been studied recently to understand their role in cell aging. Many of the chromosomal elements have been used (ARS, centromere CEN, TEL) to construct useful vectors for yeasts. Some yeasts such as *S. pombe* contain many genes with introns.

16.3.2 Extrachromosomal Materials

S. cerevisiae contains an autonomously replicating plasmid, the 2 μm plasmid, in its nucleus which is present in about 60–100 copies. The plasmid has an origin of replication but does not appear to confer any selective advantage to the host as cir^0 cells (lacking the plasmid) are indistinguishable from the cir^+ wild type cells. However, the chromosomal mutation *nib1* causes a reduction in growth of cir^+ strains, due to abnormally high copy number of the 2 μm plasmid (Sherman, 2006). While the plasmid itself has not been used for expression of foreign proteins, the vectors derived from it are extensively being used. The plasmid encodes four genes *FLP* (A), *REP1* (B), *REP2* (C) and (D), an origin of replication, STP locus (for stabilization) and two 599 bp inverted repeat sequences. FLP encodes for a site specific recombinase which promotes flipping about FLP recombination targets (FRT) within the inverted repeats and converts A form of the plasmid to B form in which gene order has been rearranged by intramolecular recombination. Therefore cells contain two forms of 2 μ, A and B (Hollenberg et al., 1976). The function of (D) is not known. Occurrence of the recombination event right after the onset of bidirectional replication leads to the replication forks pointing in the same direction. Such an event leads to formation of large number of plasmid molecules which can be resolved by another round of recombination. Other yeasts, such as *K. lactis*, some species of *Zygosaccharomyces* have been found to contain plasmids as well but these do not bear any sequence similarity to the 2 μm plasmid nor have these been used so extensively for vector construction.

Mitochondria contain the mitochondrial DNA which is 70–76 kbp in length and is present in 50 copies/cell. It has 15 genes that code for the proteins involved in the mitochondrial translational machinery, subunits of the respiratory chain and, some tRNA genes. About 15% of the mitochondrial proteins are coded for by this

DNA while the remaining are coded for by chromosomal DNA. Lack or mutations in the mitochondrial DNA lead to *rho*(ρ-) phenotype and these cells are respiratory deficient due to lack of subunits of some respiratory enzymes (cytochrome b, cytochrome c oxidase, ATPase complexes). These cells, however, are viable and still retain mitochondria, although morphologically abnormal.

All yeast strains contain double stranded (ds) RNA viruses that constitute about 0.1% of the total nucleic acids. The viruses have been categorized into three categories, L-A, L-BC, and M. Two other categories (till now considered non-viral) T and W are also found. M dsRNA encodes a toxin, and L-A encodes the major coat protein and components required for the viral replication and maintenance of M. The M and L-A dsRNA's are packaged into virus particles separately with the common capsid protein coded for by L-A. These particles are also transmitted cytoplasmically during vegetative growth and conjugation in a non-chromosomal inheritance pattern. L-B and L-C are similar to L-A, have an RNA dependent RNA polymerase and are present in intracellular particles. Lack of M dsRNA leads to *KIL*-o phenotype and these cells are readily induced by growth at elevated temperatures, and chemical and physical agents (Sherman, 2006). Yeast also contains a 20S circular single-stranded RNA that appears to encode RNA –dependent RNA polymerase that acts as an independent replicon. The segregation of this nucleic acid follows the non-Mendelian route.

16.3.3 Yeast Manipulations

The yeast *S. cerevisiae* is an ideal organism accessible to various genetic manipulations due to a well defined life cycle, development of transformation protocols, availability of suitable auxotrophic strains and availability of vector systems. All these properties have been put together for the production of foreign proteins for medical, research and industrial use. Recombinant protein expression was first described in 1981 for human α-interferon (Hitzeman et al., 1981) followed by many other examples in the subsequent years. In this section, the features required for heterologous expression of proteins in *S. cerevisiae* and some other yeast hosts are described in detail.

16.3.3.1 Molecular Transformation

Exogenously added DNA molecules can be introduced into yeast cells by three different methods which overcome the permeability barrier of thick cell wall. Molecular transformation of yeast was first achieved by a technique which involved the production of spheroplasts (wall-less yeast cells) with enzymes such as β-glucuronidase or zymolyase in an osmotically stabilized medium containing sorbitol (Hinnen et al., 1978; Beggs, 1978). The yeast spheroplasts are fused with PEG in the presence of $CaCl_2$ and DNA and finally plated out under selective conditions embedded in a top agar to facilitate regeneration of cell wall. This method is very commonly used and

is most efficient in terms of transformation frequencies per microgram of transforming DNA (10^5–10^6/μg DNA). It suffers from three disadvantages which are (i) the diploid and the triploid cells are formed by cell fusion using PEG, (ii) the screening procedure becomes difficult as the transformants are embedded in the agar overlay, and (iii) the method is time consuming and laborious. The method is significantly affected by the phase in which the cells are taken. Also, the exposure to enzyme has to be carefully monitored. Hence, this method finds application only for special purposes such as transformation with gene libraries and introduction of very large Yeast Artificial Chromosomes (YAC) based plasmids into the cell.

The more convenient methods were developed later in which the yeast cells were made competent by lithium salts (Ito et al., 1983) or use of electroporation (Becker and Guarante, 1991). Both these methods are more convenient than the spheroplast method as colonies grow on the surface of the agar plate used for selection. The former method does not give significantly any higher frequencies of transformation but is less laborious and does not require any incubation at −70°C. The electroporation method has to be optimized for each yeast species used and depends on the availability of an electroporator. An order of magnitude higher transformation frequency has been reported using this method. The freeze method has also been reported to work efficiently for *S. cerevisiae* as well as many other yeasts (Klebe et al., 1983). It has a distinct advantage in that it is independent of the yeast strain used and the cells can be used for long term. A rapid and convenient method which uses cells directly from agar plate involves agitation of cells with glass beads to introduce competence (Constanzo and Fox, 1988). All yeast strains are not equally susceptible to transformation, and the transformability of a single strain may vary from method to method.

The newly introduced DNA can meet one of the two fates inside the cell depending upon if it has an origin of replication (ORI) or not. If it contains an ORI (derived from the 2 μm plasmid) or the CEN sequence of the chromosome, it can be maintained independently in the cytoplasm. Since the incoming DNA has an auxotrophic marker, it can be maintained by growing the transformed cells on a selective medium. The only disadvantage is that in the absence of a selection pressure the plasmid is likely to be lost. In the second method, since the plasmid does not contain an origin of replication, the newly introduced DNA becomes integrated into the chromosome following homologous recombination. In such a case, the plasmid is inherited stably in spite of lack of a selection pressure. This method also finds applications in the production of heterologous proteins in yeast and will be discussed later.

16.3.3.2 Selectable Markers

16.3.3.2.1 Auxotrophic Selectable Markers

The most commonly used markers include *HIS3, LEU2, LYS2, TRP1*, and *URA3*, which complement specific auxotrophic mutation in yeast, such as *his3-Δl, leu2-Δl, lys2-201, trp1-Δl and ura3-52* (Beggs, 1978; Rose et al., 1984; Struhl et al., 1979; Tschumper and Carbon, 1980). These mutants have low reversion rates, hence give

Table 16.1 Selectable markers used in *S. cerevisiae* transformation

Markers	Marker type: dominant (D)/ Auxotrophic(A)	Comments	References
URA3	A	(a) Selection possible in casamino acid (CAA)	
		(b) Counter selection with 5- fluoro-ortic acid (5-FOA)	Boeke et al., 1989
		(c) URA3-d for high copy no. selection	Loison et al., 1989
LYS2	A	(a) Counter selection using α-amino adipate	Barnes and Thorner, 1986; Chattoo et al., 1979; Fleig et al., 1986
TRP1	A	Selection in CAA	
HIS3	A		
LEU2	A	LEU2-d for high copy no. selection	
Cm^r(Chloramphenicol-resistance) gene	D	(a) Selection using chloramphenicol in glycerol medium	Hadfield et al., 1986
		(b) Effective only using yeast promoter	
Herpes simplex virus thymidine Kinase gene [HSV TK]	D	(a) Thymidine/ Sulphanilamide/ amethopterin selection	Zealey et al., 1988
		(b) The level of resistance dependent on gene dosage	
S. pombe triose phosphate isomerase gene	D	(a) Marker used in *S. cerevisiae* tpi^- host	Kawasaki, 1986
		(b) autoselection in glucose	
Tn903 Kam^r	D	(a) Selection using G418	Hadfield et al., 1990

low background in transformation. The selection requires minimal growth media lacking the relevant nutrient. A description of selectable markers employed for *S. cerevisiae* transformation is given in Table 16.1.

LYS2 and *URA3* are versatile as both positive and negative selections are possible. The positive selection is carried out by auxotrophic complementation of the *ura3* and *lys2* mutation. Negative selection of the *lys2* and *ura3* cells is based on toxic antimetabolites a-aminoadipic acid and 5-fluoro-ortic acid respectively,

which prevent growth of the prototropic strains and allows mutants to grow (Boeke et al., 1984; Barnes and Thorner, 1986; Chattoo et al., 1979; Fleig et al., 1986). The use of these genes requires a suitably marked recipient strain. As the mutations are recessive, therefore the recipient is haploid or a specifically made homozygous diploid. Industrial strains are often polyploid and their genetics is poorly defined. A suitable recipient is difficult to obtain, therefore dominant selectable markers need to be employed when dealing with industrial yeast strains.

16.3.3.2.2 Dominant Selectable Markers

The dominant markers increase the range of host strains and can be used for selection in rich medium. Some examples are resistance to G418 conferred by Kanamycin resistance gene of Tn903 which codes for neomycin phosphotransferase II (Webster and Dickson, 1983), chloramphenicol acetyl transferase (Jimenez and Davies, 1980), hygromycin β phosphotransferase (Gritz and Davies, 1983), copper resistance conferred by copperthionein *CUP*^{R} gene (Fogel and Welch, 1982).

Autoselection: Autoselection systems have also been developed to ensure plasmid selection irrespective of culture conditions. The expression of cDNA encoding the yeast killer toxin and immunity gene could be used for self selection of transformants of industrial and research laboratory yeast since plasmid containing cells kill plasmid free cells (Bussey and Meaden, 1985).Another system used *ura3 fur1* strains as host cells for plasmids containing the *URA3* gene. Both, the *de novo* and salvage pathway for uridine 5 -monophosphate synthesis are blocked, hence these are non-viable, therefore maintenance of a *URA3* plasmid is obligatory for viability even in uracil containing media. The transformant was obtained by mating a *fur1* strain with an *ura3* strain containing the *URA3* plasmid and selecting the plasmid containing *ura3 fur1* progeny (Loison et al., 1986).

16.3.3.3 Yeast Vectors

16.3.3.3.1 Yeast Integrating Plasmids (YIp)

These are simple plasmids which do not replicate autonomously but integrate into the yeast genome by homologous recombination (Orr-Weaver et al., 1983). They lack ARS sequences and must carry at least one region homologous to a yeast chromosomal sequence. The transformation with YIp is not very efficient and < 10 transformants per μg of transforming DNA are obtained. However, the recombinant strains obtained with YIp are highly stable, even in the absence of selective pressure.

The YIp vectors integrate as a single copy. Multiple integration occurs at low frequencies. YIp plasmids with two yeast segments such as *YFGI* and *URA3* markers, can integrate at either of the two genomic loci. Repetitive DNA sequences, such as the rDNA and Ty elements in the vector can promote integration at any of the multiple sites in the genome. Multi-copy integrants are relatively stable and have

been used in gene dosage studies (Cashmore et al., 1986). These are also preferred for high level of expression of recombinant proteins. YIp is the vector of choice for the experiments in which recombinant yeast must retain the cloned gene for long periods in culture. Transplacement, an alternative type of integration, makes use of double homologous recombination to replace yeast chromosomal DNA (Rothstein, 1983). Such vectors contain the exogenous DNA and selection marker flanked by yeast DNA homologous to 3′ & 5′ regions of chromosomal DNA to be replaced. Before transformation, the vector is digested with restriction enzymes which liberate the transplacing fragment with 5′ and 3′ homologous ends.

A hybrid vector system comprising of the YIp and two terminal direct repeat (δ) sequences of the yeast retrotransposon Ty was constructed. The Ty element has two δ sequences one of which remains at the original site after transposition of the Ty retrotransposon. These sequences are distributed on each chromosome of most laboratory strains of *S. cerevisiae* and provide sites for single and multi-copy integration. Expression of several foreign proteins such as α-amylase, human nerve growth factor was reported using this system (Sakai et al., 1991). The integration was stable over a period of 50 generations under non-selective conditions.

16.3.3.3.2 Episomal Vectors (YEp Plasmids)

The vectors derived from the 2 μm plasmid are called Yeast Episomal Vectors (YEps). The copy number of these vectors varies from 10–40 per cell. Some YEp vectors contain the 2 μm Ori and the *REP3* gene whereas others contain the entire 2 μm plasmid. Plasmids of the former type must be propagated in cir^+ hosts containing the entire 2 μm plasmid as action of other genes (such as *REP1* and *REP2*) of the plasmid is required for accurate portioning of the plasmids into daughter cells. The most commonly used expression vectors are *E. coli* - yeast shuttle vectors based on the 2 μm plasmid (Armstrong et al., 1989; Broach, 1983; Parent et al., 1985). The first YEp vectors were pJDB219 and pJDB248 (Beggs, 1978). These vectors consist of the 2 μm plasmid combined with pBR322 for replication in *E. coli* and *LEU2* gene for selection. They are relatively stable and transform with high frequency of 10^4–10^5 transformants μg^{-1} DNA. PJDB207 (Beggs, 1981) and YEp13 (Broach, 1983) are smaller vectors containing only origin and STB locus. They are relatively stable and transform with high efficiencies. Many other YEP plasmids are reported to be unstable and tend to be lost at the rate of 1/100 or more cells after each generation. Under conditions of selective growth, only 60–95% of the cells retain the YEp plasmids (Sherman, 2006). Mutations in several key genes have been designed to develop high copy number plasmids of the YEp type.

16.3.3.3.3 Yeast Autonomously Replicating Plasmids (YRps)

These vectors contain the yeast autonomously replicating sequences (ARS sequence) instead of the 2 μm plasmid origin of replication and allows the transformed plasmids

to propagate several hundred folds. YRp7 is an example of such type of replicative plasmid. It is made up of pBR322 and a yeast gene *TRP1*. *TRP1* is involved in tryptophan biosynthesis and located adjacent to chromosomal origin of replication. YRps give 10^3–10^4 transformants μg^{-1} of DNA. These vectors are highly stable and present in multiple copies per cell (1–20). The plasmid free cells accumulate at a rate of up to 20%/generation without selection. This is due to inefficient transmission to daughter cells during cell division (Murray and Szoatak, 1983). The proportion of plasmid-containing cells can be very low even when grown under selection.

16.3.3.3.4 Yeast Centromere Plasmids (YCps)

Yeast ARS vectors can be made stable by addition of yeast centromeric sequences (CEN). In this case, copy number is reduced to 1–2 per cells (Clarke and Carbon, 1980). The CEN function is dependent on three conserved domains, all of which are required for stabilization of the YCp plasmids. Yeast ARS vectors are hardly ever used for foreign gene expression. However, ARS/CEN vectors are used where low level expression is required. These mimic the natural chromosomes in that they segregate to two of the four ascospores in the ascus. The low-copy number & stability of YCp vectors makes them ideal for cloning and construction of genomic DNA libraries. ARS1 is most commonly used ARS element for YCp vectors. Among the centromeres, CEN3, CEN4 and CEN11 are the ones which can be manipulated conveniently. YCp plasmids generally segregate in a Mendelian fashion during meiosis. The vector YCp50 contains ARS1 and CEN4. Some commonly used yeast transforming vectors are shown in Fig. 16.1.

Disintegration Plasmids: Disintegration vectors are also based on the 2 µm plasmid (Chinery and Hinchcliffe, 1989). The sequences necessary for propagation in bacterial cells are bound by targets for the 2 µm FLP gene product. The excision and consequent loss of these bacterial sequences occurs upon transformation of yeast cells. The expression cassette is cloned into REP1 locus of the 2 µm circle. Such constructs are completely stable and are present in high copy number. Some commonly used yeast vectors are listed in Table 16.2.

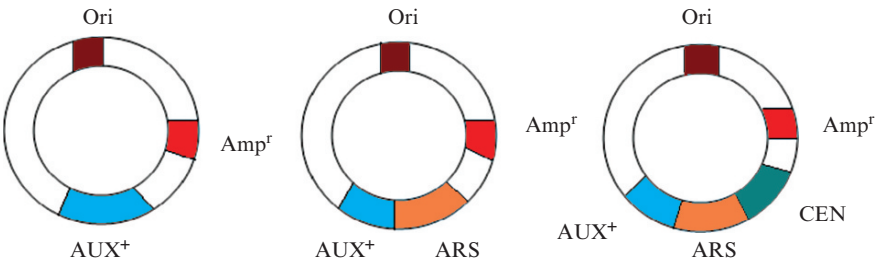

Fig. 16.1 Yeast transforming vectors. Ori: origin of replication; Amp^r: ampicillin resistant gene**;** AUX$^+$: wild type allele of yeast auxotrophic marker; ARS: autonomous replication sequence**;** CEN: centromere sequences from yeast

Table 16.2 Some commonly used *S. cerevisiae* vectors and their important features

Vector	Yeast sequences	Copy number/ cell	Transformation frequency/μg DNA	Stability	Advantages	Disadvantages	Reference
Yip	Homologous DNA	≥ 1	10^1–10^2	Less than 1%	• Provide most stable maintenance of cloned genes • Integrated YIp behave as genetic marker • Used to introduce inversions, deletions & transpositions	• Transformation frequency low	Hinnen et al., 1978
Yep (2μ based)	ORUI, STB, REP1, REP2, FLP	50–200	10^4	1%	• High copy no. plasmid • High transformation frequency • Readily recovered from yeast cells • Useful for complementation studies	• Novel recombinants can generate in vivo by recombination with endogeneous 2μm plasmids	Futcher and Cox, 1984
YCp	ARS/CEN	1–2	10^2–10^4	Less than 1%	• High transformation frequency • Useful for complementation studies • Show Mendelian segregation at meiosis • Low copy no is useful if product of gene is deleterious to cells	• Low copy no. • Recovery of vector is more difficult than YEp & YRp plasmids	Clarke and Carbon, 1980
YRp	ARS	1–20	10^3–10^4	20%	• High copy no. • Readily recovered from yeast	• Transformants are unstable	Murray, 1987
Ty/YIp	Ty & DNA	Depends on the vectors used to introduce Ty into the cell	≤ 20	Stable	• Amplification following chromosomal integration	• Needs to be introduced into cell in another vector	Sakai et al., 1991; Shuster et al., 1990

YAC	TEL, ARS, CEN	Stability depends upon length longer the YAC is more stable it is	• Very long DNA molecules > 40 Kb can be cloned	• Difficult to map by standard techniques	Sambrook and Russell, 2001

16.3.3.4 Yeast Artificial Chromosomes (YACs)

YACs are specialized vectors capable of accommodating extremely large fragments of DNA (100–1000 kb). Libraries of mammalian genomic DNA have been constructed that contain over 1 Mb of DNA (Sambrook and Russell, 2001). The architecture of these vectors mimics that of the eukaryotic chromosome in that it contains an ARS, a centromere, two telomeres and two yeast selectable markers separated by unique restriction sites. Each arm also contains a nutritional marker and an appropriately oriented DNA sequence that functions as a telomere. One of the two arms contains the ARS sequence. These also contain sequences for replication and selection in *E. coli*. YACs are linear molecules when propagated in sequences between the tips of the telomere for propagation in bacteria. Large YAC constructs are as stable as natural chromosomes. The transformants containing the YAC can be identified by plating on minimal medium. The insertion of foreign DNA into the cloning site interrupts a tRNA suppressor gene resulting in the formation of red colonies in strains carrying an ochre mutation in the *ADE2* gene. The sequences which are not possible to clone in *E. coli* cosmid and Lambda vectors can be cloned successfully in YAC vectors. They are powerful vectors for construction of genomic DNA libraries even from complex genomes such as human genome (Burke et al., 1983). The important components of the YAC are shown in Fig. 16.2.

16.3.3.5 Yeast Expression Vectors

Yeast expression vectors employ a number of promoters and terminators in addition to gene of interest in the vector backbone.

Yeast promoter: The first requirement for an expression vector is an efficient promoter. Table 16.3 lists some of the promoters which are in common use for expression of heterologous proteins in *S. cerevisiae.* Constitutive promoters are derived from the genes of glycolytic pathway such as alcohol dehydrogenase (*ADH1*), pyruvate kinase (*PYK1*), phosphoglycerate kinase (*PGK1*) and enolase (*ENO*). They lead to high level of transcriptional expression. On the other hand, regulated promoters (some derived from galactose utilizing pathway, acid phosphatase) can be controlled by controlling the availability of certain nutrients. Some heterologous promoters have also been found to be effective in *S. cerevisiae*. Downstream of the promoter there must be convenient sites for restriction enzymes for insertion of genes to be expressed. There is no particular difficulty in finding a suitable poly-linker, though an A-rich environment should be sought (Kingsman et al., 1985).

Yeast terminator: Transcriptional termination of yeast mRNA is less well understood than in bacteria and higher eukaryotes. It appears that yeast mRNA follows the same pattern of termination, processing and polyadenylation of pre-mRNA as higher eukaryotes. In yeast, these processes are tightly coupled and occur within a short distance near the 3′ end of the gene (Butler et al., 1990). A number of consensus sequences have been implicated as part of mRNA terminator, especially the tripartite sequence TAG.

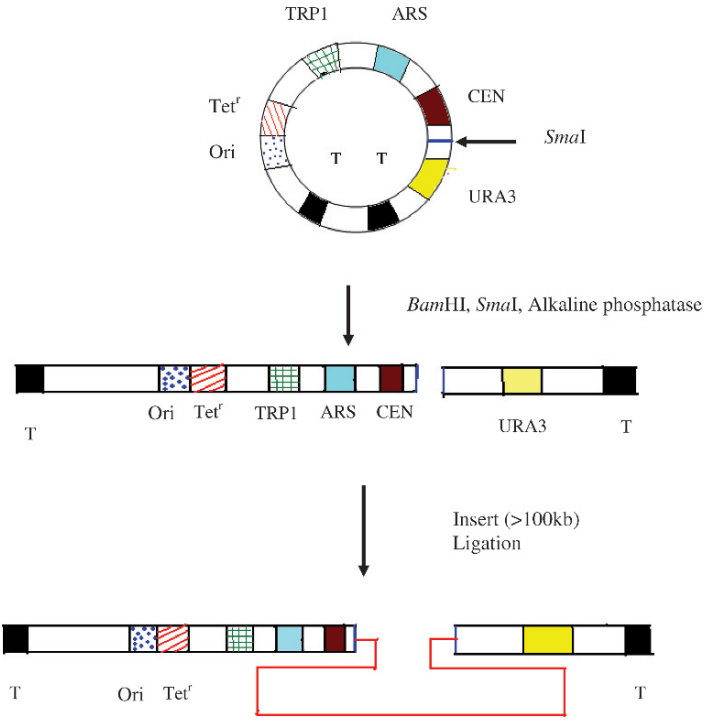

Fig. 16.2 YAC cloning system- pYAC has *E.coli* ori and selectable marker gene (Tet^r); and yeast DNA sequences TRP1, URA3, ARS and CEN. T represents telomeric sequences. URA3 is a gene in uracil biosynthesis pathway and TRP1is a gene of Tryptophan biosynthesis pathway

(T-rich)..TA (T) GT..(AT-rich)..TTT (Zaret and Sherman, 1984) and TTTTTATA (Henikoff and Cohen, 1984). Terminators from a number of genes have been used in expression vectors, e.g. *ADH1, CYC1, GAP, MF1*, *TRP1,* etc. (Urdea et al., 1984; Mumberg et al., 1995; Hitzeman et al., 1981; Rosenberg et al., 1984; Brake et al., 1984). Table 16.4 lists the eukaryotic therapeutic proteins expressed in *S. cerevisiae.*

16.4 Expression of Proteins in Non-*S. cerevisiae* Yeasts

In many cases, *S. cerevisiae* expression system is disadvantageous for large-scale production of many foreign proteins. The major drawbacks were the lack of very strong, tightly regulated promoters, need for fed-batch fermentation for attaining high cell densities and hyper-glycosylation of proteins. Therefore, other yeast expression systems have been developed. Some of the most commonly used ones are described below.

Table 16.3 Promoter systems used for expression of heterologous proteins in *S. cerevisiae*

Promoter	Gene	Protein encoded	Regulation	Strength	References
Constitutive	*ADH1*	Alcohol dehydrogenase1		+++	Hitzeman et al., 1981; Bennetzen and Hall, 1982
	PYK1	Pyruvate kinase	20-fold induced by glucose	+++	Burke et al., 1983
	PGK1	Phosphoglycerate kinase		++++	Tuite et al., 1982; Dobson et al., 1982
	ENO	Enolase	10-fold induced by glucose		Holland et al., 1981
Regulated	*ADH2*	Alcohol dehydrogenase 2	1000 fold-induced by galactose	++	Johnston and Davis, 1984
	GAL1.10.7	Galactose metabolic enzymes	100-fold repressed by glucose	+++	
	GALS	Galactokinase variant	-	+++	Mumberg et al., 1995
	MET25	O-acetyl homoserine sulphydrylase	200-fold repressed by phosphate	+	
	CUP1	Copper metallothionein	20- fold induced by Cu^{2+}	+	Karin et al., 1984
	PHO5	Acid phosphatase	100 to 200-fold induction with inorganic phosphate	++	Meyhack et al., 1982; Kramer et al., 1984
	tetO-CYC1	Tetracycline promoter	1000-fold induction with tetracycline	+++	Gari et al., 1997
Heterologous	*CaMV*	Cauliflower mosaic virus 35S promoter	RAS/cAMP pathway		Ruth et al., 1992
	ARE	Androgen response element	Dihydrotestosterone/testosterone		Eldridge et al., 2007

Table 16.4 Eukaryotic therapeutic proteins expressed in *S. cerevisiae*

Recombinant protein	Commercial name	Company	Therapeutic indication
Recombinant blood factor			
Hirudin/lepirudin	Refludan	Hoechst Marion Roussel (US)	Anticoagulant for heparin-associated thrombocytopenia
Hirudin/desirudin	Revasac	Canyon pharmaceuticals	Prevention of venous thrombosis
Recombinant hormone			
Insulin	Novolog	Novo Nordisk	Diabetes mellitus
Insulin	Exubera	Pfizer(New York) Aventis(Kent, UK)	Diabetes mellitus
Insulin	Apidra	Aventis (Germany)	Diabetes mellitus
Insulin	Liprolog	Eli Lilly	Diabetes mellitus
Somatotropin	Valtropin	Biopartners	Growth disturbances in children and adults
Glucagon	Glucagen	Novo Nordisk	Hyperglycemia
Recombinant enzyme			
r urate oxidase	Fasturtec	Sanofi-Synthetalco	Hyperuricemia
Recombinant Vaccine			
Hepatitis B	Ambirix	Glaxo Smith Kline	Immunization against hepatitis A and B
Hepatitis B	Pediarix	Glaxo Smith Kline	Immunization against hepatitis B
Hepatitis B	HBVAXPRO	Aventis Pharma	Immunization against hepatitis A and B
Hepatitis B	Infanrix-Penta	Glaxo Smith Kline	Immunization against diphtheria, tetanus, pertussis, polio and hepatitis B
Hepatitis B	Procomvax	Aventis Pasteur	Immunization against *H. influenzae* type b and hepatitis B
Hepatitis B	Primavax	Aventis Pasteur	Immunization against diphtheria, tetanus and hepatitis B
Hepatitis B	Twinrix	Glaxo SmithKline	Immunization against hepatitis A & B

16.4.1 Pichia pastoris

The most extensively developed system is based on *P. pastoris*. Molecular genetics method for *P. pastoris* like transformation, gene replacement, gene targeting and cloning by complementation are similar to the methods described for *S. cerevisiae.* This methylotrophic yeast has two key advantages over *S. cerevisiae* as a host for foreign

protein production (Cregg et al., 1993). Firstly, the promoter used to transcribe foreign gene is derived from methanol regulated *P. pastoris* alcohol oxidase 1 gene (*AOX1* promoter). In cells exposed to methanol as a sole source of carbon, transcription initiation at *AOX1* promoter is highly efficient and comparable to that of promoters which are derived from highly expressed glycolytic pathway genes (Waterham et al., 1997). Unlike glycolytic promoters, *AOX1* promoter is tightly regulated and highly repressed under non-methanol growth conditions. The second advantage of *P. pastoris* is that it is not a strong fermenter like *S. cerevisiae*. During fermentation, *S. cerevisiae* produces ethanol extracellularly, which in high density cultures, can rapidly build to toxic levels. This is called as Crabtree effect. *P. pastoris* strains are relatively easy to culture at cell densities of 100 g l^{-1} dry cell weight or greater (Siegel and Brierley, 1989). Strains have been developed for large-scale high yield production of single cell proteins using defined medium containing methanol (Wegner, 1983). Various process parameters such as pH, aeration, carbon source, feed rate likely to affect protein productivity, can be controlled in this yeast. It can also carry out post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing, similar to higher eukaryotic systems. Proteins which can not be expressed in bacteria or *S. cerevisiae*, such as G protein-coupled receptors, have been expressed in this system (Cereghino et al., 2002). Various aspects of the *P. pastoris* expression system have been described. The detailed protocols describing the construction of *P. pastoris* expression strains and foreign gene expression in this system have been given in the Invitrogen *Pichia* expression manual or readers are referred to Higgins and Cregg (1998). All *P. pastoris* expression vectors are shuttle vectors and some of the commonly used expression vectors are listed in Table 16.5.

Various factors affect the level of expression when *P. pastoris* integration vectors are used. In the first report of foreign gene expression in *P. pastoris*, higher proportion of immunogenic Human Hepatitis B surface antigens were produced in Mut^s (methanol-utilization slow) compared to Mut^+, even though expression levels were similar in both cases (Cregg and Madden, 1987). The various parameters affecting expression of tetanus toxin fragment C were investigated and equal levels were produced in Mut^+ and Mut^s hosts even when levels reached to 28% of total cell protein (Clare et al., 1991). The levels of expression were also not dependent on the type (single vs double cross-over) and the site of integration (*AOX1 vs HIS4*). A Mut^s integrant expressed *Bordetella pertussis* pertactin at 2% of total cell protein in shake flasks and 10% in fermenters, while a Mut^+ strain gave equal levels (5–6%) in shake flasks and fermenters (Romanos et al., 1991).

A combination of the *AOX1* promoter with *GAP* (glyceraldehyde 3-phosphate dehydrogenase) promoter in a strain expressing human granulocyte-macrophage colony-stimulating factor (hGM-CSF) resulted in a 2-fold increase in the production (180 mg/l) of recombinant protein (Wu et al., 2003b).The second alcohol oxidase promoter *AOX2* has also been used for production of recombinant human serum albumin (80 mg/l) by induction with 0.01% oleic acid (Kobayashi et al., 2000). Some other promoters that have been used are of the genes *FLD1* (formaldehyde dehydrogenase) and *ICL1* (isocitrate lyase). FLD1 is a key enzyme in metabolic pathway of methanol assimilation, is a tightly regulated promoter (like the *AOX1*)

Table 16.5 Commonly used *P. pastoris* expression vectors and their important features

Vector	Comments	Marker	Reference
pA0815	Expression cassette is between *Bam*HI & *Bgl*II for generation of multi copy expression vector	HIS4	Thill et al., 1990
pPIC3K	MCS for foreign gene expression; G418 selection for multicopy strains	Kan^r	Scorer et al., 1993b
pHIL-D2	*Not*I sites are present for *AOX1* gene replacement	*HIS4*	Sreekrishna, 1993
PHW 010	Constitutive promoter *GAP* controls the expression	*HIS4*	Waterham et al., 1997
pP1C9K	*AOX1* is fused to α-MF prepro signal sequence; *Not*I, *Sna*I1, *Eco*RI, *Xho*I, *Avr* II restriction sites for foreign gene insertion	*HIS4* & Kan^r	Scorer et al., 1993b
pGAP	*GAP* promoter fused to α-MF pre-pro signal sequence	ble^r	Invitrogen (Carlsbad CA)
pPICZα	MCS for cloning, *AOX1* promoter fused to α-MF pre-pro signal sequence	ble^r	Higgins and Cregg, 1998

and has good transcription efficiency (Shen et al., 1998). Nitrogen source also influences expression of proteins that are linked to the methanol dependent *FLD1* promoter. Higher expression levels of 19 U/ml were reported for *Rhizopus oryzae* lipase in *P. pastoris* which were higher than when only methanol and ammonium were used (Resina et al., 2004). While expression of dextranase using the *ICL1* promoter has been reported (Menendez et al., 2003), more work needs to be done to establish the usefulness of this promoter in *P. pastoris* expression system.

P. pastoris is capable of secreting high levels of foreign proteins. *S. cerevisiae SUC2* gene product invertase accumulated to 2.5 g l^{-1} in the medium (Tschopp et al., 1987b). In terms of post-translational modification, it is a better host for heterologous protein production than S. *cerevisiae* since the secreted products have been found to contain shorter (8–15) N-linked carbohydrate side chains than those secreted in *S. cerevisiae* (> 45). In addition to this, secreted proteins didn't contain terminal α1,3-mannose linkage (Trimble et al., 1991) which are present in *S. cerevisiae* and have been known to be immunogenic (Ballou, 1970). About 35% of N-linked oligosaccharides of endogenous glycoproteins from *P. pastoris* have less than 14 mannose residues (Grinna and Tschopp, 1989). Some proteins including bovine lysozyme (0.55 g l^{-1}) aprotonin (0.93 g l^{-1}) human serum albumin (3 g l^{-1}) and mEGF (0.45 g l^{-1}) have been secreted at high levels from *P. pastoris* (Digan et al., 1989; Thill et al., 1990; Clare et al., 1991).

Another advantage of *Pichia* system is ease of high-density growth and scale up with reduction in specific productivity (Cregg and Madden, 1987). This is important as concentration of secreted products in the medium increases with an increase in cell density. Since recombinant proteins produced in heterologous hosts can be proteolytically unstable, several process strategies can be applied to overcome this.

This includes altering the pH of the medium upon induction e.g. raising the pH to 6.0 (Clare et al., 1991b) or lowering the pH to 3.0 (Brierley et al., 1994; Koganesawa et al., 2002), providing amino acid rich supplements, lower cultivation temperature (which seems to favor protein stability), lowering specific growth rate, addition of protease inhibitors or a combination of these parameters. Several of these have been reviewed recently (Macauley-Patrick et al., 2005).

One of the major challenges using this expression system is the production and recovery of the membrane proteins which are largely hydrophobic. We have also successfully expressed extracellularly large (~90 kDa) cell wall bound proteins of related yeast in this system (Baranwal and Mishra, unpublished results).

16.4.2 Hansenula polymorpha

Apart from *P. pastoris*, methylotrophic yeast, *H. polymorpha* has also been used for heterologous protein expression. Several groups have developed a successful transformation system for *H. polymorpha* using *LEU2* and *URA3* genes from *S. cerevisiae* (Gellisson et al., 1991; Roggenkamp et al., 1986). The plasmid containing these markers complements a *leu H. polymorpha* mutant defective in enzyme β-iso-propylmalate dehydrogenase coded by *LEU2* in *S. cerevisiae.* The gene encoding methanol oxidase (*MOX*) was isolated and the promoter used to express foreign gene (Ledeboer et al., 1985). During growth on methanol, the enzyme accounts for 30–40% of total cell protein which is sequestered in peroxisomes (Giuseppin et al., 1988). In *P. pastoris* there is an absolute requirement for methanol in order to obtain significant expression of gene, however expression in *H. polymorpha* gene is de-repressed significantly during glucose limitation or in the absence of glucose (Eggeling and Samm, 1978; Egli et al., 1980). Therefore tight regulation of promoter is lost in the conditions used for high biomass fermentations (Gellisson et al., 1991). This expression system has been extensively reviewed (Gellissen, 2000; Kang and Gellissen, 2005).

A CoMed vector system has been recently reported (Steinborn et al., 2006) containing the pCoMed basic vector for integration of ARS, selection markers, rDNA sequences and expression cassettes. Various modules can be integrated in this system. The rDNA elements derived from *A. adeninivorans* and *H. polymorpha* clusters were assessed for their suitability as targeting sequences. The different combinations of the ARS, rDNA regions, selection markers and the expression cassettes have been reviewed (Steinborn et al., 2006). This gives the user flexibility to try a number of hosts simultaneously.

16.4.3 Kluyveromyces lactis

K. lactis is another well accepted system for production of foreign proteins of human use as it has been used in food industry for several years for production of β-galactosidase. It can grow on cheap substrates like lactose. This further increases

its potential as a host for production of foreign proteins, especially for low-value products. Other advantages are its faster growth and lack of hyperglycosylation of proteins (Hsieh and Da Silva, 1998).

S. cerevisiae ARS and 2 μm do not replicate in *K. lactis*, therefore transformation systems were developed by isolating *K. lactis* ARS (Das and Hollenberg, 1982; Sreekrishna et al., 1984). In *K. lactis* killer strains, two cytoplasmic linear plasmids k1 (8.9 kbp) and k2 (13.4 kbp) are present. They are stably maintained at 100–200 copies per cell. They have been considered as potential vector system. The region which encodes killer toxin in k1 can be deleted without affecting maintenance (Stark et al., 1990). By fusing markers like *LEU2* to k1 promoter, recombinant stable linear plasmids have been generated (Kamper et al., 1989; Tanguy-Rougeau et al., 1990). While this system can be used for foreign gene expression, the k1/k2 promoters are weak.

High copy number stable expression vectors of *K. lactis* have been constructed. They are based on *Kluyveromyces drosophilarum* plasmid, pKD1 which is very much similar to the 2 μm plasmid of *S. cerevisiae*. Vectors based on pKD1 have been constructed which show similarity to 2 μm vector (Bianchi et al., 1987). Various promoters have been used in *K. lactis* expression vectors e.g. *LAC4, GAL1, GAL7, GAL10, GAP*, alcohol dehydrogenase and *S. cerevisiae PGK* and *PHO5*.

A number of foreign proteins have been efficiently secreted in *K. lactis*. Prochymosin, which was poorly secreted in *S. cerevisiae* was efficiently secreted by *K. lactis* in soluble form using single copy integration vector (Van den Berg et al., 1990). Approximately 80% of the protein was secreted without a signal peptide, however, highest levels were obtained when native leader peptide or the α-factor pre-pro sequences from *K. lactis* or *S. cerevisiae* were used. The product is used on commercial scale for manufacturing various milk products. Using pKD1-derived vector, secretion of Human serum albumin (HSA) was also described (Fleer et al., 1991). Highest expressing strains produced 300 mg l^{-1} of protein in shake flasks using *S. cerevisiae PGK* promoter. High density fed batch fermentations were used to produce several g l^{-1} of HSA. Several other promoter systems, such as *K1ADH4*, have been used for ethanol-dependent production of HSA (Saliola et al., 2004). HSA-CD4 fusion protein, a potential therapeutic agent against HIV infection has also been produced using this system. The secretion of interleukin IL-1β has been reported by *K. lactis* (Fleer et al., 1991). Tissue specific inhibitor of metallo-proteinases (TIMP) and variants of tissue plasminogen activator (tPA) were secreted using secretion signal (Yeh et al., 1990).

A number of hydrolytic enzymes have been expressed in this system (Bergquist et al., 2002). Other enzymes include laccases from various white rot fungi (Piscitella et al., 2005). When compared to the production in *S. cerevisiae*, the recombinant product from *K. lactis* was secreted more efficiently. Another species, *Kluyveromyces marxianus,* has also been explored as a host for expression of heterologous proteins (Pecota and Da Silva, 2005) based on its short generation time and high growth rate at elevated temperature. A number of foreign genes have been expressed successfully in this yeast (Almeida et al., 2003; Ball et al., 1999; Bartkeviciute et al., 2000; Bergkamp et al., 1993; Pecota and Da Silva, 2005). Recently, a number of thermo-stable

cellulase genes were simultaneously successfully expressed in this organism under the control of high-expression promoters (Hong et al., 2007).

16.4.4 Schizosaccharomyces pombe

The fission yeast, *S. pombe* is well characterized and an intensely studied yeast. Its life cycle and growth characteristics are very well suited for genetic and biochemical analysis. It is genetically tractable and a number of fission-yeast specific plasmids have been developed to aid molecular manipulation of this yeast. Transformation of *S. pombe* was described using lithium salts (Heyer et al., 1986), spheroplasts (Beach and Nurse, 1981) and electroporation (Hood and Stachow, 1990). A very efficient method made use of lipofectin to enhance uptake of DNA by spheroplasts (Allshire, 1990). *S. pombe* expression vectors contain sequences from *S. pombe* ARS1 or from the 2 μm plasmid of *S. cerevisiae*. The ARS vectors have copy number of about 30/cells. Stability and copy number of ARS vector is enhanced by *S. pombe* derived STB sequences (Heyer et al., 1986).

A number of heterologous proteins have been expressed in *S. pombe*. Active factor XIIIa was produced at 2 mg l^{-1} using high copy number, alcohol dehydrogenase expression vector (Broker and Bauml, 1989). Epoxide hydrolase, human liver microsomal enzyme were expressed and isolated from microsomal fraction of the yeast (Jackson and Burchell, 1988). The expression of functional single-chain antibody molecules (Davis et al., 1991), large polyprotein of infectious bursal disease virus (Strasser et al., 1989), bacterio-opsin (Hildebrandt et al., 1989), xylose isomerase (Chan et al., 1986) and β-glucuronidase (Pobjecky et al., 1990) have been described. *S. pombe* galactosylates glycoproteins like acid phosphatase (Dibenedetto and Cozzani, 1975) and invertase (Moreno et al., 1985) have also been described. Ubiquinone, a component of the electron transfer system in many organisms, has been successfully expressed in this yeast (Zhang et al., 2007). The recombinant yeast could be cultivated under high-cell-density fermentations leading to production of the protein to the level of 23 mg l^{-1}. Plasmid stability was also maintained at high level throughout the fermentations.

16.4.5 Yarrowia lipolytica

Y. lipolytica is a dimorphic yeast which is unicellular in minimal medium containing glucose or n-hexadecane and forms mycelia in minimal medium containing olive oil and casein and gives a mixture of both forms in complex medium. It is very useful in various industrial processes like bioconversions of alkane and fatty acids to alcohols, production of secondary metabolites (citric acid, mannitol etc.) and production of SCP from n-paraffins. The yeast also secretes high molecular weight

proteins like lipases, proteases, ribonuclease and an alkaline extracellular protease. The inherent property of secretion has made it a subject of intense research for foreign gene expression. This yeast combines the facility of single cell use, high secretion abilities and availability of efficient tools for post-translational modifications. Multi-copy strains have been constructed facilitating large–scale production of foreign proteins. Mutant stains lacking extracellular proteases and lipases have also been constructed enabling high protein purity in the extracellular supernatant (Fickers et al., 2005b; Nicaud et al., 2002; Pignede et al., 2000a).

Transformation of *Y. lipolytica* was first achieved with lithium acetate which permeabilized the yeast cells (Davidow et al., 1985). The homologous *LEU2* gene was used to transform the cells. Vectors containing random *Y. lipolytica* genomic fragments inserted into the upstream region of *S. cerevisiae LYS2* gene for selection of transformed spheroplasts were also used (Gaillardin et al., 1985). In addition to *LEU2*, many other genes of this yeast have been used as markers, e.g. *LYS1, LYS5, ADE1, HIS1* and *URA3*. Several plasmids for gene expression and secretion have been developed for this yeast (Nicaud et al., 2002; Madzak et al., 2004). Alkaline extracellular protease pre-pro sequences have been used for secretion of bovine prochymosin (Franke et al., 1988) and porcine α-interferon (Heslot et al., 1990; Nicaud et al., 1991).

Proteins from viruses and different organisms, ranging in size from 6 to 116 kDa, have been expressed successfully in this yeast (Madzak et al., 2005).

16.4.6 Arxula adeninivorans

A. adeninivorans, a non-conventional dimorphic thermo- and salt-resistant yeast has been developed as a host for heterologous gene expression. The yeast is able to assimilate and ferment many compounds as sole source of carbon and/or nitrogen. It utilizes n-alkanes and degrades starch efficiently. *A. adeninivorans* is able to grow at cultivation temperatures of up to 48°C in media containing up to 20% NaCl. Additionally, the dimorphism of the yeast is unusual. *Arxula* grows at temperatures of up to 42°C as budding cells and turns into mycelia at higher temperatures. When the cultivation temperature is decreased below 42°C, the dimorphism is reversed and budding is re-established (Wartmann and Kunze, 2000). Alteration of morphology correlates with changes in secretion behavior. Mycelial cultures accumulate two-fold higher protein concentrations and contain two- to five-fold higher glucoamylase and invertase activities in the medium than budding cells. Based on these unusual properties, *A. adeninivorans* has been used for heterologous gene expression and as a gene donor to construct more suitable yeasts for biotechnology. Glucoamylase gene of *A. adeninivorans* was successfully expressed in *K. lactis* and *S. cerevisiae*.

Transformation system is used for heterologous gene expression. The transformants obtained are mitotically stable. Some of the unusual biochemical properties support its usage for production of many recombinant proteins. This

system primarily leans on integration vectors containing an expression cassette which contains the gene for the heterologous protein, a suitable selection marker and the rRNA targeting sequences. The basic *A. adeninivorans* transformation/expression vector pAL-HPH1 has been equipped with the *E. coli*-derived *hph* gene, conferring hygromycin B resistance, and the 25S rDNA from *A. adeninivorans* for rDNA targeting. Recombinants are based on the integration of linearized DNA fragments in 2–10 copies, e.g. into the 25S rDNA of *A. adeninivorans* by homologous recombination. Transformants were obtained for both budding cells and mycelia. In both cell types, similar expression levels were achieved and the green fluorescent protein was localized in the cytoplasm while more than 95% of the HSA accumulated in the culture medium (Wartmann et al., 2001). The undesired use of toxic hygromycin B for selection of the transformants forced the development an *ALEU2/AILV1* selection system. However, these were reported to be unstable.

Recently, a host/vector expression system based on *atrp1* gene disruption mutant has been developed. This host was transformed with the plasmid pAL-ATRP1-amyA (containing the *ATRP1* gene as the selection marker, 25S rRNA sequences for targeted insertion). Amylase (amyA of *Bacillus amyloliquifaciens*) production served as a marker for assessment of protein production. Good product levels were detected in the culture medium (Steinborn et al., 2007a) by integration of a single copy of the amylase gene. A fused vector element (consisting of the ATRP1 selection marker fused to a newly generated truncated ALEU2 promoter of 53 bp) allowed for multiple insertion of the vector containing the amyA protein. This resulted in superior productivity of the secreted recombinant α-amylase (Steinborn et al., 2007b). Clearly, a number of different strategies or a combination of these can be used to achieve higher expression of foreign proteins.

A partial list of expressed foreign proteins is given in Table 16.6 to provide the reader an idea of types of proteins that can be expressed using non- *S. cerevisiae* yeasts.

16.5 Industrial Enzymes

Over 500 different enzymes covering about 50 applications as in detergents, wine and beer making etc. are currently used in various industrial sectors. Due to different methods available in protein engineering, the scope of using enzymes is increasing. The enzymes can be grouped into three categories (Cherry and Fidantsef, 2003). The largest, comprising 65% of the sales are technical enzymes and include enzymes used in detergents, starch, textile, leather, pulp and paper and personal care products. The next segment (25%) is of food enzymes and includes enzymes used in dairy, brewery, wine juices, fats and oils, baking industries. The next (10%) are the feed enzymes. Today, over 90% of the enzymes are produced by recombinant organisms to maximize product purity and economy of production. As much as 40 g l^{-1} concentrations can be achieved.

Table 16.6 Partial List of foreign proteins expressed in non-*Saccharomyces* yeasts

Protein	Location	Promoter	References
P. pastoris			
HSA	Secreted	*AOX1*	Sreekrishna et al., 1989
Human EGF	Secreted	*AOX1*	Brierley et al., 1994
Murine EGF	Secreted	*AOX1*	Clare et al., 1991
S. cerevisiae invertase	Secreted	*AOX1*	Tschopp et al., 1987
Bovine lysozyme	Secreted	*AOX1*	Digan et al., 1989
Aprotinin	Secreted	*AOX1*	Thill et al., 1990
β-galactosidase	Intracellular	*AOX1,* DHAS	Tschopp et al., 1987
HBsAg	Intracellular	*AOX1*	Cregg and Madden, 1987
Tetanus toxin fragment C	Intracellular	*AOX1*	Clare et al., 1991
Pertactin	Intracellular	*AOX1*	Romanos et al., 1991
Streptokinase	Intracellular	*AOX1*	Hagenson et al., 1989
TNF	Intracellular		Sreekrishna et al., 1989
Hansenula polymorpha			
HBsAg	Periplasmic	*MOX, FMD*	Shen et al., 1989; Janowicz et al., 1991
PreSl -S2-HBsAg	Periplasmic	*MOX*	Janowicz et al., 1991
Human serum albumin	Secreted	*FMD*	Hodgkins et al., 1990
Glucoamylase	Secreted	*FMD*	Gellisson et al., 1991
Kluyveromyces lactis			
HSA, HSA-CD4	Secreted	*LAC4, S. cerevisiae PHO5, S. cerevisiae PGK*	Fleer et al., 1991
Prochymosin	Secreted	*LAC4*	Van den Berg et al., 1990
Schizosaccharomyces pombe			
Factor XIIIa	Intracellular	*adh*	Broker and Bauml et al., 1989
IBD virus VP3	Intracellular	*adh*, *S. cerevisiae ADH1*	Jagadish et al., 1990
Single chain antibody	Intracellular	*adh*	Davis et al., 1991
Antithrombin III	Secreted	*S. cerevisiae ADH1, S. cerevisiae CYC1*	Broker et al., 1987
Yarrowia lipolytica			
Bovine prochymosin	Secreted	*XPR2, LEU2*	Franke et al., 1988
Porcine IFN	Secreted	*XPR2*	Heslot et al., 1990; Nicaud et al., 1991
S. cerevisiae invertase	Secreted	*XPR2*	Nicaud et al., 1991

16.5.1 Yeast as a Source of Industrial Enzymes

Yeasts have great potential for production of microbial enzymes in the food and related sectors as this is considered as a GRAS organism and has been used for human consumption for a long time. These are unicellular, fast growing and can be

cultivated in reactors much like the bacterial cells. These can grow in simple media where growth can be monitored by simple optical density measurement method allowing for an in-depth study of growth, mass-energy balance studies and product formation kinetics.

Yeasts produce a large number of hydrolytic enzymes such as glucouronidase, glucosidases, lipases, xylanases, pectinases. Several genera such as *Candida, Saccharomyces, Pichia, Schizosaccharomyces, Kluyveromyces* are known to produce β–glucosidases. The enzymes are used as a supplement for cellulose hydrolyzing enzymes. These have also been reported (Pandey and Mishra, 1997; Bhatia et al., 2002b; Wallecha and Mishra, 2003) to carry out synthetic reaction leading to formation of glycoconjugates and will be discussed in the next section. Lipases are serine hydrolases and can be divided into following four groups according to their specificity in the hydrolytic reactions: substrate specific lipases, fatty acid specific lipases, regio- and stereo-specific lipases. They are useful for their hydrolytic as well as synthetic activities. Large amounts (nearly 1000 tonnes) of lipases are used in the detergent industry for removal of oil-based stains. Several synthesized structural lipids find application in infant formula and nutraceuticals. In the last decade, lipases are also finding a number of applications in the manufacture of pharmaceuticals, pesticides, single cell protein, biosensor preparation and in waste management. Lipases belonging to the genus of *Candida* (*Candida antarctica*, *Candida cylindracea* ATCC 14830, *Candida lipolytica*, *Candida rugosa*) and *Geotrichium candidum* are used as source of industrial lipases. *C. rugosa* lipase is one of the most extensively used lipase in the industry (Redondo et al., 1995). The steryl lipase activity of yeast *C. rugosa* finds applications for hydrolysis of steryl esters, resin acids found in wood, which would otherwise have negative impact on paper machine run ability and quality of paper. For a recent review of yeast lipases, the reader is referred to Vakhlu and Kour (2006).

Many yeasts are also reported to produce xylanases. These are *Candida guilliermondii*, *Cryptococus adeliae, Cryptococcus albidus, Filobasidium floriforme, Trichosporon cutaneum* SL 409. The pH optimum of these ranges from 4.5–5.5 and none of these is thermophilic. This low pH optimum makes it very unsuitable for application in pulp and paper sector. An extracellular acetyl esterase was isolated from *C. guilliermondii* which exhibited maximum activity at pH 7.5 and 50–60°C (Basaran and Hang, 2000) but this is more of an exception for the yeast enzymes. Some species of *Pichia* and *Kloeckera* have also been reported to produce low quantities of xylanases. Many yeasts belonging to the genera of *Debaryomyces* (*Debaryomyces hansenii*), *Hancornia* (*Hancornia speciosa*), *Stephanoaseus* (*Stephanoaseus smithiae*), *Kluyveromyce*s (*Kluyveromyces wickerhamii*) produce pectinolytic enzymes (da Silva et al., 2005). Several classes are reported under this category such as lyases (EC 4.2.2.10), pectate lyases (EC 4.2.2.2), polygalactouronase (EC 3.2.1.15 and EC 3.2.1.67). Pectinases are of major importance in beverage industry as these improve pressing and clarifications of fruit juices. Some enzymes are also used in production of wine, extraction of olive oil and fermentation of tea, coffee and cocoa.

16.5.2 β-Glucosidases from Pichia etchellsii

P. etchellsii is a yeast of oenological origin and produces multiple inducible β-glucosidases. While good ethanol production has been observed by this yeast on glucose, cellobiose and xylose, its use has been limited due to restricted knowledge of many of its enzymes. When grown on cellulase hydrolyzed Avicel cellulose, the yeast was found to produce ethanol equal to the expected theoretical yield (unpublished data). One of the advantages is that the temperature for saccharification and growth of the fungus can be adjusted as the yeast grows well between 40–50°C. The efficient wall bound β-glucosidases produced by this yeast aid in conversion of cellobiose and probably other short-chain cellodextrins to glucose. The sugars are then directly converted into ethanol.

Two wall bound inducible β-glucosidases, BGL I and BGL II, were purified from this yeast which exhibited differential substrate specificity on *p*-nitrophenyl–D-glucoside (*p*NPG) and cellobiose (Wallecha and Mishra, 2003). While the internal peptide sequences of BGL I were very similar to the putative β-glucosidase of *K. lactis* and β-glucosidase of *Kluyveromyces marxianus,* the peptide sequences of BGL II were similar to an unnamed protein of *Debaryomyces* (unpublished data). Both the enzymes have been placed in family 3 of the glycosyl hydrolase families. By way of cloning and expression, two more enzymes, namely Bgl I (Pandey and Mishra, 1997) and Bgl II (Sethi et al., 2002; Bhatia et al., 2005) were identified and studied. These multiple enzymes have been used for synthesis of various short chain cellooligosaccharides and other glycoconjugates. The synthesis of cellooligosaccharides (degree of polymerization 2–6) was demonstrated using Bgl I and Bgl II with glucose and cellobiose as the substrates. The separation of individual oligosaccharide is still a challenging problem in such synthetic reactions. We have reported some success in separation of these using a combination of charcoal adsorption and thin layer chromatography (Bacchawat et al., 2004). Transglycosylation reaction using *p*NPG as a donor and β-1-N-acetamido-D-glucopyranose, which is a glycosylasparagine mimic, as acceptor was also explored for synthesis of some special disaccharides. The yield of the reaction was 3% and both (1→3) and (1→6) linked disaccharides were synthesized using Bgl II (Kannan et al., 2004). The synthesized disaccharides β-D-Glc-(1→3)-β-D-Glc and β-D-Glc-(1→6)-β-D-Glc are important fragments of phytoalexin-elicitor oligosaccharides, which are involved in plant defense mechanisms. These oligosaccharides are regarded as essential players in the cellular communication between fungal pathogens and various plants (Geurtsen et al., 1999). In fact, the yeast cell wall contains 60% of these in their core structure. Many of these synthesized molecules may also serve to dislodge the pathogens from plants.

Another category of compounds, namely alkyl glucosides, are non-ionic surfactants which are useful for solubilization of membranes and for reconstitution of lipid vesicles (due to the amphoteric nature of the alkyl glucosides). The enzymatic synthesis of these molecules is of interest as the traditional chemical route involves a number of protection and de-protection steps. The use of toxic chemical catalysts

which remain at the end of the synthetic reactions is also not desirable. Very few microbial enzymes have been used for such synthesis and much of the work reported is with the almond β-glucosidase. We have reported synthesis of octyl-β-D-glucoside using BGL I (Wallecha and Mishra, 2003), BGL II (Wallecha, 2002) and Bgl II (Bacchawat et al., 2004). The molar yield of octyl glucoside was about 12% using BGL I in dimethyl sulfoxide stabilized single-phase reaction microenvironment. The yield of this compound was still increased to 40% (molar ratio with respect to glucose) by control of water activity in the system which favored the synthetic reactions over the hydrolytic ones (Mishra et al., 2007). The enzyme was also used successfully for driving the synthetic reactions with carbon chain length up to 12 to synthesize dodecanol glucosides.

The flavour compounds in various fruits are the various glucosides of terpene alcohols. There has been considerable interest in synthesis of these molecules using β-glucosidases. It is also expected that these will serve as a source of flavor precursors in fruit juices. We (Bacchawat et al., 2004) and others (Gunata et al., 1994) have reported synthesis of various monoterpene glucosides using β-glucosidase.

While the use of β-glucosidase for hydrolytic and synthetic reactions is widely accepted, a new finding could propel these enzymes into a different application. Recently, a new β-glucosidase-like activity has been reported from *P. etchellsii*. The purified enzyme of 50 kDa molecular mass was active on methyl-β-D-glucopyranoside (MUG), but very little activity was detected on *p*NPG, which is the most commonly used substrate for detection of β-glucosidase activity. Further, the gene sequence did not bear any significant sequence identity with either members of family1 or family 3 β-glucosidases (Roy et al., 2005). Apparently, the enzyme has a domain of hydrolytic activity similar to generic β-glucosidases but has some sequence similarity to GTP binding domains of other proteins (Mishra and Mishra, unpublished data). The sequence contained large stretches of Ser-Asp (SD), a feature shared with SD rich sequences of cell surface associated proteins from human pathogenic *Staphylococcus aureus* (McDevitt et al., 1994). The latter enzymes have been shown to bind to fibrinogen and assist in adhesion to human cells. Thus, the presence of SD repeat containing proteins in yeast cells is likely to throw new light on yeast cell surface properties and associated enzymes.

16.6 Conclusions

In this review, an overview of the yeast genetic material and its organization is presented. This has been largely possible due to the genome project. Out of nearly 6000 annotated genes, functional nature of only 40–45% is known. Some unique features of *S. cerevisiae* are lack of introns in the chromosomal genes whereas in the related *Sch. pombe* relatively large number of intron containing genes has been discovered. A close examination of the genomes could give features unique to Baker's yeast. Through an extensive collaborative project, single gene knockout mutants are commercially available. This is significant for two reasons (Astromoff

and Egerton, 1999): (i) it may allow to define the minimum genome size and (ii) the function of each of the putative genes can be elucidated. Although many of these genes may not have a specific 'phenotype' but their effect on the overall transcriptome and proteome can be analyzed in greater detail with the use of modern technologies such as DNA micro-array and Tandem mass spectrometry. These studies will also result in a better understanding of regulatory circuits operating in the cell. The vast amount of data has already propelled development of new computational methodologies.

The production of many valuable enzymes by different yeasts has been discussed. Since many of the yeasts are amenable to genetic manipulations, have a well-defined transformation system and have good vectors and hosts available, the enzymes can be produced in large scale.

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Chapter 17
A Wide-Range Integrative Expression Vector (CoMed) System for Yeasts

Gerhard Steinborn, Gotthard Kunze, and Gerd Gellissen

Contents

Abstract No single yeast-based platform exists which is optimal for every protein. It is advisable to assess several platform candidates in parallel for optimal expression characteristics in a given case. For this approach, a wide-range yeast vector has been established that can be targeted to the various yeast host strains. The vector is built up in a modular way. In its basic form, it contains conserved rDNA-derived segments for targeting. For heterologous gene expression control, it is equipped with a promoter that is functional in all yeast species tested so far. For selection, a range of dominant and auxotrophic selection markers can be employed. Examples are presented applying vector variants with dominant or auxotrophic selection markers to the comparative simultaneous integration and expression of single or multiple foreign genes in a range of yeast platforms.

Keywords Vector, modular way, auxotrophic selection markers, dominant selection markers, yeast platforms

17.1 The Need for a Wide-Range Expression Vector System

In the previous chapters of this book a plethora of yeast species and expression platforms based on these yeasts have been presented. Yeasts include a great diversity of organisms. In general, fungi are excellent hosts for the production of recombinant proteins, as detailed in the previous chapters. They offer a desired ease of genetic manipulation and rapid growth to high cell densities on inexpensive media (Romanos et al., 1992; Heinisch and Hollenberg, 1993; Sudbery, 1996; Gellissen,

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2000, 2002). As eukaryotes, they are able to perform multiple posttranslational modifications, thus producing even complex foreign proteins that are often identical or very similar to native products from plant or mammalian sources (Ruetz and Gros, 1994; Gilbert et al., 1994; Wittekindt et al., 1995; Vozza et al., 1996; Gellissen, 2000, 2002; Valenzuela et al., 1982; Sudbery, 1996). Only few examples are available for the production of the same protein in a range of fungal species and it is thus difficult to evaluate the advantages or disadvantages of a platform for a particular product development. Constituting a diverse group yeasts exhibit differences in productivity, processing or glycosylation. Some examples of such differences are briefly listed in the following; for a more detailed description the reader is referred to other chapters of the book describing individual fungal systems. The first yeast expression platform was based on the traditional baker's yeast *S. cerevisiae*. Although successfully applied to the production of pharmaceuticals like insulin or hepatitis B vaccines, some important disadvantages became quickly apparent, which limit its general use in biotechnology. Glycoproteins are often over-glycosylated, and terminal mannose residues in N-linked glycans are added by an α-1,3 bond which is suspected to be allergenic (Jigami and Odani, 1999; Guengerich et al., 2004). Instead, non-allergenic terminal α-1,2 bonds are found to be present in *Hansenula polymorpha* and *Pichia pastoris* (Montesino et al., 1998, 1999; Bretthauer and Castellino, 1999; Guengerich et al., 2004). In *Arxula adeninivorans* patterns of O-glycosylation vary depending on morphological status thereby potentially providing an option to produce a foreign protein with or without O-glycosylation in an identical strain (Wartmann et al., 2002a). The protease content differs among yeasts: in a recent comparative study it was shown that the cytokine IL-6 is correctly processed from a MFα1 leader/IL-6 fusion in *A. adeninivorans*, but that N-terminally truncated cytokines are secreted from *H. polymorpha*, *P. pastoris* and *S. cerevisiae* hosts (Steinborn et al., 2006, 2007). The narrow substrate specificity of *S. cerevisiae* hampers fermentation design (Bruinenberg, 1986; Romanos et al., 1992). Most other biotechnologically applied species can grow on a much wider range of substrate thereby providing a high versatility for the selection of attractive promoter elements and as a consequence enabling various options for fermentation design. In individual cases, hydrophobic proteins may impose problems to a particular host, but not to others. The two methylotrophic species *H. polymorpha* and *P. pastoris* differ in their methanol requirement for the activation of promoters derived from genes of the methanol pathway (Guengerich et al., 2004; Kang and Gellissen, 2005). These few arbitrary examples already illustrate the necessity of carefully considering a range of fungal organisms before deciding on an expression platform. All of them have particular favourable characteristics and specific product examples attest to the advantages of the individual platform. However, all systems have drawbacks and limitations: sometimes attempts to produce a heterologous protein fail completely; in other cases, productivity, secretion or modification and processing are severely impaired as pointed out before, thereby preventing the development of a competitive production process or a marketable product. It is evident that no single yeast system is optimal for all proteins. Hence, predictions for a successful strain and process development

can only be made to a certain extent, and misjudgments cannot be excluded. This in turn means that the initial selection may result in costly time- and resource-consuming failures. It is therefore desirable to assess several selected yeast platforms in parallel for criteria like appropriate protein processing or secretion in a given case. A vector that can be targeted to the various platform candidates greatly facilitates such a comparison.

17.2 Design and Essential Elements of a Wide-Range Expression Vector

The design of a vector suited for a wide range of fungal organisms has to meet several prerequisites. Such a plasmid has to contain a targeting element suitable for all test species. The promoter that drives heterologous gene expression has to be functional in all these organisms. The vector/host system has to employ a dominant selection marker or a sequence that can complement the auxotrophy in all selected organisms.

Obvious targeting elements that are highly conserved among the various yeasts are sequences of the rDNA genes encoding ribosomal RNAs. These genes are present in high copy number and are typically clustered as head-to-tail tandem arrays of identical units (rDNA). Transcription occurs in a special compartment in the nucleus, called the nucleolus (Warner et al., 1972). They are readily accessible to all component required for an efficient transcription. The copy number of rDNA repeats in yeasts ranges from 30–50 in *H. polymorpha* (Waschk et al., 2002) to 200 in *S. cerevisiae* (Maleszka and Clark-Walker, 1993).

The rDNA repeats are in most instances organized as arrays of rRNA genes and non-coding intergenic spacer regions as detailed in Fig. 17.1.

Each rRNA gene is transcribed into a single precursor molecule by RNA polymerase I. Subsequently, this precursor is processed to form the 18S, 5.8S and 25S (28S) rRNAs. During this process, the external transcribed spacer (ETS) and the internal transcribed spacers (ITS1/2) are excised. Precursor transcription starts at the leader sequence of the 5′ ETS and stops at the 3′ end of the 25S (28S) rRNA gene. The intergenic non-transcribed spacers (NTS1/2) include promoter, enhancer and suppressor elements to control RNA polymerase I (Pol. I)-directed transcription (Udem and Warner, 1972). In yeasts, the coding region for the 5S subunit transcribed by RNA polymerase III (Pol. III) is either located in the NTS located between successive rRNA precursors, as has been shown for *S. cerevisiae* (Johnston et al., 1997), *Ashbya gossypii* (Wendland et al., 1999) and *H. polymorpha* (Klabunde et al., 2002) or represented elsewhere in the genome as it is the case with *A. adeninivorans* (Steinborn et al., 2005).

The rDNA sequences are highly conserved during evolution. This conservation is restricted to sequences encoding the various rRNA species; the sequences of the non-coding segments can be quite divergent. Therefore, elements derived from coding sequences or elements containing extended segments of conserved sequences

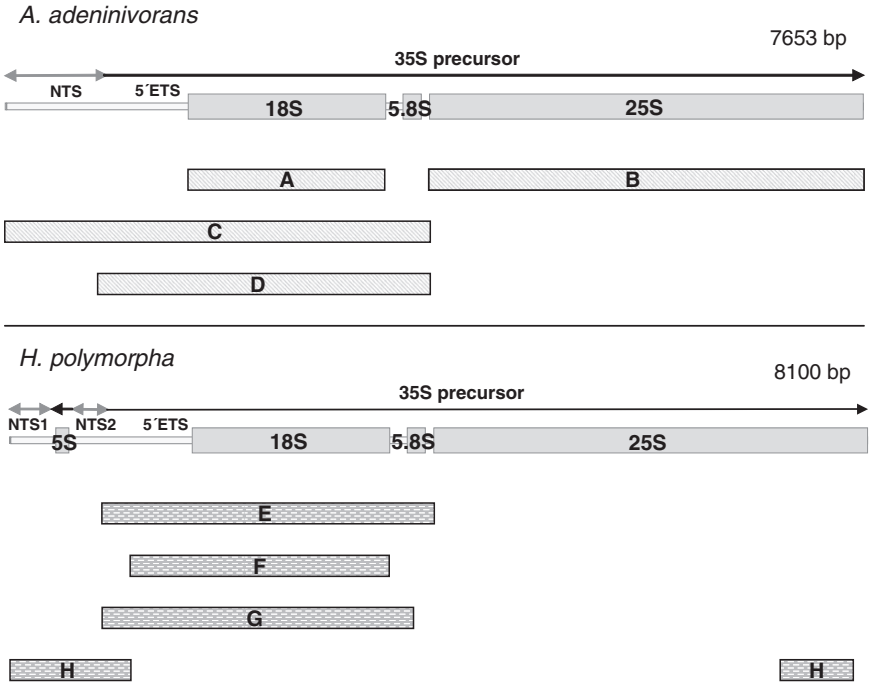

Fig. 17.1 *A. adeninivorans* and *H. polymorpha* rDNA units and the position of derived rDNA-targeting elements. The rDNA unit contains genetic elements in the following order: NTS1 (non-transcribed spacer), 5S rRNA, NTS2, the sequence of 35S precursor with the ETS1 (external transcribed spacer), followed by the sequence of the 18S, 5.8S and 25S rDNA. This gene order has been confirmed for *S. cerevisiae*, *H. polymorpha* and other yeast species, for *A. adeninivorans* the 5 S RNA is excluded from the unit represented elsewhere in the genome. (E-H) represents the position of the targeting segments of *H. polymorpha* and (A-D) that of *A. adeninivorans* assessed for transformation. Inclusion of this element in targeting vectors resulted in low transformation efficiency and unstable transformants in case of (F), in high transformation efficiency and stable transformants in case of (A-E,G,H)

have to be employed in the construction of a vector for wide-range application. Elements exclusively consisting of non-coding sequences are likely to function in a species-specific manner. However, non-conserved sequences that are important for mitotic stability have been described for *S. cerevisiae* (Lopes et al., 1996), and sequences that modulate expressibility have been postulated in *H. polymorpha* (Klabunde et al., 2003). Most of the regulatory elements in the non-coding sequences have been analyzed in *S. cerevisiae.* In light of the low extent of homology and the lack of conclusive experimental data, it can only be assumed that such functional sequences are also present at equivalent locations in the *H. polymorpha* and in other yeast species. rDNA targeting with different sequences has been described for a range of yeast species including *S. cerevisiae* (Lopes et al., 1996), *A. adeninivorans* (Rösel and Kunze, 1996), *H. polymorpha* (Klabunde et al., 2005),

Kluyveromyces lactis (Bergkamp et al., 1992) and *Yarrowia lipolytica* (Le Dall et al., 1994; Madzak et al., 2005). Only recently, rDNA sequences of *A. adeninivorans* and *H. polymorpha* have been defined as targeting elements with appropriate characteristics comprising both coding and non-coding sequences (Steinborn et al., 2005).

For selection, a range of dominant selection markers like the *E. coli*-derived *hph* gene conferring resistance against hygromycin B (Wartmann et al., 2002b; Rösel and Kunze, 1998) can be used. Alternatively, genes of different sources that complement auxotrophies of respective host strains can be chosen. Examples are the *A. adeninivorans*-derived *LEU2* gene or the *H. polymorpha*- or *S. cerevisiae*-derived *URA3* genes (Steinborn et al., 2006). A new attractive element is the *A. adeninivorans*-derived *TRP1* gene (Steinborn et al., 2007). Obviously, use of such complementation markers is restricted to the existing range of respective auxotrophic strains.

For expression control of the heterologous gene, a *TEF1* promoter like that derived from *A. adeninivorans* (Rösel and Kunze, 1996) can be employed when addressing a large number of platforms. Other control elements are likely to elicit appropriate expression levels in a restricted number of yeasts or in a single species only.

The basic design of a wide-range yeast vector with a selection of components is provided in Fig. 17.2 and Table 17.1.

It is built up in a modular way. By easy exchange of modules, such a vector can be converted into a plasmid that is optimal for an individual platform, for instance by inserting an expression cassette with a *MOX* promoter element that elicits efficient gene expression in methylotrophic yeasts only. Variants of this basic vector for the secretory production of certain compounds are under development. In yet another design, it is possible to linearize the plasmids in a way that leaves behind all bacterial DNA sequences.

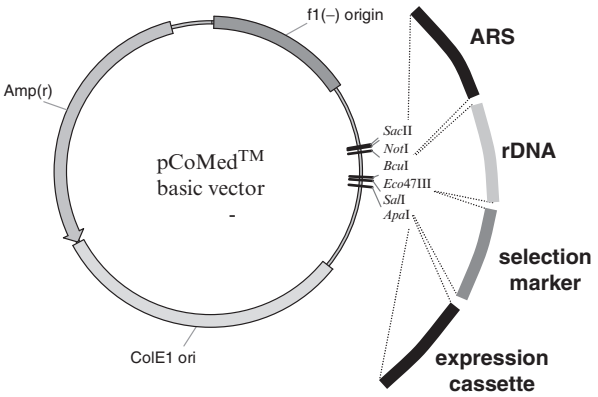

Fig. 17.2 Basic design of the CoMed™ vector. The CoMed™ basic vector contains all *E. coli* elements for propagation in the *E. coli* system and a MCS for integration of ARS, rDNA, selection marker and expression cassette modules. For this purpose, ARS fragments are flanked by *Sac*II and *Bcu*I restriction sites, rDNA regions by *Bcu*I and *Eco*47III restriction sites, selection markers by *Eco*47III and *Sal*I restriction sites and promoter elements by *Sal*I and *Apa*I restriction sites

Table 17.1 ARS, rDNA regions, selection markers and promoter elements of the CoMed vector system

Region/gene	Donor organism	Reference
■ ARS		
■ 2 μm DNA	*S. cerevisiae*	Beggs et al. (1976)
■ ARS1	*S. cerevisiae*	Gullov and Friis (1985)
■ HARS	*H. polymorpha*	Kang and Gellissen (2005)
■ SwARS	*Schw. occidentalis*	Piontek et al. (1998)
■ rDNA region		
■ NTS2-ETS-18SrDNA-ITS1	*H. polymorpha*	Ilgen et al. (2005)
■ 25S rDNA	*A. adeninivorans*	Rösel and Kunze (1998)
■ 18S rDNA	*A. adeninivorans*	Steinborn et al. (2005)
■ ITS-5S-ETS-18S-ITS-5,8S-ITS	*A. adeninivorans*	Steinborn et al. (2005)
■ NTS2-ETS-18SrDNA-ITS1	*A. adeninivorans*	Steinborn et al. (2005)
■ Selection marker		
■ *URA3*	*S. cerevisiae*	Rose et al. (1984)
■ *LEU2*	*S. cerevisiae*	Froman et al. (1984)
■ *ALEU2m*	*A. adeninivorans*	Wartmann et al. (2003a)
■ *ATRP1*	*A. adeninivorans*	Steinborn et al. (2006)
■ *HIS4*	*P. pastoris*	Thill et al. (1990)
■ Expression cassette (promoter)		
■ *FMD* promoter	*H. polymorpha*	Gellissen (2000)
■ *MOX* promoter	*H. polymorpha*	Gellissen (2000)
■ *TPS1* promoter	*H. polymorpha*	Amuel et al. (2000)
■ AOX1 promoter	*P. pastoris*	Raschke et al. (1996)
■ *TEF1* promoter	*A. adeninivorans*	Wartmann et al. (2002b)
■ *AHSB4m* promoter	*A. adeninivorans*	Wartmann et al. (2003b)
■ *GAA* promoter	*A. adeninivorans*	Bui et al. (1996)
■ *ALIP* promoter	*A. adeninivorans*	Böer et al. (2005)
■ *AINV* promoter	*A. adeninivorans*	Böer et al. (2004a)
■ *AXDH* promoter	*A. adeninivorans*	Böer et al. (2004b)
■ *RPS7* promoter	*Y. lipolytica*	Müller et al. (1998)

17.3 Application of the Wide-Range Vector to Protein Production in Various Yeasts

For proof a concept, GFP production was assessed in a range of yeast platforms transforming the different platform candidates in parallel with a single plasmid. For this purpose, a *GFP* reporter gene was employed which was inserted between the constitutive *A. adeninivorans*-derived *TEF1* promoter and the *S. cerevisiae*-derived *PHO5* terminator for expression control. Again, the resulting plasmid (pAL-HPH-TEF-GFP) was successfully used to transform *A. adeninivorans, S. cerevisiae, D. hansenii, D. polymorphus, H. polymorpha* and *P. pastoris* strains. It was found to be integrated in low copy numbers in all transformants.

Transformants were tested for the recombinant product either by Western Blot analysis or by fluorescence microscopy. The amounts varied only slightly among various transformants (Terentiev et al., 2004a; Fig. 17.3). In a second example, an expression/integration vector was constructed for the secretion of the pharmaceutically important cytokine interleukin-6 (IL-6), now combining an rDNA targeting sequence and an *A. adeninivorans*-derived *LEU2* gene (Wartmann et al., 2003a) for selection. For assessment, we inserted an expression cassette harbouring an ORF for a MFa1/IL-6 fusion protein under control of the *TEF1* promoter described before and transformed *leu2* auxotrophic strains of *A. adeninivorans*, *H. polymorpha* and *S. cerevisiae*. Again, mitotically stable strains were generated. Representatives of the three derived strain collections efficiently secreted the recombinant cytokine into the medium. In this case, product differences could be observed when comparing the secretion products of the different yeast species: the *H. polymorpha* and *S. cerevisiae*-derived molecules were found to be of smaller size than that secreted from the *A. adeninivorans* host. A more detailed comparative MS analysis of tryptic peptides revealed an N-terminal truncation at position Arg8 in *H. polymorpha* and *S. cerevisiae*, but a correctly processed mature IL-6 in *A. adeninivorans* (Steinborn et al., 2006, 2007). This is probably due to the lack of a thiol protease in this dimorphic species. The result emphasizes the need of a careful early pre-selection of a platform for the development of a production process.

17.4 Wide-Range rDNA Integration of Multiple Expression Cassettes

Following a previous observation that the integrated heterologous DNA can be present as multiple clusters inserted in the rDNA, it was demonstrated that rDNA plasmids, each equipped with the identical targeting element and the identical selection marker, but bearing different reporter genes could be integrated simultaneously into the rDNA of *H. polymorpha* (Klabunde et al., 2002) Thus, this approach provides an attractive tool for the rapid generation of recombinant strains from a diverged background that simultaneously co-produce several proteins in desired stoichiometric ratios. The following example describes the comparative assessment of different yeasts for the production of polyhydroxyalkanoates (PHA) co-integrating and co-expressing three genes of the PHA synthetic pathway from *Ralstonia eutropha*. For simultaneous assessment, three different yeasts, namely *D. polymorphus*, *D. hansenii* and *A. adeninivorans,* were selected. For introduction of the new metabolic pathway, wide-range expression vectors were equipped with the genes *phb*A*, phb*B and *phb*C of the polyhydroxyalkanoate (PHA) biosynthetic pathway of *Ralstonia eutropha* encoding β-ketothiolase, NADPH-linked acetoacetyl-CoA reductase and PHA synthase under control of the *A. adeninivorans*-derived *TEF1* promoter. Following the previous examples, the vectors were further equipped with an rDNA sequence and the *E. coli*-derived *hph* gene for wide-range integration and selection. Representatives of the three resulting strain collections were found to

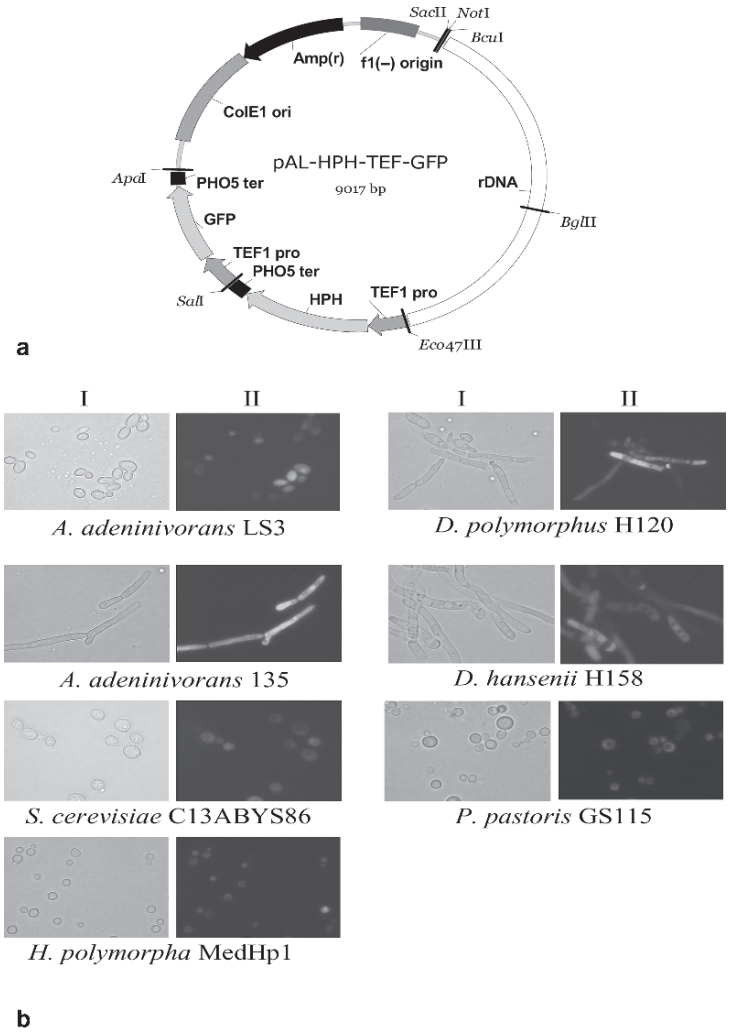

Fig. 17.3 Comparative assessment of GFP production in various yeasts. (A) Physical map of the expression/integration vector pAL-HPH-TEF-GFP used in this study. The vector contains the 25S rDNA sequence of *A. adeninivorans* (rDNA, white box) and an expression cassette for the *E. coli*-derived *hph* gene as selection marker in the order *A. adeninivorans*-derived *TEF1* promoter (TEF1 pro, grey segment), the *hph*-coding sequence (HPH, grey segment), *S. cerevisiae*-derived *PHO5* terminator (PHO5 ter, black bar). The vector further contains a second expression cassette with *TEF1* promoter – *GFP* ORF – *PHO5* terminator elements and an unique *Bgl*II site within the rDNA sequence for linearization. (B) Detection of recombinant GFP-producing yeast cells by fluorescence microscopy. Transformants were cultured for 48 h in YEPD medium at 30 °C and subsequently used for fluorescence analysis. (I) transmission, (II) GFP-fluorescence

contain all three heterologous genes as single copies mitotically stable integrated into the genome. In fed-batch cultivations in minimal medium supplemented with 1% ethanol as carbon source, the recombinant *A. adeninivorans* cells were able to convert efficiently the substrates acetyl-CoA and propionyl-CoA to PHA (2.2% of dry weight). In contrast, this level is relatively low with 4.2×10^{-3}% and 4.8×10^{-3}% of dry weight at the recombinant *D. hansenii* and *D. polymorphus* strains, respectively. Here, further optimization of the cultivation condition should improve this situation (Terentiev et al., 2004b; Steinborn et al., 2006, Fig. 17.4)

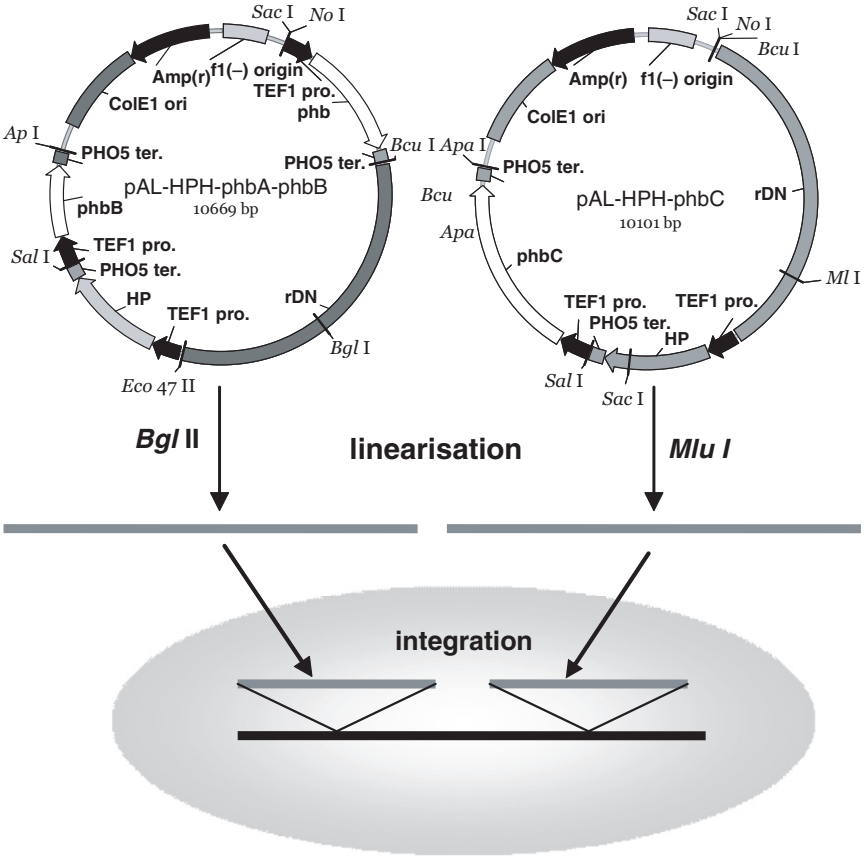

Fig. 17.4 PHA production in *A. adeninivorans, D. hansenii and D. polymorphus*. Transformation procedure based on simultaneous integration of the plasmids pAL-HPH-phbA-phbB and pAL-HPH-phbC into the 25S rDNA of *A. adeninivorans* LS3, *D. hansenii* H158 and *D. polymorphus* H120. The two plasmids pAL-HPH-phbA-phbB and pAL-HPH-phbC containing the expression cassettes with *phbA, phbB* and *phbC* genes are linearised by *Bgl*II or *Mlu*I digestion, respectively. The resulting fragments flanked by 25S rDNA sequences are co-integrated into the 25S rDNA by homologous recombination. Transformants are selected firstly by resistance to hygromycin B. In a second step, after PCR, these transformants were selected containing both plasmids (pAL-HPH-phbA-phbB and pAL-HPH-phbC)

In yet another example, the co-integration approach was applied to the improvement of IFN-γ secretion. IFN-γ was found to poorly secrete as overglycosylated proteins from various hosts (Gellissen et al., 2002). For potential improvements, strains were generated in which the gene for the cytokine was co-integrated and co-expressed together with candidate genes that could potentially influence and improve secretion and glycosylation. Of several candidate genes tried, the *H. polymorpha*-derived *CNE1* gene encoding calnexin was found to improve secretion of the cytokine considerably. The size of the secreted product corresponded to that of core-glycosylated molecules (Steinborn et al., 2006).

17.5 Conclusions and Perspectives

Integration of heterologous DNA into a range of yeast hosts in parallel has been successfully demonstrated for transformation with a single plasmid and for co-transformation with several plasmids. The existing catalogue of elements will be continuously supplemented by new promoter elements and a range of selection markers and the respective auxotrophic hosts. The CoMed vector system is very versatile and easy to handle. This important tool makes possible the simultaneous assessment of a wide range of yeast for a particular product and process development. Having it at hand, a high probability of success for an anticipated development can be envisaged.

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Chapter 18
Advances in Gene Expression in Non-Conventional Yeasts

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Contents

Abstract Yeast has been a favoured lower eukaryotic system for the expression and production of recombinant proteins for both basic research and practical applications, and the demand for foreign-gene expression systems is increasing rapidly. Despite the vast amount of information on the molecular biology and physiology of *Saccharomyces cerevisiae*, which has consequently been the first choice as host

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system for recombinant protein production in the past, several limitations have been identified in this expression system. These limitations have recently been relieved by the development of expression systems in other yeast species known as 'non-conventional yeasts' or 'non-*Saccharomyces*' yeasts. With the increasing interest in the biotechnological applications of these yeasts in applied and fundamental studies and processes, the term 'non-conventional' yeast may well soon become redundant. As there is no universal expression system for heterologous protein production, it is necessary to recognize the merits and demerits of each system in order to make a right choice. This chapter will evaluate the competitive environment of non-conventional expression platforms represented by some of the best-known alternative yeasts systems including *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Hansenula polymorpha*, *Pichia pastoris* and more recently, *Arxula adeninivorans*.

Keywords Recombinant proteins, expression systems, non-conventional yeast, heterologous protein, expression platforms

18.1 Yeasts as Eukaryotic Expression Systems

As large numbers of new genes become available due to the progress of various genome projects, methods for highly efficient expression and production of heterologous proteins, for both industrial and academic purposes, are currently under intense investigation.

Gerngross (2004) summarized five primary parameters for evaluating protein expression platforms:

- The cost of manufacturing and purification,
- The ability to control the final product, including its post-translational processing,
- The time required from gene to protein,
- The regulatory path to approve a drug/protein produced on a given expression platform,
- The overall royalties associated with the production of a recombinant product in a given host.

Yeasts are unicellular eukaryotic organisms and have been used for the expression of a wide variety of heterologous proteins from diverse origins. This popularity is due to the fact that it combines the advantages of bacterial expression systems in terms of ease of manipulation and established fermentation technologies with the ability to perform eukaryotic processing of polypeptides expressed by these systems (Gellissen and Hollenberg, 1997). Unlike higher eukaryotic cell lines producing recombinant proteins, yeasts can produce high protein titers (up to 14.8 g l^{-1}) growing on relatively inexpensive chemically defined media (free from animal derived supplements) in a short fermentation process (lasting only a few days) that is easily scalable to 100 m^3, thereby yielding a rapid turnaround from gene to protein (Gerngross, 2004).

The emerging of protein-based therapeutics as the largest class of new chemical entities being developed by drug companies (Walsh, 2003), lead to renewed interest in the production of these therapeutics by systems that could be engineered to produce recombinant proteins exhibiting properties similar to native proteins. One of these properties is the correct glycosylation of the therapeutic protein to allow the recombinant protein to be therapeutically equally effective when compared to the native counterpart. Yeasts have the ability to glycosylate glycoproteins, but unfortunately *N*-glycosylation in yeast is generally coupled with hypermannosylation of the core oligosaccharides (Gerngross, 2004), yielding proteins with suboptimal folding, function and stability in human serum (Helenius and Aebi, 2001). Fortunately, the abundance of molecular biology tools and techniques available in yeasts made it possible for researchers at GlycoFi Inc. (USA) and the Korean Research Institute of Bioscience and Biotechnology (KRIBB, Korea) to humanize the *N*-glycosylation patterns of glycoproteins produced by *Pichia pastoris* and *Hansenula polymorpha*, respectively (Wildt and Gerngross, 2005; Gellissen et al., 2005). Solving the non-human glycosylation problem would most possibly allow yeast to be one of the major hosts systems for the production of overall therapeutic proteins.

Another problem, occurring when a host was used for the heterologous expression of foreign proteins, was the difference in codon bias and usage between the donor organism and the recipient host. The non-codon optimized gene expression resulted in an apparent poor expression record for several host strains that exhibited a different in codon bias compared to the foreign gene being expressed (Yadava and Ockenhouse, 2003; Gustafsson et al., 2004). Recent advances in the quality as well as the reduced production costs of oligonucleotide synthesis allowed for the artificial synthesis of genes and even complete genomes (Smith et al., 2003). This artificial synthesis, together with codon preference tables available on the internet (http://www.kazusa.or.jp/codon/), allows for custom designed, host codon optimized genes – an approach exploited by several commercial companies (GENEART, Germany; Codon Devices, USA), that provides a cost-effective (from US$ 0.79/bp), rapid turnaround service to the biotechnology community.

In this chapter, we will briefly highlight the latest developments in *Saccharomyces cerevisiae* followed by a more comprehensive analysis of the available expression systems in the most widely used non-conventional yeasts. These are *Arxula adeninivorans, Kluyveromyces lactis, Yarrowia lipolytica, Hansenula polymorpha* (*Pichia angusta)* and *Pichia pastoris*. We also briefly discuss two additional yeast species (*Schwanniomyces occidentalis* and *Pichia stipitis)* which, due to specific properties, have the potential to also become widely used in heterologous expression.

18.2 Saccharomyces cerevisiae

Saccharomyces cerevisiae has been used for thousands of years for the purpose of brewing and baking and has been granted GRAS (generally recognized as safe) status. An overwhelming wealth of information on genetics, molecular biology and

physiology has been accumulated on this organism (Rose and Harrison, 1989; Broach et al., 1991), making this traditional species the best-characterized eukaryotic system today (Watson et al., 1987). Several limitations, in terms of using *S. cerevisiae* as a tool for heterologous production, have been reported in the past (Buckholz and Gleeson, 1991). These included reduced biomass yield due to aerobic alcohol fermentation, very low yields (with a maximum of 1–5% of total protein), hyperglycosylation, plasmid instability, and the retention of protein in the periplasmic space. It is due to these limitations that attention was redirected to non-*Saccharomyces* or non-conventional yeasts as hosts for heterologous production of proteins.

Researchers have, however, not abandoned *S. cerevisiae* due to these limitations, but invented strategies to overcome most of these limitations, thereby making *S. cerevisiae* the most utilized yeast system for the production of biopharmaceutical products (Graumann and Premstaller, 2006). Researchers at Novozyme Delta Ltd. in the UK (previously Delta Biotechnology Ltd. before being acquired by Novozymes) solved the yield problem, by producing recombinant proteins of up to 40% (w/v) total intracellular protein, or secreting recombinant proteins up to 5.5 g l^{-1}. To obtain these values, the native 2 μm plasmid had to be engineered to be stably propagated under non-selective conditions with elevated copy numbers (copy number was increase from 60 to 100 copies per genome), together with an additional multiple cloning site in the vector for co-expression of an additional gene (Sleep et al., 2001; C. Finnis, personal communication). A recent comprehensive review of approved biopharmaceuticals in the United States and/or Europe indicated that 24% of approved biopharmaceutical products were expressed in *S. cerevisiae* (Fig. 18.1) (Melmer, 2005), even though hyperglycosylation of glycoproteins still presents a major obstacle in the use of *S. cerevisiae* in therapeutic protein production. The reason for this is that the ratio of glycosylated proteins to non-glycosylated

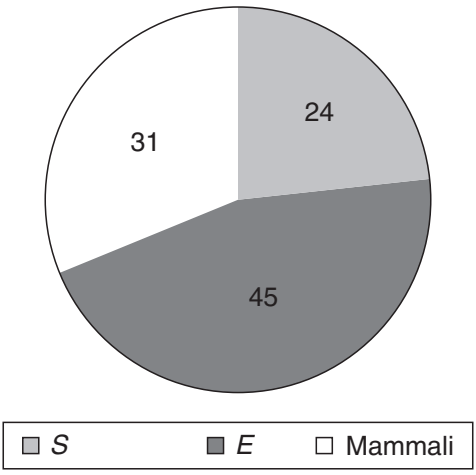

Fig. 18.1 Chart indicating the percentage of biopharmaceutical products (approved in the US and/or Europe) produced in the three most popular expressions host platforms (Melmer, 2005)

proteins for therapeutic use is 60:40, allowing the theoretical use of hosts such as *S. cerevisiae* and *Escherichia coli* for the production of 40% of the therapeutic protein market (Gerngross, 2004).

The deciphered genome of *S. cerevisiae*, together with the myriad of tools and techniques developed for manipulating and dissecting gene function in *S. cerevisiae*, allowed several scientists to move beyond normal recombinant protein expression studies (Goffeau et al., 1996). Recent literature reported on the engineering and manipulation of complex metabolic pathways to allow the accumulation or over-production of pathway intermediates, even improving process performance and cellular properties, again exploring the unknown for others to follow (Wada et al., 2006; Ostergaard et al., 2000; Mutka et al., 2006; Ro et al., 2006).

18.3 Arxula adeninivorans

18.3.1 History

Arxula adeninivorans is a non-pathogenic, xerotolerant, ascomycetous, anamorphic, arthroconidial yeast which was first known as *Trichosporon adeninovorans* (Middelhoven et al., 1984). In 1990 it was renamed as *Arxula adeninivorans (*van der Walt et al., 1990). The genus *Arxula* consists of two species, *Arxula terrestre* [(van der Walt and Johanssen) van der Walt, Smith and Yamada (1990)], which is the type species of the genus, and *A. adeninivorans* [(Middelhoven, Hoogkamer-Te Niet and Kreger van Rij) van der Walt, Smith and Yammada (1990)].

18.3.2 Introduction

Middelhoven et al. (1984, 1991, 1992); van der Walt et al. (1990) and Gienow et al. (1990) demonstrated that this yeast species is able to assimilate and ferment many compounds as the sole source of carbon and energy. Both *Arxula* species, *A. adeninivorans* and *A. terrestre*, are conspicuous due to their utilization of nitrate. They are able to grow on adenine, uric acid, butylamine, pentylamine or putrescine as sole source of carbon and energy (Wartmann and Kunze, 2000). However, *A. terrestre*, in contrast to *A. adeninivorans*, has no fermentative ability and does not assimilate soluble starch, melibiose, melizitose, propylamine or hexylamine (Middelhoven et al., 1984). *A. adeninivorans* is able to use a wide range of sugars as substrates, which is enabled by the secretion of enzymes such as glucoamylase, acid phosphatase I and II, trehalase, cellobiase I and II, β-D-xylosidase, 3-phytase and invertase (Wartmann and Kunze, 2000). In addition, *A. adeninivorans* assimilates polyalcohols and organic acids used in the conventional carbon assimilation test, except for L-rhamnose, inulin, lactose, lactate and methanol. Likewise, all conventionally

used nitrogen compounds, except creatine and creatinine, are suitable nitrogen sources. Several nitrogen compounds, like amino acids and purine derivatives, many primary *n*-alkyl-amines and terminal diamines, are metabolized as sole carbon, nitrogen and energy sources. In the case of alcohols, dialcohols, carboxylic acids, dicarboxylic acids and other nitrogen-less analogous compounds and intermediates of the general metabolism are also assimilated. In addition, *A. adeninivorans* also degrades some phenols and hydroxybenzoates (Gienow et al., 1990; Middelhoven et al., 1991, 1992).

Not only can *A. adeninivorans* grow on many different carbon sources, but produces and secretes several extracellular enzymes into the culture medium during cultivation. Besides RNAse, some proteases, various glucosidases such as glucoamylase, β-glucosidases, pectinases, xylosidase, some acid phosphatases, trehalose, some cellobiose, invertase and phytase could be detected (reviewed by Wartmann and Kunze, 2000).

Furthermore, an interesting property of *A. adeninivorans* is its osmotolerance. This yeast can grow in minimal as well as rich media containing up to 3.32 osmomol kg^{-1} water in the presence of ionic (up to 20% NaCl), osmotic (PEG400) and water stress (ethylene glycol) (Gellissen et al., 2005). The influence of NaCl concentration of up to 10% NaCl on growth parameters is weak, but NaCl concentrations of higher than 10% cause a decrease in the specific growth rate, a longer adaptation phase and a lower cell count during the stationary growth phase (Yang et al., 2000). In addition to the tolerant behaviour of *A. adeninivorans* in high-salt conditions, results obtained by Wartmann and co-workers (2003a) demonstrated that the *A. adeninivorans AHSB4* gene (encoding histone H4) is strongly expressed in the presence and absence of NaCl. The maintenance of a strong expression profile under high-salt conditions is an unprecedented characteristic not reported for any other fungal histone H4 gene. Therefore, the *AHSB4* promoter is an attractive control element for heterologous gene expression, especially when defining fermentation condition with media of high osmolarities (up to 10% NaCl).

18.3.3 Arxula adeninivorans Strains

Besides the aforementioned properties, the temperature-dependent dimorphism of *A. adeninivorans* is of biotechnological interest. Dimorphism in fungi refers to two states of the same organism, namely budding cells and mycelia. It is a characteristic of several fungi (Shepherd, 1988) and can be influenced by changes in a variety of parameters as described by San Blas and San Blas (1984). Although the pathogenic yeast *Candida albicans* is generally used as a model organism in the analysis of dimorphism, the isolation of mutants and the genetic and molecular biological handling of *C. albicans* are difficult since it is an asexual diploid organism (Hubbard et al., 1986; Gil et al., 1990). The *A. adeninivorans* strain LS3 can grow at temperatures of up to 48°C without previous adaptation (Wartman et al., 1995). Wartmann et al. (1995) found that temperatures above 42°C induce a morphological transition from budding to mycelial form, concomitantly with an altered gene expression pattern.

This environmentally conditioned dimorphism is reversible, and budding is re-established when the cultivation temperature is decreased to below 42°C. This environmentally conditioned dimorphism in conjunction with the molecular biological characteristics (Gienow et al., 1990) and the haploidy of *A. adeninivorans* LS3 provide the possibility of selecting a large number of mutants (Samsonova et al., 1989, 1996). Temperature shift-induced dimorphism has already been described for the fungi *Histoplasma capsulatum* (Medoff et al., 1981; Marcesca and Kobayashi, 1989), *Blastomyces dermatitidis* (Burg and Smith, 1994) and *Paracoccidoides brasiliensis* (San Blas and San Blas, 1993). In contrast to *A. adeninivorans*, these fungi grow as mycelia at lower temperatures (25°C) and they form budding cells after an increase in the cultivation temperature to 37°C. Other well-characterized dimorphic yeasts with a temperature-dependant dimorphism, e.g. *C. albicans* and *Y. lipolytica*, need an additional factor to induce morphological shifts such as a change in pH or the addition of serum to the medium (Buffo et al., 1994; Orlowski, 1994; Saporito-Irwin et al., 1995). This is in contrast to *A. adeninivorans*, for which the temperature-regulated dimorphism is independent of the medium and the carbon source used (Wartmann et al., 1995). Because *A. adeninivorans* LS3 is non-pathogenic haploid yeast with many biotechnological important properties, the secretion behavior of budding cells and mycelia was analyzed in detail (Wartmann et al., 2000). In order to analyze whether the dimorphism observed in *A. adeninivorans* is exclusively influenced by high temperatures or also by other parameters, Wartman and coworkers (2000) selected, identified and characterized a large number of mutants with altered behaviour in dimorphism. These mutants formed mycelia at 30°C and therefore enabled the distinguishing of temperature-mediated and morphology-related effects on gene expression. The analysis of these mutants showed that most of the dimorphic mutants were not temperature-sensitive, since morphological changes did not correlate with changes in temperature. This indicates that morphology rather than temperature is the decisive factor in the analyzed process (Wartmann et al., 2000).

Protein secretion plays an important role in filamentous fungi. In particular, the high levels of native proteins secreted by fungi make these organisms excellent hosts for the expression of recombinant proteins. In addition, yeast species that form either budding cells or pseudomycelia, e.g. *S. cerevisiae*, *P. pastoris* and *H. polymorpha*, have also been used as hosts for heterologous gene expression (Sudbery, 1996; Gellissen and Hollenberg, 1997). Similarly, mycelial cultures of *A. adeninivorans* accumulate two-fold higher protein concentrations and contain two- to five-fold higher glucoamylase and invertase activities in the medium than budding cells. Cell morphology was also found to influence the post-translational modifications of the Afet3p component of the iron transport system, an observation of potential impact for heterologous gene expression. *O*-glycosylation was found in budding cells only, whereas *N*-glycosylation occurred in both cell types. The characteristics of differential *O*-glycosylation may provide an option to produce heterologous proteins in both *O*-glycosylated and non-*O*-glycosylated form and to compare the impact of its presence on properties such as biological activity or immunological tolerance (Wartmann et al., 2002a).

18.3.4 Genetics and Molecular Biology

The DNA content of *A. adeninivorans* cells is similar to that of haploid cells of *S. cerevisiae* and other ascomycetous yeasts (Samsonova et al., 1989; Gienow et al., 1990; Wartmann et al., 2000). The first transformation system for *A. adeninivorans* was developed by Kunze and Kunze (1996) using the *LYS2* genes from *A. adeninivorans* or *S. cerevisiae* as selective markers. A second transformation procedure was based on a dominant selection marker, such as the *E. coli hph* gene, which allows resistance against the toxic hygromycin B (Rösel and Kunze, 1998). To avoid the employment of an undesired dominant marker gene and the use of toxic compounds or antibiotics during strain development, auxotrophic strains and the respective gene sequences for complementation should be available. The haploidy of *A. adeninivorans* enabled the selection of mutants by *N*-methyl-*N*Î-nitro-*N*-nitrosoguanidine (NG) and UV mutagenesis. A large number of auxotrophic mutants and mutants with altered catabolite repression that were resistant to 2-deoxy-D-glucose were selected and characterized by Samsonova et al. (1989, 1996) and Büttner et al. (1989). These mutants are characterized by a high synthesis of some extracellular enzymes in the presence of different carbon sources.

Wartmann et al. (1998) described the *AILV1* gene encoding threonine deaminase as a selection marker for transformation of an *A. adeninivorans ailv1* mutant strain. Using the *A. adeninivorans*-derived 25S rDNA–targeting approach, recombinant strains were generated harboring one to three copies of the foreign DNA integrated into the 25S rDNA. However, this system suffered several drawbacks rendering it unsuitable for efficient heterologous gene expression. These drawbacks included that all identified *ailv1* mutant strains showed an atypical anamorphic phenotype causing an inefficient separation of the yeast transformants from the media after centrifugation. In addition, the large size of the transformation plasmid prevented simple integration of expression cassettes (Wartmann et al., 2003b). However, a range of auxotrophic *A. adeninivorans* mutants, showing a typical growth phenotype, have been identified (Klabunde et al., 2002), such as the *aleu2* mutant. Subsequently, Wartmann et al. (2003b) established a host-vector system based on complementation of this auxotrophy. The respective *ALEU2* gene was isolated and incorporated into suitable transformation vectors. For targeting, the plasmid additionally contained the *A. adeninivorans* 25S rDNA similar to, like the previously designed vector. However, a disadvantage of the *A. adeninivorans* platform is the low copy number of the heterologous DNA targeted to the 25S rDNA (Wartmann et al., 2002b, 2003b). This results in comparably low productivities, and results obtained by Wartmann and co-workers (2003a) confirmed a gene-dosage effect on heterologous gene expression. When strains were co-transformed with two rDNA integration vectors equipped with two different selection markers it resulted in two mitotically stably integrated copies, with transformants displaying a considerable increase in productivity. Use of defective promoters in the complementation gene may result in higher copy numbers. However, in all instances mitotic stability under non-selective conditions was found to be high. In previous studies (Wartmann et al., 2002b), based on the

plasmid pAL-HPH1, the cassette '*TEF1* promoter - heterologous gene - *PHO5* terminator' has already been successfully applied for heterologous gene expression. The *TEF1* promoter provides a strong and constitutive expression of a heterologous gene (Müller et al., 1998; Wartmann et al., 1998) even when present in low copy numbers. This was confirmed in a study by Wartmann et al. (2003a) using *GFP* and *HSA* as model genes. In this respect, the *A. adeninivorans* system differs from most other non-conventional yeast systems, in which higher copy numbers are required to obtain comparable high expression levels (Juretzek et al., 2001; Gatzke et al., 1995). Current developments by Wartmann and co-workers (2003a) are aimed at further increasing the copy number and thereby potentially improving the productivity of recombinant *A. adeninivorans* strains.

A. adeninivorans is a non-conventional yeast with a range of properties attractive for both basic and applied research. The very extensive range of substances that can be utilized as carbon and/or nitrogen sources, its growth and secretion behaviour, thermo- and halotolerance and temperature-dependent dimorphism makes this yeast an attractive organism for biotechnological research. In addition, it is an excellent host for heterologous gene expression and might replace or supplement other yeast-based systems.

18.4 Kluyveromyces lactis

18.4.1 History

Kluyveromyces lactis belongs to the family Saccharomycetaceae in the genera *Kluyveromyces* and consist of two varieties; *Kluyveromyces lactis* (Dombrowski) van der Walt var. *lactis* and *Kluyveromyces lactis* var. drosophilarum (Shehata, Mrak and Phaff) (Sidenberg and Lachance, 1986). The former is heterothallic and ferments lactose and the latter is homothallic and does not ferment or assimilate lactose (Lachance, 1998). Most of the published work that has been performed on *K. lactis,* was performed on the variety *lactis.* In the genera *Kluyveromyces* there are 15 accepted species (*Kluyveromyces* van der Walt emend. (van der Walt in Lachance (1998)). The key character for the genus *Kluyveromyces* is ascus deliquescence, however, (Kurtzman, 2003) showed that this character have no phylogenetic basis. Phylogenetic analysis based on 18S, 5.8S, ITS and 26S rDNAs, translation elongation factor 1-α, mitochondrial small-subunit rDNA and COXII showed that species of *Kluyveromyces* are found in six clades indicating the polyphyly of the genus as presently defined. It was also previously proposed that the six known species of the *K. marxianus* clade (including *K. lactis*) should be placed under the genus *Zygofabospora* (Naumov and Naumova, 2002). It was however proposed that changing the specie name of the biotechnological important species *K. lactis* and *K. marxianus* is inconsistent with nomenclature stability of well known species (Kurtzman et al., 2001; Kurtzman, 2003).

18.4.2 Introduction

K. lactis has been studied since the early 1960s. The natural habitat of this species is diverse, but many strains were originally isolated from milk-derived products in which the major carbon source is lactose. However, study of this yeast was performed in a far lesser extend in comparison to its closely related neighbor *S. cerevisiae*. In the 1980s more prominence was given to *K. lactis* due to the identification of a DNA plasmid based killer system, lactose metabolism and secretion of recombinant proteins.

K. lactis is amenable to genetic studies, having four spores in an evanescent ascus, so that single-spore cultures can be easily obtained. The ability of this yeast to secrete high molecular weight protein, specifically a killer toxin, increased the interest in the use of this yeast as a host for heterologous expressed proteins (Wésolowski-Louvel et al., 1996). Because of its distinctive physiological properties, *K. lactis* has become an important alternative to the classical *S. cerevisiae*. *K. lactis* is well known for its ability to produce β-galactosidase and as an expression host for the production of the milk clotting enzyme bovine chymosin (van den Berg et al., 1990). This yeast is also used to commercially produce the native enzyme lactase that is sold under the trade name Maxilact™ by DSM Food Specialties, Delft, The Netherlands (van Ooyen et al., 2006).

18.4.3 Kluyveromyces lactis Strains

Most of the initial work in the 1960s conducted on *K. lactis* was performed by the group of Harlyn O. Halvorson at Madison, Wisconsin (then called *Saccharomyces lactis*) (Fukuhara, 2006). The initial work was based using two isolates, a *MATa* mating type strain NRRL Y-1140 (CBS 2359) (the proposed reference strain for genetic and molecular studies; (Wésolowski-Louvel et al., 1996)) and a *MATα* mating type strain NRRL Y-1118 (CBS 6315). The major genetic background seems to come from these two strains together with the *MATα* strain NRRL Y-1205 (CBS 2360) (Wésolowski-Louvel et al., 1996; Fukuhara, 2006). The main industrial strain is a prototrophic wild-type isolate GG799 that has an excellent protein expression track record. The main advantage of this strain is the low amount of glucose repression of the *LAC4* promoter; therefore *LAC4*-based heterologous expression is high in media containing glucose as carbon source (van Ooyen et al., 2006).

18.4.4 Genetics and Molecular Biology

The complete genomic sequence of *K. lactis* has been determined through the Génolevures project. Strain CLIB 210 which is an auxotrophic derivative of strain CBS 2359/NRRL Y-114 was used as source for genomic DNA. The *K. lactis*

genome consists of approximately 10.6 Mb (not including the rDNA) and is organized as six chromosomes ranging in size from 1 to 3 Mb, containing 5329 open reading frames (Bolotin-Fukuhara et al., 2000; Dujon et al., 2004).

A pair of linear plasmids (pGKL1 and pGKL2) has been isolated in certain strains of *K. lactis* and confers a killer phenotype (Gunge, 1986; Gunge and Kitada, 1988; Volkert et al., 1989; Stark et al., 1990). pGKL1 is a 8.8 kb double-stranded DNA plasmid and contains the genes that encode the killer toxin subunit proteins. pGKL2 is a 13.4 kb plasmid and is needed for the maintenance of pGKL1. Attempts have been made to use these plasmids in heterologous expression; however, expression levels were low in comparison with results obtained when circular vectors were used.

There are no naturally-occurring circular plasmids in *K. lactis* (*Kluyveromyces lactis* var. *lactis*). Vectors derived from the 2μ plasmid of *S. cerevisiae* can be used to transform *K. lactis* strains, but are very unstable, requiring maintenance of selective pressure. The most widely used replicating vector system specific for *K. lactis* is based on the plasmid pKD1 isolated from *Kluyveromyces drosophilarum* (*Kluyveromyces lactis* var. *drosophilarum*) (Chen et al., 1986; Falcone et al., 1986). pKD1 belongs to the 2μ family plasmids sharing the same type of gene organization, although there is little homology of nucleotide sequence. Analysis of individual functional elements of pKD1 has also been described (Bianchi et al., 1991, 1992). This plasmid is maintained in the region of 60 to 80 copies per cell, but reduction in copy number to approximately 20 copies per cell have been observed when containing some heterologous genes (Falcone et al., 1986; Morlino et al., 1999). Instability of this plasmid expressing a human lysozyme was also shown by Iwata et al. (2004) where only 17% of cells retained the plasmid after growth in rich media (YPD) for 20 generations. At the same time an integrated expression vector was retained by more than 90% of the cells producing the same enzyme. Furthermore, a pKD1 based plasmid containing a *K. lactis* centromeric region was retained by at least 70% of transformed cells after 20 generations.

Transformation of *K. lactis* can be achieved by most standard methods (Iborra, 1993; Wésolowski-Louvel et al., 1996). The standard *S. cerevisiae* markers such as *TRP1, URA3* and *LEU2* genes have been conveniently used in *K. lactis*, because corresponding mutations in *K. lactis* can be complemented by these genes under their native promoter. Study of non-conventional yeasts often suffers from the absence of available auxotrophic mutants as transformation hosts. Sensitivity of many yeasts, including *K. lactis*, to the antibiotic G418 (geneticin) allows the use of the kanamycin resistance gene (coding for 3′-aminoglycoside-phosphotransferase) as a transformation marker (Sreekrishna et al., 1984). A selection marker free system based on acetamidase was developed and patented by Selton et al. (2000). Cells transformed with a vector system containing this marker gene are grown in media lacking nitrogen source (for example yeast carbon base) but supplemented with acetamide. Expression of acetamidase converts acetamide to ammonia which acts as a source of nitrogen for the cells. The advantage of such a selection mechanism is that multi-copy integrants are enriched leading to higher levels of heterologous protein production. Apart from the use of this marker gene in *K. lactis,* Selton and co-workers (2000) also demonstrated its use in *S. cerevisiae*,

E. coli, *Bacillus subtilis* and *Bacillus licheniformis*. The main criterion for the use of this system is the absence of any acetamide activity in the host strain. Another advantage of this marker system is that it can be recycled through a counter selection using fluoroacetamide. Cells expressing acetamidase converts fluoroacetamide to the toxic compound fluoroacetate. If the acetamidase encoding gene is flanked by sequences that allow recombination (e.g. *Lox* cassette) cells can be selected that have lost the marker gene (Selton et al., 2000; van Ooyen et al., 2006). This system is similar to the *S. cerevisiae URA3* counter selection using 5-FOA (5-Fluoroorotic Acid) (Boeke et al., 1987).

Most of the promoters that are available and that have been used successfully for heterologous expression are *S. cerevisiae* promoters. These include constitutive (e.g. *PGK*) as well as regulatable promoters (*GAL1, GAL7, ADH2* and *PHO5).* The most extensively used *K. lactis* promoters are the *KlADH4* and the inducible *LAC4* promoter (Gellissen et al., 1992; van Ooyen et al., 2006). The *KlADH4* is a mitochondrial located alcohol dehydrogenase and strains with impaired sugar fermentation (i.e. Rag^-) need the addition of exogenous ethanol for induction. Furthermore, this gene is not repressed by glucose and is induced in strains that do produce ethanol from fermentable carbon sources. Transcriptional activity can be further increased in these strains with the addition of exogenous ethanol. The advantage of such a promoter in heterologous driven expression is that expression can be regulated with the addition of the inducer, in this case ethanol, independently of the carbon source used (Saliola et al., 1999). Saliola et al. (1999) identified an ethanol-responsive element in the promoter region of *KlADH4*. When this element was inserted into either the native *LAC4* promoter or the *S. cerevisiae PGK* promoter it conferred ethanol-dependent induction of these promoters. The *LAC4* gene encodes a native lactase (β-galactosidase) in *K. lactis*. The *LAC4* promoter (P_{LAC4}) is often used in heterologous expression due to its strength and inducible expression. One negative aspect of this promoter is its functionality in *E. coli* which can be problematic in the assembly of an expression vector in this bacterium especially if the heterologous gene product is deleterious. Colussi and Taron (2005) constructed a P_{LAC4} promoter lacking the sequences on this promoter that resemble the bacterial Pribnow box element (designated $P_{LAC4\text{-}PBI}$). The mutated promoter reduced expression of a reporter protein (GFP) by approximately 87% in *E. coli* and expression of human serum albumin in *K. lactis* were comparable to expression using the wild-type promoter. Furthermore, a number of proteins that are toxic in *E. coli* (including the protease bovine enterokinase and the mouse transthyretin) were cloned using this altered promoter (Colussi and Taron, 2005).

K. lactis can also be used as a host for production of heterologous proteins as such proteins may be readily excreted into the medium. For this, two types of strategies are considered: one is based on the expression of chromosomally integrated foreign protein genes (which are highly stable through mitosis), and the other relies on multi-copy plasmid vectors carrying the foreign gene (maintained at a high gene dosage). Both approaches have given successful examples: prochymosin (*LAC4* integration) (van den Berg et al., 1990), glycoprotein E2 from hepatitis C virus (*TRP1* integration) (Mustilli et al., 1999) and α-galactosidase (rDNA integration)

(Bergkamp et al., 1992) was produced from chromosomally integrated genes. pKD1 plasmid-based vectors were employed to express, for example, human serum albumin (Fleer et al., 1991a), human interleukin-1 beta (Fleer et al., 1991b), cellulose, lipase and polygalacturonase (Müller et al., 1998). Multiple integration of a plant α-galactosidase gene into ribosomal DNA produced a high level of secreted production of this enzyme, 250 mg l^{-1} in comparison to 90 mg l^{-1} when expression was pKD1 based (Bergkamp et al., 1992). Targeted integration is however not as efficient as is the case of *S. cerevisiae* and efficiency of integration often depends on the targeted loci (Zeeman et al., 1998). It was shown recently that targeted integration can be dramatically increased with the deletion of the *KlKu80* (Kooistra et al., 2004). Using the *KlADE2* as targeted loci with varying lengths of homologous sequences, site specific integration efficiency in wild-type *K. lactis* varied from 0% to 80%. In a *KlKu80* deletion mutant, correctly targeted integration efficiency was improved to > 97% independent of the length of the homologous flanking regions. Targeted integration does however have the disadvantage of reduced number of heterologous gene copies in comparison to multicopy vectors. One method to ensure multi-copy integration is through the targeting of the rDNA loci (Bergkamp et al., 1992).

In addition to the above-mentioned heterologous proteins, more that 40 proteins have been produced with *K. lactis*. These proteins derived from bacteria, fungi, viruses, plants, and mammals showing the importance of this yeast in heterologous protein production (reviewed by van Ooyen et al., 2006).

Colussi and Taron (2005) reported on the development of an integration vector (pKLAC1) containing a number of advances discussed. This vector, developed at New England Biolabs, Inc. and DSM Biologics Company B.V., contains the modified *K. lactis* $P_{LAC4\text{-}PBI}$ promoter, DNA encoding the *K. lactis* α-mating factor (KL-α-MF) secretion domain, a multiple cloning site, the *K. lactis LAC4* transcription terminator (TT), and the *Aspergillus nidulans* acetamidase selectable marker gene (amdS) expressed from the *S. cerevisiae ADH2* promoter. An *E. coli* replication origin (ori) and ampicillin resistance gene (ApR) are present for propagation of pKLAC1 in *E. coli*. This vector forms part of the *K. lactis* protein expression kit together with the industrial strain GG799 commercially sold by New England Biolabs Inc. (USA). This expression system can be used for research purposes; however, a license should be obtained from New England Biolabs, Inc. (USA) or DSM Biologics Company B.V. (The Netherlands) for commercial purposes.

18.5 Yarrowia lipolytica

18.5.1 History

Yarrowia lipolytica [(Wickerham, Kutzman and Herman) van der Walt and von Arx] is the only ascosporic member of the genus *Yarrowia* van der Walt and von Arx. *Y. lipolytica* was identified as the teleomorph of *Candida lipolytica* and originally

described as *Endomycopsis lipolytica* then as *Saccharomycopsis lipolytica* and finally as *Yarrowia lipolytica* (van der Walt and von Arx, 1980). *Yarrowia lipolytica* is heterothallic displaying dimorphic growth forming yeast cells, true hyphae and pseudohyphae, depending on growth conditions. *Y. lipolytica* strains are frequently isolated from dairy products and meat products, do not ferment sugars and are not considered as a pathogenic species. This lack of fermentative capabilities allows their easy elimination from for example dairy products (Barth and Gaillardin, 1997; Kurtzman, 1998a).

18.5.2 Introduction

Interest into the potential of *Y. lipolytica* as a heterologous protein producer and secretor aroused from the fact that this yeast naturally secretes several proteins, including proteases, lipases, phosphatases, RNAse and esterase. Yield of alkaline extracellular protease (AEP) secreted under inductive conditions by the wild-type organism ranged between 1–2 g l^{-1}. The good yield and secretion capability, together with the fact that *Y. lipolytica* is considered as non-pathogenic and that several processes based on the yeast were awarded GRAS status by the FDA, made *Y. lipolytica* an excellent candidate for genetic exploration and exploitation as a alternative expression host (Barth and Gaillardin, 1997).

Most of the groundbreaking work in terms of genetic elucidation, manipulation and tools to assist in manipulation of *Y. lipolytica* was and still is being performed at the laboratories directed by Prof. Claude Gaillardin in Grignon, France. Inbreeding programs resulted in genetically tractable lines, allowing complementation and linkage studies to become more feasible. *Y. lipolytica* can be transformed with either replicative vectors (containing centromeric as well as replicative functions) or integrative vectors (integration occurs usually by recombination between the plasmid sequence homologous to a chromosomal target sequence) (Vernis et al., 2001; Gaillardin et al., 1985).

The elucidation of the genomic organization and finally the complete sequence of the genome of an organism are necessary in order to fully utilize the power of molecular biology and molecular manipulation. Studies into the genomic organization of several *Y. lipolytica* strains revealed the presence of six chromosomes and that the overall structure of the genome is conserved between different isolates (Casarégola et al., 1997). Two isolates of *Y. lipolytica* were subjected to genome sequencing driven by the Génolevures projects, resulting in the assembly of the complete genome of 20.5 Mb in size (excluding the rDNA) containing approximately 6700 open reading frames (Casarégola et al., 2000; Dujon et al., 2004). The *Y. lipolytica* genome was found to be the largest of the hemiascomycetous species, but with the lowest gene density due to the amount of non-coding DNA present in the genome. The elucidation and annotation of the genome, opens the possibility for intricate pathway engineering by laying down the blueprint describing each component involved in cellular processes.

Y. lipolytica has two major advantages over the established *S. cerevisiae* system when it comes to the production of human therapeutic proteins. The first is that it allows for predominantly co-translational translocation of newly synthesized proteins into the endoplasmic reticulum (also the case in higher eukaryotic organisms), in contrast to the post-translational translocation predominant in *S. cerevisiae* (Biosramé et al., 1998). The second advantage is that recorded glycosylation of a protein from human origin indicated short oligosaccharide chains (8–10 mannose residues added to the core oligosaccharides), compared to the long oligosaccharide chains (50–150 mannose residues) added by *S. cerevisiae* (Madzak et al., 2005).

Müller and co-workers (1998) performed an evaluation of different yeast expression platforms for the production of heterologous proteins encoded by genes originating from three filamentous fungal strains. Evaluation criteria included transformation efficiency, degree of glycosylation, growth of recombinant host and the amount of active recombinant enzyme produced by the host. *Y. lipolytica* was found to be the most promising host tested, thereby outcompeting *S. cerevisiae*, *Schizosaccharomyces pombe*, *K. lactis* and *H. polymorpha.*

18.5.3 Yarrowia lipolytica Strains

A detailed review on the characteristics and properties of all *Y. lipolytica* strains used for protein expression was published by Madzak and co-workers (2004). This section would only indicate the highlights in the construction of the most widely used strain and its derivatives.

Several *Y. lipolytica* host strains were engineered for the expression of recombinant proteins. Engineering of the strains entailed the identification and subsequent disruption of a wide variety of marker genes and the use of antibiotic resistance markers, allowing manipulation of industrial *Y. lipolytica* strains not harbouring any auxotrophic markers (Madzak et al., 2004). The importance of *Y. lipolytica* as a secretor of heterologous proteins was advanced by the construction of the W29 wild-type derived Po1d strain (le Dall et al., 1994). The strain contained an alkaline extracellular protease (AEP) deletion, which was necessary since AEP was produced in abundance and could cause havoc by degrading secreted recombinant proteins. This strain also shared the same high secretion capability present in W29 wild-type and its derivatives. It also contained an integrated copy of the *SUC2* gene from *S. cerevisiae*, facilitating sucrose utilization as an alternative carbon source and allowing efficient and cost-effective growth of the recombinant strain on molasses. Further improvements to the strains in terms of heterologous protein production included deletion of the acidic extracellular protease, effectively eliminating the major proteases secreted into the culture medium. Another derivative (Po1g) was fitted with a pBR322 docking platform to allow easy integration of pBR322 derived expression constructs (Madzak et al., 2000; Madzak, 2003).

18.5.4 Genetics and Molecular biology

Functional gene expression in *Y. lipolytica* is derived from the expression cassette, including a promoter, the recombinant gene to be produced (with or without a signal peptide) and a terminator region recognized by the transcription machinery of *Y. lipolytica*. The expression cassette is also linked to a marker gene (either auxotrophy complementation or antibiotic resistance) that allows selection of transformants carrying the expression cassette and flanked by either a target region that allows recombination of the expression cassette with homologous regions in the genome (for integrative vectors) or by regions allowing replicative and centromeric functions (for episomal maintenance of the plasmid). Most of the expression construct attention was focused on integrative vectors instead of episomal vectors, due to the limited copy number of the episomal vectors (1–3 copies/cell), together with the requirement for continual selective pressure and limited gene expression (Madzak et al., 2000).

Transformation of competent *Y. lipolytica* with linearized integrative expression cassettes based on a single crossover recombination event (using the lithium acetate method) resulted in transformation efficiencies of up to 10^6 transformants μg^{-1} DNA where more than 80% of the transformants will harbor a single copy of the expression cassette integrated at the correct site into the genome (Xuan et al., 1988; Barth and Gaillardin, 1996). The integrative vectors were further optimized in such a way that the transformation construct that would integrate into the genome would be free of any bacterial DNA (Pignéde et al., 2000). These 'auto-cloning' vectors consist of a yeast cassette, flanked by a ≥8 bp restriction site, separating the bacterial part from the yeast cassette. Removal of the bacterial DNA is achieved by digesting the circular plasmid with the appropriate enzyme, separation of the two moieties using agarose gel electrophoresis, isolation of the yeast cassette for transformation of the host to yield a transformant devoid of bacterial DNA and resistance markers that could complicate commercial and industrial applications. A typical example of such an auto-cloning integrative expression construct used in *Y. lipolytica*, together with the most common elements used in the construct, is graphically illustrated in Fig. 18.2.

Le Dall and co-workers (1994) set out to increase expression cassette copy number by creating different sets of vectors targeted to the conserved and highly abundant rDNA regions in the genome of *Y. lipolytica*. These vectors contained sequentially truncated promoter regions of the *URA3* marker gene, thereby effectively preventing growth of the transformants when a single copy of the marker gene is present under selective conditions. Up to 60 copies of the expression cassette integrated into the genome when the *ura3d4* defective allele (containing only 6 bp or the original promoter upstream from the initiator ATG codon) was used as a marker gene for selection of positive transformants - albeit with a very low transformation efficiency of only 10 transformants μg^{-1} DNA. Integration into the genome occurred mostly as tandem repeats of the cassette into two different chromosomes, with some dispersed copies also observed. Induction of the reporter gene, AEP, however resulted in a decrease and stabilization in copy number of ~10 copies/genome. A good correlation between copy number and AEP production was

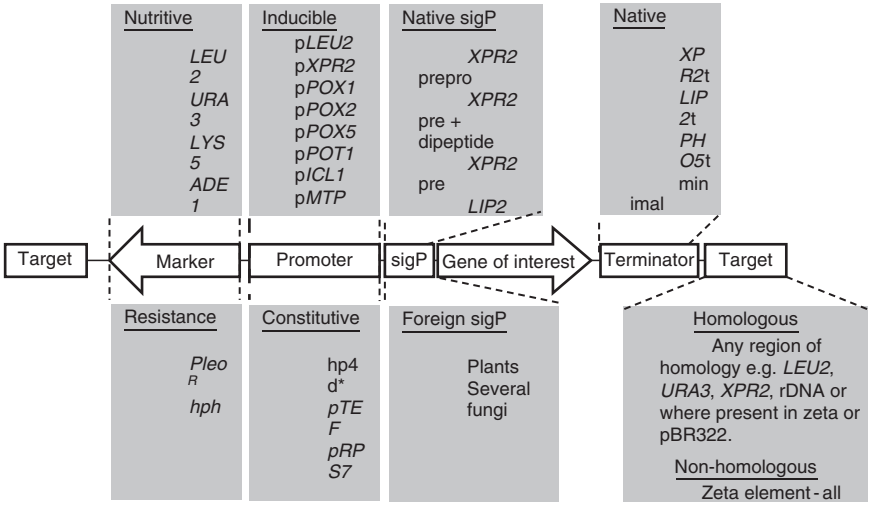

Fig. 18.2 Graphical representation of a typical auto-cloning integrative expression construct used for transformation of *Y. lipolytica*. All elements listed have been reviewed and described in detail by Madzak and co-workers (2004)

observed for up to 10 copies/genome, without causing expression cassette instability and affecting strain viability. This was confirmed in a study performed by Juretzek and co-workers (2001) where results indicated that multi-copy integration occurred for all the target regions predominantly as tandem repeats, with stable copy number of 10–13 copies/genome after induction. It was also observed that transformants exhibiting lower copy number (3–6 copies/genome) showed an increase to the level of 10–13 copies/genome after successive cultivation on selective media. The conclusion of the study indicated that the determining factor for multi-copy transformants seems to be the requirement for multiple copies of the defective marker to restore the ura− phenotype, and not the presence of multiple target sites for integration.

The use of a PCR-mediated gene deletion/disruption method employing the counter selectable *URA3* marker gene for use in *Y. lipolytica* has been described by Nicaud and co-workers (1998). The method relies on constructing a promoter-*URA3*-terminator for gene replacement, verification of deletion and use of a promoter-terminator cassette in a second round of transformation, followed by selection of ura− transformants on 5-fluoroorotic acid (5-FOA). The presence of a functional intact *URA3* in the genome will allow the cells to metabolize the 5-FOA where the metabolite becomes toxic to the cells, thereby allowing only cells without the functional intact *URA3* to grow. The *URA3* can then be re-used for the next gene deletion (Boeke et al., 1987). This method has been successfully employed in *Y. lipolytica* for the functional analysis of acyl-coA oxidase family as well as functional analysis of some genes involved in the glycosylation pathway (Wang et al., 1999; Barney-Verdier et al., 2004). The major disadvantages of this method are that it is limited to the *URA3* marker and that it is laborious and time consuming.

With the publication of the first series of sequences from the Génolevures project, an efficient system was developed for the rapid gene disruption and marker recycling in *Y. lipolytica* (Fickers et al., 2003). The system is based, like the 5-FOA system, on a promoter-marker-terminator cassette to facilitate in the gene disruption, but in this method the marker is flanked by *lox* sequences. The *lox* sequences serve as template for heterologous expressed *Cre* recombinase (transcribed from an episomal plasmid of which the recipient strain can be cured of), which will, with high efficiency, recombine the two *lox* sites, effectively looping out the marker gene. Any marker gene can be used with this system, making multiple gene disruptions, followed by a once off marker rescue, possible. The use of the antibiotic resistance marker gene *hph* allows for the manipulation and engineering of all *Y. lipolytica* strains, especially industrial strains that often lack the conventional auxotrophic marker. The only disadvantage of the method is that every recombination event of the *lox* sites, leaves a recombined *lox* scar in the genome. Multiple deletions would result in several *lox* sites, which could theoretically be recombined when *Cre* recombinase is expressed in the system, allowing for the possibility of looping out of stretches of non-essential DNA that could affect the experimental approach.

Buckholz and Gleeson (1991) reviewed yeast systems for the commercial production of heterologous proteins and indicated that a mere five recombinant heterologous proteins were reportedly produced using *Y. lipolytica* as an expression hosts. A recent comprehensive review on *Y. lipolytica* as a heterologous expression host listed 42 heterologous proteins produced in the same system, with this number continually increasing (Madzak et al., 2004). These protein encoding genes originated from viruses, eubacteria, cyanobacteria, fungi, plants and mammals, covering proteins with molecular weights of 10 kDa–116 kDa in size. The secretion machinery of *Y. lipolytica* also recognized several foreign secretion signals, processed them and secreted functional protein into the extracellular environment. The production of laccase from *Pycnoporus cinnabarinus* and α-foetoprotein from *Homo sapiens* was roughly three- and two-fold, respectively, higher in *Y. lipolytica* when compared to *P. pastoris. Y. lipolytica* also outperformed *K. lactis* during the production of anti-Ras scFv.

Recombinant protein yields in *Y. lipolytica* have been moderate with the maximum yield of 1 g l^{-1} obtained for the *E. coli* derived amylolytic protein. The low production efficiencies of recombinant human proteins in this system could be explained by the difference in codon usage by the host, since these heterologous genes have not been codon optimized for *Y. lipolytica* (Madzak et al., 2004). A recent paper indicated the overproduction *LIP2* from *Y. lipolytica* in up to 3 g l^{-1} quantities using a *Y. lipolytica* strain with a multi-copy expression cassette (Aloulou et al., 2007). It should however be mentioned that at present, no industrial processes for the production of recombinant proteins by *Y. lipolytica* exist (Gellissen et al., 2005).

Y. lipolytica has been extensively researched and adapted as a host for the production of heterologous recombinant proteins from a wide variety of sources. Strains have been optimized for secretion, to accommodate several vector systems and allow stable integration into the genome of the organism. Tools to perform

yeast system engineering on DNA level have also been developed, to allow stable and rapid deletion, disruption and replacement of targets in the genome. The completion of the *Y. lipolytica* genome sequence will facilitate studies into pathway engineering, factors effecting high level expression of recombinant proteins, and assist in the identification of new production friendly promoters. Recent work done by Song and co-workers (2007) solved the first step in the production of therapeutic glycoproteins containing N-linked human-compatible sugars in *Y. lipolytica* by deleting the *Y. lipolytica OCH1* gene, resulting in a strain that can be used to produce glycoproteins lacking the outer chain mannoses.

Y. lipolytica as an expression system has also made its debut as being commercially available through a biotechnology company. This system, known as the YLEX Expression kit, has been commercialized by researchers at INRA (France) in collaboration with Yeastern Biotech Co. located in Taiwan (http: //www.yeastern.com) (C. Madzak, personal communication). The system offers a yeast strain (Po1g), two vectors (for intra- and extracellular expression), two primers (for insert sequencing) and a one-step transformation kit. This commercialized product should lead to the increased use of *Y. lipolytica* as an alternative expression host.

All the aspects in terms of *Y. lipolytica* as a heterologous producer of recombinant proteins have been optimized in order to make *Y. lipolytica* a definite candidate for the expression of any given protein than needs to be produced.

18.6 Schwanniomyces occidentalis

Schwanniomyces occidentalis (formerly known as *Schwanniomyces castellii*) is an amylolytic species that belongs to the same subfamily (*Saccharomycoideae*) as the genera *Saccharomyces*, *Kluyveromyces*, *Pichia* and *Hansenula*. *Sw. occidentalis* is important because of its ability to degrade starch completely. Starch is degraded by two secreted amylases, an α-amylase (encoded by thy *AMY1* gene) and a glucoamylase (encoded by the *GAM1* gene) (Boze et al., 1989). The production of enzymes is induced in the absence of glucose by the presence of maltose or starch. The ability of this species to grow in inexpensive media makes it a useful organism for production of heterologous proteins (Spencer et al., 2002). In addition, *Sw. occidentalis* is a potentially important host for large-scale commercial production of heterologous gene products because it can secrete proteins greater than 140 kDa efficiently into the culture medium (Oteng-Gyang et al., 1981; Wilson and Ingledew, 1982; Sills et al., 1984; Deibel et al., 1988), does not hyperglycosylate secreted proteins, and does not secrete measurable quantities of proteases (Deibel et al., 1988).

Klein and Favreau (1988) developed an efficient transformation and cloning system for *Sw. occidentalis* based on *ade2* mutants and the *ADE2* gene from *Sw. occidentalis* as a selectable marker. This system used a modification of the spheroplast procedure described by Beggs (1978). In the transformants derived from the *ade2* mutant host, the heterologous DNA was either integrated into the

chromosome or maintained as an extrachromosomal element without detectable mitotic loss. The episomal DNA was found to be present in a variety of plasmids of different molecular mass as a result of a high level of rearrangements. Pointek et al. (1998) designed expression vectors based on the *Sw. occidentalis*-derived autonomously replicating sequence (*SwARS*) and the *S. cerevisiae*-derived *TRP5* sequence for plasmid propagation and selection in yeast hosts, an origin of replication and an ampicillin-resistance sequence for propagation and selection in a bacterial host. These vectors share components for selection and propagation suitable for *S. cerevisiae*, *Sw. occidentalis* and *P. stipitis* (Dohmen et al., 1989) and the basic design of these vectors provides the potential to assess gene expression in a wide range of tryptophan-auxotrophic yeasts. In contrast to the results obtained by Klein and Favreau (1988, 1991) regarding heterologous DNA integration, the plasmids carrying the *S. cerevisiae*-derived *TRP5* gene and *SwARS* sequences do not recombine and yield an average of 5–10 copies per cell under selective conditions in the *Sw. occidentalis* host (Dohmen et al., 1989). Vector systems for *Sw. occidentalis* have been investigated and developed to some extent (Klein and Favreau, 1991), but no *CEN* (centromere) plasmids are available at the moment.

18.7 Methylotrophic Yeasts

Methylotrophic yeasts have gained increasing interest for fundamental research and as attractive hosts for the production of biologically active proteins (Hollenberg and Gellissen, 1997). These yeasts comprise a group of microorganisms able to use methanol as carbon source and energy. Adaptation to growth on methanol is associated with induction of methanol oxidase (also referred to as alcohol oxidase), dihydroxyacetone synthase and several other enzymes involved in methanol metabolism. The most spectacular increase, however, is seen with alcohol oxidase, which is virtually absent in glucose-grown cells, but can account for over 30% of the cell protein in methanol-grown cells. Extensive proliferation of peroxisomes, accounting for over 80% of the cell volume, is also observed in methanol-grown cells (Veenhuis et al., 1983). Due to these characteristics, methylotrophic yeasts have gained the attention of biochemists, molecular biologists, cell biologists, biotechnologists, microbiologists and chemists in academics and industry.

The use of these organisms in fundamental research is mainly related to studies of peroxisome homeostasis and nitrate assimilation (van der Klei and Veenhuis, 1996; Perez et al., 1997; Veenhuis et al., 2000). The peroxisomes contain the key enzymes involved in methanol metabolism, namely alcohol oxidase (AOX), dihydroxyacetone synthase (DHAS) and catalase (CAT). The two proteins AOX and DHAS may constitute over 60% of total cellular protein under these conditions. This illustrates that the genes encoding these proteins are controlled by very strong promoters. This feature contributed to recognizing the methylotrophs as attractive hosts for the production of heterologous proteins.

18.8 Hansenula polymorpha (Pichia angusta)

18.8.1 History

Hansenula polymorpha is one of the species of *Hansenula* that were transferred to the genus *Pichia* based on the formation of hat-shaped ascospores (Kurtzman, 1984). This lead to the proposed name change from *H. polymorpha* to *Pichia angusta* [(Teunisson, Hall and Wickerham) (Kurtzman, 1984)]. With the move to the *Pichia* genus a nomenclatural difficulty arose. The name *Pichia polymorpha* was previously used for the species currently known as *Debaryomyces polymorpha*. Due to the unavailability of the name *polymorpha, Pichia angusta* was selected because *Hansenula angusta* is an obligate synonym of *H. polymorpha* (Kurtzman, 1998b). However, most researchers prefer the original name of *H. polymorpha* and although taxonomically incorrect we will for the sake of simplicity refer to this yeast as *H. polymorpha*.

18.8.2 Introduction

H. polymorpha has some specific advantages over other methylotrophic yeasts (such as *P. pastoris* and *C. boidinii*), being more thermotolerant and capable to grow at higher rates on simple, defined media. The relatively high optimal growth temperature for *H. polymorpha* (37°C–43°C) may be favorable for the production of mammalian proteins and furthermore has the advantage that it allows for better management and reduces the risk of contaminations in large scale fermentations (Gellissen, 2000).

Extensive proliferation of peroxisomes, accounting for over 80% of the cell volume, is observed in methanol-grown cells (Veenhuis et al., 1983). A characteristic feature of peroxisomes is that they are inducible. In *H. polymorpha*, peroxisomes can be induced by methanol, ethanol, primary amines, D-amino acids, L-α hydroxyl acids and purines (Veenhuis and Harder, 1988). The advantage of the accumulation of proteins in peroxisomes is obvious in cases when expressed proteins are toxic for the host organism, where the peroxisomal membrane forms a barrier, and thereby preventing that the proteins can exert their toxic activity to the yeast cell. Another potential advantage of storage in peroxisomes is the absence of modifying enzymes which prevent undesired modifications such as glycosylation. In addition, proteins which are sensitive to proteolytic degradation are protected from proteolysis inside the matrix of the peroxisome.

H. polymorpha also proved an excellent model by which to study the nitrate assimilation pathway and its transcriptional regulation (Brito et al., 1999). All these facts have promoted important development of the genetic analysis and molecular biology tools for *H. polymorpha* (Rezaee, 2003).

18.8.3 Genetics and Molecular Biology

An essential tool for the construction of recombinant *H. polymorpha* strains are *E. coli–H. polymorpha* shuttle vectors. An important feature of these vectors is the selectable marker genes, which can functionally complement various auxotrophic *H. polymorpha* strains. Commonly used marker genes are *H. polymorpha LEU2, URA3, TRP3* and *ADE11*, the *S. cerevisiae* genes *LEU2, URA3* as well as *C. albicans LEU2* (Roggenkamp et al., 1986; Merckelbach et al., 1993; Agaphonov et al., 1994; Bogdanova et al., 1995). Unlike *S. cerevisiae, H. polymorpha* does not harbour any natural plasmids, but with the isolation of auxotrophic mutants, like ura− strains (Roggenkamp et al., 1986) and leu− strains (Gleeson et al., 1986), and complementation by the respective genes form *S. cerevisiae*, introduction of plasmid DNA could be established. In addition to auxotrophic markers a number of alternative selection systems have been used successfully including G418 (Liu et al., 2005), phleomycin (Zurek et al., 1996) and zeocin (Song et al., 2003).

Production systems based on *H. polymorpha* rely on the use of various promoter elements, both inducible and constitutive. Commonly used promoter elements are those derived from genes of the methanol metabolism pathway, which are strongly inducible. The methanol oxidase promoter, dihydroxyacetone synthase promoter and the promoter of formate dehydrogenase gene are fully repressed by excess glucose and are strongly induced by methanol. With the employment of the abovementioned promoters, various attractive induction strategies can be designed. For instance, in the case of harmful recombinant proteins, first biomass can be generated, followed by an alteration in composition of the growth medium, thereby inducing the expression of the heterologous gene (Rezaee, 2003). Derepression of these promoters is also possible under glucose- or glycerol-limiting conditions (e.g. in carbon-limited chemostat cultures). Other inducible promoter elements that have been identified in *H. polymorpha* include elements derived from genes of the nitrate metabolism, *YNT1, YNR1* and *YNL1* (Avila et al., 1996; Brito et al., 1996; Perez et al., 1997) and the repressible acid phosphatase (*PHO1*) promoter (Phongdara et al., 1998). These promoters could be used as new control elements for protein production in *H. polymorpha*. Promoters for constitutively expressed gene products have also been identified in *H. polymorpha*, such as the promoter of the plasma membrane H^+-ATPase (P*PMA1*) (Cox et al., 2000) and the promoter of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*PGAP*) (Heo et al., 2003).

Efficient and reliable transformation procedures for *H. polymorpha* have been developed. Yeast cells can be transformed using whole-cell methods according to the lithium acetate-dimethylsulfoxide method described by Hill et al. (1991), by adding PEG (Dohmen et al., 1991) or by electroporation (Faber et al., 1994). In *H. polymorpha*, a high frequency of plasmid integration is observed despite the presence of an *ARS* (autonomously replicating sequence) (Sohn et al., 1996) on a vector. Transformation results in mitotically stable strains containing different copies of an integrated expression cassette exhibiting a head-to-tail arrangement (Gellissen and Hollenberg, 1997). They are integrated by non-homologous recombination and strains with up to 100 copies have been identified (Janowicz et al.,

1991; Gellissen et al., 1994; Gatzke et al., 1995). Targeted integration is achieved through recombination following transformation of linear plasmids containing homologous sequences which are targeted to specific loci in the genome. A variety of target sequences have been used successfully including the *LEU2* gene and *HARS36* (Agaphonov et al., 1999) or the *MOX/TRP* locus (Agaphonov et al., 1995; Machin et al., 2001; Song et al., 2003).

Recently a number of researchers have shown that integration at the rDNA loci is very good alternative (Cox et al., 2000; Klabunde et al., 2002, 2003, 2005; Liu et al., 2005). The advantage of integration into the rDNA loci includes the use of such a vector for the transformation of variety of yeast species. Klabunde et al. (2003) designed a vector (pTHpH181Hp) with the aim of transforming *H. polymorpha*, *S. cerevisiae*, *P. stipitis* and *A. adeninivorans*. Apart from a bacterial moiety the vector contained the *TEF1* promoter from *A. adeninivorans* linked to the *hph* gene from *E. coli* followed by the *PHO5* terminator from *S. cerevisiae.* This region, obtained from the pAL-HPH1 plasmid (Rösel and Kunze, 1998) served as selection against hygromycin in the different yeast species. Different regions of the *H. polymorpha* rDNA unit were tested and it was shown that the inclusion of the putative promoter region of the 35S rDNA precursor together with the ETS region and the full-length 18S rDNA sequence allowed successful transformation of all the yeast species tested. In addition to the 18S rDNA region, the 25S region was also shown to allow integration of homologous rDNA containing plasmids using the *S. cerevisiae* derived *URA3* gene as selectable marker (Klabunde et al., 2002) or the geneticin (G418) as resistance gene (Liu et al., 2005). No difference was seen in terms of expression of a firefly luciferase (Luc) reporter gene when transformed *H. polymorpha* was tested using either an 18S or 25S containing vector (Liu et al., 2005).

H. polymorpha is currently used as an expression system by Rhein Biotech GmbH (Germany) to produce a recombinant hepatitis B vaccine which is sold under the name Hepavax-Gene®. This company uses the *Hansenula* expression system for production of recombinant proteins, and also licenses the technology to other companies. The complete genomic sequence of *H. polymorpha* was determined by Rhein Biotech who commissioned Qiagen Genomic Services (Qiagen, GmbH, Germany). The *H. polymorpha* genome consists of approximately 9.5 Mb organized as six chromosomes ranging in size from 0.9 to 2.2 Mb containing approximately 5933 open reading frames (Ramezani-Rad et al., 2003). Currently the genome data is not in the public domain and access to the genome data can be obtained following a material transfer agreement. Part of the *H. polymorpha* genome sequence was also determined through the Génolevures project. In this instance coverage of 0.5 genome equivalents was completed and approximately 2500 novel protein-coding genes were identified (Blandin et al., 2000).

The need to establish a reliable system for the production of heterologous membrane proteins is emphasized by the observation that many human diseases are caused by the malfunctioning of membrane proteins, therefore, such a system would be of the utmost importance for various reasons, such as the facilitation of rational drug design. In addition, fundamental research such as resolving the structure and functionality of important membrane proteins rely on the availability of

relative large amounts of biologically active membrane proteins. In *H. polymorpha*, excessive peroxisomal membranes are easily developed that hardly contain protein components and thus are an excellent storage place for heterologous membrane proteins (Veenhuis et al., 1990). Localization to these membranes can be mediated by the targeting signal of the *H. polymorpha* peroxisomal membrane protein Pex3p (Baerends et al., 1996) which suggests that *H. polymorpha* could be a significant tool in the production of membrane proteins.

18.9 Pichia pastoris

18.9.1 History

Pichia pastoris [(Guilliermond) Phaff] is a methylotrophic yeast that can grow on methanol as sole carbon and energy source. The Phillips Petroleum Company was the first to develop media and protocols for cultivating *P. pastoris* on methanol in continuous cultures. An efficient ultra-high cell density (>130 g dry cell weight per liter) (Cereghino and Gregg, 2000) fermentation process with high biomass productivity (>10 g l^{-1} h^{-1}) was developed for this yeast (Sreekrishna and Kropp, 1996). During the 1970s, *P. pastoris* was considered as a potential source of single-cell protein for feedstock due to its ability to utilize methanol as sole carbon source. However, the oil crisis of the 1970s caused a dramatic increase in the cost of methane (the source of the methanol) and the economics of this process, while impressive from a fermentation standpoint (approximately $5 per pound of protein), was clearly an order of magnitude higher in comparison to the cost of a pound of soybeans, which was the major alternative source of animal feed. This resulted in Phillips Petroleum Company investing its efforts in developing this yeast as an expression system for the production of recombinant proteins proving to be a worthwhile endeavor (Sreekrishna and Kropp, 1996). *P. pastoris* has gained widespread attention as an expression system because of its ability to express high levels of heterologous proteins. As a result, recombinant vector construction, methods for transformation, selectable marker generation and fermentation methods have been developed to exploit the potential of this system (Rosenfeld, 1999). Research Corporation Technologies (Tucson, Arizona, USA) are the current holders of the patent for the *P. pastoris* expression system and Invitrogen Corporation (Carlsbad, California, USA), has an exclusive license to sell the *Pichia* Expression Kit to scientist for academic research purposes (Macauley-Patrick et al., 2005; www.invitrogen.com).

18.9.2 Introduction

P. pastoris is a homothallic, ascomycetous yeast that can be manipulated by classical genetic methods (Cregg and Madden, 1988; Cregg et al., 1998). The conceptual basis for the *P. pastoris* expression system stems from the observation that some of the

enzymes required for methanol metabolism are present at substantial levels only when cells are grown on methanol (Egli et al., 1980; Veenhuis et al., 1983). Unlike homothallic strains of *S. cerevisiae*, which are diploid, *P. pastoris* remains haploid unless forced to mate. Strains with complementary markers can be mated by subjecting them to a nitrogen-limited medium. After one day on this medium, cells are shifted to a standard minimal medium supplemented with nutrients designed to select for complementing diploid cells (not self-mated or non-mated parental cells). The resulting diploids are stable as long as they are not subjected to nutritional stress. To obtain spore products, diploids are returned to the nitrogen-limited medium, which stimulates them to proceed through meiosis and sporulation. Spore products are handled by random spore techniques rather than micromanipulation, since *P. pastoris* asci are small and difficult to dissect. Yet most standard classical genetic manipulations, including mutant isolation, complementation analysis, backcrossing, strain construction and spore analysis, can be accomplished (Cereghino and Gregg, 2000).

18.9.3 Genetics and Molecular Biology

Techniques required for the genetic manipulation of *P. pastoris*, such as DNA-mediated transformation, gene targeting, gene replacement and cloning by functional complementation, are similar to those described for *S. cerevisiae*. *P. pastoris* can be transformed by electroporation (Becker and Guarente, 1992), a spheroplast generation method (Cregg et al., 1985; Sreekrishna et al., 1987), or whole cell methods such as those involving lithium chloride (Ito et al., 1983) and polyethylene glycol (Dohmen et al., 1991). The introduced DNA can establish itself in two ways: integration into chromosomal DNA by homologous recombination or autonomous replication as a circular plasmid. As in *S. cerevisiae*, *P. pastoris* exhibits a tendency for homologous recombination between genomic and artificially introduced DNA's. Cleavage of a *P. pastoris* vector within a sequence shared by the host genome stimulates homologous recombination events that efficiently target integration of the vector to that genomic locus (Cregg and Madden, 1987). Gene replacements occur at lower frequencies than those observed in *S. cerevisiae* and appear to require longer terminal flanking sequences to efficiently direct integration (Cregg and Russel, 1998).

The majority of heterologous protein production in *P. pastoris* is based on the fact that enzymes required for the metabolism of methanol are only present when cells are grown in methanol (Egli et al., 1980). Therefore, although other promoter options are available for the production of foreign proteins in *P. pastoris* (extensively reviewed by Cereghino and Gregg (2000)), the *AOX* promoters have been the most widely used (Cereghino et al., 2001). However, there are circumstances in which the *AOX* promoter may not be suitable. Promoters that are not induced by methanol are preferred for the production of food related products since the petroleum related compound methane, is a source of methanol. A second consideration is that methanol is a potential fire hazard, especially with the large quantities that

are needed for large-scale fermentations (Cereghino and Gregg, 2000). Alternative promoters to the *AOX1* promoter include the *P. pastoris GAP* (glyceraldehyde 3-phosphate dehydrogenase), *FLD1* (glutathione-dependent formaldehyde dehydrogenase), *PEX8* (a peroxisomal matrix protein), *YPT1* (a GTPase involved in secretion) and more recently, *ICL1* (isocitrate lyase) promoters (Cereghino and Gregg, 2000; Macauley-Patrick et al., 2005).

Few selectable marker genes have been described for *P. pastoris.* Initially, markers were limited to biosynthetic pathway genes including *HIS4* from either *P. pastoris* or *S. cerevisiae*, *ARG4* from *S. cerevisiae* and the *Shble* gene from *Streptoalloteichus hindustanus* which confers resistance to the bleomycin-related drug zeocin (Cregg et al., 1985, 1989; Higgins et al., 1998). An additional set of biosynthetic markers that includes the *P. pastoris ADE1*, *ARG4*, and *URA3* genes have also been described. Each of these selectable markers have been incorporated into expression vectors and a series of host stains containing all possible combinations of *ade1, arg4, his4* and *ura3* auxotrophies has been generated (Cereghino and Gregg, 2000; Cereghino et al., 2001).

The commercial *P. pastoris* Expression Systems supplied by Invitrogen Corporation have a number of expression vectors available. Currently three types of expression kits are available that includes *P. pastoris* strains, expression vectors, transformation reagents, sequencing primers and media. The vectors contain either the inducible *AOX1* or the constitutive *GAP* promoters and the *HIS4* gene for selection. Expressed proteins can be directed to the extracellular environment with either the *S. cerevisiae* α-factor or the native *P. pastoris* acid phosphatase signal sequence (*PHO1*) or alternatively expressed intracellularly.

The *P. pastoris* expression system has gained acceptance as an important host organism for the production of foreign proteins (for an extensive list of heterologous proteins expressed successfully in *P. pastoris*, refer to the review by Cereghino and Gregg (2000) or visit the website: http: //faculty.kgi.edu/cregg/index.htm, for an updated version of the list). It is noteworthy to indicate that *P. pastoris* can secrete a large variety of proteins, with yields of up to 14.8 g l^{-1} as reported by Werten and co-workers (1999).

A number of proteins synthesized in *P. pastoris* are being tested for use as human pharmaceuticals in clinical trials. However, a major limitation of *P. pastoris* and other yeast expression systems in the production of therapeutic proteins is that they were unable to replicate the exact post-translational modifications of the proteins as they passed through the protein processing pathway (Cereghino and Gregg, 2000). One of these modifications, correct glycosylation of the therapeutic proteins, was found to be critical in therapeutic efficacy of the protein, since non-human glycosylation can reduce the half-life of the protein and eliciting an immunogenic response to the foreign carbohydrate moiety (Helenius and Aebi, 2001). Researchers at Dartmouth College (Hannover, NH, USA) and GlycoFi Inc. (Lebanon, NH, USA) succeeded in manipulating the glycosylation pathway of *P. pastoris* to secrete human glycoproteins with uniform complex *N*-glycosylation patterns (Hamilton et al., 2003). In order to humanize the *P. pastoris* glycosylation pathway, the endogenous yeast glycosylation pathways had to be eliminated together with the introduction of five eukaryotic proteins involved in glycosylation. Correct localization of these

proteins in the glycosylation pathway was critical and involved complex combinatorial approaches between signal sequences and catalytic domains in order to correctly localize active enzymes in the pathway (reviewed by Wildt and Gerngross (2005)). Li and co-workers (2006) used several of these engineered *P. pastoris* strains to study the effect of glycosylation on monoclonal antibodies and observed improved results when the yeast derived antibodies were compared to commercial, mammalian cell-line derived antibodies. This was followed by advancing the system even further to allow the production of terminally sialylated glycoproteins (Hamilton et al., 2006). The construction of the strain allowing terminal sialylation of the complex human *N*-glycosylated proteins, included the deletion of four yeast genes involved in the yeast specific glycosylation pathway followed by the introduction, expression and correct localization of 14 heterologous genes – effectively mimicking the sequential steps of human glycosylation. GlycoFi Inc. was recently acquired by Merck and Co. Inc. shortly after announcing the potential of yeast derived uniform glycosylation to improve expression of therapeutic proteins.

The yeast *P. pastoris* is a useful system for the expression of milligram to several gram quantities of proteins for both basic laboratory research and industrial manufacture. This methylotrophic yeast is particularly suited to foreign protein expression for reasons such as ease of genetic manipulation encompassed by gene targeting, high-frequency DNA transformation, cloning by functional complementation, high levels of protein expression, both intra- and extracellularly, and the ability to perform higher eukaryotic protein modifications, such as glycosylation, disulfide bond formation and proteolytic processing (Cregg et al., 2000). Integrated Genomics completed the sequence coverage of the 9.4 Mb *P. pastoris* genome (although publicly still unavailable), containing approximately 6000 ORF sequences, thereby allowing fast tracking any future developments of the *P. pastoris* expression system due to genomic data availability (http://www.integratedgenomics.com/pichia.html). Simple purification of secreted recombinant proteins is possible due to the relatively low levels of native secreted proteins (Cregg et al., 1993). The humanization of the *N*-glycosylation pathway in *P. pastoris*, the powerful genetic tools, together with its economy of use, make *P. pastoris* the system of choice for heterologous protein expression of industrial as well as biopharmaceutical importance.

18.10 *Pichia stipitis*

The respiratory yeast *P. stipitis* does not produce ethanol during aerobic cultivation (Skoog et al., 1992; Passoth et al., 1996) suggesting that this yeast is suitable for heterologous protein production since it can effectively convert a carbon source to cell mass. More importantly, this yeast can use xylose, the major hemicellulose component in plant cell walls (and the second most abundant renewable carbon source in nature) as sole energy and carbon source. Xylose has to be isomerized to xylulose in a two-step reaction by a NADP(H)-linked reduction to xylitol (catalyzed by xylose reductase encoded by the *XYL1* gene), followed by the NAD^+-linked

oxidation to xylulose (catalysed by the xylulose dehydrogenase encoded by the *XYL2* gene) (Barnett, 1976). Both these genes have been cloned and characterized (Kötter et al., 1990; Amore et al., 1991).

The main emphasis of heterologous expression in *P. stipitis* is based on the improvement of hemicellulosic sugar utilization. Successful expression and/or improved xylose utilization were obtained with expression of the *Cryptococcus albidus* endo1,4-β-xylanase (Morosoli et al., 1993; Passoth and Hahn-Hägerdal, 2000; Görgens et al., 2005), the *Trichoderma reesei* β-xylanase II (*xyn2*) gene (den Haan and van Zyl, 2001) the *Trichoderma reesei* xyn2 and the *Aspergillus kawachii* xynC (both encoding a β-1,4-endoxylanase) and the *Aspergillus niger* xlnD (encoding a β-xylosidase) genes (den Haan and van Zyl, 2003).

Pointek and co-workers (1998) developed a expression system for *P. stipitis* to test different promoter elements using either the native inducible xylose reductase encoding *XYL1* promoter or the *S. cerevisiae* derived *PDC1* and *ADH1* promoter elements together with the *Schwanniomyces occidentalis GAM1* (encoding a glucoamylase) terminator. Propagation and selection was accomplished through the *Sw. occidentalis* autonomous replication sequence (SwARS) and the *S. cerevisiae* tryptophan syntase (*TRP5*) gene and the cellulose encoding *celD* gene from *Clostridium thermocellum* was used as reporter gene. Enzymatic determination of the reporter gene showed that the native *XYL1* promoter provided the highest productivity. Furthermore, the vector system share components for selection and propagation in *P. stipitis, Sw. occidentalis* and *S. cerevisiae* which can potentially be used to assess heterologous expression in these yeast and potentially in other tryptophan auxotrophic yeasts.

Den Haan and van Zyl (2001) evaluated the *P. stipitis XYLI and TKL* (transketolase gene) promoters as well as the *S. cerevisiae PGK1* (phosphoglycerate kinase) promoter using the *T. reesei xyn2* gene as reporter. In addition to these components the vectors also contained the *P. stipitis ARS2* and *URA3* for propagation and selection. In this study it was shown that the *XYL1* promoter is inducible in the presence of xylose and the *TKL* promoter to be constitutive in the presence of either xylose or glucose. The *S. cerevisiae PGK1* promoter was shown to be non-functional in *P. stipitis*. More important, the recombinant β-xylanase enzyme corresponds to the size of the native *T. reesei* enzyme indicating no or very little glycosylation. The availability of a gene expression system utilizing different promoters together with the indication that *P. stipitis* have a low level of glycosylation makes this yeast a good alternative as an expression host. Furthermore, the 12 Mb genome sequence of *P. stipitis* strain CBS 6054 (containing 5841 ORFs) was recently completed by the DOE Joint Genome Institute (JGI) (http: //genome.jgi-psf.org/) making this yeast even more genetically accessible (Jeffries et al., 2007).

18.11 Conclusions

Since no single yeast-based expression platform exists which is optimal for every protein, a suitable host has to be defined for each heterologous gene to be expressed. It would however be ideal to assess several selected organisms as expression platforms

in parallel for optimal product characteristics. The wide-range integrative yeast expression vector systems based on *A. adeninivorans*- and *H. polymorpha*-derived elements (Ilgen et al., 2005; Klabunde et al., 2003, 2005; Terentiev et al., 2004) fulfill the criteria expected of a vector system that could be targeted to the various test species. These criteria include a targeting sequence (such as the conserved NTS2-ETS-18SrDNA-ITS1 region from *H. polymorpha* or the 25S rDNA region from *A. adeninivorans*), a promoter element for expression control of reporter sequence (e.g. the *A. adeninivorans*-derived *TEF1* promoter) and a selection marker that function in all selected organisms (*E. coli*-derived *hph* gene conferring hygromycin B resistance). Heterologous gene expression from these expression platforms was assessed with green fluorescent protein (GFP), phytase from *Aspergillus* or *E. coli lacZ* as reporter gene. The plasmids were found to be integrated into the genome of *A. adeninivorans, S. cerevisiae, H. polymorpha, P. pastoris, P. stipitis, Debaryomyces hansenii* and *Debaryomyces polymorphus* (Gellissen et al., 2005). Such a single expression system that can be used to utilize the power of a number of yeasts together with the complete genome sequence of a number of non-conventional yeasts available that allow specific pathway engineering and tweaking of several metabolic pathways to improve any given process, becomes a reality.

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Chapter 19
A Comparative Study of RNA Polymerase II Transcription Machinery in Yeasts

Nimisha Sharma and Surbhi Mehta

Contents

Abstract The control of gene expression, predominantly at the level of transcription, plays a fundamental role in biological processes determining the phenotypic changes in cells and organisms. The eukaryotes have evolved a complex and sophisticated transcription machinery to transcribe DNA into RNA. RNA polymerase II enzyme lies at the centre of the transcription apparatus that comprises nearly 60 polypeptides and is responsible for the expression and regulation of protein-encoding genes. Much of our present understanding and knowledge of the RNA polymerase II transcription apparatus in eukaryotes has been derived from studies in *Saccharomyces cerevisiae*. More recently, *Schizosaccharomyces pombe* has emerged as a better model system to study transcription because the transcription mechanism in this yeast is closer to that in higher eukaryotes. Also, studies on components of the basal transcription machinery have revealed a number of properties that are

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common with other eukaryotes, but have also highlighted some features unique to *S. pombe*. In fact, the fungal transcription associated protein families show greater species specificity and only 15% of these proteins contain homologues shared between both *S. cerevisiae* and *S. pombe*. In this chapter, we compare the RNA polymerase II transcription apparatus in different yeasts.

Keywords Transcription, transcription machinery, RNA polymerase II, *S. cerevisiae*, *S. pombe*

19.1 Introduction

Regulated expression of protein-coding genes underlies fundamental biological processes, including development, differentiation, morphogenesis and oncogenesis. Most of this regulation occurs predominantly at the level of transcription initiation. More than 60 different proteins coordinate with each other to fine tune the spatial and temporal pattern of gene expression. These proteins can be grouped into three different classes (Fig. 19.1).

- General or basal transcription factors (GTFs) that are ubiquitous and bind to core promoter DNA sequences. These proteins enable the recruitment of RNA polymerase II (pol II) to the specific promoter sequences of protein-coding genes;

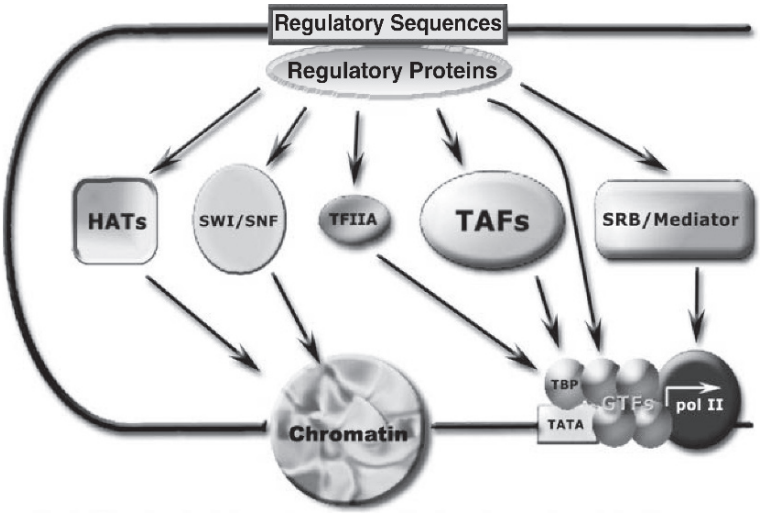

Fig. 19.1 Schematic representation of the key players involved in expression and regulation of protein encoding genes (see text for details)

- Regulatory proteins that bind to proximal promoter elements, enhancers or silencers in a sequence-dependent manner. They activate or repress transcription of target gene(s) either in a cell-type/developmental stage-specific manner or in response to external stimuli;
- Co-activators and co-repressors - proteins which interact with the regulatory proteins, and mediate their effects on the basal transcription machinery. These can be further sub-divided into two groups-those which affect chromatin structure, such as Swi/Snf complex and HATs (Histone Acetyl Transferases) and those which serve as an interface, integrating information from the different transcription regulatory proteins and transmitting it to the general transcription machinery. The latter category of protein is collectively referred to as 'mediators'.

Much of our current knowledge of the mechanism of transcription and the transcription machinery has come from biochemical and genetic studies carried out in the budding yeast, *Saccharomyces cerevisiae*. However advances in structural biology over the past few years, have helped in unveiling the structures of the *S. cerevisiae* pol II enzyme alone and in complex with several transcription factors. Specifically, structures of the 10 subunit pol II enzyme alone, i.e. lacking the Rpb4 and Rpb7 subunits (Cramer et al., 2000, 2001), the 12 subunit enzyme alone (Armache et al., 2003, 2005; Bushnell and Kornberg, 2003), the 10 subunit enzyme in the form of a transcribing complex with the general transcription factor TFIIB (Chen and Hahn, 2004; Bushnell et al., 2004), and also in complex with the transcription elongation factor TFIIS (Kettenberger et al., 2003, 2004) have been resolved. Lower resolution EM structures have also been determined for the pol II–mediator complex (Davis et al., 2002) and for pol II–TFIIF complex (Chung et al., 2003). Determination of structures of these large multiprotein complexes, have added a new dimension to our analysis of the different steps of the transcription reaction (reviewed by Hahn, 2004; Woychik and Hampsey, 2002).

Recently, fission yeast, *Schizosaccharomyces pombe*, has emerged as a complementary model system to study many of the biological processes. Cross-species comparisons between *S. cerevisiae* and *S. pombe* have proven a valuable tool in analyzing cell division cycle control, DNA repair and recombination (reviewed by Sunnerhagen, 2002). Several lines of evidence indicate that the mechanism of transcription initiation by *S. pombe* RNA polymerase II is more similar to higher eukaryotes than that of *S. cerevisiae*. Initiation of transcription by pol II occurs 25–30 bp downstream from the TATA box of the core promoter in both *S. pombe* and mammalian cells; but this distance may vary between 40 bp and 120 bp downstream of the TATA box in *S. cerevisiae* (Li et al., 1994). These observations raise the possibility that both *S. cerevisiae* and *S. pombe* may use different mechanisms to identify transcription start sites. It was also observed that transcriptional initiation from mammalian promoters introduced into *S. pombe* occurred at the same site as in mammalian cells (Toyoma and Okayama, 1990). Furthermore, the AP2 and CTF transcriptional activators did not stimulate transcription in *S. cerevisiae*, whereas they activated transcription in *S. pombe* and humans. This implies that the

transcription activation mechanism in *S. pombe* is closer to that of humans than *S. cerevisiae* (Remacle et al., 1997).

The scope of this chapter is to provide an overview of the structural features and functions of the various proteins that function in RNA pol II-mediated transcription initiation in yeast, with more emphasis on the evolutionary distant *S. cerevisiae* and *S. pombe*. To grasp the relationship between these proteins, it is imperative to briefly describe the steps involved in the transcription of protein-coding genes.

19.2 Overview of Pol II Transcription

Transcription of RNA polymerase II-dependent genes begins with the assembly of a pre-initiation complex at the promoter. The pre-initiation complex or PIC is a conglomerate of six different basal transcription factors-TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH and the RNA polymerase II enzyme (Fig. 19.2).

Formation of the PIC is followed by promoter melting or separation of the two strands of the template DNA to expose the transcription initiation site and the synthesis of the first phosphodiester bond in the nascent RNA. Subsequently many short RNA transcripts, 3 to 10 bases long, are transcribed by pol II and released. This phenomenon is referred to as 'abortive initiation'. Finally, longer transcripts of approximately 30 bases are synthesized and transcription switches from being abortive to productive, i.e. RNA polymerase II is released from the proteins

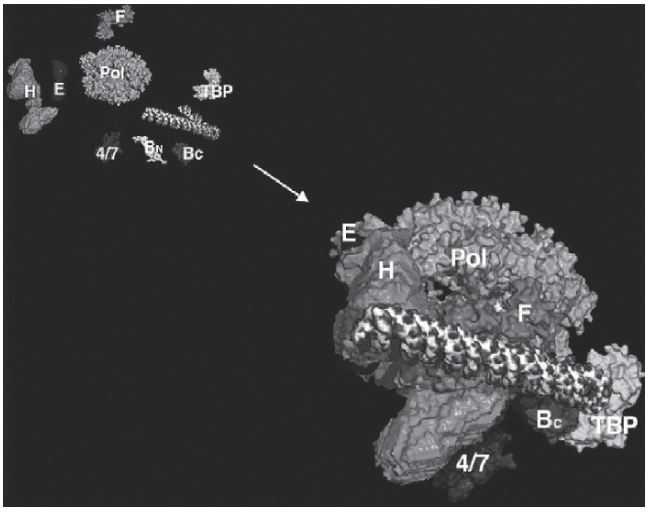

Fig. 19.2 RNA polymerase II transcription initiation complex. X-ray and electron microscopic structures (upper left) were assembled in a complete transcription initiation complex (lower right). Reprinted by permission of Federation of the European Biochemical Societies from Structural basis of eukaryotic gene transcription, by Boeger et al., 2005. *FEBS Lett.* 579: 899–903

assembled at the promoter (known as 'promoter clearance') and enters the stage of transcription elongation. Once the enzyme reaches the termination site, it dissociates from the DNA template and the nascent RNA transcript is released (reviewed by Orphanides et al., 1996).

The key players involved in transcription initiation of RNA pol II genes are described below:

19.3 RNA Polymerase II Core Enzyme: The 'CPU' of mRNA Synthesizing Machinery

RNA polymerase II core enzyme lies at the centre of the transcription apparatus responsible for decoding the information stored in DNA into its usable form, the mRNA. It resembles the CPU or the central processing unit of a computer receiving inputs from various internal and external stimuli, processing these inputs and then giving an output in the form of expression of specific genes or specific classes of genes (Fig. 19.3).

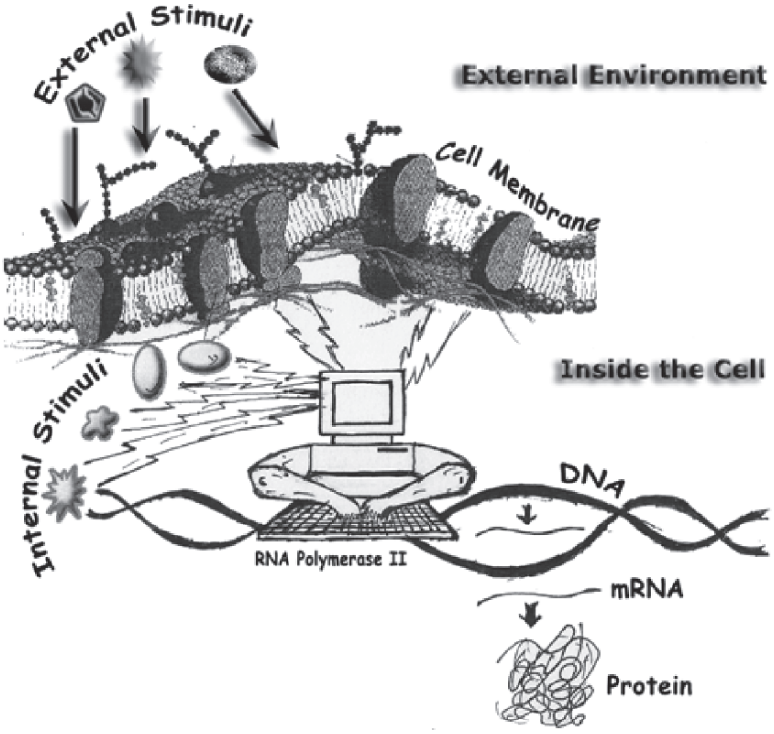

Fig. 19.3 A cartoon depicting the RNA polymerase II enzyme as the Central Processing Unit of the computer

Pol II from both *S. cerevisiae* and *S. pombe* consist of twelve subunits (reviewed by Kolodziez et al., 1990; Young, 1991; Mitsuzawa and Ishihama, 2004). These subunits, designated as Rpb1 to Rpb12, can be divided into three overlapping categories: (i) core subunits-Rpb1, Rpb2, Rpb3 and Rpb11; (ii) shared or common subunits-Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12; and (iii) pol II unique subunits-Rpb4, Rpb7 and Rpb9. Pol II has also been isolated from other yeasts, including *Candida albicans* and *Candida utilis*. Interestingly, *Candida albicans* pol II contains only nine subunits, with apparent molecular weights of 170, 145, 120, 80, 62, 58, 45, 40 and 20 kDa. The mobility of these subunits on SDS-PAGE is different from the corresponding subunits from *S. cerevisiae* or *C. utilis* (Patturajan et al., 1999). In comparison to the *C. albicans* pol II, the enzyme from *C. utilis* comprises ten subunits, with molecular weights ranging from 205 kDa to 14 kDa (Patturajan, 1995). Interestingly, pol II from *C. utilis* could initiate transcription accurately upon addition of cell extracts from both *C. utilis* and *S. cerevisiae.* Moreover, the GTFs were also functionally interchangeable between these two yeasts (Patturajan et al.,1994). Both prokaryotic and eukaryotic RNA polymerases are zinc containing metalloproteins (reviewed by Archambault and Friesen, 1993). The *S. cerevisiae* enzyme contains two molecules of zinc, whose removal damages the enzyme conformation irreversibly (Mayalagu et al., 1997). In comparison, both *C. albicans* and *C. utilis* pol II contain five molecules of zinc bound to them. It was also shown that the three largest subunits of *C. albicans* pol II had the ability to bind zinc, whereas only the largest subunit of *C. utilis* pol II could bind zinc (Patturajan et al., 1999). In case of the *S. cerevisiae* enzyme, five subunits have been demonstrated to possess zinc binding ability (reviewed by Archambault and Friesen, 1993).

Following sections describe our current status of understanding of the different subunits of *S. cerevisiae* and *S. pombe* RNA polymerase II. Table 19.1 shows a comparison of some of the features of these subunits in *S. cerevisiae* and *S. pombe*.

Table 19.1 Comparison between orthologous subunits in *S. cerevisiae* and *S. pombe*

Subunit	Molecular weight (kDa) in *S. cerevisiae*	Molecular weight (kDa) in *S. pombe*	Deletion viability in *S. cerevisiae*	Deletion viability in *S. pombe*	Identity in *S. pombe*
Rpb1	192	194	Essential	Essential	59
Rpb2	139	138	Essential	Essential	67
Rpb3	35	34	Essential	Essential	47
Rpb4	25	15.4	Nonessential	Essential	20
Rpb5	25	24	Essential	Essential	56
Rpb6	18	16	Essential	Essential	54
Rpb7	19	19	Essential	Essential	52
Rpb8	17	14	Essential	Essential	34
Rpb9	14	13	Nonessential	Essential	47
Rpb10	8	8.3	Essential	Essential	72
Rpb11	14	14	Essential	Essential	44
Rpb12	8	7.2	Essential	Essential	39

19.3.1 Core Subunits: Rpb1, Rpb2, Rpb3 and Rpb11

The subunits- Rpb1, 2, 3 and 11, form the core catalytic domain of RNA polymerase II. Rpb1 and Rpb2 are homologous to the largest and second largest subunits of RNA polymerases I and III, and also share sequence homology with the β′ and β subunits respectively of the eubacterial RNA polymerase. Not surprisingly, this sequence similarity also extends to functional similarity: the Rpb1 and β′ subunits bind DNA, whereas the Rpb2 and β subunits are involved in binding nucleotide substrates. Specific mutations have been isolated in *S. cerevisiae* Rpb1 and Rpb2 subunits that affect accuracy of transcription initiation, thereby implying a role of these subunits in start site selection. Other mutations in these subunits confer sensitivity to 6-azauracil (6-AU), a phenotype linked to defects in transcription elongation, suggesting that these subunits help in overcoming transcriptional arrest (reviewed by Archambault and Friesen, 1993).

A unique feature of the pol II Rpb1 subunit is the presence of the carboxyl terminal repeat domain (CTD) containing tandem repeats of a heptad motif with the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Although the presence of CTD is a ubiquitous feature of all eukaryotic RNA polymerase II enzymes, the number of heptapeptide repeat varies between different organisms. The budding yeast pol II CTD contains 26 repeats (Allison et al., 1985), whereas fission yeast pol II CTD has 29 repeats (Azuma et al., 1991). Complete removal of the CTD from budding yeast pol II is lethal, but truncation of the number of repeats present in the CTD to 10–12, results in conditional phenotypes like cold sensitivity and the inability to grow on a variety of carbon sources (Nonet et al., 1987; Nonet and Young, 1989). A genetic screen carried out to isolate suppressors of the cold-sensitive phenotype identified mutations in nine different genes and these suppressors were called SRBs or Suppressors of RNA Polymerase B (Nonet and Young, 1989). The CTD plays myriad roles in coordination and regulation of transcription initiation, elongation and termination, DNA repair, mRNA processing and mRNA export, by interacting with proteins directly involved in these processes (Shilatifard et al., 2003). It is largely unstructured in the absence of interacting proteins (Cramer et al., 2001) and the binding of proteins depends on the phosphorylation status of the CTD. It undergoes extensive phosphorylation and dephosphorylation during the transcription cycle at serine residues present at positions 2 and 5 in each heptapeptide repeat. In both the budding yeast and the fission yeast, cyclin-dependent kinases are involved in CTD phosphorylation. RNA polymerase II containing an extensively phosphorylated CTD (designated form IIo) is found in the elongating complex, while the dephosphorylated form (designated as form IIa) preferentially enters the PIC. A phosphatase called Fcp1 (TFIIF-associating CTD phosphatase) has been identified in *S. cerevisiae* (Chambers and Kane, 1996) and *S. pombe* (Kimura et al., 2002), which predominantly dephosphorylates serine 2 in the CTD. Fcp1 interacts with TFIIB, TFIIF and the Rpb4 subunit of the *S. pombe* polymerase (Kimura et al., 2002). Another CTD phosphatase has also been isolated in *S. cerevisiae* called Ssu72, which can dephosphorylate serine 5 in vitro (Krishnamurthy et al., 2004).

Sakurai and Ishihama (2002) reported that the intracellular concentration of the pol II in fission yeast remains constant but phosphorylation of the serine, threonine and tyrosine residues in its carboxy terminal domain varies depending on the phase and rate of growth.

The Rpb2 subunit of the budding yeast features a strong negative charge cluster in the third quartile of the protein, comprising residues 665 to 724: there are 23 acidic residues with no basic residues (Brendel and Karlin, 1994). Recently, Kato et al. (2005) isolated a mutation in the Rpb2 subunit of fission yeast that resulted in loss of heterochromatic histone modifications, accumulation of pericentromeric transcripts and loss of siRNAs, indicating that RNA polymerase II may couple pericentromeric transcription with siRNA processing and heterochromatin assembly.

Rpb3 and Rpb11 subunits form a heterodimer. They also share sequence similarity with the bacterial RNA polymerase α subunit. Mutational analysis of Rpb3 revealed its role in the assembly of *S. cerevisiae* pol II (Kolodziez and Young, 1991) and it is likely that the pol II assembly is initiated by the formation of the Rpb3/Rpb11 heterodimer. More recently, Benga et al. (2005) have shown that the formation of *S. cerevisiae* Rpb3/Rpb11 heterodimer critically depends on the presence of the C-terminal region of Rpb11. Rpb3 is also important in activator-dependent transcription in *S. cerevisiae* (Tan et al., 2000). In case of *S. pombe*, exposure to 6M urea results in the dissociation of the pol II into Rpb2-Rpb3-Rpb11 subcomplex. This ternary complex is considered to be an intermediate in the assembly of *S. pombe* pol II (Kimura et al., 1997). Far western blot and GST-pull down assays with different Rpb3 deletion mutants demonstrated that amino acid residues 105 to 263 of Rpb3 were involved in binding to the Rpb5 subunit and amino acid residues 105 to 297 were required for binding the Rpb11 subunit of *S. pombe* pol II. In fact binding of Rpb5 stabilized the Rpb3-Rpb11 heterodimer (Yasui et al., 1998). In another study, analysis of temperature -sensitive mutants of *S. pombe* Rpb3 provided further evidence for a role of this subunit in assembly of pol II in *S. pombe* and also in transcription activation (Mitobe et al., 1999, 2001).

To examine if the *S. pombe* core subunits could functionally complement their respective *S. cerevisiae* counterparts, Shpakovski et al. (2000) replaced the core subunits of *S. cerevisiae* pol II with their respective *S. pombe* homologs. Interestingly, no heterospecific complementation was observed for the two largest subunits, Rpb1 and Rpb2. In contrast, the Rpb3 and Rpb11 subunits partially complemented the defect. They supported growth at 30°C but not at either high (37°C) or low (16°C, 25°C) temperatures. However, growth at these temperatures was restored by increasing the gene dosage of the *S. cerevisiae* Rpb11 or Rpb10 subunits.

19.3.2 Shared Subunits: Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12

The five small subunits, Rpb5, -6, -8, -10 and -12 are common to all the three eukaryotic RNA polymerases. Despite accumulation of data from genetic, biochemical and structural experiments, the precise functions of each of these subunits

are still largely unclear! In *S. pombe*, it has been estimated that on an average 10–20% of these subunits are assembled in pol II and the remaining are either assembled in RNA polymerase I and III or exist as unassembled subunits (Sakurai and Ishihama, 2002). The Rpb5 subunit of *S. pombe* pol II complements the absence of the Rpb5 subunit in *S. cerevisiae* (Shpakovski et al., 2000). It has also been reported to form a binary complex with the Rpb3 subunit. These two subunits also bind to the Rpb1 and Rpb2 subunits independently (Miyao et al., 1996). Chemical crosslinking and far western blotting experiments carried out to determine subunit-subunit interactions within the *S. pombe* pol II have shown that the Rpb5 subunit associates with Rpb3 and Rpb6 subunits and stimulates the formation of the Rpb3-Rpb11 heterodimer (Kimura and Ishihama, 2002). The *S. cerevisiae* Rpb5 subunit interacts with the CTD of Rpb1 subunit and both have been proposed to have overlapping functions in transcription activation (Miyao and Woychik, 1998). Furthermore, *S. cerevisiae* Rpb5 has been shown to directly interact with the RAP30 subunit of the basal transcription factor, TFIIF (Wei et al., 2001).

The Rpb6 subunit is considered to be a homolog of the bacterial w subunit and both Rpb6 and the w subunits have been implicated in subunit assembly (Nouraini et al., 1996; Minakhin et al., 2001). However, Rpb6 is essential for pol II activity and cell viability, whereas the w subunit is neither required for the survival of bacterial cells nor for in vivo or in vitro transcription. The budding yeast Rpb6 subunit contains a highly acidic N-terminus (14 acidic residues among the 3–31 amino acid residues with no basic residue). It also displays a C-terminal alternating charge pattern with mostly hydrophobics intervening the charges (Brendel and Karlin, 1994). *S. pombe* Rpb6 gene can rescue the growth defect associated with the absence of the corresponding gene in *S. cerevisiae* (Shpakovski, 1994). Mutational and overexpression studies have implicated Rpb6 in transcription elongation in *S. pombe* (Ishiguro et al., 2000).

Alignment of the budding yeast and fission yeast Rpb8 amino acid sequence shows that a central segment spanning amino acid residues 65 and 88 in the budding yeast Rpb8 is absent in the *S. pombe* homolog. This segment is not essential for growth (Voutsina et al., 1999). *S. pombe* Rpb8 also did not rescue the growth defect associated with the lack of Rpb8 subunit in *S. cerevisiae* (Shpakovski et al., 2000). However, growth was recovered in the presence of a high genetic dosage of the largest subunit of RNA polymerase III (Voutsina et al., 1999). Yeast two-hybrid screen using *S. cerevisiae* Rpb8 as the 'bait' showed that it interacted with the 516–639 amino acids of the Rpb1 subunit of RNA polymerase I, II and III, which is in agreement with the pol II crystal structure (Cramer et al., 2001). A nucleoprotein, Nup82, was also identified as one of the Rpb8-interaction partners in this screen and extragenic suppression analysis identified Rpb6 as one of the physiological partners of Rpb8 (Briand et al., 2001). Kimura and Ishihama (2000) demonstrated a direct interaction between the Rpb8 and the Rpb3 subunits of *S. pombe* RNA polymerase II. They further showed that Rpb8 causes an enhancement of the Rpb1-Rpb3 interaction, albeit at a low level.

The genes encoding the other two common subunits, Rpb10 and Rpb12, have been cloned from both the budding and fission yeasts. Heterologous complementation

studies have shown that the *S. pombe* gene encoding the Rpb10 subunit is proficient in supporting the growth of an *rpb10* mutant of *S. cerevisiae* (Shpakovski et al., 2000). But very little information is available about their in vivo function(s). Apparently, both Rpb10 and Rpb12 play an indispensable role in assembly and maintenance of pol II, forming sub-complexes with the Rpb3-Rpb11 heterodimer during the early stages of the assembly of RNA pol II (Lalo et al., 1993).

19.3.3 Unique Subunits: Rpb4, Rpb7 and Rpb9

Rpb4, -7 and -9 subunits are unique to RNA polymerase II and Rpb4 and Rpb9 are the only two subunits that are dispensable for growth of budding yeast cells under optimal growth conditions (Woychik and Young, 1989; Woychik et al., 1991). In contrast, both these subunits are essential for viability in the fission yeast (reviewed by Mitsuzawa and Ishihama, 2004). The Rpb4 subunit also differs markedly in these two yeasts in several other aspects (Sakurai et al., 1999)

- The *S. pombe* subunit is smaller in size (135 amino acids), lacking several regions present in the *S. cerevisiae* subunit (221 amino acids);
- Stoichiometric amount of Rpb4 is present in the *S. pombe* pol II, whereas only 20% of the *S. cerevisiae* pol II prepared from log-phase cells contain Rpb4;
- Rpb4 is more tightly associated with the pol II in *S. pombe* as compared to its *S. cerevisiae* counterpart.

Despite all these differences, *S. pombe* Rpb4 could rescue the growth defect associated with the lack of Rpb4 in *S. cerevisiae* (Shpakovski et al., 1994). The Rpb4 subunit also forms a heterodimer with the Rpb7 subunit in archaebacteria, yeast, plants and humans (reviewed by Choder, 2004). Using deletion analysis, the regions involved in interaction between the *S. cerevisiae* Rpb4 and Rpb7 subunits have been delineated (Sareen et al., 2005). The in vivo functions of the Rpb4 subunit alone and in a complex with the Rpb7 subunit have been quite well characterized in *S. cerevisiae* (reviewed in Choder, 2004). Convergence of data from several studies suggests that this complex is essential for stress response and stress survival. More recently it has been shown that while Rpb4 promotes sporulation, Rpb7 enhances psudohyphae formation in budding yeast (Singh et al., 2007). Earlier studies provided evidence that Rpb4 is not required for constitutive transcription, but is important for activated transcription from a subset of promoters (Pillai et al., 2001). It plays a significant role in carbon and energy metabolism at moderate temperatures and sporulation (Pillai et al., 2003). It also has a dual role in controlling sub-pathways of transcription-coupled DNA repair-repressing the Rpb9-mediated sub-pathway and facilitating the Rad26-mediated subpathway (Li and Smerdon, 2002). Recently, the involvement of Rpb4 in the decay of specific class of mRNAs has been reported in *S. cerevisiae* (Lotan et al., 2005). In contrast, we are only beginning to decipher the functions of the Rpb4 subunit in *S. pombe*. Kimura et al. (2002) provided evidence that it interacts with the CTD phosphatase, Fcp1 and may play a role in the assembly of the Fcp1-pol II complex, thus stimulating

CTD dephosphorylation for the recruitment of pol II in a new cycle of transcription in *S. pombe*. Whole genome expression analysis in *S. pombe* has uncovered a new function for Rpb4 in cell separation (Sharma et al., 2006).

The Rpb7 subunit is one of the most highly conserved subunits of RNA polymerase II. The *S. cerevisiae*, *S. pombe* and *C. albicans* Rpb7 orthologs display a high sequence similarity in the central stretch of 20 amino acids and also in both the N-terminal and C-terminal regions of the protein (Sadhale and Woychik, 1994). The similarity between the *S. cerevisiae* and *S. pombe* protein is 75%, while the extent of similarity between the *S. cerevisiae* and *C. albicans* orthologs is 79% (Singh et al., 2004). Overexpression of *S. cerevisiae* Rpb7 in an *rpb4* deletion mutant rescues some of the phenotypes linked with the lack of Rpb4 in *S. cerevisiae*, thus suggesting that Rpb4 may also play a role in stabilizing the association of Rpb7 with the remaining pol II (Sharma and Sadhale, 1999; Sheffer et al., 1999; Pillai et al., 2003). These *rpb4*-deletion phenotypes could also be partially rescued by overexpression of either *C. albicans* Rpb7 or *S. pombe* Rpb7 (Singh et al., 2004). The *C. albicans* and *S. pombe* orthologs could also complement for the absence of *S. cerevisiae* Rpb7. In summary, these observations imply that the high sequence similarity seen in the Rpb7 orthologs from different yeasts also extends to a functional conservation. The budding yeast Rpb4/7 complex also binds single-stranded nucleic acids and mediates a post-recruitment step in transcription initiation (Orlicky et al., 2001). Pull down assays carried out in *S. pombe* identified glyceraldehyde-3-phosphate dehydrogenase and actin as proteins interacting with *S. pombe* Rpb7 subunit (Mitsuzawa et al., 2005). Another report showed that the Rpb7 protein associates with the Seb1 protein in fission yeast and with the Nrd1 protein in budding yeast, thus linking the Rpb7 protein to transcription termination of small nuclear and small nucleolar RNAs (Mitsuzawa et al., 2003). A recent study discovered a novel role for *S. pombe* Rpb7 in RNAi- directed chromatin silencing pathway (Djupedal et al., 2005).

The functions of the Rpb9 subunit have been investigated in detail in *S. cerevisiae*. Hull et al. (1995) demonstrated that *S. cerevisiae* cells lacking the Rpb9 subunit exhibited an upstream shift in the position of the start site. It was later shown that this alteration is associated with an impaired interaction between Rpb9 and TFIIF (Ziegler et al., 2003). Several studies provided evidence for the role of Rpb9 in transcription elongation in vitro and in vivo (Awrey et al., 1997; Hemming and Edwards, 2000; Hemming et al., 2000). Moreover, Mullem et al. (2002) observed that *rpb9* null mutants failed to grow when they also lacked the histone acetyl transferase activity of either the elongator or the SAGA complex, adding another facet to the role of Rpb9 in transcription elongation. They also showed a direct physical interaction between Rpb9 and the large subunit of TFIIE, and proposed that this may be the mechanism by which Rpb9 may contribute to the recruitment of TFIIE to pol II. Recent observations have illuminated the role of Rpb9 in maintaining transcriptional fidelity (Nesser et al., 2006). The Rpb9-encoding cDNA from *S. pombe* has also been cloned. From the cDNA sequence, the *S. pombe* Rpb9 subunit was found to consist of 113 amino acids with a molecular mass of approximately 13 kDa. It also possesses 47% identity in amino acid sequence with *S. cerevisiae* Rpb9 (Sakurai et al., 1998). Interestingly although both *S. cerevisiae* and *S. pombe*

differ in the position of the transcription initiation site, Rpb9 is readily exchangeable between these two yeasts. This suggests that the Rpb9 subunit does not directly determine the differences in start site selection between these yeasts.

19.3.4 Structure of pol II

As mentioned in the introduction, structure of the *S. cerevisiae* 10-subunit RNA polymerase II (lacking the Rpb4/7 subcomplex) in the absence of DNA has been determined at different resolutions. It is formed of four mobile elements, known as core, clamp, shelf and jaw lobe, which move relative to each other. The core element comprising the Rpb3, -10, -11, -12 and those parts of Rpb1 and Rpb2 that form the active centre, accounts for approximately half the mass of pol II and is composed predominantly of subunits shared among the three polymerases. A deep cleft is located at the centre of the enzyme, where incoming DNA enters from one side and the active site is buried at the base. This cleft is formed by all the four mobile elements and is present in both the open and closed conformations in the 10-subunit enzyme. The shelf and jaw lobe move relatively less and can rotate parallel to the active site cleft. In comparison, the clamp which is connected to the core through a set of flexible switches can move with a large swinging motion of upto 30A° to open and close the cleft.

Subsequently, structure of the complete 12-subunit enzyme from budding yeast was derived independently by two different groups (Armache et al., 2003; Bushnell and Kornberg, 2003). The polymerase models presented by both these groups were essentially identical, revealing the location of Rpb4/7 heterodimer in a pocket formed by the subunits, Rpb1, Rpb2 and Rpb6, at the base of the clamp. The position of Rpb7 in this pocket functions not only as a wedge to lock the clamp in a closed conformation, but both Rpb4 and Rpb7 provide a surface for binding of other transcription factors and also for RNA exiting the elongating Pol II.

19.4 General Transcription Factors: Pol II Helper Proteins

Unlike the *E. coli* RNA polymerase, eukaryotic RNA polymerase II does not have the ability to recognize the promoter. Thus, it requires the help of accessory proteins called the basal or general transcription factors (GTFs). Six GTFs have been purified from different systems, though the human and budding yeast proteins remain the most well characterized proteins. These GTFs assemble at the promoter along with pol II to form the PIC that initiates transcription. Two different pathways have been proposed to explain the assembly of the PIC in vivo. According to the ‘sequential’ or the step-wise’ assembly pathway, each GTF enters the assembling transcription apparatus individually and sequentially. The assembly is nucleated by the binding of TFIID, through the direct interaction of its TATA-box

binding protein (TBP) with the promoter. This interaction also depends on other factors, referred to as TAFs or the TBP-associated factors. Binding of the TFIID is followed by the sequential binding of TFIIA, TFIIB, TFIIF, RNA pol II, TFIIE and TFIIH, thus completing the assembly of the PIC.

An alternative pathway was proposed when several groups discovered that pol II could be isolated as a preassembled 'holoenzyme'. Although the exact composition of the holoenzyme complex varied according to the method of purification, it was isolated with or without a subset of the GTFs, but with mediator proteins and other proteins involved in chromatin-remodelling, mRNA processing, DNA repair and DNA replication. This holoenzyme is then recruited to the promoter as a single, large complex (reviewed by Lee and Young, 2000; Myers and Kornberg, 2000). We still do not know which of the two pathways operates in a cell, but it is possible that either both pathways exist or a mechanism in between these two extremes exists in vivo.

The properties and functions of the GTFs have been discussed below.

19.4.1 TFIID

TFIID is the first GTF that recognizes and associates with both TATA-containing and TATA-less promoters, to begin the assembly of the PIC. It is a multi-protein complex containing the TATA-binding protein (TBP) and TBP-associated Factors (TAFs). Besides its role in promoter binding, TFIID functions as a co-activator in mediating interaction between activators and the basal transcription machinery; it interacts with other basal transcription factors to enhance PIC assembly; and also acts as an enzyme to post-translationally modify chromatin and protein factors involved in transcriptional control.

Specifically, it is the TBP subunit of TFIID that has the ability to recognize the TATA box sequence in promoters. Crystal structures of budding yeast TBP in complex with the TATA box showed that TBP binding caused a severe bend in the DNA (Kim et al., 1993). In the bound and the unbound states, TBP resembles a molecular 'saddle' with a pair of 'stirrups' flanking the DNA-binding surface which help in bending the DNA. TBP associates with 14 different TAFs in the *S. cerevisiae* TFIID complex (Sanders and Weil, 2000). The elucidation of the functions of TAFs has been an area of intense research (reviewed by Albright and Tjian, 2000; Thomas and Chiang, 2006). Four TAFs in the *S. cerevisiae* TFIID complex-TAF17, TAF60, TAF48 AND TAF61, have domains similar to histones H3, H4, H2A and H2B respectively and these TAFS can form an octameric structure in vitro (Selleck et al., 2001). Studies in *S. cerevisiae* revealed that depletion or inactivation of individual TAFs does not have a global effect on transcription activation of many genes (Moqtaderi et al., 1996; Walker et al., 1996). Additional evidence supporting the view that TAFs may not be universally required for gene expression in vivo came from whole genome expression analysis with *S. cerevisiae* TAF mutants (Holstege et al., 1998; Lee et al., 2000). In vitro studies have also confirmed

that TAFs may not be essential for transcription of every gene. TBP, not bound to TAFs, has been found in *S. cerevisiae* (Kuras et al., 2000; Li et al., 2000) and can be biochemically separated from TAFs during in vitro fractionation of budding yeast TFIID (Sanders et al., 2002). Interestingly, some TAFs have also been found in other complexes, like TBP-free TAF_{11}–containing complex (TFTC), TFTC-related GCN5 complexes, SAGA complex and SAGA-like complexes. These TBP-lacking TAF-containing complexes are involved in diverse aspects of pol II-mediated transcription. In fact yeast genes have been categorized into two distinct classes-TAF-dependent and TAF-independent, based on the requirement of TAFs for their expression (Kuras et al., 2000; Li et al., 2000).

The gene-encoding *S. pombe* TFIID homolog has also been cloned (Hoffman et al., 1990). *S. pombe* TFIID contains 231 amino acids and shares a 93% identity with the *S. cerevisiae* ortholog. Not surprisingly, the *S. pombe* TFIID can complement a disruption of the *S. cerevisiae* TFIID (Fikes et al., 1990). The carboxy-terminal three quarters of the *S. pombe* TFIID protein exhibits an extraordinary degree of amino acid sequence homology with a corresponding region of *S. cerevisiae* TFIID. This region is necessary and sufficient for TATA-box-binding and basal transcription activation. In contrast, the amino-terminal region of *S. pombe* TFIID differs markedly in amino acid sequence and composition from its *S. cerevisiae* counterpart (Hoffman et al., 1990). *S. pombe* TFIID consists of TBP and 14 TAFs (reviewed by Thomas and Chiang, 2006). Out of these 14 TAFs, only five TAFs have been identified biochemically-TAF111, TAF72, TAF73, TAF50 and Ptr6. The TAF72-encoding gene was cloned using sequence homology by Yamamoto et al., 1997. Later, the genes encoding the TAF72 and TAF73 were isolated as high-copy number suppressors of cell cycle mutations (Mitsuzawa et al., 2001). Both these TAFs contain WD repeat motif and are components of the TFIID complex. TAF72 is also a component of the SAGA complex. *S. cerevisiae* TFIID contain only one TAF (TAF90) with a WD repeat motif and it has a stoichiometry of two (Sanders et al., 2002). This raises the possibility that the single species of the WD repeat TAF in *S. cerevisiae* is present in two copies in *S. pombe* TFIID (Mitsuzawa et al., 2001). TAF 50 was identified as a protein that interacts with TAF72 (Mitsuzawa and Ishihama, 2002). TAF50 also possesses limited homology to histone H4. The gene encoding Ptr6 (poly A+ RNA transport) was identified in a screen for mutants defective in mRNA export (Shibuya et al., 1999) and it is considered to be a homolog of the budding yeast TAF67. The *S. pombe* counterparts of the remaining TAFs were identified by a search of the *S. pombe* genome (Mitsuzawa and Ishihama, 2004). Tamayo et al. (2004) purified a TAF-containing complex from *S. pombe* and demonstrated that TAFs are not required for basal or activated transcription in vitro.

19.4.2 TFIIA

S. cerevisiae TFIIA is composed of two subunits, with apparent molecular masses of 32 and 13.5 kDa (Ranish and Hahn, 1991). The genes encoding these two subunits, TOA1 and TOA2, are essential for cell viability (Ranish et al., 1992). The role of

TFIIA as a general transcription factor is controversial. Early in vitro experiments revealed that TFIIA was required for transcription, while later studies showed that it was dispensable for basal transcription (reviewed by Orphanides et al., 1996). Subsequent studies suggested that TFIIA is more likely to function as an anti-repressor, rather than a basal transcription factor. It stabilizes the TBP-DNA binding (Weideman et al., 1997) by competing with the N-terminal domain of TAF145 that occludes the DNA binding surface of TBP when TFIID is not bound to DNA (Kokubo et al., 1998; Sanders et al., 2002). TFIIA can also compete with the negative regulatory factors, Mot1 and NC2, to stimulate TBP binding in vitro (Xie et al., 2000). In addition to its role as an anti-repressor, TFIIA also functions as a co-activator to stimulate overall transcription by interacting with various activators, and other components of the transcriptional machinery. It has also been reported to interact directly with TAF40 both in vivo and in vitro (Kraemer et al., 2001). Mutational studies abolishing the interaction of TFIIA with TBP have shown that TFIIA is essential for transcription of only a subset of genes (Stargell et al., 2000). Recently, Kraemer et al. (2006) examined the transcriptional profiles of different *S. cerevisiae* TFIIA mutants in order to further characterize the functions of TFIIA in the regulation of gene expression by pol II. It was observed that approximately 11–27% of the expressed genes exhibited altered expression levels depending on the particular TFIIA mutant. Surprisingly, all these affected genes contained the binding site for the Yap1, a transcription factor involved in oxidative stress. The dependence of Yap1 on TFIIA was also demonstrated in genetic and biochemical experiments, thus highlighting a novel role for TFIIA in response to oxidative stress.

19.4.3 TFIIB

S. cerevisiae TFIIB (also called factor e) is a monomer of approximately 41 kDa, encoded by the *SUA7* gene (Tschochner et al., 1992). Pinto et al. (1992) observed that mutations in the *SUA7* gene shifted the transcription start site downstream of the normal site, indicating a role for TFIIB in transcription start site selection in vivo. Berroteran et al. (1994) provided evidence that mutations in the Rpb1 encoding gene also resulted in alterations in the start site of transcription. Moreover, a functional interaction was demonstrated between TFIIB and the Rpb2 subunit of RNA polymerase II (Chen and Hampsey, 2004). Thus, the selection of the start site may depend on both TFIIB and pol II. By superposition of the structures of TFIIB-RNA pol II and DNA-TBP-TFIIB, it was observed that TFIIB acts as a bridge between TBP and RNA polymerase II such that the DNA template need only follow a straight path from the TATA box to position the start site in the active centre of pol II (Leuther et al., 1996). TFIIB interacts directly with TBP and enters the PIC after TBP. It is also a prerequisite for recruitment of pol II (Buratowski et al., 1989). TFIIB is also a direct target of many transcription activators and recruitment of TFIIB is the mechanism by which many activators stimulate transcription (Lin et al., 1991). In addition, a post-assembly function for TFIIB was also revealed by

the isolation of TFIIB mutants competent for assembly of the transcription complex, but defective for in vitro transcription (Cho and Buratowski, 1999).

The cloning, expression and functional characterization of *S. pombe* TFIIB was reported in 2002 by Tamayo and Maldonado. It is a 340 amino acid long protein with a calculated molecular mass of 37.4 kDa. It displays 38.8% identity to its *S. cerevisiae* homolog and 40.1% identity to its human counterpart. Earlier fission yeast TFIIB was also purified from cell extracts as a 35 kDa protein. It has been shown that pairwise replacement of TFIIB and pol II from *S. cerevisiae* by their respective *S. pombe* counterparts was sufficient to shift the start sites from the pattern characteristic of *S. cerevisiae* to the pattern characteristic of *S. pombe* (Li et al., 1994).

19.4.4 TFIIE

TFIIE (known as factor a), is a two subunit protein in *S. cerevisiae*. The apparent molecular weights of these subunits are 66 and 43 kDa. The genes encoding these subunits, TFA1 and TFA2, are present in a single copy and are essential for cell survival. Two functionally distinct domains have been identified by mutational analysis of the TFA1-encoded subunit: mutations in the N-terminal half confer growth defects at high temperatures, whereas mutations in the C-terminal half confer growth defects at low temperatures. TFIIE is able to bind single-stranded DNA, thus explaining the dispensability of TFIIE for transcription initiation from pre-melted template DNA (Holstege et al., 1995). TFIIE influences recruitment of TFIIH and subsequent control of TFIIH activities. Both TFIIE and TFIIH are required for ATP-dependent formation of the open complex before formation of the first phosphodiester bond. TFIIE and TFIIH, in cooperation with TFIIF, suppress promoter-proximal stalling, thereby facilitating early events in the transition of RNA pol II to productive elongation (Dvir et al., 1997). The functional link between TFIIE and TFIIH was elegantly demonstrated by the inability of the *S. cerevisiae* TFIIE to functionally substitute the *S. pombe* TFIIE ortholog in a constituted transcription system, unless they were exchanged as a TFIIE-TFIIH pair (Li et al., 1994). *S. pombe* TFIIE is made up of two subunits, α and β. The α subunit contains 434 amino acids, with a calculated molecular weight of 49.1 kDa. It shares a 26% amino acid sequence identity and a 50% similarity with its *S. cerevisiae* ortholog. The smaller β subunit contains 285 amino acids with a calculated molecular weight of 32.2 kDa, sharing a 38% amino acid sequence identity and 49% similarity with the *S. cerevisiae* ortholog (Hayashi et al., 2005). The genes encoding both these subunits have been cloned and are essential for cell viability as seen in *S. cerevisiae*. The functions of TFIIE have been investigated by biochemical and genetic approaches in *S. pombe* (Hayashi et al., 2005). Chromatin immunoprecipitation assays revealed that TFIIE was localized to the promoter and promoter-proximal regions. It was also observed that mutation of the C-terminal residues of fission yeast TFIIE β subunit conferred cold sensitivity. These mutations had earlier been shown to be linked to transcription defects, either at initiation or at the transition from initiation to elongation phase (Watanabe et al., 2003). These findings

confirm the role of TFIIE in transcription initiation and transition from the initiation to the elongation phase in fission yeast. Far western studies have shown that *S. pombe* TFIIE formed an α_2 β_2 heterotetramer with a molecular weight of 180 kDa in vitro. Further characterization of the binding specificities revealed that the β subunit of *S. pombe* TFIIE interacts predominantly with the Rpb2 and Rpb12 subunits of pol II and also weakly with the Rpb1 subunit. In comparison, the α subunit mainly binds to the Rpb5 subunit of pol II.

19.4.5 TFIIF

Three different subunits, designated as Tfg1, Tfg2 and Tfg3, constitute the *S. cerevisiae* TFIIF (known as factor g). The Tfg1 (105 kDa) and Tfg2 (54 kDa) are considered to be homologous to the RAP74 and RAP30 subunits of human TFIIF respectively. Tfg1 and Tfg2-encoding genes are essential for cell viability, whereas the gene encoding the Tfg3 subunit (30 kDa) is dispensable. Archambault et al. (1997) showed that the CTD phosphatase, Fcp1 binds to Tfg1. Cryo-electron microscopy resolved the structure of *S. cerevisiae* pol II in complex with the TFIIF (Chung et al., 2003). TFIIF interacts with a highly extended surface of pol II along the edge of the clamp element and also with the Rpb4/7 subcomplex. Tfg1 also interacts with the Rpb9 subunit of pol II in budding yeast (Ziegler et al., 2003; Ghazy et al., 2004). This stable Tfg1-pol II complex accounts for almost 50% of pol II isolated from *S. cerevisiae* nuclear extracts and is active in supporting multiple rounds of transcription (Rani et al., 2004).

Two subunits of TFIIF have been identified in *S. pombe* (Tamayo et al., 2004). The α subunit has 490 amino acids and shows a 33% amino acid identity with its *S. cerevisiae* counterpart, while the β subunit contains 301 amino acids and shares 37% identity with the *S. cerevisiae* ortholog. *S. pombe* TFIIF had earlier been isolated as part of a complex containing Fcp1 and pol II (Kimura et al., 2002) and further characterization of the complex showed that the *S. pombe* homolog of Tfg3 was indeed a constituent of the Fcp1/pol II/TFIIF complex. Deletion of Tfg3 in *S. pombe* is associated with temperature-sensitive phenotype and other stress-related phenotypes. Interaction of Tfg3 with TFIIB and TBP has also demonstrated (Kimura and Ishihama, 2004). Infact, the Tfg3 subunit is not only present in TFIID, but is also a component of the Swi/Snf and NueA complexes (Cairns et al., 1996). Therefore, it can be speculated that the Tfg3 subunit of TFIIF may act as an intermediary protein, facilitating interactions between Swi/Snf complex and the general transcription apparatus.

19.4.6 TFIIH

TFIIH, also called factor b, has a host of enzymatic activities. These include DNA dependent ATPase, two ATP-dependent DNA helicases with opposite polarity

(called Rad3 and Rad25 in humans) and CTD kinase (Cdk7-cyclin H). TFIIH can be separated into two sub-complexes- core TFIIH and the cyclin-kinase complex. In addition to its role in transcription, core TFIIH plays an important role in nucleotide excision repair. Till recently, the *S. cerevisiae* TFIIH was considered to contain nine different subunits. Ranish et al. (2004), discovered a tenth subunit called Tfb5 involved in DNA repair function of TFIIH. Mutations in genes encoding the different components of the core TFIIH, i.e. Tfb1, Tfb2, Ssl1 and Tfb4, caused defects in responding to UV irradiation, thus implying a role of these subunits in DNA damage. A ubiquitin-ligase activity associated with Ssl1 has been discovered recently because of the presence of a RING finger domain at its C-terminal region encompassing amino acid residues 403 to 454 (Takagi et al., 2005) and this activity can be enhanced by addition of another TFIIH RING-finger containing subunit Tfb4. The electron-crystal structure of core TFIIH has been solved at 13A° resolution (Chang and Kornberg, 2000). Many activators, including Gal4-VP16, have been demonstrated to bind to TFIIH (reviewed by Zurita and Merino, 2003).

The gene encoding the fission yeast homolog of the budding yeast TFIIH subunit, Ssl1, was cloned by Adachi et al. (1999) and the gene product was called p47. Although deletion of the p47 gene was not lethal, but it was required for normal growth. In contrast, an *ssl1* null mutation in budding yeast was lethal. A comparison of the primary amino acid sequence of Ssl1 and p47 revealed that p47 has a 45% identity to Ssl1. Both these yeast homologs contain a charged cluster in the most N-terminal region. The amounts of charged residues are 55% in p47 and 57% in Ssl1. However, the polarity as a whole within the charged cluster is acidic in Ssl1 and basic in p47. The *S. pombe* Tfb1 subunit of TFIIH contains 457 amino acids and shares a 29% amino acid sequence identity with its *S. cerevisiae* ortholog (Tamayo et al., 2004). Also, the Ssl2 subunit of *S. cerevisiae* is encoded by the *ptr8* gene in *S. pombe*, which is involved in mRNA transport (Mitsuzawa and Ishihama, 2004).

19.5 Mediator: The Link Between Transcriptional Regulators and pol II Machinery

Mediator is a multi-protein complex that provides the interface between gene-specific regulatory proteins and the general RNA polymerase transcription machinery. The first evidence for its existence came from squelching experiments in *S. cerevisiae* (Gill and Ptashne, 1988). In these experiments, the ability of one activator to inhibit transcription by another activator could not be rescued by addition of excess GTFs, but was rescued by addition of a partially purified yeast fraction. The factor(s) present in the partially purified fraction with the ability to support activated transcription was termed as the ‘mediator’. Our current understanding of the mediator complex in terms of its subunit composition, its structure and its role in regulation of gene expression can be summarized as follows.

19.5.1 Subunit Composition

The *S. cerevisiae* core mediator complex comprises 21 proteins as its 'bona fide' members (reviewed by Biddick and Young, 2005; Björklund and Gustaffsson, 2005). In addition to these bonafide members, four Srb proteins, Srb8-11, form a distinct sub-complex which may sometimes found to be associated with the mediator (Liao et al., 1995; Borggrefe et al., 2002). Many of these mediator components have been identified in genetic screens for mutations that influence transcription. The mediator subunits-Srb2, Srb4, Srb5 and Srb6 were identified as dominant suppressors of the cold sensitive phenotype associated with the truncation of the CTD of the Rpb1 subunit of pol II (reviewed by Myers and Kornberg, 2000), while the Srb8-Srb11 were identified as recessive suppressors of this phenotype (Hengartner et al., 1995; Liao et al., 1995). Genetic analysis demonstrated that the complex comprising the Srb8-11 subunits is involved in the negative regulation of transcription of a subset of genes (Holstege et al., 1995). When budding yeast cells were grown under conditions of nutrient limitation, the Srb8-11 module was degraded. This suggests that the conversion of an Srb8-11 containing mediator into a smaller active mediator may be a regulated event. Srb 10 and Srb11 form a cyclin-kinase pair. Srb10 has the unique ability to phosphorylate the CTD prior to formation of the initiation complex on promoter DNA, consequently inhibiting transcription (Hengartner et al., 1998). Eight other subunits of the mediator, i.e. Gal11, Nut2, Rgr1, Rox3, Sin4, Med3, Med9 and Med10, were identified in different genetic screens for positive as well as negative transcriptional regulators (reviewed by Carlson, 1997). The remaining seven subunits were identified as novel proteins present as part of the biochemically isolated mediator complex from the budding yeast (Kim et al., 1994). These subunits were called Med1, Med2, Med4, Med6, Med7, Med8 and Med11. Since different mediator subunits were identified in different screens by different groups of investigators, there is a lot of variation with respect to their nomenclature. Bourbon et al. (2004) have proposed a unified nomenclature in which all mediator subunits are designated MED followed by a number.

Purification and characterization of the RNA polymerase II holoenzyme from *S. pombe* led to the identification of the proteins constituting the fission yeast mediator complex (Spahr et al., 2000, 2001). 13 individual subunits were identified as mediator components. Ten of these subunits were homologs of the *S. cerevisiae* Rgr1, Nut2, Med4, Med6, Med7, Med8, Rox3, Srb4, Srb6 and Srb7 proteins. Three subunits, Pmc2, Pmc3 and Pmc6, lacked homologs in the *S. cerevisiae* mediator. Gene disruption experiments showed that the genes encoding the Pmc3 and Pmc6 subunits are non-essential for the viability of *S. pombe* cells. In comparison, deletion of genes encoding the *S. pombe* homologs of Srb4, Med4, Med7 and Med8 resulted in lethality (Spahr et al., 2001). The mediator present in the *S. pombe* RNA pol II holoenzyme stimulated phosphorylation of the CTD by TFIIH isolated from *S. pombe*. But if TFIIH was isolated from *S. cerevisiae*, this stimulation was not seen, demonstrating that the stimulation of CTD by TFIIH was species specific

(Spahr et al., 2000). It has been speculated that the essential subunits conserved between *S. cerevisiae* and *S. pombe* may constitute a core mediator, interacting with RNA polymerase II and TFIIH. *S. pombe* homologues of the Srb8-11 module of *S. cerevisiae* were identified later by Samuelsen et al. (2003). It was observed that the mediator containing these proteins was present in a free form, without RNA polymerase II. On the other hand, mediator lacking these proteins could associate with the polymerase. The Srb10 homolog has also been identified from *Kluyveromyces lactis* (Nunez et al., 2004) and it contains 593 amino acids and can complement the phenotypes of a *S. cerevisiae* haploid *srb10* null mutant. Orthologs of various mediator subunits across different species have been identified using a genome-wide search (Boube et al., 2002). A comparison of the primary sequence of mediator subunits present in the yeasts- *S. cerevisiae*, *S. pombe* and *C. albicans*, with that of their metazoan counterparts suggest that the overall subunit composition and therefore, the structural organization of mediator exhibits a remarkable degree of conservation.

19.5.2 Structure

The *S. cerevisiae* mediator complex is organized into three functionally and physically distinct sub-complexes or modules- the head module, the middle domain and the tail domain (Asturias et al., 1999; Dotson et al., 2000). Seven mediator subunits, i.e. Med6, Med8, Med11, Med17, Med18, Med20 and Med22, constitute the head domain, while Med1, Med4, Med5, Med7, Med9, Med10 and Med21 comprise the middle domain, and the tail domain contains Med2, Med3, Med15 and Med16. The Med 14 subunit connects the middle domain to the tail domain. The structure of the mediator alone and in complex with RNA polymerase II have been determined. Figure 19.4 shows a schematic representation of the pol II–mediator complex.

The mediator structure reveals that it can exist in two different conformational states- an elongated structure seen in the presence of pol II and a compact form observed in the absence of pol II. It remains in the elongated state even if the CTD is truncated, providing evidence that the mediator makes multiple contacts with the polymerase, in addition to the CTD. Recent findings show that over-expression of the proteins comprising the head module of the budding yeast mediator could complement the absence of a ‘headless’ mediator in transcription initiation in vitro. Interestingly, the head module interacted with the RNA pol II-TFIIF complex, but not with either of the two components separately and this interaction was lost in the presence of DNA template and associated RNA transcript. Also, disruption of the head module in vivo resulted in the release of the middle and the tail domains from a transcriptionally active promoter. In summary all these observations suggest that the head module regulates the interaction of the mediator with pol II and also with the promoter (Takagi et al., 2006).

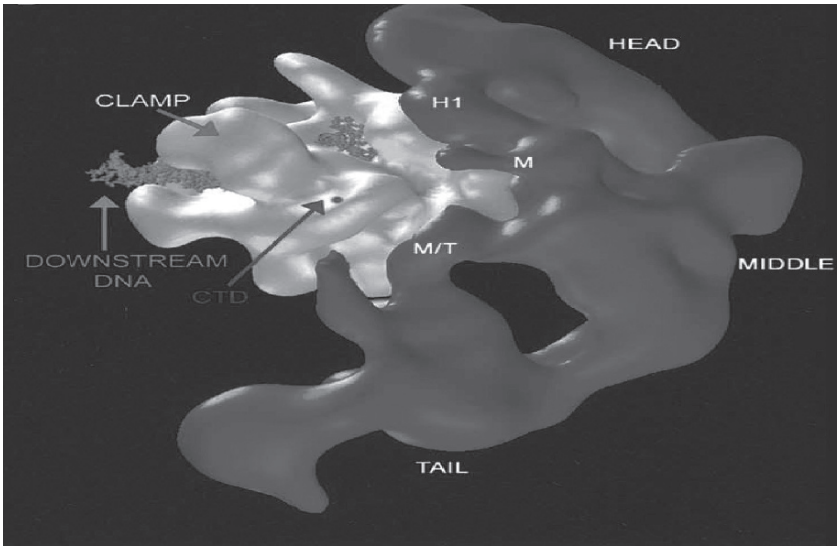

Fig. 19.4 RNA polymerase II-mediator complex. Reprinted by permission of Federation of the European Biochemical Societies from Structure of the yeast RNA polymerase II holoenzyme: Mediator conformation and polymerase Interaction by Davis et al. 2002. *Mol. Cell* 10: 409–415

19.5.3 Functions

Different approaches used to understand the functions of the mediator complex have led to the proposal that it acts as a global regulator of transcription. Although most evidence suggests that it functions as a co-activator, but several observations also point towards its negative role in transcription (reviewed by Biddick and Young, 2005).

Regulatory proteins directly interact with the mediator complex, but the specific subunit of the mediator that is contacted depends upon the regulatory protein. In *S. cerevisiae*, three acidic-rich transcriptional activators (Gal4, Gcn4 and VP16) interact with the mediator and this interaction requires the proteins constituting the tail module. In case of *S. pombe*, these mediator subunits are absent. Therefore, to determine if the *S. pombe* mediator had the ability to interact with these acidic activators, Spahr et al. (2001) carried out GST pull down assays to test the interaction between the mediator and VP16. These assays demonstrated that the mediator could still interact with VP16, suggesting that other subunits of the mediator could substitute for the missing subunits in *S. pombe*. In addition to its role in activator-dependent transcription, mediator is also known to stimulate basal pol II transcription in *S. cerevisiae* and it functions like any other general transcription factor involved in initiation of transcription (Takagi and Kornberg, 2006). Earlier work

had demonstrated that mediator was critical for the formation of a stable PIC (Koleske et al., 1992; Ranish et al., 1999). Subsequently, results of Nair et al. (2005) suggested that mediator may perform this function by incorporating and stabilizing TFIIH in PIC and re-initiation scaffold. Several studies have also demonstrated that mediator is recruited to the promoter separately from RNA polymerase II and general transcription factors (Kuras et al., 2003) and it continues to remain at the promoter even after transcription is initiated (Yudkovsky et al., 2000). Thus, it may serve as a scaffold for the assembly and re-assembly of the transcription complex during each cycle of transcription. This proposition was further supported by the existence of pol II-free mediator in *S. cerevisiae*, which was also the most abundant form of the mediator (Takagi et al., 2005). This view was challenged by the findings of Fan et al. (2006), which suggest that the mediator is not a stable component of the basic pol II transcription apparatus that binds to promoters in vivo and the intact mediator complex may not be required for transcription of many genes in wild type cells.

The role of mediator in stimulation of basal transcription has also been shown in *S. pombe*. Spahr et al. (2003) showed that the mediator lacking the Srb8-11 module had a stimulatory effect on basal transcription, while the mediator containing this module repressed basal transcription. An RNA polymerase II holoenzyme containing both the mediator and the RNA pol II enzyme was isolated from *S. pombe* that was more active in basal transcription in vitro than pol II alone and supported activated transcription in the absence of TAFs with proline-rich (AP2 and CTF) and acidic (VP16) activators, but not with Sp1 (Tamayo et al., 2004). Zhu et al. (2006) used chromatin immuno-precipitation assays and DNA microarrays to study genome-wide localization of mediator complex lacking the Srb8-11 module and the Srb8-11 sub-complex in *S. pombe*. Both of these complexes showed similar binding patterns and their interactions with promoters and UAS correlated with increased transcription activity. The mediator was also seen to interact with the downstream coding region of many genes.

Finally as mentioned before, the mediator also acts as a co-repressor of gene transcription, as exemplified by the Srb8-11 module of the mediator, but no universal mechanism has been proposed to explain this role of the mediator.

19.6 Elongation and Termination

Over the last 35 years, considerable effort has been invested in characterizing the early events of transcription, involving the GTFs- their interactions with each other, with RNA pol II and other regulatory proteins. But relatively little is known about the other events in the transcription cycle, like promoter clearance, elongation and termination. Several transcription elongation factors have been identified which enhance productive RNA synthesis, RNA processing, RNA export and chromatin modelling. Otero et al. (1999) isolated a novel protein complex, called elongator, as the major component of the elongating RNA polymerase II holoenzyme.

They proposed an interesting hypothesis, according to which the elongator is the counterpart of the mediator complex and it may be exchanged for the mediator, as transcription moves from the initiation to the elongation phase. However, to gain a complete understanding of the mechanism of transcription elongation, we still need to identify the entire repertoire of elongation factors, and define their precise functions. Also, in the light of growing evidence that some activator proteins function by enhancing the elongation efficiency and also that the elongating pol II interacts with proteins involved in mRNA processing, future studies will elucidate the role of pol II elongation complex not only as a target of regulatory proteins but also as a regulator of downstream steps in transcription of genes.

19.7 Conclusions and Future Perspective

Several groups identified components of the transcription machinery by fractionation of cell extracts, guided by transcription assays with naked DNA in vitro. The RNA pol II transcription machinery defined in this way consists of three different components- the 12-subunit RNA polymerase II, a set of GTFs and the mediator complex. Owing to the growing wealth of information, it is almost impossible to describe all the aspects of all the components of pol II transcription machinery. Hence, in this chapter we have attempted to review the information about the above mentioned components in different yeasts. RNA polymerase II shows a remarkable degree of conservation in terms of its structure and subunit composition. But the functions of the five common subunits in transcription still need to be dissected in detail. The observation that some of the subunits in *S. pombe* are essential for cell survival in contrast to their *S. cerevisiae* orthologs suggest that they may have more important roles to play in gene expression and regulation. Thus, future studies may entail a more detailed functional characterization of the *S. pombe* pol II subunits. The GTFs are obviously the most highly conserved components of the transcriptional machinery. Analysis of the structure of a PIC containing the 12-subunit *S. cerevisiae* pol II and GTFs bound to promoter DNA has revealed the specific roles of the GTFs: TBP configures DNA to pol II surface; TFIIB directs the DNA to the active site of pol II and stabilizes the transcription complex; TFIIE recognizes the closed complex of pol II and helps recruit TFIIH; TFIIF captures the template strand DNA when the double-stranded DNA melts to form the transcription bubble. Finally, the TFIIH helicase introduces negative supercoils into the promoter DNA, helping the pol II enzyme to move away from the promoter (Boeger et al., 2005). The most unique and intriguing component of the RNA polymerase II transcriptional machinery is the mediator. The subunits of the mediator complex exhibit low levels of primary sequence conservation, which may reflect a functional flexibility required to interact with specific transcriptional regulators in different systems. It is interesting to see how the combination of genetics, molecular genetics, biochemistry and structural biology have resulted in a nearly complete picture of the transcription initiation complex. However, in the future it will be important to

address the specific roles of mediator subunits and whether and how the mediator subunits are themselves regulated. Additional challenges will be to understand how the mediator receives and transduces signals to pol II transcriptional machinery, and finally to integrate all the information to build a step-by-step picture of one of the most fascinating processes of life!

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Chapter 20
Non-Genetic Engineering Approaches for Isolating and Generating Novel Yeasts for Industrial Applications

P.J. Chambers, J.R. Bellon, S.A. Schmidt, C. Varela, and I.S. Pretorius

Contents

Abstract Generating novel yeast strains for industrial applications should be quite straightforward; after all, research into the genetics, biochemistry and physiology of Baker's Yeast, *Saccharomyces cerevisiae*, has paved the way for many advances in the modern biological sciences. We probably know more about this humble eukaryote than any other, and it is the most tractable of organisms for manipulation using modern genetic engineering approaches. In many countries, however, there are restrictions on the use of genetically-modified organisms (GMOs), particularly in foods and beverages, and the level of consumer acceptance of GMOs is, at best, variable. Thus, many researchers working with industrial yeasts use genetic engineering techniques primarily as research tools, and strain development continues to rely on non-GM technologies. This chapter explores the non-GM tools and strategies available to such researchers.

Keywords Adaptive evolution, strain development, non-GM strategies, yeasts, hybrid yeasts, spheroplast fusion, continuous culture

20.1 Introduction

Humans have utilised yeasts for millennia in brewing, baking and winemaking, and over this time have inadvertently and deliberately isolated and selected strains with traits that are desirable for the industries that they serve. This has led to the generation of large collections of yeasts that can be used to deliver reliability, diversity and

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novelty in yeast-generated processes and products. In more recent times, genetic engineering techniques have been harnessed to build even greater diversity into industrial yeast genomes and provide enormous potential for shaping phenotypes for industrial applications. However, in many countries there are legal restrictions on the use of genetically modified organisms (GMOs), particularly in foods and beverages, and the level of consumer acceptance of these organisms is, at best, variable. Generating novel yeasts with improved characteristics for industry might, therefore, have to rely on non-recombinant approaches for some time to come; at least until consumers are more accepting of products made using genetically engineered strains and legislation is less prohibitive. While this is, at times, frustrating for yeast biotechnologists there is in fact considerable genetic variation in existing yeasts and this can be increased further using non-genetic engineering approaches such as chemical or UV mutagenesis. This means there is a great deal of potential for tailoring yeasts to particular ends without resorting to recombinant DNA techniques; indeed for complex quantitative genetic traits that involve large numbers of genes, such as ethanol tolerance and fermentation performance (see Pretorius, 2004), it is unlikely at this time that genetic engineering techniques would be able to contribute a great deal anyway.

20.2 Strategies for Generating Novel, Non-GM Industrial Yeasts

This section explores the range of non-recombinant approaches available to scientists for the isolation or selection of improved yeast strains for industrial applications. These approaches essentially involve either screening for desired variants or using adaptive evolution to drive change in a particular direction. However, both approaches are absolutely dependent on genetic variation (i.e. diversity) in the yeasts that scientists have at their disposal, or on the potential to increase variation if there is little of it available. Thus, the starting point for this chapter will be to focus on how yeast scientists can access or increase genetic variation without resorting to genetic engineering technologies. We will then assess approaches to screen for and generate desired phenotypes from this diversity. While the strategies and methods described in the following are not exclusive, for the sake of clarity and convenience they are each covered under discrete sub-headings.

20.2.1 Genetic Variation: The Source of Novel Genotypes and Variants

Charles Darwin and Alfred Russel Wallace independently formulated theories of evolution by natural selection, their ideas being presented in back-to-back papers at a meeting of the Linnean Society in London in 1858. What led these two natural historians

to arrive at the same mechanism as a driving force for evolutionary change? Both were travelers at the time of developing their ideas and were exposed to natural environments, the likes of which they had not previously encountered. This opened their eyes to the amount of variation one finds in natural populations. They coupled this observation with the knowledge that most organisms produce a great deal more offspring than can possibly survive and reasoned that, in the ensuing struggle for survival, the variants that are best suited to their environment are the ones most likely to succeed and reproduce. Thus, according to this paradigm for evolutionary change, variation is the raw material for selection; variation is the clay that selection moulds into adaptations, thereby generating new forms. Without genetic variation there can be no selection or, for that matter, evolution. And so it is for yeast.

Most industrial yeasts are, of course, domesticated species, having been shaped over millennia by artificial selection (i.e. selection driven by human choice); a form of selection Darwin dedicated a chapter to in his *magnum opus*, 'The Origin of Species'. Darwin was interested in domesticated organisms because he found in them clear evidence for rather extreme variation, and he used this as supportive evidence for his theory of descent with modification by natural selection. Darwin recognised that if there is variation in a species, and only 'selected' members reproduce, evolution has to occur, whether this be artificial or natural.

If we want to generate novel yeast varieties we will, of course, require access to a pool of genetic variation. Where do yeast scientists find this genetic variation for the isolation or generation of strains with desirable phenotypes? Essentially, they can either rely on existing, or generate new, variation.

20.2.1.1 Using Existing Genetic Variation to Isolate Yeasts with Desirable Phenotypes

There are two major sources of existing genetic variation in yeasts that scientists can, at least potentially, exploit: the repository of yeast strains stored in culture collections around the world and wild yeasts. There are probably hundreds (perhaps even thousands) of different yeast strains stored in the world's culture collections, and this represents a huge reservoir of genetic material. However access to culture collections is variable and depends on how open the custodians and owners are to sharing their resources; with good reason, most industries are usually protective of the strains that generate their income. Thus, there will be limits to how much of this genetic variation can be accessed, but there are nonetheless many strains available through collections held by organisations such as ATCC (American Type Culture Collection), NCYC (National Collection of Yeast Cultures, UK), CBS (Centraalbureau voor Schimmelcultures, The Netherlands), Phaff Yeast Culture Collection (University of California Davis).

Genetic variation in wild yeasts is more of an unknown quantity but there is undoubtedly an enormous reservoir in existence. In fact, it is estimated that there are over 600,000 undescribed species of yeast, about 10% of which will be ascomycetes (the group that includes all industrial yeasts) (Walker, 1998; Verstrepen

et al., 2006). It is not known how much of the genetic diversity in this natural resource will be accessible to geneticists to extend the gene pools of already available yeast species without the use of genetic engineering techniques. However, as covered later in this chapter, it is possible to generate interspecific hybrids of yeasts, so the genomes of at least some of these, as yet unidentified, strains may well be exploitable by generating hybrids between them and species such as *Saccharomyces cerevisiae*.

20.2.1.2 Using Mutagenesis to Increase Genetic Diversity

Mutation is the fundamental source of new genetic variation for evolutionary processes to work on and, therefore, for artificial selection. Mutations arise spontaneously but at a relatively low frequency (typically about $1:10^8$ nucleotides per cell cycle), thus mutagenesis techniques that increase available variation are important for yeast breeding programs. Mutagenesis requires the use of mutagenic agents such as UV or X-ray irradiation, or chemicals such as ethylmethane sulfonate (EMS), nitrosoguanidine (NTG), or diethyl sulfonate, (DES) (Lawrence, 1991), all of which induce changes in DNA. The dose of mutagen delivered is critical: too much will be lethal and most of the population being targeted will die and, more importantly, survivors are likely to have many mutations, only some of which are desirable (Pretorius, 2000). Suboptimal doses will obviously create less diversity than is desirable to generate the traits being sought (Sauer, 2001). Thus, it is important to find the dose that is optimal, and this will vary depending on the strain and conditions being used. For tried and tested mutagenesis protocols, the reader is directed to Lawrence (1991); Spencer and Spencer (1996) and Amberg et al. (2005).

Mutagenesis has been used to generate many industrial yeast mutants with improved phenotypes. Examples of publications describing such work are listed in Table 20.1.

20.2.1.3 Generating Novel Variants by Creating Hybrid Yeasts

As any plant or animal breeder knows, one of the best ways of generating desirable phenotypes in domesticated organisms is to produce hybrids by mating genetically distinct individuals. Most commonly, a hybrid is the progeny arising from sexual reproduction between two members of the same species; such progeny are known as intraspecific hybrids. But hybrids can also be generated from crosses between members of different species, although the progeny of such crosses are, by definition, sterile. These interspecific hybrids might be the products of natural matings if the parents are from species that are phylogenetically closely related, as is seen when a horse and donkey, which are members of the same genus, mate to produce a mule. Generating hybrids from crosses between phylogenetically more distant species is less straightforward, and is very unlikely to happen in nature. It can,

Table 20.1 Applications of mutagenesis to generate novel industrial yeasts

Aim of work	Industrial application	Reference
To isolate mutants with improved freeze tolerance	Baking	Teunissen et al. (2002)
To isolate mutants that produce reduced quantities of H_2S	Winemaking and brewing	Rupela and Tauro, 1984; Spencer and Spencer, 1983
To isolate mutants that produce reduced levels of diacetyl	Brewing	Spencer and Spencer (1983)
To isolate high-glycerol producing strains	Glycerol production	Zhuge et al. (2005)
To raise mutants with higher pigmentation	Pigment production	Schroeder et al. (1996)
To raise non-foaming mutants	Saké production	Ouchi and Akiyama (1971)
To isolate strains that produce reduced levels of urea	Saké production	Kitamoto et al. (1993)
To obtain strains with improved sedimentation properties	Sparkling wine production	Snow (1983)
To raise mutants with improved autolysis	Sparkling wine production	González et al. (2003)
To generate ethanol-tolerant wine yeasts	Winemaking	Snow (1983)

however, sometimes be made to happen in plants and microorganisms in the laboratory using spheroplast fusion.

20.2.1.3.1 Generating Interspecific Yeast Hybrids by Mating

Most industrial yeasts, and this largely means members of the *Saccharomyces* sensu stricto group, which includes *Saccharomyces cerevisiae* (baker's yeast, wine yeast and ale yeast), *Saccharomyces pastorianus* (lager yeast) and *Saccharomyces bayanus*, can reproduce sexually and, therefore, can be mated with other members of the same species to generate interspecific hybrids. The sexual cycle of such yeasts is illustrated in Fig. 20.1.

In summary, diploids sporulate (undergoing meiosis) to produce an ascus containing four haploid gametes known as ascospores. Each ascospore will be either an 'a' or an 'α' mating-type, capable of fusing with another spore of the opposite mating-type ('a' with 'α' and vice versa) to generate a/α diploids. These diploids can again sporulate to generate another generation of haploid spores; the four spores in each ascus comprising two 'a's and two 'α's (see Haber, 1998 for a description of the intricacies of the yeast mating-type system). The methods employed in the laboratory to bring about mating of yeast cells are covered thoroughly in Sherman (2002) and Amberg et al. (2005).

The description above of the mating system in *Saccharomyces* sensu stricto yeasts reflects what has been learnt from studies on well-behaved laboratory strains of *S. cerevisiae*. However, laboratory yeasts are typically heterothallic, meaning that the mating-type of haploids is stable: 'a' types do not change to 'α' and 'α's also

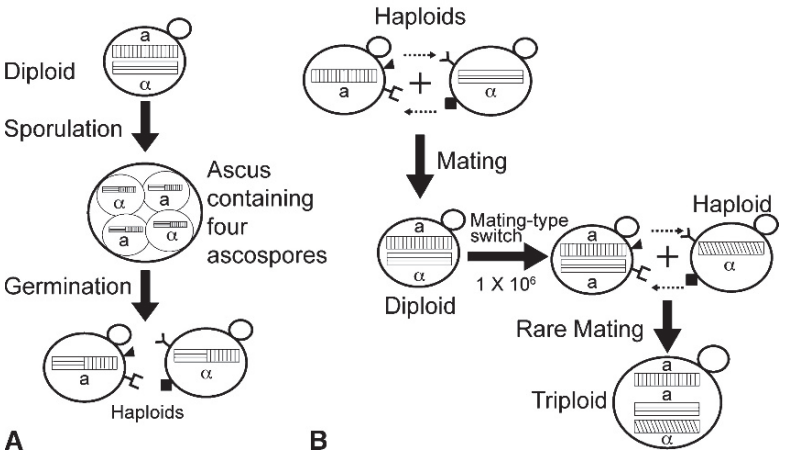

Fig. 20.1 Illustration of the sexual cycle and mating system in *Saccharomyces* sensu stricto yeasts. (**A**) Diploid cells can sporulate to generate four haploid spores contained in an ascus. (**B**) Haploid cells produce mating hormones, 'a' or 'α', illustrated as ▶ and ■ respectively. They also produce cell surface receptors for the opposite mating-type hormone. Binding of hormone to its cognate receptor induces a cell to fuse with a yeast cell of the opposite mating type. Diploids are 'a/α', and as such cannot mate. However, rare mating-type switches occur generating diploids that are homozygous for the mating-type locus. These diploids can then mate with haploids of the opposite mating type to produce triploids. The generation of triploids in this way is known as rare mating

remain true to form. In contrast to this, most wild and industrial *Saccharomyces* spp. are homothallic, readily switching mating-type (Haber, 1998). As a result, it is difficult to isolate haploid industrial yeasts because, following a mating-type switch, a cell will rapidly mate with a neighbouring 'sibling', thereby diploidising.

In addition to this, many industrial yeast strains are recalcitrant when it comes to sporulation or their spores have poor viability, and therefore, are difficult to mate (see for example Johnston et al., 2000); if one cannot generate gametes, sexual reproduction becomes difficult. The reason for this recalcitrance and poor spore viability is probably that many industrial (and natural) yeasts are not simple diploids, but might be polyploid or aneuploid. Bakalinsky and Snow (1990), for example, found wine yeast strains that were diploid for most of their chromosomes but were triploid or even tetraploid for others. Segregation of chromosomes during meiosis will, of course, be problematic for organisms that do not have a diploid complement of chromosomes, leading to a failure in gamete production or, at least, failure to produce viable spores.

Mating of industrial yeasts can, nonetheless, be accomplished in the laboratory. If the parents to be mated have complementary selectable phenotypic traits and the strains can be encouraged to sporulate, selection for hybrids should be straightforward and, therefore, a random spore mating approach can be used (see, for example, Miklos and Sipiczki, 1991). This requires very little hands-on work for the mating step, however, much time can be taken up attempting to find a selection pressure that will be specific for selecting hybrids. Most industrial strains are wild-type with

respect to auxotrophic markers and, therefore selection typically has to rely on the parents' abilities to grow under different conditions. For example, one strain might be capable of growing on a carbohydrate source that the other cannot, while the other strain might be able to grow at a temperature that is not possible for the first. This method has been used extensively in brewing yeast programs (Johnson, 1965; Bilinski et al., 1987) and, to some degree, in wine yeast breeding programs (Van der Westhuizen and Pretorius, 1992).

If there is not a simple way of selecting for hybrids and they cannot be easily identified, mating techniques can be problematic. Nonetheless, if the mating frequency is sufficiently high it might be possible to screen a mated population for hybrids using molecular techniques such as colony pick Polymerase Chain Reaction (PCR) with primers targeting regions of the genome that differ between the mated strains (Burke et al., 2000). Sites that can be targeted for this include the δ sequence of the TY1 retrotransposon (Ness et al., 1993).

As an alternative to random mating, individual spores from the strains to be crossed can be placed adjacent to each other on a nutrient agar plate. Resultant colonies are micromanipulated to attain clonal populations, which are subsequently analysed to confirm the hybrid status of progeny. Analysis typically involves PCR-based DNA fingerprinting and suitable plate-based assays (Winge and Lausten, 1938; Naumov et al., 1986). While this procedure requires a dissection microscope and micromanipulator, and it can be quite tedious, it does deliver results. A technique known as two-colour flow cytometric cell sorting has also been used successfully to identify hybrids arising from a random mating (Bell et al., 1998); one parental strain was labeled with a fluorescent green stain, the other with a fluorescent orange stain and hybrids were selected on the basis of their duel orange and green fluorescence.

As mentioned previously, many industrial yeast strains do not sporulate, and therefore would not be expected to mate, but this situation can be overcome. Diploid *Saccharomyces* spp. cells undergo mating-type switches, to generate homozygous a/a or α/α cells from heterozygote a/α types, albeit at a frequency of 10^{-6} or less (Pomper et al., 1954; Gunge and Nakatomi, 1972; de Barros Lopes et al., 2002). The resultant single mating-type diploid cells can mate with haploids of the opposite mating-type. However, because the frequency of this type of mating is so rare, it is crucial to have strong selection for the resultant hybrid; it would not be feasible to use colony PCR or to screen millions of colonies for a desired phenotype. Rare mating, as it is known, has enabled researchers to mate non-sporulating diploid strains with either haploids or spores from a diploid strain (see, for example, de Barros Lopes et al., 2002); in fact, as early as 1958, polyploids were produced by rare mating triploid and diploid yeast (Mortimer, 1958).

20.2.1.3.2 Generating Interspecies Yeast Hybrids by Mating

All species of the *Saccharomyces* sensu stricto complex have the same a/α mating system and, therefore, have the potential to interbreed. In fact, several natural interspecific yeast hybrids have been identified. For example, the lager yeast, *S. pastorianus*

(synonym *S. carlsbergensis)* is a hybrid of *S. cerevisiae* and a *S. bayanus*-like yeast (Kielland-Brandt et al., 1995); the commercial wine yeast, S6U, is a hybrid of *S. cerevisiae* and a *S. bayanus* strain (Masneuf et al., 1998; de Barros Lopes et al., 2002); the cider yeast, CID1, appears to carry genetic information from three different species, *S. cerevisiae*, *S. bayanus* and *Saccharomyces kudriavzevii* (Masneuf et al., 1998; Groth et al., 1999; de Barros Lopes et al., 2002), and recently several commercial wine yeast strains and wine yeast isolates have been found to be hybrids of *S. cerevisiae* and *S. kudriavzevii* (Bradbury et al., 2006; González et al., 2006). Researchers have been able to exploit the capacity of *Saccharomyces* spp. to produce interspecific hybrids (Naumov, 1987; Zambonelli et al., 1993; Hunter et al., 1996; de Barros Lopes et al., 2002), to increase dramatically the amount of genetic variation available within a species.

Whilst the incompatibility of two genomes in an interspecies hybrid leads to sterility, this should not be an impediment to the use of such hybrids in an industrial setting because yeast numbers (and therefore biomass) increase by asexual budding. However, in addition to infertility, incompatibility of genomes might lead to a level of genome instability, and this may be of concern for industrial applications. Studies on plant evolution have demonstrated that hybrid genomes undergo a wide range of genomic modifications including gene loss and chromosomal translocations. It is thought that these changes can lead to the elimination of gene redundancy and/or genome incompatibility (Soltis and Soltis, 1999), eventually leading to stability. Presumably this is what occurred in the evolution of the natural hybrids mentioned above as they each carry only remnants of their ancestral parents and are now stable. The issue of stability is clearly something that should be kept in mind when generating interspecific hybrid yeasts for industrial applications.

As one might guess, interspecific hybrids are produced at a low frequency and, therefore, it is important in interspecific matings to have strong and clear selection for the hybrid. However, even with a clear selection system, the status of the hybrid should be confirmed using molecular techniques such as PCR-generated Restriction Fragment Length Polymorphisms (PCR/RFLP), targeting the ribosomal DNA (rDNA) internal transcribed spacer (ITS) region (Esteve-Zarzoso et al., 1999). In a complete hybrid there should be evidence of rDNA ITS regions from both parents. However, because of the inherent instability of interspecific hybrids it is possible that the rDNA ITS region from one of the parents could be lost; this has been observed for hybrids generated in our laboratory (unpublished data). Thus it is probably worth checking more than one region of the genome if the rDNA ITS region appears to come from only one source in a putative hybrid.

While not recommended as a general method for generating hybrid yeasts, researchers have shown that the hostile environment of an invertebrate's digestive tract can provide a setting for the generation of hybrids between *S. cerevisiae* and *Saccharomyces uvarum* strains (Pulvirenti et al., 2002). This unusual procedure was devised in order to verify that animals can promote the formation of new yeast strains, the experiments relying on the invertebrate's digestive tract to degrade the ascus wall. Different species (freshwater worms and fruit flies) were fed a diet of different sporulated yeasts and their faeces collected. Yeast propagated from the faeces were analysed to confirm their hybrid nature.

20.2.1.3.3 Generating Hybrid Yeasts Using Spheroplast Fusion

If mating strategies cannot be made to work, hybrids (intra- and interspecies) can be generated by removing the walls of the yeast cells to be crossed then fusing the resultant spheroplasts[1] (Table 20.2). Removal of the cell wall is accomplished using any one of a range of commercially available lytic enzymes including: Zymolyase™ (Zymo Research), purified from culture fluid of the bacterium *Arthrobacter luteus*; Novazyme (Novazyme Corp), extracted from the fungus *Trichoderma harzianum*; or Helicase (Industries Biologique), found in the secreted digestive juice of the snail *Helix pomatia*. Spheroplasts are osmotically labile and have to be kept in an osmotically buffered solution (0.8 M sorbitol or 0.6 M potassium chloride) to prevent bursting. To bring about fusion, spheroplasts are mixed together and, in the presence of calcium ions, induced to fuse using either an electric field or a solution of polyethylene glycol (PEG). PEG has been shown to induce the formation of non-specific cell aggregates, allowing intimate contact between small localized areas of the plasma membranes of adjacent cells (Knutton, 1979).

After a short incubation to allow fusion, the PEG solution is removed and replaced with buffer containing either sorbitol or potassium chloride for osmotic stability. The fusion 'mix' is then plated in (i.e. spheroplasts are suspended and poured in) cooled, molten, osmotically stabilised agar, where the spheroplasts regenerate their cell walls and subsequently grow into colonies.

As was the case for interspecific and rare mating, the frequency of hybrid production in spheroplast fusion is very low. Therefore it is again important to have strong and clear selection to enable rapid and easy isolation of hybrids. This can be achieved as described in Spencer and Spencer (1980, 1981) by using a respiratory deficient mitochondrial mutant strain as one parent. The respiratory-competent fusion products were selected on non-fermentable carbon and on the basis of using a carbon source utilised by the petite parent and not by the other. This approach enabled Spencer and colleagues to cross *S. cerevisiae* strains with a number of diverse yeasts such as *Candida pseudotropicalis, Hansenula capsulata* and *Pichia membranaefaciens.*

In the absence of a clear selection system of the type described above, hybrids are more difficult, but not impossible, to isolate. For example, Katsuragi et al. (1994) used fluorescence cell sorting as a means of distinguishing and separating hybrids from unfused regenerated parental cells.

As was the case for the generation of interspecific hybrids by mating, no matter how good the selection system available or the means of distinguishing fused from non-fused cells, it is advisable to validate the status of putative hybrids using molecular techniques, as described in section 2.1.3.2. It is also important to again appreciate that interspecific hybrids might well be genetically unstable, at least initially.

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[1] Spheroplasts and protoplasts are cells that have had their walls partially or almost completely removed, respectively. The terms are often used interchangeably but, technically, they are not the same thing. Successful fusion of cells is thought to be best achieved using spheroplasts, probably because there is more cell wall left intact, and this is thought to increase the chance of regeneration following fusion.

Table 20.2 Examples of hybrid yeasts generated for industrial applications

Nature of hybrid	Aim of work	Industrial application	Reference
Intraspecific protoplast fusion: *Candida tropicalis*	To increase phenol-degrading ability	Wastewater treatment	Chang et al. (1995)
Intergeneric protoplast fusion: *S. cerevisiae* X *Kluyveromyces fragilis*	To produce ethanol from lactose	Biofuel production	Farahnak et al. (1986)
Intergeneric protoplast fusion: *Saccharomycopsis fibuligera* X *S. cerevisiae*	To produce ethanol from starch	Biofuel production	Kishida et al. (1996)
Intraspecific spore mass mating: *S. cerevisiae*	To combine maltose utilization and osmo-tolerance	Breadmaking	Higgins et al. (2001)
Intraspecific spore mass mating: *S. cerevisiae*	To increase flocculation and lower diacetyl production	Brewing	Gjermansen and Sigsgaard (1981)
Intergeneric protoplast fusion: *Kluyveromyces lactis* X *S. cerevisiae*	To generate resistance to killer activity	Brewing	Gunge and Sakaguchi 1981
Intraspecific protoplast fusion: *S. cerevisiae*	To combine osmotolerance with increased ester production	Brewing	Mukai et al. (2001)
Interspecific protoplast fusion: *Penicillium album* X *Penicillium caseicolum*	To improve industrial performance	Dairy industry	Reymond and Fevre (1986)
Intraspecific spore-haploid cell mating: *S. cerevisiae*	To eliminate undesirable foaming property	Winemaking	Thornton (1978)
Intraspecific spore mating: *S. cerevisiae*	To increase flocculation and reduce H_2S formation	Winemaking	Romano et al. (1985)
Intraspecific spore mass mating: *S. cerevisiae*	To improve fermentation efficiency and desirable oenological properties	Winemaking	van Wyk and Pretorius (1990)
Intergeneric protoplast fusion: *S. cerevisiae* X *Schizosaccharomyces pombe*	To increase ability of wine yeast to degrade L-malic acid	Winemaking	Carrau et al. (1990)
Interspecific spore mating *S. cerevisiae* X *Saccharomyces bayanus*	To increase fermentation vigor over a wide temperature range and reduce levels of acetic acid production	Winemaking	Zambonelli et al. (1997)

An additional problem that is peculiar to spheroplast fusion and was evident from some of its early applications, is that nuclei of mitotically growing cells are not necessarily competent to fuse (van Solingen and van der Plaat, 1977; Svoboda, 1978; Russell and Stewart, 1979); cells ready to mate have increased competence in this regard. Curran and Carter (1983) and Rose et al. (1986) however described a way around this. They found there was an increased fusion rate between *S. cerevisiae* haploid strains of the same 'a' mating-type when the cells were pre-incubated with 'α' mating factor prior to spheroplasting. Mating factors are hormones; 'α' mating factor is produced by 'α'-type cells to stimulate neighbouring 'a'-type cells to mate, and vice versa. These mating factors have been shown to be integral players in cell membrane and nuclear envelope fusion (Rose et al., 1986) presumably by stimulating karyogamy following fusion.

20.2.2 Isolating Novel Yeast Variants for Industrial Applications: Screening and Selection

'Over all these causes of change I am convinced that the accumulative action of selection, whether applied methodically and more quickly, or unconsciously and more slowly, but more efficiently, is by far the predominant power'. From: Charles Darwin (1859), *The Origin of Species by Means of Natural Selection*, Chapter 1: Variation Under Domestication.

When utilising genetic variation (accessed or generated as described in Section 2.1) to obtain novel variants with sought-after traits, screening is relatively straightforward if the desired phenotype confers a selective advantage under defined conditions. Genetic variants are grown in the presence of the selective conditions and survivors harvested. This is most easily achieved on plates, as it enables the isolation of single (i.e. clonal) colonies. Typically, several such colonies would be picked and tested for overall performance. For example, if working with brewing yeast one would confirm that novel variants not only have the trait selected for, but also retain the necessary brewing properties of the parent yeast. This is very important because the methods described in Section 2.1 for producing variation might generate incidental mutations that impact on attributes required of the yeast in its industrial application.

If the sought-after trait does not confer a selective advantage, isolation of desirable variants can be considerably more time-consuming and convoluted. For example, when attempting to isolate beer or wine yeasts that impart desirable flavour attributes to beverages, it would be necessary to have a screen that enables large numbers of variants to be tested; the discriminatory qualities of the approach and technologies used are essential to its success. An analytical chemistry-based approach can be employed to speed up the process, but one would have to know something about the chemistry of the trait being sought; for example, one might screen for the presence of known desirable flavour molecules.

Thus, screening genetic variants for desirable traits comes down to using selection or assessing numerous variants with the hope of finding one with the desired

phenotype. However, there is another way of using selection as a means of generating novel strains with sought-after, selectable traits, namely adaptive evolution[2].

20.2.2.1 Using Adaptive Evolution to Generate Novel Yeast Strains: The Theory and The Practice

Adaptive evolution strategies consist of extensive phases of selection to 'drive' evolutionary change; selection is not just a 'one-step' means of screening, but rather is carried out over many hundreds of generations. These strategies include serial transfers of batch cultures, continuous culture (Fig. 20.2) and even plate selection. The

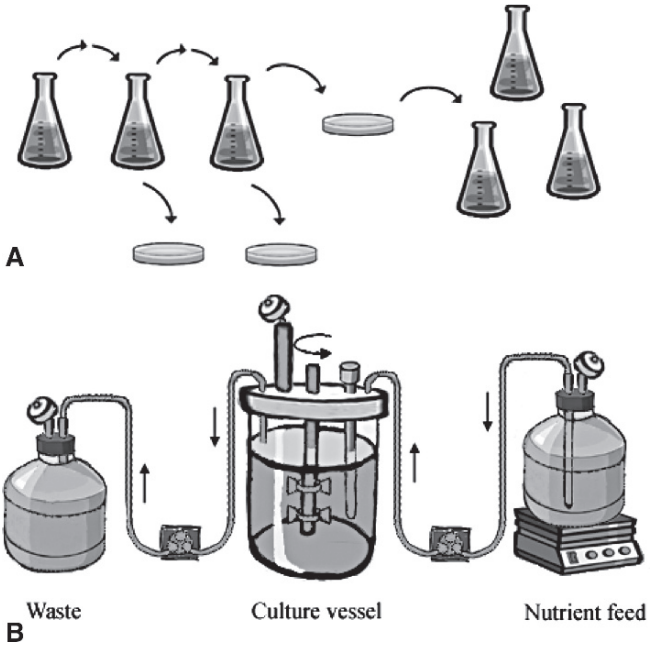

Fig. 20.2 Adaptive evolution: a microbial culture is grown under selection pressure for hundreds or thousands of generations. (**A**) Serial transfers of batch cultures: batch cultures are passaged so that each time a culture reaches stationary phase a sample is inoculated into fresh selective medium. This is repeated over hundreds of generations. Individual colonies can be isolated after each transfer and tested for the desired phenotype. (**B**) Continuous culture: a microbial population is grown under steady-state conditions in the presence of a suitable selection pressure. Growth is controlled by dilution rate.

[2]In the context of strain development, the term 'adaptive evolution' is used to describe methods that involve the use of iterative or ongoing selection to drive the establishment of genetic change. The terms 'directed selection' and 'evolutionary engineering' are sometimes used to describe the same thing, but in recent times they have taken on other, more specific, meanings.

method utilises the continuing emergence of mutants and selection of variants with improvements in the desired phenotype, and these might arise spontaneously or can be generated using methods described in Section 2.1. Thus, during selection, genetic variation is continuously 'topped up' and selection acts on this as it arises. Mutants, even with only a marginal selective advantage, outgrow others in the culture and thereby take-over (Dykhuizen and Hartl, 1983; Jansen et al., 2005). This approach is particularly powerful when attempting to isolate phenotypes that are shaped by multigenic determinants; long-term continuous or iterative selection enables the accumulation of mutations that confer an increasing selective advantage, thus progressively pushing the phenotype in the desired direction. In an adaptive regime, the rate of evolution is determined by mutation rate, population size and the strength of selection. In most situations the mutation rate is governed by growth rate which, in chemostats[3], can be manipulated by modulation of the dilution rate (Novick and Szilard, 1950).

The effect of population size on evolutionary rate is a little more complex. Wick et al. (2002) pointed out that given a large enough population it is not necessary to wait for the evolution of a beneficial mutation but that it is only necessary to wait for existing favourable mutations to take over the population. For example, the rate of advantageous mutations in *S. cerevisiae* is estimated to be $\sim 1 \times 10^{-11}$ per generation (Zeyl, 2004) and, given a population size of 1×10^{11} cells, there would be at least one favourable mutation every generation.

Thus, in theory, an adaptive evolution program can be accelerated by working with large populations, and this can generally be achieved using chemostats. Unfortunately, although the number of advantageous mutations is less limiting in large populations, the rate of fixation decreases with increasing population size (Paquin and Adams, 1983). This is at least in part due to competition from individuals with other, different, advantageous mutations in systems with large populations (Wahl and Krakauer, 2000). Furthermore, the rate of evolution in diploid populations is particularly dependent on whether the trait under selection is dominant or recessive. If an advantageous mutation exhibits only partial dominance then there is a high chance of it being lost before it is fixed (Anderson et al., 2004). Theoretically, if a strong selection pressure can be applied this will accelerate fixation of a mutation, particularly when the fitness advantage of that mutation is small (Wahl and Krakauer, 2000; Wick et al., 2002).

When using serial batch transfer for adaptive evolution experiments one is generally working in flasks or test tubes with small populations. These cultures will inevitably carry less genetic variation but, as discussed above, this should enable adaptive mutations to be more rapidly fixed, particularly for diploids (Orr and Otto, 1994), and diversity can be increased using the techniques covered in section 2.1. Furthermore an adaptive mutation will almost always be fixed long before a second

[3] A chemostat is a vessel having an input aperture for the influx of sterile nutrient medium from a reservoir and an overflow aperture for the efflux of exhausted medium, living cells and cellular debris. The growth rate is controlled via the concentration of the growth-limiting nutrient in the medium, which is controlled by dilution rate.

mutation appears. Thus, almost all favourable mutations arise among descendants already carrying favourable mutations. However the cost of working with smaller populations is that the outcomes of such evolutionary programs will exhibit an increase in 'stochasticity' with a resultant loss of repeatability (Wick et al., 2002), not an insignificant consideration when a method is applied in development programs for organisms destined for industrial use.

The following examples give an indication of the dynamics of evolutionary change in yeast populations over time utilising different adaptive evolution strategies. Sonderegger and Sauer (2003) made use of long-term adaptive chemostat cultures to generate strains of metabolically engineered *S. cerevisiae* that could utilise xylose anaerobically. After 460 generations, the best isolates were further evolved using serial batch growth in shake flasks. Measurable improvements in anaerobic growth rate could be detected following a further 40 generations. However, this study used a haploid *S. cerevisiae* strain, and haploids are generally expected to evolve in a shorter time than diploid or polyploid yeasts. In a subsequent study, Sonderegger et al. (2004) found this to be the case when evolving a diploid, industrial, xylose-utilising *S. cerevisiae* to anaerobiosis by means of the same approach as that used for the haploid strain. While the metabolically-engineered haploid laboratory strain TMB3001 required only 120 generations to adapt to micro-aerobic conditions (Sonderegger and Sauer, 2003), 390 generations were required for an industrial strain to reach the same point (Sonderegger et al., 2004).

Zeyl (2005) mapped evolutionary dynamics of *S. cerevisiae* over long time frames (2000 generations) in serially propagated 10 mL cultures of a laboratory strain in glucose limited medium. The seed population size for these experiments was ~2.7×10^6 cells. Fitness of the evolving population was estimated from experi ments involving competition between the ancestral genotype and the evolved populations under conditions identical to those in which the population evolved; genotype frequencies were estimated after 24 and 48 h. Zeyl (2005) showed a constant increase in fitness for the first 1000 generations after which the population stabilised with no further changes for the next 500 generations. However, fitness increases were again observed during the last 500 generations of the experiment, approximating quantitatively the increases over the first 1000 generations. These findings illustrate beautifully the dynamics of adaptive change and highlights again the time scales that should be anticipated when designing a program for strain improvement. Zeyl (2005) found that, on average, evolved isolates each carried three to four advantageous mutations.

Recently, Attfield and Bell (2006) demonstrated how mating and iterative selection on plates followed by serial propagation in flasks, could be used to raise *S. cerevisiae* mutants capable of utilising pentose sugars. This was a significant advance in generating yeasts that are able to utilise agricultural waste for bioethanol production. Pentose sugars comprise a major part of lignocellulose in agricultural wastes and as such are a potential source of carbon for the production of renewable fuel from an otherwise discarded material. However, ethanologenic yeasts are unable to utilise pentoses, and those yeasts that can, do not make ethanol. The approach Attfield and Bell (2006) took was to create a large pool of genetic variants of *S. cerevisiae* by

sporulating and mating industrial, wild-type and laboratory strains and plating these onto a pentose agar medium. Cells showing signs of growth were harvested, remated and again plated on xylose. This process was repeated several times over 569 days, after which the growth rate and yield of the heterogeneous population had increased to the point that culturing in liquid media became possible. The authors then continued cycles of mating and growth in a pentose-based liquid medium for a further 894 days. After a total of 13 cycles of mating and selection over 1463 days, the authors isolated 30 individuals with doubling times in exponential growth phase ranging from 5 to 8 h (mean = 5.49 h) using xylose as a sole carbon source. The reported ethanol accumulation for this strain grown aerobically in xylose minimal medium was 0.58 g/L, which is similar to that achieved using metabolically engineered strains under similar assay conditions (Pitkanen et al., 2005).

While iterative plate selection was ideal for the early stages of the selection of pentose utilising yeast, batch and chemostat cultures would generally be the methods of choice for directed evolution strategies. An example of serial batch selection for directed evolution of an industrial yeast strain can be seen in the work of Higgins et al. (2001). These authors used mass mating to generate variation and then selected strains for strong maltose utilisation and hyperosmotic tolerance. This approach enabled the isolation of baking yeasts capable of leavening both unsugared and sweet doughs, which typically would require two different yeasts.

During growth in batch culture, a microbial population passes through the different phases of lag, exponential and stationary growth, so evolutionary events may arise from advantages in any of these phases. In contrast, continuous culture systems provide a constant environment: nutrients are supplied to the culture at a constant rate, and in order to maintain a constant volume, an equal volume of cell culture is removed. Thus, microbial growth takes place under steady-state conditions, which means that growth occurs at a constant rate and in a constant environment. As will be seen from the following, this can be achieved using computerised feedback control systems that monitor and regulate growth and/or metabolic rate of the culture, constantly responding to the changing physiology of the population and automatically regulating the feed rate and intensity of the selection pressure.

20.2.2.1.1 Interactive Continuous Culture as a Means of Driving Adaptive Evolution

When a yeast population is propagated asexually under long-term continuous culture, it normally experiences a series of adaptive shifts whereby clones able to cope better with the culture conditions, successively outgrow and replace less fit ancestral populations (Ferea et al. 1999). By manipulating environmental conditions to modulate selective pressure, adaptive shifts can be directed in favour of the desired phenotype.

The use of continuous culture systems to drive selection of mutants generally requires some knowledge of the level of resistance or tolerance of the parental strain to the selective pressure being used. This enables the investigator to estimate

the appropriate level of the selection agent to be applied. However, the optimal level will increase as the culture evolves greater tolerance to the selective agent (Butler et al.,.1996), necessitating continuous monitoring of the evolving culture and responses that can only be 'best guesses' of what level of selection to employ next. The main risks of such an approach are over- and under-estimation of the level of selective agent to be used, leading to death of the culture, failure to evolve, or the generation of undesirable mutants (Butler et al., 1996; Lane et al., 1999).

Brown and Oliver (1982) overcame the problems of over- and under-addition of inhibitor by applying an Interactive Continuous Selection (BOICS) system using a measurable metabolic parameter (in this case, liberation of CO_2) to control the addition of the selective agent (ethanol). The authors demonstrated the utility of the method in the generation of ethanol-tolerant variant of *S. cerevisiae*. First, they established continuous culture conditions using a medium with little ethanol added. Once this culture was stable, a feedback control system was initiated, which added ethanol to the culture in response to CO_2 production exceeding a predetermined level. The system continued adding ethanol until it reached a concentration at which metabolism was compromised, and therefore CO_2 output was reduced. This triggered the ethanol-feed pump to be switched off. Dilution by continual addition of fresh medium gradually reduced the ethanol concentration, allowing cells to recover and thus increase their rate of CO_2 production. Eventually the CO_2 concentration again exceeded the set point resulting in addition of more ethanol. This iterative process was continued for over 160 generations and ultimately led to the recovery of yeast mutants with an enhanced ethanol tolerance (Brown and Oliver, 1982).

In principle, the technique of Brown and Oliver (1982) can be used to obtain mutants that are tolerant to any toxic or inhibitory substance, environmental stress or growth medium condition, and any measurable growth parameter can be used for automatic feedback control (Lane et al., 1999; Sauer, 2001). For example, Markx and Kell (1995) replaced the CO_2 control loop with a culture output permitivity sensor to monitor the concentration of viable cells in a culture carrying a large proportion of dead cells. This approach enabled them to obtain yeast mutants tolerant to cytotoxic chemicals (Markx and Kell, 1995).

The two principal advantages of using the BOICS approach are: the versatility of the system (any parameter measurable online can be used to control the level of selective agent), and decisions on the level of selective agent to be used are not arbitrary or 'best guesses', but rather based on the physiology of the evolving population.

20.2.2.1.2 The Importance of Medium Composition on Evolutionary Outcomes in Adaptive Evolution

Strains adapt to the environment in which selection takes place and not solely to the selection 'pressure' that is applied. Therefore, an ill-considered medium design might push strain evolution in undesirable directions, generating strains that are not suitable for industrial applications. A recent review of the role of medium composition

on industrial yeast strain development has been published by Hahn-Hägerdal et al. (2005), to which the reader is directed. The following examples illustrate the importance of appropriate medium choice.

Ho et al. (1998) took a metabolic engineering approach to develop a xylose-metabolising yeast for the purpose of converting spent sulfite liquor (SSL) into ethanol for use as fuel. The authors tested their new yeast strain's ability to convert xylose in a yeast extract and peptone-based laboratory medium. However, when the capacity of the strain was tested in the SSL industrial medium it was found that xylose fermentation was completely inhibited (Helle et al., 2004). Ultimately, the group showed that fermentation of xylose from SSL origin was possible but that pH adjustment, yeast extract and peptone addition, and thorough aeration were required for xylose fermentation to occur. A similar result was obtained by Butler et al. (1996) when they used a laboratory medium for directed evolution to generate an antibiotic hyper-producing strain of the bacterium *Streptomyces griseus* using BOICS. While the authors were successful in producing a strain that made more antibiotic than the parent in the medium used for selection, the phenotype was not maintained in an industrial medium. These examples highlight the importance of medium composition in determining the evolved phenotypes. The probability of such adverse outcomes could be minimised by employing an evolutionary environment that mimics as closely as possible the characteristics of the industrial environment in which the microorganism will be used.

20.2.2.1.3 Examples of Strain Development Using Adaptive Evolution in Continuous Culture

One of the first uses of continuous culture to study experimental evolution in yeast was described by Paquin and Adams (1983). These authors studied haploid and diploid yeast evolution in glucose limited chemostats for several hundred generations in which the appearance and proliferation of adaptive mutants was followed by monitoring fluctuations in the frequencies of neutral genetic markers (Paquin and Adams, 1983). Final adaptive clones showed a dramatic increase in the glucose uptake rate after selection (Adams et al., 1985). A significant change in cell shape was also observed, leading to a more elongated longitudinal section and an overall 13% increase in the ratio of surface area to volume, probably a more favourable ratio for glucose uptake (Dykhuizen and Hartl, 1983; Adams et al., 1985). Chemostats can select for mutants tolerant to extremely low concentrations of the limiting nutrient, in this particular case a carbon source, but it could be nitrogen, phosphorous, sulfur, etc. (Lane et al., 1999).

Trying to understand the mechanism(s) responsible for strain fitness changes in long-term chemostats under glucose limitation, Brown et al. (1998) compared a clone propagated from the diploid yeast strain used to initiate the experiment of Paquin and Adams (1983) with a clone isolated from the population after 450 generations of continuous culture. Relative to the original clone, the evolved population was able to sustain growth at lower steady-state glucose concentrations with clearly

enhanced cell yield per mole of glucose, significantly enhanced high-affinity glucose transport and greater relative fitness in competition experiments (Brown et al., 1998). A more detailed analysis revealed that the evolved yeast strain carried multiple tandem duplications of the high-affinity hexose transport genes *HXT6* and *HXT7*. A correlation between the change in copy number and a significant increase in mRNA levels of the *HXT6/7* transcript was also observed. The authors suggested that the evolved strain not only transported the limiting substrate more rapidly when provided with lower steady-state concentrations, but also utilised it more efficiently in the production of biomass (Brown et al., 1998).

Interestingly, not all of the mutants isolated by Brown et al. (1998) with the above phenotype had duplications of the *HXT6* and *HXT7* region. Southern blot analysis of restriction digests, probed with an *HXT* sequence, revealed that some mutants were indistinguishable from the parent strain. These findings indicate that there are multiple adaptive solutions to limitations in glucose availability in a continuous culture environment and demonstrates the existence of a heterogeneous population at the 'end' of selection. The level of heterogeneity necessitates that a representative of individual clones from such populations must be examined to identify individuals that will be best suited to their industrial application (Sauer, 2001).

Aarnio et al. (1991) carried out one of the first experimental evolution studies with a commercially available industrial strain. These authors attempted to enhance the acetic acid tolerance of baker's yeast in order to reduce costs and improve the quality of sourdough bread production; greater tolerance to acetic acid in baker's yeast reduces the amount of yeast required to leaven dough and decreases the risk of development of yeast-like off-flavours. To this end, seven bioreactors were inoculated with a commercial baker's yeast and the populations were grown in a continuous turbidostat[4] with manually regulated, increasing concentrations of acetic acid as the selection pressure. After 40–45 generations, isolated clones were grown in a medium without selection pressure and stable variants were isolated. Just one of the populations delivered variants showing increased tolerance to acetic acid and improved raising power in sour dough (Aarnio et al., 1991). Although the approach used was successful in providing a yeast strain with the desired phenotype in a short time, longer cultivation times and a more 'automated', responsive approach to the addition of acetic acid might have delivered a larger number of variants with even greater tolerance to acetic acid.

Other types of continuous culture system have also been used successfully to generate novel yeast variants. Jimenez and Benitez (1988), for example, used a pH-stat[5] to raise an ethanol-tolerant wine yeast. Genetic variation was generated at the outset by crossing spores from a wine yeast with a haploid laboratory strain. The resultant hybrids were initially cultured with no ethanol added. Then the feed was changed

[4] A turbidostat is a continuous culture system that maintains a constant turbidity (cell density) of an exponentially growing culture by means or an optical sensor that regulates the nutrient inflow.

[5] A pH-stat is a continuous culture system in which the pH of the medium is maintained close to a predetermined set point by regulating the nutrient feed pump.

using the same medium but containing 8% ethanol; thus the culture was exposed to a continuously increasing ethanol concentration. During this stage, the rate of feed (and, therefore, the rate of increasing ethanol concentration) was regulated in response to the pH of the culture; *S. cerevisiae* acidifies the medium when it is metabolically active and, therefore, pH is an indicator of metabolic rate. As ethanol concentration was increased from 0% to 8%, the pH of the medium was maintained close to a predetermined set point by switching on and off the nutrient (and therefore ethanol) feed to the chemostat in response to pH changes; when the pH dropped, the feed would automatically switch on. Once the culture reached steady state at 8% ethanol the feed bottle was again changed, this time with the same medium but with 18% ethanol; thus the medium would gradually increase from 8% to 18% ethanol. As the culture approached 13% ethanol, however, the authors found that metabolism came to a halt. Thus the culture was harvested and this led to the isolation of a highly ethanol-tolerant hybrid 'wine' yeast strain.

Baker's yeast strains that produce increased levels of amino acids, and thereby enrich products made from leavened doughs, have been generated using an interesting selection pressure. Martinez-Force and Benitez (1992) added toxic amino acid analogues to the feed in a pH-stat carrying baker's yeast. The rationale behind this is that mutants resistant to amino acid analogues are likely to be over-producers of the amino acids that the analogues mimic (Alix, 1982). Martinez-Force and Benitez (1992) first used ethionine as the mimic to isolate mutants that overproduce methionine. Mutants isolated using this approach accumulated up to 150-times more methionine and showed growth rates similar to, or even higher than, those of the parent strain. The latter was possible since continuous culture selects cells that grow (i.e. divide) at the highest rate. Amino acid over-producers generated by other means are often unhealthy and, in general, slow growing (Ramos et al., 1991).

Ethionine-resistant mutants were then subjected to a second round of selection, this time in batch cultures supplemented with hydroxynorvaline, which is a toxic analogue of threonine, leading to the generation of mutants that produced 37-times more threonine than the wild type strain. However, this round of selection caused a reduction in the yeast's capacity to produce methionine. Nonetheless, these 'double' mutants were still able to produce 18-times more methionine than the original parental strain (Martinez-Force and Benitez, 1992).

In a similar fashion, a pH-stat supplemented with increasing concentrations of a toxic analog of lysine, *S*-2-aminoethyl-L-cysteine (AEC) was used to generate a baker's yeast that produced 3–17 times as much lysine as the wild-type strain, depending on the nitrogen source used (Gasent-Ramírez and Benitez, 1997).

Adaptive evolution strategies that target secreted proteins require special consideration. This is because the evolution of any advantageous mutation will benefit not only the individual cell producing the protein but also other inhabitants in the culture. Fan et al. (2005) explored solutions to this dilemma in a theoretical study of *S. cerevisiae* cultured on a growth-limiting substrate. The authors concluded that a situation in which the protein was tethered to the cell surface was the only scenario that could overcome the limitation. Theoretically, the optimal selective environment was one in which the substrate was also immobilized.

An earlier publication by Francis and Hansche (1972) demonstrated that an adaptive evolution strategy can be used to improve the performance of a secreted enzyme. The enzyme in question, an acid phosphatase, is periplasmic and, therefore, improvements in its performance would be of limited advantage to neighbouring cells. These authors cultured a haploid laboratory strain of *S. cerevisiae* in a chemostat with phosphate as a limiting factor, in a culture medium buffered at pH 6, which was suboptimal for acid phosphatase activity. Selection was based on the capability of acid phosphatase to catalyze the hydrolysis of the ß-glycerophosphate, and increase orthophosphate uptake and phosphate metabolism efficiency. After 290 generations, the authors identified a genetic modification of the acid phosphatase gene resulting in an increase in enzyme activity and a shift in the pH optimum for the enzyme. Interestingly, it was noted that the activity of the evolved acid phosphatase actually decreased with regard to certain substrates. On the basis of this observation, the authors speculated that it might be possible to alter enzyme specificity to the extent that it is active on a new substrate (Francis and Hansche, 1972, 1973).

20.2.2.2 Risk Minimisation and Quality Assurance When Generating Novel Yeast Strains

The methods described in Section 2.1 for generating genetic variation are random in that they do not target particular genes, and will probably introduce several changes across the genome. In addition, very often the strong selective pressures used in adaptive evolution experiments generate genome rearrangements, especially amplifications and deletions (Brown et al., 1998; Dunham et al., 2002). Thus, there might be considerable collateral damage to the genome when using some of the strategies described in the preceding sections. On top of this, when adaptive evolution is used, unintended selective pressures might inadvertently be introduced (e.g. in the growth medium composition) that select for phenotypic differences over and above what was planned. In light of all of these potential problems it is critical to assess the performance of mutant yeasts before they are used in a commercial setting. Typically, this would involve assessing new strains and the quality of the product they make, in small- and at pilot-scale. The stability of the strain should also be assessed to confirm that the new trait is still evident after handling in an industrial setting. As mentioned in Sections 2.1.3.2 and 2.1.3.3, this is particularly important when one has generated interspecific hybrids as part of the strain development strategy, as these can be genetically unstable.

An additional problem one might encounter when using continuous culture to drive evolutionary change concerns the ecology of evolving populations. In most, if not all, cases the culture will carry a mixture of different genotypes and, in some instances, these differences might complement each other by, for example, co-metabolising the selective reagent. In an industrial setting this would not be desirable; generally one would rather work with single, clonal strains than with mixed cultures. To ensure that evolutionary adaptation during continuous selection

proceeds in the desired direction in individual cells, it is necessary to monitor evolutionary progress at the single cell, clonal level. To avoid, or at least minimise, the risk of generating co-metabolism and/or unfavourable combinations of adaptive mutations it is also recommended to occasionally inoculate a new culture with the best clone from an earlier stage of the selection (Sauer, 2001).

These basic checks and quality assurance measures will, in most cases, suffice but it might also be of interest in a research setting to examine novel strains further to gain an increased understanding of how the new trait works. In other words, determine the genetic and metabolic characteristics that make the new strain desirable. Since the changes usually affect several genes, pathways and/or metabolic processes, global approaches such the use of DNA microarrays and metabolic profiling would be methods of choice to characterise new strains.

Microarray-based comparative genomic hybridisation (array CGH) has been used to identify and characterise gene rearrangements and also to accurately detect aneuplodies (Dunham et al., 2002; Myers et al., 2004). The types of rearrangement that can be detected using these techniques include gene amplifications, changes in chromosome copy number and intrachromosomal and interchromosomal translocations (Dunham et al., 2002).

DNA microarrays have been used to study differences between evolved and parental yeast transcriptomes (Ferea et al., 1999; Jansen et al., 2004; van Maris et al., 2004). Depending on the study, very few to several hundred genes have been reported to show differences in expression. Most of the changes appear to be consistent with the selection pressure applied. For example, selection in glucose-limited chemostats resulted in an altered regulation of central metabolism that favoured complete glucose oxidation over glucose fermentation (Ferea et al., 1999). However, other changes have been more obscure and difficult to explain (van Maris et al., 2004; Jansen et al., 2005).

A novel approach to identify adaptive mutations at single nucleotide level was described by Gresham et al. (2006). High-density yeast tiling microarrays (YTMs), which provided complete, 5-fold redundant coverage of the entire yeast genome, were used to detect single-base pair substitutions, and deletions and even single-nucleotide polymorphisms between strains (Gresham et al., 2006). Using YTMs, the authors identified the changes in two strains evolved from long-term chemostats limited in sulfur.

While the resolving power of chip-based technologies continues to improve it is likely that, in the not-to-distant future, it will become feasible to sequence novel strains more routinely than is currently the case. This would clearly be the method of choice for strain characterisation.

20.3 Future Perspectives and Conclusions

While genetic engineering has the potential to revolutionise industrial yeast breeding programs, community perception of GMOs is such that products derived from recombinant sources run the risk of consumer rejection, and this is particularly the

case in the food and beverage industries. In addition, many industrially important traits of yeast, such as stress tolerance, are genetically complex, involving the contributions and interactions of numerous, perhaps hundreds, of genes. Such traits are therefore not readily amenable to GM approaches. In light of these problems it is likely that industrial yeast breeding programs will continue, at least for the immediate future, to rely on 'traditional' approaches. While this might be limiting, particularly when attempting to generate strains with novel traits that do not have a selective advantage, this chapter illustrates that non-GM strategies, nonetheless, provide a very powerful means of generating novel yeasts.

Ideally we would be able to use GM and non-GM approaches in yeast breeding programs, reaping the benefits of both and using them complementarily. In some countries, including the USA and Canada, this is already the case and has led, for example, to the availability of GM wine yeasts for industrial application. However, at the time of writing this chapter, consumer acceptance of wines made using these yeasts has yet to be tested. In the meantime, in many parts of the world, GM approaches will be used primarily to aid research and, thereby, to generate knowledge. Application of this knowledge to generate novel yeast variants will continue to rely largely on non-GM approaches.

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Chapter 21
Yeast Proteome Analysis

Andrea Matros and Hans-Peter Mock

Contents

Abstract Yeast organisms, and specifically *Saccharomyces cerevisiae*, have become model systems for many aspects in fundamental and applied research. Consistently, many papers have been published applying proteome techniques to study these organisms. The review will give an overview on the proteome research performed on yeast systems so far; however, due to the large number of publications, only selected reports can be cited neglecting many more interesting ones in the interest of space. The review will focus on research involving mass spectrometry as a basic proteome technique, although many more approaches are relevant for the functional characterization of proteins in the cell, e.g. the yeast two-hybrid system. We will provide an overview on yeasts as models in the context of proteome analysis, and explain the basic techniques currently applied in proteome approaches. The main part of the review will deal with a survey on the current status of proteomic studies in yeasts. In a first part of this chapter, we will deal with the currently available proteome maps of yeasts, and in the following part we will discuss studies dealing with fundamental aspects, but also mention proteome studies related to applied microbiology. Finally, we will envisage future perspectives of the proteome technology for studying yeasts, and draw major conclusion on the current status reached in this field of functional genomics.

Keywords Proteome, protein separation, 2-D gel electrophoresis, liquid chromatography, mass spectrometry, post-translational modifications

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21.1 Introduction

In the recent years, techniques for the multiparallel analysis of transcripts and proteins have been introduced and permanently improved. For a number of reasons *Saccharomyces cerevisiae* has proven as a valuable model organism for these and other techniques as major tools for functional genomic studies of eukaryotes (Suter et al., 2006). It is a unicellular organism with a short life cycle and easy to grow. Baker's yeast was also the first eukaryotic organism to be fully sequenced with roughly 6.500 open reading frames. The availability of the nucleotide sequence information is a prerequisite for the successful identification of proteins of interest by mass spectrometry in proteome analyses. Proteome approaches have gained considerable interest and a vast number of papers have been published on the proteomics of *S. cerevisiae*, but also many other yeast organisms have been analysed by these technologies. Current achievements reflect the technical state of the proteome techniques and their limitations to cover a whole proteome. The availability of large collections of knock-out mutants and of genetic and protein arrays, the introduction of the yeast two-hybrid system and of the tandem affinity purification system are other examples highlighting the usefulness of this model organism to perform comprehensive functional studies and address a plethora of questions of cell biology and physiology.

21.2 Techniques of Proteome Analysis

The analysis of the whole complement of cells, of tissues or of whole organisms is based on a few core technologies. In the first instance, the extract of proteins has to be separated effectively. In many laboratories the method of choice is still 2-D gel electrophoresis (2-DE), despite severe limitations to cope with the complexity of eukaryotic proteomes. This method combines separation according to the iso-electric point in the first dimension with SDS-PAGE resolving the set of proteins then by molecular weight in the second dimension. With the standard gel formats commercially available, visualization of 1000–2000 protein spots can be regularly achieved, depending on sample composition and preparation. Working with crude extracts, this set of proteins normally comprises the more abundant proteins within the cell. To overcome limitations of the separation capacity, narrow pH-gradients can be applied for iso-electric focusing (IEF). Also, complex protein extracts might be pre-fractionated by preparative IEF or by using chromatographic techniques such as ion exchange or affinity chromatography. Another way of reducing the initial complexity of a proteome is the isolation of organelles or defined sub-cellular structures. In combination, these approaches will increase the fraction of proteins out of the initial proteome covered by the analysis, but require more starting material and multiply labour efforts.

Subsequent to 2-DE, staining and image analysis are performed to select the proteins of interest in a given study. Common stains include Coomassie, silver nitrate

and a range of fluorescent dyes. As with protein separation itself, limitations of staining are the dynamic range, covering to three or four orders of magnitude in protein abundance. Sophisticated programs are offered from a number of companies, to support the researcher in the evaluation and comparative analysis of 2-DE images. These programs must take into account technical variations of spot positions. Guided by the image analysis, spots are then selected for identification by mass spectrometry (MS). Peptides and proteins have been made accessible to MS analysis by the introduction of appropriate ionisation techniques such as MALDI (matrix assisted laser desorption ionisation) and ESI (electro-spray ionisation). Identification of candidate proteins is achieved either by peptide mass fingerprinting (PMF) or by acquisition of additional peptide sequence information. With the current instrumentation, spots excised from 2-D gels normally will lead to spectra of sufficient quality for database searches. However, unequivocal identification of proteins is not only dependent on the quality of MS analysis, but also on the availability of sequence information for the particular organisms. For organisms with fully sequenced genome, identification can rely on PMF for many proteins, but when working with organisms for which only limited nucleotide database entries are currently available, additional sequence information will be necessary. The availability of EST (extended sequence tags) is helpful for protein identification in non-model organisms when of sufficient length and quality for matching with MS data.

Within recent years, a sophisticated technique named DIGE (differential in gel electrophoresis) has been introduced to overcome technical limitations of 2-DE, such as by distortions of protein patterns. For comparison of two proteomes, extracts are labelled with two different fluorescent dyes, and samples then loaded on one gel; by using a third fluorescent dye, an internal standard can be added, rendering quantitative evaluation more reliable.

Due to the limitations of 2-DE, separation techniques based on liquid chromatography were developed and are constantly improved, either as an alternative to 2-DE or as a complementary approach. One of these approaches has been termed MudPIT (for multi-dimensional protein identification technology), and is based on the use of two independent chromatographic phases in capillary columns for the separation of complex peptide mixtures by 2-D-LC prior to MS/MS analysis (Link et al., 1999). Proteome analysis of *S. cerevisiae* by MudPIT technology identified nearly 1500 proteins (Washburn et al., 2001).

Quantitative proteomics based on 2-D-LC has been gained momentum by the development and improvement of isotope-coded tags for protein labelling such as ICPL (see Schmidt et al., 2005, and references therein). In principle, the techniques are based on the differential isotopic labelling of samples to be compared allowing relative quantification by peptide signal ratios in the MS analysis.

Recently, label-free quantitative comparison of complex peptide mixtures has been introduced (Silva et al., 2006). With the availability of these and other techniques, liquid chromatography based separation methods are becoming more and more popular in proteome studies.

For a deeper insight into technical and conceptual aspects of proteome techniques, the reader is referred to recent monographs (e.g. Simpson, 2003; Walker, 2005; Humphery-Smith and Hecker, 2006).

21.3 Critical Review of Proteomic Studies Performed in Yeast

21.3.1 Proteome Maps of Yeasts

2-DE is still the most generally applicable technique for a global screening of protein composition as well as for the analysis of the expression level of a proteome affected during different physiological processes and divergent genotypes, and thus routinely used in yeast proteomic studies. Proteome maps of *S. cerevisiae* and other yeasts based on 2-DE are available on several web pages; see e.g. http://www.ibgc.u-bordeaux2.fr/YPM/ and links therein.

The 2-DE proteome map of *S. cerevisiae* is currently the most comprehensive and has been refined over years by several laboratories. A French group has identified 401 proteins corresponding to 279 different genes (Perrot et al., 1999). Proteins in the alkaline range were made accessible for the 2-DE map by applying extracts to IEF gradients up to pH 12 and subsequent identification by MALDI-TOF MS (Wildgruber et al., 2002). 2-DE based proteome maps have also been established for industrial yeast strains. Lager brewing yeast strains which are now common for beer production combine different genomes. The proteome of lager brewing yeasts can be explained by superimposing the proteome of *S. cerevisiae* with the gene products corresponding to a divergent *Saccharomyces* species most likely with similarity to *S. pastorianus* (Joubert et al., 2000; Joubert et al., 2001).

A further model yeast is *Schizosaccharomyces pombe* as the genome is sequenced, and due to easy access for genetic manipulation. A recent study of *Sch. pombe* revealed more than 1500 spots by 2-DE with silver staining by using a pH-gradient of 3–10 for IEF and 1000 spots when applying extracts to strips from 4–7 in the first dimension. Analysis of spots by MALDI-TOF MS combined with nanoLC-MS/MS led to the identification of 364 proteins, among them 123 proteins identified by both approaches. Classification of identified proteins showed that two-fifth were associated with primary metabolism. As *Sch. pombe* also contains many genes and regulatory aspects with similarity to mammals, further proteome analysis of this organism might contribute to the functional characterization of basic cellular mechanisms of eukaryotes (Hwang et al., 2006).

For *Candida albicans* hyphal forms a reference 2-DE proteome map was also established. Of 106 spots excised from the 2-D gels, 43 were identified. Further refinement of this map will provide a tool for the study of differential protein expression in this human pathogen (Hernandez et al., 2004).

Additional coverage of the proteome of *S. cerevisiae* can be achieved by separate analysis of sub-proteomes. The organelle proteome of mitochondria was investigated by a combination of several independent methods for protein separation, namely 2-DE, 1-DE, nanoLC-MS/MS and multi-dimensional liquid chromatography (Sickmann et al., 2003; Reinders et al., 2006). In the initial study, the acquisition of more than 20 million MS spectra enabled the identification of 750 different proteins, indicative of various biochemical pathways and cellular processes, such as oxidative phosphorylation and the TCA cycle. It was calculated

that the set of identified proteins represents about 90% of the mitochondrial proteome. The functions of almost one quarter of the proteins remained undefined at that stage of analysis. Refinement of this workflow led to the identification of 851 mitochondrial proteins (Reinders et al., 2006). Also a 2-DE based reference map was established for mitochondria with about 800 spots from a group in Finland, (Ohlmeier et al., 2004, www.biochem.oulu.fi/proteomics/). Out of these spots, 459 proteins were identified, representing 253 individual proteins and including low-abundant proteins as well as proteins with previously tentative annotations.

Global analysis of protein localization via a green fluorescent protein (GFP) tagging approach identified 527 proteins for yeast mitochondria (Huh et al., 2003). An integrative approach identifying proteins from purified mitochondria by LC-MS combined to a variety of genomic tools such as systematic deletion phenotype screening, expression profiling, sub-cellular localization studies, protein interaction analysis and computational predictions provided evidence for about 700 proteins of mitochondrial localization (Prokisch et al., 2004).

A general problem in 2-DE based proteomics is the poor analytical access to membrane proteins. To compensate this bias, targeted analysis of membrane proteins can be performed. Critical to the analysis is the purity of the membrane fractions. A protocol was adopted for the enrichment of plasma membrane vesicles from *S. cerevisiae* to get rid of cytosolic contaminations and to reduce other membranes (Navarre et al., 2002). A specific cationic detergent was applied for the first dimension of 2-DE, and led to the identification of 50 proteins. Among these identified spots, two ABC transporters, a P-type ATPase, a glucose transporter, a low-affinity iron transporter and a kinase were noticed, demonstrating the validity of the approach (Navarre et al., 2002). In an independent study by Delom et al. (2006), proteins from detergent-treated plasma membrane vesicles were subjected to ion exchange chromatography, and fractions analysed by 1-DE. In the study, 90 plasma membrane proteins were identified.

As an alternative approach to 2-DE based overall proteome analysis of yeast, LC-based techniques without or in combination with gel-based techniques have been introduced (Washburn et al., 2001; Wei et al., 2005; Breci et al., 2005). Multidimensional liquid chromatography enabled the identification of nearly 1500 yeast proteins (Washburn et al., 2001). Further development of the methodology by applying on-line three-dimensional liquid chromatography coupled with MS/MS analysis led to the identification of 3019 unique yeast proteins, using soluble, urea-solubilised and SDS-solubilised protein extracts for separation (Wei et al., 2005). A combination of MudPIT technique with the analysis of SDS-PAGE gel slices resulted in the greatest amount of yeast proteins identified when testing different separation approaches (Breci et al., 2005).

First mapping of some yeast phosphoproteins was described as part of a general sampling of the yeast proteome by a 2-DE based approach (Futcher et al., 1999). In a global analysis of the *S. cerevisiae* phosphoproteome, enriched by immobilized metal-affinity chromatography (IMAC) and analysed by nanoHPLC-MS/MS, more than 1,000 phosphopeptides were detected from whole cell lysate (Ficarro et al.,

2002). A total of 216 peptide sequences defining 383 phosphorylation sites were determined, 18 of which had been previously identified.

21.3.2 Functional Proteomics

The proteome of cells or organisms is a dynamic entity, reflecting the influence of developmental programs, environmental impact such as responses to stress factors and nutrient conditions, as well as tissues specificity. Therefore, proteome analysis will provide valuable insights into cellular processes and the final adaptations occurring in response to environmental or developmental stimuli. Many of the proteome studies can be considered as perturbation analysis, where a specific parameter is changed, and the responses of the system are observed on the protein level, e.g. after the application of a stress factor, or as a consequence of modified expression of genes. The proteome analysis provides information on the overall regulatory circuits of a biological system, and also helps to define the functional context of individual genes. Functional proteomic analysis most often focuses on the differences observed between two or more samples to be compared, rather than analysing the whole inventory of cells. Techniques to better visualize the differences between the sets of proteins to be compared are of specific interest, such as the DIGE technology already mentioned. Post-translational modifications (PTMs) of proteins are important features of cellular control mechanism and are detectably by general proteome analysis or by targeted proteomics. PTMs include events such as phosphorylation, glycosylation and ubiquitination to name just a few among many others. PTMs might modify the separation properties of the protein or related peptides, so that the modified fraction of the protein appears e.g. as a novel spot on 2-DE gels. A number of methods have been developed to monitor phosphorylation events, and the reader is referred to reviews (e.g. Reinders and Sickmann, 2005; Jensen, 2006).

Culturing conditions of valuable yeast strains influence the metabolic state of the cells and hence might limit the production of desirable compounds. A comparative proteome analysis of the methylotrophic *Hansenula polymorpha* strains DL1 and A16 was performed to monitor the changes in metabolism when shifting from glycerol to methanol as a carbon source (Kim et al., 2004). The facultative methylotroph *H. polymorpha* has been shown to be a good host system for the expression of heterologous proteins, and is in use for industrial production of proteins. Image analysis was used to categorize the patterns of proteins, including spots which were strain-specific and constitutively expressed irrespective of carbon source, strain specific proteins which were methanol inducible, strain-specific proteins only abundant in one strain, and proteins commonly expressed. Proteins specific for the DL1 strain, but constantly expressed under both carbon sources included glucose-6-phosphate dehydrogenase, isocitrate lyase, scuccinyl-CoA synthetase and glycerol-3-phsophae dehydrogenase. These findings correlated with the higher rate of glycerol and methanol consumption in DL1 strain (Kim et al., 2004).

Analysis of the limiting supply of glucose or ethanol in chemostat cultures of *S. cerevisiae* revealed only changes in the central metabolism (Kolkman et al., 2005). In the glucose-limited culture, enzymes of glycolysis were significantly more abundant, whereas components of the TCA cycle were more prominent in the ethanol-limited system. Key enzymes in the glyoxylate cycle, isocitrate lyase and malate synthase, and the gluconeogenic enzyme phosphoenolpyruvate carboxykinase were only detected with ethanol as a carbon source, consistent with the metabolic routes predominant under these nutrient conditions. Comparison of parallel datasets obtained for transcripts indicated that glycolytic enzymes are preferentially regulated on the proteome level with the exception of hexokinase I, for which regulation on the transcript level was evident. Collectively, the study demonstrates the capability of the approach to gain an overview on major metabolic routes in response to culturing conditions as valuable information to select appropriate conditions for cultivation of production strains.

Cellular responses to amino acid limitation were studied in *C. albicans* and *S. cerevisiae* (Yin et al., 2004). *S. cerevisiae* activates general amino acid control (GCN) in response to amino acid limitation, and other fungi such as *C. albicans* share elements of this response. Comparative proteomic analysis identified 55 spots from *C. albicans* and 65 spots in extracts from *S. cerevisiae* responding to the histidine analogue 3-aminotriazole treatment in a Gcn4p-dependent manner. Gcnp4 is a bZIP transcription factor, which stimulates amino acid biosynthesis via GCRE-lie promoter elements. Identification of spots demonstrated that significant aspects of the overall GCN response were conserved between both yeasts, mainly the effects on amino acid biosynthesis and carbon metabolism. However, enzymes of purine biosynthesis were induced in *S. cerivisiae*, but not in *C. albicans* (Yin et al., 2004).

Sulfur metabolism was investigated in *S. cerevisiae* by combining metabolic profiling with a proteome approach (Lafaye et al., 2005). The increased synthesis of glutathione, indispensable for the detoxification of cadmium, was provided on the expense of sulphur-containing enzyme synthesis. Kinetic analysis demonstrated considerable changes in the pools of intermediates consistent with the proteome analysis. In contrast to cadmium responses, proteome and metabolite analysis showed negative correlation when analysing the effects of methionine supplementation or sulphate starvation. These differences were related to alternative mechanisms in the regulation of the activator of sulphur pathway, Met4. Activity of Met4 is controlled by cysteine content when responding to sulphur sources and availability, but in a cysteine-independent way after exposure to cadmium stress. This study exemplifies the necessity to supplement proteome or transcriptome data sets with metabolic analysis to obtain a full insight into the cellular responses.

For analysis of *S. cerevisiae* grown in the presence of sorbic acid an approach combining phosphoprotein affinity enrichment with 2-DE and MS analysis was used (Makrantoni et al., 2005). Altered phosphorylation patterns of 17 proteins have been demonstrated, 13 of which had previous evidence of phosphorylation or ATP binding activity.

A combined proteomics and metabolomics approach aimed at the elucidation of the hydrogen peroxide- and Sty1p-dependent stress response in *Sch. pombe* (Weeks et al., 2006). The stress-activation pathway of this organism also shares homology with higher organisms, and possesses Sty1p as a central element in the protein kinase cascade activated by stress. Sty1p shares homology with mammalian p38 kinase. Processes controlled by Sty1p include phosphorylation of the bZIP transcription factor Atf1p, but other proteins and pathways yet have to be identified. As an approach, the authors have further studied the functional context of Sty1p by analyzing its contribution to the response towards peroxide stress. Proteome patterns of control and stress-treated cells were compared in wild-type and *sty1Δ* mutant cells using DIGE technology. In total 260 differentially expressed proteins were found in all comparisons, with the largest number of differences between wild-type and mutant in response to stress treatment. 47 protein spots were up regulated in wild-type versus 14 in the mutant in response to hydrogen peroxide, whereas the number of down regulated proteins was similar. Examples include the redox enzymes catalase, thioredoxin reductase, and thioredoxin peroxidase, which were up regulated in wild-type cells, but not in the *sty1Δ* mutant cells. Nearly half of the differentially regulated proteins in response to stress were regulated similarly in the mutant cells compared with wild-type, indicating the restricted impact of the Sty1p mutation on the overall cellular responses. Another cluster included 20 proteins not affected by stress, but de-repressed in the *sty1Δ* mutant. Comparison of proteomic data with earlier transcript data provided poor overall correlation.

Response of the proteome of *S. cerevisiae* to hydrogen peroxide was recently analysed by making use of isotopically labelled leucine to quantify relative changes in protein abundance based on peptide signal intensities. In the kinetics of cellular responses, one of the first events was the repression of glycolysis, but restored after longer exposure after the added hydrogen peroxide was detoxified (Jiang and English, 2006).

Changes in the proteome of the halophilic black yeast *Hortea werneckii* in response to steroids were assessed by applying the DIGE approach. Twenty proteins were at least two-fold up regulated, among them protein kinase C-like 2 (Pck2), cAMP-independent regulatory protein (Pac2), Hsp70, aspartyl protease, quinone oxidoreductase and PCNA, a proliferating cell nuclear antigen. Further analysis demonstrated that the phosphorylation status of Pac2 and Pck2 was altered due to progesterone application, indicating interaction of the steroid hormone with cell growth and reproduction signalling (Matis et al., 2005).

The osmotolerant *Candida magnoliae* has been isolated from honey comb and has the potential for production of erythritol, a noncarcinogenic, low calorie sweetener (Lee et al., 2003). Chemical mutagenesis has been performed to obtain strains with improved erythritol yield. A proteomics approach was now applied to monitor the physiological basis for the improved capacity of the mutant strain relative to the parental isolate. Six proteins out of nine differentially expressed proteins could be identified, among them citrate synthase, succinyl-CoA ligase, fumarase, pyruvate decarboxylase and enolase as an initial result to establish a metabolic network database (Lee et al., 2003).

The involvement of the Sfn1 kinase pathway in the diauxic shift in *S. cerevisiae* was assayed by generating a mutant strain deleted for *SNF4* gene encoding an activator domain for this kinase (Haurie et al., 2004). In the mutant strain, 82 spots were affected at the diauxic shift, an half of the proteins with reduced abundance are encoded by genes controlled by the transcriptional activator Cat8p, a target of Snf1p. Among the proteins increased in the mutant strain were enzymes of glycolysis, whose synthesis is down regulated when wild-type cells enter the phase of diauxic shift, suggesting that Snf1 exerts a negative control on their expression (Haurie et al., 2004).

An organelle proteome analysis of the diauxic shift from fermentation to respiration was performed for mitochondria (Ohlmeier et al., 2004). After change of the conditions, 18 mitochondrial proteins were found to be altered in abundance, 17 increased and one decreased. Spots increased included proteins of the tricarboxylic cycle, proteins contributing to the respiratory chain and two proteins of unknown function. The constant abundance of the majority of the mitochondrial proteins during the diauxic shift is in contrast to previous transcriptional analysis, which suggested a heterogeneous up- and down regulation of individual transcripts. This discrepancy might however be related to altered turnover rates of proteins (Ohlmeier et al., 2004).

Functional characterization of dihydroxyacetone kinases (DAKs) was performed by proteome analysis of deletion and over-expressing strains (Molin et al., 2003). The two DAKs homologs of *S. cerevisiae* are presumably involved in the detoxification of dihydroxyacetone, and *DAK1* is induced by a wide range of stresses. DAK activity is fully repressed in the double deletion mutant, but is stimulated 250fold in a strain over-expressing *DAK1*. Results of the proteome analysis were indicative for a two-domain structure of DAK1 protein (Molin et al., 2003).

For the pathogenicity of *C. albicans* in causing systemic infections in humans, the morphological transition from the yeast into the hyphal stage is of outmost importance. During this process, cell walls are relevant for the contact of the pathogen with the host. A comparative study of a cell wall enriched fraction of the yeast and the hyphal stage was initiated and revealed 14 proteins which where up regulated and 10 which were down regulated in hyphae (Ebanks et al., 2006).

Drug-induced changes in the *C. albicans* proteome were followed after application of antifungal agents, namely a β-(1,3)-glucan synthase inhibitor, and of two triazoles. Similar changes in the proteome were monitored for the triazoles, demonstrating that this approach can differentiate between antifungal drugs with contrasting mode of action (Bruneau et al., 2003).

The effect of Calcofluor white, an antifungal component disturbing the synthesis of chitin microfibrils, on the plasma membrane proteome was studied in *S. cerevisiae* (Delom et al., 2006). Although the patterns of plasma membrane proteins were quite similar between controls and treated cells, subtle changes were monitored in response to Calcofluor white treatment. These changes included two sphingolipid long-chain base-responsive inhibitors of protein kinases involved in signalling for integrity of the cell wall, and Rho1p, a small GTPase. The former proteins were discussed to be involved in regulation of β-(1,3)-glucan synthase,

an enzyme related to the biosynthesis of another macromolecule of the cell wall (Delom et al., 2006).

In the analysis of the eukaryotic transcription machinery, multidimensional mass spectrometry revealed proteins associated with components of the yeast general transcription factor TFIID consisting of TATA-binding protein TPB and TPB associated factors (Sanders et al., 2002). By systematic immunopurification with antisera against known subunits of TFIID, a number of novel protein-protein associations were observed, such as association between TPB and the RSC chromatin remodelling complex, the TAF17p-dependent association of the Swi6p transactivator protein with TFIID, but also the identification of three novel subunits of the SAGA acetyltransferase complex. These findings show the suitability of the proteome approach to further define the eukaryotic transcription apparatus (Sanders et al., 2002).

For functional characterisation of multiprotein complexes that assemble on centromeric DNA and attach chromosomes to spindle microtubules, named kinetochores, a comprehensive study combining biophysical techniques, affinity purification, mass spectrometry and in vivo assays was performed (De Wulf et al., 2006). With this approach the state of association of 31 centromere-binding proteins, including six proteins newly recognized as kinetochore subunits of *S. cerevisiae* were examined. Evidence for a hierarchical assembly of the yeast kinetochore from at least 17 discrete sub-complexes was described. In addition two kinetochore complexes have been newly identified, and different functions regarding force generation and microtubule attachment were proposed.

A proteome-wide approach has been taken to study the localization of proteins (Kumar et al., 2002). By using genome-wide transposon mutagenesis, 60% of the proteome were epitope-tagged and allowed the sub-cellular localization of 2744 yeast proteins by high-throughput immunolocalization (Kumar et al., 2002). Based on these results, it was concluded that the yeast possesses roughly 5.000 soluble and more than 1.000 membrane-bound proteins. With 47% the majority of the proteins were localized in the cytosol, whereas 13% were allocated to mitochondria, and another 13% appeared to be exocytic, and 27% were nuclear/nucleolar (Kumar et al., 2002).

21.4 Future Perspectives

Biological processes are not only controlled by abundance of proteins, but also by their spatial distribution, sub-cellular localization, and by their interaction with other proteins in complexes. A first genome-wide screen for complexes using affinity purification and mass spectrometry was provided for budding yeast (Gavin et al., 2006). By a systematic tagging approach, most of the complexes were isolated several times indicative for the comprehensiveness of the experimental set-up. Analysis of the data suggests a high modularity of the yeast protein complexes, with an estimated number of 800 cores being present in most isoforms, and attachments being present only in some of them. The cores ranged from 1 to 23

proteins in size, with an average of about three proteins (Gavin et al., 2006). Such an experimental platform paves the way to establish comprehensive models for entire biological systems, along with other techniques for systems biology approaches including proteomics and transcriptomics. Integration of large and diverse datasets, visualization of data sets and data mining by bioinformatical approaches will be fundamental to evaluate the full information from such studies. The specific contribution of proteomics will remain to reveal regulatory aspects on the protein level including complex formation, and in particular, to monitor post-translational modifications, such as phosphorylation or glycosylation among many others. Apart from such comprehensive approaches for systems biology, proteomics will remain a valuable approach to study specific issues in fundamental and applied research. In all fields, proteomics will benefit from current developments in instrumentation, including protein or peptide separation and MS analysis. The implementation of improved application techniques, e.g. novel tools for sample preparation, novel chemical tags etc., will similarly improve proteome technology.

21.5 Conclusions

Proteomics has experienced a vast increase in interest in recent years, based on the developments in mass spectrometry, such as MALDI-TOF and nanoHPLC in combination with ESI-MS/MS, and since the rapid increase in nucleotide sequence information for many organisms. In the front of this progress in general, analysis of *S. cerevisiae* has served as a model for eukaryotic organisms, providing a large set of tools and knowledge for fundamental and applied research of other yeasts. Within a few years, a vast number of publications have appeared, demonstrating the capacity of the proteomics approach to gain novel insights into various biological processes. Therefore, broader use of this technique can be anticipated in the field of yeast research, alone or in combination with other methods of functional genomics.

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Chapter 22
Yeast Genomics for Bread, Beer, Biology, Bucks and Breath

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Contents

Abstract The rapid advances and scale up of projects in DNA sequencing during the past two decades have produced complete genome sequences of several eukaryotic species. The versatile genetic malleability of the yeast, and the high degree of conservation between its cellular processes and those of human cells have made it a model of choice for pioneering research in molecular and cell biology. The complete sequence of yeast genome has proven to be extremely useful as a reference towards the sequences of human and for providing systems to explore key gene functions. Yeast has been a 'legendary model' for new technologies and gaining new biological insights into basic biological sciences and biotechnology. This chapter describes the awesome power of yeast genetics, genomics and proteomics in understanding of biological function. The applications of yeast as a screening tool to the field of drug discovery and development are highlighted and the traditional importance of yeast for bakers and brewers is discussed.

Keywords Yeast, fermentation, genome, drug discovery, protein–protein interaction

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22.1 Introduction

Complete genome sequences are providing a framework to allow the investigation of biological processes by the use of comprehensive approaches. Genome analysis is also having a dramatic impact on medicine through its identification of genes and mutations involved in disease and the elucidation of entire gene sets. Yeast has long been in the vanguard of genomic research. It was the first eukaryotic organism (*Saccharomyces cerevisiae*) from which a complete chromosome was sequenced Oliver et al. (1992). The genome of budding yeast (*Saccharomyces cerevisiae*), was also the first complete eukaryotic genome, to be completely sequenced Goffeau et al. (1996).

The *S. cerevisiae* genome is 12.8 Mb in size and is ~200 times smaller than the human genome. It is less than 4 times bigger than *E. coli* and is packaged into 16 well characterized chromosomes ranging in size from 250 Kb to >2500 Kb. The number of recognized genes in yeast hovers around 6000, remaining in flux due to continued research on which of these are spurious and what additions should be made Kumar et al. (2002). Nevertheless, by comparison to most eukaryotes, coding regions are simple to identify in yeast: about 70% of the genome encodes proteins and only about 4% of yeast genes contain introns usually as a small insertion near the 5′ end of the coding region Sakharkar and Kangueane (2004), Sakurai et al. (2002).

Yeast has great utility as a surrogate system to study aspects of mammalian biology. Fundamental studies in yeast have made a considerable contribution to our present understanding of conserved biological processes Snyder and Kumar (2002). In addition, yeast has proven to be a valuable experimental tool, in particular in the development of genomic and proteomic technologies. This chapter describes on the utility of these approaches towards the understanding of biological function. The applications of yeast as a screening tool to the field of drug discovery and development are highlighted and the traditional importance of yeast for bakers and brewers is discussed.

22.2 Yeast in Food

Saccharomyces is the principal yeast used in modern fermentation processes including winemaking, bread making and brewing Mortimer (2000). Recently, Cavalieri et al. reported on presence of ribosomal DNA from *S. cerevisiae* from inside of the earliest known wine jars in Egypt. This confirmed that this organism was probably responsible for wine making by at least 3150 B.C. Cavalieri et al. (2003). This is an important evidence for the use *S. cerevisiae* in ancient wine. Besides the traditional industrial uses of yeast in the making bread, wine and beer, yeasts are also a rich source of a range of industrially important enzymes such as invertase Tucker (1996). Another enzyme from yeast converts fatty acids to lactones for use in flavorings for margarines and in fruit flavors. In addition, yeast extract has important uses as a source of B and D vitamins, in flavor enhancement and is a common ingredient of routine microbiological culture media Luce and Maclean (1925), Martin et al. (2002). The sequential expression and regulation of several genes associated with carbon, nitrogen and sulfur metabolism and genes required for tolerance of high sugar concentration, low pH, ethanol and nutrient

deficiency during wine and beer fermentations has been elaborated for *S. cerevisiae* Varela et al. (2005), Brejning et al. (2005). Also, as yeast grows in food and beverages, they utilize carbon and nitrogen substrates and generate a vast array of volatile and non-volatile metabolites that determine the chemosensory properties of the product and its appeal to the consumer Nathalie (2002), Zoecklein et al. (1998). Some yeasts produce extracellular proteases, lipases, pectinases and amylases that influence product texture and flavor. The biochemistry of these reactions is generally known Swiegers et al. (2005). The genome data of *S. cerevisiae* has added a new dimension and has provided a unique opportunity to yeast researchers to seize upon the information and direct their research in an ever-more '*omic*' fashion towards commercially applicable directions. Brewing, baking and wine making involve the exposure of yeast to environmental stresses (high osmotic and hydrostatic pressure, high alcohol concentration, anaerobiosis and temperature fluctuations). These can affect the vitality and viability of the culture. Yeast also exhibits a complex array of stress responses when subjected to conditions that are less than physiologically ideal. Genomic studies have helped couple these responses to expression and regulation of individual genes Bond and Blomerg (2006). The brewing industry provides an interesting example of how strain improvement is approached. As the taste and look of the beer (once served in a glass) are paramount for the brands commercialization, development of yeast strains which produce less depth of froth on fermentation vessels and have increased osmotic tolerance and produce high strength beer, are hot topics in the world of brewing beer and just some of the advancements in biotechnology of brewing. When yeast cells flocculate in beer, they are easier to remove and, thus, satisfy the preference of many consumers for clear over cloudy beer. Recently, Verstrepen et al. reported that genes that control flocculation—the tendency of yeast (or other) cells to adhere to one another, then clump to the bottom or float to the top—contain many tandem repeats Verstrepen et al. (2004), Verstrepen et al. (2005). They reported that many genes in the *S. cerevisiae* genome carry tandem repeats, and 75% of them are in genes encoding cell surface proteins. Concurrently, their group has also proposed the use of immobilized yeast cell systems for continuous fermentation applications that offer economical advantage in comparison with traditional systems Verbelen et al. (2006). Alongside, it was observed that fungal pathogens such as *Candida* depend on similar sets of genes containing tandem repeats to stick to and invade cells of susceptible hosts Verstrepen et al. (2005), Fink (2005). Through the selection of strains and development of propagation techniques, more specific applications of yeast are now being found in many different industries, including brewing, malting, farming (animal feeds), and more importantly pharmaceuticals which are elaborated below.

22.3 Yeast in Bucks 'N' Biology

The early stage of drug discovery process in the pre-clinical phase starts with the isolation of relevant target genes involved in a certain disease and the subsequent identification of lead compounds that affect the function or the product of such target genes in a desired manner. Cell based screening is used to identify chemicals that can suppress or enhance particular cellular phenotypes and valuable to study

complex cellular processes. They are often attractive candidates for drug development. Here, we list some of the several endearing attributes including the ability to fill the laboratory with a pleasant 'warm-bread' odor.

22.3.1 Yeast as a Model

The yeast, *S. cerevisiae* has been proposed as a tool for identifying human drug targets because >40%of the yeast proteins share some conserved sequence with at least one known or predicted human protein, including several hundred genes implicated in human disease. 31% of yeast genes have a mammalian homolog and additional 30% of yeast genes have domain similarity Rubin et al. (2000). Conversely, nearly 50% of human genes implicated in heritable diseases have a yeast homolog Hartwell (2004). A comparison between the predicted proteins of the *S. pombe* and *S. cerevisiae* and 289 human disease proteins, found 182 *S. cerevisiae* proteins with significant similarity to 50 probable orthologs Wood et al. (2002).

Caspases are important class of enzymes in apoptosis and cell death and have implications in cancer biology. The identification of caspase-type protein(which was earlier thought to be absent) in yeast and demonstration of its orthology to metazoan caspases Uren et al. (2000), Madeo et al. (2002) has provided a step forward in the direction of understanding cell death and apoptosis using yeast as a model. Recently, we identified that 267 of the highly curated disease genes have a homolog in *S. cerevisiae* genome Sakharkar et al. (2007) (Fig. 22.1). Thus, yeast and human cells are very similar at the molecular level and yeast is now being modeled for several human diseases.

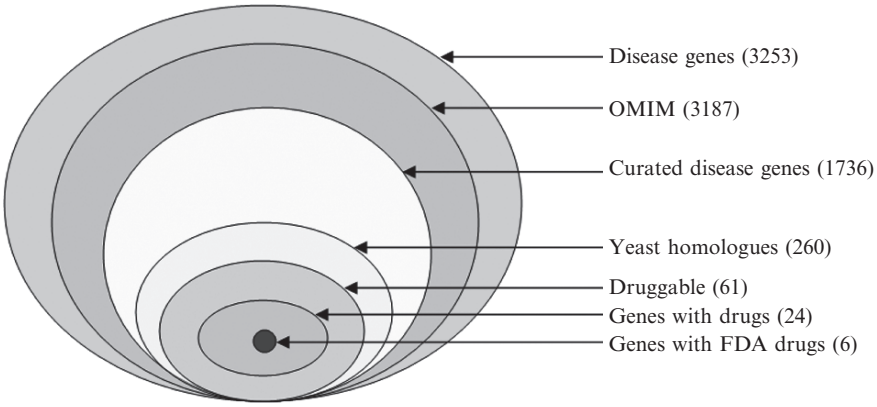

Fig. 22.1 The Druggable Human genome and yeast orthologs: The effective number of exploitable drug targets can be determined by the intersection of the number of genes linked to diseases and the druggable subset of the human genome. Though 260 of the genes involved in human diseases have a yeast ortholog, only 24 genes have drugs available for them and only about 6 of the genes have FDA approved drugs available for them. Hence, there is a need to explore the remaining protein-fold space of disease genes with reference to yeast

22.3.2 Yeast – The Model of Choice

Yeast has a compact genome. Unlike other eukaryotes, only 263 of yeast genes have introns, which simplify the process of computer based gene identification Goffeau et al. (1996). Yeast cells are advantageous for high throughput analysis because they are robust, fast growing and easy to manipulate. During its short life cycle of 90 min, yeast exists in a haploid or diploid state. This biological feature allows phenotype analysis of recessive mutations, which are normally masked in a diploid state by the wild-type allele. Assignment of distinct genes to different pathways can be obtained through epistasis analysis in which the phenotype of a double mutant strain is compared to the corresponding single mutant strain. Yeast, among other eukaryotes, has the unique advantage of permitting rapid genetic manipulation. Thus, it is a genetically tractable organism, amenable to modifications such as gene disruption, gene marking, mutation or gene-dosage effects. Yeast also has a highly versatile transformation system that allows for choice of linear, circular or integrating vector with high or low copy number, a positive or negative selection system and expression of gene of interest from several regulated promoters. Also, homologous recombination occurs with high efficiency that allows the integration of transformed DNA into precise locations, replacing or deleting host DNA as desired. Additionally, unlike mammalian cell culture, culturing yeast does not require elaborate sterile techniques or complex media and yeast can be stored temporarily (weeks to months) in readily usable refrigerators.

Yeast is classified as a GRAS (generally recognized as safe), and contains a multitude of selective markers that add to its experimental tractability including markers for nutritional selection (e.g. HIS3 and URA3), drug resistance (e.g. KanMX, patMX, natMX) and drug susceptibility (URA3, cyhR). Although single celled, it can occasionally display group characteristics such as pseudohyphal growth and inter-cellular signaling.

The above characteristics have made experimental strategies using yeast unravel disease-related molecular events and discover novel medicinal compounds and ‘crowned’ yeast as a model of choice for molecular genetics studies.

22.3.3 Yeast in Understanding of Human Diseases: Parkinson’s and Cancer

Yeast models are used in the study of genetic diseases because they offer researchers a simple system that allows them to elucidate how genes work e.g. to understand the molecular mechanisms behind neurodegenerative disorders such as Parkinson’s disease (PD) Outeiro and Lindquist (2003). Outeiro and Lindquist created a yeast model that expresses the alpha-synuclein gene, that has implications in PD. The study was conducted by creating a yeast that expresses wild type synuclein, using the normal gene, and another yeast that expresses two mutant forms, using a mutated version of the gene found in patients with PD. Outeiro and Lindquist,

studied the gene's actions under normal conditions and under abnormal conditions to learn how and when the gene's product, alpha-synuclein, becomes harmful to the surrounding cells. These studies gave insight into important changes that happen when alpha-synuclein is over-expressed in Parkinson's patients.

On the other hand fission yeast *S. pombe* and the budding yeast *S. cerevisiae* have become valuable tools for the study of basic cellular functions of eukaryotic cells, including DNA repair mechanisms and cell cycle control. As alterations of genes involved in the cell cycle control and regulation of the cell death process are common genetic changes in human tumor cells, studies with yeast have contributed greatly to our knowledge on the regulation of eukaryotic cell division, including the cancer-related disturbances thereof Smardova et al. (2005), Hartwell (2004).

22.3.4 Yeast in Drug Screening

The availability of genome sequence data has a great impact on the field of genetics, especially for diseases. Chemical genetics has received considerable attention as it can help identify drugs that affect the molecular mechanisms of different diseases. Using the set of non-essential yeast gene deletion (approximately 4500) strains, very recently it was demonstrated that chemical-genetic analysis provides a fast and efficient way to determine the targets of inhibitory compounds Parson et al. (2004). This was done by analyzing the growth of the yeast gene deletion strain colonies in the presence of the sub-lethal concentration of a bioactive compound. Analyses of the gene mutations that cause hypersensitivity to the compounds were then used to identify the cellular targets of the bioactive compounds.

In another extensive series of experiments haploid gene deletion mutants were screened for hypersensitivities towards 12 inhibitory compounds Parsons et al. (2003), Parson et al. (2004), Hartwell et al. (1997). The yeast system has provided a powerful cellular approach for assessing the effect of specific genetic alterations on the ability of the cell to respond to chemotherapeutic agents. Thus, although the yeast cannot completely replace human cells for pharmacological studies, it may be a valuable model system specifically for drug screening and in particular for identifying new drugs acting against a specific target or eukaryotic genes that control chemosensitivity.

22.4 Genomic and Proteomic Technologies in Yeast

22.4.1 Gene Expression Profiling Using DNA Microarray

In the mid to late 1990s, technology (DNA microarray) that allowed to assess gene expression for the entire genome was developed Shalon et al. (1996). DNA microarray technologies since then have had a profound impact on biological research, pharmacology, and medicine and have bridged the fields of biology and medicine.

The ability to obtain quantitative information about the complete transcription profile of cells has provided an exceptionally powerful means to explore basic biology, diagnose disease, facilitate drug development, tailor therapeutics to specific pathologies, and generate databases with information about living processes Young (2000).

Yeast was the first organism for which whole genome microarrays were available. Yeast DNA arrays are used to elucidate the mode of action of new drug candidates on whole cells and to identify effects of drugs that could not be seen by more conventional in vitro screening assays Gray et al. (1998). Marton et al. (1998) demonstrated and quantified the ability of a compound to inhibit pathways other than its intended target providing a means to group and rationally select desirable chemotypes Marton et al. (1998). After creating a large dataset of response profiles generated by mutations and compounds of known mechanism, Dimster-Denk and colleagues were able to use clustering algorithms to categorize new compounds or compounds presented 'blind' into functional groups. Complexities of blocking a pathway such as isoprene synthesis at different steps was revealed Dimster-Denk et al. (1999). Also, more than 400 RNAs whose levels are significantly modulated as the cell progresses through the cell cycle were identified by DNA chips Cho et al. (1998). In combination with the sequence of yeast, this information made possible the finding of potential regulatory elements important for the use of different genes at different stages and allowed new insight into the relationship between the local organization of genes in the genome and their temporal regulation. Thus, expression profiling holds great promise for high-throughput genome functional analysis and helps understand the genes and pathways involved in biological processes. It has transformed research, by allowing for gene expression analyses associated with relatively simple biological experiments in a fully comprehensive way. This comprehensiveness has stimulated both experimental and theoretical approaches to understanding regulatory networks and other features of yeast biology at the system level Hartwell et al. (1999), Ideker et al. (2001).

22.4.2 The Deletion Collection

The ease of gene disruption and single step gene replacement is unique in *S. cerevisiae* that offers an outstanding advantage for experimentation. A unique collection of mutant yeast strains, each bearing a defined deletion in one of yeast's 5998 potential protein coding genes has been constructed Winzeler et al. (1999). These genome-wide approaches have provided a wealth of information about the function of eukaryotic proteins. These deletion strains are extremely useful for characterizing disruption phenotypes. In addition several studies have used targeted gene deletions to characterize drug responses and drug targets on a genome-wide scale. Phenotypic screens for genes involved in rapamycin sensitivity Chan et al. (2000), proteasome inhibition by PS-341 Fleming et al. (2002), and DNA repair Ooi et al. (2001) are available. Deletion collection has also been used for 'haploinsufficiency' screening of drug targets Giaever et al. (2002). A cell is haploinsufficient when

lowering the dosage of a gene encoding a potential drug target from two copies to one copy confers hypersensitivity to the drug.

Two nonessential genes that cause lethality when mutated at the same time form a synthetic lethal interaction. Such genes are often functionally associated and their encoded proteins may also interact physically. This type of genetic interaction has also been studied in an all-versus-all approach in yeast Tong et al. (2001).

22.4.3 Protein Localization

One aspect that is critical for the understanding of protein function is protein localisation within distinct sub-cellular compartments and microenvironments. A first large scale localization study in *S. cerevisiae* determined the sub-cellular localisation of nearly half of the yeast proteins by genome wide epitope tagging and immuno-histochemistry Kumar et al. (2002). A more recent study investigated the localization pattern of proteins involved in lipid metabolism by GFP tagging and high resolution confocal laser scanning microscopy Natter et al. (2005).

22.4.4 Protein–Protein Interactions

Proteins interact with each other in a highly specific manner and protein interactions play a key role in many cellular processes; in particular, the distortion of protein interfaces may lead to the development of many diseases. Protein interactions determine the outcome of most cellular processes Legrain et al. (2001), Giot et al. (2003), Li et al. (2004), Krogan et al. (2006), Gavin et al. (2006). Protein–protein interactions also regulate a wide variety of important cellular pathways, and therefore represent a highly populated class of targets for drug discovery. The detected phenotypes in many diseases are caused from dysfunction in protein–protein, protein–DNA and receptor–ligand interactions. Hence, determination of these molecular interactions followed by designing or screening the compounds to target these interactions provides a significant challenge in drug development. Identifying and characterizing protein–protein interactions and their networks is essential for understanding the mechanisms of biological processes on a molecular level. Thus, comprehensive protein–protein interaction maps promise to reveal many aspects of the complex regulatory network underlying cellular function and may contribute towards the understanding of health and disease states. Also, knowledge accumulated on proteins and their interactions potentially reveals pathways and allows the selection of various potential drug targets.

The use of the yeast two-hybrid system in determination of protein–protein interactions and its possible outcomes in pharmaceutical research is well known. 50% of all interactions published in Medline are derived from yeast two-hybrid screens Xenarios et al. (2000). The yeast two-hybrid system has been utilized to identify

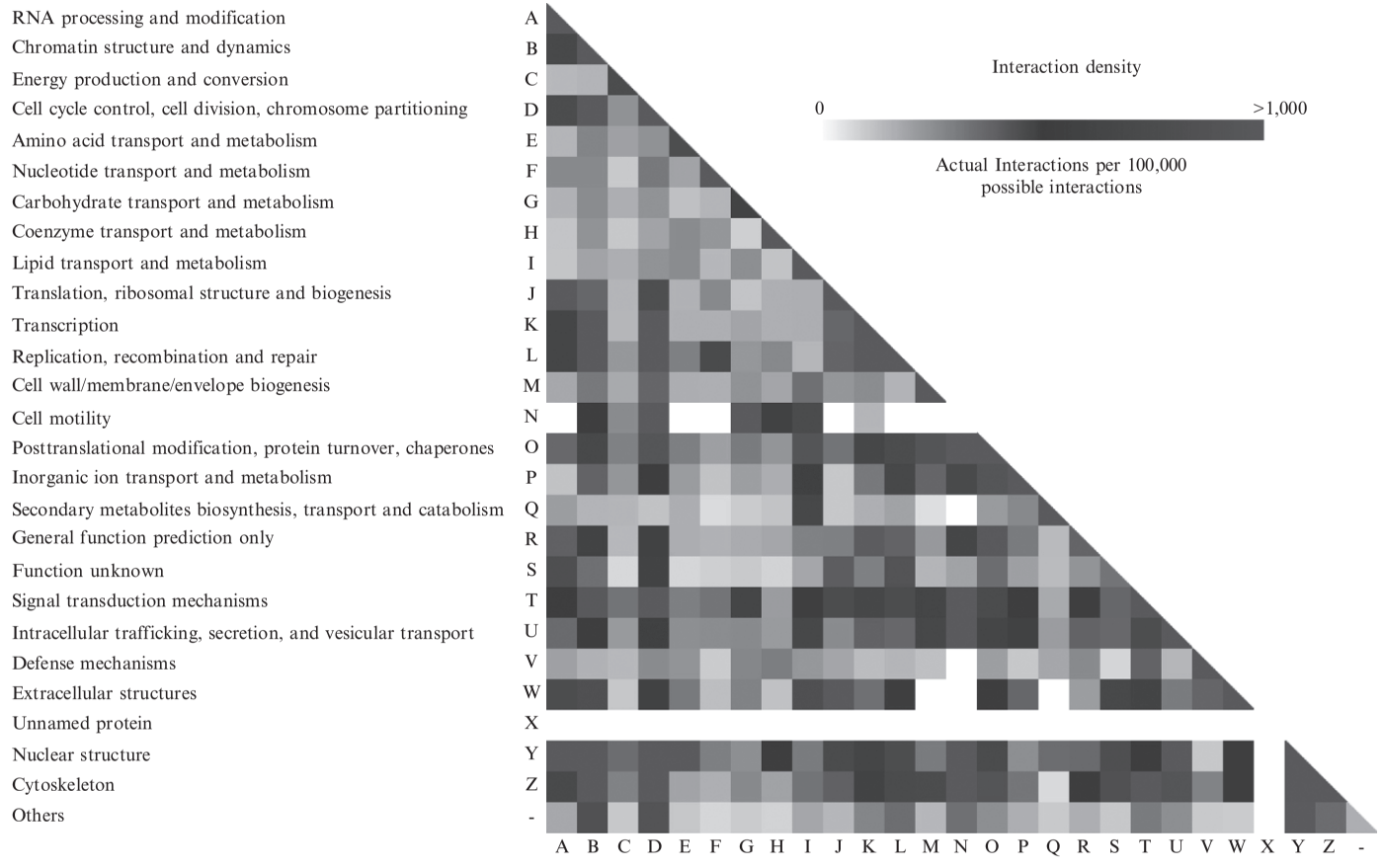

Fig. 22.2 Large scale interaction data and the distribution of interactions according to functional categories. The matrix shows the distribution of interactions (interaction density) by color Ge et al. (2001). Each axis represents the entire yeast genome interaction data (based on BioGRID) divided into functional categories

inhibitors of protein–protein interactions. Small molecule three-hybrid is used to detect small molecule protein interactions instead of protein–protein interactions Baker et al. (2003). Several different versions of this small molecule three hybrid have been used for screening binding partners of a given compound Henthorn et al. (2002). High-throughput screening technologies are aiding in the accumulation of protein–protein interaction data and their availability as a database resource – BIND Alfarano et al. (2005), DIP Salwinski et al. (2004), HPRD Peri et al. (2003), IntAct Hermjakob et al. (2004), MINT Zanzoni et al. (2002), MIPS Mewes et al. (2004), and BioGRID Stark et al. (2006). These data will pave the way for generation of protein interaction networks that is crucial to post-genomic systems biology.

A distribution of interactions with respect to functional categories of interacting proteins (data derived from KOG) for yeast data from BioGRID database is presented (Fig. 22.2).

The data reveal a strong enrichment for interaction within the majority of functional categories endorsing the earlier proposed hypotheses that interacting proteins are expected to have the same sub-cellular localization and indicating that interactions generally occur between proteins residing in the same sub-cellular compartment Gandhi et al. (2006). Thus, the identification of novel protein interaction modules contributes not only to the studies of the particular pathway but also to wider fields of biomedical research.

It should be noted that recent studies of protein–protein interactions, in particular, those involved in signal transduction, uncovered a number of protein-binding domains or motifs, which are evolutionarily conserved and used in various signaling pathways Pawson and Nash (2000). A large set of protein–protein interaction data will facilitate the search of such modules by both experimental and computational means. Thus, yeast-based functional genomics and proteomics technologies have revealed that genes and their products interact in complex biological networks. Perturbations of local and global properties of these networks contribute to the disease state. The ability to measure and modulate multiple parameters in yeast has made it a natural test bed for systems biology Ideker et al. (2001).

22.5 Future Perspectives and Conclusions

Genomics has provided the impetus to develop and implement new techniques to manage and exploit the sequence information leading to the creation of a new generation of ‘omics’ offsprings, which emphasize comparative and functional aspects of genomics, transcriptomics, proteomics, metabolomics, infectomics, pharmacogenomics, immunoproteomics, and importantly ‘econ-omics’. However, major challenges remain as these ‘omics’ often stand in isolation. There is a need to pull them together to gain an understanding of the system at a higher level, with its complex collection of networks and pathways. This will guide the path from genomics to functional genomics which is a major bottleneck in the path towards greater success for genomics approaches and their applications for human health and basic biology.

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Part III
Biotechnology Applications

Chapter 23
Ethanol Production from Traditional and Emerging Raw Materials

Andreas Rudolf, Kaisa Karhumaa, and Bärbel Hahn-Hägerdal

Contents

Abstract The ethanol industry of today utilizes raw materials rich in saccharides, such as sugar cane or sugar beets, and raw materials rich in starch, such as corn and wheat. The concern about supply of liquid transportation fuels, which has brought the crude oil price above 100$/barrel during 2006, together with the concern about global warming, have turned the interest towards large-scale ethanol production from lignocellulosic materials, such as agriculture and forestry residues. Baker's yeast *Saccharomyces cerevisiae* is the preferred fermenting microorganism for ethanol production because of its superior and well-documented industrial performance. Extensive work has been made to genetically improve *S. cerevisiae* to enable fermentation of lignocellulosic raw materials. Ethanolic fermentation processes are conducted in batch, fed-batch, or continuous mode, with or without cell recycling, the relative merit of which will be discussed.

Keywords Ethanol, lignocellulosics, baker's yeast, fermentation, saccharides

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23.1 Traditional Ethanol Production

Presently Brazil together with the United States is the world's largest ethanol producer (Table 23.1), producing more than 16 million L (4 billion gallons) of ethanol annually. In Brazil, the raw material for ethanol production is sugar cane, whereas in the United States, corn is the major raw material. Both sources contain large fractions of saccharides, the sucrose content of sugar cane being almost 20%. Sucrose from sugar beets is also used in a growing ethanol industry in Western Europe (www.suedzucker.de, www.nedalco.com).

Ethanol production is growing rapidly in many countries, promoted by tax incentives and favorable legislation. The US Energy Policy Act requires oil companies to blend 7.5 billion gallons of biofuels into gasoline by 2012, and the European Union strongly promotes increased use of bioethanol in the transportation sector (Gray et al., 2006; Hahn-Hägerdal et al., 2006).

23.1.1 Saccharides

The disaccharide sucrose is readily fermented to ethanol by *S. cerevisiae*, after hydrolysis into glucose and fructose by the enzyme invertase, which is naturally present in this yeast (Fig. 23.1). When ethanol is produced from sugar cane, either molasses, the viscous saccharide-rich residue left after sucrose crystallization, or cane juice, are used as the raw material (Kosaric and Vardar-Sukan, 2001). Due to its high osmolality, molasses has the advantage that it can be stored for extended periods of time without microbiological spoilage. It is diluted prior to fermentation to facilitate pumping and to avoid inhibitory ethanol concentrations in the fermentation step. In Brazil, fed-batch fermentation in combination with yeast-recycling is frequently used (Fig. 23.2; Table 23.2). Because the fermentation is carried out with high cell densities, fermentation time is relatively short, 6–11 h. Almost exclusively *S. cerevisiae* is used for fermentation (Amorim et al., 2004). Another disaccharide

Table 23.1 Annual world ethanol production by country

Country	2004 (billion L)	2005 (billion L)
Brazil	14.8	16.0
USA	13.4	16.2
China	3.7	3.8
India	1.8	1.7
France	0.83	0.91
Russia	0.75	0.75

(adapted from www.ethanolrfa/industry/statistics)

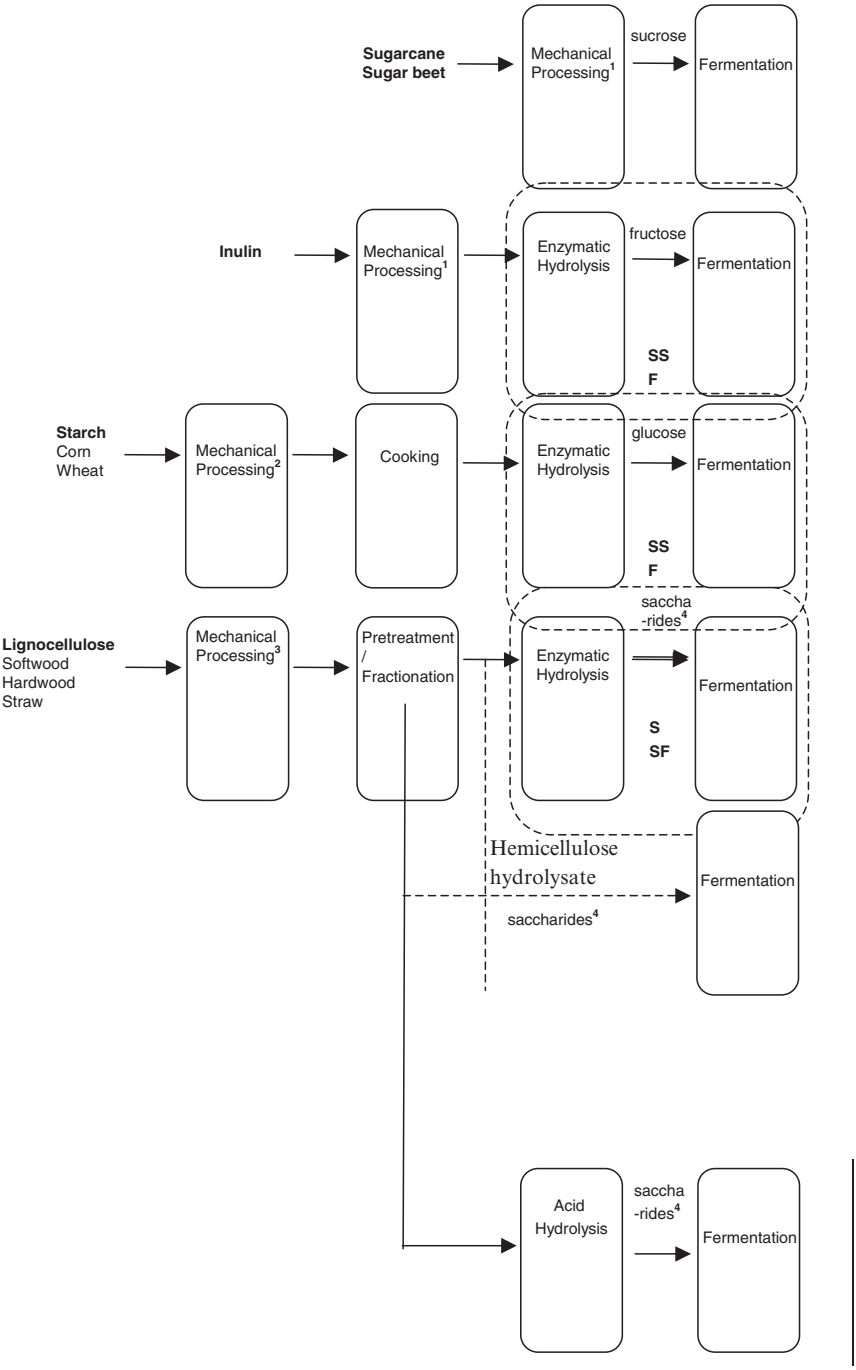

Fig. 23.1 Raw materials and their processing for ethanol production
[1]Slicing and extraction
[2]Milling (Dry-mill process), steeping (Wet-mill process)
[3]Chipping or milling
[4]Glucose, mannose, xylose, galactose, arabinose, cellobiose and oligosaccharides

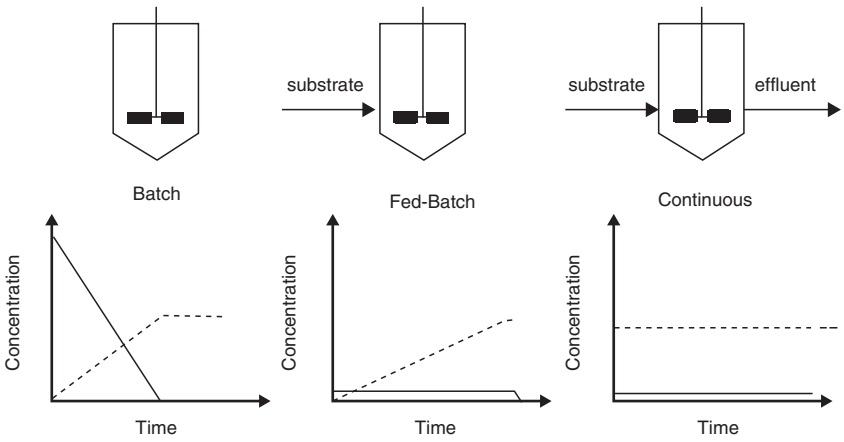

Fig. 23.2 The three main fermentation modes and typical substrate (—) and product (---) profiles

Table 23.2 Advantages and disadvantages for different modes of fermentation operation

	Advantages	Disadvantages
Batch	Robust: can be sterilized and cleaned frequently	Labour intensive
		Much idle time
	Complete conversion of the substrate is possible	Risk of contamination due to low initial yeast and ethanol concentration
Fed-batch	Robust: Can be sterilized and cleaned frequently	Labour intensive
	Possible to carefully control substrate addition	Much idle time
	Complete conversion of the substrate is possible	Some risk of contamination due to low initial yeast and ethanol concentration
Continuous	High productivity Low labour cost and good utilization of the reactor Constant high yeast and ethanol concentrations prevent contamination	Interruptions caused by contamination and mutation of the production strain are costly

that can be used for ethanol production is lactose, which is present in whey, a by-product from cheese production. Lactose cannot be fermented by *S. cerevisiae* since it lacks β-galactosidase activity. It can, however, ferment the hydrolysis products of lactose: galactose and glucose. Strains of *Kluyveromyces marxianus* are commonly used in commercial ethanol production from whey (Coté et al., 2004; Mawson, 1994). *S. cerevisiae* could be a better choice due to its superior ethanol tolerance and high ethanol yield. Two methods for hydrolyzing lactose to galactose

and glucose have been evaluated: enzymatic hydrolysis and acid hydrolysis. Enzymatic hydrolysis with β-galactosidase is hardly possible because of the high enzyme costs, whereas acid hydrolysis of lactose is a cheaper option. However, by-products formed during acid hydrolysis may inhibit fermentation (Coté et al., 2004).

23.1.2 Starch

Ethanol is produced from corn starch by wet mill or by dry mill processes (Fig. 23.1) (Bothast and Schlicher, 2005; Elander and Putsche, 1996). In a wet mill process corn is initially steeped with water at 49–53°C, which softens the hulls and causes the grains to swell. Subsequently, the wet grain is milled and germs, fibres and gluten are separated, after which the starch is dried. In a dry mill plant, the raw material is initially ground to increase the surface area without separating the different grain components. Starch itself is composed of amylose and amylopectin, the former a linear α-1,4 polymer of glucose and the latter α-1,4 polymer of glucose with α-1,6 branches. Its hydrolysis is rather similar in wet mill and dry grind plants.

During the liquefaction step, performed at elevated temperature, swollen starch is hydrolyzed by α-amylase to dextrins of varying chain-length. Subsequently, dextrins are hydrolyzed to glucose by glucoamylase. After an initial hydrolysis stage, the temperature is lowered to 30–35°C and yeast is added, the glucoamylase will continue to hydrolyse dextrins during the fermentation (Bothast and Schlicher, 2005; Elander and Putsche, 1996). Energy costs are significantly reduced if the initial hydrolysis is omitted and hydrolysis and fermentation are performed together in simultaneous saccharification and fermentation (SSF) (Fig. 23.1).

In dry mill plants fermentation is normally run batch-wise, whereas in wet mill plants a continuous configuration is more common (Fig. 23.2; Table 23.2). The fermenter size ranges from 100,000 to 6,000,000 l. The final ethanol concentrations are in the range of 8–18% (v/v) with productivities of 2–6 g/(l·h). Even though yeast recycling is an obvious method to increase productivity it is not universally employed.

Often, the yeast produced during fermentation is enough for sustaining operation over extended time periods. The fermenting organism in large scale corn-to-ethanol processes is almost exclusively *S. cerevisiae*, which has high tolerance to low pH and can tolerate ethanol concentrations above 10%, properties, which permit non-sterile process operation. The yeast is produced on site from a seed culture (Senn and Pieper, 2001). In plants with a continuous mode of operation, yeast is propagated in an aerated prefermenter and the yeast containing liquid is then cascaded to the fermentation vessels.

Whereas continuous fermentation is generally questioned in textbooks and publications, industry has chosen this operation mode for large-scale alcohol production (Table 23.2; Fig. 23.2). The better part of global alcohol production is

actually produced continuously. Provided that the fermentation process has been properly designed, plants can be sterilized and cleaned during operation, which prevents interruptions caused by contamination. Vogelbusch reports successful design and operation of MULTICONT plants in USA, Europe, Asia and Canada over the past 20 years including dry-milling plants.

23.2 Second-Generation Ethanol Production

To satisfy the ever increasing demand for fuel ethanol and to respond to the demand for reducing green house gas emission (Farrell et al., 2006), ethanol for transportation has to be produced from other raw materials than saccharides and starch. Lignocellulosic feed stocks constitute by large the most abundant alternative raw material (Claassen et al., 1999). However, in certain countries also inulin is considered as raw material for fuel ethanol production (Claassen et al., 1999), which will be briefly discussed.

Lignocellulose can be obtained from agricultural and forestry by-products. In addition, the indirect use of fossil fuels for ethanol production can be significantly reduced when lignocellulose is the raw material compared to current ethanol production based on saccharide and starch-rich raw materials (Farrell et al., 2006). There are, however, a number of challenges for the development of entirely new process technology (Hahn-Hägerdal et al., 2006), including the development of enzymes for hydrolysis of lignocellulosics, ethanologenic microorganisms with a wide substrate utilization range and innovative approaches to process integration. Ethanol production from lignocellulosic raw materials was already developed in the 1920s by Scholler (Fig. 23.1) (Faith, 1945; Keller, 1996). The process was commercially operated in the former Soviet Union until the late 1980s.

The low hydrolysis yield of the original dilute-acid hydrolysis process (50–60%) (Jones and Semrau, 1984) has been the incentive to develop enzymatic hydrolysis processes (Fig. 23.1), in which hydrolysis yields of 80–90% of theoretical have been achieved (Söderström et al., 2003; Bura et al., 2003). However, no full-scale ethanol production plants based on enzymatic hydrolysis of lignocellulosic raw materials are yet in operation. Several demonstration/pilot-plants exist: Iogen Corporation has a demonstration plant in Ottawa, Canada, which can produce 3 million L of ethanol from wheat-straw based on enzymatic hydrolysis (www.iogen.ca); Abengoa Bioenergy operates a pilot-plant in York, Nebraska, USA, in which residual cellulose and hemicellulose in corn fibers is enzymatically hydrolyzed and fermented to ethanol; and a demonstration plant is built in Salamanca, Spain, which can produce 5 million L of ethanol per year, mainly from wheat-straw (www.abengoabioenergy.com). Furthermore, a pilot-plant capable of producing 190,000 L of ethanol from softwood was built in Örnsköldsvik, Sweden, in 2004. The choice of operation mode in this plant is flexible and both dilute-acid hydrolysis and enzymatic hydrolysis can be evaluated (www.etek.se). In addition, a highly flexible and complete process development unit (PDU), equipped with a 10 L-pre-

treatment reactor and with 25–50 l-bioreactors, is operated at Lund University, Sweden (www.chemeng.lth.se).

Presently, commercialization of lignocellulosic ethanol production is occurring worldwide. The US Department of Energy expects to invest $385 million in the development of six commercial biorefineries in the US during the next few years. The plants will produce 130 million gallons of ethanol annually from lignocellulosic waste-products (www.renewableenergyacess.com). Also the Dutch Company Nedalco has announced plans to construct a plant in Sas van Gent, the Netherlands, capable of producing 200 million l of ethanol per year from lignocellulosic waste products (www.nedalco.nl).

23.2.1 Lignocellulose

Lignocellulose is composed of cellulose, hemicellulose and lignin, which are associated to each other in a complex plant cell wall matrix. Cellulose is a polymer of β-1,4-linked glucose units, where the repeating unit is the disaccharide cellobiose. Cellulose chains associate very strongly to each other by hydrogen bonds and give cellulose a highly crystalline structure (Delmer and Amor, 1995). Hemicellulose, in turn, is a complex heteropolymer consisting of different saccharides, both hexose and pentose sugars. The composition of hemicellulose varies widely between different plant species. Hemicellulose from herbaceous plants and hardwood contains large fractions of xylan and arabinan, whereas softwood hemicellulose has a low content of xylan but is rich in mannan. Lignocellulose also contains lignin, a complex hydrophobic polymer of substituted aromatic rings, that is very resistant to chemical or biological degradation (Lee, 1997). Table 23.3 summarizes the carbohydrate composition of potential ligno cellulosic raw materials for ethanol production.

Cellulose and hemicellulose must hydrolyzed to monosaccharides by thermochemical and enzymatic methods to enable ethanolic fermentation by yeast (Fig. 23.1). A first hydrolysis step in which hemicellulose is converted to oligo-, di- and mono-saccharides may also be referred to as a pre-treatment and fractionation step (Fig. 23.1; Table 23.4).

Table 23.3 Carbohydrate composition of typical lignocellulosic feed-stocks

	Salix*(hardwood) Sassner et al. (2006)	Spruce* (softwood) Söderström et al. (2003)	Corn Stover* Öhgren et al. (2006, 2007)	Bagasse* Rudolf (2007)
Glucan	43	50	42	43
Galactan	2.0	2.3	1.1	0.4
Mannan	3.2	12	-	-
Xylan	15	5.3	20	26
Arabinan	1.2	1.7	2.9	1.5

*Values shown as percentages based on dry raw material. The remainder constitutes mainly lignin and ash.

Table 23.4 Methods for pre-treatment/fractionation of lignocellulosic raw materials

Method	Description	Ref.
Steam explosion	Treatment with high pressure steam (170–220°C) followed by rapid decompression. An acid catalyst is often used (H_2SO_4 or SO_2)	Nguyen et al. (2000); Ramos et al. (1992); Schell et al. (2003); Söderström et al. (2002); Söderström et al. (2003); Tengborg et al. (1998); Torget et al. (1996); Torget et al. (1990); Wayman and Parekh (1988)
Hot water treatment	Treatment with liquid water at temperatures above 200°C	Bonn et al. (1983)
Wet oxidation	Treatment with hot water and oxygen	McGinnis et al. (1983)
AFEX (ammonia freeze explosion)	Impregnation with high-pressure ammonia followed by decompression	Holtzapple et al. (1991)
Organosolv	Cooking in aqueous alcohols. An acid catalyst is often used	Arato et al. (2005); Pan et al. (2006)

23.2.1.1 Acid Hydrolysis of Lignocellulose

Acid hydrolysis of lignocellulose has been known for over hundred years. In 1819 the French chemist Henri Braconnot discovered that cellulose could be hydrolysed into fermentable saccharides by acid (Fig. 23.1) (Sherrard and Kressman, 1945). Initially, concentrated acid hydrolysis was used. Processes based on concentrated acids give high sugar yields, however, corrosion and costly acid recovery constitute major disadvantages (Jones and Semrau, 1984). Acid consumption is considerably reduced in dilute-acids hydrolysis. One of the most widely operated full-scale dilute-acid processes was the Scholler process, originally developed in Germany in the late 1920's (Faith, 1945; Keller, 1996). It was a single-stage dilute-acid process (0.4% H_2SO_4), which in addition to ethanol was designed to produce fodder yeast and glycerol. Despite the low acid consumption in dilute-acid processes, the high hydrolysis temperature causes corrosion and saccharide degradation (Jones and Semrau, 1984). Saccharide degradation can be reduced by applying a two-stage process in which hemicellulose is hydrolyzed in a first step (150–190°C) and cellulose subsequently is hydrolyzed in a second step at more severe conditions (190–230°C) (Nguyen et al., 1999; Wayman et al., 1984). The two-stage process may also be considered a thermo-chemical pre-treatment (see below), followed by an acid hydrolysis of the cellulose fraction (Fig. 23.1).

23.2.1.2 Enzymatic Hydrolysis of Lignocellulose

Thermo-chemical pretreatment followed by enzymatic hydrolysis (Fig. 23.1) is considered the most promising method for the saccharification of hemicellulose and cellulose. The specificity of the enzymes and the mild conditions of hydrolysis minimize saccharide degradation, and enables high hydrolysis yields and reduced by-product/inhibitor formation (Jones and Semrau, 1984).

To enable efficient enzymatic cellulose hydrolysis, the lignocellulose material has to be pretreated to disrupt the cellulose-hemicellulose-lignin matrix (Fig. 23.1; Table 23.4) (Galbe and Zacchi, 2002). The material is first mechanically processed to reduce size and to improve mass transfer, and is then thermo-chemically treated to hydrolyze hemicellulose to its monomeric saccharides. Methods involving high-pressure steam are frequently employed, and referred to as steam-explosion. Acid and alkaline catalysts accelerate hemicellulose hydrolysis, whereas lignin largely remains non-solubilised. Steam-pretreatment generates a fibrous slurry, of which the liquid phase mainly contains hemicellulose mono-, di- and oligosaccharides, and the solid phase mainly contains cellulose and lignin. In the organosolv process the material is treated with an organic solvent – mostly ethanol - at elevated temperature, which in contrast to steam-pretreatment solubilises lignin.

Despite the crystallinity of cellulose and its resistance to degradation, a number of organisms, among them many filamentous fungi, excrete enzymes, which degrade cellulose, so called cellulases (Reese et al., 1950). Most commercial cellulase preparations originate from the filamentous fungus *Hypocrea jecorina* (formerly *Trichoderma reesei*). These enzyme mixtures contain different types of cellulolytic activity, which hydrolyze the cellulose-chains in a synergistic manner (Mandels and Reese, 1963; Mandels and Sternberg, 1976; Spano et al., 1976). Cellulose hydrolysis is product-inhibited by both cellobiose and glucose. Therefore the accumulation of these end-products of hydrolysis results in lower conversion of cellulose (Beltrame et al., 1984; Rao et al., 1989; Teleman et al., 1995). Furthermore, most commercial cellulase preparations contain insufficient activity of the enzyme β-glucosidase, which hydrolyses cellobiose to glucose, and hence additional β-glucosidase has to be supplied to prevent cellobiose accumulation (Sternberg et al., 1977).

23.2.1.3 Fermentation

Due to the inhibitory nature of lignocellulose hydrolysates and dilute-acid hydrolysates in particular (see below), *S. cerevisiae* is the preferred fermenting organism. The early Scholler process employed continuous fermentation (Fig. 23.2; Table 23.2), where agitation was limited and yeast slowly deposited on the bottom of the fermentation tank. The deposit was resuspended by agitation or aeration or alternatively removed and dried (Scholler and Eikmeyer, 1937; Scholler and Seidel, 1940). In the United States the Scholler process was further developed and renamed the Madison process (Harris et al., 1946), in which yeast was aerobically grown on

molasses and subsequently used in batch or fed-batch fermentation of the dilute-acid hydrolysate. Yeast recycling after each batch improved production rates.

Fermentation in a process based on enzymatic cellulose hydrolysis can be performed separately, or in combination with the enzymatic hydrolysis (Fig. 23.1). In separate hydrolysis and fermentation (SHF), the hydrolysis step is performed at the optimum temperature of the cellulolytic enzymes (>50°C) and fermentation at the optimum temperature for *S. cerevisiae* (28–32°C). SHF permits yeast recirculation since insoluble matter can be removed after hydrolysis, before yeast separation. The disadvantage of SHF is additional investment in separate hydrolysis and fermentation vessels and the inhibition of cellulose hydrolysis by accumulating glucose and cellobiose.

In simultaneous saccharification and fermentation (SSF) (Takagi et al., 1977; Wyman et al., 1992), hydrolysis yields are significantly higher than in SHF (Stenberg et al., 2000; Wright et al., 1988). In SSF, glucose is fermented at the same time as it is enzymatically released, which prevents inhibition of hydrolysis by glucose and cellobiose accumulation (Fig. 23.3).

Low glucose levels in SSF are also beneficial for the fermentation of hemicellulose-derived mono-saccharides such as pentose sugars (Jeffries et al., 1985; Karhumaa et al., 2007a; Meinander et al., 1999; Öhgren et al., 2006). The disadvantage of the SSF configuration is the compromise in operation temperature (33–37°C), which reduces the initial hydrolysis rate compared with SHF. Development of thermotolerant yeast strains may enable elevated operation temperatures (see below). However, SSF of acid/base pretreated material does not permit cell-recycling due to the accumulation of insoluble lignin. This in turn requires that SSF is performed with low yeast concentration, since yeast biomass production consumes sugar otherwise fermented to ethanol.

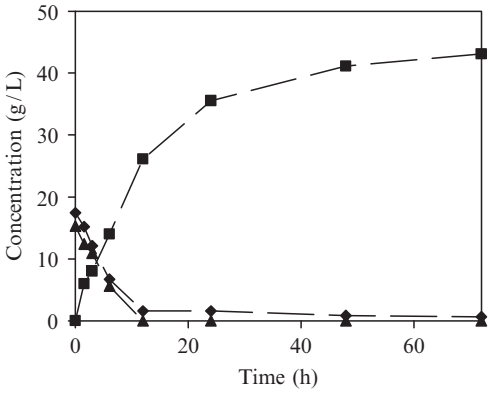

Fig. 23.3 Ethanol (-■-), glucose (-●-) and mannose (-▲-) concentrations during SSF of steam-pretreated spruce with a commercial cellulose preparation, *Saccharomyces cerevisiae* (5 g/l) and 10% water insoluble contents

23.2.1.4 Fermentation Inhibition

During pre-treatment/fractionation and acid hydrolysis of lignocellulose, a fraction of cellulose, hemicellulose and lignin is converted to low molecular weight fatty acids, furans, and aromatic compounds (Fig. 23.4), the relative amounts of which are dependent on the severity of the treatment (Larsson et al., 1999a). These compounds are biocidal and inhibit subsequent enzymatic hydrolysis and fermentation steps (Almeida et al., 2007; Luo et al., 2002). Acetic acid is an inherent component of hemicellulose, and it is released during hydrolysis (Fig. 23.4A). Levulinic acid and formic acid are products of saccharide degradation. These acids inhibit cell growth, especially at low pH when the undissociated form diffuses across the cell membrane and decreases the cytosolic pH, which depletes the cell of energy and reduces cell viability (Imai and Ohno, 1995; Verduyn et al., 1992; Viegas and Sá-Correia, 1995). Therefore, fermentation of lignocellulose hydrolysates is performed around pH 5.0, whereas fermentation based on saccharides and starch usually can be performed at a lower pH.

At high temperatures and acidic conditions, furfural and hydroxymethyl furfural (HMF) (Fig. 23.4B) are formed from pentose and hexose sugars, respectively. These compounds inhibit cell growth and at higher concentration also fermentation (Banerjee and Viswanthan, 1976; Dunlop, 1948; Palmqvist et al., 1999; Sanchez and Bautista, 1988; Sárvári Horváth et al., 2001; Taherzadeh et al., 2000a).

Degradation of lignin results in a variety of aromatic compounds (examples given in Fig. 23.4C), which also inhibit cell growth and fermentation (Ando et al., 1986; Buchert et al., 1989). The mechanism of inhibition is unclear, however, it is

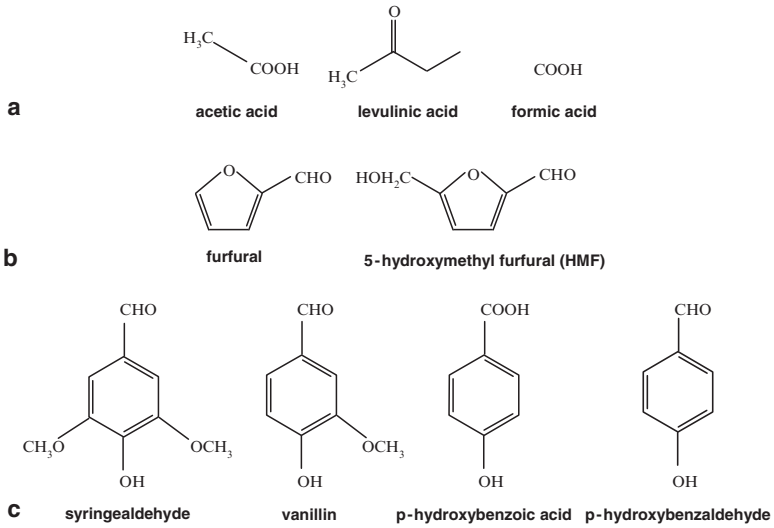

Fig. 23.4 Compounds inhibitory to cell growth and fermentation found in lignocellulose hydrolysates. **a**: acids, **b**: inhibitors from sugars, **c**: aromatic compounds

believed that partition into cell membranes disrupts membrane barrier properties (Hage et al., 2001; Heipieper et al., 1994).

To circumvent the inhibitory nature of lignocellulose hydrolysates, three principally different strategies have been investigated:

- Fermentation technology
- Strain development
- Detoxification

Fed-batch and continuous fermentation (Fig. 23.2; Table 23.2) of lignocellulose hydrolysates exploit the inherent ability of yeast to convert inhibitors present in hydrolysate to less toxic compounds. By controlling the substrate addition rate, the hydrolysate is detoxified by the yeast cells and superior ethanol productivity compared to batch fermentation can be achieved (Fig. 23.5).

In a fed-batch set-up, the feed rate can be controlled by an easily measured metabolic signal, such as the carbon dioxide evolution rate (CER) (Nilsson et al., 2002; Nilsson et al., 2001; Rudolf et al., 2004; Taherzadeh et al., 2000b). In a SSF set-up, the fed-batch mode has the additional benefit that the initial high viscosity of the pretreated material is avoided (Ballesteros et al., 2002; Rudolf et al., 2005).

Selecting naturally inhibitor-tolerant strains significantly improves fermentation of inhibitory substrates as demonstrated with *S. cerevisiae* strains isolated from a spent sulphite liquor plant (Lindén et al., 1992). A similar effect is obtained by strain adaptation, which exploits the ability of yeast to adapt to inhibitory hydrolysates. Even a short pre-cultivation on hydrolysate dramatically improves fermentation performance (Alkasrawi et al., 2006). The same result can be achieved by repeated transfer of yeast to increased concentrations of hydrolysate (Keller et al., 1998). Genetic modification by introducing or increasing the expression of genes encoding proteins procuring inhibitor tolerance is another option, which was recently reviewed (Almeida et al., 2007).

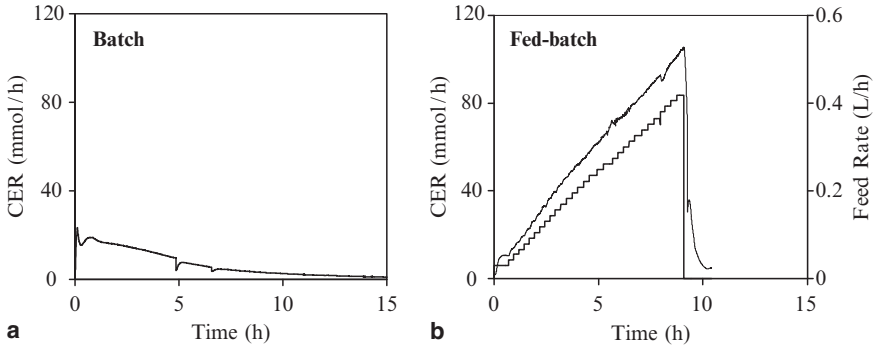

Fig. 23.5 Carbon dioxide evolution rate (CER) during lab-scale batch (**a**) and fed-batch (**b**) fermentation of dilute-acid hydrolysate from spruce with *Saccharomyces cerevisiae* using a feed rate control developed by Nilsson et al. (2001)

Chemical detoxification of inhibitory hydrolysates increases their fermentability. The methods available include treatment with alkali, ozone, ion-exchange resins and enzymes (Alriksson et al., 2006; Jönsson et al., 1998; Nilvebrant et al., 2003; Nilvebrant et al., 2001; Santos et al., 2003). However, in addition to adding extra costs to the production of lignocellulosic bioethanol, most detoxification procedures also potentially reduce the saccharide content of the hydrolysate (Larsson et al., 1999b; Nilvebrant et al., 2003).

23.2.2 Inulin

In addition to the far more abundant lignocellulosic feed-stocks, inulin-rich plants, such as Jerusalem artichoke, have been considered for fuel ethanol production. Inulin is a reserve carbohydrate abundant in roots and tubers of plants belonging to the families of *Compositae* and *Gramineae* (Vandamme and Derycke, 1983). These crops are highly productive and - more importantly - can be grown on relatively poor soil. Inulin is a polymer of β-2,1 linked fructose units and its conversion to ethanol resembles that of starch (Fig. 23.1). Some yeast species such as *Kluyveromyces marxianus* and *Saccharomyces rosei* can ferment inulin directly to ethanol (Duvnjak et al., 1981; Margaritis and Merchant, 1983). Inulin can also be hydrolysed with inulinases and simultaneously or subsequently fermented to ethanol (Kazuyoshi et al., 1993; Vandamme and Derycke, 1983).

23.3 Fermenting Organisms

23.3.1 S. cerevisiae

Beyond comparison, baker's yeast *S. cerevisiae* is the most used organism for industrial ethanol production. Compared to bacteria, other yeast and filamentous fungi, *S. cerevisiae* has a number of physiological characteristics advantageous in the industrial context (Hahn-Hägerdal et al., 2007). *S. cerevisiae* grows both under aerobic and anaerobic conditions, and performs well in industrial fermentation conditions. It tolerates a wide range of pH, with an optimum at acidic pH, which makes *S. cerevisiae* fermentation less susceptible to infection than e.g. bacterial fermentation. *S. cerevisiae* tolerates temperatures up to around 40°C, with an optimal temperature around 30–35°C. The theoretical yield of ethanol from sugar is 0.51 g g^{-1} and in the industrial context 90–95% of this can generally be achieved by *S. cerevisiae* when traditional saccharide and starch feed-stocks are used. The theoretical specific productivity of *S. cerevisiae* is around 2 g ethanol g^{-1} cells and hour, which makes it a much faster production organism than e.g. filamentous fungi (Skoog and Hahn-Hägerdal, 1988).

Since *S. cerevisiae* has been used since ancient times in the production of goods for human consumption it has GRAS (generally regarded as safe) status, and *S. cerevisiae* processes are well accepted by the general public. Because of its long industrial history and consequential "domestication" this yeast is also tolerant to high concentrations of sugar and ethanol, and it tolerates relatively high osmotic pressures. *S. cerevisiae* strains isolated from industrial environments display tolerance to compounds inhibitory to fermentation processes (Hahn-Hägerdal et al., 2005; Lindén et al., 1992), which illustrates the ability of this yeast to adapt to different conditions.

S. cerevisiae can utilize sucrose, glucose, fructose, galactose and mannose (Lindén et al., 1992; Nilsson et al., 2002). However, in the perspective of using novel feed-stocks for ethanolic fermentation, *S. cerevisiae* lacks the ability to ferment a number of mono-, di-, and trisaccharides derived from starch, cellulose and hemicellulose. It can not readily utilize starch, cellulose and hemicellulose, nor the disaccharides cellobiose and xylobiose, features suggested to be thermodynamically advantageous (Lynd et al., 2002; Zhang and Lynd, 2005), neither can it ferment the pentose sugars xylose and arabinose.

Despite its long history, existing industrial ethanolic fermentation suffers from some drawbacks related to the metabolism of *S. cerevisiae*. Under anaerobic conditions a considerable amount of glycerol is formed as a consequence of the regeneration of the reduced co-factor NADH (Oura, 1973). Also, the hexose sugar galactose is only consumed upon glucose depletion, which prolongs the fermentation time of galactose-rich substrates (Johnston and Carlson, 1992). Stuck fermentation, that is, when the fermentation process comes to a standstill despite considerable concentrations of remaining fermentable sugars, is mainly considered a problem in the wine industry (Lucero et al., 2002). It is believed to be related to nutrient depletion, which among other inactivates sugar transporters (Cooper, 2002).

23.3.2 Other yeasts

Yeasts other than *Saccharomyces* sp. are misleadingly referred to as nonconventional yeast (NCY) (Boekhout and Kurtzman, 1996). In fact NCY constitute the majority of yeast species, whereas *Saccharomyces* sp. has very unusual qualities due to its adaptation to industrial use. Among NCY are yeast that consume the pentose sugars xylose (Skoog and Hahn-Hägerdal, 1988), arabinose (Dien et al., 1996; Fonseca et al., 2007; McMillan and Boynton, 1994), starch (Spencer-Martins and van Uden, 1977) and starch and lignocellulose derived di- and trisaccharides (Parekh, 1986; Ryabova et al., 2003). Among NCY are also those that endure much lower pH than *Saccharomyces* sp., such as *Zygosaccharomyces* sp. (Thomas and Davenport, 1985) as well as those that perform ethanolic fermentation at temperatures above 40°C, such *Hansenula polymorpha* (Ryabova et al., 2003). Whereas NCY will probably not be exploited for ethanolic fermentation due to their lack of robustness, their versatile bioconversion reactions are considered for industrial

production of chemicals and chemical intermediates (Abbas, 2003). Thus, NCY have and will continue to be a source of genes to be introduced into *S. cerevisiae* to improve its industrial performance in ethanolic fermentation (Hahn-Hägerdal et al., 2001, 2007; Jeffries and Jin, 2004).

23.4 Genetic Modification of *S. cerevisiae*

The advent of recombinant DNA technology and the increasing number of sequenced genomes (www.ncbi.nlm.nih.gov) have opened up entirely new avenues for the improvement of industrial ethanolic fermentation. To the best of the authors' knowledge none of the novel recombinant strains have so far been introduced into large-scale ethanolic fermentation processes.

23.4.1 Engineering of S. cerevisiae for Traditional Ethanolic Fermentation

During ethanolic fermentation, *S. cerevisiae* produces the by-product glycerol, which is a consequence of accumulation of intracellular NADH under anaerobic conditions (Oura, 1977). As much as 4% of the fermentable carbon source is lost to glycerol formation, which has been the incentive for several targeted metabolic engineering strategies to redirect the carbon flux from glycerol to ethanol. Bacteria regulate their intracellular concentrations of NADH and NADPH with transhydrogenase enzymes, which catalyze the reversible reaction

$$\mathrm{NADH + NADP^{+} <=> NAD^{+} + NADPH.}$$

Yeast does not harbour such an enzyme activity (Dijken and Scheffers, 1986). One strategy has therefore been to express bacterial transhydrogenase enzymes in *S. cerevisiae* (Nissen et al., 2001; Anderlund et al., 1999). However, this approach was not successful since the heterologous enzymes favoured the reverse reaction. More successful was the creation of an artificial transhydrogenase activity (Nissen et al., 2000), where the NADPH-consuming glutamate synthesis pathway was replaced by an NADH and ATP consuming synthesis pathway. Similar metabolic engineering strategies were later combined with a heterologous pentose assimilation pathway in *S. cerevisiae* (Roca et al., 2003; Verho et al., 2003).

The domestication of *S. cerevisiae* has lead to preferential consumption of glucose over other carbon sources, generally referred to as carbon catabolite repression (Gancedo, 1998). In industrial fermentation of mixed sugar raw materials, this leads to prolonged fermentation time. Metabolic engineering efforts have been made to overcome this. The prime engineering target has been the regulatory protein Mig1, known to regulate a cascade of signaling pathways (Klein et al., 1998). The deletion of *MIG1* (Klein et al., 1996), as well as both *MIG1* and *MIG2*

(Klein et al., 1999), reduced glucose repression of sucrose, galactose and maltose utilization. The manipulation of the *MIG1* gene has more recently also been combined with xylose utilization in recombinant *S. cerevisiae* (Roca et al., 2004; Thanvantri Gururajan et al., 2007). Here the effect was less pronounced, probably because laboratory strains were used instead of industrial production strains.

23.4.2 Expanding the Substrate Range

23.4.2.1 Polymeric Substrates

Genes encoding amylolytic and cellulolytic enzymes from numerous organisms have been successfully introduced into *S. cerevisiae* (Lynd et al., 2002). However, relatively few investigations have characterized the consumption of the polymeric substrates and the corresponding product formation (Den Haan et al., 2007; Eksteen et al., 2003; Knox et al., 2004). Degradation and simultaneous fermentation of polymeric substrates by the same biocatalyst is hampered by diffusion limitation and, in the case of a non-soluble substrate such as cellulose, mass transfer limitation. To overcome such limitation, a laboratory strain of *S. cerevisiae* was engineered to co-display glucoamylase and α-amylase on the cell surface using the endogenous anchor proteins a-glutinin and Flo1p (Shigechi et al., 2004). About 60 g l^{-1} ethanol was produced from raw starch with more that 85% yield. In contrast, only 3 g l^{-1} ethanol was produced when the three enzymes constituting the cellulolytic enzyme system were co-expressed on the surface of a yeast cell (Fujita et al., 2004). The order of magnitude difference illustrates the fundamental difference between the soluble starch raw material and the insoluble cellulose raw material.

23.4.2.2 Di- and Trisaccharides

Enzymes that hydrolyze di- and trisaccharides derived from starch, hemicellulose and cellulose have been successfully expressed in *S. cerevisiae* (Ostergaard et al., 2000a; van Rooyen et al., 2005). Such constructs have proven relatively efficient since they are not hampered by diffusion and mass transfer limitations. When the expression of the hydrolytic enzymes is directed towards the cellular periplasmic space, i.e. space between the cell wall and the membrane, the degradation of substrate becomes independent of the efficiency of the transport across the cell membrane (van Rooyen et al., 2005). More recently, cellooligosaccharide assimilation was combined with xylose assimilation in a single strain (Katahira et al., 2006).

Molasses may contain up to 2% of the tri-saccharide raffinose (Rosen, 1987), which is hydrolysed by invertase into fructose and melibiose. Melibiose in turn is hydrolyzed into glucose and galactose by the enzyme melibiase (Naumov et al., 1996). While invertase is naturally present in *S. cerevisiae,* only a few species harbour the enzyme melibiase. Melibiose utilization was increased when the *MEL1*

gene encoding melibiase was expressed in *S. cerevisiae* (Ostergaard et al., 2000b). Furthermore, the disaccharide galactose is not only a component of molasses but also of hemicellulose. Its uptake was improved by deleting general regulatory genes such as *MIG1* as well as specific galactose regulatory genes (Ostergaard et al., 2000c).

23.4.2.3 Pentose Sugars

Generating pentose-utilizing *S. cerevisiae* strains has been the focus of considerable research effort (recently reviewed by Jeffries, 2006, and by Hahn-Hägerdal et al., 2007). Xylose-utilising strains of *S. cerevisiae* have been generated by introducing heterologous xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Pichia stipitis*, in addition to overexpressing the endogenous *S. cerevisiae* xylulokinase (XK) (Table 23.5; Fig. 23.6) (Ho et al., 1998; Wahlbom et al., 2003).

As an alternative pathway, also the enzyme xylose isomerase (XI) has been used. The first XI actively expressed in *S. cerevisiae* originated from the bacterium *Thermus thermophilus* (Walfridsson et al., 1996), however, its specific activity was very low. More recently was a eukaryotic gene encoding XI from an anaerobic rumen fungus *Piromyces* sp. expressed in *S. cerevisiae* with much higher activity (Kuyper et al., 2003). However, also for this enzyme, the specific activity was too low to permit chromosomal integration (Karhumaa et al., 2007b). Arabinose-utilizing strains of *S. cerevisiae* have been developed much more recently. Both in bacteria

Table 23.5 The most significant metabolic engineering strategies successfully applied to *S. cerevisiae* for pentose fermentation

	Modification	References
Initial pentose uptake pathways	XR-XDH	Kötter and Ciriacy (1993) Tantirungkij et al. (1993)
	XI	Walfridsson et al. (1996) Kuyper et al. (2003)
	XK	Deng and Ho (1990)
	Fungal arabinose pathway	Richard et al. (2003)
	Bacterial arabinose pathway	Becker and Boles (2003)
	Fungal xylose and bacterial arabinose pathway	Karhumaa et al. (2006)
Additional modifications	Aldose reductase *Gre3* deletion	Träff et al. (2001)
	Oxidative PPP disruption	Jeppsson et al. (2002)
	Overexpression of the non-oxidative PPP	Johansson and Hahn-Hägerdal (2002b)
	Redox metabolism	Roca et al. (2003) Verho et al. (2003) Jeppsson et al. (2003)
	Xylose transport	Hamacher et al. (2002) Leandro et al. (2006)

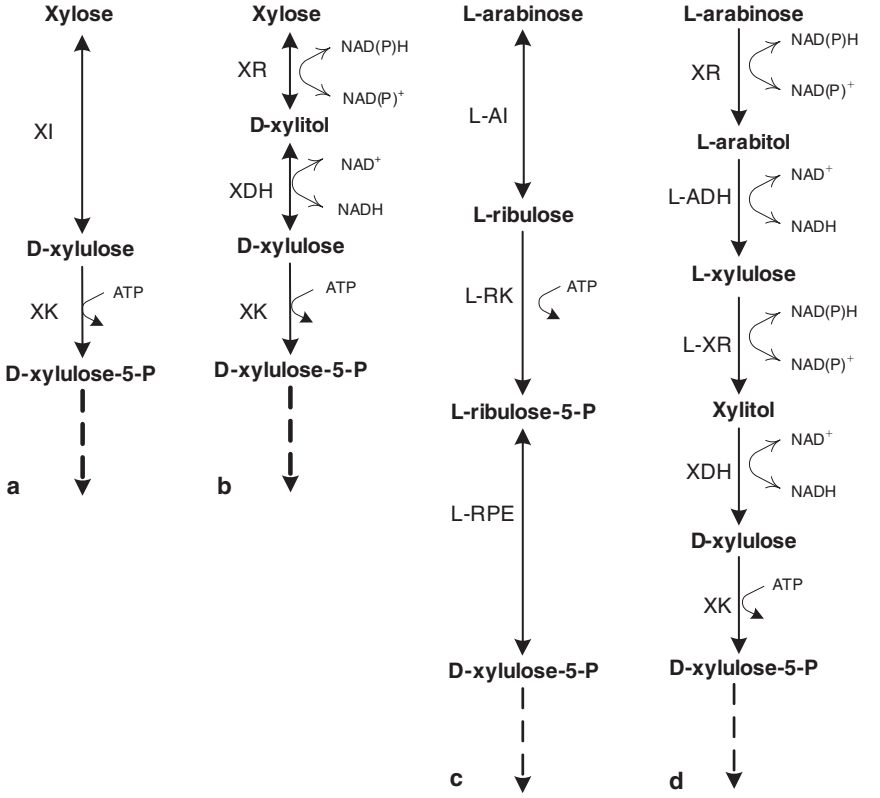

Fig. 23.6 Xylose (**a**,**b**) and arabinose (**c**,**d**) utilization pathways in bacteria (**a**,**c**) and in fungi (**b**,**d**)

and in fungi, the conversion of arabinose into metabolites of the pentose phosphate pathway (PPP) involves several more steps than the conversion of xylose (Fig. 23.6).

The first trial of expressing the *E. coli* arabinose pathway in *S. cerevisiae* did not result in arabinose utilisation (Sedlak and Ho, 2001), and only when the L-arabinose isomerase from *E. coli* was substituted by the *Bacillus subtilis* L-arabinose isomerase, a functional arabinose pathway was established (Becker and Boles, 2003). Recently, the bacterial arabinose pathway was successfully combined with the fungal xylose pathway in a single industrial *S. cerevisiae* strain (Karhumaa et al., 2006). Once the initial pentose utilization pathways had been established, metabolic engineering has been used to change additional cellular functions including sugar transport (Fig. 23.7; Table 23.5; Hamacher et al., 2002; Leandro et al., 2006), optimized initial pentose metabolism (Eliasson et al., 2001; Karhumaa et al., 2007a, b), the pentose phosphate pathway (PPP) (Johansson and Hahn-Hägerdal, 2002a, b), and inhibitor tolerance (Almeida et al., 2007). In addition to rational metabolic engineering strategies, adaptation, selection and evolutionary engineering (Sauer, 2001)

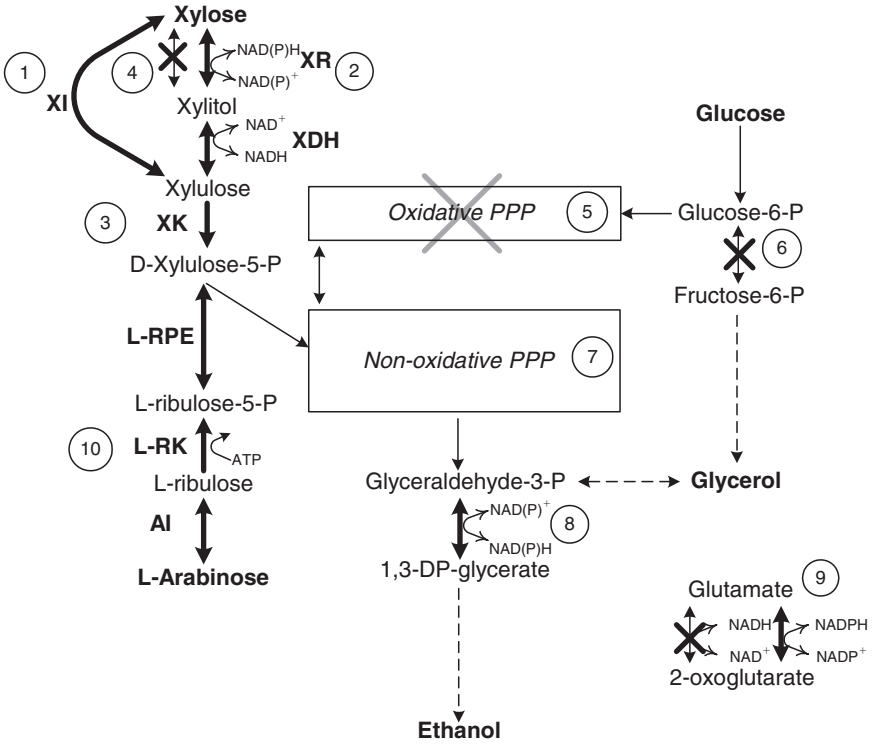

Fig. 23.7 Schematic illustration of metabolic steps/pathways engineered for ethanolic xylose and arabinose fermentation. Enhanced activity is indicated with bold lines; reduced activity is indicated with crosses. (1) xylose isomerase, (2) xylose reductase – xylitol dehydrogenase, (3) xylulokinase, (4) aldose reductase, (5) oxidative pentose phosphate pathway, (6) phosphoglucose isomerase, (7) non-oxidative pentose phosphate pathway, (8) glyceraldehyde 3-P dehydrogenase, (9) glutamate dehydrogenase, (10) arabinose isomerase, L-ribulokinase and ribulose-5-P 3-epimerase

have been extensively employed to obtain improved ethanolic fermentation of xylose (Ho et al., 1998; Karhumaa et al., 2005; Sonderegger and Sauer, 2003; Wahlbom et al., 2003) and arabinose (Becker and Boles, 2003).

The drawback in pentose fermentation is that ethanolic pentose fermentation by recombinant strains of *S. cerevisiae* generally proceeds one to two orders of magnitude slower than the fermentation of hexose sugars (Hahn-Hägerdal et al., 2007). In addition, fermentation of xylose and arabinose frequently results in excretion of a considerable fraction of xylitol and arabitol, respectively. The excretion of xylitol and arabitol has been ascribed to an intracellular lack of reduced co-factors (Bruinenberg et al., 1984). It is however also controlled by the carbon flux such that rapid sugar consumption prevents by-product formation (Karhumaa et al., 2007a). This can be seen in terms of metabolic control analysis (MCA), which predicts that

metabolism is controlled by demand of metabolic intermediates (Cornish-Bowden et al., 1995). High glycolytic activity may generate such a demand, since it has been frequently demonstrated that simultaneous pentose and glucose consumption at low glucose concentration improves pentose fermentation (Jeffries et al., 1985; Karhumaa et al., 2007a; Meinander et al., 1999; Öhgren et al., 2006).

The ultimate goal of the development of pentose-fermenting recombinant *S. cerevisiae* is to use them in large-scale fermentation process based on lignocellulosic raw materials. This requires that the strains are robust and tolerant towards the inhibitors generated in the pre-treatment and fractionation processes (Fig. 23.4; Almeida et al., 2007). Thus, the fermentation of lignocellulosic raw materials requires that the metabolic engineering strategies developed in laboratory strains are translated to industrial yeast strains. Such strains may be isolated from industrial environments (Lindén et al., 1992) and are often aneuploid. Whereas introduction of novel genes and novel pathways in laboratory strains is relatively routine technology, the fine-tuning and deletion of genes in industrial strains may be much more cumbersome due to the presence of multiple chromosomes and unknown sequences. So far, a limited number of recombinant pentose-fermenting industrial *S. cerevisiae* strains have been described in literature (Ho et al., 1998; Wahlbom et al., 2003). Even more rarely has the fermentative performance of these strains been described under simulated industrial conditions (Hahn-Hägerdal et al., 2006, 2007; Hahn-Hägerdal and Pamment, 2004; Sonderegger et al., 2004).

23.5 Conclusions

The production of ethanol from saccharide and starch raw materials is a well developed large scale industrial process, while the exploitation of lignocellulosic raw materials for the production of ethanol is a great multi-task challenge. The pretreament/fractionation and hydrolysis of the raw material must be developed in synergy with the development novel recombinant strains of *S. cerevisiae* able to ferment the whole range of lignocellulose-derived oligo-, di- and mono-saccharides. Similar to the traditional fermentation processes in the baker's yeast, wine, beer and potable alcohol industry, it is foreseeable that different raw materials and different process designs will require different fermenting strains. One of the great metabolic engineering challenges will be to translate existing molecular and physiological knowledge to robust industrial production strains designed for the needs of particular fermentation processes.

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Chapter 24
Potentiality of Yeasts in the Direct Conversion of Starchy Materials to Ethanol and Its Relevance in the New Millennium

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Contents

Abstract In recent years, the use of renewable and abundantly available starchy and cellulosic materials for industrial production of ethanol is gaining importance, in view of the fact, that ethanol is one of the most prospective future motor fuels, that can be expected to replace fossil fuels, which are fast depleting in the world scenario. Although, the starch and the starchy substrates could be converted successfully to ethanol on industrial scales by the use of commercial amylolytic enzymes and yeast fermentation, the cost of production is rather very high. This is mainly due to the non-enzymatic and enzymatic conversion (gelatinization, liquefaction and saccharification) of starch to sugars, which costs around 20% of the cost of production of ethanol from starch. In this context, the use of amylolytic yeasts, that can directly convert starch to ethanol by a single step, are potentially suited to reduce the cost of production of ethanol from starch.

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Research advances made in this direction have shown encouraging results, both in terms of identifying the potentially suited yeasts for the purpose and also their economic ethanol yields. This chapter focuses on the types of starch and starchy substrates and their digestion to fermentable sugars, optimization of fermentation conditions to ethanol from starch, factors that affect starch fermentation, potential amylolytic yeasts which can directly convert starch to ethanol, genetic improvement of these yeasts for better conversion efficiency and their future economic prospects in the new millennium.

Keywords Ethanol, starch, starchy substrates, fermentable sugars, amylolytic yeasts

24.1 Introduction

More than 95% ethanol is produced commercially by the yeast fermentation method, utilizing the cheap raw material like blackstrap molasses. Less than 5% of overall output is accounted for by the synthetic feedstocks. The ethanol production is directly dependent on the availability of molasses, and the output varies according to the annual production of sugar cane. To overcome the shortage of alcohol, it is sometimes imported. The shortfall in ethanol production could also be met by utilizing alternative renewable sources like sugary, starchy and cellulosic substrates for economic reasons. Sugary substrates are comparatively expensive, but could be easily and readily employed for ethanol production. Table 24.1 shows the data on world ethanol production, which is around 32.96 billion liters for a population of more than 6.5 billion. If ethanol were to be used as motor fuel available quantity must be at least four- to fivefold more than the present.

The increased concern for the security of the oil supply and the negative impact of fossil fuels on the environment, particularly greenhouse gas emissions, has put pressure on society to find renewable fuel alternatives. The most common renewable fuel today is ethanol produced from sugar or grain (starch); however, this raw material base will not be sufficient (Hahn-Hagerdal et al., 2006). It has been predicted by several experts that by 2010, one half of agricultural output may be directed towards non-food usage.

Recent years have seen the introduction of large-scale processing in the bioconversion of biomass resources, especially starchy materials, to ethanol, which is expected to find a wide range of uses, including as potable, motor fuel and as the chemical feed stock in the new millennium. However, the present process for ethanol production from starchy materials via fermentation is laborious, cost intensive and consists three steps namely, liquefaction, saccharification and fermentation. Besides, the fermentation itself requires improvement if it is to realize efficient production at low cost. There are two main reasons for the present high cost: one is that, as the yeast *Saccharomyces cerevisiae* cannot utilize starchy materials, large amounts of amylolytic enzymes, namely, glucoamylase (EC 3.2.1.3) and α-amylase

Table 24.1 Country-wise world ethanol production in 1000 h l

Country	2001	2000	1999	1998	1997
France	8,000	8,120	7,540	7,788	7,671
Germany	2,950	2,850	3,400	3,640	3,750
Italy	1,900	2,056	2,009	2,251	2,297
Spain	2,250	1,450	1,250	1,400	1,360
UK	4,300	4,350	4,100	4,220	4,100
Other EU	2,221	1,871	1,853	1,876	2,020
EU	21,621	20,697	20,152	21,175	21,198
Czech Republic	900	900	900	1,050	1,090
Hungary	510	553	481	494	531
Poland	1,580	1,600	1,700	2,080	2,400
Russia	11,700	11,500	12,800	12,000	11,800
Ukraine	2,200	1,960	1,740	1,560	2,470
Other Europe	3,023	3,032	2,952	3,045	3,587
Europe	41,534	40,242	40,725	41,404	43,076
Argentina	1,530	1,710	1,735	1,766	1,610
Brazil	119,000	114,000	129,821	141,221	154,934
Canada	2,380	2,380	2,000	1,500	1,500
Cuba	850	840	800	795	1,100
Ecuador	627	375	321	313	263
Guatemala	600	600	450	450	500
Mexico	701	671	562	531	532
USA	75,800	70,500	66,050	64,500	58,860
Other Americas	4,180	4,042	3,867	3,828	3,770
Americas	205,668	195,118	205,606	214,904	223,069
China	30,900	29,700	28,600	28,000	26,900
India	17,800	17,200	16,900	16,881	16,470
Indonesia	1,650	1,600	1,500	1,650	1,740
Japan	1,360	1,100	1,040	1,020	1,040
Saudi-Arabia	3,900	4,100	3,900	3,700	3,900
Thailand	1,500	1,000	3,200	2,650	3,750
Other Asia	2,485	2,613	2,653	2,820	2,763
Asia	59,595	57,313	57,793	56,721	56,563
Australia	1,540	1,200	1,000	900	850
New Zealand	174	195	176	190	178
Other Oceania	80	80	80	80	80
Oceania	1,794	1,475	1,256	1,170	1,108
Malawi	120	120	122	119	154
South African CU	3,852	3,800	3,900	4,100	4,300
Zimbabwe	293	257	263	221	252
Other Africa	1,059	1,036	1,048	991	1,089
Africa	5,324	5,213	5,333	5,431	5,795
World	313,915	299,361	310,713	319,630	329,611

Source: *World Fuel Ethanol Analysis and Outlook* by Dr. Christoph Berg (April 2004).

(EC 3.2.1.1), need to be used in the process; the other is that the starchy materials need to be cooked at a high temperature (60 to 120°C) for gelatinization to facilitate the enzymatic conversion to sugars, and then to obtain a high ethanol yield by yeast fermentation.

Basically starch is the storage carbohydrate in plants, and it serves as an important energy and carbon source in biotechnological processes. Starch is made up of long chains of glucose units joined by α-1, 4 linkages and joined at branch points by α-1, 6 linkages. Many microorganisms, including the industrially used *S. cerevisiae*, are not able to degrade starch since they do not produce starch-decomposing enzymes such as α-amylase (which cleaves α-1, 4-glycosidic bonds), β-amylase (which cleaves maltose units from the nonreducing end of starch), pullulanase or isoamylase (debranching enzymes that hydrolyze α-1, 6-glycosidic bonds), and glucoamylase (which hydrolyzes glucose units from the nonreducing end of starch) (Table 24.2). Hence, it is necessary to add starch-degrading enzymes to the starch in order to convert it to sugars before fermentation.

Since molasses being the cheapest and the richest sugary raw material in countries like Brazil and India, the ethanol produced from it is however, apparently the cheapest in its cost. Since the sugary crops such as sugarcane and sugar beet are utilized for food purposes, their availability for the larger-scale production of ethanol is meager. Therefore, the starchy substrates such as cereals, tubers and cacti, though used for food purposes, are abundantly available and a considerable portion could be diverted for ethanol production. Direct or one-step starch fermentation not requiring the separate treatments could be competitive in the economic production of industrial ethanol. With a view to reduce the cost of production of ethanol from starchy substrates, investigations are going on the promising yeasts that can produce both amylases and ethanol for a one-step process or to use a recombinant yeast strain that produces starch-degrading enzymes as well. In this article, we are focusing mainly on amylolytic yeasts and the genetic improvements made on some of the promising strains of yeasts for one-step fermentation of starch to ethanol.

Table 24.2 Starch-hydrolyzing enzymes and their end products

Name	E.C.No.	Substrate	Bond(s) hydrolyzed	End product (s)
α-Amylase	3.2.1.1	Starch and related Polysaccharides	α-1,4	Di and/or oligosaccharides
β-Amylase	3.2.1.2	Starch and related Polysaccharides	α-1,4	β-D-Maltose
Glucoamylase	3.2.1.3	Starch and related Polysaccharides	α-1,4 or α-1,6	β-D-Glucose
Pullulanase	3.2.1.41	Starch and pullulan	α-1,6	Maltotriose, dextrins
Isoamylase	3.2.1.68	Oligo and polysaccharides	α-1,6	Oligosaccharides
Cyclodextrins glucosyltransferase	2.4.1.19	Oligo and polysaccharides	α-1,4	α-,β- and γ-Cyclodextrins
α-Glucosidase	3.2.1.20	Oligosaccharides	1,4and α-1.6	α-D-Glucose

Source: Pretorius, I.S., Lambrechts, M.G., Marmur, J. 1991. *Crit Rev Biochem Mol Bio*l. 26: 53–76.

24.2 Starchy Substrates

Starch is the second most abundant compound produced in higher plants after cellulose. While cellulose is a structural component of plants, starch mainly serves as a compound to temporarily store energy that can be accessed at a later time point. Generally starch is deposited as semi-crystalline granules, which consists of two types of molecules, namely amylose and amylopectin. Both molecules are polymers of glucose residues and contain the same chemical linkages. Amylopectin is a much larger molecule than amylose and consists of shorter chains of α-1,4 linked glucose residues that are connected by α-1,6 glycosidic linkages resulting in a branched structure. Amylose is an essentially linear molecule of α-1,4 linked glucose with few branches. However, the α-1,4 glucan chains have a much higher average degree of polymerization as compared to amylopectin (Table 24.3).

The amylose and amylopectin ratio in starch depends on the source of starch and the maturity of the crop plant, but amylose generally accounts for 20 to 25% of starch. However, waxy starch, which contains less than 1% (w/w) amylose, shows typical levels of molecular ordering and crystallinity suggesting that the characteristic internal structure of the granule may be due to the amylopectin component of starch. The gelatinization temperature of starch, generally influenced by the ratio of amylose and amylopectin content (Table 24.4).

Starch is a cheap, nontoxic, renewable carbon source. It is abundant in cereals and root crops. Among them corn is the most viable feedstock due to variety of reasons including high crop yields in short periods, broad geographical range and having C_4 mechanism of photosynthesis. Starch is also not susceptible to spoilage; therefore, storage conditions require minimum attention. Starch content of corn is reported to be 65–70% on dry weight basis. In tropical countries like India the production of tapioca tubers is enormous. As compared with corn, tapioca is not a cash crop; it is commonly used for direct human consumption, because of its rapid perishability and cyanogenic glucosides (Shukla et al., 1989). Brazilian

Table 24.3 Properties of amylose and amylopectin

Properties	Amylose	Amylopectin
Molecular mass	10^5–10^6	10^7–10^9
Basic structure	Linear	Branched
Colour with iodine	Dark blue	Purple
λ Max of iodine complex	650 nm	540 nm
Iodine affinity (g iodine bound to 100 g of amylose or amylopectin)	19–20%	<1%
Films	Strong	Week
Gel formation	Firm	Soft
Solubility in water	Variable	Soluble
Stability in aqueous solution	Retrogrades	Stable

Source: Whistler, R.L. and Bemiller, J.N. 1984. *Starch: Chemistry and Technology (2nd edition)*. Academic Pr ISBN: 0127462708.

Table 24.4 Amylose and amylopectin content of commercial starchy substrates and their gelatinization temperature

Starchy substrates	Amylose %	Amylo-pectin %	Gelatinization temperature°C
Maize (*Zea mays* L.)	26.0	74.0	62–72
Waxy maize (*Zea mays* L.)	1.0	99.0	63–72
Amylo maize (*Zea mays* L.)	74.5	25.5	67–100
Tapioca (*Mannihot esculenta* Cranz)	17.0	83.0	51–65
Wheat (*Triticum* sp.)	25.0	75.0	58–64
Rice (*Oryza sativa*)	19	81	–
Potato (*Solanum tuberosum* L)	24.0	76.0	58–67

Source: Whistler, R.L. and Bemiller, J.N. 1984. *Starch: Chemistry and Technology (2nd edition)*. Academic Pr ISBN: *0127462708.*

National Alcohol Programme recognized cassava as one of the best alternative crops for ethanol production (Schenberg and Pinto da Costa, 1987).

Even the aflatoxin contaminated corn can be economically converted to ethanol. Bothast et al. (1982) described a process for converting aflatoxin-contaminated corn to ethanol by combining ammonia inactivation with the liquefaction step of the ethanol fermentation process. Better ethanol yields were obtained when ammonia was added during liquefaction than when no ammonia was added. Aflatoxin B_1 levels were reduced more than 80 to 85% by the process.

Enormous amounts of starch is processed from cultivated crops annually and subsequently used for food as well as non-food purposes. In the year 2000 around 48.5 million tons was produced worldwide with the most important crop plants for extraction being corn, wheat and potato (LMC International, 2002). Corn is by far the biggest crop corresponding to 80% of total starch production. The main part of the produce is converted into syrups of sugars to be used as sweeteners or substrate for fermentation processes where in the end product may be ethanol and more recently lactic acid for the production of PLA (polylactic acid) (Colman, 2003). Starch is used in both food and non-food applications. As an ingredient in foods, its main function is as a thickener to add viscosity and texture to food products; but there are also other uses such as coatings and extruded products (Jobling, 2004). In non-food applications the larger part of the starch is consumed in the paper industry (Röper, 2002). It has uses in the wet end parts as a retention aid and to bind the cellulose fibres but is also applied for coating of paper. Other non-food uses are, for example, adhesives and in oil drilling.

24.3 Potentiality of Amylolytic Yeasts

Amylolytic yeasts like *Lipomyces* and *Schwanniomyces* were evaluated for the production of single cell protein (SCP) from starch with an average yield up to 0.52 g /g. In the “Symba-process” a two- stage fermentation process overcomes the

drawback of slow growth. In the first stage only *Endomycopsis fibuligera* is continuously cultivated and in the second stage the main part of the starch containing medium is converted to microbial biomasses by a mixed cultures consisting of fodder yeast *Candida utilis,* and full-grown culture of *E. fibuligera* is continuously added to this (Thi Son and Behrens, 1977). Similar protocol has been successfully employed for *Schw. castellii* (Moresi et al., 1983), *S. diastaticus* (Odgen and Tubb, 1985), *Schw. castellii* and *E. fibuligera* (Rossi and Clementi, 1985), *Sacch. fibuligera* and *Candida utilis* (Pasari et al., 1989), and for a patented fermented food product using *Schw. occidentalis* (Galzy et al., 1988).

Yeast strains are known to produce α-amylase and glucoamylase except a few, which produce only α-amylase or glucoamylase or pullulanase. These enzymes are produced extracellularly. So far, there is no report on β-amylase production by yeasts. Several workers observed that amylose and amylopectin could be completely degraded by a combination of α-amylase and glucoamylase. Many species of yeasts belonging to *Aerobasidium, Brettanomyces, Candida, Chalara, Debaryomyces, Endomycopsis (Saccharomycopsis), Filobasidium, Hansenula, Lipomyces*, *Leucosporidium, Pichia, Saccharomyces, Schwanniomyces, Torulopsis* and *Wingea* produce varied types of amylases (DeMot and Verachtert, 1985).

Laluce et al. (1988) isolated yeast strains capable of fermenting starch and dextrin to ethanol from samples collected from Brazilian factories in which cassava flour is produced. One strain (DI-10) fermented starch rapidly and secreted 5 times as much amylolytic enzyme than that observed for *Schwanniomyces alluvius* UCD 54-83. This strain and three other similar isolates were classified as *S. cerevisiae var diastaticus* by morphological and physiological characteristics and molecular taxonomy.

The constitutive nature of glucoamylsae produced and its lack of catabolite repression, resistance to ethanol inhibition and the nature of starch hydrolytic products were the important attributes enabling the *E. fibuligera* NRRL 76 strain to efficiently convert starch directly to ethanol (Reddy and Basappa, 1993). An amylolytic strain of *S. cerevisiae* growing optimally at pH 5.0 and 60°C was isolated from yam tuber, and the results are discussed in relation to the potential use of this amylolytic yeast in the brewing industry in Nigeria (Olasupo et al., 1996).

Fifteen yeast strains were isolated from natural sources including fruits, soil, molasses, honey and a variety of indigenous fermented foods. Screening of these strains for growth, ethanol production and glucoamylase activity led to selection of a yeast strain SM-10 identified as *S. diastaticus* having maximum glucoamylase activity (80 U ml^{-1}) and ethanol production from starch (3.5%). Ethanol production from wheat flour was found to be 1.75% which could be increased to 5.2% after treatment of wheat flour with pepsin, diastase and glucoamylase (Sharma et al., 2002).

Marcha or *Murcha* is a traditional amylolytic starter used to produce sweet-sour alcoholic drinks, commonly called *Jnaar* in the Himalayan regions of India, Nepal, Bhutan, and Tibet (China). Twenty yeast strains were isolated from six samples of *marcha* and identified by genetic and phenotypic methods. They were first classified into four groups (Group I, II, III, and IV) based on physiological features using an API test. Phylogenetic, morphological, and physiological characterization led to

the identification of the isolates as *Saccharomyces bayanus* (Group I), *Candida glabrata* (Group II), *Pichia anomala* (Group III), and *Saccharomycopsis fibuligera*, *Saccharomycopsis capsularis* and *Pichia burtonii* (Group IV). Among them, the Group I, II, and III strains produced ethanol. The isolates of Group IV had high amylolytic activity. Because all *marcha* samples tested contained both starch degraders and ethanol producers, it was hypothesized that all four groups of yeast (Group I, II, III, and IV) contribute to starch-based alcohol fermentation. (Tsuyoshi et al., 2005).

24.3.1 Production and Characterization of Yeast Amylases

Among variety of yeasts, *Saccharomycopsis fibuligera*, *Schwanniomyces castellii* and *Saccharomyces diastaticus* have been found to be more promising amylase (Clementi et al., 1980; Sandhu et al., 1987) and ethanol producers. Amylases of *Lipomyces, Torulopsis* and *Schwanniomyces alluvius* have also been studied to some extent. Different substrates like maltose, soluble starch and glucose were used as substrates (de Mot et al., 1984) and effect of medium composition and culture conditions were studied on amylases production (Oteng-Gyang et al., 1980; Boze et al., 1987).

24.3.1.1 Effects of Carbon Sources

In assimilation or fermentation of starch, the regulatory roles of constitutive and inducible enzymes are important. If high levels of amylase activity accumulated with non-inducing carbon sources, such as glucose, then the major production appears to be constitutive enzyme as in the case of *Pichia polymorpha* (Moulin et al., 1982) and *Saccharomycopsis fibuligera* (Volkova et al., 1978). With several other species, substrates containing α-1, 4 linked glucose units including maltose and dextrins were capable of inducing significantly enhanced levels of amylase (Moulin and Galzy, 1978; de Mot and Verachtert, 1986b). Methylglucoside and cyclodextrins were also found to be stimulating amylase secretion (de Mot and Verachtert, 1986a). Compounds having no α-1, 4 linked glucose such as melezitose for *Schwanniomyces occidentalis* (Clementi and Rosi, 1986) and melibiose, cellobiose and glycerol for *Candida antarctica* (de Mot and Verachtert, 1987b) can also induce amylase accumulation even higher than with starch.

Reduced synthesis of constitutive or inducible enzyme in presence of glucose or other readily utilizable carbon sources is known as catabolite repression. This phenomenon is equally involved in the regulation of amylase production by several yeasts, including *Filobasidium capsiligenum* (de Mot and Verachtert, 1987a), *Lipomyces starkeyi* (Moulin and Galzy, 1979), *Saccharomyces diastaticus* (Searle and Tubb, 1981), *Saccharomycopsis fibuligera* (Afanaseva and Burd, 1980) and *Schwanniomyces occidentalis* (Wilson et al., 1982).

24.3.1.2 Effect of Different Nitrogen Sources, Aeration and Temperature

Nitrogen requirement for optimal amylase production is species dependent (de Mot et al., 1984). This may be supplemented with ammonium sulfate, peptone, yeast extract and yeast nitrogen base. Wheat and rice brans also improve the accumulation of yeast amylases. The enzyme secretion is not coupled with active cell multiplication and maximum levels are typically obtained in stationary phase cultures. In continuous culture, the optimum conditions for biomass production may differ from those for amylase production. Mycelial forms secrete amylases more actively than the corresponding yeast forms. Phosphate enhanced starch hydrolysis by *S. diastaticus* through release of glucoamylase (Kleinmann et al., 1988; Kumar and Satyanarayana, 2001). Excretion of these enzymes by *Schw. alluvius* was stimulated by dissolved oxygen (Calleja et al., 1986). Effect of growth temperature on amylase production by *Lipomyces kononenkoae* showed that accumulation of enzyme is impaired with temperature closer to maximum (Estrela et al., 1982).

24.3.1.3 Determination of Amylase Activity

Protocols for amylase assay, physico-chemical characteristics, catalytic properties and regulatory aspects of extra cellular enzymes produced by yeasts have been reviewed by de Mot (1990). These methods are classified mainly into amyloclastic detection of reducing sugars, enzymatic determination of hydrolyzed products and use of chromogenic substrates. Each method is having its own merits and demerits. When the organism is producing more than one type of amylases, one will interfere in the estimation of other enzyme. Several workers have used certain compounds to arrest the interference in amylase assays (Miyoshi, 1975; Irshad and Sharma, 1986).

24.3.1.4 Purification and Characterization of Amylases and Killer Protein

Amylases have been purified from cultures of bacteria, fungi and yeasts by using conventional protein purification techniques, such as salting out, solvent precipitation, molecular sieving, ion exchange and affinity chromatography. In the affinity method starch and glycogen are used as adsorbents. High and specific affinity of glucoamylases for acarbose was used as ligand in affinity chromatography, using starch (Schwimmer and Balls, 1949) and glycogen (Schramm and Loyter, 1966) as adsorbents.

Both α-amylase and glucoamylase were purified and characterized from amylolytic yeasts like *E. fibuligera* (Sukumavasi et al., 1975; Ueda and Saha, 1983; Gogai et al., 1987) and *Schw. alluvius* (Wilson and Ingledew, 1982) using conventional purification techniques. Raw starch digesting yeast α-amylase from *L. starkeyi* was also studied (Punpeng et al., 1992). Secretion of α-amylase and multiple forms of glucoamylase by *Trichosporon pullulans* were studied by de Mot and Verachtert (1986).

Electrophoretic analysis showed that α-amylase activity was due to a single monomeric protein. Glucoamylase, however, occurred in multiple forms. The four glucoamylases and the α-amylase were glycoproteins. Modena et al. (1986) attempted biochemical and immunological characterization of the STA2-encoded extracellular glucoamylase from *S. diastaticus.*

Cryptococcus sp. S-2 raw-starch-digesting and thermostable α-amylase (AMY-CS2) was purified, characterized, cloned and sequenced. An open reading frame of the cDNA specified 611 amino acids, including a putative signal peptide of 20 amino acids. The N-terminal region of AMY-CS2 had 49.7 percent similarity with the whole region of α-amylase from *A. oryzae* (Taka-amylase), whereas the C-terminal region had a sequence similar to the C-terminal region of glucoamylase G1 from *A. niger*. A mutant AMY-CS2 lacking the C-terminal domain lost not only its ability to bind or digest raw starch, but also its thermo stability (Iefuji et al., 1996).

The glucoamylase from *E. fibuligera* NRRL 76 culture was purified using ion - exchange chromatography and gel filtration. The molecular mass of this enzyme was 40 kDa. The pH and temperature optima for its activity were 5.5 and 55°C, respectively. It was inhibited by Cu and Hg ions. The enzyme hydrolyzed amylopectin more specifically and maximally but not amylose. The K_m values for amylopectin and soluble starch were 2.8 and 4.0 respectively indicating that the enzyme had more affinity to amylopectin (Reddy and Basappa, 2000).

Chen et al. (2000) isolated, purified and characterized a killer protein from *Schwanniomyces occidentalis*. It produces a killer toxin lethal to sensitive strains of *S. cerevisiae*. Killer activity is lost after pepsin and papain treatment, suggesting that the toxin is a protein. They have purified the killer protein and found that it was composed of two subunits with molecular masses of approximately 7.4 and 4.9 kDa, respectively, but was not detectable with periodic acid-Schiff staining. A BLAST search revealed that residues 3 to 14 of the 4.9 kDa subunit had 75% identity and 83% similarity with killer toxin K2 from *S. cerevisiae* at positions 271 to 283. Maximum killer activity was between pH 4.2 and 4.8. The protein was stable between pH 2.0 and 5.0 and inactivated at temperatures above 40°C. The killer protein was chromosomally encoded. Mannan, but not β-glucan or laminarin, prevented sensitive yeast cells from being killed by the killer protein, suggesting that mannan may bind to the killer protein. Identification and characterization of a killer strain of *Schw. occidentalis* may help reduce the risk of contamination by undesirable yeast strains during commercial fermentations.

24.3.1.5 Inhibitors

Substances that affect the activity of fungal glucoamylases are α-glucosides, maltitol, amino alcohols and deoxy nojirimycin. They have similar effect on *C. antarctica* enzyme. Amylase inhibitors produced by *Actinoplanes* and *Streptomyces* species have been isolated and implicated in therapy of diabetes and obesity (Starch blockers).

These are acarbose, aplanin, trestatins and amylostatins and are characterized as pseudo-oligosaccharides. Both α-amylase and glucoamylase from *C. antarctica* were inhibited by aplanin and trestatins (de Mot and Verachtert, 1987). Inhibition of glucoamylase by acarbose was demonstrated in *C. tsukubaensis* (de Mot et al., 1985) and *F. capsuligenum* (de Mot and Verachtert, 1985).

24.3.1.6 Physico-Chemical Characteristics

Relative molecular mass (Mr) of α-amylases and glucoamylases vary between 40,000 to 80,000. The high molecular weight forms (670,000) contain up to 80% carbohydrate and may occur as dimers. In general carbohydrate moieties enhance thermal stability. Amino acid composition has been determined for the amylases produced by *C. antarctica* (de Mot and Verachtert, 1987) and *S. diastaticus* (Erratt and Stewart, 1981; Tucker et al., 1984).

24.3.1.7 Effect of pH and Temperature

The pH optima for the activity of most amylases lie in the range of 4.0 to 6.5. Optimum temperature for α-amylase (40–50°C) is lower than that for glucoamylase (50–60°C). Truly thermostable amylases, as produced by several bacteria, are not yet reported for yeasts. Thermolabile enzymes like yeast amylases are particularly important when they are intended for use in low-calorie beer production, as the added enzyme is inactivated during pasteurization (Sills et al., 1983).

24.3.1.8 Substrate Specificity

High molecular weight starchy substrate is better for both α-amylase and glucoamylase. The glucoamylases of *S. diastaticus* have relatively high activity on smaller malto-oligosaccharides, but the debranching activity is low (Tucker et al., 1984). The glucoamylase of *E. fibuligera* could digest different kinds of raw starch. Waxy starches were more easily digested than cereal starches. Cassava starch was easily digested than cornstarch. Debranching isoamylase activity was found only in *L. kononenkoae* (Spencer-Martins, 1982). Pullulanase debranching activity was demonstrated in several yeast species such as *F. capsuligenum* and *Sacch. fibuligera* (Kato et al., 1976). α-glucosidase with significant activity on starch and amylopectin was secreted together with α-amylase by *L. starkeyi* (Kelly et al., 1985). An extracellular transglucosidase from *Sacch. capsularis* with activity on maltose and malto-oligosaccharides was characterized by Ebertova (1966) and its presence in commercial glucoamylase is undesirable because it converts maltose and malto-oligosaccharides into polymer (Fogarty and Kelly, 1980). A cyclodextrinase from *L. kononenikoae* was also identified by Spencer-Martins (1984).

24.3.1.9 Enzyme Adsorption and Degradation of Raw Starch

Adsorption, an important feature of raw starch hydrolysis has received little attention as far as yeast amylases are concerned. This phenomenon is well known for various amylolytic enzymes from other sources (Fuwa, 1982; Clarke and Swenson, 1984; Akaki et al., 1984). It helps in reducing inputs in the form of heat energy involved in gelatinization of starch. Digestion of starch granules by yeast glucoamylases has been reported for *C. antarctica*, *Rhodosporidium* spp and *Sacch. fibuligera* (Takaya et al., 1982; Ueda and Saha, 1983). Characterization of a novel α-amylase (raw starch degrading) from *L. kononenkoae* and expression of its gene (*LKA1*) in *S. cerevisiae* was studied by Steyn and Pretorius (1995).

24.4 Ethanol Production from Starch

Although many sugars are fermented and assimilated by yeasts, the ability to degrade starch is not widespread among them (Table 24.5). However yeasts that are capable of degrading starch have been investigated as promising microorganisms for the conversion of starchy materials to single cell protein and ethanol. Several starch-degrading yeasts are currently recognized. The extent of starch degradation and amylase secretion is strain dependent (de Mot et al., 1984).

24.4.1 Conventional Method of Fermentation

The conversion of starchy substrate to ethanol is a three-step process involving liquefaction with α-amylase, saccharification with glucoamylase and alcoholic fermentation of the resultant glucose by non-amylolytic *S. cerevisiae* strain. The enzyme sources are generally from bacteria and fungi. The cost of production of the isolated enzymes is generally high. Several approaches have been made to reduce the cost of the process in the recent years. Ethanol production from fresh tubers, flour and starch of tapioca was studied by simultaneous saccharification and fermentation procedure. In this process 20 percent slurry yielded 8–9% ethanol amounting to 95% fermentation efficiency (Srikanta et al., 1987). Simultaneous solid phase fermentation and saccharification of cassava fibrous residue yielded more ethanol compared to the fermentation of liquid hydrolysate (Jaleel et al., 1988).

Several workers used sweet potato as a substrate since it contains considerable amount of starch and reducing sugars. The acid-liquefied tuber starch was saccharified by utilizing *Rhizopus niveus* as the source of glucoamylase, prior to alcoholic fermentation by *S. cerevisiae* (Sreekantaiah and Satyanarayana, 1980). The enzyme liquefied and saccharified tubers were fermented by *S. cerevisiae* after the pretreatment with pectinase (Toyama et al., 1984). William and Francis (1982) studied the effect of lye - peeling conditions on phenolic destruction, starch hydrolysis and

Table 24.5 Ethanol from dextrin/starch by amylolytic yeasts

Yeast strain	Substrate (%)	Ethanol (% w/v)	Yield (% theoretical)	Productivity (g/l/h)	Reference
C. shehatae IGC 3504	Dextrin (22.5)	6.5	50.9	–	de Mot et al. (1985)
S. diastaticus	Liquefied Starch (16.3)	10.5	92.0	1.12	Tamaki (1986)
S. diastaticus	Liquefied Starch (40)	12.0	–	1.25	Laluce and Matton (1984)
S. diastaticus NCYC 625	Dextrin (40)	12.0	58.0	–	Amin et al. (1985)
Schw. occidentalis ATCC 26074	Soluble starch (2.5)	1.4	99.3	0.20	Calleja et al. (1982)
Schw. occidentalis CBS 2863	Dextrin (30)	4.2	27.5	–	Amin et al. (1985)
S. diastaticus NCYC 625 + *Schw. occidentalis* CBS 2863	Dextrin (30)	10.9	71.3	–	Amin et al. (1985)
Sacch. fibuligera NRRL Y-1062	Mashed wheat (20)	3.7	54.0	0.38	Wickerham et al. (1944)
S.cerevisiae NRRL Y-25 + *Sacch. fibuligera* NRRL Y-567	Mashed wheat (20)	5.5	78.3	0.57	Wickerham et al. (1944)
S. diastaticus NCYC 625 + *Sacch. fibuligera IGC* 3961-18	Dextrin (25)	8.0	62.9	9.6	Amin et al. (1985)
S. cerevisiae	Sweet potato (50)	6.6	87.7	–	Saha and Ueda (1983)
Schw. castellii ATCC 26077	Cassava starch (20)	7.35	79	–	Pasari et al. (1989)
E. fibuligera NRRL76	Cassava starch (20)	7.35	77	–	Reddy and Basappa (1993)
E. fibuligera NRRL76	Soluble starch (20)	4.4	60	–	Reddy and Basappa (1993)
Candida tropicalis	soluble starch (9)	4.31	–	0.65	Jamai et al. (2007)

Abbreviations: *Asp - Aspergillus*; *C - Candida*; S - *Saccharomyces*; *Sacch - Saccharomycopsis*; *Schizo - Schizosaccharomyces;* Schw - *Schwanniomyces.*

carotene loss in sweet potato. Manlan et al. (1985) developed a process for the manufacture of instant drum dried flakes. McArdle and Bowkamp (1986) suggested that rapid heating of sweet potato mash to 80°C optimized conversion of starch to sugar and then to ethanol, since the heat induced β-amylase activity inherent in sweet potato helped in the formation of maltose.

Alcoholic fermentation of raw sweet potato was carried out in a one-step process, which combined the conventional process of liquefaction, saccharification and fermentation to ethanol, by several researchers. Matsuoka et al. (1982) used *Rhizopus* glucoamylase for raw starch digestion. Ueda and Saha (1983) used *E. fibuligera* glucoamylase for raw starch digestion in the fermentation to ethanol by yeast. A detailed study on red variety of sweet potatoes was carried by Reddy and Basappa (1997). It contained 14–15% starch, 3–5% free sugars and 1.0 to1.2% pectin on wet weight basis. Hand- peeling method of steamed sweet potato yielded more dry flour (5.1 kg) from 20 kg fresh sweet potato than lye- peeling (4.1 kg) and abrasive-peeling (4.8 kg) methods. Addition of pectinase at 60 U ml^{-1} followed by gelatinization before mashing of sweet potato or its flour, facilitated conversion of starch to fermentable sugars by amylases. Fermentation of the mash with *S. cerevisiae* by modified Toyama's method yielded more ethanol than that of CFTRI method and Toyama's method. A wine-like product with ethanol up to 8.6 percent (w/v) with pleasant aroma and colour was also prepared (Reddy and Basappa, 1997).

24.4.2 Immobilized Yeast and Amylases for Ethanol Fermentation to Starch

Immobilized microorganisms have been tried for the production of beer (White and Portno, 1978) and wine (Gestrelius, 1982). Basically these processes are quite similar to the production of ethanol from immobilized cells. In the later case, specific ethanol productivity and final ethanol concentration are the major parameters for evaluating the process, which depend on temperature and microbial contamination. Immobilization techniques can be put into three major groups. The carrier binding method can be based on physical, ionic or covalent binding. Immobilization by entrapment represents another method and is achieved by retaining the microorganisms within the small beads of highly polymerized gel or by covering them with semi-permeable membrane. The third procedure involves cross-linking. These methods generally used to produce ethanol using *S. cerevisiae* (Bandhopadhyay and Ghose, 1982). Mc Ghee et al. (1984) immobilized *S. cerevisiae* and glucoamylase on calcium alginate for ethanol fermentation using 10% starch hydrolysate as a substrate. Immobilization of cells of *S. diastaticus* and *E. fibuligera* in polyurethane foam cubes has been used for conversion of dextrin to ethanol by Amin et al., (1985). Several other researchers have used hydrolysates of Stover and Israel artichoke as substrates with immobilized *S. cerevisiae* for ethanol production (Silton and Gaddy, 1980; Joshi and Yamazaki, 1984). Schafhauser and Story (1993) used co- immobilization of amyloglucosidase and pullalanase on granular chicken

bone for enhanced starch fermentation. Lee et al. (1993) have studied the co-immobilization of *A. awamori* and *Z. mobilis*, and *R. japonicus* and *Z. mobilis* for direct ethanol production from raw starch. Chaudhary and Chincholkar (1996) reviewed the cell immobilization techniques for ethanol production.

S. diastaticus cells were immobilized onto beech wood chips of different particle size (1.84–1.92) and at three pH values (5.0–6.0) had a positive effect on the immobilization process. The chosen carrier, 1.84 mm sized wood chips adsorbed 150 mg dry cell mass per g dry carrier mass. The kinetics of immobilized cell systems in ethanol production has been studied in a packed bed-reactor. Ethanol production and the respiration quotient were at a maximum at a dilution rate of 0.16 h^{-1}. The reactor was operated under steady-state conditions for 30 days at the dilution rate 0.16 h^{-1} (Razmovski et al., 1996).

The performance of co-immobilized *S. cerevisiae* and amyloglucosidase was evaluated in a fluidized-bed reactor. Soluble starch and yeast extracts were used as feed stocks. Conversion of soluble starch streams to ethanol has potential practical applications in corn dry and wet milling and in developmental lignocellulosic processes. The biocatalyst performed well, and demonstrated no significant loss of activity or physical integrity during 10 week of continuous operation. The reactor was easily operated and required no pH control. No operational problems were encountered from bacterial contaminants even though the reactor was operated under nonsterile conditions over the entire course of experiments. Productivities ranged between 25 and 44 g ethanol/l/h. The experiments demonstrated that ethanol inhibition and bed loading had significant effects on reactor performance (Sun et al., 1997).

Giordano et al. (2000) studied the conditions for achieving a stable biocatalyst to be used in the production of ethanol from starch. Different pellets were used depending on which characteristic of the biocatalyst was being studied: (a) *S. cerevisiae* entrapped in pectin or calcium alginate gel particles; (b) silica containing immobilized glucoamylase entrapped in pectin gel particles; or (c) pectin gel particles, with the silica-enzyme derivative and yeast coimmobilized. The influence of several variables on the mechanical resistance of the particle, on the viability of the microorganism, and on the rate of substrate hydrolysis was studied with biocatalyst. The best conditions found were 6% pectin gel, 2 mm particle diameter, and cure in 0.2 M $CaCl_2$ $2H_2O$/60 mM acetate buffer, pH 4.2, for gel preparation; and 6.0 g l^{-1} of $CaCl_2$ $2H_2O$ in the fermentation medium. Biocatalyst (c) was successfully tested for the production of ethanol from liquefied manioc flour syrup.

Simultaneous saccharification and fermentation (SSF) experiments were performed using small, uniform kappa-carrageenan beads (1.5–2.5 mm in diameter) of co-immobilized glucoamylase and *Z. mobilis*. They obtained very low levels of glucose were observed in the reactor, which indicating that saccharification was the rate-limiting step. But separate hydrolysis and fermentation (SHF) experiments, dextrin feed solutions of 150–160 g l^{-1} were first pumped through an immobilized-glucoamylase packed column. At 55°C and a residence time of 1 h, greater than 95% conversion was obtained, giving product streams of 162–172 g glucose l^{-1} (Krishnan et al., 1999). Co-immobilized cells of *S. diastaticus* and *Z. mobilis*

produced a high ethanol concentration as compared to immobilized cells of *S. diastaticus* during batch fermentation of liquefied cassava starch. In repeated-batch fermentation using co-immobilized cells, the ethanol concentration increased. The co-immobilized gel beads were stable up to seven successive batches. Continuous fermentation using co-immobilized cells in a packed bed column reactor was operated and it exhibited maximum ethanol productivity (Amutha and Gunasekaran, 2001).

A simultaneous saccharification and fermentation (SSF) process was investigated by Fujii et al. (2001) to produce ethanol using two kinds of cellulose carriers that were respectively suitable for immobilization of *A. awamori* and *Saccharomyces pastorianus*. The maximum ethanol concentration attained by the batch operation was 25.5 g l^{-1}. Under suitable conditions, both cellulose carriers with immobilized cells could be reused efficiently for three cycles. The total amount of ethanol production was 66.0 g (per 1 litre working volume) after the repeated operation. Ethanol productivity mainly depends on a saccharification process. There is a limit in durability in the repeated batch operation, and it is important to maintain high activity of the fungus in order to produce ethanol efficiently.

Candida tropicalis is capable of fermenting starch at a low rate. To enhance corn soluble starch utilization and increase the rate of alcohol production, it was pretreated with alpha-amylase. Starch liquefaction was sufficient to drive the fermentation and to convert 96% substrate to ethanol. Indeed, in the presence of exogenous alpha-amylase, 9% (w/v) soluble starch was converted to 43.1 g ethanol/l in 65 h with a productivity of 0.65 g/l h. Thus, bio-ethanol production using free and calcium alginate-immobilized *C. tropicalis* does not require the saccharification step. Furthermore, fed-batch fermentation by free *C. tropicalis* cells increased the final concentration to 56 g ethanol/l, reaching published values for *S. cerevisiae* recombinant strains expressing both alpha-amylase and glucoamylase (Jamai et al., 2007).

24.4.3 Fermentation of Starch to Ethanol by Monoculture

In a comparative study of most known starch degrading yeasts, it was shown that they were not completely suitable for direct ethanol fermentation of starch (de Mot and Verachtert, 1985). In addition, some amylolytic yeasts were unable to ferment (Kreger-van Rij, 1984) glucose, maltose, and dextrins. *E. fibuligera* and *Schw. castellii* are the two promising species known to directly ferment starch to ethanol (Frelot et al., 1982). Amyloglucosidase rather than α-amylase was the limiting factor in these cases (Casey et al., 1984). Strains of *E. fibuligera* on wheat mash gave more ethanol production (54.4%) under aerated conditions than the non-aerated cultures (5.8%) (Wickerham et al., 1944). Calleja et al. (1982) observed that *Schw. occidentalis* fermented 2.5% soluble starch to almost completion (99.3%) with little unwanted metabolites. However, it was demonstrated subsequently that it is not suitable at higher concentration of starch for economic recovery of ethanol (Amin et al., 1985; Malfait et al., 1986). On the other hand, when *Schwanniomyces* cells or their enzyme preparation (Malfait et al., 1986) was used along with non-amylolytic yeast; higher

yield of ethanol was obtained. The fermentation characteristics of a large number of starch degrading yeasts were compared by de Mot et al. (1985).

Fermentation of dextrins to ethanol by monocultures of yeast has resulted in better yield. The strains of *S. diastaticus* produced 10.5–12% (w/v) ethanol from dextrins (Laluce and Matton, 1984; Amin et al., 1985). Among a wide range of starchy substrates used in ethanol production by the yeast strains, cassava starch and waxy rice were found to be the best (Reddy and Basappa, 1993). When *E. fibuligera* NRRL 76 was grown optimally at pH 6 and 30°C for 1 day under aeration followed by 3 days without aeration, maximum ethanol concentration was 92.0 g l^{-1} and 73.5 g l^{-1} in media containing 300 g l^{-1} and 200 g l^{-1} of cassava starch respectively, as compared to 73.5 g l^{-1} and 77.3 g l^{-1} of ethanol by another promising strain of *Schw. castellii* ATCC 26077 under similar set of conditions. This process employing *E. fibuligera* NRRL 76 was found to be better than other methods reported earlier. *E. fibuligera* NRRL 76 maintained its activity at high concentrations of cassava starch (400 g l^{-1}) and waxy rice (250 g l^{-1}) (Reddy and Basappa, 1993).

24.4.4 Fermentation of Starch to Ethanol by Mixed Cultures

In view of the low yield of ethanol from starch and dextrin by monocultures, mixed culture fermentation was tried. Monoculture fermentation of mashed wheat by *Sacch. fibuligera* gave a theoretical yield of 54% of ethanol as compared to 78.3% in conjunction with *S. cerevisiae* (Laluce and Matton, 1984). Abouzied and Reddy (1986) showed that mixed inoculum of *A. niger* and *S. cerevisiae* fermented soluble starch to ethanol with conversion efficiency of 96% of the theoretical maximum. They further demonstrated that synergistic co-cultures of an amylolytic yeast *Sacch. fibuligera* and *S. cerevisiae* fermented soluble starch to ethanol with conversion efficiency of over 90% of the theoretical maximum and the synergistic effect of mixed inoculum was further enhanced under anaerobic condition. Such beneficial effects were also shown in co-culturing of *Sacch. fibuligera* and *L. kononenkae* (Abouzied and Reddy, 1987). Similar encouraging results were obtained with combination of *Schw. occidentalis, S. diastaticus and Sacch. fibuligera* on dextrin (Amin et al., 1985). Dostalek and Haggstrom (1983) used the mixed culture of *Sacch. fibuligera* and *Z. mobilis* on starch using oxygen as a regulator. Glucose production from starch was the rate-limiting step in the above system causing a lower ethanol production.

Addition of *Sacch. fibuligera* enzymes to sweet potato and fermentation by *S. cerevisiae* gave encouraging percentage of theoretical yields of ethanol ranging from 82.0 to 87.7% (Erratt and Stewart, 1981). Pirselova et al. (1993) reported static fermentation of starch to ethanol by a co-culture of *Sacch. fibuligera* and *S. cerevisiae* without addition of nutritional supplements. Mixed culture of *E. fibuligera* NRRL76 and *Z. mobilis* ZM4 could directly and more efficiently ferment cassava starch (22.5% w/v) to ethanol (10.5% w/v) than the monocultures (Reddy and Basappa, 1996). Combination of culture filtrates of *E. fibuligera* containing

amylases and *Z. mobilis* efficiently saccharified and fermented cassava starch to ethanol. Addition of 0.1% glucoamylase further enhanced the efficiency to 90% of the theoretical value, indicating that this enzyme is a rate- limiting factor in *E. fibuligera* (Reddy and Basappa, 1996).

Potatoes exploded at high pressure were hydrolyzed into a low molecular liquid starch and then easily converted to ethanol by simultaneous saccharification and fermentation, using a mixture of the amylolytic, *A. awamori*, and fermentative *S. cerevisiae* cultures. Maximal ethanol production was 4.2 g l^{-1} in batch and continuous culture produced 3.6 g l^{-1} (Kobayashi et al., 1998).

Farid et al. (2002) studied the influence of different agitation speeds on alcoholic fermentation by free and immobilized cells of *S. cerevisiae* in a co-culture with free cells of *A. awamori* was investigated. Starch hydrolysis and glucose, glucoamylase and α-amylase accumulation in the fermentation medium was affected by agitation speed. Maximum amounts of the enzymes were obtained at 150–200 rpm after 72 h where, maximum growth and glucose accumulations were noticed at 200–300 rpm. Alcohol production was stimulated by low agitation speed (50 rpm) and decreased at higher speeds. The results also showed that the amount of produced alcohol was affected by the time of yeast inoculation. When the inoculation of yeast was carried out after the growth of fungi for 72 h, the amounts of produced alcohol increased by 84, 75, 89 and 68% at 50, 100, 150 and 200 rpm, respectively than that produced when the two organisms were inoculated together at the beginning of the fermentation process. A batch culture of the two organisms produced about 2% (v/v) alcohol from 12% (w/v) corn starch in 72 h at 50 rpm. On the other hand, the immobilized yeast and suspended *A. awamori* produced more alcohol reaching 3.7% (v/v) at 200 rpm under the same cultivation conditions.

24.4.5 Raw Starch Fermentation

As in case of moulds, raw starch digesting enzymes are also found in yeasts. Use of such enzymes reduces 30–40% of the total heat energy required in gelatinization of starch. In a single step process of raw starch fermentation utilizing the glucoamylase of *A. niger* (Ueda and Koba, 1980; Ueda et al., 1981), *Rhizopus* (Matsuoka et al., 1982) and *Endomycopsis* (Ueda and Saha, 1983), it is possible to produce ethanol by using yeasts. Thammarutwasik et al. (1986) produced ethanol (12.9% v/v) from raw sorghum (35% w/v) after hydrolysis with *A. niger* glucoamylase. They showed that addition of enzymes like cellulosin HC reduced the fermentation time and increased alcohol yield. Saha and Ueda (1983) studied the alcoholic fermentation of raw sweet potato by a nonconventional method using the glucoamylase of *E. fibuligera*. Svendsby et al. (1982) observed that pectin depolymerase allowed glucoamylase to act easily on sweet potato by reducing the viscosity of the broth.

Raw corn-starch fermented by *sake* yeast after hydrolysis with glucoamylase of *Chalara paradoxa* gave higher yield of 92.1% (Mikuni et al., 1977). Similar results

were also obtained with uncooked sweet potato (Svendsby et al., 1982). The *Rhizopus* Koji, which contained both glucoamylase and yeast cells fermented raw cassava starch yielding ethanol up to 14% (w/v). The productivity (2.3 g/ l/ h) of this fermentation was about 50% as compared to glucose yeast system (Fujio et al., 1985). Saccharifying enzyme from *Corticium rolfsii* and simultaneous fermentation using *Schizo. pombe* yielded high concentration of ethanol (18.5%) from 30% raw corn-starch (Hariantono et al., 1991). Ueda et al. produced aromatic red rice wine by fermenting uncooked red rice containing anthocyanin pigments (Ueda et al., 1991).

Singh et al. (1995) used crude glucoamylase preparation from *Rhizoctonia solani* to saccharify raw and cooked starch. Various concentrations of potato starch and wheat flour from 10–40% (w/v) was used for mashing but 30% was found to be the optimal and economical. The saccharified mash yielded 5.89% (v/v) ethanol in a simultaneous saccharification and fermentation process using a yeast strain *S. cerevisiae* (SC-39) at 35°C for 4 days. Removal of inhibitory substances from the fermenting broth through dialysis caused considerable increase in ethanol production.

Ethanol production, by a simultaneous saccharification and fermentation process from raw wheat flour, has been performed by *S. cerevisiae* and a low level of amyloglucosidase enzyme. The fermentation time was about 60 h after a 6 h pre-saccharification, with an amyloglucosidase (AMG) level of 270 AGU. kg^{-1} starch, but only 31 h with a simultaneous saccharification fermentation process (SSF). When an AMG level of 540 AGU kg^{-1} starch was used, the time decreased to 21 h, giving an ethanol concentration of 67 g l^{-1}. Sugar composition of the wort after the liquefaction may be responsible for the difference between these two processes. Maltose, a fermentable sugar, was produced in high concentration during the liquefaction, allowing a shorter process period, counteracting the effect of the slow starch hydrolysis at 35°C (Montesinos and Navarro, 2000).

Shigechi et al. (2004) succeeded in producing ethanol directly from raw corn starch using yeast strain YF207/pGA11/pUFLA, which co displays *R. oryzae* glucoamylase and *S. bovis* α-amylase, using α-agglutinin and Flo1p with no time lag in the decrease in corn starch. In sequential reactions of α-amylase and glucoamylase co displayed on the cell surface, raw corn starch was hydrolyzed to glucose. The non-cooking fermentation system using a cell surface-engineered yeast strain, thus, promises to be a very effective method in reducing the production costs of ethanol. In 72 h fermentation, this strain produced 61.8 g of ethanol l^{-1}, with 86.5% of theoretical yield from raw corn starch.

24.4.6 Factors Impairing Starch Fermentation

The degradation and fermentation of starch to ethanol is a multifactor-controlled process. Starch degradation is impaired by retrogradation. Amylose is unstable with a tendency to precipitate spontaneously because of its molecular shape and linear chain configuration, which align them by hydrogen bonding, to form aggregates.

This process is essentially irreversible. Retrograded amyloses will only redissolve in alkaline solution. Selecting non-retrograding amylopectin rich substrates like waxy rice varieties may solve this problem.

Non-*Saccharomyces* amylolytic species including *Schw. occidentalis* have lower ethanol tolerance and hence are not economical in ethanol production from starch (Demot et al., 1985). In *Schw. occidentalis*, the activity of α-amylase is impaired, much more strongly than glucoamylase, by increased ethanol concentration (Malfait et al., 1986). Besides, ethanol represses the biosynthesis of amylases in non-growing cells. In view of high initial starch concentrations used in commercial production, the yeasts amylase production need to be very high and relatively insensitive to repression by hydrolysis products. The culture conditions used in ethanol fermentation also have an effect on amylase production. *Schw. occidentalis* requires definite level of dissolved oxygen for active amylase secretion (Calleja et al., 1986). However, *S. diastaticus* grown in wort secretes more glucoamylase under anaerobic conditions (Searle and Tubb, 1981). In order to improve the one-step fermentation of starch to ethanol there are certain factors need to be understand properly. Incomplete knowledge on ethanol tolerance, genetics and molecular biology of amylolytic yeasts, low amylase production and sensitivity to repression to high starch hydrolysis products and unfavorable anaerobic conditions that exist in ethanol fermentation, are some of the constraints in the one-step fermentation of starch to ethanol. These factors should receive due cognizance while developing such a process.

The specific ethanol production rate of raw starch by arming yeast cells displaying α -amylase and glucoamylase increased significantly when the cells and starch granules settled together. The specific ethanol production rate also increased when the size distribution of starch granules was almost same as that of the yeast cells. These results indicate that the surface contact between starch granules and yeast cells is important for increasing the apparent specific activity of α-amylase, which was the rate-limiting factor of the direct fermentation (Khaw et al., 2007).

24.4.7 Improved Methods for Corn Starch Fermentation

Krishnan et al. (1999) studied the production of ethanol from industrial dry-milled corn starch in a laboratory scale fluidized-bed bioreactor using immobilized biocatalysts. Saccharification and fermentation were carried out either simultaneously or separately. Simultaneous saccharification and fermentation (SSF) experiments were performed using small, uniform kappa-carrageenan beads (1.5–2.5 mm in diameter) of co-immobilized glucoamylase and *Z. mobilis*. Dextrin feeds obtained by the hydrolysis of 15% dry milled corn starch were pumped through the bioreactor at residence times of 1.5–4 h. Single-pass conversion of dextrins ranged from 54–89%, and ethanol concentrations of 23–36 g l^{-1} were obtained at volumetric productivities of 9–15 g/l/h. Very low levels of glucose were observed in the reactor, indicating that saccharification was the rate-limiting step. In separate hydrolysis

and fermentation (SHF) experiments, dextrin feed solutions of 150–160 g l^{-1} were first pumped through an immobilized-glucoamylase packed column. At 55°C and a residence time of 1 h, greater than 95% conversion was obtained, giving product streams of 162–172 g glucose l^{-1}. These streams were then pumped through the fluidized-bed bioreactor containing immobilized *Z. mobilis*. At a residence time of 2 h, 94% conversion and ethanol concentration of 70 g l^{-1} were achieved, resulting in an overall process productivity of 23 g/l/h. At residence times of 1.5 and 1 h, conversions of 75 and 76%, ethanol concentrations of 49 and 47 g l^{-1}, and overall process.

Ponnampalam et al. (2004) conducted ethanol fermentations using both whole corn, and corn with 100% of the germ, and a portion (approximately 74%) of the fiber removed. Ethanol production increased to11% in the germ and fiber-removed corn which was more than that of the whole corn. The protein content of distiller's dried grains and solubles increased from 30 to 36%, and phosphate levels were 60% lower in corn with germ and fiber removed vs. whole corn. Removal of germ and fiber prior to fermentation allows higher starch loading and results in increased ethanol production. The integration of germ and fiber removal in the dry-grind ethanol industry could increase capacity and add valuable co products, resulting in increased productivity and profits.

Rajagopalan et al. (2005) developed an Aspen Plus modeling platform to evaluate the performance of the conversion process of degermed and defibered corn (DDC) to ethanol in 15 - and 40-million gallons per year (MGPY) dry mill ethanol plants. Upstream corn milling equipment in conventional dry mill ethanol plants was replaced with germ and fiber separation equipment. DDC with higher starch content was fed to the existing saccharification and fermentation units, resulting in higher ethanol productivity than with regular corn. The results of the DDC models were compared with those of conventional dry mill ethanol process models. A simple financial analysis that included capital and operating costs, revenues, earnings, and return on investment was created to evaluate each model comparatively. Case studies were performed on 15- and 40-MGPY base case models with two DDC process designs and DDC with a mechanical oil extraction process.

Ethanol has been utilized as a motor fuel source in the United States since the turn of the century. However, it has repeatedly faced significant commercial viability obstacles relative to petroleum. Renewed interest exists in ethanol as a fuel source today owing to its positive impact on rural America, the environment and United States energy security. Today, most fuel ethanol is produced by either the dry grind or the wet mill process. Current technologies allow for 2.5 gallons (wet mill process) to 2.8 gallons (dry grind process) of ethanol per bushel (8 gallons) of corn. Valuable co-products, distillers dried grains with solubles (dry grind) and corn gluten meal and feed (wet mill), are also generated in the production of ethanol. While current supplies are generated from both processes, the majority of the growth in the industry is from dry grind plant construction in rural communities across the Corn Belt. While fuel ethanol production is an energy-efficient process today, additional research is occurring to improve its long-term economic viability. Three of the most significant areas of research are in the production of hybrids with

higher starch content or a higher extractable starch content, in the conversion of the corn kernel fiber fraction to ethanol, and in the identification and development of new and higher-value co-products (Bothast and Schlicher, 2005).

24.5 Genetic Manipulation of Amylolytic Yeasts

Not much is known on the genetics of amylolytic yeasts except *S. diastaticus*, which is closely related to the industrial species of *S. cerevisiae*. Characterization of this species for its amylolytic genes, their polymeric nature (Erratt and Stewart, 1978; Erratt and Stewart, 1981) assessment of allelism and currently the STA nomenclature are required to designate the genes coding for glucoamylase production. However, this strain does not possess α-amylase and requires its addition to aid starch digestion. Details regarding regulatory genes (*AMY, STA 10, MAT*), control of export of glucoamylase into extra cellular medium (*CDX1*), homology of *STA* genes, nucleotide sequencing of *STA1*, physical structure of *STA* genes family, have been reviewed (de Mot, 1990). Pretorius et al. (1991) reviewed the glucoamylase multigene family in *S. diastaticus.* According to them starch utilization in the yeast *S. diastaticus* depends on the expression of the three unlinked genes, *STA1* (Chr. IV), *STA2* (Chr. II), and *STA3* (Chr. XIV), each encoding one of the extra cellular glycosylated glucoamylase isozymes *GAI*, *GAII*, or *GAIII*, respectively. The restriction endonuclease maps of *STA1, STA2*, and *STA3* are identical. These genes are absent in *S. cerevisiae*, but a related gene, *SGA1*, encoding an intracellular, sporulation-specific glucoamylase (SGA), is present.

Amylolytic yeasts such as *S. diastaticus, E. fibuligera, Schw. castellii* and *Schw. alluvius* are capable of fermenting starch and dextrin to ethanol, with low conversion efficiency. To overcome this problem, various genetic procedures including mutagenesis, hybridization, rare mating, spheroplast fusion, protoplast fusion, somatic hybridization and gene cloning have been used.

24.5.1 Mutagenesis

Due to polyploid nature of commercial strains, the usefulness of mutagenesis is limited. Auxotrophs can be selected for genes, which confer a certain phenotype. The common mutagens used are UV, X-rays, ethyl methane sulfonate (EMS), N-methyl, N-nitro-N-nitrosoguanidine (NTG), N-nitrosourea or diethylstilbestrol (Spencer and Spencer, 1983). In the case of amylolytic yeasts, UV and NTG mutagenesis in combination with 2-deoxy-D-glucose was used to develop catabolite-derepressed mutants, which were superior when compared to parent strains (Stewart et al., 1985; van Uden et al., 1980; Jones et al., 1986; Cabeca-Silva, 1982; Dhawale and Ingledew, 1983; Kate et al., 1984). For site specific genes whose structure has been elucidated, targeted or site specific, mutagenesis is possible. Auxotroph

mutants are selected in order to construct strains for use in spheroplast and protoplast fusion experiments. Lambrechts et al. (1991) studied the primary structure and regulation of a glucoamylase-encoding gene (*STA2*) in *S. diastaticus*.

Sogaard et al. (1993) studied the site-directed mutagenesis of histidine 93, aspartic acid 180, glutamic acid 205, histidine 290, and aspartic acid 291, at the active site and tryptophan 279 at the raw starch binding site in barley α-amylase1. Acarbose had high affinity for the active site and low affinity for a secondary site in barley α-amylase1. Mutants of putative catalytic residues, D180N, E205Q, and D291N, were inactive and displayed low affinity for acarbose-sepharose. H93N and H290N mutants, at invariant residues, had kcat K_m^{-1} for p-nitrophenyl maltoheptaoside of 0.3 and 1.2 percent of wild type. A corresponding 370 and 85-fold increased K_i for acarbose and lack of shifts in pH activity profiles indicated that these histidine residues participate in transition state stabilization. This finding agrees with H- bonding to OH groups of the valienamine ring of acarbose in the three-dimensional structure. Loss of inhibition above pH 6, supports that acarbose is most potent in protonated form. The low affinity site contains Trp278 and Trp279, known to bind cyclomaltoheptose. While the W279A mutant had 10-fold decreased affinity for starch granules, production of Trp278 mutants failed. The invariant Trp278 is perhaps critical for stability or folding in cereal α-amylases**.**

A 100% -respiration-deficient nuclear petite amylolytic *S. cerevisiae* NPB-G strain was generated, and its employment for direct fermentation of starch into ethanol was investigated. In a comparison of ethanol fermentation performances with the parental respiration-sufficient WTPB-G strain, the NPB-G strain showed an increase of ca. 48% in both ethanol yield and ethanol productivity (Toksoy et al., 2005).

24.5.2 Hybridization

It is not used to manipulate the yeast genome but to verify the genetic composition of recombinants. However it is possible to study gene dosage and gene suppression effects using hybridization. Intra-strain and inter-strain crosses of seven strains of *Schwanniomyces* spp. did not yield any recombination (Elzbieta et al., 1980). Hybridization of haploid strains of *S. diastaticus* and *S. cerevisiae* with a rapidly fermenting strain SD2 yielded diploid strains, some of which fermented α-amylase digested crude manioc starch to almost 100% conversion efficiency, when the predigested starch was supplemented with adequate minerals and growth factors (Abouzied and Reddy, 1986). Tamaki (1986) studied the genetic analysis of intergeneric hybrids of *S. cerevisiae* and *Schw. castellii* obtained by protoplast fusion.

Polymeric genes *DEX1, DEX2* and *STA3* control the glucoamylase production in *S. diastaticus*. However the beer produced by this species had a phenolic unpalatable aroma. This is due to the *POF* gene, which converts ferulic acid to 4-vinyl glycol (4-VG). Most brewer's yeast lacks this enzyme. It has been found that genes segregate independently when hybridized between α-*DEX-POF* haploid and α-*DEX-POF* haploid. This allowed selection of *DEX*-positive and *POF* negative

strains, which fermented dextrins, but did not produce the phenolic aroma. But the strains were sluggish resulting in slow hydrolysis of maltose by the glucoamylase instead of α-glucosidase (Panchall et al., 1984).

24.5.3 Rare Mating

If non-mating strains are mixed with a high cell density, a few hybrids with fused nuclei emerge. Based on this principle, the killer factor from haploid strains has been transferred to brewing yeast strains. The killer activity of the recombinant is directed towards other strains of contaminating yeasts and not towards other microorganisms (Stewart, 1981).

Kim and Kim (1996) developed a yeast strain that is able to produce ethanol directly from starch. A α-amylase cDNA (originated from mouse salivary glands) was introduced into the haploid *S. diastaticus* cells secreting glucoamylase by using a linearized integrating vector. The integrating vector contains a *LEU2* gene and the inside of the *LEU2* gene was cut by *Kpn*I to make the linearized vector. One of the transformants exhibited 100% mitotic stability after 100 generations of cell multiplication. To improve its ethanol-fermentability, the haploid transformant was raremated with a polyploid industrial strain having no amylase activity. The resulting hybrid RH51 produced 7.5% (w/v) ethanol directly from 20% (w/v) soluble starch and its mitotic stability was 100% at the end of fermentation.

24.5.4 Spheroplast Fusion

Spheroplast fusion technique was used to develop flocculent osmotolerant and dextrin/starch utilizing strains. *S. diastaticus* spheroplast fusion product reduced the consumption of added glucoamylase to 50% in dextrinized cassava starch fermentation (Russell et al., 1986). The dextrin utilization property of *S. diastaticus* was introduced into *S. uvarum* and *S. cerevisiae* by using spheroplast fusion (Barney et al., 1980).

24.5.5 Protoplast Fusion

This technique resembles spheroplast fusion, except that the protoplasts are completely devoid of cell wall. Protoplast fusion is a simple and rapid approach and could be used for intergeneric and interspecific crosses. Several types of interspecific crosses of yeast have yielded fusion products with improved properties such as higher fermentation productivity, possessing killer toxin and more amylases. The starch is usually predigested by α-amylase for efficient (> 95%) ethanol fermentation by

S. diastaticus in order to overcome the fermentation of dextrins and to increase the solubility of starch (Mattoon et al., 1987).

Intergeneric fusion products of *Schw. occidentalis* with *Saccharomyces* spp. have been obtained (Wilson et al., 1982) but such hybrids were not stable and lacked efficient amylase secretion. Current knowledge on genetic properties of *Schw. occidentalis* is very limited. Protoplast fusion of *S. diastaticus* has been attempted with various other species of yeasts (Dhawale and Ingledew, 1983). Fusion of *S. diastaticus* with a baker's yeast, yielded a fusion product capable of using dextrin and starch (de Figueroa et al., 1984) and with a flocculent strain of *S. cerevisiae* gave a fusion product of better ethanol fermentation (de Figueroa et al., 1985). Gautam et al. (2000) improved hyper amylase production and fermentation efficiency through protoplast fusion of *Torulospora delbruechii* and *K. marxianus*.

Fusion products that can ferment dextrins and produce killer toxin were produced by Janderova et al. (1986). Suitable crosses between *Saccharomyces* spp and stable fusion products with improved ethanol from cassava were also obtained by Ribeiro et al. (1989). Somatic hybridization of a lager brewing strain with *S. diastaticus*, a hybrid was constructed which is suitable for the production of low calorie beer (Hansen et al., 1990).

24.5.6 Gene Cloning

Greater emphasis is being laid on incorporating amylolytic capability into industrial strains of *S. cerevisiae* for direct fermentation of starch to ethanol. Techniques that are used for gene cloning have been adopted in the improvement programme. Besides this suitable vectors such as autonomously replicating sequences (ARS) giving rise to yeast replicating plasmid (YRP), 2 μm plasmid based vectors called yeast episomal plasmid (YEP) and yeast integrating vectors (YIP) are also available for transformation of yeasts with desired traits. The frequency of transformation with YIP vectors is low (1–10 transformants μg^{-1} DNA) as compared to YRP (10–10,000 transformants μg^{-1} DNA) and YEP (1000–100,000 transformants μg^{-1} DNA).

Several amylolytic derivative strains of *S. cerevisiae* and *Schizo. pombe* have been constructed recently by gene cloning and transformation (Table 24.6). They produce either a glucoamylase or α-amylase. Successful cloning and expression of both α-amylase and glucoamylase from *Schw. castellii* into *S. cerevisiae* has also been reported (Alexander et al., 1988). It has been claimed that the transformants had the ability to degrade starch with high fermentation rate and ethanol tolerance. It has not been established whether the engineered strains would be superior to existing strains with regard to amylase production and starch conversion into ethanol or single cell protein (SCP).

It is possible to clone α-amylase cDNA from even wheat (Rothstein et al., 1984) and human sources (Nakamurua et al., 1986; Sato et al., 1986) into *S. cerevisiae* by

Table 24.6 Construction of recombinant amylolytic yeasts for ethanol production from starch

Enzyme	Source of gene	Recipient	Reference
α-Amylase	Wheat	*S. cerevisiae*	Rothstein et al. (1984)
α-Amylase	Human	*S. cerevisiae*	Nakamurua et al. (1986)
α-Amylase	*Sacch. fibuligera*	*S. cerevisiae*	Yamashita et al. (1985)
Glucoamylase	*A. awamori*	*S. cerevisiae*	Innis et al., 1985
Glucoamylase	*Rhizopus sp.*	*S. cerevisiae*	Ashikari et al. (1985, 1986); Tanaka et al.(1986)
Glucoamylase	*Sacch. fibuligera*	*S. cerevisiae*	Yamashita and Fukui (1983)
Glucoamylase	*S. diastaticus (STA1)*	*Schizo. pombe*	Yamashita and Fukui (1983)
Glucoamylase	*S. diastaticus (DEX1)*	*S. cerevisiae*	Meaden et al. (1985)
Glucoamylase	*S. diastaticus (DEX 1)*	*Brewers yeast*	Meaden et al. (1985)
Glucoamylase	*S. diastaticus*	*S. cerevisiae* *Schizo. pombe*	Ashikari et al. (1985)
α-Amylase Glucoamylase	*Schw. castellii*	*S. cerevisiae*	Alexander et al. (1988)
α-Amylase gene	Plasmid (SwARS1) carrying gene cloned from *Schw. Occidentalis*	*Schw. occidentalis*	Dohmen et al., 1989
α-Amylase	*Schw. occidentalis* (*AMY*)	*S. cerevisiae*	Tsung et al., 1989
α-Amylase	*Barley*	*S. cerevisiae*	Sogaard and Svensson (1990)
α-Amylase	*B. amyloliquefaciens*		
Glucoamylase	*S. diastaticus*	*S. cerevisiae*	Steyn and Pretorius (1991)
Glucoamylase	*S. diastaticus (STA1)*	*S. cerevisiae*	Hammond (1995)
α-Amylase	*L. kononenkoae* (*LKA1*)	*S. cerevisiae*	Steyn and Pretorius (1995)
α-Amylase	Bacterial *(AMY1)*	*S. cerevisiae*	Janse and Pretorius (1995)
Glucoamylase	Yeast *(STA2)*		
Pullulanase	Bacterial *(pulA)*		
α-Amylase	*B. stearothermophilu*	Cell-surface engineered *S. cerevisiae*	Murai et al. (1999)
Glucoamylase	*R. oryzae*		
Amylase (SWA2)	*Schw. occidentalis*	*S. cerevisiae*	Marin et al., 2001
α-Amylase Glucoamylase	*L. kononenkoae, E. fibuligera*	*S. cerevisiae*	Eksteen et al. (2003)
Glucoamylase	*Rhizopus oryzae*	*S. cerevisiae*	Shigechi et al. (2004)
α-Amylase	*Streptococcus bovis*		
Glucoamylase	*A. niger*	*S. cerevisiae*	Latorre-Garcia et al. (2005)
Glucoamylase		cell-surface engineered *S. cerevisiae*	Khaw et al., 2005

Abbreviations: *Asp - Aspergillus*; *C - Candida*; *S - Saccharomyces*; *Sacch - Saccharomycopsis*; *Schizo - Schizosaccharomyces*; *Schw - Schwanniomyces*.

encouraging transformation efficiency. Similarly α-amylase gene of *Sacch. fibuligera* has been cloned and expressed in *S. cerevisiae* (Yamashita and Fukui 1985a, b); but the transformant grew poorly in starch rich medium. This could be due to the presence of maltose and maltotriose in the medium. Amylolytic strains of *S. cerevisiae* were constructed by transformation with expression plasmids containing cDNAs encoding barley amylase *AMY1* (clone E) and barley amylase *AMY2* (clone pM/C). The α-amylases were efficiently secreted into the culture medium directed by their own signal peptides (Sogaard and Svensson, 1990).

One-step enzymatic hydrolysis of starch using a recombinant strain of *S. cerevisiae* producing α-amylase, glucoamylase and pullulanase was constructed. The genes of the above enzymes were introduced into *S. cerevisiae* in different combinations and the resulting amylolytic *Saccharomyces* transformants were compared to *Schw. occidentalis*. Introduction of *PUL1* into a *S. cerevisiae* strain containing both *STA2* and *AMY1* resulted in 99% assimilation of starch (Janse and Pretorius, 1995).

Eksteen et al. (2003) conducted starch fermentation experiments by using recombinant *S. cerevisiae* strains expressing the α-amylase and glucoamylase genes from *L. kononenkoae* and *S. fibuligera*. This strain was also the most efficient at starch utilization in batch fermentations, utilizing 80% of the available starch and producing 0.61 g/100 ml of ethanol after 6 days of fermentation.

Latorre Garcia et al. (2005) have constructed a hybrid glucoamylase-encoding gene by in-frame fusion of the *S. cerevisiae STA1* gene and DNA fragment that encodes the starch binding domain of *A. niger* glucoamylase. The hybrid enzyme resulting from expression of the chimeric gene in *S. cerevisiae* has substrate binding capability and hydrolyses insoluble starch, properties not present in the original yeast enzyme.

24.5.6.1 Amylolytic Yeast

Schw. occidentalis cells that were transformed with an autonomously replicating plasmid (SwARS1) carrying the cloned α-amylase gene from *Schw. occidentalis* secreted about five times more α-amylase than the wild type without additional copies of the α-amylase gene. Both the chromosomal copies as well as the plasmid-carried copies of the α-amylase genes were repressed in presence of glucose. This transformation system provides a possibility to improve starch degradation by *Schw. occidentalis* (Dohmen et al., 1989).

24.5.6.2 Brewer's Yeast

Industrially useful polyploid yeasts such as the brewing yeasts do not possess any auxotrophic genetic markers, and hence are not easily amenable to plasmid-mediated DNA transformations. In an attempt to obtain genetic markers, a number of useful *Saccharomyces* sp. strains and some amylolytic *Schwanniomyces* sp. strains were tested for their susceptibility to the antibiotic Geneticin G418, a 2-deoxystreptamine,

reported to be active against bacteria, yeasts, and plant and animal cells. All of the *Saccharomyces* sp. strains, including the brewing strains, were found to be susceptible to G418 in the concentration range of 150 to 500 μg ml^{-1}. Of the three *Schwanniomyces* species investigated, only *Schw. castellii* (strain 1402) was found to be resistant to G418 at concentrations up to 1 mg ml^{-1}. Resistance was exhibited both in liquid media and on glycerol-peptone-yeast extract agar plates. This finding is interesting in view of the possibility of using this strain as a DNA donor for transformations aimed at introducing the amylolytic capability into brewing yeasts (Panchal et al., 1984). Hollenberg and Strasser (1990) also succeeded in constructing a *S. cerevisiae* brewers strain that is able to hydrolyze starch by introduction of both the *GAM1* gene and the *AMY1* gene, which encode a glucoamylase and an α-amylase, respectively, both originating from the yeast *Schw. occidentalis*.

It is an essential step in the manufacture of low calorie beer to convert dextrins to alcohol. The glucoamylase gene (*DEX*) from *S. diastaticus* which is required for the above has been cloned into *S. cerevisiae* and transformed through a recombinant plasmid (Perry and Meaden, 1988). The transformant was capable of super-attenuating the wort and produced good quality beer. Similar attempts were made to clone and transform *A. niger* glucoamylase. Hammond (1995) reviewed the recombinant-DNA techniques; specific breeding of new brewing yeast strains has become widespread. Strains have been produced with the ability to ferment a wider range of carbohydrates, altered flocculation properties and imparted pleasant flavour. One such modified strain has already been approved for commercial application.

24.5.6.3 Ethanol Yeast

Genetic transformation of *S. cerevisiae* for direct conversion of starch to ethanol was achieved by using *A. awamori* glucoamylase (Innis et al., 1985). However, the rate of fermentation was slow towards the end due to the accumulation of disaccharides and dextrins (Inlow et al., 1988). Ashikari et al. (1985, 1986) and Tanaka et al. (1986) have been reported high rate expression of *Rhizopus* glucoamylase (raw-starch digesting) gene from cDNA library in *S. cerevisiae*. The recombinant glucoamylase got adsorbed to gelatin as well as raw starch and degraded them more efficiently. This suggested that *Rhizopus* glucoamylase consist of two domains, one for causing adsorption and the other for catalyzing degradation.

Cloning and expression of glucoamylase gene of *Sacch. fibuligera was* successfully done in *S. cerevisiae* (Yamashita and Fukui, 1983), and this transformant fermented starch and secreted more glucoamylase than the parent. The glucoamylase genetics of *S. diastaticus* is worked out satisfactorily; the gene is cloned and expressed in several industrial species of yeast. In fact, the complete nucleotide sequence of the *STA1* gene of glucoamylase of *S. diastaticus* has been determined**,** cloned and expressed successfully into *S. cerevisiae* and *Schizo. pombe* (Yamashita and Fukui, 1984). The transformant of *S. cerevisiae* produced 5–10 folds the

amount of glucoamylase compared with *S. diastaticus*. Transformation of brewing yeast and *S. cerevisiae* (Meaden et al., 1985) has also been carried out using DEX1 glucoamylase gene of *S. diastaticus*.

α-amylase gene (*AMY*) was cloned from *Schw. occidentalis* CCRC 21164 into *S. cerevisiae* AH22 by inserting *Sau*3AI-generated DNA fragments into the *Bam*HI site of YEp16. The 5 kilobase insert was shown to direct the synthesis of α -amylase. After subclones containing various lengths of restricted fragments were screened, a 3.4 kilobase fragment of the donor strain DNA was found to be sufficient for α-amylase synthesis. The concentration of α-amylase in culture broth produced by the *S. cerevisiae* transformants was about 1.5 times higher than that of the gene donor strain. The secreted α-amylase was shown to be indistinguishable from that of *Schw. occidentalis* on the basis of molecular weight and enzyme properties (Wang et al., 1989).

A recombinant plasmid YEP13 of *S. diastaticus* was introduced into *S. cerevisiae* and *Schizo. pombe,* but the resultants had very low glucoamylase activity. Tetrad analysis of crosses between *S. cerevisiae* and *S. diastaticus* showed that the enzyme was sporulation specific (Stewart, 1981). Molecular cloning of α-glucosidase gene isolated from a *C. tsukubaensis* genomic library in *S. cerevisiae* was reported. On the basis of its substrate specificity profile, the cloned enzyme was classified as α-glucosidase having pH optimum of 4.2–4.6, temperature optimum at 58°C and was readily inactivated at pasteurization temperature 60°C. Southern blot analysis did not reveal any homology between the cloned gene and genomic DNA isolated from other well-characterized amylolytic yeasts (Kinsella et al., 1991). Steyn and Pretorius (1991) studied the co-expression of a *S. diastaticus* glucoamylase-encoding gene and *B. amyloliquefaciens* α-amylase encoding gene in *S. cerevisiae.*

Murai et al. (1999) constructed a recombinant strain of *S. cerevisiae* that displayed glucoamylase and α-amylase on its cell surface and its starch-utilizing ability was evaluated. The gene encoding *R. oryzae* glucoamylase, with its own secretion signal peptide and a truncated fragment of the α-amylase gene from *B. stearothermophilus* with the prepro-secretion signal sequence of the yeast α-factor, respectively, were fused with the gene encoding the C-terminal half of the yeast α-agglutinin. The constructed fusion genes were introduced into the different loci of chromosomes of *S. cerevisiae* and expressed under the control of the GPD promoter. The glucoamylase and α-amylase activity was detected in the cell pellet and not in the medium. The transformant strain co-displaying glucoamylase and α-amylase could grow faster on starch as the sole carbon source than the transformant strain displaying only glucoamylase.

An amylolytic industrial yeast strain of *S. cerevisiae* containing the *Schw. occidentalis* SWA2 amylase gene was generated. The new strain contained DNA derived exclusively from yeast and expressed high starch hydrolyzing activity. The SWA2 enzyme was constitutively expressed under the *ADH1* promoter. The growth, substrate utilization and fermentative capacity of this organism have been described (Marin et al., 2001).

Four types of cell-surface-engineered yeast *S. cerevisiae* displaying glucoamylase, namely, systems A, B, C, and D, were constructed to evaluate their performance in

direct ethanol fermentation from raw corn starch. Systems A and B were glucoamylase-displaying nonflocculent yeast (YF237) types that secrete α-amylase into the culture medium and co display α-amylase on the cell surface, respectively. Systems C and D were flocculent yeast counterparts (YF207) for systems A and B, respectively. In batch fermentations, the specific ethanol production rates of systems A, B, C, and D were 0.18, 0.06, 0.06, and 0.04 g/g cell/h, respectively. In repeated fermentations, the specific ethanol production rate of system. A decreased with the number of repetitions, whereas, that of system B was maintained. In all systems, the rate-limiting step was the conversion of starch to oligosaccharide because oligosaccharide and glucose were not accumulated throughout the fermentations. (Khaw et al., 2005).

All the above attempts to genetically-engineer a super yeast strain that can directly and economically ferment starch to ethanol in one-step, though yielded somewhat encouraging results, coordinated and concerted efforts involving alcohol fermentation industry and petrochemical industry on one hand and academic and research institutions on the other are essential to achieve the goal.

24.6 Conclusions

In view of the dwindling reserves and ever escalating cost of the fossil fuels, it is imperative to look for a renewable biofuel like ethanol in the new millennium. In fact, ethanol is already being used extensively as an additive to petroleum in countries like Brazil and USA. However, in order to use ethanol as a long-term automobile fuel, its abundant availability and reasonable lower price are to be ensured. The proposal to use ethanol-blended petrol has necessitated cost effective production of large quantities of ethanol. Although, sugarcane molasses form a cheaper substrate for the production of ethanol, its availability is limited. Alternatively, the domestic and farm wastes containing fairly high amount of starch, as well as surplus starchy grains, provide a cheap and widely available substrates for alcohol production. Fermentation of these substrates by yeasts not only yields ethanol but also single cell protein. The conventional three-step fermentation process involving liquefaction, saccharification and fermentation is highly energy and cost intensive. A direct one -step process employing amylolytic yeasts holds the key to this problem. The protocol is technically feasible and economically viable on a commercial scale. All that is needed is to identify and develop genetically-engineered super amylolytic-alcohol-producing yeast strains, which perform with equal efficiency on a variety of starchy substrates. Concerted efforts by researchers towards achieving the goal would pay great dividends to the motor fuel-hungry public all over the world in the new millennium.

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Chapter 25
Thermotolerant Yeasts for Bioethanol Production Using Lignocellulosic Substrates

Chand Pasha and L. Venkateswar Rao

Contents

Abstract No other sustainable option for production of transportation fuels can match ethanol made from lignocellulosic biomass with respect to its dramatic environmental, economic, strategic and infrastructure advantages. Substantial progress has been made in advancing biomass ethanol (bioethanol) production technology to the point that it now has commercial potential, and several firms are engaged in the demanding task of introducing first-of-a-kind technology into the marketplace to make bioethanol a reality in existing fuel-blending markets. In order to lower pollution India has a long-term goal to use biofuels (bioethanol and biodiesel). Ethanol may be used either in pure form, or as a blend in petrol in different proportions. Since the cost of raw materials, which can account up to 50% of the total production cost, is one of the most significant factors affecting

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the economy of alcohol, nowadays efforts are more concentrated on using cheap and abundant raw materials. Several forms of biomass resources exist (starch or sugar crops, weeds, oil plants, agricultural, forestry and municipal wastes) but of all biomass cellulosic resources represent the most abundant global source. The lignocellulosic materials include agricultural residues, municipal solid wastes (MSW), pulp mill refuse, switchgrass and lawn, garden wastes. Lignocellulosic materials contain two types of polysaccharides, cellulose and hemicellulose, bound together by a third component lignin. The principal elements of the lignocellulosic research include: i) evaluation and characterization of the waste feedstock; ii) pretreatment including initial clean up or dewatering of the feedstock; and iii) development of effective direct conversion bioprocessing to generate ethanol as an end product. Pre-treatment of lignocellulosic materials is a step in which some of the hemicellulose dissolves in water, either as monomeric sugars or as oligomers and polymers. The cellulose cannot be enzymatically hydrolyzed to glucose without a physical and chemical pre-treatment. The pre-treatment processes normally applied on the different substrates are acidic hydrolysis, steam explosion and wet oxidation. A problem for most pretreatment methods is the generation of compounds that are inhibitory towards the fermenting microorganisms, primarily phenols. Degradation products that could have inhibitory action in later fermentation steps are avoided during pre-treatment by wet oxidation. Followed by pre treatment, hydrolysed with enzymes known as cellulases and hemicellulases, which hydrolyse cellulose and hemicellulose respectively. The production of bioethanol requires two steps, fermentation and distillation. Practically all ethanol fermentation is still based on *Saccharomyces cerevisiae*. The fermentation using thermotolerant yeasts has more advantageous in that they have faster fermentation rates, avoid the cooling costs, and decrease the over all fermentation costs, so that ethanol can be made available at cheaper rates. In addition they can be used for efficient simultaneous saccharification and fermentation of cellulose by cellulases because the temperature optimum of cellulase enzymes (about 40°C to 45°C) is close to the fermentation temperature of thermotolerant yeasts. Hence selection and improvement of thermotolerant yeasts for bioconversion of lignocellulosic substrates is very useful.

25.1 Introduction

Energy is a factor of the well being of the people and is a production factor of the commercial and industrial sectors. Demand for energy and transportation fuels is growing steadily worldwide over the last century. As a result, energy is a prime mover of the country's competitive edge and economic development in the long term. In order to attain continuous and sustainable economic development, it is essential that energy supplies be adequate and secure, at reasonable prices. Indian annual requirement of oil is 114 million tones. Significant part of this is consumed in the transportation sector. We produce only about

25% of our total requirement. The import cost today of oil and natural gas is over Rs. 180,000 crores in 2005. Oil and gas prices are escalating making alternative fuels most urgent.

25.2 Critical Review

25.2.1 Fuels

Vehicle fuels can be classified in three groups: (a) traditional fuels, (b) oxygenated fuels and (c) alternative fuels (AFDC, 1997). The most important fuels in those groups are listed in Table 25.1. Gasolines are complex mixtures of several hundred types of hydrocarbons. Oxygenated gasoline is created by adding ether or alcohol oxygenates. Ether oxygenates are methyl-tertiary-butyl ether (MTBE), ethyl-tertiary-butyl ether (ETBE) and tertiary-amyl-methyl ether (TAME). Alcohol oxygenates are ethanol, methanol and tertiary-butyl alcohol (TBA) (Williams et al., 2003).

The transportation sector by using fossil fuel contributes up to 30% of CO_2, 67% of carbon monoxide, 41% of nitrogen oxides, 51% of reactive hydrocarbon and 23% of particulate matter emissions (MacKenzie, 2003). The DOE (U.S. Department of Energy) estimates that bioethanol usage could reduce the sulfur oxide emissions by 60% to 80% and the volatile organic compound emissions by 13% to 15% when ethanol is used as 95% blend with gasoline (Himmel et al., 1997). As the transportation sector is the major consumer of petroleum, it significantly affects environmental quality suggesting replacing fossil fuels by clean-burning ethanol fuel.

25.2.1.1 Ethanol

Ethyl alcohol or ethanol is a well known substance that has a wide range of applications. It can be used in the chemical industry as a solvent or agent for

Table 25.1 Available and potential vehicle fuels. (AFDC, 1997)

Traditional fuels	Oxygenated fuels	Alternative
Gasoline	Methyl tertiary butyl ether (MTBE)	Liquefied petroleum gas (LPG)
Diesel	Ethyl tertiary butyl ether (ETBE)	Compressed natural gas (CNG)
	Tertiary amyl methyl ether (TAME)	Ethanol 85% (E85)
	Ethanol, 10% (E10)	Ethanol 95% (E95)
	Methanol	Methanol 85% (M85)
	Tertiary butyl alcohol (TBA)	Methanol absolute (M100)
		Electricity
		Hydrogen
		Biodiesel

chemical reactions and uses include the manufacture of industrial products like detergents, paint, varnish and cosmetic products. Ethanol is also used in food industry to produce vinegar and spirits and has for long been playing an important role in the medical sector as an antiseptic and drug formulae component. It is also utilized as feedstock material to produce ethyl esters (Biodiesel) and to manufacture industrial-grade diamonds.

Although the referred uses have been the traditional driving forces that fostered the international trade of ethanol, its expanded use as an automotive fuel has created a new market segment that is viewed as the fastest growing and most important market for the product in the foreseeable future (Pimentel, 2003).

25.2.1.2 Bioethanol as a Fuel

Bioethanol is an alcohol made from sugar, starch, cellulose and products containing sugars. It is fermented, distilled and used as a substitute or partial substitute for petrol. Bioethanol is a clean-burning, non-petroleum liquid fuel. Countries' dependence on imported oil; environmental issues and employment in rural areas (Farrell et al., 2006) has been reasons for the consideration of the replacement of fossil fuels by bioethanol. Ethanol can be used as a transportation fuel as well as a gasoline fuel extender and an octane enhancer. The ethanol can be mixed with gasoline in 10% (E10), 20% (E20) and 22% (E22) blends without engine modifications (Bullen et al., 2006) but for higher-level blends (such as 85% or 95%) require some engine modification (Von Blottnitz and Curran, 2006). Ethanol has an octane number of 113, allowing a higher compression ratio in the gasoline engine. Ethanol serves as an octane-booster in itself and in the form of ethyl tertiary butyl ether (ETBE), thus it can replace the toxic additive benzene. Using ETBE blended at 15–16% level with gasoline, the carbon dioxide, nitrogen oxides and some aromatic compounds emissions diminish, but aldehyde emission increases at a low level (<1%). As a fuel additive, ethanol provides oxygen to the fuel and thus improves fuel combustion and reduces tailpipe emission of carbon dioxide and unburned hydrocarbons. It gives further advantages to the fuel because of its high heat of vaporization, low flame temperature, greater gas volume change, high specific energy, lower volumetric energy content, which means shorter distance travel compared to gasoline (Gautam and Martin, 2000; Ragauskas et al., 2006).

In fossil fuel uses a fair amount of hydrocarbon may end up as 'half-burned' products – chief among these are the *polyaromatic hydrocarbons*, which are implicated in carcinogenesis (causing cancer). Whereas ethanol can help, ensure the complete combustion of other compounds in a fuel mix.

Besides these carbon compounds, incomplete burning also generates a lot of carbon monoxide (CO). Though not a greenhouse gas, this is a toxic and nasty compound that is a major cause of air-pollution related malaise in urban centers. Providing oxygen within the fuel in the form of an oxygen-containing compound can greatly reduce the amount of CO produced.

The main sources of ethanol for use in fuel are sugar (obtained from sugar cane), starch (obtained chiefly from corn) and cellulosic biomass. These are renewable

resources on the timespan of a human lifetime, unlike fossil fuels. In terms of drawbacks of the ethanol-blended fuels, the most important ones are probably the increased vapor pressure and the water absorption. Ethanol blends must furthermore be handled with care since they are sensitive to water in the gasoline distribution systems.

A key barrier to ethanol's expanded role in fuel consumption is its price differential with gasoline. Since a major part of the total production cost is the cost of feedstock, reducing feedstock costs could lead to lower wholesale ethanol costs (Wyman, 1996; Andersson et al., 2006). The way to reduce the cost of the feedstock is to switch from the traditional starch-based feedstocks (Ericsson et al., 2004; Wright, 2006) to cellulosic biomass. For this reason, there is a great deal of interest in producing ethanol from cellulosic feedstocks. However, low-priced feedstock requires costly preparation, which makes it necessary to develop effective and relatively inexpensive pretreatment procedures. The expensive price of the commercial cellulase enzyme induces researchers to develop more affordable solutions, such as the production of cheaper own cellulase (Katzen and Monceaux, 1995) from the same cellulosic biomass substrate that is used for ethanol production.

25.2.2 World Ethanol Production and Trade

Many countries are taking an increased interest in ethanol as an alternative fuel, with the USA and Brazil currently leading the way. In 2005, Brazil produced 4.8 million gallons of ethanol and the CO produced 3.9 billion gallons, making these countries the two largest producers in the world, accounting for over 90 percent of the total world production. There are a limited number of studies on ethanol markets, as the industry has experienced a boom only in recent years (Gallagher et al., 2006). The recent increase in crude oil and gasoline prices has also opened a new market for ethanol as a fuel extender (Eidman, 2006).

Brazil is currently the world's largest producer of ethanol, deriving its supply from sugarcane. Brazil is one of the first countries to promote ethanol widely through its National Alcohol Program, which was launched in late 1975 in response to high oil prices and declining sugar prices (Bolling and Suarez, 2001). The cost of ethanol from sugarcane in Brazil, at 83¢ per gallon of fuel, is lower than the cost from corn in the U.S., at $1.09 per gallon (von Lampe, 2006). Besides the higher cost of production, there are additional costs in the U.S. associated with transporting ethanol from the production locations in the Midwest to major population areas, particularly in the coastal regions. This has led to an increase in the competitiveness of Brazilian ethanol imports despite steep tariffs in U.S.

Pure ethanol (100% ethanol) is used in approximately 40% of the cars in Brazil. The remaining vehicles use blends of 24% ethanol with 76% gasoline. Brazil consumes nearly 4 billion gallons of ethanol annually (Gallagher et al., 2006).

Ethanol came into active use in USA way back in 1977, following the promulgation of the clean air act. About 4 billion liters of ethanol are produced from corn annually with ethanol blends making up nearly 12% of the total gasoline market today. As per the futuristic projections, about 14 billion gallons of bioethanol is

expected to replace around 348 billion barrels of oil in US by 2020. The possible total bioethanol production from crop residues and crops in the world is 491GL/year, about 16 times higher than the current world ethanol production. Asia is the largest potential producer of bioethanol from crop residues and wasted crops that could produce up to 291 GL/year of bioethanol (Kim and Dale, 2003). The demand for ethanol is approximately divided into 68% for fuel, 21% for industries and 11% for beverages. Approximately 9% of the ethanol is produced synthetically and consequently, fermentation is responsible for 91% of global ethanol production (Wheeler et al., 1991).

25.2.2.1 Indian Strategies of Biofuel Program

India is not self-sufficient in petroleum and has to import about two-thirds of its requirement. India's known crude oil reserve is estimated to last only for about 21 years. With insufficient oil resources, India cannot rely on imported oil, which will seriously affect on economic development and sovereignty in international political relations. The prices have crossed US$ 70 per barrel and the expenditure on crude purchase is in the range of Rs.1800 billion per year, impacting in a big way, the country's foreign exchange reserves (Chandel et al., 2007a, b).

A blend of ethanol in fossil fuel will, make a big difference both in our import as well as in environmental management. In India, ethanol is produced mostly from sugarcane molasses, a byproduct of the sugar industry. The Government of India through its Planning Commission has initiated a national program to cultivate vast areas of waste/degraded lands by plantation of oil-bearing and bio energy trees. India inspired by a desire to reduce greenhouse gas emissions in order to meet Kyoto Protocol targets, have turned to ethanol fuels as a cheap and proven alternative to reduce vehicular emissions. Political instability in the Middle East has further motivated to develop our own fuel supply to ensure the security of supply and promote internal economic growth. Persistent lobbying from agricultural groups has also encouraged governments to pursue aggressive energy policies to create crop price stability and reduce the need for government subsidies on exported surpluses. After successful field trials, the government made it mandatory to blend 5% biofuel in fossil fuel for the supply in 9 states (1.Andhra Pradesh 2. Goa. 3. Gujarat 4. Haryana 5. Karnataka 6. Maharashtra 7. Punjab 8.Tamilnadu and 9. Uttar Pradesh) and four contiguous union territories (1. Daman and Diu 2. Dadra Nagar Haveli 3. Chandigarh 4. Pondicherry) with effect from January 1, 2003. (Kapoor et al., 2006).

In January 2003, it planned to make this mandatory throughout the country later, but back-pedalled on the plan due to poor output and high costs. Oil companies had needed 363 million litres of ethanol in the 2003/04 year to satisfy the mandate, but only 196 million litres had been available due to declining sugarcane output. Infrastructure and shortage of anhydrous ethanol saw a scaled down launch of India's national program for introducing ethanol-blended petrol or gasohol across the country in stages.

According to many analysts, the major obstacle to ethanol fuels becoming more widespread is simply a question of cost. However, there is great potential that these costs can be considerably reduced as more widespread ethanol use generates increased attention towards the science and economics behind ethanol fuels.

25.2.3 Ethanol Production

Ethanol may be produced synthetically or by direct fermentation of sugars or from other carbohydrates that can be converted to sugar such as starch and cellulose.

25.2.3.1 Synthetic Ethanol

Indirect hydration of ethylene is the oldest process among the two major ethanol production methods from ethylene and started more than one hundred years ago. The ethanol is prepared from ethylene in a three-step process using sulfuric acid (John, 1969). In the first step, the hydrocarbon feedstock containing 35–95% ethylene is exposed to 95–98% sulfuric acid in a column reactor to form mono- and diethyl sulfate.

$$CH_2 = CH_2 + H_2SO4 \rightarrow CH_3CH_2OSO_3H$$
$$2(CH_2 = CH_2)_2SO_2 + H_2SO_4 \rightarrow (CH_3CH_2O)_2SO_2$$

This is subsequently hydrolyzed with enough water to give 50–60% aqueous sulfuric acid solution:

$$CH_3CH_2OSO_3H + H_2O \rightarrow CH_3CH_2OH + H_2SO_4$$
$$(CH_3CH_2O)_2SO_2 + 2H_2O \rightarrow 2CH_3CH_2OH + H_2SO_4$$

The ethanol is then separated from the dilute sulfuric acid in a stripper column. The last step of this process is to concentrate the sulfuric acid and recycle to the process.

Direct hydration of ethylene was commercialized in 1947. In this process, an ethylene-rich gas is combined with water and passes through a fixed-bed catalyst reactor, in which ethanol is formed according to the following reaction (Nelson and Courter, 1954).

$$CH_2 = CH_2 + H_2O \rightarrow CH_3CH_2OH$$

The ethanol is then recovered in a distillation system. Both direct and indirect hydration of ethylene gives rise to undesirable by-products such as diethyl ether, which reduce the quality of ethanol. Other processes to make ethanol synthetically are not commercially important.

25.2.3.2 Ethanol Production by Fermentation

Ethanol can be produced by fermentation from different kinds of raw materials. The raw materials are classified into three categories: Saccharine, starchy and cellulosic materials (Table 25.2). Some sugars can be converted directly to ethanol, whereas starch and cellulose must first be hydrolyzed to sugar before conversion to ethanol.

25.2.4 Raw Materials

The factors affecting the choice of raw materials are as follows (Cowling and Kirk, 1976):

- Materials should be inexpensive and have consistent quality a year round availability
- Ease of handling in along with associated transport and storage costs.
- Sterilization requirements and any potential denaturation problem.
- The quantity of target product and its rate of formation should be high.
- The levels and range of impurities should be less.
- Overall health and safety implications.

The cost of ethanol production is highly sensitive to the delivered feedstock cost and the operating scale. Lignocellulosic perennial weed crops are promising feedstock because of high yields, low costs, good suitability for low quality land (which is more easily available for energy crops) and low environmental impact. Most ethanol conversion systems encountered in literature, have been based on a single feedstock. But considering the hydrolysis fermentation process, it is possible to use multiple feedstock types. This may even be necessary to achieve the desirable large scale towards the future.

Most of the polymeric raw materials are available at prices lower than refined sugars. However, transportation costs of the raw materials make it necessary to use locally available raw material. Consequently, each country should develop ethanol production based on the available raw material in that country (Butterworth, 2006).

Table 25.2 Major resources for fermentative production of ethanol

Saccharines	Starchy materials	Cellulosis materials
Sugarcane	Grains	Wood
Sugarbeet	Potatoes	Agricultural residue
Molasses	Root crops	Municipal solid waste
Fruit		Waste paper
		Crop residues

25.2.4.1 Saccharine Materials

Fermentation involves microorganisms that use the fermentable sugars for food and in the process produces ethanol and other byproducts. These microorganisms can typically use the 6-carbon sugars, one of the most common being glucose. Therefore, biomass materials containing high levels of glucose or precursors to glucose are the easiest to convert to ethanol. One example of a sugar feedstock is sugarcane (also molasses). Other biomass feedstocks rich in sugars (saccharides) include sugar beet, sweet sorghum and various fruits. However, these materials are all in the human food chain and except for some processing residues are generally too expensive to use for fuel ethanol production.

25.2.4.2 Starchy Materials

Another potential ethanol feedstock is starch. Starch molecules are made up of long chains of glucose molecules. Examples of starchy materials commonly used around the world for ethanol production includes cereal grains, potato, sweet potato and cassava. Cereal grains commonly used for ethanol production include sorgam, maize, barley and wheat. Starchy materials require a reaction of with water (hydrolysis) to break down the starch into fermentable sugars (saccharification). Mixing the starch with water to form slurry, which is then stirred and heated, performs hydrolysis. Specific enzymes (amylase) that will break the chemical bonds are added at various times during the heating cycle.

25.2.4.3 Lignocellulosic Materials

Lignocellulosics are abundant sources of carbohydrate, continuously replenished by photosynthetic reduction of carbon dioxide by sunlight energy (Parikka et al., 2004). They are the most promising feedstock for the production of energy, food and chemicals (Solomon et al., 1999; Kim and Dale, 2004). In India, the ongoing research in Department of Microbiology, Osmania University, Hyderabad by Prof. L.V. Rao and Dr. Pasha and Department of Microbiology, University of Delhi South Campus, New Delhi by Prof. R. C. Kuhad and co-workers, for the evaluation of ethanol production from wild plant materials namely *Lantana camara*, *Prosopis juliflora* and *Saccharum spontaneum* has opened new choices for cheaper and sugar rich substrates with improved saccharification and subsequently fermentation methodologies. This nationwide project on fuel ethanol production from lignocellulosic feedstock has been financially supported by Department of Biotechnology, Ministry of Science and Technology, Govt. of India and is supposed to determine the Indian bioethanol future scenario. Agricultural wastes and in fact all lignocellulosics can be converted into products that are of commercial interest such as ethanol, glucose xylitol, vinegar and single cell protein (Hood, 2004; Das and Singh, 2004).

Lignocellulosic materials are formed by three main polymeric constituents, cellulose, hemicelluloses and lignin (Lynd et al., 2005) in various proportions (Table 25.3).

The bioconversion of cellulosic materials has been receiving attention in recent years. It is now a subject of intensive research as a contribution to the development of a large-scale conversion process beneficial to mankind (Ragauskas et al., 2006). Such process as suggested by Fan et al. (1987) and Kumakura (1997) would help alleviate shortages of food and animal feeds, solve modern waste disposal problem and diminish man's dependence on fossil fuels by providing a convenient and renewable source of energy in the form of glucose. Cellulose is the cheapest raw material on earth, which is available domestic agricultural and industrial waste. The global production of plant biomass of which over 90% is lignocellulose, amounts to about 200×10^9 tons of the primary biomass remains potentially accessible (Berndesa et al., 2003).

Table 25.3 Composition (%) of agricultural and wood residues

Substrates	Cellulose	Hemicellulose	Lignin
Bagasse	33	30	29
Barley straw	40	20	15
Birch	40	33	21
Corn cobs	42	39	14
Corn stakes	35	15	19
Groundnut shells	38	36	16
Oat straw	41	16	11
Pine	41	10	27
Rice straw	32	24	13
Rice hulls	36	15	19
Saw dust	55	14	21
Sorghum straw	33	18	15
Wheat straw	30	24	18
Hard wood stems	40–55	34–40	18–25
Prosopis stem	48.5	21.7	26.10
Lantana wood	45.1	17.0	27.25
Soft wood stems	45–50	25–35	25–35
Grasses	25–40	35–50	10–30
Paper	85–99	0	0–15
Cotton seed hairs	80–95	5–20	0
Newspaper	40–55	25–40	18–30
Waste papers – chemical pulps	60–70	10–20	5–10
Primary wastewater solids	8–15	NA	24–29
Sine waste	6.0	28	NA
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

Source: Cheung and Anderson (1997), Boopathy et al. (1998) and Dewes and Hunsche (1998).

25.2.4.3.1 Cellulose

It is the most abundant cell wall component forming a supporting matrix for the cell membrane. It is a regular linear homopolymer of repeating D-glucose units in 1-4 linkage. (Sluiter et al., 2004). However the basic building block of cellulose is actually cellobiose, a dimmer of two glucose units (Katzen and Monceaux, 1995). As glucose units are linked together into polymer chains, a molecule of water is lost, which makes the chemical formula $C_6H_{10}O_5$ for each monomer unit of 'glucan'. Each monomer unit of cellulose can form two hydrogen bonds with a monomer in a neighboring chain. This causes the chain to be packed together in larger units called 'micro fibrils'. The hydrogen bonds are present between the chains in each layer. The secondary and tertiary conformation of cellulose as well as its close association with lignin and hemicellulose, starch, protein and mineral elements make cellulose, a hydrolysis resistant molecule. Native crystalline cellulose is insoluble and occurs as fibers of densely packed hydrogen bonded anhydrous chains of 12000 glucose units (Sluiter et al., 2004).The exposed crystalline cellulose is still guile resistant chemical or enzymatic hydrolysis (Yunqiao et al., 2006).

25.2.4.3.2 Hemicellulose

The second major structural component, hemicelluloses (polyoses), has an intermediate degree of complexity and is made up of different pentose and hexose residues. Typically, hemicelluloses in softwood are glucomannans, whereas those in hardwoods are mainly xylans together with variable percentages of galactose, arabinose, rhamnose and methylglucuronic acid units. Hemicelluloses are usually classified according to the sugar residue present, for example D-galactan, D-mannan, D-xylan, L-arabinan etc, (Sluiter et al., 2004). The principal pentose sugar in hemicellulose, Xylose (i.e. b-D-xylopyranose as found in xylan) is the simplest general representation of a normal hemicellulose. The other common pentose sugar is arabinose, which is usually linked to xylose and galactose in arabinoxylan and arabinogalactan, respectively. Xylan is by far the major class of hemicellulose present in several lignocellulosic residues. D-xylan comprises 15 to 30% of annual plants, 20 to 25% of hardwoods and 7 to 12% of softwoods.

25.2.4.3.3 Lignin

Lignin is a three-dimensional network built up of dimethoxylated (syringyl, S), monomethoxylated (guaiacyl, G) and non-methoxylated (p-hydroxyphenyl, H) phenylpropanoid units, derived from the corresponding p-hydroxycinnamyl alcohols, which give rise to a variety of subunits including different ether and C-C bonds. Acetylated lignin units have been identified in non-woody plants using analytical

pyrolysis. Lignin is highly resistant towards chemical and biological degradation and confers mechanical resistance to wood. The highest concentration of this strong polymer is found in the middle lamella, where it acts as a cement between wood fibers, but it is also present in the layers of the cell wall (especially the secondary cell-wall), forming, together with hemicelluloses and protected against biodegradation (Balakshin et al., 2003; Sluiter et al., 2004). The utilization of lignocelluloses requires pre-treating the biomass to open the lignocellulosic polymers, to allow their hydrolysis by either enzymes or acids. The residual lignin can be used as a boiler fuel or as a starting material for higher value products (phenolics, polymers, etc.).

Extensive research has been carried out in this field for decades, but practicable processes have yet to be demonstrated. Indeed several problems hamper such a processes: pretreatment is cumbersome; enzyme hydrolysis is slow and requires large number of enzymes, while acid hydrolysis is fast, but results in poor yields and many degradation products form, that inhibit fermentations and the fermentations themselves are complicated by the two types of sugars present as well as the potential for contamination. As regards contamination, the sterilizable fermenters used in laboratory research and even most demonstration projects, are not practical for large-scale commercial operations. In conventional cornstarch fermentations, contamination is minimized by the relatively rapid batch fermentation process, selective conditions (e.g. low pH), inoculation and clean (though not sterile) start-up and operating conditions. The key problem for ethanol fermentation of lignocellulosics is the long times required for the enzymatic saccharification which is preferred over acid hydrolysis as it results in higher yields. The exponential increase of contamination problems with cycle time is a major limitation of the enzyme hydrolysis process. Research has concentrated on the development of improved processes for enzymatic hydrolysis and fermentation of the pentose sugars using genetically engineered microbes.

25.2.5 Pretreatment of Lignocelluloses

A major obstacle to the effective utilization of lignocellulose is the chemically unreactive nature of cellulose. The lignin-hemicellulose matrix, cellulose crystallinity and its low surface area make the lignocellulose very resistant to enzymes (Alex et al., 2006). Making cellulose accessible to the enzyme is an essential factor in order to increase the rate of hydrolysis (Philippids and Smith, 1995). Therefore, pretreatment is necessary before enzymatic hydrolysis (Wyman et al., 2005). Enzymatic hydrolysis has been recognized as an attractive method for hydrolysis of cellulose and hemicelluloses contained in pretreated biomass suspension (Himmel et al., 1996). The methods of lignocellulose pretreatment can be classified in three groups: chemical, physico-chemical and biological pretreatment.

Various research groups all over the world have attempted different pretreatment methods. Some of the methods have been demonstrated to be effective in disrupting the lignin-carbohydrate complex and others in disrupting the cellulose structure itself (Gamble et al., 2000).

25.2.5.1 Physical Pretreatment

25.2.5.1.1 Mechanical Breakage

Waste materials can be broken down by a combination of chipping, grinding and milling to reduce cellulose crystallinity. The sizes of the materials are usually 10–30 mm after chipping and 0.2–2 mm after milling or grinding. Vibratory ball milling has been found to be more effective in breaking down the cellulose crystallinity of spruce and aspen chips and improving the digestibility of the biomass than ordinary ball milling (Millet et al., 1976).

25.2.5.1.2 Pyrolysis

When the materials are treated at temperatures greater than 300°C, cellulose rapidly decomposes to produce gaseous products and residual char (Shin et al., 2001). The decomposition is much slower and less volatile products are formed at lower temperatures. Mild acid hydrolysis (1 N H_2SO_4, 97°C, 2.5 h) of the residues from pyrolysis pretreatment has resulted in 80–85% conversion of cellulose to reducing sugars with more than 50% glucose (Fan et al., 1987).

25.2.5.1.3 Autohydrolysis

Autohydrolysis is the process of converting lignocellulose into fermentable sugars by exposure to high temperature steam (Vazquez et al., 2006). In this method, chipped biomass is treated with high-pressure saturated steam and then the pressure is swiftly reduced, which makes the materials undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160–260°C for several seconds to a few minutes before the material is exposed to atmospheric pressure. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis. Ninety percent efficiency of enzymatic hydrolysis has been achieved in 24 h for poplar chips pretreated by steam explosion, compared to only 15% hydrolysis of untreated chips (Grous et al., 1986). Addition of H_2SO_4 (or SO_2) or CO_2 in steam explosion can effectively improve enzymatic hydrolysis, decrease the production of inhibitory compounds and lead to complete removal of hemicellulose (Kim and Hong, 2001; Bura et al., 2002).

Limitations of steam explosion include destruction of a portion of the xylan fraction, incomplete disruption of the lignin-carbohydrate matrix and generation of compounds that may be inhibitory to microorganisms used in downstream processes. Because of the formation of degradation products that are inhibitory to microbial growth, enzymatic hydrolysis and fermentation, pretreated biomass needs to be washed by water to remove the inhibitory materials (Vazquez et al., 2006). The water wash decreases the overall saccharification yields due to the removal of soluble sugars such as those generated by hydrolysis of hemicellulose.

25.2.5.2 Chemical Pretreatment

25.2.5.2.1 Acid Hydrolysis

There are two basic types of acid processes: concentrated acid and dilute acid.

25.2.5.2.1.1 Concentrated-Acid Hydrolysis

Hydrolysis of lignocellulose by concentrated sulfuric or hydrochloric acids is a relatively old process. Braconnot in 1819 first discovered that cellulose could be converted to fermentable sugar by concentrated acid (Sherrard and Kressman, 1945). Concentrated-acid processes are generally reported to give higher sugar yields compared to dilute-acid processes. However, dilution and heating of the concentrated acid during the hydrolysis process makes it extremely corrosive. Therefore, the process requires either expensive alloys or specialized non-metallic constructions such as ceramic or carbon-brick lining. The high investment and maintenance costs have greatly reduced the commercial potential for this process. Furthermore, the environmental impact strongly limits the application of concentrated acids (Katzen and Monceaux, 1995).

The concentrated acid process uses relatively mild temperatures and the only pressures created by pumping materials from vessel to vessel used. The primary advantage of the concentrated process is the high sugar recovery efficiency, which can be approximately over 90% of both hemicellulose and cellulose sugars. The low temperatures and pressures employed also allow the use of relatively low cost materials such as fiberglass tanks and piping. Unfortunately, it is a relatively slow process and cost effective acid recovery systems have been difficult to develop. Without acid recovery, large quantities of lime must be used to neutralize the acid in the sugar solution. This neutralization forms large quantities of calcium sulfate, which requires disposal and creates additional expense.

25.2.5.2.1.2 Dilute-Acid Hydrolysis

Dilute-sulfuric-acid hydrolysis is a favorable method for either the pretreatment before enzymatic hydrolysis or the conversion of lignocellulose to sugars (Qureshi and Manderson, 1995; Lloyd and Wyman, 2005). The first established dilute-acid

hydrolysis process was probably the Scholler process (Faith, 1945). It was a batch process, in which the wood waste was kept in 0.5% sulfuric acid at 11–12 bars for approximately 45 minutes. Thereafter, some other plants were built in Germany, Russia, Italy and Korea to produce ethanol, glycerol and fodder yeast from the provided sugar solution. The process was able to produce 0.17 g of ethanol per g of dry wood. Almost all the dilute-acid hydrolysis processes are performed in a batch mode with a residence time of a few minutes. However, continuous hydrolysis in plug flow reactors could be an option (e.g. Church and Wooldridge, 1981). Dilute-acid hydrolysis in two stages is an improvement of the process. The first stage may involve a mild condition to convert hemicellulose to sugar monomers. The residual solid can then be separated from the solution and be hydrolyzed under more severe conditions to make glucose from cellulose (Harris et al., 1984). Compared to other pretreatment methods, it is especially useful for the conversion xylan in hemicellulose to xylose that can be further fermented to ethanol by many microorganisms Most dilute acid processes are limited to a sugar recovery efficiency of around 50%. The reason for this is that at least two reactions are part of this process. The first reaction converts the cellulosic materials to sugar and the second reaction converts the sugars to other chemicals. Unfortunately, the conditions that cause the first reaction to occur also are the right conditions for the second to occur. Thus, once the cellulosic molecules are broken apart, the reaction proceeds rapidly to break down the sugars into other products most notably furfural. Not only does sugar degradation reduces sugar yield but also the furfural and other degradation products can be poisonous to the fermentation microorganisms.

25.2.5.2.2 Ozonolysis

Ozone degrades lignin and hemicellulose in many lignocellulosic materials such as wheat and cotton straw, bagasse, green hay, peanut, pine (Ben-Ghedalia and Shefet, 1983) and poplar sawdust (Vidal and Molinier, 1988). The degradation was essentially limited to lignin and hemicellulose was slightly attacked, but cellulose was hardly affected. The rate of enzymatic hydrolysis increased by a factor of 5 following 60% removal of the lignin from wheat straw in ozone pretreatment (Vidal and Molinier, 1988). Enzymatic hydrolysis yield increased from 0% to 57% as the percentage of lignin decreased from 29% to 8% after ozonolysis pretreatment of poplar sawdust (Vidal and Molinier, 1988). Ozonolysis pretreatment has the following advantages: (1) it effectively removes lignin; (2) it does not produce toxic residues for the downstream processes and (3) the reactions are carried out at room temperature and pressure (Vidal and Molinier, 1988). However, a large amount of ozone is required, making the process expensive.

25.2.5.2.3 Alkaline Hydrolysis

Some bases can also be used for pretreatment of lignocellulosic materials and the effect of alkaline pretreatment depends on the lignin content of the materials

(Kaar and Holtzapple, 2000). The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds cross linking xylan hemicelluloses and other components. Dilute NaOH treatment of lignocellulosic materials causes swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates and disruption of the lignin structure (Fan et al., 1987). The digestibility of NaOH-treated hardwood increased from 14% to 55% with the decrease of lignin content from 24–55% to 20%. However, no effect of dilute NaOH pretreatment was observed for softwoods with lignin content greater than 26% (Millet et al., 1976).

Ammonia was also used for the pretreatment to remove lignin. Iyer et al., (1996) described an ammonia recycled percolation process (temperature-170°C; ammonia concentration- 2.5–20%; reaction time, 1 h) for the pretreatment of corn cobs/stover mixture and switchgrass. The efficiency of delignification was 60–80% for corncobs and 65–85% for switchgrass.

25.2.5.2.4 Oxidative Delignification

The pretreatment of cane bagasse with hydrogen peroxide greatly enhanced its susceptibility to enzymatic hydrolysis (Crestini et al., 2006). About 50% lignin and most hemicellulose were solubilized by 2% H_2O_2 at 30°C within 8 h and 95% efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45°C for 24 h (Crestini et al., 2006). Bjerre et al., (1996) used wet oxidation and alkaline hydrolysis of wheat straw (20 g straw/l, 170°C, 5–10 min) and achieved 85% conversion yield of cellulose to glucose.

25.2.5.3 Biological Pretreatment

In biological pretreatment processes, microorganisms such as brown, white and soft-rot fungi are used to degrade lignin and hemicellulose in waste materials (Herpoel et al., 2002). Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are the most effective basidiomycetes for biological pretreatment of lignocellulosic materials (Kornaros and Lyberatos, 2006). The white-rot fungus *P. chrysosporium* produces lignin-degrading enzymes, lignin peroxidases and manganese-dependent peroxidases, during secondary metabolism in response to carbon or nitrogen limitation (Boominathan and Reddy, 1992). Both enzymes have been found in the extra cellular filtrates of many white-rot fungi for the degradation of wood cell walls (Waldner et al., 1988). Other enzymes including polyphenol oxidases, laccases, H_2O_2 producing enzymes and quinone-reducing enzymes can also degrade lignin (Blanchette, 1991). The advantages of biological pretreatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment is very low. Inhibition of fermentation has been shown to decrease when phenolic monomers and phenolic acids were specifically removed from a willow hemicellu-

lose hydrolysate by treatment with the lignin-oxidizing enzyme laccase (Jonsson et al., 1998).

25.2.6 Enzymatic Hydrolysis of Cellulose

Enzymatic hydrolysis of cellulose is carried out by highly specific cellulase enzymes (Suurnakki et al., 2000). Different kinds of 'cellulases' may be used to cleave the cellulose.

A cellulosic enzyme system consists of three major components: endo cellulase (EC 3.2.1.4), exo-cellulase (EC 3.2.1.91) and -glucosidase (EC 3.2.1.21) (Gan et al., 2003; Adsul et al., 2005).

The mode of action of each of these being:

(1) Endo- cellulase, 1,4- -D-glucan glucanohydrolase, CMCase, Cx: 'random' scission of cellulose chains yielding glucose and cello-oligo saccharides.
(2) Exo- cellulase, 1,4- -D-glucan cellobiohydrolase, Avicelase, C1: exo-attack on the non-reducing end of cellulase with cellobiose as the primary structure.
(3) -glucosidase, cellobiase: hydrolysis of cellobiose to glucose.

In addition to the three major groups of cellulase enzymes, there are also several ancillary enzymes that attack hemicellulose, such as glucuronidase, acetyl esterase, xylanase, beta-xylosidase, galactomannanase and glucomannanase (Duff and Murray, 1996). During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented. Various factors have been found to affect the enzymatic hydrolysis of the pretreated lignocellulosics - including (a) accessibility and adsorption characteristics of the cellulose, (b) reactivity of the cellulose and (c) adsorption characteristic of the lignin present (Converse and Optekar, 1993).

25.2.6.1 Cellulase

Successful utilization of cellulosic materials as renewable carbon sources is dependent on the development of economically feasible process technologies for cellulase production and for the enzymatic hydrolysis of cellulosic materials. In the biomass-to-ethanol processes, the cost of cellulase enzyme production constitutes a 43.4% of the total processing cost (Galbe and Zacchi 2002; Zheng et al., 2006). The enzyme production cost varies with the carbon source used, so it could be lowered by using less expensive carbon sources - such as lignocellulosic materials (Doppelbauer et al., 1987). Approaches to reduce enzyme costs include: 1) the recovery of enzymes (Eklund, 1994), 2) utilization of lignocellulosic substrates for cellulose production 3) development of high cellulase-producing mutant strain (Awafo et al., 1996; Pasha et al., 2005) and 4) the optimization of production and hydrolysis conditions

Cellulolytic enzymes isolated from various source differ in their molecular weight amino acid sequence and composition, isoelectric point, carbohydrate content, adsorbability onto cellulose, catalytic activity and substrate specificity

(Yu Cao and Huimin Tan, 2002; Karlsson et al., 2002; Gustafsson et al., 2003). Some cellulases particularly of bacterial origin are known to be strongly associated with microbial cells. Some cellulases are organized in supra molecular structures called cellulosomes as found in bacteria.

25.2.7 Screening of Cellulase-Producing Microorganisms

Although many microorganisms are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of cell-free enzymes capable of completely hydrolysing crystalline cellulose in vitro. Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. Bacteria belonging to *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora* and *Streptomyces* can produce cellulases (Bisaria, 1989). Cellulomonas fimi and Thermomonospora fusca have been extensively studied for cellulase production. Although many cellulolytic bacteria, particularly the cellulolytic anaerobes such as *Clostridium thermocellum* and *Bacteroides cellulosolvens* produce cellulases with high specific activity, they do not produce high enzyme titers (Vladimir et al., 2005). Because the anaerobes have a very low growth rate and require anaerobic growth conditions, most research for commercial cellulase production has focused on fungi (Duff and Murray, 1996).

Fungi that have been reported to produce cellulases include *Sclerotium rolfsii, P. chrysosporium and species of Trichoderma, Aspergillus, Schizophyllum* and *Penicillium* (de Vries and Visser, 2001; Mach and Zeilinger, 2003; Krogh, et al., 2004). Fungi of the genera Trichoderma and Aspergillus are thought to be potent cellulase producers and crude enzymes produced by these microorganisms are commercially available for agricultural use. Microorganisms of the genus *Trichoderma* produce relatively large quantities of endo and exo cellulase, but only low levels of -glucosidase, while those of the genus *Aspergillus* produce relatively large quantities of endo cellulase and -glucosidase with low levels of exo- -celluase production.

25.2.8 Improving Enzymatic Hydrolysis

The factors that affect the enzymatic hydrolysis of cellulose include substrates, cellulase activity and reaction conditions (temperature, pH). To improve the yield and rate of the enzymatic hydrolysis, research has focused on optimizing the hydrolysis process and enhancing cellulase activity (McCarthy et al., 2004).

25.2.8.1 Substrate Size

Substrate concentration is one of the main factors that affect the yield and initial rate of enzymatic hydrolysis of cellulose. At low substrate levels, an increase of

substrate concentration normally results in an increase of the yield and reaction rate of the hydrolysis (Cheung and Anderson, 1997). However, high substrate concentration can cause substrate inhibition, which substantially lowers the rate of the hydrolysis and the extent of substrate inhibition depends on the ratio of total substrate to total enzyme (Penner and Liaw, 1994).

25.2.8.2 Cellulase Loading

Increasing the dosage of cellulases in the process, to a certain extent, can enhance the yield and rate of the hydrolysis, but would significantly increase the cost of the process. Cellulase dosage of 10 FPU/g cellulose is often used in laboratory studies because it provides a hydrolysis profile with high levels of glucose yield in a reasonable time (48–72 h) at a reasonable enzyme cost (Gregg and Saddler, 1996).

Enzymatic hydrolysis of cellulose consists of three steps: adsorption of cellulase enzymes onto the surface of the cellulose, the biodegradation of cellulose to fermentable sugars and desorption of cellulase. The irreversible adsorption of cellulase on cellulose is partially responsible for cellulase deactivation (Converse and Optekar 1993). Addition of surfactants during hydrolysis is capable of modifying the cellulose surface property and minimizing the irreversible binding of cellulase on cellulose. The surfactants generally used in the enzymatic hydrolysis include nonionic Tween - 20, 80 (Wu and Ju, 1998).

The addition of -glucosidases into the T. reesei cellulases system achieved better saccharification than the wild system without -glucosidases (Xin et al., 1993). -Glucosidases hydrolyze the cellobiose, which is an inhibitor of cellulase activity. A mixture of hemicellulases or pectinases with cellulases exhibited a significant increase in the extent of cellulose conversion (Arrizubieta and Polaina, 2000). Cellulases can be recovered from the liquid supernatant or the solid residues. Enzyme recycling can effectively lower the enzyme cost (Movagharnejad, 2005). The efficiency of cellulose hydrolysis decreased gradually with each recycling step.

25.2.8.3 End-Product Inhibition

Cellulase activity is inhibited by cellobiose and to a lesser extent by glucose. Several methods have been developed to reduce the inhibition, including the use of high concentrations of enzymes, the supplementation of -glucosidases during hydrolysis and the removal of sugars during hydrolysis by ultra filtration or simultaneous saccharification and fermentation (SiSF). The production of enzymes is influenced by induction and catabolite repression (de Groot et al., 2003). 2-Deoxy-D-glucose (DG), a toxic glucose analogue has frequently been employed to isolate glucose-deregulated mutants (Haq et al., 2001). Hence development of 2 deoxy glucose resistant mutants will be a solution for increased cellulase production (Spiridonov and Wilson, 2000).

25.2.8.4 Formation of Inhibitors in Hydrolysis

In addition to the sugars, several inhibitors are formed or released in the hydrolysis process. During acid hydrolysis of lignocellulosics, aliphatic acids (acetic, formic and levulinic acid), furan derivatives and phenolic compounds are formed in addition to the sugars. Furfural and 5-hydroxymethyl furfural (HMF) are the most important furans, formed by decomposition of pentoses and hexoses respectively (Zhao et al., 2005). Acetic acid is formed from hydrolysis of the acetyl groups in the hemicellulose as a consequence of deacetylation of acetylated pentosan (Lawford and Rousseau, 1993). Phenolic compounds are e.g. phenol, vanillin, vanillic acid, vanillyl alcohol, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, coumaric acid, syringaldehyde, syringic acid, cinnamaldehyde, dihydroconiferyl alcohol, hydroquinone, catechol, veratrole, acetoguaiacetone, homovanillic acid and Hibbert's ketones. These compounds are known to inhibit ethanol fermentation performance synergistically (Keating et al., 2006). Therefore, in order to improve the fermentability and quality of the hydrolysates, it is necessary to remove the inhibitory compounds before fermentation.

25.2.9 Mechanism of Inhibition by Various Hydrolysate Inhibitors

25.2.9.1 Weak Acids

The growth-inhibiting effect on microorganisms has been proposed to be due to the inflow of undissociated acid into cytosol (Zhao et al., 2004). It has been found that acetic acid, formic acid and levulinic acid are the most common carboxylic acids in the hydrolyzates. Acetic acid it self helps ethanol fermentation at low concentration but in the presence of phenolic inhibitors, reduces ethanol yield drastically (Keating et al., 2006).

25.2.9.2 Furfural and HMF

Furfural is partially metabolized by *S. cerevisiae* (Taherzadeh et al., 1998). During fermentation furfural reduction to furfuryl alcohol occurs with high yields (Palmqvist et al., 1999). Inhibition of aerobic growth of *Pichia stipitis* by furfuryl alcohol has been reported (Weigert et al., 1988) whereas only slight inhibition of anaerobic growth of *S. cerevisiae* has been detected (Palmqvist et al., 1999). HMF is also metabolized by *S. cerevisiae* (Taherjadeh, 1999). HMF converted at a lower rate than furfural, which might be due to lower membrane permeability and cause a longer lag-phase in growth (Larsson et al., 1998).

25.2.9.3 Phenolic Compounds

Phenolic compounds have been suggested to exert a considerable inhibitory effect in the fermentation of lignocellulosic hydrolysates, the low molecular weight phenolic compounds being most toxic (Buchert and Niemelä, 1991). Phenolic compounds penetrate into biological membranes and cause loss of integrity, thereby affecting their ability to serve as selective barriers (Heipieper and de Bont, 1994).

25.2.10 Removal of Inhibitors

Hydrolysis of lignocellulosic materials always goes together with the formation of by-products that inhibit the fermentation process. Although the concentration of these by-products strongly depend on the raw material hydrolysis and method, it is probably not possible to avoid them completely. There have been several studies, which deal with decreasing the by-products and thereby increasing the fermentability of the hydrolyzates. Two different approaches can be distinguished: reducing the formation of inhibitors and detoxification.

25.2.10.1 Reducing the Formation of Inhibitors

The degradation products probably are the most important inhibitors in the hydrolyzates. Consequently, modification of the hydrolysis process in order to minimize formation of the hydrolysis by-products is of interest for two reasons: (a) a higher sugar yield is obtained by decreasing the formation of by-products and (b) a better fermentability of the hydrolyzate is obtained. If dilute acid hydrolysis is made at mild conditions (temperature less than 200°C), the hydrolyzates are fermentable due to the low concentrations of inhibitors such as furfural and HMF. Mild conditions result in a fermentable hydrolyzate, but a major part of the cellulose is not depolymerized to sugars yet. On the other hand, if we apply a higher temperature (or longer time) for the hydrolysis, a better conversion of the cellulose and hemicellulose is obtained. However, this procedure causes the hydrolyzate to be more inhibiting and decrease the fermentability (Tengborg et al., 1998). Methods to increase the sugar yield in hydrolysis are e.g. the drainage technique, multistage acid hydrolysis and acid plus enzymatic hydrolysis. Basically, these methods all use different conditions for hydrolysis of the cellulose and the hemicellulose part of the lignocellulose. It is generally accepted that hemicellulose is hydrolyzed faster, or at milder conditions than cellulose. Consequently, when the cellulose is being depolymerized to glucose a major part of the hemicellulose has already been hydrolyzed degraded to by-products (Saeman, 1945). In the modified drainage technique, the liquid solution is collected from the reactor, while the solid part (cellulose and lignin) is retained. Hence, already formed monosaccharides do not remain in the reactor and degradation is decreased.

In conclusion, the enzymatic hydrolysis is probably the best and one-stage acid hydrolysis is the worst in terms of formation of inhibitors. However, we should keep in mind that the only enzymatic hydrolysis is probably not the best in terms of process economy at present stage of development (Qureshi and Manderson, 1995).

25.2.10.2 Detoxification

In order to remove the inhibitors and increase the hydrolysate fermentability, several detoxification treatments, including chemical, physical and biological methods have been used. These methods include neutralization, over liming, ion exchange resins, adsorption into activated charcoal, Tin oxides and treatments with enzymes such as peroxidase and laccase (Palmqvist and Hahn-Hagerdal. 2000). Since detoxification increases the cost of the process (Sivers et al., 1994) it is important either to overcome detoxification steps or to develop cheap and efficient methods.

25.2.10.2.1 Overliming

Overliming is one of the classical chemical detoxification methods (Leonard and Hajny, 1945). In this method, CaO or $Ca(OH)_2$ is added to the hydrolyzate to adjust the pH to 9 or 10. The most economical method of detoxification involves treatment of hydrolyzates with solid calcium hydroxide called as 'overliming' (Ranatunga et al., 2000). Furan derivatives make transient complexes with calcium ions and this complex will be removed in solids (Purwadi et al., 2004). Larsson et al. (1999) tested the detoxification effects by increasing the pH to 10 with NaOH and $Ca(OH)_2$. Both reagents increased the fermentability of the hydrolyzates, although better results were obtained with calcium hydroxide. Calcium hydroxide is stated to catalyze the condensation reactions of formaldehyde (Niitsu et al., 1992). Possibly, it can catalyze the condensation of other kinds of aldehydes in the hydrolyzates such as furfural and HMF.

Extraction with organic solvents such as ether or ethyl acetate has also been applied to remove most of the inhibitors, such as phenolics, weak acids and furans (Pasha et al., 2007a; Palmqvist, 1998).

25.2.10.2.2 Activated Charcoal Treatment

Activated charcoal treatment is an efficient and economic method of reduction in the amounts of HMF, phenolic compounds (Parajo et al., 1996), acetic acid, aromatic compounds (Parajo et al., 1997), furfural and hydroxy methyl furfural (Mussato and Roberto, 2001) normally found in hemicellulosic hydrolysates.

Biological detoxification methods: Biological detoxification is another method to enhance the fermentability of hydrolyzates. Lignin is degradable by several fungal enzymes: lignin peroxidase, Mn-dependent peroxidase and laccase or mono-phenol

oxidase (Lee, 1997). Jonsson et al. (1998) and more recently Chandel et al., 2007c, applied laccase to remove phenolic monomers and phenolic acids from willow hydrolyzates treated with stream and SO_2. Treatment with the peroxidase and laccase enzymes, obtained from the ligninolytic fungus *Trametes versicolor*, has been shown to increase the maximum ethanol productivity in a hemicellulose hydrolysate of willow (Jonsson et al., 1998).

25.2.11 Microorganisms for Ethanol Production

Ethanol fermentation is a biological process in which fermentable sugars are converted to ethanol and CO_2 by microorganisms. The microorganism is the key factor in the conversion of sugars to ethanol. There are several important characteristics of the microorganism in an industrial 'hydrolyzate-to-ethanol' process. Ideally, the microorganism should give a high ethanol yield, have a high ethanol tolerance, be resistant to inhibitors of hydrolyzates, with no oxygen requirement and a broad substrate utilization range. It is also desirable to have a strain with high sugar consumption rate and productivity, minimal nutrient requirement, high salt tolerance, high shear tolerance, thermotolerance, safety for humans and no spore formation (Picataggio and Zhang, 1996). Strain development of different organisms is on the way to achieving the criteria listed above. Although improvements have been made, no single microorganism has been developed yet which satisfies all those characteristics.

Although bacteria (Table 25.4), fungi and yeast (Table 25.5) can be used for fermentation, the most commonly used microbe has been yeast. Bioconversion of

Table 25.4 Bacterial species producing ethanol

Ethanol fermenting microbe	Ethanol yield (g/L) at 180/L sugar
Clostridium sporogenes	45–50
Clostridium idioli	85
Clostridium spenoidis	80
Clostridium soredelli	75
Zymomonas mobilis	82
Zymomonas mobilis subsps pomaceas	75
Spirochaeta aurantia	65
Spirochaeta stenostrepta	37
Spirochaeta litoralis	42
Erwinia amylovora	50
Escherichia coli KO11	35
Escherichia coli LYO1	40–50
Leuconostoc mesentroides	40
Streptococcus lactis	44
Klebsiella oxytoca	40
Klebsiella aerogenes	24

Miyamoto, 1997; Dien et al., 2003; Matthew et al., 2005; Ingram et al., 1999.

Table 25.5 Yeast species producing ethanol

Yeast species	Temp. (°C)	pH	Carbon source (g/L)	Nitrogen source g/L	Incubation time hrs.	Ethanol produced g/L	Reference
27817 *Saccharomyces cerevisiae*	30	5.5	Glucose50–200	Peptone 2 Amm. sulfate 4	18–94	5.1–91.8	Vallet et al., 1996
L-041 S.*cerevisiae*	30–35	-	Sucrose100	Urea 1 Sucrose 1–2	24	25–50	Leticia et al., 1997
181 S. *cerevisiae* (Aerobic)	30	6.0	Glucose 10	Peptone 5	40–160	-	Toder and Tsonka, 2002
UO-1-S *cerevisiae* (Aerobic)	30	5.0	Sucrose 20	Amm. sulfate1	60–96	-	Camacho et al., 2003
V5- *S. cerevisiae*	24	-	Glucose 250	-	36	-	Viriginie et al., 2001
ATCC, 24860 S. *cerevisiae*	30	4.5	Molasses1.6–5	Amm. sulfate 0.72–2.0	24	5–18.4	Ergun and Mutlu, 2000
Baker's yeast *S. cerevisiae*	28	4.5	Sugar 150–300	-	192	53	Roukas, 1996

various agro residues into ethanol also carried out by different yeasts (Nigam, 2001; Krishna et al., 2001; Ali et al., 2004; Sharma 2004; Palmarola-Adrados et al., 2005; Chandel et al., 2007c).

Saccharomyces is also generally recognized as safe (GRAS) as a food additive for human consumption and is therefore ideal for producing alcoholic beverages and for leavening bread. *Saccharomyces cerevisiae*, which can produce ethanol to give concentration as high as 18 percent of the fermentation broth, is the preferred one for most ethanol fermentation. Theoretically, 100 grams of glucose will produce 51.11 g of ethanol and 48.8 g of carbon dioxide. However, in practice, the microorganisms use some of the glucose for growth and the actual yield is less than 100%. *Saccharomyces cerevisiae* has several essential and desirable characteristics for fermentation of hydrolyzates, which are widely cited in the literature. But the main disadvantage is its limited substrate utilization range, specially its inability to ferment xylose. This is particularly disadvantageous when fermenting hardwood hydrolyzates. Still, *S. cerevisiae* is probably the first choice for most hydrolyzates, especially from softwoods, where glucose and mannose constitute the largest part of the sugars. Some identified xylose fermenting microorganisms are *Pachysolen tannophilus, Pichia stipitis, Candida shehatae* yeast strains and the most widely applied recombinant bacterial strains are *E. coli, Klebsiella oxytocoa* and *Zymomonas mobilis* (Krishnan et al., 1997; Himmel et al., 1997). *Zymomonas mobilis* is an unusual Gram-negative microorganism that has several appealing properties as a biocatalyst for ethanol production. The microorganism has a homoethanol fermentation pathway and tolerates up to 120 g/l ethanol. It has a higher ethanol yield (5–10% more ethanol per fermented glucose) and has a much higher specific ethanol productivity (2.5×) than *Saccharomyces sp.* (Sprenger, 1996). Furthermore, *Z. mobilis* is GRAS and has simple nutritional needs. Despite its advantages as an ethanologen, *Z. mobilis* is not well suited for all the biomass resources conversion because it ferments only glucose, fructose and sucrose (Swings and Deley, 1977). Moreover, for *Z. mobilis* on synthetic medium containing glucose, fructose or sucrose, the specific rates of sugar uptake and ethanol production are at a maximum when utilizing the glucose medium. In addition, *S. cerevisiae* is still preferred by the industry because of the yeasts hardiness.

Fermentation microorganisms have also been undergone continuous improvement. Genetically engineered microorganism that can convert pentoses as well as hexose sugars simultaneously to ethanol can greatly improve ethanol production efficiency and reduce the production cost (Sun and Cheng, 2002). Both yeasts (such as Saccharomyces and Pichia species) and bacteria (*E. coli, Klebsiella* and *Zymomonas*) have been genetically engineered to ferment glucose, xylose and arabinose sugars (Ingram et al., 1999; Nigam, 2001). Though new technologies have greatly improved bioethanol production, yet there are still a lot of problems, which has to be solved out. The major problems include maintaining a stable performance of recombinant yeast in commercial scale fermentation operations (Chan et al., 2004), developing more efficient technologies for lignocellulosic biomass (conversion) and integrating best components into an economic ethanol production system.

25.2.12 Thermotolerant Yeasts

The metabolic activity of yeasts results in exothermic production of heat during ethanol fermentation (Jones et al., 1981) and the quantity of heat released per gram varies with different substrates. The fermentation of pentoses results in the release of larger quantities of heat than hexoses (Holderby and Moggio, 1960). As the substrate concentration increases, the amount of heat produced will also increase (Lyons, 1981). Without temperature control, this rise in temperature continues until the temperature of the medium becomes intolerable for growth and activity. This often results in incomplete fermentation and thereby decreases the fermentation efficiency (Roxas and Anguila, 1971). Eventually the culture enters a stationary phase, the fermentation slows down, stops and the cells die. Usually this occurs at temperatures ~35°C, however it varies depending on the strain. Yeast in general, grows at relatively low temperatures in comparison with bacteria with upper temperature limits for thermotolerant and thermophilic yeasts at 42°C and 45°C respectively (Arthur Watson, 1976). Studies dealing with thermotolerant and ethanol-tolerant yeast strains (Ballesteros et al., 1992) showed that the thermotolerant strains have a reduced ethanol tolerance at higher temperatures (Szcodrak and Targonski, 1989; Ballesteros et al., 1994), resulting in a reduction in ethanol production. Banat et al., (1992) have reported that a *Kluyveromyces marxianus* strain is able to grow at 52°C and ferment ethanol at 50°C but ethanol production rate was less.

25.2.12.1 Thermotolerant *Saccharomyces cerevisiae* VS_3 Strain

Our thermotolerant yeast VS_3 strain was isolated from samples collected in summer month (May) from hot regions of Andhra Pradesh like Thermal power station in Kothagudem, where the temperatures in summer months are more than 45°C. After mass screening program, the four isolates obtained were named as VS_1, VS_2, VS_3 and VS_4. Out of the four, VS_3 had the ability to grow on the plate culture at 42°C & 44°C, it has many advantages like good ethanol tolerance and also it has the ability to produce more biomass (KiranSree et al., 1999).

25.2.13 Improved Methods for Ethanol Fermentation

25.2.13.1 Simultaneous Saccharification and Fermentation

A process based on enzymatic hydrolysis and fermentation are today regarded as the most promising alternative in converting the carbohydrates in lignocellulosic materials into ethanol with high yields and low production cost (Galbe and Zacchi, 2002; Sassner et al., 2006). The SiSF have several advantages over performing the

steps separately like process alleviates end-product inhibition of the enzymes and is also less capital intensive (Wingren et al., 2003). In general, simultaneous saccharification and fermentation (SiSF) promises to achieve low enzyme loadings fast hydrolysis reaction rates and high product yields. The optimal temperature for SiSF is around 38°C, which is a compromise between the optimal temperatures for hydrolysis (45–50°C) and fermentation (30°C). Thermo tolerant yeasts and bacteria have been used in the SiSF to raise the temperatures close to the optimal hydrolysis temperature. The disadvantages which need to be considered for SiSF include: (1) incompatible temperature of hydrolysis and fermentation; (2) ethanol tolerance of microbes and (3) inhibition of enzymes by ethanol.

25.2.13.2 Immobilization of Cells

Adsorption of cells on the solid support and entrapment in polymer gels are efficient methods for immobilizing cells. There are several advantages of using immobilized cell system over a free cell system. First, a higher cell mass per unit fermentation volume can be achieved than with batch, continuous or cell recycle system, resulting in a corresponding increase in ethanol production. Second, there is no need for cell removal or cell recycle, making extraction of product more economical i.e. reduction in down stream processing cost. Third, maintaining a specific growth and dilution rate inherent in continuous free cell systems is not a factor in an immobilized system (Williams and Munnecke, 1981). When microorganisms are agglomerated or attached to relatively large solid supports, fluid viscosity is lowered than that corresponding to medium containing free cells. Lower viscosity contributes to better mixing and mass transfer properties in the reactor. Solid supports in the medium are likely to concentrate the nutrients at the Liquid-solid interface. Microorganisms attached to these supports are exposed, in their microenvironment, to higher nutrient concentration than exist in the bulk solution. Thus, with higher cell population, fast growth rate and rapid product formation results in higher productivity.

25.2.13.3 Adaptation of Microorganisms

Fermentation of wood-derived hydrolysates is problematic when compared with pure sugars because of the potential toxic inhibitors that are released as a result of hydrolysis (Yu et al., 1987). The fermentation of such hydrolysates has been slow, incomplete and has resulted in low ethanol concentration and poor yields (Clark and Mackie, 1984). Parekh et al., (1987) used an adapted strain of *P. stipitis* on a steam-stripped hardwood SSl and found that it yielded 46% more ethanol, with over 90% xylose utilization than steam-unstripped hardwood and that the presence of softwood SSl is a source of hexose enhanced xylose utilization. Yeast cells adapt to hyper osmotic shock by accumulating glycerol and altering expression of hundreds of genes. This transcriptional response of *Saccharomyces cerevisiae* to

osmotic shock encompasses genes whose products are implicated in protection from oxidative damage (Krantz et al., 2004).

25.2.13.4 Strain Improvement

Maintaining production strains of microorganisms without trying to improve them is futile. Periodic reisolation and evaluation under production conditions is necessary, not only to replenish stocks of primary culture sources, but also to allow selection of strains under new fermentation conditions and with fermentation raw materials that may not be constant in composition. Genetic strategies for yeast stain improvement for fuel ethanol production include adaptation, mutation and selection, spheroplast fusion, hybridization, transformation and Recombinant DNA technology. Homothallism and inability to sporulate pose a problem for sexual hybridization in yeast.

Xylose and Arabinose are the predominant pentose sugars derived from the hemicellulose of most feedstocks. Two routes for arabinose metabolism have been engineered in *S. cerevisiae* and adapted strains of *P. stipitis* have been shown to ferment hydrolysates with ethanol yields of 0.45 g/g sugar consumed so commercialization seems feasible for some applications. Native strains of *Saccharomyces cerevisiae* do not use xylose as a carbon source. *Candida utilis* or 'torula yeast' will grow on xylose, but this yeast is strictly aerobic and does not produce ethanol. In the early 1980s, following the discovery that *S. cerevisiae*, *Schizosaccharomyces pombe* and other yeasts can ferment D-xylulose to ethanol (Wang and Schneider, 1980), intensive screening efforts rapidly revealed that some could convert xylose to ethanol directly under aerobic or oxygen-limiting conditions (Jeffries, 1982). Attention focused on *Pachysolen tannophilus*, *C. shehatae* (Du Preez and vander Walt, 1983) and *Pichia stipitis*, which are the best native xylose-fermenting yeasts known (DuPreez et al., 1986). Many improvements have been made in the genetic engineering of yeasts and bacteria for the fermentation of xylose and arabinose to ethanol and other products such as lactic acid.

25.2.13.4.1 Protoplast Fusion

Protoplast fusion has made a significant contribution to our understanding of the genetics and biochemistry of the non-conventional yeasts and it has facilitated the creation of the novel strains of yeasts that display enhanced biotechnological potential. Yeast protoplast may be induced to fuse, under the appropriate physiological conditions by electrical or chemical means. Protoplast fusion provides characteristic advantage such as promotion of high frequencies of genetic recombination between organisms for which poor or no genetic exchange has been demonstrated or which are genetically uncharacterized. Heluane et al., (1993) have transferred the genes for that utilization of xylose from *P. tannophilus* to *S. cerevisiae*. The hybrids assembled the *S. cerevisiae* parent morphologically and in sugar assimilation. The technique of pulse field electrophoresis showed that the chromosome-banding

pattern was intermediate between the two parental species. These hybrids were able to utilize D-xylose with biomass yields higher than those of any other D-xylose-fermenting yeast.

In an attempt to increase yeast strains with ethanol tolerance and the ability to ferment xylose, *S. cerevisiae* has been fused with pentose utilizing strains of *C. shehatae or P. stipitis* (Pasha et al., 2007b; Guptha, 1992), but mononucleate fusants quickly segregate into their parental type strains (Yoon et al., 1996). Other researchers report fusants between *S. cerevisiae* and *P. stipitis* that show the capacity for xylose fermentation and an ability to ferment glucose in the presence of 6% ethanol (Kordowska-Wiater and Targonski, 2001). Given the instability of the hybrids, this will probably not lead to commercial yeast strains. Kida et al., (1992) constructed a thermotolerant flocculating yeast (KF-7) by protoplast fusion. However the fusant KF-7 acquired thermotolerance but its osmotolerance was found to be inferior to IR2.S. Gera et al., (1997) constructed intergeneric ethanol producing hybrids from thermotolerance *Kluyvermocyes marxianus* capable of growing upto 52°C and non-thermotolerant *Saccharomyces cerevisiae* capable of growth up to 40°C by protoplast fusion. The high production of ethanol by hybrid fusions appears to be due to consultation in the pathway of ethanol production by complementation (Takagi et al., 1983).

25.2.13.4.2 Mutations

Mutations are the ultimate fuel for evolution, but most mutations have the negative effect on fitness. Mutation is any change that alters the sequence of bases along the DNA molecule, thus modifying the genetic material. Chemical and physical mutagens treatments are used to induce mutation frequencies to detectable levels. One disadvantage of this approach is that mutagenises is a destructive process and it cause gross rearrangement of genome, resulting in numerous undesirable changes. In addition, mutagenesis is seldom employed with industrial food and beverage yeast strains. These strains are usually polyploid and after mutagenic treatment the mutations usually do not reveal themselves because of the presence of non-mutated alleles. An important point in mutant screening is the rate of mutation. This depends on the mutagen employed, the dose rate and the psychological conditions. A moderate mutagen dose (i.e. one with 20% survival rate) has been found advisable for obtaining superior polyploid strains. The chance of forming multiple mutations is reduced, which therefore upwards the frequency of isolating strains differing at a single locus. Induced mutagenesis using physical and chemical mutagens seems to be a simple and rational approach for yeast strain improvement. Selection after treatment with mutagens has been used to a considerable extent improvement of yeasts. Sridhar et al. (2000) examined mutagenesis as a means to generate thermotolerance in yeasts and enhanced ethanol production (Sridhar et al., 2002). Kida et al. (1992) used ethyl methane sulphonate for strain improvement studies. Induction of mutation by UV and MNNG followed by selection was successfully used for improvement of baking yeast strains (Pasha et al., 2007b).

25.2.13.4.3 Metabolic Engineering of Yeasts

Even though genes for xylose assimilation are present in *S. cerevisiae*, they are not expressed at a sufficient level to enable significant sugar assimilation. Metabolic engineering can alter sugar transport, assimilation, the pentose phosphate pathway, glycolysis and the terminal steps of fermentation. While a few of the changes enable xylose utilization in S. cerevisiae, most have marginal effects. Over the last decade, the development of microorganisms capable of converting xylose and other hemicellulose-derived sugars to ethanol at high yields has been one of the most significant advances in the fields of both biomass conversion and metabolic engineering. The native-substrate utilization strategy is exemplified by the work of Ingram et al. (1999) with *E. coli* and *Klebsiella oxytoca*, in which organisms that naturally use hemicellulose-derived sugars were engineered to produce high product yields. The recombinant substrate utilization strategy is exemplified by the work of Ho et al. (1999) and Hahn-Hägerdal et al., (2001) with *Saccharomyces cerevisiae*. In the work by these three groups, an organism with high product tolerance and yield was engineered so that it was able to use desired substrates.

Bacteria employ xylose isomerase (EC 5.3.1.5) to convert D-xylose to D-xylulose, whereas most yeasts, fungi, plants and animals use aldose (xylose) reductase (EC 1.1.1.21: XYL1) and xylitol dehydrogenase (EC 1.1.1.9:XYL2) with xylitol as an intermediate (Chiang and Knight, 1960). When NADPH is a cofactor in the first step, the reaction is tied to NADPH production. The second step is coupled to reduction of NAD + , which can create a cofactor imbalance when oxygen or respiration is limiting (Bruinenberg et al., 1983). Xylulokinase (XK), encoded by the gene XKS1 (Sambrook et al., 1989), phosphorylates xylulose to xylulose 5-phosphate, which is then metabolized through the pentose phosphate pathway and glycolysis. *S. Cerevisiae* has been transformed with XYL1 and XYL2 from the xylose-fermenting yeast Pichia stipitis encoding XR and XDH, respectively (Walfridsson et al., 1996). Xylose fermentation by these recombinant strains of *S. cerevisiae* yields little ethanol and xylitol is the major product (Walfridsson et al., 1996), perhaps due to limited XK activity in *S. cerevisiae* (Deng and Ho, 1990). *Saccharomyces sp*. strain 1400(pLNH32), a fusion between *Saccharomyces uvarum* and *Saccharomyces diastaticus* (D'Amore et al., 1989), which overexpresses XYL1, XYL2 and XKS1, had an estimated ethanol yield of 0.44 carbon-millimole (c-mmol)/c-mmol in complex medium (Ho et al., 1998).

25.2.14 Economic Feasibility of Biofuels

The import cost of crude oil during the year 2004–2005 was around Rs. 1,21,500 crores (Subramanian et al., 2005). Historically, the projected cost of bioethanol has dropped from US$1.22 per liter to about US$0.31 per liter because of continuous improvement in pretreatment, enzyme application and fermentation (Wyman, 1999).

Further economic analysis of the bioethanol process has yielded a projected cost of as low as US$0.20 per liter in 2015 if enzymatic processing and biomass improvement targets are met (Wooley et al., 2001). The process used for ethanol production, the uses of cellulolytic enzymes makes the process cost effective (Alzate and Toro, 2005). Recently the cost of enzymes has been reduced by a combination of protein engineering and process development. The use of novel, tailored cocktails of enzymes with higher specific activities are required for further cost reductions (Gray et al., 2006) World leaders in enzyme production, Genencore, Novozym and Iogen are actively working towards reducing cost of cellulases production for bioethanol production using new and improved enzymes developed by protein engineering and directed evolution. Iogen Corporation is now producing ethanol commercially from cellulosics from its biomass-to-ethanol demonstration facility. Iogen makes its ethanol from wheat straw and corn stover and uses steam explosion to free the cellulose from hemicellulose and lignin digesting the cellulose with cellulase. The company's newly opened demonstration plant has a capacity of 260,000 gal per year (Griffith and Atlas, 2005) and the company plans a 42-million-gallon plant with an expected completion by 2007.

In India ethanol is mainly produced from molasses but other crop, which requires less input than sugarcane like sweet sorghum can be utilized for the purposes. Looking into production cost economics ethanol from damaged food-grains has the lowest cost but the availability is insufficient to meet the demand. The next lowest cost option is corn but is used as corn flour and in other food products for human consumption thus none of it is available for ethanol production in India. Therefore the most promising alternative is agro-residues which are available plentiful in India, while unfortunately the production of ethanol from agro residues is nil. Fuel ethanol production will remain a significant industry and become a potentially self-sustainable agricultural based system for the 22nd century, if the utilization of lignocellulosics becomes a commercial reality. R&D work for converting lignocellulosic biomass into ethanol is continuing in various laboratories and more research in this area needs to be encouraged for reducing the cost of ethanol production. (Chandel et al., 2007a, b).

25.3 Future Perspectives

The use of biofuels is hampered by scarce production and high cost. If blending of biofuel in gasoline is increased from 5%; it creates further hike of price due to high demand. It is proposed that a limited subsidy should be given for an initial period. This would help in bringing awareness quickly. Established biotech companies in India like- Biocon India Ltd. Bangalore, Advance Biochemicals, Pune, Reddy's laboratories, Hyderabad and Reliance Life Sciences Ltd. should come in front to produce new technologies for production of biofuels. Government has declared broad biofuel policies. The present need is the deregulation of feedstock and its pricing, simplification of licensing for biofuel production, which will make biofuel industry strong and vibrant.

In India, currently there are no full scale or demonstration plants for the ethanol production from agro residues or forestry waste. Indian Sugar and Distillers Units should initiate their R&D program for biofuels production based on lignocellulosics materials. Though, sugar industries are doing excellent in sugar demand and ethanol production from sugarcane molasses but they need to explore the ethanol production from sugarcane bagasse. Integration of sugar production from cane juice and bioethanol production from bagasse will benefit the industry and nation as a whole.

The next generation technologies would be more advantageous in terms of low cost production economics. If we consider the environmental friendly characteristics of biofuel blended gasoline as an automobile fuel, the pricing of ethanol/biodiesel needs to be viewed not only in terms of a financial cost benefit analysis, but also in terms of an economic cost benefit analysis.

25.3.1 Thermotolerant Yeast

The idea of producing thermotolerant yeast has intrigued producers and researchers for many years. Although significant progress has been made recently in thermotolerance of yeast, it is still around 42°C. However, if yeast were available which could tolerate 60°C, the process would look completely different from the way it looks today. For example, if we were to apply typical kinetics at this higher temperature, we would expect fermentation time to be one fourth of the time required today. Although heat would have to be removed at four times the rate that it is today, heat exchange is much more efficient when there is a large driving force (temperature difference between the fermenter media and the coolant). Cooling tower water, even if it were to rise to 38°C, would easily cool these fermenters. Besides designing an organism that would withstand high temperature, there is the issue of the volatility of the ethanol from the fermenter. This would make the CO_2 scrubber a much more critical part of the process. But the flip side of this problem is that the ethanol concentration in the fermenter would be lower, so the percentage dry solids (DS) in the fermenter could be even higher than it is today.

25.3.2 Yeast Engineered to Produce Less Glycerol

Glycerol is produced as a result of osmotic or chemical stress by yeast and to a lesser extent, by metabolic leakage from inside the yeast. Dissolved salts, especially sulfites, are known to result in reduced ethanol and increased glycerol formation. Increased levels of glycerol are found to be indicators of excess sugar, salts, or sulfites in the fermenter medium. In terms of metabolism, each molecule of glycerol is a lost molecule of ethanol. In addition to robbing some yield, glycerol tends to cycle-up in backset and can participate in saponification (soap-making) reactions with caustic or soda ash to promote foaming in the water column of a still. Yeasts

which produce reduced glycerol have been developed by several approaches. Some salt-tolerant yeasts are found to under-produce glycerol due to the reduced expression of a glycerol transport protein in the yeast's membrane, conserving glycerol within the cell. Another approach is to change the yeast's chemical pathways to promote more efficient ethanol formation. Such pathway improvements, called 'metabolic flux engineering,' could lead to improvements in the usefulness of industrial microbes.

25.3.3 Thermotolerant Yeast That Utilizes Pentose and Hexose Sugars

Biomass contains cellulose and hemicellulose with both Pentose (5-carbon) and Hexose (6-carbon) sugars. If weak-acid hydrolysis is used as the chemical treatment method of the plant biomass, the hemicellulose fraction is solubilized into monomeric sugars with reasonably good yields. Progress must be made in improved pretreatment technology and C5 fermentation. The problem utilizing the pentose potential is that the commonly used yeast strains, *S. cerevisiae*, are unable to convert C5 sugars. In order to obtain a commercially acceptable conversion of plant fiber into ethanol, the sugar potential in the hemicellulose fraction must be utilized. Great efforts have been made in introducing genes into *S. cerevisiae* to enable the organism to co-ferment sugars other than glucose. Another approach being pursued is development of other types of industrially robust organisms to ferment the C5 and C6 sugars found in biomass hydrolysates. More heat is released due to fermentations of pentoses; hence thermotolerant yeasts should be developed for pentose utilization.

25.3.4 Molecular Sieves

Molecular sieve dehydration technology utilizes microporous particles such as alumino-silicates, possessing a very precise pore size. The pores make it possible to separate small molecules from large ones through selective adsorption. For example, ethanol dehydration is accomplished with molecular sieves having a pore diameter of 3Å, which entraps water molecules, which have a diameter of 2.5Å. Ethanol molecules, which have a diameter of 4Å, cannot enter and therefore flow around the material. Molecular sieves first entered the ethanol industry in the 1970s. The first designs utilized liquid phase separation. The advantages were the elimination of hazardous solvents and reduced distillation complexity. However, process equipment size restrictions kept liquid phase molecular sieves from making significant inroads into the ethanol industry. There is a need to develop online sieves to separate ethanol during the fermentation to reduce ethanol toxicity and high ethanol yields.

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Chapter 26
Applications of the Non-Conventional Yeast *Yarrowia lipolytica*

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Contents

Abstract The yeast *Yarrowia lipolytica* is often found associated to proteinaceous or hydrophobic substrates such as alkanes or lipids. To assimilate these hydrophobic substrates, *Y. lipolytica* has developed an adaptative strategy resulting in elaborated morphological and physiological changes leading to terminal and β-oxidation of substrates as well as to lipid storage. The completion of the *Y. lipolytica* genome greatly improved our understanding of these mechanisms. Three main applications of this metabolism will be discussed. The first class corresponds to bioconversion processes for the production of secondary metabolites (citric acid), of aroma (γ-lactone, green note, epoxy geraniol) and of chemicals (dicarboxylic acids). The second class leads to fine chemical production by enantio separation of pharmaceutical compounds using *Y. lipolytica* enzymes such as epoxyde hydrolase or lipase. The third one refers to production of Single Cell Oils (SCO) from agriculture feedstock. In addition to its ability to handle hydrophobic substrates, *Y. lipolytica* has also been recognised as a strong secretor of various proteins such as proteases, lipases, RNases and others. A comprehensive review of recent developments of the *Y. lipolytica* expression/secretion system will finally be presented.

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26.1 Introduction

The yeast *Yarrowia lipolytica* is often found associated to proteinaceous or hydrophobic substrates such as alkanes or lipids. To assimilate these hydrophobic substrates, *Y. lipolytica* has developed an adaptative strategy resulting in elaborated morphological and physiological changes leading to terminal and β-oxidation of substrates as well as to lipid storage. The completion of the sequence of the *Y. lipolytica* genome greatly improved our understanding of these mechanisms. Three main applications of this metabolism will be discussed. The first corresponds to bioconversion processes for the production of secondary metabolites (citric acid), of aroma (γ lactone) and of chemicals (dicarboxylic acids). The second leads to fine chemical production by enantio separation of pharmaceutical compounds using *Y. lipolytica* enzymes such as epoxide hydrolase or lipase. The third one refers to production of Single Cell Oils (SCO) from agriculture feedstock. In addition to its ability to handle hydrophobic substrates, *Y. lipolytica* has also been recognised as a strong secretor of various proteins such as proteases, lipases, RNases and others. A comprehensive review of recent developments of the *Y. lipolytica* expression/secretion system will thus finally be presented.

26.2 Yarrowia lipolytica

Yarrowia lipolytica was assigned to the *Dipodascacea* family (Kurtzman and Fell, 1998). This hemiascomycetous yeast was formerly known as *Candida*, *Endomycopsis*, or *Saccharomycopsis lipolytica* (Barth and Gaillardin, 1996, 1997). *Y. lipolytica* was for a long time the only known species in its genus, but the asexual taxon *Candida deformans* appears phylogenetically closely related (Bigey et al., 2003). Recently, several species were proposed as new members of the Yarrowia clade (Kurtzman, 2005; Knutzen et al., 2007). *Y. lipolytica* is a dimorphic fungus which forms yeast cells, pseudohyphae and septate hyphae depending on strains and growth conditions (Dominguez et al., 2000). *Y. lipolytica* exhibits various colony shapes ranging from smooth and glistening to heavily convoluted and mat. Most strains are unable to grow above 32°C and the species is strictly aerobic. It is therefore not considered as pathogenic (Holzschu et al., 1979).

Y. lipolytica is often found associated to proteinaceous or hydrophobic substrates such as alkanes or lipids (Sinigaglia et al., 1994; Barth and Gaillardin, 1997; Fickers et al., 2005a). This yeast can be commonly isolated from dairy products such as cheeses or as a contaminant from various commercial chilled foods (cheeses, yoghurt, and sausages). Strains have also been isolated from soil, sewage and oil-polluted environments.

Most natural isolates are haploids, although occasional isolation of diploid clones has been reported. Both haploid and diploid states are stable under laboratory conditions, although diploids may tend to lose chromosomes upon storage by mitotic haploidisation. Mating of haploids involves two mating types, encoded by the *MatA* and *MatB* loci without silent cassettes (Butler et al., 2004). Sporulation occurs under specific growth conditions and generates four crescent shaped spores that can be separated with a micromanipulator (Barth and Gaillardin, 1996).

26.3 Hydrophobic Substrate Utilization

Although the first studies on hydrophobic substrate (HS) utilisation by *Y. lipolytica* were published more than 50 years ago (Peters and Nelson, 1948; Klug and Markovetz, 1967), many aspects of this metabolism are still unclear.

Since alkanes, fatty acids and triglycerides are poorly water-miscible compounds, the first challenge for the cell is thus to get access to them. In order to handle these substrates, *Y. lipolytica* has elaborated sophisticated mechanisms both to modify their solubility (surfactant-mediated transport) and to remodel its own cell surface (direct interfacial transport). The surfactant-mediated transport involves solubilization of HS by an extracellular emulsifier, called liposan, which was initially described as a 27 kDa glycolipid consisting of 5% protein, 20% carbohydrate and 75% lipid (Zinjarde and Pant, 2002). More recently, the production of a different surfactant was reported; containing 43–54.3% protein, 40–35.5% carbohydrate and 16–8.4% lipid (Vance-Harrop et al., 2003). It is presently unclear whether different emulsifiers are produced depending on strain, growth and media composition, or if there is a single protein presenting different levels of carbohydrate and lipid modification. No information is available on the gene(s) coding them and on their synthesis pathways, but recent successes at optimising culture conditions for bioemulsifier production should permit progress in the near future (Albuquerque et al., 2006). The second mechanism, direct interfacial transport, entails modification of the cell surface by the production of protrusions on top of which HS droplets bind (Mlickova et al., 2004). The production of emulsifier together with the synthesis of protrusions results in an increase of the apolar properties of the cell surface, in the decrease of HS droplet sizes and in an increase of the number of HS droplets on the cell surface, all improving the contact between HS and the cells. The protrusions resemble channels that connect the cell wall to the interior of the cell and probably constitute a transport mechanism which details remain to be elucidated. In addition to those surface properties changes, we observed that the periplasmic space width increased from approximately 80 nm to 150 nm while the cell wall thickness decreased from 40 to 25 nm.

The following metabolic steps have been the subject of recent reviews and will only be summarised here (Barth et al., 2003; Fickers et al., 2005b). Once into the cell, alkanes undergo primary or monoterminal oxidation in three steps which take place in both the endoplasmic reticulum (ER) and the peroxisomes. The first step

requires terminal hydroxylation by a P450-dependent alkane monooxygenase (AMOS, encoded by *ALK* genes belonging to the *CYP52* family) and its cognate electron transfer component, an NADP-dependent P450 reductase (CPR, *NCP* gene). The second step is performed either by a fatty-alcohol dehydrogenase (FADH, *ADH* genes) located in the endoplasmic reticulum or by a fatty-alcohol oxidase (FAOD, *FAO* genes) located in the peroxisome. The final step involves oxidation of the fatty aldehyde to a free fatty acid by ER or peroxisomal fatty-aldehyde dehydrogenase (FALDH, *ALD* genes).

The β-oxidation of fatty acid occurs exclusively in the peroxisome and requires four steps. The first step is performed by an acyl-CoA oxidase (AOX, *POX* genes). The two next reactions are catalysed by a multifunctional enzyme bearing the hydratase and dehydrogenase activities (*MFE* gene). The final step is catalyzed by the thiolase (*THIO* gene) and releases an acyl-CoA shortened by two carbons (N-2) and an acetyl-CoA.

Depending on the environmental conditions, *Y. lipolytica* is able to mobilise fatty acids or to store them as triacylglycerides (TAG) and steryl esters (STE) into lipid bodies (LB). Depending on growth conditions, *Y. lipolytica* can thus accumulate large amount of lipids, and is therefore considered as an oleaginous yeast (see below).

26.4 Genomics

Recently, the complete genome sequence of the haploid *Y. lipolytica* strain E150 (CLIB99) has been determined by the Génolevure consortium (Casaregola et al., 2000; Dujon et al., 2004). More information can be found at the Génolevures web site (http:/cbi.labri.fr/Genolevures/). *Y. lipolytica* has a 20.5 Mb genome with a G + C content of 49%, composed of six chromosomes. Although linkage groups are broadly conserved, natural isolates of *Y. lipolytica* seem to propagate mostly in a clonal form: they have thus somewhat divergent genetic structures which may account for the low spore viability observed in the initial inbreeding programs (Barth and Gaillardin, 1996). Several differences were observed between natural isolates; they differ by chromosome sizes, the number of rDNA repeats and the presence or absence of the retrotransposon Ylt1 (present in the American strain CBS6142-2 and absent in the French W29 and the German H222 wild-type strains). The total number of putative CDSs is estimated to be 6,703. This low number is unexpected considering the size of the *Y. lipolytica genome*. The estimated overall coding sequence is 45.8% against 70% for *S. cerevisiae*, i.e. the gene density is one gene per 3.3 kb for *Y. lipolytica* vs. one gene per 2 kb for *S. cerevisiae*. The number of detected tRNA genes, 510, is very high, with typical eukaryotic classes which are absent from other hemiascomycetous yeasts (Marck et al., 2006). *Y. lipolytica* also departs from other hemiascomycetous yeasts studied until now with 1006 introns at least in 908 genes (or over 13% intron containing genes), against 252 introns for *S. cerevisiae*. These introns are mainly located at the 5′end of the coding sequences like in

S. cerevisiae. Genes containing more than one intron (87 cases of genes with two to four introns) are more common than in *S. cerevisiae* (4 cases only). Finally, a greater diversity of transposable elements is observed, with several Ty3/gipsy-like elements, non-LTR retrotransposon of the human L1 family and DNA transposons of the *Mutator* family (Casaregola et al., 2002; Neuveglise et al., 2002; Neuveglise et al., 2005).

A striking feature revealed by the sequence of *Y. lipolytica* is a significant expansion of protein families containing paralogues of genes involved in hydrophobic substrates utilisation. Indeed, as shown in Table 26.1, several gene families contain more members than the other yeasts, which correlate nicely with *Y. lipolytica* addiction for HS utilisation. This was confirmed by functional analysis of the acyl-CoA oxidase family (GLR.2026) in which *POX2* and *POX3* code for long- and short- chain specific acyl-CoA oxidases, respectively (Wang et al., 1999; Luo et al., 2000; Luo et al., 2002). Similarly, in the family GLR.1254 encoding lipases, *LIP2*, *LIP7* and *LIP8* are specific of fatty acid chain lengths, C18, C6 and C10, respectively (Pignede et al., 2000a; Fickers et al., 2005b). In the cytochrome P450 family (GLR.3196), *ALK1* and *ALK2* were shown to be involved in alkane hydroxylation, C10 and C16, respectively, while *ALK3*, *ALK5* and *ALK7* code for lauric acid ω-hydroxylases (Iida et al., 2000; Hanley et al., 2003; Fickers et al., 2005a). The large number of orthologues clearly complicated functional analysis, although strategies were devised to inactivate successively each member in a family (Fickers et al., 2003). Recently however, functional analysis was greatly facilitated by the availability of *Y. lipolytica* oligo-arrays (available from Eurogentec), which allow easy follow-up of the expression of each gene in a family according to the alkane or fatty acid used for growth.

26.5 Genetic Tools

Many genetic tools are nowadays available for gene manipulation for *Y. lipolytica*, ranging from conventional *in vivo* genetics (complementation, strain construction by random spore or tetrad analysis, etc.) to genetic engineering using single- or multi-copy integration at preselected sites or at random, transposon mutagenesis, replicative plasmids of controlled copy-number etc.. Available strains, plasmids, cloned genes as well as detailed methods for manipulating this yeast have been reviewed previously (Barth and Gaillardin, 1996). New cassettes for rapid gene disruption and marker rescue using auxotrophic markers, like *URA3* and *LEU2*, or antibiotic resistance markers, like *hph* which confers resistance to hygromycine-B, have been developed. Those new markers are flanked by *loxR* sites which allow very effective (98%) and precise marker excision by transformation with a plasmid containing the Cre recombinase. Those tools allow efficient gene disruption (average frequency of 45%) and greatly facilitate marker rescue and reuse (Fickers et al., 2003).

Table 26.1 Gene families involved in hydrophobic substrate utilisation showing expansion in the *Y. lipolytica* genome compared to the ascomycetous yeast *S. cerevisiae*, *C. glabrata*, *K. lactis* and *D. hansenii*. Family number*, Number of paralogues: nb, gene names, references

Family	nb	Enzymatic function	Pathway	Gene names	Comment	References
GLS.95	4	Lipase/carboxesterase	Lipid hydrolysis	*LIP1*, *LIP3*, *LIP6*	*Y. lipolytica*-specific gene family, similar to bacterial and fungal type B lipases	Domingez et al., unpublished
GLS.94	16	Lipase	Lipid hydrolysis	*LIP2* *LIP4*, *LIP5* *LIP7*, *LIP8* L*IP9* to *LIP19*	Amplified in *Y. lipolytica* (one gene in *S. cerevisiae*, *C. glabrata*, *K. lactis*, two in *D. hansenii*) Lip2p is the major extracellular lipase	Pignede et al. (2000a); Fickers et al. (2005a)
GLR.2026	6	Acyl-CoA oxidase	β-Oxidation	*POX1* to *POX6*	Amplified in *Y. lipolytica* (one gene in *S. cerevisiae*, *C. glabrata*, *K. lactis*, three in *D. hansenii*) Pox2p is long chain specific, Pox3p is short chain specific	Luo et al. (2000); Luo et al. (2002)
GLR.3196	16	Cytochrome P450	Hydroxylation of alkanes or fatty acids; hydroxylation of C-15 trichotecenes, demethylation of sterols	*ALK1* to ALK12 (CYP52) *YALI0A18062g*, *YALI0B21824g*, *YALI0B21824g*, *YALI0E14509g*	Amplified in *Y. lipolytica* Alk1p for C10 alkane, Alk2p for C16 alkane hydroxylation, *Alk3*p, 5p, 7p for ω-oxidation	Iida et al. (2000); Hanley et al. (2003); Fickers et al. (2005a)
GLR.3319	4	Dienoyl-CoA hydratase/isomerase	β-Oxidation	*YALI0D06215g*, *YALI0F22121g*, *YALI0A07733g*, *YALI0B10406g*	Amplified in *Y. lipolytica* (one gene in *S. cerevisiae*, *C. glabrata*, *K. lactis*, 2 in *D. hansenii*)	
GLC.1852	3	3-Oxoacyl (acyl-carrier protein) reductase	β-Oxidation	*YALI0C19965*g, *YALI0D05929*g, *YALI0F29975*g,	*Y. lipolytica*-specific gene family, similar to bacterial 3-oxoacyl-acyl-carrier-protein reductase	
GLC.1876	4	Acyl-CoA dehydrogenase	β-Oxidation	*YALI0C16797g*, *YALI0D15708g*, *YALI0E12573g*, *YALI0F23749g*	*Y. lipolytica*-specific gene family, similar to fungal acyl-CoA dehydrogenases Two are induced on oleic acid	

GLR.1607	3	Epoxide hydrolase	Not known	*YALI0A01441g*, *YALI0C23224g*, *YALI0E19899g*	*Y. lipolytica*-specific gene family, similar to fungal and mammalian epoxide-hydrolases Peroxisomal, SKL motif
GLC.1427	11	4-Coumarate-CoA ligase	β-Oxidation	*YALI0A1423g*, *YALI0A15103g*, *YALI0B05456g*, *YALI0B07755g*, *YALI0C05885g*, *YALI0D17314g*, *YALI0E05951g*, *YALI0E11979g*, *YALI0E12419g*, *YALI0E20405g*, *YALI0F06556g*	*Y. lipolytica*-specific gene family, similar to bacterial and plant 4-coumarate-CoA ligase Peroxisomal, SKL or AKI motif; 2 are induced on oleic acid
GLC.2144	19	Unknown function	Not known	*YALI0A01650g*, *YALI0A03201g*, *YALI0A03993g*, *YALI0A04785g*, *YALI0A05005g*, *YALI0A06347g*, *YALI0A10120g*, *YALI0A11759g*, *YALI0A20790g*, *YALI0B00748g*, *YALI0B19228g*, *YALI0C01925g*, *YALI0C07931g*, *YALI0C16126g*, *YALI0D18832g*, *YALI0D21406g*, *YALI0E07293g*, *YALI0E14707g*, *YALI0F23199g*	No homologue in *S. cerevisiae, C. glabrata, K. lactis, D. hansenii* One repressed on oleic acid

(continued)

Table 26.1 (continued)

Family	nb	Enzymatic function	Pathway	Gene names	Comment	References
GLC.2493	6	Cell wall protein	Not known	*YALI0D27214g*, *YALI0E11517g*, *YALI0E18788g*, *YALI0E26125g*, *YALI0E31108g*, *YALI0F18282g*	*Y. lipolytica*-specific gene family,	
GLC.1851	3	Peroxisomal 2–4 dienoyl-CoA reductase	β-Oxidation	*YALI0C03003g*, *YALI0D01694g*, *YALI0E14322g*	Amplified in *Y. lipolytica* (one gene in *S. cerevisiae*, *C. glabrata*, *K. lactis*, 2 in *D. hansenii*) On induced and one repressed on oleic acid, PKL and SKI motif	

* For nomenclature and description of gene families, see http://cbi.labri.fr/Genolevures/fam/ *Y. lipolytica* specific gene families have no orthologue in the four species *S. cerevisiae*, *C. galbrata*, *K. lactis* and *D. hansenii*.

26.6 Bioconversions I

The possibility of producing valuable compounds by bioconversion of HS was first demonstrated by the production of intermediate metabolites (chapter 26.6.1). Development of genetic tools and research on β-oxidation demonstrated that modified *Y. lipolytica* strains could be used for aroma production, e.g. lactone production (chapter 26.6.2). Recent advances on pathways involved in HS utilisation by *Y. lipolytica* allowed the obtention of genetically modified strains for dicarboxylic acids (DCA) production (chapter 26.6.3).

26.6.1 Secondary Metabolite

Y. lipolytica is able to grow efficiently on low-cost HS such as fats and vegetable oils, which metabolites feed into its highly efficient TCA cycle. Depending on nutrition factors used for growth limitation, different TCA-cycle intermediates accumulate.

Nitrogen exhaustion triggers production and secretion of citric (CA) and isocitric (ICA) acids. Initially, wild-type strains produced simultaneously CA and ICA. The percentage of CA versus ICA depends on the substrates used. While CA represents 90% of acids when *Y. lipolytica* is grown on glucose, glycerol or ethanol, it represents only 60% when growth is performed on HS. However this percentage can be improved by either, i) the addition of acetate to the fermentation medium (the percentage of CA increases from 60 to 80%), ii) the use of selected mutant strains which increase CA levels from 65 to 80% (Finogenova et al., 2002; Finogenova et al., 2005) or iii) the use of genetically modified strains over-expressing the isocitrate lyase encoded by the *ICL1* gene. For those strains, CA represents nearly 95% of the acids when the cells are grown on any substrate (Kruse et al., 2004). *Y. lipolytica* exhibits a high maximal production rate (up to 3 g CA l^{-1} h^{-1}) and a high substrate-related yield (up to 1.5 g g^{-1}, especially on HS). Barth and co-workers reported the production of up to 250 g l^{-1} of CA with sunflower oil as carbon source (Kruse et al., 2004; Fickers et al., 2005a). Archer Daniels Midland (Decatur, Illinois, USA) currently uses such a process for the production of CA from rape seed oil (Barth and Gaillardin, 1996).

Thiamine limitation triggers 2-ketoglutaric acid (KG) production. *Y. lipolytica* exhibits a maximal production rate of 1.4 g KC l^{-1} h^{-1} with a good yield of 0.9 g g^{-1} (Weißbrodt et al., 1988; Chernyavskaya et al., 2000).

Recent work is based on the co-production of citric acid and lipase on animal and vegetable fat as shown by the Finogenova group. Depending on the mutants used, lipase activity varied from 120 to 2760 U ml^{-1}. Four of their mutants produced more than 1200 U ml^{-1} (Svetlana et al., 2005).

26.6.2 Aroma Production

γ- and δ-lactone (4- and 5- alkanolides) exhibit fruity and oily notes, which are naturally present in fruit and some fermented food product, and are thus interesting as food additives. The different lactones produced by *Y. lipolytica* and the patents mentioning *Y. lipolytica* for the production of lactones have been recently reviewed (Wache et al., 2003).

γ-lactone, the main aroma produced by yeast fermentation is obtained by biotransformation of castor oil. *Y. lipolytica* is one of the yeast species that is able to use the castor oil as well as ricinoleic acid or its methylester, probably due to the high level of lipase and esterase produced by this yeast (Fickers et al., 2005a). The pathway involves the shortening of the ricinoleic acid (C18) by four cycles of β- oxidation to produce the 4-hydroxidecanoic acid (C10), which is then spontaneously lactonized under acidic conditions. Processes have been developed for the production of γ-lactone with the wild-type strain resulting in concentrations of several grams per litter (12 g l^{-1}; Rabenhorst and Gatfield, 2000). However, *Y. lipolytica* was shown to degrade rapidly the lactone produced due to the high level of acyl-CoA oxidase activity. Indeed, this yeast possesses a family of six acyl-CoA oxidases (Aox1-6, encoded by *POX1*-6). First attempts to improve γ-lactone production involved the use of an uracil auxotrophic strain (Nicaud et al., 1996). The auxotrophic strain was transferred at high cell density in a medium containing low amounts of uracil allowing weak proliferation and improved lactone production.

Further improvements were obtained by mastering the *POX* genotype of the strain. Interest in this enzyme family came from our first experiments showing that disruption of *POX1* resulted in an increased β-oxidation activity but in a decrease of lactone production (Pagot et al., 1998). Characterisation of the Aox family enabled us to show that Aox2p was specific for long-chain-fatty acid while Aox3p was specific for short-chain fatty acid (Luo et al., 2000; Luo et al., 2002; Wache et al., 2003). Aox4p and Aox5p exhibited a weak activity on the whole spectrum of straight chain-CoA and Aox1p and Aox6p did not exhibit any detectable activity.

The first strategy used to improve lactone production was to decrease the β-oxidation of the C10 lactone-precursor and the degradation of the aroma compound by deleting enzymes involved in β-oxidation of acyl-CoA shorter than C10. A 5- fold increase in γ lactone production was observed upon deletion of the short-chain specific Aox3p resulting most likely from decrease of 4-hydroxy decanoic acid degradation and limited γ-lactone degradation. In this strain, the main lactone accumulated is 3-hydroxy-γ-lactone which represents 2/3 of the C10 lactones produced. The highest level of γ-lactone was obtained with the *Δpox2pox3* double mutant which did not accumulate the hydroxy form (Wache et al., 2000; Wache et al., 2001). The second strategy was to increase the β-oxidation of the C18 to the C10 lactone-precursor by over expressing Aox2p involved in long chain acyl-CoA β-oxidation. Even if the lactone production was improved, the results were not fully satisfying as the Aox activity toward long chain acyl-CoA remained low and the biotransformation took place slowly, probably due to failure of the Aox complex to be correctly imported into peroxisome (Titorenko et al., 2002).

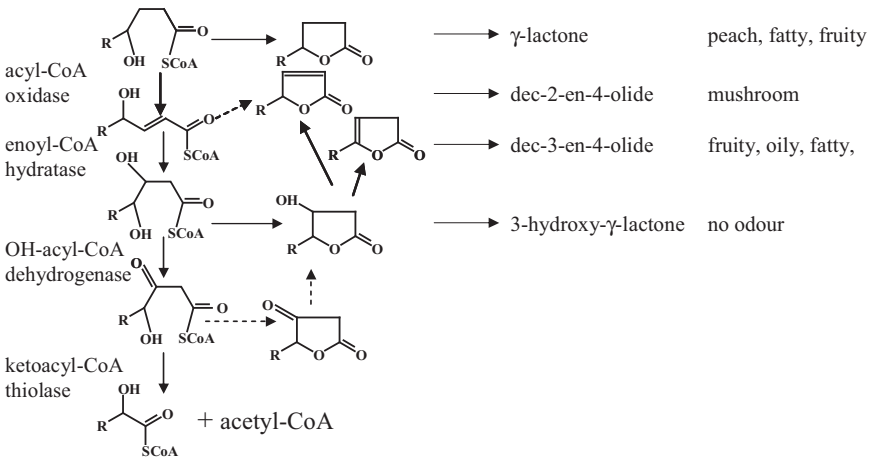

Fig. 26.1 The four steps of β-oxidation of the 4-hydroxydecanoyl-CoA, the catalysing enzymes and the lactones potentially formed at each step with their odorous note are shown. R: C6H13

Depending on the *POX* genotype, *Y. lipolytica* mutants accumulated different lactones (dec-2-en-4-olide, dec-3-en-4-olide and 3-hydroxy-γ-lactone) which seem to result from lactonisation of β-oxidation intermediates at the various steps of the C10-β-oxidation cycle (Fig. 26.1).

Their structure is closely related to that of γ-lactone, but their aromatic properties are quite different (Gatfield et al., 1993). The dec-3-en-4-olide possesses a fruity, peach-like odour more powerful than that of γ-lactone, its isomer (dec-2-en-4-olide) displays a mushroom-like note, while the 3-hydroxy-γ-lactone has no odour. Potential utilisation of those intermediates would depend on a cheap method to separate them or on the isolation of mutants affected in hydroxy-acyl-CoA dehydrogenase activity. Alternatively, limitation of this activity could be obtained by environmental parameters during bioconversion like pH and agitation.

26.6.3 DCA Production

Dicarboxylic acids (DCA), with the general chemical formula HOOC-(CH2)n-COOH, are fine chemical intermediate for the chemical industry. Nylons and other polyamides, resins, hot melt adhesives, powder coating, corrosion inhibitors, perfumes, lubricants, plasticizers, and greases are just some of the wide variety of products arising from DCA intermediates. The main DCA used are adipic acid (C6, hexanedioic acid), suberic acid (C8, octanedioic acid), sebacic acid (C10, decanedioic acid), dodecanedioic acid (C12) and brassylic acid (C13, tridecanedioic acid).

Chemical synthesis is effective with good yield for DCA smaller than C12. These can also be obtained by chemical conversion of fatty acids, such as sebacic acid which can be obtained by alkali fission of castor oil (ricinoleic acid). In order to develop alternative ways for the production of those short DCA, or to produce DCA longer

than C12 or unsaturated DCA which are difficult to produce chemically, research was initiated on bioconversion alternatives. Better characterization of the pathway involved in alkane degradation was a prerequisite. This degradation pathway involves hydroxylation of the alkane by a specific cytochrome P450 monooxygenase to produce the fatty alcohol. The alcohol is oxidized by either an alcohol oxidase or an alcohol dehydrogenase to produce the corresponding aldehyde. The aldehyde is converted to fatty acid by an aldehyde dehydrogenase. This pathway corresponds to the monoterminal-oxidation. Similar steps may occur on the fatty acid, which is called ω-oxidation (Fickers et al., 2005a). The fatty acid and the DCA produced may however be degraded through the β-oxidation pathway. Therefore, significant DCA production will necessitate mutants affected in this pathway.

The main yeast species investigated for DCA production using either classical genetics or genetic engineering are *Candida cloacae, Candida tropicalis* and *Yarrowia lipolytica*. First DCA production was investigated up to pilot plan scale by the Ajinomoto Company in Japan, using a *C. cloacae n*-alkane non assimilating mutant. When fed an *n*-alkane together with a co-substrate that supported growth of the mutant, this strain accumulated the homologous DCA (Shiio and Uchio, 1971).

The first industrial process was developed by the Nippon Mining Company (Japan), using mutants of *C. tropicalis* (Uemura, 1985). Since 1987, the company produced approximately 150 tons of brassylic acid (DCA-13) from *n*-tridecane per year, which is used in the perfume industry as a macrocyclic musk component (Uemura, 1985). At present, this process is exploited by Cathay Biotechnology of Shanghai (China) and Cognis (Düsseldorf, Germany). The mutant is defective for the two acyl-CoA oxidases and for the 3-ketoacyl-CoA thiolase (Kanayama et al., 1998).

In *C. tropicalis* DCA production was obtained by blocking the β-oxidation by deletion of the *POX* genes (Picataggio et al., 1991). Further improvement was obtained by overexpression of a cytochrome P450 monooxygenase and of the cognate cytochrome P450 reductase involved in the first hydroxylation step (Picataggio et al., 1992). However, in fermentations producing DCA from fatty acids, small amounts (ca 0.5% [wt/wt]) of ω-hydroxy fatty acid consistently accumulated in the broth. This accumulation indicated that the second step in the ω-oxidation pathway was also rate-limiting (Eschenfeldt et al., 2003).

Recently, Craft and coll. identified in *C. tropicalis*, three fatty alcohol oxidase genes (*FAO1-3*). The *FAO1* gene was shown to be highly induced during growth on fatty acids and Fao1p oxidizes ω-hydroxy fatty acid. Increasing the copy number of *FAO* genes and/or their transcriptional activity resulted in a high activity of alcohol oxydase and in an increase in the productivity of DCA (Eschenfeldt et al., 2003).

Currently, the production of DCA is studied by using *Y. lipolytica*. This yeast has the capacity to assimilate triglycerides as carbon source. The first step of this catabolism implies hydrolysis of triglycerides into free fatty acids and glycerol by the lipolytic enzymes (lipases), identified by Peters and Nelson (1948) under suitable conditions of pH. Hydrolysis of ester or oil and conversion into DCA take place simultaneously, which simplifies the operational protocol since the stage of chemical hydrolysis is eliminated.

In *Y. lipolytica*, six *POX* genes encode acyl-CoA oxidase isozymes (see above). (Smit et al., 2005) used *Y. lipolytic*a wild type strain W29 as well as double, triple and quadruple *POX*-deleted strains to investigate DCA accumulation (Fickers et al., 2005a; Smit et al., 2005). All the strains accumulated dodecanedioic acid from the diterminal functionalised 1,12-dodecane diol and 12-hydroxydodecanoic acid. The quadruple-deleted strain (*Δpox2*, *Δpox3*, *Δpox4*, *Δpox5*) was the only one able to accumulate DCA from C16 alkanol and alkanes. However, the DCA formed were rapidly degraded through the β-oxidation pathway.

By sequential disruption of the six *POX* genes, Thevenieau et al. (unpublished) constructed a sextuple *POX*-deleted strain to evaluate the DCA production. Deletion of the six *POX* genes eliminated chain shortening of the DCA produced by completely blocking of the β-oxidation pathway, increasing therefore DCA production. The DCA productivity is similar regardless of the substrate used (fatty acid, vegetable oils, fatty acid ester). To increase the specific bioconversion rate, we put the *CPR* gene (encoding the NADPH-dependent P450 reductase) under the control of the strong promoter pPOX2 induced by fatty acids. Overexpression of the target gene was carried out using single copy integrants, which were more stable than the multi-copy *C. tropicalis* strains obtained by gene amplification. A two-fold increase of the specific bioconversion rate was observed upon overexpression of the *CPR* gene, indicating that the hydroxylation was a rate-limiting step in the ω-oxidation pathway.

Among the twelve *ALK* genes encoding P450 cytochrome oxydase identified in *Y. lipolytica*, only *ALK1* and *ALK2* have a demonstrated role in alkane chain length specific hydroxylation (especially C10–, C16+) (Iida et al., 1998; Iida et al., 2000; Sumita et al., 2002). Recently, eight *ALK* genes have been expressed in plant indicating that *ALK3*, *ALK5* and *ALK7* code for ω-hydroxylase (Hanley et al., 2003). These studies demonstrated that a single P450 form is able to catalyse efficiently a cascade of sequential mono- and diterminal monooxigenation reactions form n-alkanes to diacids with high regioselectivity. A functional study of the *ALK* family has been recently undertaken by Thevenieau et al. (unpublished). Among twelve genes *ALK*, some may be specific of alkane chain length and their overexpression would make it possible to increase the production of DCA.

During the bioprocess, *Y. lipolytica* mutants store fatty acids as triglycerides in lipid bodies which are thereafter not anymore transformed into DCA, resulting in reduced bioconversion yield. Using biochemical and proteomic approaches, Athenstaedt et al. recently analyzed lipid bodies and identified proteins involved in the storage and utilization of fatty acids (Athenstaedt et al., 2006). In particular, we identified an acyl-CoA diacylglycerol acyltransferase like protein (*DGA1*, YALI0E32769g) and a lecithin cholesterol acyltransferase-like gene (*LRO1*, YALI0E16797g) which were shown to be involved in triglyceride and sterol ester accumulation in *S. cerevisiae* (Sandager et al., 2002) and in *Y. lipolytica* (Thevenieau, unpublished). Deletion of those two genes allowed further improvement of DCA production (Thevenieau et al., patent pending).

26.7 Bioconversion II

There is a growing industrial need for enantiomerically pure molecules, particularly in the pharmaceutical industry, since the legislation requires the production of molecule as single-enantiomer. Enzymatic methods for resolving racemic mixtures using whole cells or enzymes (free or immobilised) are very attractive due to their efficiency and selectivity. Whole cells or enzymes of *Y. lipolytica* have been applied in the reduction of prochiral carbonyl group and in enantioselective resolution of esters and epoxides.

26.7.1 Resolution of Ketones and Alcohols

Fantin and co-workers screened several *Y. lipolytica* strains isolated from various habitats (chilled food, sea water, butter and poultry meat) for the enantioselectivity of prochiral ketones and for the kinetic resolution of racemic secondary alcohols via oxidation (Fantin et al., 1996; Fantin et al., 2000). They recorded different yields and enantiomeric excesses for the anti-Pre-log reduction (*R*-enantiomer) depending on the structure of the ketones but also depending on the strain isolate. Best results were obtained in the reduction of hexenone and heptenone to their corresponding *R*-alcohols (Fantin et al., 1996). Lagos and co-workers in a study on the reduction of aryloxy-halo-2-propanones observed that *Y. lipolytica* displayed superior enantioselectivity compared to *S. cerevisiae*, which is widely used for this type of bioconversion (Lagos et al., 2004). They also demonstrated that *Y. lipolytica* presented a high tolerance to the substrate and that conversion and ees (enantiomeric excess) remained high with either growing or resting cell.

Kinetic resolution of cyclic and alicyclic racemic secondary alcohols was tested by Fantin and Co-worker (Fantin et al., 2000). While all ten *Y. lipolytica* strains tested were able to oxidize *endo*-bicyclo heptenol and *endo*-bicyclo octenol, only two strains were able to oxidize the *cis*-2-methylcyclohexanol and only one strain oxidized the *trans*-2-methylcyclohexanol. From those results, they proposed that *Y. lipolytica* may contain two genes coding for alcohol dehydrogenases with opposite enantioselectivity.

26.7.2 Resolution of Esters

Whole cells of *Y. lipolytica* were shown to carry out enantioselective hydrolysis of a range of racemic acetyl esters of secondary alcohols as well as enol esters (Fantin et al., 2001). As for ketones and alcohols, these authors demonstrated that the yields, enantiomeric excesses and enantioselectivity varied depending on the strain and the substrates. These activities can probably be ascribed to the membrane-bound lipases or carboxyesterases which are found in multi gene families (section 26.4).

The development of *Y. lipolytica* for ester resolution could be obtained by the construction of strains overexpressing selected enzyme activity or by the use of a purified enzyme as exemplified for the *Lip2* lipase. Strains over producing *Y. lipolytica* Lip2p were obtained by selection of overproducing strains after chemical mutagenesis and by gene amplification (Pignede et al., 2000b). Strains containing several integrated copies of the *LIP2* gene under the control of the oleic acid inducible *POX2* promoter produced 60,000 U ml^{-1} of lipase activity. Marty and co-workers showed that free Lip2p was efficient for the resolution of 2-bromo-arylacetic acid esters (Guieysse et al., 2004). Resolution of 2-bromo-*p*-tolylacetic acid ethyl ester catalyzed by Lip2p showed an enantiopreference of 28, almost equal to that obtained with *Burkholderia cepacia* lipase (E = 30). The *B. cepacia* lipase is selective to the (R)-enantiomer while the lipolytica Lip2p is selective to the (S)-enantiomer and has a higher catalytic activity. The most interesting observation is that Lip2p was the only enzyme able to catalyse the resolution of 2-bromo-*o*-tolylacetic acid ethyl ester (E = 27). By site mutagenesis and saturated mutagenesis at specific amino acid positions, mutants of Lip2p were obtained with improved enantioselectivity (E = 200) and higher activities (Fickers et al., 2005a). He and co-workers tested esterification of 2-ethyl hexanol with different fatty acids. No esterification was obtained with the immobilised Lipolase from Novo or with the free lipase from *Candida* sp 99–125 (a *Y. lipolytica* strain), while efficient esterification (about 85%) was obtained with the immobilized lipase from *Candida* sp 99–125 using saturated fatty acids with chain lengths of C8-C16 and with oleic acid. They optimized the esterification with the palmitic acid at 40°C in 10% petroleum and obtained an esterification degree of 91% (He et al., 2002).

26.7.3 Resolution of Epoxides

Optically active epoxides and vicinal diols are versatile fine chemical intermediates, useful for the production of pharmaceuticals, agrochemicals, ferro-electric liquid crystals, flavours and fragrances. Epoxide hydrolases (EHs; E.C. 3.3.2.3) catalyze the hydrolysis of epoxides to the corresponding vicinal trans-diols (Figure 26.2A).

EHs are found in a wide variety of organisms, including mammals, plants, insects, nematodes, protozoa, archaea, bacteria, fungi and yeasts. Analysis of publicly available genomes revealed 239 putative EH's from 91 taxonomically different organisms in addition to the 90 EH's previously known. Whereas about 20% of all sequenced organisms contain one or more putative EH genes, nearly 60% of Actinobacteria and fungi contain one or more putative EH genes (van Loo et al., 2006).

The biocatalytic applications of EHs have significantly increased with the overexpression of microbial and plant EH's in a variety of microbial hosts. EHs from yeasts are particularly interesting due to their high activity, selectivity and broad substrate ranges. Kinetic resolution of racemic epoxides from all structural classes as well as desymmetrisation of *meso*-epoxides are catalysed by yeast EH's (Fig. 26.2B) (Weijers and de Bont, 1999). Several host systems like *E. coli*, baculovirus and yeasts

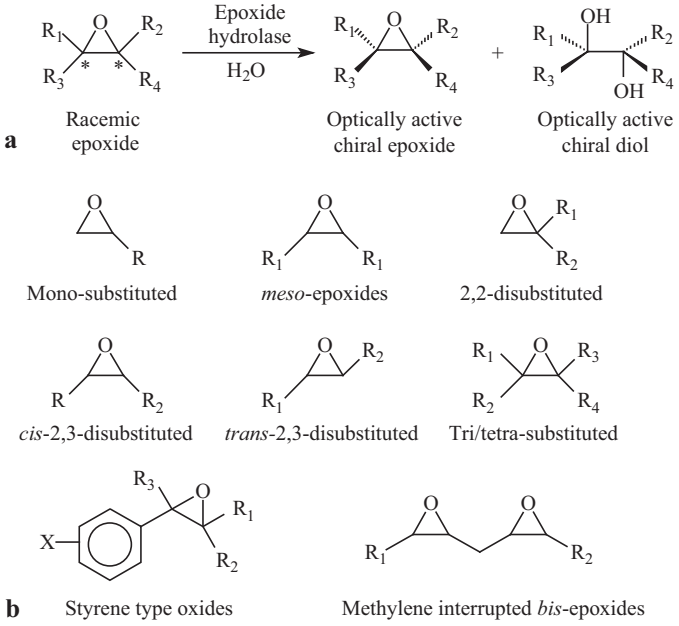

Fig. 26.2 A Hydrolysis of epoxides by EHs, B, is a depiction of different substrate types for microbial epoxide hydrolases: Tri- and tetra-substituted are shown together in where any one of the R groups = H

(*S. cerevisiae, P. pastoris*, *Y. lipolyti*ca) have been used for the expression of EHs from a variety of origins. For example, cytosolic plant EH's have been successfully expressed in *E. coli*, baculovirus systems and yeast with no apparent changes in their kinetic properties: the soluble EH from potato (StEH1) was functionally expressed in baculovirus and in *E. coli* (Li, 2006), and the EH's from *Brassica napus* and *Arabidopsis thaliana* were successfully expressed in *P. pastoris*. In contrast, microsomal epoxide hydrolases (mEH's) from zebra fish, mammals and yeasts have been expressed in different hosts with variable degrees of success. Problems were often encountered such as no expression, formation of inclusion bodies, decreased selectivity of the recombinant enzyme or impaired activity and selectivity. The mEH from zebra fish was expressed in *E. coli* with excellent activity, but no comparison of the selectivity of the recombinant enzyme with the native enzyme of zebra fish is available. Functional expression of human mEH was demonstrated in *S. cerevisiae*, but the effect on selectivity was not studied. Insect mEH were reported to be functionally expressed only in baculovirus systems (e.g. for *Manduca sexta*, *Trichoplasia ni*., *Bombyx mori*, and *Drosophila melanogaster*), while no active enzyme could be produced by cloning the *Manduca sexta* or the *Ctenocephalides felis* JHEH in *E. coli* (Keiser et al., 2002). The mEH from yeast were also expressed in different hosts with various successes. For example, the *Rhodotorula glutinis* EH was produced in *E coli*

with excellent activity, but problems were encountered with the formation of inclusion bodies and the recombinant enzyme displayed a decrease in selectivity. Expression in *S. cerevisiae* resulted in a decrease in both activity and selectivity. A 10- fold increase in activity was obtained upon expression in *Pichia pastoris*, but the selectivity was decreased compared to the native enzyme. While *E. coli* appears to be a suitable host for expression of prokaryotic and cytosolic plant EH's (often with addition of affinity tags such as 6xHis or as fusion proteins with e.g. maltose binding protein), this is not the case for the functional expression of the microsomal EH's from different eukaryotes where retention of the native kinetic properties is often poor.

Yarrowia lipolytica was recently found to efficiently express EH's from other yeasts with excellent activity and selectivity. This expression system was also used to functionally express the problematic microsomal insect EH's, as well as mammalian, bacterial, fungal and plant EH's from many different origins (Botes and Lotter, 2005; Botes et al., 2005a, b; Botes, personal communication). This highly efficient expression system is currently used for the production on an industrial scale of a wide range of chiral epoxides and diols by Oxyrane (Pty) Ltd in South Africa using multi-molar substrate concentrations in a simple, batch stirred tank reactor process. The biocatalytic process for the production of chiral epoxides and vicinal diols compares favourable with existing chemical technologies in terms of cost and productivity and it is the only single technology that can produce chiral epoxides and vicinal diols from all structural classes of epoxides (http://www.oxyrane.com/). The versatility of the epoxide hydrolase technology is further enhanced by the fact that catalysts with matching opposite selectivities can be obtained from different sources.

For example in *Y. lipolytica*, very good expression of *Rhodotorula araucariae* EH was obtained and the selectivity was conserved (Figure 26.3).

The wild-type *Rhodotorula araucaria*e EH presents an initial rate of 11.3 nmol min^{-1} mg $dryweight^{-1}$ with 1,2 epoxyoctane, while, when a single copy of the

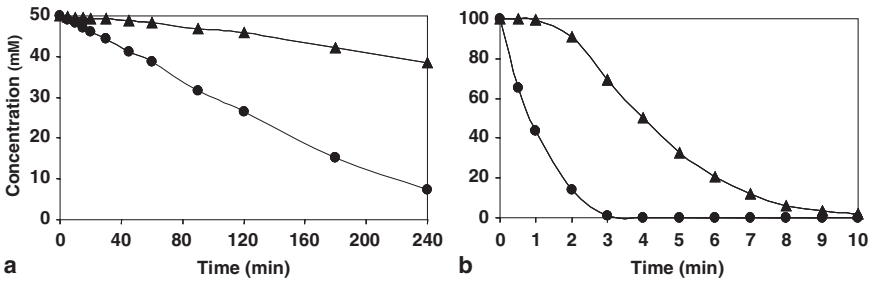

Fig. 26.3 Resolution of 1,2 epoxyoctane by Wild-type *Rhodotorula araucariae* strain (A) and by *Yarrowia lipolytica* catalyst (*Y. lipolytica* strain containing 10–13 copies of the *R. araucariae* gene expressed under the strong hybrid promoter hp4d) (B). Reaction conditions were; 100 mM (A) and 200 mM (B) of 1,2 epoxyoctane, and catalyst were 10% wet weight/v (A) and 5% wet weight/v (B). Symbols are: (R) epoxide (●) and (S) epoxide (▲)

corresponding gene was expressed in *Y. lipolytica* under the *hp4d* promoter (see section 26.9), the initial rate was 51.4 nmol min^{-1} mg $dryweight^{-1}$. Further increase was obtained by amplification of the expression cassette with marker *ura3d1* (2–3 copies) and *ura3d4* (10–13 copies) (154 and 4577 nmol min^{-1} mg $dry\text{-}weight^{-1}$, respectively, (see also section 26.9). EH from yeasts with matching opposite selectivities have been reported for 2,2-disubstituted epoxides (Botes et al., 2005c), from Archaea for meso-epoxides (eg. van Loo et al., 2006) and from bacteria for styrene-type epoxides. Enantioconvergent hydrolysis of styrene type epoxides worth a nearly 100% yield of *R*-diols was reported using the *Solanum tuberosum* plant EH (St-EH) expressed in *E. coli.* The recombinant EH hydrolysed both styrene oxide enantiomers but with opposite regioselectivities to give only the *R*-diol. In the case of mung bean EH's, two enzymes were isolated that displayed opposite enantiopreferences: mbEHA preferentially hydrolysed the *S*-epoxide by regioselective attack at the a-C (inversion of configuration) to yield the *R*-diol, while mbEHB preferentially hydrolysed the *R*-epoxide by regioselective attack at the b-C (retention of configuration) to produce the *R*-diol from styrene oxide (Xu et al., 2006). In contrast, when expressed in *Y. lipolyti*ca, the *S. tuberosum* EH displayed kinetic properties that differed from those of the enzyme made in *E. coli* (Botes, personal communication). Although it is difficult to make a direct comparison, since whole cells were used as catalyst with *Y. lipolytica*, while an enzyme extract from *E. coli* was employed in the reactor, the recombinant *Y. lipolytica* EH whole cell catalyst displayed a higher activity (in U mg^{-1}) than the *E. coli* derived enzyme extract. Furthermore, the enzyme was highly enantioselective as well as regioselective. In a typical biotransformation reaction employing 20 g l^{-1} dry wt catalyst, 100 mM (12 g l^{-1}) racemic styrene oxide was resolved within 30 minutes to yield *R*-styrene oxide (enantiomeric excess, ee 99% with a yield of 45%) and *R*-diol (ee 99%, yield 45%). The *Rhodopsoridium paludigenum* EH expressed in *Y. lipolytica* displayed matching opposite selectivity and regioselectivity for styrene-type epoxides: under the same reaction conditions as above, the catalyst yielded *S*-styrene oxide (ee 99%, yield 45%) and *R*-diol (ee 99%, yield 45%). These enzymes can thus be used together in an enantioconvergent fashion to produce (*R*)-diol from racemic styrene-type epoxides (Botes, personal communication).

Y. lipolytica contains 3 epoxide hydrolases, a situation which apparently did not compromise the selectivity of the heterologously expressed EH's from other sources. To confirm this, the enzymes were cloned and overexpressed in the same host PO1h. None of these native EH's displayed activity or selectivity for the vast majority of epoxides tested for enantioselective hydrolysis by the yeast epoxide hydrolases, except for epoxides bearing carboxylic acids next to or remote from the epoxide moiety (Botes, personal communication). This means that the host can be used as a production system for heterologous expression of epoxide hydrolases without any need to disrupt the native EH for most applications. Interestingly, when the peroxisomal targeting sequence (terminal SKL motif) was removed and then expressed, the *Y. lipolytica* epoxide hydrolases displayed excellent activity and selectivity for a large number of epoxide substrates (Nicaud and Botes, unpublished results).

26.8 Lipid Accumulations by *Y. lipolytica*

Lipids are important storage compounds in plants, animals and fungi. Storage lipids, usually found within special organelles called lipid particles or lipid bodies (LB), are composed of triacylglycerol (TAG) and steryl esters (SE). Some yeasts, called oleaginous yeasts, are able to store lipids in LB which represent more than 20% and up to 70% of the total lipid content of the cell (Ratledge, 1994). Microbial lipids, termed Single Cell Oil (SCO), have often been considered as an alternative to agricultural and animal oil and fat sources. Indeed, SCO from microorganisms are not too different from oils and fats obtained from plant seeds (Ratledge, 1991; Certick and Shimizu, 1999; Ratledge, 2005). Application of SCO will depend on the production of specific oils and on the use of cheap or nearly zero-cost raw materials. This will also depend on the isolation of mutants with improved lipid content or accumulating of specific lipids.

Lipid accumulation in *Y. lipolytica* was investigated by Aggelis and co-workers using industrial glycerol in a single-stage continuous culture. They obtained yeasts containing 43% of lipids in dry biomass with a maximum volumetric productivity of 0.12 g lipid l^{-1} h^{-1}. *Y. lipolyti*ca was also tested for the production of Cocoa Butter Equivalent fat (CBE) using different substrates such as corn oil or stearin (Papanikolaou and Aggelis, 2002). Bati and co-workers evidenced a major effect of dissolved oxigen, nitrogen/carbon ratio, pH and amount of oil substrate on lipid accumulation, resulting in yeast having from 37% up to 70% of lipid (Bati et al., 1984). Papanikolaou and co-workers analysed lipid accumulation and composition when yeasts were grown on agro-industrial residues consisting in a mixture of industrial animal fat (stearin), technical glycerol and glucose. They could modulate the level of polyunsaturated fatty acids and obtained a SCO presenting a CBE-type lipid composition (Papanikolaou et al., 2001; Papanikolaou and Aggelis, 2003a; Papanikolaou et al., 2003). This was probably due to a difference in selective uptake and fatty acid utilisation by *Y. lipolytica* (Papanikolaou and Aggelis, 2003b).

It has been demonstrated that lipid accumulation could be modulated also by the modification of the β-oxidation flux depending on the *POX* genotype. Indeed, in *Y. lipolytica* there are six *POX* genes encoding acyl-CoA oxidase isozymes. A strain deleted for *POX2*, *POX3* and POX5 was shown to accumulate small amounts of TG. In contrast, a strain overexpressing *POX2*, coding for the long chain specific acyl CoA oxidase, accumulated more lipid than the wild-type (Mlickova et al., 2004).

Recently, Damude and co-workers demonstrate that *Yarrowia lipolytica* could be used for the production of long-chain polyunsaturated fatty acids (LC-PUFAs). They expressed in *Y. lipolytica* bifunctional *Δ*2/ω3 desaturases from *Fusarium* species. Expression of *Fusarium moniliformis Fm1* gene under the strong constitutive glyceraldehyde 3-phosphate dehydrogenase promoter results in strains accumulating α-linolenic acid (ALA, 18:3 $\Delta^{9,12,15}$) at 28.1% of total fatty acids (Damude et al., 2006). This demonstrates that *Y. lipolytica* has good potential to be used as a SCO producer, that lipid accumulation level could be further improved and that the type of accumulated lipids could be modified for the production of CBE and LC-PUFA.

26.9 Protein Expression/Secretion

Yarrowia lipolytica was one of the first yeast after *S. cerevisiae* that was considered for heterologous protein production (Davidow et al., 1987). It naturally secretes various proteins at high yield, including proteases, lipases, RNases, each under specific conditions. Various promoters, either constitutive or regulated were developed over the years (Madzak et al., 2004). The most commonly used ones nowadays are: (i) an artificial construct called *hp4d* which is based on four copies of the upstream activating sequences of the alkaline protease gene promoter placed in front of the *LEU2* TATA box (Madzak et al., 2000), (ii) the *POX2* promoter derived from the major acyl-CoA oxydase encoding gene, which is induced on oleic acid and the *ICL1* promoter derived from the isocitrate lyase gene. Comparative data concerning these promoters have been published (Juretzek et al., 2000).

The secretion signals of the alkaline extracellular protease (encoded by *XPR2*) and of the major extracellular lipase (encoded by *LIP2*) are routinely used to drive secretion of foreign proteins. These two highly expressed and secreted enzymes are synthesized as precursors with a short signal sequence, followed by a stretch of X/Ala-X/Pro dipeptides, probable substrate of a diamino peptidase encoded by YALI0B02838g, the only homologue of *S. cerevisiae STE13*, and by a pro region ending by a Lys-Arg (KR) motif cleaved by the endoprotease encoded by the *XPR6* gene (see Figure 26.4). A large number of host strains and vectors for heterologous gene expression have been constructed. Strains carrying non-reverting auxotrophic markers (*leu2, ura3*) and devoid of all known extracellular protease activities (alkaline and acidic) are available. Some of those strains contains pBR333 or zeta docking platforms for the insertion of plasmids in single copy at a specific locus.

Although replicating plasmids have been sometimes used, most experiments use integrative vectors (Fig. 26.5B) that can be targeted to selected sites in the genome by homologous recombination with an integration platform: this facilitates comparison of different constructs which are thus stably integrated as single copies in a defined genomic environment.

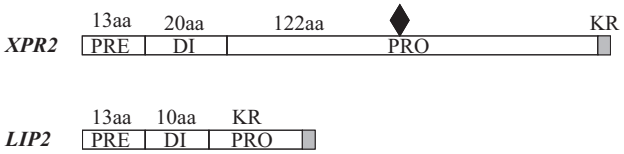

Fig. 26.4 Schematic representation of the targeting sequence of the alkaline extracellular protease (Aep) encoded by the *XPR2* gene and of the major extracellular lipase (Lip2) encoded by the *LIP2* gene. Shown are the putative 13-aa signal sequence (PRE), followed by a stretch of X/Ala-X/Pro dipeptide (DI) (five for Aep and four for Lip2), and a pro region (PRO) ending by a Lys-Arg (KR) motif (122 amino acid for Aep and 12-aa for Lip2). The Aep pro region contains an asparagine-linked glycosylation site indicated by a diamond

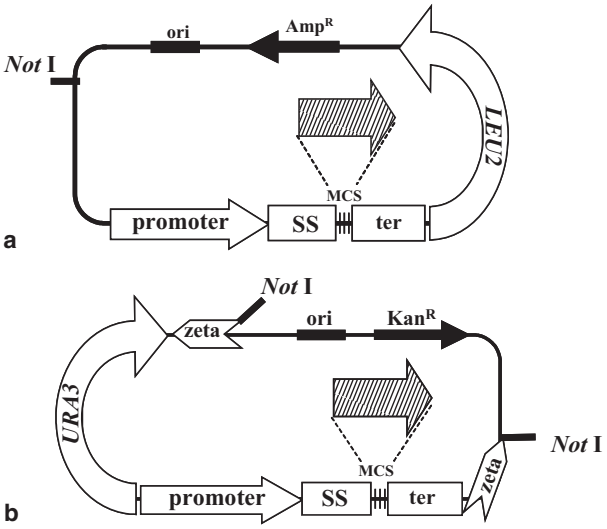

Fig. 26.5 Schematic maps of typical integrative (A) and auto-cloning vectors (B) for gene expression in *Yarrowia lipolytica*. The bacterial moiety from the vectors, represented by a thick line, containing the replicon origin (ori) and the selection markers conferring ampicillin (Amp^R) and kanamycin resistance (KanR), is based on pBR322 and pHSS6, respectively. They contain the *LEU2* and the *URA3* marker, respectively, for selection in *Y. lipolytica*. A multiple cloning site (MCS) is present for the insertion of the gene of interest (hatched arrow), it ends with *XPR2* or *LIP2* terminator (ter). The vectors for secretion contain either the *XPR2* or the *LIP2* targeting region (SS). The integrative vectors are digested by NotI, which cuts in the pBR322 moiety, for targeting the plasmid at the docking platform. The auto-cloning vectors are digested by NotI, which separates the yeast cassette from the bacterial moiety. This yeast cassette integrates at random in the genome

To avoid bacterial DNA and antibiotic resistance genes, autocloning vectors (Fig. 26.5B) are used, which integrate at random in the genome by non homologous recombination. Autocloning vectors carrying defective markers (such as the *ura3d* series) permit targeting from 2–3 to 6–10 copies at these platforms (Juretzek et al., 2001), whereas *Zeta* based vectors permit a dispersed, multicopy integration. Reports have been published on more than 40 different proteins of various origin successfully expressed in this system. For more details, the reader is referred to recent reviews on this subject (Madzak et al., 2004, 2005). A schematic structure of integrative and auto-cloning vectors is presented in Figure 26.5.

An expression kit is commercialised by the Yeastern Company (Taiwan) containing the vectors and recipient strains as well as details on how to use them (see http://www.yeastern.com/).

For fermentation at high cell density, a synthetic medium was developed which fulfilled *Y. lipolytica* requirements for protein expression driven by the oleic acid inducible promoter *POX2* (developed in the Biotechnology - Bioprocess Laboratory in Toulouse by Alain Marty and Jean-Louis Uribellarrea groups). The culture is

conducted in a fed-batch mode. 60 g l^{-1} dry-weight biomass production is obtained with glucose as carbon source for 15 hours at 28°C and pH 5,6 (growth rate: 0.27 $h-^{1}$). Then substrate is switched to an exponential oleic acid feed for protein expression. Fermentation lasts for about 50 hours and a final biomass concentration of 100 g l^{-1} was reached with the production of 60.000 lipase U ml^{-1} (1 U = 1 micromole triolein hydrolyzed per minute) (Marty, to be published).

A new recombinant protein expression system for high-throughput screening was developed for *Y. lipolytica*. We constructed strain JMY1212, auxotrophic for uracile and containing a docking zeta platform derived from the LTR of the Ylt1 retrotransposon, for the integration in mono copy of the expression cassette. JMY1212 derives from W29 which contains no endogenous Zeta nor Ylt1 sequences. The expression cassette can be obtained from a range of autocloning vectors, which are designed to avoid insertion of bacterial sequences into the transformants (Pignede et al., 2000b; Nicaud et al., 2002; Madzak et al., 2004). After directed mutagenesis or error-prone PCR, the cassettes transform JMY1212 at a frequency of about 8000 transformants per microgram of DNA. Conditions for transformant picking, for growth and induction in 96-well microplates and for enzymatic test were optimized (Bordes et al., 2007).

26.10 Conclusions

Whereas the initial industrial interest in *Y. lipolytica* stemmed from its ability to utilize n-paraffins as a carbon source for single cell protein production (Klug and Markovetz, 1967), these aspects did not elicit much interest after the oil crisis of 1973. Rather, it was the ability of this yeast to produce organic acids like citric acid or to secrete proteins that attracted much of the industrial and academic interest. Although these aspects still elicit considerable interest, the recent years have seen a renewal of researches focussing on hydrophobic substrate utilization, the main application targets concerning bioconversion and formation of high added value compounds. The availability of sophisticated genetic tools, the completion of the genome analysis facilitating global approaches (transcriptomics and proteomics) should permit rapid advances in this exciting field.

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Chapter 27
Arxula adeninivorans (Blastobotrys adeninivorans) – A Dimorphic Yeast of Great Biotechnological Potential

Erik Böer, Gerhard Steinborn, Kristina Florschütz, Martina Körner, Gerd Gellissen, and Gotthard Kunze

Content

Abstract The dimorphic ascomycetous yeast *Arxula adeninivorans* exhibits some unusual properties. Being a thermo- and halotolerant species it is able to assimilate and ferment many compounds as sole carbon and/or nitrogen source. It utilises n-alkanes and is capable of degrading starch. Due to these unusual biochemical properties *A. adeninivorans* can be exploited as a gene donor for the production of enzymes with attractive biotechnological characteristics. Examples of *A. adeninivorans*-derived genes that are overexpressed include the *ALIP1* gene encoding a secretory lipase, the *AINV* encoding invertase, the *AXDH* encoding xylitol dehydrogenase and the *APHY* encoding a secretory phosphatase with phytase activity.

The thermo- and halotolerance as well as differential morphology-dependent glycosylation and the secretion characteristics render *A. adeninivorans* attractive as host for heterologous gene expression. A transformation system has been established based on homologous integration of linearised DNA fragments. Successful expression examples like that of the *E. coli*-derived *lacZ* gene, *GFP* and human *HSA* and *IL6* genes add to the attraction of *A. adeninivorans* as host for heterologous gene expression.

Keywords Dimorphic yeast, *Arxula adeninivorans*, phosphatase, heterologous gene expression, thermotolerance, halotolerance

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27.1 Introduction

Yeasts are simply organized ubiquitous unicellular eukaryotes that are able to adapt rapidly to alterations of environmental conditions. In addition to the traditional baker's yeast *Saccharomyces cerevisiae* a wide range of non-conventional yeast species exists with attractive characteristics and growth properties. These species can be exploited for biotechnological applications and can serve as suitable model organisms for plant or animal research. They are either used as donor for genes encoding interesting gene products or employed as excellent hosts for the production of recombinant proteins. The range of yeast species that have been developed as platforms for heterologous gene expression includes the traditional baker's yeast *Saccharomyces cerevisiae*, *Kluyveromyces lactis, Pichia pastoris, Yarrowia lypolytica, Hansenula polymorpha*, *Arxula adeninivorans* (Gellissen, 2005; Gellissen et al., 2005; Wolf, 1996; Wolf et al., 2003) and others described in this book.

The first description of *A. adeninivorans* was provided by Middelhoven et al. (1984) who selected a yeast species from soil by enrichment culturing designating it as *Trichosporon adeninovorans*. A particular strain CBS 8244T was found to exhibit unusual biochemical activities being able to assimilate a range of amines, adenine and several other purine compounds as sole energy and carbon source.

At the same time a second strain, LS3 (PAR-4) with characteristics similar to the CBS strain (Gienow et al., 1990) was isolated from wood hydrolysates in Siberia (Kapultsevich, Institute of Genetics and Selection of Industrial Microorganisms, Moscow, Russia).

In 1990, three additional *Tr. adeninovorans* strains were isolated from chopped maize herbage ensiled at 25 or 30°C in the Netherlands, yet another four strains were detected in humus-rich soil in South Africa (Van der Walt et al., 1990). A new genus name *Arxula* Van der Walt, M.T. Smith & Yamada (*Candidaceae*) was proposed for all these strains. They all share properties like nitrate assimilation and xerotolerance. All representatives of the newly proposed genus are ascomycetous, anamorphic and arthroconidial (Van der Walt et al., 1990).

Kurtzmann and Robnett (2007) accomplished phylogenetic analyses of the ascosporic yeast genera *Sporopachydermia, Stephanoascus, Trichomonascus, Wickerhamiella* and *Zygoascus* and the associated anamorphic genera *Arxula, Blastobotrys, Sympodiomyces* and *Trigonopsis* comparing sequences derived from the large-subunit rDNA genes, the mitochondrial small-subunit rDNA genes, and the genes for cytochrome oxidase II. They deduced that *Arxula, Blastobotrys* and *Sympodiomyces* are members of the *Trichomonascus* clade, with the genus *Blastobotrys* having taxonomic priority for anamorphic states (Fig. 27.1).

The genus *Blastobotrys* includes now the both type species of the genus *B. terrestris* (Van der Walt & Johanssen) Kurtzman & Robnett comb. nov. (Basionym: *Arxula terrestris*) and *B. adeninivorans* (Middelhoven, Hoogkamer Te-Niet & Kreger van Rij) Kurtzman & Robnett comb. nov. (Basionym: *Arxula adeninivorans*).

Eukaryot **(Superkingdo)**
Fungi **(Kingdom**
Ascomycot **(Phylu)**
Saccharomycotin **(Subphylu)**
Saccharomycet **(Clas)**
Saccharomycetal **(Order)**
Mitospori *Saccharomycetal*
Blastobotry **(Genus**
Blastobotry adeninivoran **(Specie)**

Fig. 27.1 Taxonomy of *B. adeninivorans* (synonym: *A. adeninivorans*) (Kurtzmann and Robnett, 2007)

27.2 Physiology and Temperature Dependent Dimorphism

A detailed physiological description of the yeast was provided by Gienow et al. (1990), Middelhoven (1993) and Middelhoven et al. (1984, 1991, 1992). *A. adeninivorans* is able to assimilate nitrate like *H. polymorpha* employing nitrate reductase and nitrite reductase for metabolism. It can utilize a range of compounds as sole energy and carbon source including adenine, uric acid, butylamine, pentylamine, putrescine, soluble starch, melibiose, melicitose, propylamine or hexylamine. It rapidly assimilates all sugars, polyalcohols and organic acids used in conventional carbon compound assimilation tests, except for L-rhamnose, inulin, lactose, lactate and methanol. Likewise all nitrogen compounds are suitable nitrogen sources with the exception of creatine and creatinine. Several nitrogen compounds, like amino acids and purine derivatives, are metabolized as sole energy, carbon and nitrogen source, furthermore many primary n-alkylamines and terminal diamines. In case of alcohols, dialcohols, carboxylic acids, dicarboxylic acids and other nitrogen-free analogous compounds, metabolic intermediates are assimilated. In addition, *A. adeninivorans* degrades some phenols, hydroxybenzoates, tannic acid and is able to assimilate urotropine as sole nitrogen source (Middelhoven and van Doesburg, 2007).

For substrate utilization *A. adeninivorans* produces numerous secretory enzymes including RNases, proteases, glucoamylase, lipase, tannase, some acid phosphatases, trehalase, some cellobiases, invertase, -glucosidase, xylosidase and phytase. Table 27.1 lists these secretory enzymes and summarizes some of their properties.

Special features of biotechnological impact are the thermotolerance- and temperature-dependent dimorphism which is especially pronounced in the Siberian wild strain *A. adeninivorans* LS3. This strain can grow at temperatures of up to 48°C without previous adaptation to elevated temperatures and is able to survive some hours at a temperature of 55°C (Böttcher et al., 1988; Wartmann et al., 1995a). Strain LS3

Table 27.1 Properties of secretory enzymes of *A. adeninivorans* (Böer et al., 2004b, 2005b; Büttner et al., 1987, 1988, 1989, 1990a, c, 1991a, b; Büttner and Bode, 1992; Büttner et al., 1992a, b; Kunze and Kunze, 1994b; Sano et al., 1999; Wartmann et al., 1995b)

	Optimum		Molecular mass	
Enzyme	Temperature	pH	k_m value	(Da)
Glucoamylase (1.4-α-D-glucan glucohydrolase, EC 3.2.1.3)	60–70°C	4.0–5.0	1.2 g/L for starch 11.1 mM for maltose	225,000
Acid phosphatase I and II (ortho-phosphoric-monoester phospho-hydrolase, EC 3.1.3.2)				
I	50–55°C	5.2–5.5	3.5 mM for p-nitro-phenylphosphate	320,000
II	50–55°C	5.2–5.5	5 mM for p-nitro-phenylphosphate	250,000
Trehalase (α,α-trehalose-gluco-hydrolase, EC 3.2.1.28)	45–55°C	4.5–4.9	0.8–1.0 mM for trehalose	250,000
Cellobiase I and II (β-D-gluco-sidase, EC 3.2.1.21)				
I	60–63°C	4.5	4.1 mM for cellobiose	570,000
II	60–63°C	4.5	3.0 mM for cellobiose	525,000
Invertase (β-D-fructofura-noside fructohydrolase, EC 3.2.1.26) 36 mM raffinose	50–60°C	4.5	40–60 mM for sucrose	600,000
β-D-xylosidase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37)	60°C	5.0	0.23–0.33 mM for p-nitro-phenyl-β-xylopyranoside	60,000
3-Phytase (*myo*-inositol hexakis phosphate 3-phosphohydro-lase EC 3.1.3.8)	75°C	4.5	0.23 mM for phytata	n.d.
Lipase (triacylglycerol acylhydro-lases, EC 3.1.1.3)	30°C	7.5	0.4 mM *p*NP-caprate	100,000
Tannase (tannin acyl hydrolase, EC 3.1.1.20)	35–45°C	5.0–6.5	0.14 mM for gallotannin	320,000

exhibits a temperature-dependent morphological dimorphism. At temperatures above 42°C a reversible transition from budding cells to mycelial forms is induced. Budding is re-established when cultivation temperature is decreased below 42°C (Fig. 27.2).

Wartmann et al. (2000) selected mutants with altered dimorphism characteristics. These mutants grow already as mycelia at 30°C thus enabling a distinction between temperature-mediated and morphology-related effects on gene expression and protein accumulation. In analogy to other dimorphic yeasts *A. adeninivorans* budding cells and mycelia differ in their contents of RNA and soluble protein and in their dry weight. During the middle and the final phases of the exponential growth mycelia are found to be of lower RNA and protein content. In contrast, synthesis of secreted proteins including the enzymes glucoamylase and invertase is more pronounced in mycelia resulting in a two-fold higher extracellular protein accumulation. This indicates that morphology, rather than temperature, is the decisive factor for the observed differences (Table 27.2).

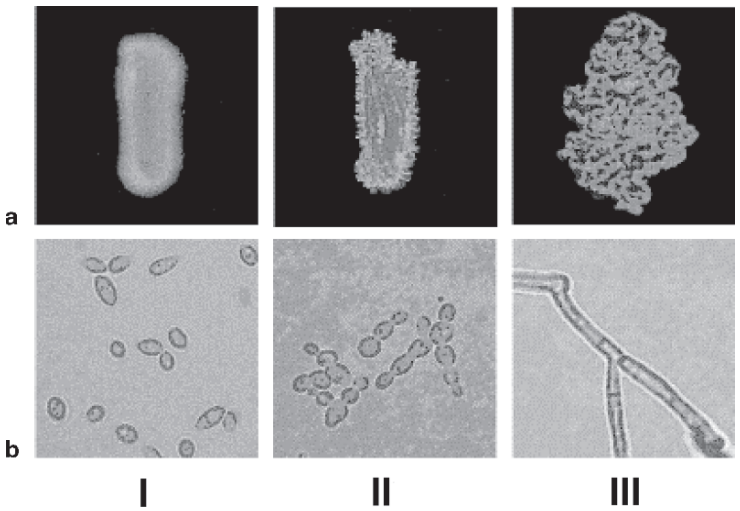


Fig. 27.2 (**a**) Colony form and (**b**) cell morphology of *A. adeninivorans* LS3 grown at 30°C (I), 42°C (II) and 45°C (III). The cells were cultured in YEPD medium for 18 h

Table 27.2 DNA and RNA content, dry weight and amount of soluble protein of *A. adeninivorans* LS3 cultured at 30°C (budding cell) and at 45°C (mycelium) and of *A. adeninivorans* 135 cultured at 30°C (mycelium) in yeast minimal medium (Tanaka et al., 1967) with 1% maltose as carbon source (Wartmann et al., 2000). The values are means ± SD from three separate cultures each with three batches in parallel

Budding cell	Mycelium LS3-30°C	Mycelium LS3-45°C	135-30°C
Content (fg)			
DNA	25.3 ± 0.5	23.6 ± 2.4	24.8 ± 0.9
RNA (45 h)	118.0 ± 15.2	56.4 ± 9.1	44.7 ± 8.8
max. RNA	142.5 ± 18.0 (45 h)	73.0 ± 21.0 (36 h)	57.5 ± 9.6 (36 h)
Dry weight (pg, 45 h)	18.2 ± 0.2	22.4 ± 0.8	23.3 ± 0.3
Soluble protein (fg, 45 h)	169.2 ± 16.3	107.5 ± 20.2	76.9 ± 0.3
Max. soluble protein (fg)	234 ± 5.5 (60 h)	150.1 ± 17.9 (60 h)	125.8 ± 11.6(60 h)*A.*

A. adeninivorans is provided with two transport systems that differ in iron affinity. A strong correlation exists between the morphological status and the iron uptake. In the presence of high Fe(II) concentrations (>2 μM), budding cells accumulate iron concentrations up to seven-fold higher than those observed in mycelia, while at low Fe(II) concentrations (< 2 μM), both cell types accumulate similar amounts of iron. The copper-dependent Fe(II) oxidase (Afet3p) and the respective *AFET3* gene, components of the high affinity transport system, were analyzed in more detail. In this case gene expression strongly depends on iron concentration but is independent

of the morphological stage. However cell morphology was found to influence the posttranslational modifications of Afet3p, an observation of potential impact for heterologous gene expression. O-glycosylation was found in budding cells only, whereas N-glycosylation occurred in both cell types. The characteristic of differential O-glycosylation may provide an option to produce heterologous proteins in both, O-glycosylated and non-O-glycosylated form and to compare the impact of its presence on properties like biological activity or immunological tolerance (Wartmann et al., 2002b).

In addition to temperature the presence of compounds like Cd^{2+}, tocopherol, NaCl and tunicamycin as well as anaerobic cultivation conditions cause alterations in the cell morphology whereas dimorphism is not affected by Ca^{2+}, pH-value, carbon source or substrate limitations (Table 27.3).

A further interesting property of *A. adeninivorans* is its osmotolerance. It can grow in minimal as well as rich media containing up to 3.32 osmomol kg^{-1} H_2O in presence of ionic (NaCl), osmotic (PEG400) and water stress (ethylene glycol). In strain LS3 the influence of NaCl on the growth characteristics was investigated in more detail. Supplementation with up to 3.4 M (10%) NaCl was of limited influence on growth only. However, at NaCl concentrations higher than 3.4 M a decrease of the specific growth rate, a longer adaptation phase and a lower cell count during the stationary growth phase was observed (Yang et al., 2000).

As in other yeast species, this tolerance is elicited by components of the high osmolarity glycerol (HOG) response pathway. However, in contrast to organisms of moderate osmo-resistance, which activate the HOG pathway by enzyme phosphorylation only, this species of high osmo-resistance also induces the expression of HOG pathway genes, such as MAPKK kinase-encoding *ASTE11* and MAP kinase-encoding *AHOG1*. Phosphorylated Ahog1p induces the expression of genes for the synthesis of compatible solutes, such as glycerol, erythritol and

Table 27.3 Environmental factors and their influence on the dimorphism of *A. adeninivorans* LS3

Transition budding cells → mycelia	⇒ Temperature ≥ 43°C
	⇒ 0.1 mM $CdSO_4$
	⇒ Tocopherol
Transition mycelia → budding cells ⇒ temperature < 43°C	
	⇒ NaCl Concentration ≥ 10%
	⇒ Anaerobic conditions
	⇒ Tunicamycin ≥ 8 μg mL^{-1}
Without influence	⇒ pH value
	⇒ Cultivation media (YEPD, YMM)
	⇒ Carbon source (glucose, fructose, maltose, sucrose, xylose, cellobiose, glycerol)
	⇒ Sub- and emers cultivation
	⇒ Substrate limitation
	⇒ Ca^{2+}

mannitol. While glycerol and erythritol levels correlate directly with the osmolarity of the culture media, intracellular mannitol is accumulated 50 fold in an osmolarity-independent manner. The combination of these effects seems to provide a better adaptation during the transition from low to high osmolarity conditions (Böer et al., 2004a).

27.3 Genetics and Molecular Biology

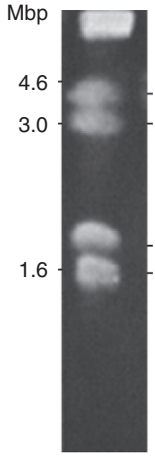
The DNA content of *A. adeninivorans* cells is similar to that of haploid cells of *S. cerevisiae* and other ascomycetous yeasts (Gienow et al., 1990; Samsonova et al., 1996; Wartmann et al., 2000). A relatively high frequency of auxotrophic mutants that is obtained after nitrosoguanidine mutagenesis (Samsonova et al., 1989, 1996), quantitative analysis of chromosomal DNA and determination of genome size (Gienow et al., 1990) further sustain the conclusion for *A. adeninivorans* to be a haploid organism.

The complexity of the nuclear genome of *A. adeninivorans* was analyzed by DNA re-association studies; furthermore karyotyping was performed by pulsed field gel electrophoresis (PFGE) by Gienow et al. (1990) and Kunze and Kunze (1994a). Genome sizes of 16.1×10^9 and 16.9×10^9 Da were calculated from reassociation kinetics of chromosomal DNA from both, *A. adeninivorans* wild type strains LS3 and 8244T, respectively. These are the highest values reported for a yeast species so far - much higher than the 9.2×10^9 Da determined for *S. cerevisiae* under identical conditions. The share of repetitive sequences was determined as 33.1% in LS3 and 35.9% in CBS 8244T. These values are higher than those reported for other yeasts, too. Karyotype polymorphisms were observed among the wild type strains tested demonstrating the existence of four chromosomes ranging between 1.6 and 4.6 Mb in size.

Genetic studies were promoted by the isolation of mutants and by the development of special techniques. After UV-induced mutagenesis or after treatment with nitrosoguanidine a large number of auxotrophic mutants and of mutants with an altered catabolite repression (resistance to 2-deoxy-D-glucose) have been selected and characterized (Böttcher and Samsonova, 1983; Büttner et al., 1990b; Samsonova et al., 1989, 1996).

Since no sexual cycle could be observed, mapping techniques based on parasexual mechanisms had to be applied to establish a genetic map of *A. adeninivorans*. After polyethylene glycol-induced fusion of spheroplasts heterozygous diploids were obtained from auxotrophic mutants of strains LS3 and CBS8244T (Büttner et al., 1990b; Samsonova et al., 1996). Segregation of these diploids was achieved by treatment with benomyl, a drug known to induce haploidization, without affecting other mitotic recombination events (Böttcher and Samsonova, 1983). This permitted the linkage analysis of various markers.

In this way 32 genes could be assigned to four linkage groups thus meeting the chromosome number of the *A. adeninivorans* genome. This was confirmed by



Chromosomes	1	2	3	4
	ARG7	*MET2*	*ARG4*	*ARG6*
	CYS1	*MET6*	*ADE1*	*ARG1*
	LEU2	*MET17*	*INO1*	***AILV1***
	LEU4	*ade-96*	*AINV*	*AEFG1*
	LYS5	*ade-109*		*AFET3*
	LYS2	*NIC4*		*APHO1*
	LYSI 4	***GAA***		
	PRO1	*TEF1*		
	PRO3	*ATRP1*		
	ADE2			
	NIC1			
	NIC2			
	NIC3			
	NIC8			
	PDX			
	ARFC3			
	AHSB4			
	AHOG1			
	ALIP1			
	AXDH			

Data obtained by linkage group analyses are normal bold, by PFGE are underlined and by linkage group analyses as well as PFGE are extra bold

Fig. 27.3 *A. adeninivorans* chromosomes separated by PFGE and chromosomal gene localization by linkage group analyses and PFGE followed by DNA hybridization

relating the analyzed 32 auxotrophic mutations to particular chromosomes by PFGE and subsequent DNA hybridization with specific probes (Samsonova et al., 1996; Fig. 27.3). Sequencing of the genome of strain LS3 is approaching completion at the time this manuscript was written (Gaillardin, personal communication).

27.4 *Arxula adeninivorans* as Gene Donor

Several *A. adeninivorans* genes were isolated from gene libraries containing either cDNA or chromosomal DNA from *A. adeninivorans* strain LS3 by PCR amplification with specific consensus primer sequences, among others *AEFG1, AFET3, AHOG, AHSB4, AINV, ALIP1, ALYS2, APHO1, ARFC3, ATAL, AXDH* and *TEF1*as well as the complete rDNA repeat (Böer et al., 2004a, 2004b, 2005b, 2005c; El Fiki et al., 2007; Kaur et al., 2007; Kunze and Kunze, 1996; Rösel and Kunze, 1995, 1996; Steinborn et al., 2005; Stoltenburg et al., 1999; Wartmann et al., 2001, 2002b, 2003a).

The presence of introns is more common in the *A. adeninivorans* genome than in other yeasts like *S. cerevisiae*. *AHSB4, ARFC1* and *AHOG1* were found to contain an intron. The comparison of 5′-splice site (DS/GUARGU), branch site (HRCUAAC) and 3′-splice site (HAG/R) sequences demonstrate that the resulting consensus sequences are similar to that of *S. cerevisiae* and filamentous fungi (Böer et al., 2005a).

The complementation of respective *E. coli* and *S. cerevisiae* mutants was used as an approach for the isolation of additional genes, namely the *ALYS2*, *AILV1, ALEU2* and *ATRP1* genes which are suitable selection markers for the *A. adeninivorans*-based platform (Kunze and Kunze, 1996; Steinborn et al., 2007b; Wartmann et al., 1998, 2003b).

The *GAA* gene encoding glucoamylase was identified from a cDNA library using an anti-glucoamylase antibody as probe for product detection. When heterologously expressed in *S. cerevisiae* and *Kluyveromyces lactis* more than 90% of the synthesized glucoamylase was found to be secreted. The level of secreted enzyme was 20 times higher in *Kl. lactis* than that observed in *S. cerevisiae* transformants using a similar construct for transformation (Bui et al., 1996a, b).

In parallel biotechnologically important secretory enzymes like lipases were synthesized as recombinant proteins in *A. adeninivorans*. This temperature-sensitive protein with a pH - optimum at 7.5 hydrolyses ester bounds in triglycerides. Thereby fatty acids with middle-sized chains are more efficiently hydrolysed than those with short- or long-chains, with the highest activity on C8/C10 fatty acid esters *p*NP-caprylate, *p*NP-caprate and tricaprylin (Böer et al., 2005b).

The *AINV* gene provides another example of an interesting enzyme gene. The encoded invertase preferentially hydrolyzes -D-fructofuranosides and could be applied to the hydrolysis of sugar cane molasses or sugar beet molasses on an industrial scale. The *AINV* gene was obtained by screening a cDNA and a chromosomal library with a PCR amplificate corresponding to a particular gene segment. The isolated gene was expressed in recombinant *A. adeninivorans* strains fusing the coding sequence to the strong constitutive *TEF1* promoter. The resulting transformants were found to secrete the enzyme in high concentrations independent of the carbon source used for cultivation (Böer et al., 2004b).

An example for an interesting intracellular protein is a temperature-sensitive xylitol dehydrogenase which oxidizes polyols like xylitol and D-sorbitol and reduces simultaneously D-xylulose, D-ribulose and L-sorbose. Due to its optima at low temperatures and weak basic pH values the enzyme is of potential interest for application to food manufacturing processes. The respective *AXDH* gene was isolated and overexpressed in *A. adeninivorans* (Böer et al., 2005c).

A last example is the *ATAL* gene encoding a temperature-sensitive transaldolase with an acidic pH optimum. The preferred substrates for the enzyme include D-erythrose-4-phosphate and D-fructose-6-phosphate. Based on these properties the enzyme could be applied to C-C bounding and enantio-specific synthesis of novel sugars, as previously demonstrated for the *S. cerevisiae*-derived transaldolase (El Fiki et al., 2007).

27.5 The *A. adeninivorans*-Based Platform

27.5.1 Transformation System

A. adeninivorans provides an attractive novel gene expression platform. A first transformation system based on this dimorphic yeast species has been established more than a decade ago using *S. cerevisiae* and *A. adeninivorans*-derived *LYS2* genes for selection (Kunze et al., 1990; Kunze and Kunze, 1996). In these instances, transformation vectors either integrated into the chromosomes as single copy or in low copy numbers or were of episomal fate.

Improvements of the transformation system were introduced by Rösel and Kunze (1998) using a vector type (pAL-HPH1) that employed an rDNA targeting sequence for stable integration and the *E. coli*-derived *hph* gene (conferring hygromycin B resistance) under the control of the *A. adeninivorans*-derived *TEF1* promoter for dominant selection. As rDNA targeting sequence a 25S rDNA fragment from *A. adeninivorans* was used. The resulting hygromycin B-resistant transformants were found to harbour 2–10 plasmid copies stably integrated into the ribosomal DNA (Fig. 27.4a).

Employment of the dominant marker gene results in the undesired need for toxic compounds or antibiotics during the strain development. This can be avoided using auxotrophic strains and the respective gene sequence for complementation. As such the *AILV1* and *ALEU2* genes were isolated as described before and the respective auxotrophic strains were selected. In several examples the *A. adeninivorans ailv1* or *aleu2* hosts were transformed with the plasmids pAL-AILV1 containing the *AILV1* gene and pAL-ALEU2m containing the *ALEU2* gene for complementation (Fig. 27.4b and c). Transformants generated in this way were found to harbor 1–3 copies of the heterologous DNA mitotically stable integrated into the rDNA unit by homologous recombination (Steinborn et al., 2005; Wartmann et al., 1998, 2003b).

The *ailv1* and *aleu2* mutant strains selected after *N*-methyl-*N*Í-nitro-*N*-nitrosoguanidine treatment reverted to leucine/isoleucine prototrophy at a frequency of 10^{-6} (Samsonova et al., 1989, 1996). To eliminate this disadvantage, a *Δ atrp1* gene disruption mutant was generated. For disruption an amplified DNA fragment containing the *ALEU2m* gene flanked by *ATRP1* gene sequences of some 750 bp was employed (Fig. 27.5).

The generated auxotrophic host strain *A. adeninivorans* G1212 [*aleu2 atrp1:: ALEU2*] excels by mitotic stability during cultivation in both rich and minimal medium. The strain was transformed with the plasmid pAL-ATRP1, which contains the *ATRP1* gene as selection marker and for targeting the 25S rDNA. The resulting transformants contained a single chromosomal copy of the pAL-ATRP1 DNA (Steinborn et al., 2007b;– Fig. 27.4d).

For further platform improvement a novel vector element has been constructed that provides multicopy integration in *A. adeninivorans* G1212 [*aleu2 atrp1:: ALEU2*]. The element consists of the *ATRP1* coding sequence under control of a newly generated truncated *ALEU2* promoter of 53 bp. In several examples 8 and

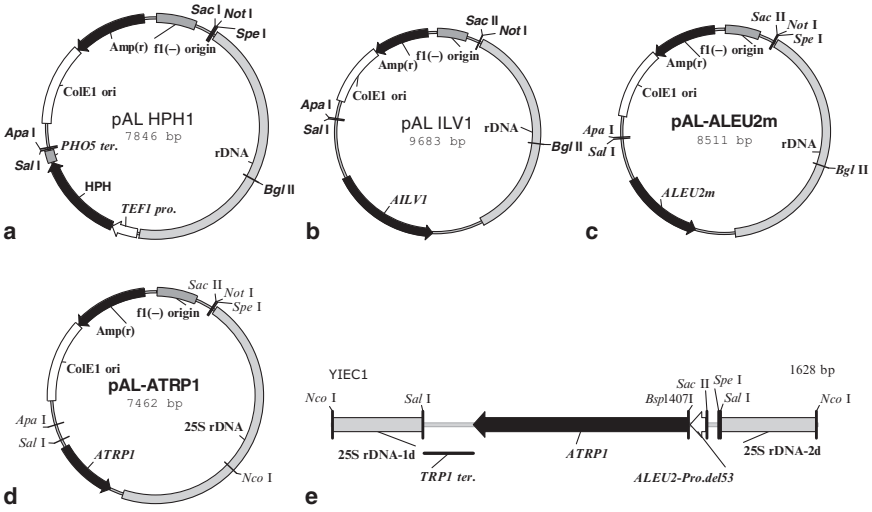


Fig. 27.4 Physical maps of vectors for the *A. adeninivorans*-based expression platform. (**a**) The vector pAL-HPH1 contains the following elements: a 25S rDNA sequence (rDNA) for chromosomal targeting, an expression cassette for the *E. coli*-derived *hph* gene in the order *A. adeninivorans*-derived *TEF1* promoter (TEF1 pro.), the *hph*-coding sequence (HPH), *S. cerevisiae*-derived *PHO5* terminator (PHO5 ter.). The vector further contains unique *Apa*I and *Sal*I restriction sites for the insertion of the expression cassettes and an unique *Bgl*II site within the rDNA sequence for linearization. The vectors (**b**) pAL-AILV1, (**c**) pAL-ALEU2m and (**d**) pAL-ATRP1 contains the selection marker *AILV1* (AILV1), *ALEU2m* (ALEU2m) or *ATRP1* (ATRP1) instead of the expression cassette for the *E. coli*-derived *hph* gene. (**e**) Yeast integration-expression cassettes (YIEC1), a novel vector type for multicopy transformation of *A. adeninivorans* lacking an *E. coli* part. The YIEC1 flanked by *Nco*I sites contains the 25S rDNA sequences and the selection marker *ATRP1* fused to the deleted 58 bp *ALEU2* promoter

more copies of the vector were now found to be integrated in the genome of the recombinant strains instead of a single copy. In addition the vector design enables the integration of a small vector fragment that consists of yeast DNA only (yeast integration-expression cassette - YIEC) providing high transformation frequencies and a high mitotic stability (Steinborn et al., 2007a).

27.5.2 Heterologous Gene Expression

The construction of expression plasmids follows a two-step cloning strategy. First the heterologous genes are inserted between the respective *A. adeninivorans*-derived promoter and fungal terminator elements like *PHO5* from *S. cerevisiae* and *trpC* from *Aspergillus nidulans*. Subsequently the resulting expression cassettes (*A. adeninivorans* promoter – heterologous gene - fungal terminator) are integrated

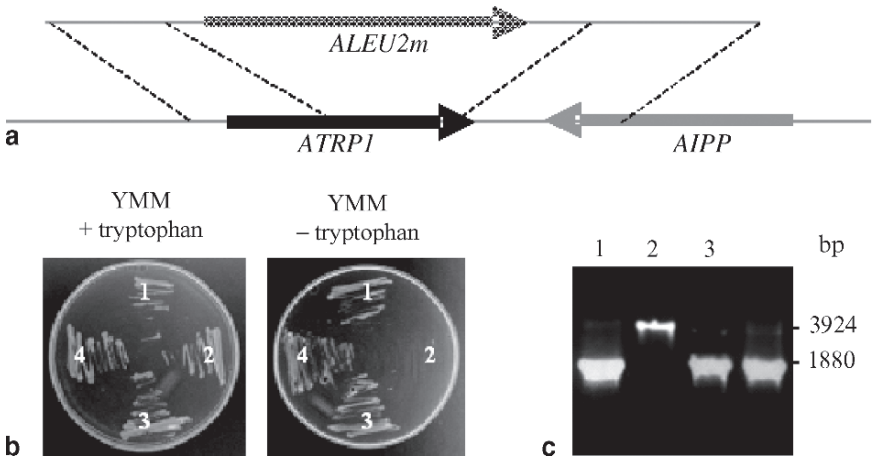


Fig. 27.5 *ATRP1* gene disruption and analysis of the *Δatrp1* mutants (**a**) The *ATRP1* gene replacement strategy by gene disruption. The *Δatrp1* fragment with 789 bp 5′-*atrp1* region – *ALEU2m* gene - 948 bp 3′-*atrp1* region was amplified by PCR and used to transform *A. adeninivorans* strain G1211 for sequential disruption of the chromosomal *ATRP1* copy. For this purpose the transformants were first selected for leucine prototrophy, second for tryptophan auxotrophy. (**b** and **c**) Analysis of the selected leucine-prototroph transformants for tryptophan auxotrophy and correct integration of the *Δatrp1* DNA fragment. *A. adeninivorans* G1211 (1) and the selected transformants (2–4) were spotted onto solid agar plates of YMM with and without tryptophan. The plates were incubated for 3 days at 30°C prior to photographic documentation. In parallel the genomic DNA of the analysed strains was isolated and used as template for amplification of the 3924 bp fragment with 5′-*atrp1* region – *ALEU2m* gene - 948 bp 3′-*atrp1* region with the primers ATRP1-1 and ATRP1-2

into the respective *A. adeninivorans* expression plasmid. For this purpose the cassettes are flanked by unique restriction sites (*Apa*I – *Sal*I, *Apa*I – *Xho*I, *Spe*I – *Sac*II, *Spe*I – *Not*I) allowing a directional integration (Fig. 27.4).

An increasing number of heterologous genes have been expressed in *A. adeninivorans*. As a first example the *XylE* gene from *Pseudomonas putida* encoding the catechol-2,3-dioxygenase was expressed under control of the *AILV1* promoter (Kunze et al., 1990; Kunze and Kunze, 1996; Table 27.5), followed by successful examples of genes expressed under control of the strong constitutive *TEF1* promoter. As such *GFP* and *HSA* gene sequences were inserted into the vectors pAL-HPH1 and pAL-ALEU2m and used to transform *A. adeninivorans* wild type and mutant strains. The recombinant strains contained 1–2 copies of the heterologous DNA integrated in 25S rDNA region. In case of *GFP* expression the recombinant protein was localized in the cytoplasm rendering the cells fluorescent. In case of *HSA*, the expression based on an ORF including the native 5′-signal sequence. Accordingly, the recombinant HSA was secreted to more than 95% into the culture medium. In fermentation trials of a single copy-transformant on a 200 ml shake flask scale maximal HSA product levels of 50 mg l^{-1} were observed after 96 h of cultivation.

Table 27.4 Isolated and sequenced genes of the yeast *A. adeninivorans* LS3

Gene	Gene product	Accession no.	Reference
AEFG1	Mitochondrial elongation factor G	AJ312230	(Wartmann et al., 2001)
AFET3	Copper-dependent Fe(II) oxidase	AJ277833	(Wartmann et al., 2002b)
AHOG1	Mitogen-activated protein kinase	AJ626723	(Böer et al., 2004a)
AHSB4	Histone H4	AJ535732	(Wartmann et al., 2003a)
AILV1	Threonine deaminase	AJ222772	(Wartmann et al., 1998)
AINV	β-Fructofuranoside fructohydrolase	AJ580825	(Böer et al., 2004b)
ALEU2	β-Isopropylmalate dehydrogenase	AJ488496	(Wartmann et al., 2003b)
ALIP1	Lipase	AJ879165	(Böer et al., 2005b)
ALYS2	Amino-adipate reductase	Not sequenced	(Kunze and Kunze, 1996)
APHO1	Acid phosphatase	AM231307	(Kaur et al., 2007)
ARFC3	Replication factor C component	AJ007712	(Stoltenburg et al., 1999)
ATAL	Transaldolase	AM400899	(El Fiki et al., 2007)
ATRP1	Phosphoribosyl anthranilate iso-merase	AM261500	(Steinborn et al., 2007b)
AXDH	Xylitol dehydrogenase	AJ748124	(Böer et al., 2005c)
GAA	Glucoamylase	Z46901	(Bui et al., 1996a)
TEF1	Elongation factor 1	Z47379	(Rösel and Kunze, 1995)
25S rDNA	25S rRNA	Z50840	(Rösel and Kunze, 1996)
18S rDNA	18S rRNA	Z50840	(Steinborn et al., 2005)
5.8S rDNA	5.8S rRNA	Z50840	(Steinborn et al., 2005)

Budding cells as well as mycelia secreted similar levels demonstrating a morphology-independent productivity (Wartmann and Kunze, 2003; Wartmann et al., 2003b; Table 27.5).

In addition to the *TEF1* promoter the strong constitutive *AHSB4* promoter was successfully assessed for suitability and was found to elicit similar expression levels (Wartmann et al., 2003a; Table 27.5).

Since *A. adeninivorans* is a dimorphic yeast, recombinant proteins can be produced in cells of different morphological stages. A *MF 1-IL6* fusion was expressed under control of the strong *TEF1* promoter in *A. adeninivorans* budding cells and mycelia. In contrast to other yeast species (*S. cerevisiae, H. polymorpha*) the recombinant interleukin-6 (IL-6) was correctly processed from the MF 1-IL6 precursor and was accumulated to more than 95% in the culture medium. In cultivation on a shaking flask scale a productivity of ca. 210 mg l^{-1} was observed in budding cell cultures and 145 mg l^{-1} in mycelial cultures (Böer et al., 2007; Fig. 27.6).

An approach to introduce simultaneously several genes was taken to established new metabolic pathways in *A. adeninivorans*. As an example the genes *phb*A, *phb*B and *phb*C of the polyhydroxyalkanoate (PHA) biosynthetic pathway of *Ralstonia eutropha* encoding -ketothiolase, NADPH-linked acetoacetyl-CoA reductase and PHA synthase were introduced for construction of a recombinant biocatalyst. *A. adeninivorans* strains initially transformed with the PHA synthase gene (*phb*C) plasmids alone were able to produce PHA. However, the maximal content of the polymer detected in these strains was just 0.003% (w/w) poly-3-hydroxybutyrate (PHB) and 0.112% (w/w) poly-3-hydroxyvalerate (PHV). The expression of all

Table 27.5 Examples of heterologous gene expression in *A. adeninivorans* LS3

Gene	Donor organism	Gene product	Promoter	Vector	Recombinant protein level	Reference
lacZ	*E. coli*	β-Galactosidase	*GAA*	pAL-HPH1	350 kU mg^{-1}	(Wartmann and Kunze, 2000)
lacZ	*E. coli*	β-Galactosidase	*AHOG1*	pAL-HPH1	350 U mg^{-1}	(Böer et al., 2004a)
GFP	*Aequorea victoria*	Green fluorescent protein	*TEF1*	pAL-HPH1	n.d	(Wartmann et al., 2002a)
GFP	*Aequorea victoria*	Green fluorescent protein	*TEF1*	pAL-ALEU2m	n.d	(Wartmann et al., 2003b)
GFP	*Aequorea victoria*	Green fluorescent protein	*AHSB4*	pAL-ALEU2m	n.d	(Wartmann et al., 2003a)
HAS	*Homo sapien*	Human serum albumin	*TEF1*	pAL-HPH1	50 mg l^{-1}	(Wartmann et al., 2002a)
HAS	*Homo sapiens*	Human serum albumin	*TEF1*	pAL-ALEU2m	50 mg l^{-1}	(Wartmann et al., 2003b)
HAS	*Homo sapiens*	Human serum albumin	*AHSB4*	pAL-ALEU2m	50 mg l^{-1}	(Wartmann et al., 2003a)
XylE	*Ps. putida*	Catechol 2,3-dioxygenase	*AILV1*	I1-ALYS2	0.4 pkat mg^{-1}	
XylE	*Ps. putida*	Catechol 2,3-dioxygenase	*AINV*	pAL-ALEU2m	4.5 pkat mg^{-1}	(Böer et al., 2004b)
AINV	*A. adeninivorans*	Invertase	*TEF1*	pAL-ALEU2m	500 μkat l^{-1}	(Böer et al., 2004b)
PhbA	*R. eutropha*	β-Ketothiolase	*TEF1*	pAL-HPH1	2.2% PHA*	(Terentiev et al., 2004)
PhbB	*R. eutropha*	Cetoacetyl CoA reductase	*TEF1*	pAL-HPH1	2.2% PHA*	(Terentiev et al., 2004)
PhbC	*R. eutropha*	PHA synthase	*TEF1*	pAL-ALEU2m	2.2% PHA*	(Terentiev et al., 2004)
PhbC	*R. eutropha*	PHA synthase	*TEF1*	pAL-HPH1	2.2% PHA*	(Terentiev et al., 2004)
GFP	*Aequorea victoria*	Green fluorescent protein	*AXDH*	pAL-HPH1	n.d	(Böer et al., 2005c)
AXDH	*A. adeninivorans*	Xylitol dehydrogenase	*TEF1*	pAL-ALEU2m	900 mkat l^{-1}	(Böer et al., 2005c)
ALIP1	*A. adeninivorans*	Lipase	*TEF1*	pAL-ALEU2m	3,300 U l^{-1}	(Böer et al., 2005b)
amyA	*B. amyloliquefac.*	α-Amylase	*TEF1*	pAL-ALEU2m	150 μkat l^{-1}	(Steinborn et al., 2005)
hERα	*Homo sapiens*	Estrogene receptor α	*TEF1*	pAL-HPH1	n.d.	(Hahn et al., 2006)
phyK	*Klebsiella sp.ASR1*	Extracellular phytase	*GAA*	pAL-ALEU2m	75 mkat l^{-1}	(Hahn et al., 2006)
amyA	*B. amyloliquefac.*	α-Amylase	*TEF1*	pAL-ATRP1	300 μkat l^{-1}	(Steinborn et al., 2007b)
APHO1	*A. adeninivorans*	Acid phosphatase	*TEF1*	pAL-ALEU2m	70 mkat l^{-1}	(Kaur et al., 2007)
ATAL	*A. adeninivorans*	Transaldolase	*TEF1*	pAL-ALEU2m	35 mkat l^{-1}	(El Fiki et al., 2007)
HSA	*Homo sapiens*	Human serum albumin	*ATAL*	pAL-ALEU2m	0.6 mg l^{-1}	(El Fiki et al., 2007)
IL6	*Homo sapiens*	Interleukin-6	*TEF1*	pAL-ALEU2m	220 mg l^{-1} (b.c.) 145 mg l^{-1} (m.)	(Böer et al., 2007) (Böer et al., 2007)

*) % final product per dry weight n.d.) not detected b.c.) budding cell culture μ.) mycelial culture

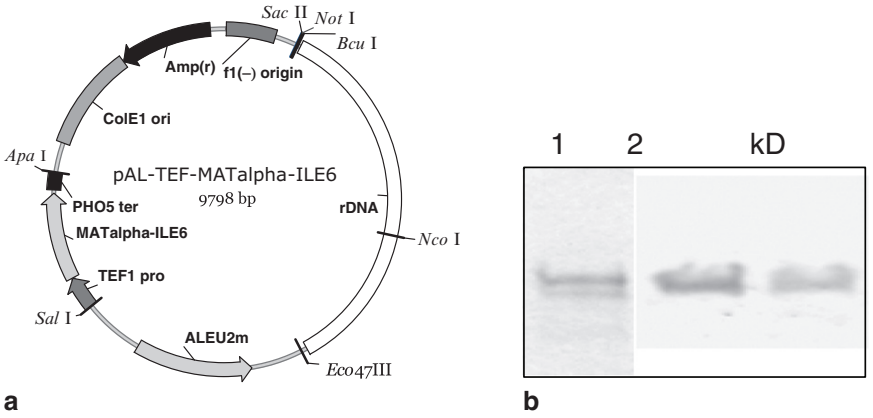


Fig. 27.6 (**a**) Physical map of the expression/integration vector pAL-ALEU2m-TEF-MATalpha-IL6. The vector contains the 25S rDNA sequence of *A. adeninivorans* (rDNA, white box), the selection marker *ALEU2m* (grey segment) and an expression cassette for the *IL6* gene in the order *A. adeninivorans*-derived *TEF1* promoter (TEF1 pro, grey segment), the *IL6*-coding sequence, *S. cerevisiae*-derived *PHO5* terminator (PHO5 ter, black bar) as selection marker. The vector contains a unique *Nco*I site for linearization within the rDNA sequence (**b**) IL6 accumulation in recombinant *A. adeninivorans* budding cell and mycelial cultures. The strains were cultured in YMM supplemented with 2% glucose for 72 h at 30°C (budding cells) or 45°C (mycelia). 20 µl aliquots of culture media were separated on SDS-PAGE (11%) gels, transferred to nitrocellulose filters and probed with anti-IL-6 antibodies. The concentration of recombinant IL-6 was calculated from the signal intensity of an IL-6 standard. (1) IL-6 standard (*E. coli*), (2) *A. adeninivorans* G1211/pAL-ALEU2m-TEF-MATα-IL6 – budding cells (205 mg l^{-1}), (3) *A. adeninivorans* G1211/pAL-ALEU2m-TEF-MATα-IL6 – mycelia (144 mg l^{-1}) (**c**) N-terminus of IL-6 secreted from recombinant *A. adeninivorans* (budding cell and mycelial cultures).genes was able to accumulate up to 2.2% (w/w) PHV and 0.019% (w/w)

three genes (*phb*A, *phb*B, *phb*C) resulted in small increases in the PHA content only. However, under controlled conditions, using minimal medium and ethanol as the carbon source for cultivation, the recombinant yeast containing all three *phb* PHB (Terentiev et al., 2004; Fig. 27.6 and 27.7).

The *A. adeninivorans* transformation/ expression system can be used for promoter assessment. For this purpose the *lacZ* gene from *E. coli,* the *GFP* gene from *Aequorea victoria,* the *phyK* gene from *Klebsiella* spec. and the *XylE* gene from *Ps. putida* can be employed as reporter genes. In previous examples the expression cassettes containing the *GAA, AHOG1, AINV, AXDH* as well as the *ATAL* promoter – reporter gene – *PHO5* terminator were analyzed. The characteristics of the selected promoters could be assessed for aspects like dependence on carbon source, osmolarity of the medium or morphological stage (Böer et al., 2004a,

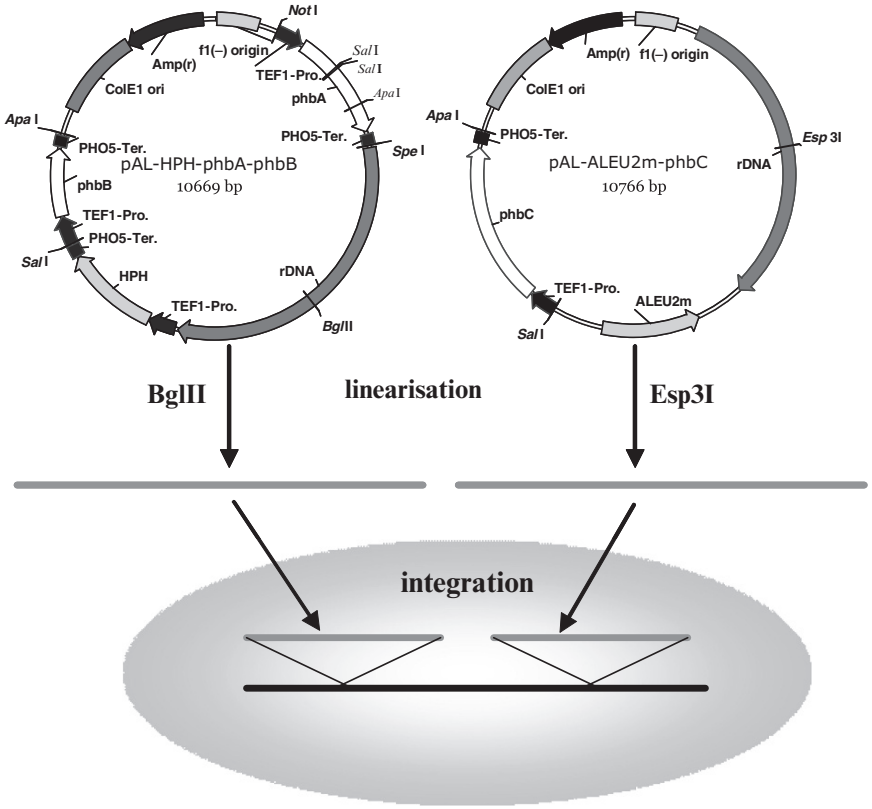


Fig. 27.7 Transformation procedure based on simultaneous integration of the plasmids pAL-HPH-phbA-phbB and pAL-ALEU2m-phbC into the 25S rDNA of *A. adeninivorans* G1211 (*aleu2*). The two plasmids pAL-HPH-phbA-phbB and pAL-ALEU2m-phbC containing the expression cassettes with *phbA, phbB* and *phbC* genes are linearised by *Bgl*II or *Esp*3I digestion, respectively. The resulting fragments flanked by 25S rDNA sequences are co-integrated into the 25S rDNA by homologous recombination. Transformants are selected either by resistance to hygromycin B (plasmid pAL-HPH-phbA-phbB) or the complementation of the *aleu2* mutation (plasmid pAL-ALEU2m-phbC)

2004b, 2005c; El Fiki et al., 2007; Hahn et al., 2006; Wartmann and Kunze, 2000; Table 27.5).

As another application of recombinant strains a novel estrogen biosensor has been developed. For this purpose, recombinant *A. adeninivorans* strains were engineered co-expressing the human estrogen receptor (hER) and a *Klebsiella*-derived phytase (*phyK*) reporter gene under control of an *A. adeninivorans*-derived glucoamylase (*GAA*) promoter modified by insertion of estrogen-responsive elements (EREs). In response to the presence of estrogenic compounds, two estrogen-hER complexes dimerize and bind to estrogen-responsive elements (ERE)

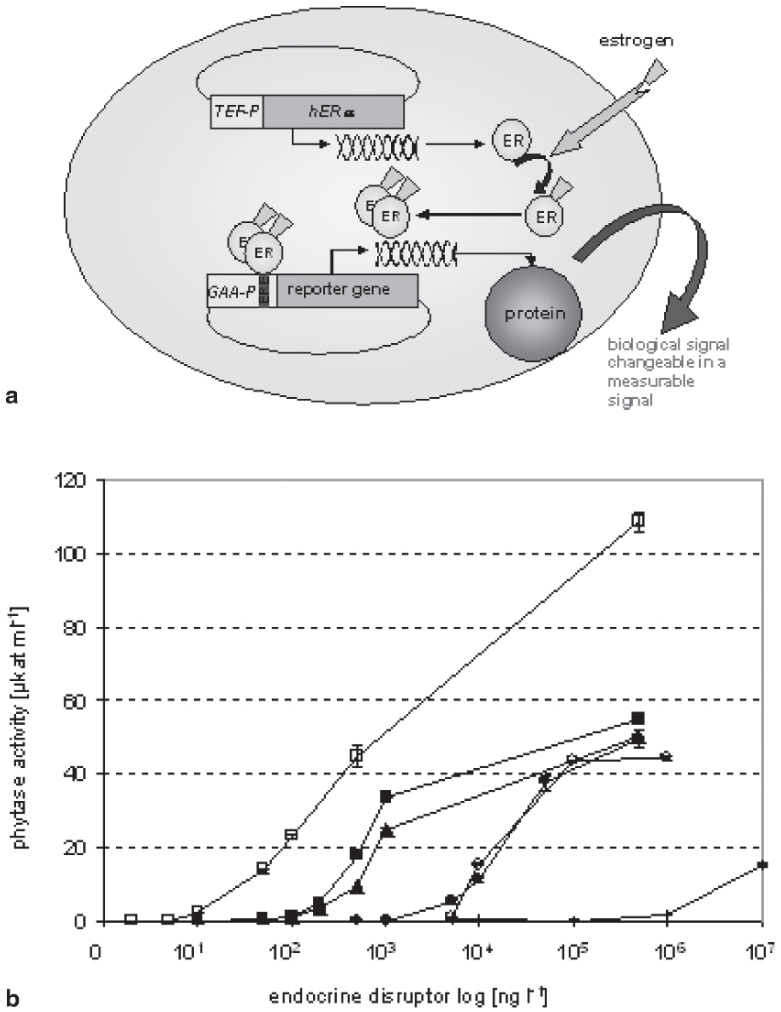


Fig. 27.8 (**a**) Principle of an estrogen sensor based on recombinant *A. adeninivorans* strains (A-YES). *A. adeninivorans* G1211 transformed with the plasmids pAL-HPH-hERα and pAL-ALEU2m-GAA(2xERE-107)-phyK (G1211/pAL-HPH-hERα - pAL-ALEU2m-GAA(2xERE-107)-phyK) is the bio-component of the A-YES. It expresses the estrogen receptor gene (*TEF1* promoter – *hERα* gene – *PHO5* – terminator) constitutively and produces a relatively constant level of recombinant hERα independent of the estrogen concentration. In the presence of estrogen or estrogen analogues, however, hERα forms a hERα-estrogen-dimer complex, which binds to the ERE-region of the *GAA* promoter located in the second reporter gene expression cassette. The cassette (*GAA-ERE* – promoter – *phyK* gene – *PHO5* terminator) is activated, the *phyK* gene is expressed and phytase is synthesized. Since this enzyme contains a native signal sequence it is secreted and accumulates extracellularly. The recombinant phytase level can then be quantified using a simple biochemical method. (**b**) Specificity of the A-YES based on *A. adeninivorans* G1211/pAL-HPH-hERα - pAL-ALEU2m-GAA(2xERE-107)-phyK for a range of steroids and steroid metabolites. The graphs depict the log concentration of 17α-ethynylestradiol (α), 17 -estradiol (■), estrone (▲), estriol (●), coumestrol (○) and bisphenol A (+) plotted against the recombinant phytase activity of the medium after 30 h incubation

within the promoter to subsequently induce the expression of the reporter gene. The insertion of different numbers of EREs in three alternative positions within the promoter and its effect on reporter gene expression were assessed. In a particular construct, a detection limit of 5 ng l^{-1} and a quantification limit of 10 ng l^{-1} for 17 -estradiol-like activity could be achieved. A convenient photometric assay enables estrogen monitoring in sewage samples within 30 hrs (Hahn et al., 2006; Fig. 27.8).

27.6 Conclusions and Perspectives

A. adeninivorans is a haploid, dimorphic, non-pathogenic, ascomycetous, anamorphic, arthroconidial yeast. It is an attractive organism for both, basic and applied research. The very broad range of substrates which can be used as carbon and/or nitrogen sources, the growth and secretion characteristics, the thermo- and osmotolerance as well as the temperature-dependent dimorphism make this yeast an attractive organism for biotechnological application. *A. adeninivorans* is an interesting host for the synthesis of special products because all essential prerequisites and components for heterologous gene expression are available. In addition, the exceptional properties make *A. adeninivorans* a potential donor for genes underlying such properties to equip traditional biotechnologically applied organisms with new attractive capabilities.

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Chapter 28
Biotechnological Applications of Dimorphic Yeasts

N. Doiphode, C. Joshi, V. Ghormade, and M.V. Deshpande

Contents

Abstract The dimorphic yeasts have the equilibrium between spherical growth (budding) and polarized (hyphal or pseudohyphal tip elongation) which can be triggered by change in the environmental conditions. The reversible growth phenomenon has made dimorphic yeasts as an useful model to understand fungal evolution and fungal differentiation, in general. In nature dimorphism is clearly evident in plant and animal fungal pathogens, which survive and most importantly proliferate in the respective hosts. However, number of organisms with no known pathogenic behaviour also show such a transition, which can be exploited for the technological applications due to their different biochemical make up under different morphologies. For instance, chitin and chitosan production using dimorphic *Saccharomyces, Mucor, Rhizopus* and *Benjaminiella*, oil degradation and biotransformation with yeast-form of *Yarrowia* species, bioremediation of organic pollutants, exopolysaccharide production by yeast-phase of *Aureobasidium pullulans,* to name a few. *Myrothecium verrucaria* can be used for seed dressing in its yeast form and it produces a mycolytic enzyme complex in its hyphal-form for the biocontrol of fungal pathogens, while *Beauveria bassiana* and other entomopathogens kill the insect

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pest by producing yeast- like cells in the insect body. The form-specific expression of protease, chitinase, lipase, ornithine decarboxylase, glutamate dehydrogenases, etc. make *Benjaminiella poitrasii, Basidiobolus* sp., and *Mucor rouxii* strains important in bioremediation, nanobiotechnology, fungal evolution and other areas.

Keywords Dimorphic yeasts, dimorphism, biocontrol, yeast-form, hyphal-form, bioremediation

28.1 Introduction

The second largest group of species on earth is of yeasts and filamentous fungi. The estimated fungal species are 15,00,000 in number among which 72,000 are known. The use of this highly differentiating group for various biotechnological applications needs no emphasis. In addition to the conventional applications, yeast, *Saccharomyces* is a well-known model to understand human genome and human genetic-disorders. The phenomenon of yeast dimorphism is known for over a century as ability of a fungal cell to differentiate reversibly in two different morphological forms in response to the environmental perturbations. The dimorphic ability of fungi from all the taxonomic classes is conventionally regarded as a reversible change between the two vegetative forms, yeast and hypha. Furthermore, it has been reported that a zygomycetous fungus *Benjaminiella poitrasii* showed dimorphic response during asexual and sexual spore germination (Ghormade and Deshpande, 2000a). For instance, the zygospores germinated into budding yeast when subjected to yeast-form supporting conditions while hyphal-form favoring conditions gave rise to true hyphae. Similarly, the asexual spores displayed a dimorphic response during germination.

The morphological outcome is decided by the chemical composition and the deposition pattern of the cell wall polymers (Chitnis and Deshpande, 2002). Indeed all the fungal species exhibit three cell wall deposition patterns sometime or the other in their life cycle. In the yeast form regulated non-polarised deposition pattern is prevalent, while regulated polarized during hyphal elongation and occasionally deregulated polarized deposition pattern triggers irregular growth (Fig. 28.1). Based on the chitin synthase analysis, Sburlati and Cabib (1986) suggested the intermediate position for established dimorphic fungi from all the taxonomic groups between filamentous fungi and highly evolved yeasts.

In addition to use of dimorphism to understand fungal pathogenesis and evolution this phenomenon has potential for biotechnological applications. Production of certain form specific metabolites/products in yeast or hyphal form and the knowledge of the specific conditions favoring these forms make dimorphic yeasts easy to manipulate for applications to biotechnological processes. Dimorphic yeasts are more advantageous than the organisms where dimorphism is not established. In addition to the advantages of the morphology *per se*, the physiological make up of

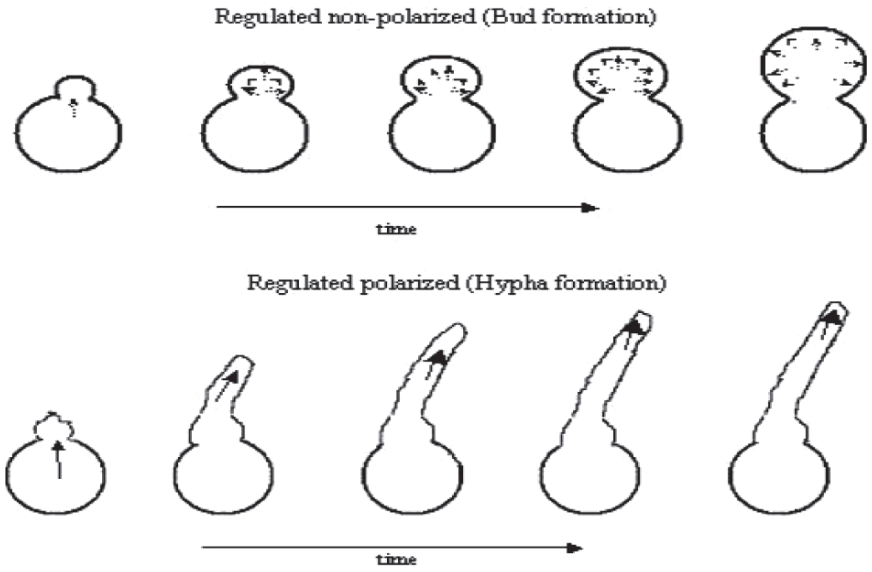

Fig. 28.1 Cell wall disposition pattern in yeast and hyphal-form of *B. poitrasii* in 4 h

the cells also contribute in the applications. The levels of intracellular effectors such as cAMP, Ca-calmodulin (Ca-CaM) involved in the recognition of environmental signals can be modulated to favour yeast or hyphal forms. The quantitative differences in the yeast or hyphal cell wall composition differentially affect the extracellular release of enzymes.

In the following sections, the importance of the dimorphic behaviour and the role played by different morphologies in the biotechnological applications are discussed.

28.2 Physiological Differences in the Yeast and Hyphal Forms of Dimorphic Yeast

In most of the dimorphic yeasts, the physiological make up of both the forms is different which plays an important role in the life cycle of the organisms and in turn can be used for the distinct applications (Deshpande, 1992, 1996). The form- specific expression of certain proteins has also been reported. For instance, in *Benjaminiella poitrasii,* a zygomycetous fungus, form-specific expression of NADP-glutamate dehydrogenases was reported (Amin et al., 2004). Ghormade et al. (2005) found the increased levels of ornithine decarboxylase (ODC) enzyme, involved in nitrogen metabolism, in the hyphal form. Earlier Khale et al. (1990) observed the extracellular production of cellulases and amylases in the presence of carboxymethyl

cellulose and starch, respectively in the hyphal form while alcohol dehydrogenase activity was significant in the yeast form. In addition, Ghormade et al. (2000) reported higher levels of endo-chitinase and *N*-acetylglucosaminidase activities in hyphal form while Chitnis et al. (2002) suggested form-specific expression of chitin synthases in *B. poitrasii.*

Zygomycetous *Mucor* species such as *Mucor rouxii* and *Mucor racemosus,* are normally unable to utilize disaccharides, such as maltose, cellobiose and trehalose anaerobically i.e. in yeast form. In case of *M. rouxii*, different groups of researchers reported α- glucosidase (Flores-Carreon et al., 1970), β- glucosidase (Borgia and Mehnert, 1982), and aminopeptidases and carboxypeptidases (DiSanto and Logan, 1989) mainly from hyphal- form. While Paveto and Passeron (1977) reported 30–40 times more phosphofructokinase in yeast-form than the hyphal- form.

Establishing other means to control the morphology would be a distinct advantage in the development of a fermentation process for a particular organism for the production of heterologous proteins. The possibility to induce specific physiological state and in turn, morphological form in a dimorphic *Mucor circinelloides* was studied. *M. circinelloides* produced multipolar budding yeast under an anaerobic atmosphere and in the presence of high glucose. The anaerobic conditions were maintained by applying 30% CO_2 and 70% N_2 (Lubbehusen et al., 2003). It was found that supplementing cultures with mixtures of ergosterol and Tween 80 resulted in yeast-like growth under 100% N_2 (Bartnicki-Garcia, 1968).

Basidiobolus, a zygomycetous entomophthoralean fungus produced high levels of alkaline protease active at pH 10 in its yeast-like form while hyphal form had little secretion of the enzyme (Ingale et al., 2002).

Studies on the overall expression of glycolytic enzymes in the yeast and the hyphal form of the ascomycete *Candida albicans*, extensively researched dimorphic yeast, revealed that steady-state mRNA levels were subject to variations. These studies included the genes encoding pyruvate kinase, alcohol dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, phosphofructokinase and secretory aspartyl proteases (Hube et al., 1994; Lorberg et al., 1999; Swoboda et al., 1993). The levels of pyruvate kinase (*PYK*), alcohol dehydrogenase (*ADH1*), phosphoglycerate kinase (*PGK1*) and phosphoglycerate mutase (*GPM1*) mRNAs measured during the yeast-to-hypha transition in *C. albicans* revealed that the changes in the expression of these genes was not dependent on alterations to cell shape (Swoboda et al., 1993). However, Lorberg et al. (1999) reported that in *C. albicans*, specific phosphofructokinase activity was decreased two fold upon induction of hyphal growth. Members of the secretory aspartyl proteases were differentially expressed in yeast and the hyphal forms (Hube et al., 1994).

Saccharomyces cerevisiae, an ascomycete and one of the most studied model organisms to understand eucaryotic systems, also exhibited differential levels of certain enzymes in its yeast and pseudo- or true hyphal form. The co-regulation of starch hydrolysis and dimorphism in *S. cerevisiae* was extensively reviewed by Melane et al. (1997). Production of α-amylase and glucoamylase was observed in the pseudo hyphal form (Melane et al., 1997) while alcohol dehydrogenase, 6-phosphogluconate dehydrogenase, phosphogluco isomerase and NADP-glutamate

dehydrogenase were produced significantly in its yeast form (Palecek et al., 2000; Wilkinson et al., 1996).

A pullulan expolysaccharide producer, *Aureobasidium pullulans*, an ascomycete produced xylanase, glucoamylase, glucohydrolase in its hyphal form (Federici and D'Elia, 1983; Leathers, 1986; Leathers, 2003) while enzymes involved in pullulan production are expressed in the yeast-form cells.

28.3 Intracellular Effectors and Their Role in Dimorphism

Intracellular effectors such as cAMP and Ca-CaM have been assigned a role in transducing external environmental stimuli into specific intracellular responses (Gadd, 1995). These secondary messengers transmit their effect via various cellular signalling pathways. In dimorphic fungi yeast-hypha morphological change is marked by change in levels of these intracellular effectors. The knowledge of the role of these effectors and their triggers initiating morphogenesis is useful for manipulation of morphology of fungi for specific biotechnological applications.

In the plant pathogenic fungi *Ceratocystis ulmi*, the causal agent of Dutch elm disease the yeast form showed higher levels of Ca-CaM than the hyphal form (Muthukumar et al., 1987). Higher Ca-CaM levels were also reported in the yeast-form of *C. albicans* and TFP (trifluoperazine) an inhibitor of Ca-CaM promoted hyphal formation (Paranjape et al., 1990).

cAMP levels were reported to be higher in yeast form of *U. maydis*, *M. rouxii, M. racemosus* and *B. poitrasii* (Bolker, 2000; Cantore et al., 1980; Khale and Deshpande, 1993; Larsen and Sypherd, 1974). Whereas lower cAMP levels were observed in yeast form of *C. albicans*, *S. cerevisiae*, *A. pullulans*, *Myrothecium verrucaria, Yarrowia lipolytica* and *C. ulmi* (Niimi et al., 1980; Cooper et al., 1985; Brunton and Gadd, 1991; Joshi et al unpublished data, Zaragosa and Gancedo, 2000). The higher levels of cAMP in yeast- or hyphal – form can be attributed to the survival form of the organism and not the morphology, *per se.*

In case of *U. maydis*, it has been suggested that polyamines are important effectors of dimorphic transition as the null mutants for ODC could form hypha in presence of high polyamine levels (Ruiz-Herrera, 1994; Ruiz-Herrera and Martinez-Espinosa, 1998). While in *Magnaporthe grisea*, causal agent of rice blast, it has been suggested that polyamines may reduce intracellular cAMP levels, leading to the inhibition of appressorium formation (Choi et al., 1998). In *B. poitrasii* hyphal forms showed higher polyamine contents in comparison to the yeast form (Ghormade et al., 2005). The exogenous addition of cAMP or polyamines or their inhibitors can be used to control the morphological form in dimorphic fungi.

The following section highlights the contribution of each morphological form for the specific application.

Table 28.1 Biotechnological applications of some dimorphic fungi

	Hypha/pseudohypha	Yeast
A. pullulans	Cellulose/hemicellulose degradation	Exopolysaccharide and pullulan production
B. poitrasii	Hyphal tip bursting test	Production of germ-tubes
	Cellulose/starch degradation	Ethanol production
	Chitin deacetylase production	Biomass for chitosan production; deacetylation of chitin
M. anisopliae	Penetration through cuticle	Proliferation in hemocoel
M. circinelloides	Heterologous protein production	Biomass
M. verrucaria	CDE/ME complex production	Formulation for biocontrol application
P. fumosoroseus	Penetration through cuticle	Formulation, hemocoel blockage
S. cerevisiae	Starch hydrolysis	Ethanol production
Y. lipolytica	Survival under anaerobic condition	Hydrocarbon degradation

28.4 Biotechnological Applications

Table 28.1 enumerates different organisms, which use two different morphologies to achieve the desired application.

28.4.1 Heterologous Protein Production

Wolff and Arnau (2002) suggested that the yeast-hypha transition of *Mucor* species can be exploited for the production of heterologous proteins such as glucose oxidase, glyceraldehyde-3-phosphate dehydrogenase and others. The biomass production was predominantly in the yeast form while the hyphal form had protein secretory capacity.

Filamentous fungi are capable of secreting large amounts of extracellular proteins from growing hyphal tips (Gordon et al., 2000; Wosten et al., 1991). However filamentous fungi tend to form aggregates or pellets in fermentors causing inadequate mixing and aeration as well as deprived growth. The dimorphic *M. circinelloides* grew rapidly as multipolar yeasts allowing efficient biomass production in a homogenous culture. After sufficient biomass was obtained, the yeast to hyphal transition was induced that provided optimal conditions for protein secretion. In *M. circinelloides*, the dimorphic yeast to hyphal morphological change was used for development of an expression system for the recombinant protein (Wolff and Arnau, 2002). Control of biochemical and genetic factors affecting the morphology will be the approaches useful for optimization and subsequent production of recombinant proteins by fermentation. For instance, morphological shift from yeast to filamentous growth can be achieved by changing anaerobic environment to aerobic condition. Exogenous addition of cAMP resulted in the constitutive yeast growth in *M. circinelloides* (Orlowski, 1991). cAMP is used by cAMP dependant protein kinase A (PKA) which consists of two regulatory subunits

(PKAR) that bind to and inhibit the activity of two catalytic subunits (PKAC). Expression levels of *pkaR* and *pkaC* were found more in anaerobic yeast culture whereas overexpression of *pkaR* resulted in hyper-branching morphologies (Wolff and Arnau, 2002). By constructing genetically engineered strains morphology can be controlled for the production of proteins in desired morphological form (Wolff and Arnau, 2002).

28.4.2 Form Specific Expression of Different Enzymes

Fungi use morphological shift governed by the changes in the level of metabolic enzymes to survive and proliferate during changes in the atmosphere. These metabolic enzymes are directly or indirectly connected to the cell wall synthesis pathway. Chitin**,** a 1,4-β-D-*N*-acetylglucosamine-linked polymer is the major polymer of fungal cell wall. The chitinase complex comprising endochitinase (EC 3.2.1.14) and *N*- acetylglucosaminidase (EC 3.2.1.52) act on chitin breaking it down to *N*-acetylglucosamine residues. Ghormade et al. (2000) reported that in *B. poitrasii* the *N*-acetylglucosaminidase activity increased up to 17 fold during yeast to hypha transition, whereas endochitinase activity increased by 12 fold. It was suggested that *N*-acetylglucosamine residues contributed to the hyphal proliferation. Enzymes associated with nitrogen metabolizing pathways such as NAD-glutamate dehydrogenase (NAD-GDH, EC 1.4.1.2), NADP-glutamate dehydrogenase (NADP-GDH, EC 1.4.1.4) and ornithine decarboxylase (ODC, EC 4.1.1.17) were reported to play significant role in yeast to hypha transition (Amin et al., 2004; Khale et al., 1992; Martinez-Pacheco and Ruiz-Herrera, 1993). In *B. poitrasii*, NAD-GDH enzyme levels were found to be 10 fold lower in the yeast form cells than the hyphal form, whereas NADP-GDH were found to be seven fold higher in the yeast form cells (Khale et al., 1992). It has also been reported that there are two NADP-GDH enzymes, one expressed in yeast and another in hypha (Amin et al., 2004). Most pathogenic fungi use dimorphic change for infection and pathogenesis (Molero et al., 1998). The differential expression of these enzymes provides a suitable target for antifungal compounds. The inhibitor of ODC, difluromethyl-ornithine, has been used for control of plant pathogenic fungi such as *Rhizoctonia solani*, and *Helminthosporium oryzae* (Kumria et al., 2000). GDH as a target for antifungal compounds will provide a new mean of controlling plant pathogenic fungi as the plants use alternate GS/GOGAT nitrogen assimilating pathway.

Form specific expression of certain proteins has been observed in case of other dimorphic fungi as *Histoplasma capsulatum*, *S. cerevisiae* and *C. albicans* (DeLuna et al., 2001; Hube, 1998; Maresca and Kobayashi, 1989). *YPS-3* has been associated with the yeast form of *H. capsulatum* while form specific expression of NADP-GDH has been reported in *S. cerevisiae*. In *C. albicans* 9 secretory aspartyl protease genes have been reported and *SAP1-3* are differentially expressed in yeast form while *SAP4-6* are expressed in the hyphal form (Hube et al., 1994; Hube, 1998). It has been suggested that the differential expression of multiple protease genes might indicate their different functions in infection during specific stage and type of host tissue (Hube et al., 1994).

28.4.3 Polysaccharide Production

Chitosan, a linear polymer of β-1,4 linked glucosamine is a deacetylated form of chitin, a polymer of β-1,4-linked *N*-acetyl-β-D-glucosamine. Chitosan being polycationic, non-toxic, biodegradable finds numerous applications especially in the agriculture, food and pharmaceutical industries.

Mucoraceous fungi contain chitosan as one of the main cell wall components. The contents vary from 1–10% of the dry weight of cells. Different fungal species such as *Absidia coerulea, Rhizopus delemar, Cunninghamella blackesleeana* and *Mortierella isabelina* were found to contain 6.7–10.4% chitosan of the dry weight of cells (Miyoshi et al., 1992). Fungi of this group can be readily grown in the laboratory on cheap nutrients and the wall material can be recovered by simple chemical procedures (McGahren et al., 1984). Therefore chitosan can be produced in a controlled environment all year round and be independent of seasonal shellfish industry, that currently provides the raw material for chitosan production (Pochanavanich and Suntornsuk, 2002; Chatterjee et al., 2005).

In dimorphic *B. poitrasii* cell wall analysis showed that the total hexosamine contents were 1.7 times higher in the hyphal (H, 26.6%) form than the yeast (Y,17.3%) form (Khale and Deshpande, 1992). However, the proportion of deacetylation was found to be more in the yeast form than the hyphal form. Moreover, in the submerged fermentation the yeast biomass can be produced in large quantities in the presence of glucose and complex nitrogen that makes it more suitable for growth in fermentor for recovery of chitosan on large scale. Alternately, the hyphal form can be used for chitosan production using a chitin deacetylase (EC 3.5.1.41) treatment for the conversion of chitin to chitosan.

An exopolysaccharide, pullulan is a linear homopolysaccharide of glucose that is often described as α- (1-6) linked maltotriose, secreted primarily by strains of a dimorphic fungus *A. pullulans*. The unique polymer has distinctive physical traits, including adhesive properties, capacity to form fibers, compression moldings and strong oxygen impermeable films. Pullulan and its derivatives have numerous demonstrated uses in foods, pharmaceuticals, manufacturing and electronics (Leathers, 2003)

The dimorphic ascomycete fungus *A. pullulans* is an important industrial microorganism due to its capability to produce the polysaccharide pullulan and a wide range of different enzymes, of which the most studied include xylanase, glucoamylase and fructofuranosidase (Karni et al., 1993). Importantly, growth in the yeast form is the prerequisite for the pullulan production. Depending on the environmental conditions, particularly the carbon and nitrogen source of the culture medium, *A. pullulans* exists as either filamentous and /or cellular forms (blastospores, swollen cells and chlamydospores). It was reported that Zn^{2+} concentration in the medium affected the morphology and pullulan production (Jurgensen et al., 2001). In the presence of low Zn^{2+} concentration (<0.45 μM) the yeast form was favoured predominantly (Reeslev et al., 1993). Therefore, the fermentation can be designed to obtained yeast form and in turn pullulan.

However, for the production of pullulan using hemicellulosic agro-waste such as wheat bran, paddy husk, and rice straw the filamentous form of *A. pullulans*, which secretes xylanase can be exploited to utilize xylan present in abundant in agro-waste, to convert it into simple sugar. Conditions can be manipulated to obtain hyphal form which secretes xylanases and subsequently the hexoses produced can be used by the yeast form for pullulan production.

28.4.4 Ethanol Production

Production of ethanol by the fermentation of carbohydrates in fruits, grains and other biomass by *S. cerevisiae* is an important process for a wide range of products from fine wines to gasoline additives (Bothast et al., 1999). In *S. cerevisiae* the conversion of yeast to pseudohypha in response to nitrogen starvation was discovered by Gimeno et al. (1992). Poorly utilized carbon sources also induced filamentous growth in *S. cerevisiae*. One of the important observations made was that some starch-degrading strains of *S. cerevisiae* have an ability to form pseudohyphae and to grow invasively into a starch-containing medium (Vivier et al., 1997). Furthermore, pseudohyphal phase of *S. cerevisiae* was reported to produce enzymes such as, α-amylase and glucoamylase (Vivier et al., 1997). *S. cerevisiae* can be grown on starch containing medium as a carbon source, which is otherwise poorly utilized. The pseudohyphal form is capable of converting starch into glucose, which could be useful for the production of ethanol by the yeast-form.

Since *S. cerevisiae* is most studied organism, it is vastly used as a model organism for the heterologous protein expression in yeast form by fermentation. This organism can be used more effectively if the morphology could be manipulated by constructing mutants for the genes involved in dimorphic transition. *SSY1* amino acid permease or the *PTR3* peptide permease regulator is essential for the growth in yeast form (Klasson et al., 1999). Similarly, mutation in NADP-glutamate dehydrogenase (*GDH3*) (Wilkinson et al., 1996), isomerase (*PGI1*), 6-phosphogluconate dehydrogenase (*GND1*) and alcohol dehydrogenase (*ADH1*) induced pseudohyphal growth in haploid *S. cerevisiae* strain (Palecek et al., 2000).

Khale et al. (1990) studied effect of different carbon source on yeast to hypha transition of *B. poitrasii*. The conversion of complex compounds such as starch, cellulose and lactose to glucose and further to ethanol can be made by use of dimorphic transition in a step-wise manner. In the presence of starch, lactose and cellulose *B. poitrasii* grew in the hyphal form and breakdown of the complex compounds resulted in glucose formation. Glucose acts as a trigger for the conversion of hypha into yeast form that converts sugar in to ethanol. Such a strategy can be used by identifying other efficient dimorphic organisms too.

28.4.5 Protease Production

Proteases are classified in four groups depending on their mechanism of action: serine proteases, cysteine proteases, metal proteases and acid proteases (North, 1982). Serine proteases and acid proteases are produced by most of the fungi, whereas cysteine proteases and metal proteases are more limited. Proteases play major part in the growth and differentiation of fungi (Deshpande, 1992). Proteases are also of great commercial value in detergents, leather and food industries (Rao et al., 1998).

High alkaline protease activity was reported in the member of order entomophthorales, *Conidiobolus coronatus* (Phadtare et al., 1997) and *Basidiobolus* (Ingale et al., 2002). Especially in *Basidiobolus* species it has been reported that number of inorganic salts triggered the darmform morphology in the submerged fermentation (Ingale et al., 2002). This transition was associated with the significant increase in the levels of alkaline protease activity whereas growth as mycelial pellets was associated with less extracellular protease (Ingale et al., 2002). This study could be helpful for developing a technology for the production of alkaline protease based on submerged fermentation, which could also be useful to solve a problem of formation of large pellets in submerged growth.

28.4.6 Hydrocarbon Degradation

Oil pollution in the marine environment occurs mainly due to the routine shipping operations, coastal oil refinery effluents, industrial and municipal waste disposal, oil spills caused by tanker accidents and blowouts from off shore oil well platforms. Several bacteria, unicellular yeasts and filamentous fungi play an important role in hydrocarbon degradation in the marine environment. *Y. lipolytica*, an ascomycete fungus, was found to degrade the aliphatic fraction of crude oil (Zinjarde, 1996). It has been observed that the yeast form of *Y. lipolytica* was predominant during alkane degradation as well as in emulsifier production whereas, hyphal form appeared only under condition of partial anaerobiosis and alkane degradation is highly an aerobic process. This organism forms yeast cells in the presence of air with or without alkanes (Zinjarde et al., 1998).

The anaerobic environment is usually created due to oil spill. The marine isolate *Y. lipolytica* degrades oil under aerobic condition, but requires the hyphal form for its survival in partial anaerobic conditions.

28.4.7 Screening of Antifungal Agents

Most of the pathogenic fungi use morphological shift to facilitate pathogenesis in the human. Due to the increase in the number of immuno-compromised patients, newer antifungal targets are of great importance. Some of plant pathogens such as

Taphrina weisneri, U. maydis, and *C. ulmi* are also known to use morphological change for the infection in plant (Ghormade and Deshpande, 2000b). Arrest in these dimorphic transitions was suggested to be a useful strategy for the development of antifungal drugs (Georgopapadakou and Walsh, 1994). For the rapid screening the test introduced by Hutter et al. (1965) was based on morphological changes of the hyphae of *Botrytis cinerea*. However, the sensitivity of the test was limited to antibiotics with strong action against *B. cinerea*. Later on, same group reported the use of zygospore formation inhibition test using *Mucor hiemalis* to screen antifungal antibiotics (Kneifel et al., 1974). Thrautomycin, a nucleoside antibiotic produced by *Streptomyces exfoliates*, was identified using this test. It was observed that (+) and (−) hyphae coming together for zygospore formation had different sensitivities towards thrautomycin. The morphological yeast-hypha and reverse transition exhibited by pathogens can be a model phenomenon to screen antifungal agents. In most of the cases it is not possible to use pathogens for the initial screening. Alternately, non-pathogenic dimorphic fungi exhibiting morphological transitions in response to the environmental perturbations similar to the pathogens can be the choice. In this regard, the use of a non-pathogenic dimorphic fungus *B. poitrasii* was reported (Patil et al., 2001; Salunke et al., 2004).

Apoptosis and other programmed cell death processes (PCD) could potentially have applications in biotechnology as a novel strategy for controlling food-spoilage organisms and food borne diseases, agricultural pathogens and fungal diseases. The conventional methods of controlling fungi, which produce mycotoxin, are same. PCD could be an ideal target for new antifungal strategies. An antifungal agent that overrides the PCD regulation and induces cell death could be very potent since PCD are usually induced at low concentrations of stimulating agent. Since, morphological transition in organism is an intermediate stage between growth and cell death (Denmeade and Isaacs, 1996) non-pathogenic dimorphic fungi could be a good model for study of apoptosis and screening apoptosis inducing agents.

28.4.8 Insect and Fungal Pest Control

Entomopathogenic fungi, such as *Metarhizium* and *Beauveria* spp., which commonly parasitise insects, penetrate the insect cuticle and then proliferate in a single celled form in the circulating fluids of the host, are commonly used for biocontrol. In most of the mycopesticide preparations conidia are used as an infective propagule. The pathogenesis is initiated by adhesion of a conidium to the insect cuticle which further produces a germ tube that penetrates the host. The fungal germlings are continually moving through different environments during cuticular penetration. They respond to these changes by invoking adaptive biochemical processes and cellular differentiation to form a series of specific morphological structures. The germ tubes of entomopathogenic fungi develop appressoria (located at the cuticle surface), infection pegs (in the epicuticle),

penetrant hyphae and penetrant plates (in the procuticle), and yeast-like hyphal bodies (blastospores) for dispersal through the hemocoel (Deshpande, 1999; St.Leger et al., 1987). However, the formation of yeast like cells in the host body is poorly studied. It was reported that a *Metarhizium anisopliae* isolate that presented a fast dimorphic change to form more number of yeast like cells was more virulent for the adult locusts (Toriello et al., 2003). In other words, two morphological forms, hyphal for penetration and yeast for proliferation in the hemo-lymph can be used effectively in the biocontrol of insect pest.

Paecilomyces fumosoroseus produced hyphae, blastospores and yeast-like cells in liquid culture (Goettel and Roberts, 1992). Blastospores were reported to be more effective than conidia as infective propagules to control silverleaf whiteflies, *Bemisia argentifolii* (Jackson et al., 1997; Lacey et al., 1999). The blastospores transit in to germ tubes rapidly on the cuticle than conidia for penetration and further pathogenesis.

Myrothecium verrucaria is a saprophytic, deuteromycetous fungus that produces extracellular hydrolytic enzymes like chitinase, β-1,3 glucanase, lipase and protease involved in the degradation of fungal cell wall and insect cuticle (Vyas and Deshpande, 1989). In other words, the enzyme complex can be used for the effective control of both the fungal pathogens of plants such as *Sclerotium rolfsii, Fusarium oxysporum* and insect pests. *M. verrucaria* in the vegetative cycle exhibits unicellular yeast-like and hyphal form under different nutritional conditions (Deshpande, 1999). The yeast-like cells of *M. verrucaria* can be produced easily within 72 h in submerged fermentation, therefore are useful to prepare different formulations for seed dressing and soil application. After application in the soil (at pH 6.0), they transit in to hyphal form which produce extracellular cuticle degrading and mycolytic enzymes within 24 h. Thus the two morphological forms, one for preparation of formulation and the other for slow release of enzymes, are effective in the biocontrol strategy (Patil et al., 2001).

28.4.9 Fungal Evolution

The significance of yeasts in the phylogeny of fungi has been reviewed extensively (Prillinger et al., 1993). In evolution process, yeast possibly has evolved from filamentous fungi by a series of changes in mechanisms of cell wall growth and deposition pattern. The induction of yeast like vegetative growth in the zygomycetous dimorphic fungi such as *Mucor* and *Rhizopus* was reported to be significant in the phylogeny of Eumycota (Prillinger, 1987).

Chitin synthase is the key enzyme that synthesizes chitin for the fungal cell wall and its role in evolution of fungi has been studied. Class I, II and IV chitin synthase enzymes are present in the dimorphic yeasts *S. cerevisiae* and *C. albicans* whereas Class I-V chitin synthase enzymes are present in filamentous fungi like *Aspergillus fumigatus* and *Aspergillus nidulans* (Chitnis et al., 2002).

Rhizopus oligosporus Class II genes (*CHS1* and *CHS2*) are reported to function mainly in the growing hyphal form. Different levels of chitin synthase genes *UmCHS1* and *UmCHS2* were reported in case of *U. maydis* and higher levels of *UmCHS2* transcripts were observed in the hyphal form (Xoconostle-Cazares et al., 1996). The dimorphic fungus *B. poitrasii* was reported to have 8 chitin synthase genes with *BpCHS1 – 4* belonging to Class II, *BpCHS5-6* and *8* of Class IV and *BpCHS7* of Class V (Chitnis et al., 2002). Among 8 chitin synthase genes reported for *B. poitrasii* 2 genes *BpCHS2* and *BpCHS3* appeared to be mycelium specific. Chitin synthase Class II enzymes are reported to synthesize septal chitin in *S. cerevisiae* cells and *C. albicans* whereas it had additional role in lateral wall synthesis in the later (Bulawa and Osmond, 1990; Silverman et al., 1988; Munro et al., 2001). The Class II enzyme of *A. nidulans* was suggested to be involved in conidium formation and hyphal wall synthesis (Culp et al., 2000). Class V chitin synthase of *A. fumigatus* synthesized 25% of hyphal wall chitin and was also reported to be involved in sporulation (Aufavre-Brown et al., 1997). Presence of multiple chitin synthase genes in the *B. poitrasii* a dimorphic fungus suggests that such fungi may have an intermediate position in comparison to filamentous fungi and more advanced yeasts.

28.5 Epilogue

The morphological outcome in fungi is dependent on the physiological make up of the cell. For the biotechnological applications these differences can be effectively used and the morphology is the indicator for them. Indeed there are certain applications in which morphology, *per se* is important. For instance, biocontrol formulations can be made easily with yeast-like cells and the hyphal form is for the secretion of the hydrolytic enzymes; hyphal form is for the penetration and the yeast form is for the proliferation. The change in the morphology for the survival, proliferation and dispersal in fungi can be used for effective biotechnological applications by manipulating environmental conditions. As this is the outcome of cell wall composition and deposition pattern, the knowledge of cause-effect relationship between environmental perturbations, signal transducing intracellular effectors and the biochemical correlates of cell wall synthesis will be useful for the effective use of any yeast for a specific application.

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Chapter 29
Extracellular Polysaccharides Produced by Yeasts and Yeast-Like Fungi

Inge N.A. Van Bogaert, Sofie L. De Maeseneire, and Erick J. Vandamme

Contents

Abstract Several yeasts and yeast-like fungi are known to produce extracellular polysaccharides. Most of these contain D-mannose, either alone or in combination with other sugars or phosphate. A large chemical and structural variability is found between yeast species and even among different strains. The types of polymers that are synthesized can be chemically characterized as mannans, glucans, phosphomannans, galactomannans, glucomannans and glucuronoxylomannans. Despite these differences, almost all of the yeast exopolysaccharides display some sort of biological activity. Some of them have already applications in chemistry, pharmacy, cosmetics or as probiotic. Furthermore, some yeast exopolysaccharides, such as pullulan, exhibit specific physico-chemical and rheological properties, making them useful in a wide range of technical applications. A survey is given here of the production, the characteristics and the application potential of currently well studied yeast extracellular polysaccharides.

Keywords Exopolysaccharide, pullulan, glucans, glucomannans, rheological properties

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29.1 Introduction

Formation of extracellular polysaccharides is a well studied characteristic among bacteria and some of them are now commercially produced i.e. xanthan, dextran, gellan and hyaluronan. A few fungal polysaccharides have also been examined with respect to their interesting properties and application potential especially in the medical and technical field (e.g. schizophyllan and scleroglucan). However, exopolysaccharides produced by yeasts or yeast-like fungi have rather been neglected, with the exception of those from *Aureobasidium pullulans* and *Cryptococcus neoformans*. Extracellular polysaccharides have been described to occur in the following yeast genera: *Aureobasidium*, *Bullera*, *Candida*, *Cryptococcus*, *Pichia, Phomopsis, Exophiala*, *Lipomyces*, *Rhodotorula*, *Sporobolomyces*, *Tremella* and *Trichosporon* (De Baets et al., 2002b; Leathers, 2002). Only those genera of which relevant published information is available will be discussed here and ordered based on recent taxonomy. The types of polymers which are synthesized by these yeasts can be chemically characterized as mannans, glucans, phosphomannans, galactomannans, glucomannans and glucuronoxylomannans. Some of these extracellular polymers are very similar to the yeast cell wall polysaccharides, suggesting they are derived from them or at least synthesized by the same mechanism. Other structures are totally different and thus are produced separately from the cell wall. In all cases, these polysaccharides offer interesting perspectives with respect to application in various speciality fields ranging from the medical, food, feed, cosmetic and chemical sector.

29.2 Ascomycetous Yeasts

29.2.1 Candida spp.

Several members of the *Candida* genus produce heteropolysaccharides which have a wide diversity of component sugars. Do Carmo-Sousa and Barroso-Lopes (1970) performed a comparative study of the extracellular and cell wall polysaccharides of the several *Candida* species. They were found to produce an extracellular polysaccharide, which contains galactose, glucose, mannose, fucose and rhamnose. The cell wall sugar composition was similar, indicating that the same enzyme system might be responsible for the synthesis of both polymers.

Candida albicans is a medically important yeast, causing candidiasis and candidemia in immunocompromised persons. The cells produce a water soluble fraction (CAWS), suggested to be a mannoprotein β-glucan complex and composed of mannan, (1→3)-β-D-glucan (only a small part) and (1→6)-β-D-glucan. Comparison with NMR-data of cell wall polysaccharides confirmed that the yeasts release a portion of the β-glucans in their cell wall in the form of soluble β-1,3-glucan fragments. This water soluble fraction can be detected in the blood of mycosis

patients, where they tend to accumulate because the (1→3)-β-D-glucan portion can not be metabolized by the human body. The water soluble fraction activates the factor G cascade (Uchiyama et al., 1999) and was found to exhibit various other biological activities. It is able to provide a shock model, an endogenous septicemia model and an angiitis model. Those models are valuable for use as animal models for the treatment of refractory diseases. Based on the Limulus G-activating property, the Fungitec G Test MK was recently developed as a serological diagnostic tool for *C. albicans* and certain other fungi or yeasts causing deep mycosis (Ohno, 2003). The exopolysaccharides produced by *C. albicans* also seem to play an important role in biofilm formation, a growing issue in the treatment of persistent hospital infections on bioprosthetic materials.

Another interesting *Candida* extracellular polysaccharide is the glucomannan of *C. utilis*. The major components are D-mannose and D-glucose, linked in an α-1,3; α-1,4 and α-1,6 way. Also for this species a significant protein fraction of about 12% could be detected (Bukova et al., 2002). The extracellular glucomannans of *C. utilis* exhibit an antioxidative and antimutagenic effect, based on their ability to complex metal ions and to scavenge different reactive oxygen species (Bukova et al., 2002). Their good water solubility, relatively small molecular weight (15–30 kDa) and bioprotective activity against chemical compounds differing in mode of action, also upon oral administration, make them a promising protective agent.

A mixture of *C. utilis* and bacterial cells was used as a probiotic for young chickens. The probiotics enhanced the intestinal mucosal immunity of the chickens, probably in part due to the produced exopolysaccharides and other components of the yeast cell wall (Yang et al., 2005).

29.2.2 Pichia spp.

The phosphomannan synthesized by *Pichia holstii* NRRL Y-2448 (former name *Hansenula holstii*) was already patented in 1962 by Benedict et al. (1962). They described the polymer to consist solely of D-mannose. The main chain has several side chains attached to it by α-1,2 or α-1,4 linkages. The side chains were terminated by a d-mannose unit carrying a phosphate group at C_6. The structural differences between the mannan extracted from the cell wall and the exocellular mannan led to the conclusion that both polymers are synthesized independently (San Blas and Cunningham, 1974).

Two important factors influence the structure and composition of the polymers synthesized by this *Pichia* strain: the haploid/diploid phase of the yeast and the phosphate content of the medium. San Blas and Cunningham (1974) examined both the cell wall and extracellular polymer of the haploid strain NCYC 560 and found that the exopolysaccharides synthesized by strain NCYC 560 resembled better the cell wall mannan of *Saccharomyces cerevisiae* than the extracellular mannan produced by the diploid *P. holstii* NRRL Y-2448.

The phosphorous content of the mannan depends largely on the phosphate level in the medium. When phosphate is present in the medium, an exocellular phosphomannan is formed with an α-1,6-linked backbone to which α-1,2 and α-1,3 side chains are attached (Fig. 29.1), which in turn may be branched resulting in a tree like structure resembling glycogen or starch. Phosphate is present as a monoester and probably this polymer serves as a phosphorous-reservoir when concentrations in the medium turn low (San Blas and Cunningham, 1974).

Phosphate limitation in the yeast culture medium leads to the production of structurally different mannans (San Blas and Cunningham, 1974), with almost undetectable traces of phosphor. The extracellular mannan has a branched arrangement similar to its related phosphomannan, but contains fewer and longer side chains in which occasionally β-linkages occur. Variation of the nitrogen source did not seem to influence the D-mannose to phosphate ratio.

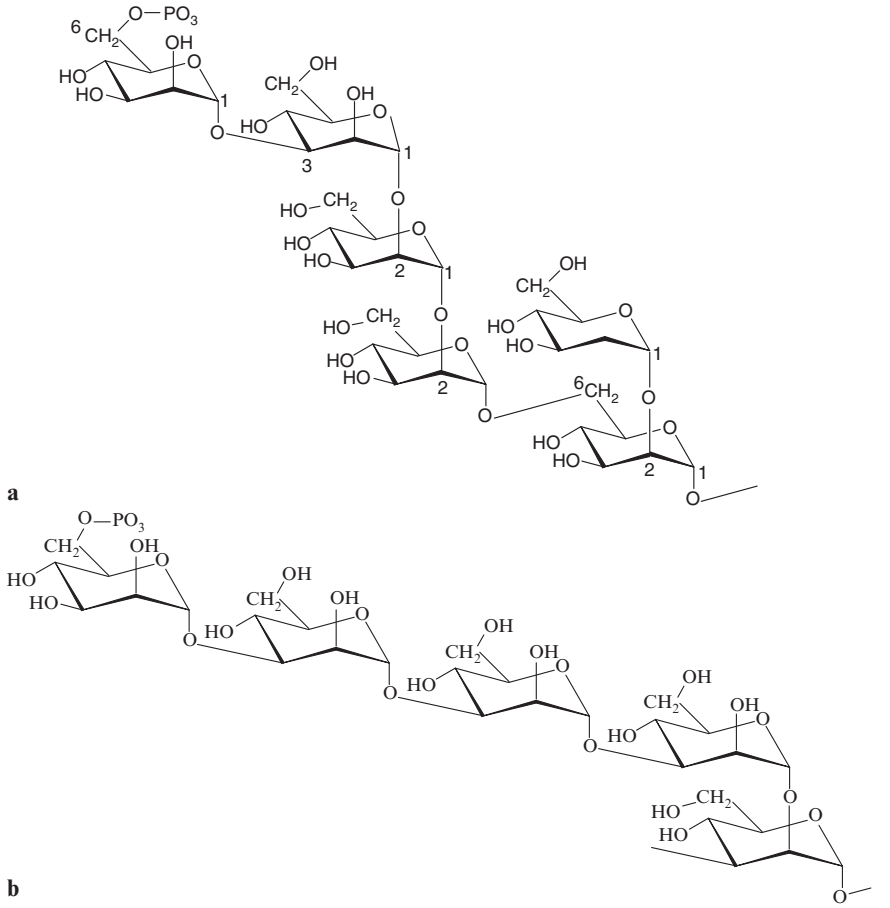

Fig. 29.1 *Pichia holstii* NRRL Y-2448 phosphomannan core (a) and side chain (b) repeating units (Parolis et al., 1996, 1998)

The chemical structure of the polymer synthesized by *P. holstii* NRRL Y-2448 has further been elucidated by Parolis et al. (1996). They studied the phosphomannan backbone after hydrolytic removal of the oligosaccharide phosphate side chains. This backbone corresponds to the high molecular weight component found after mild acid hydrolysis. Since this phosphomannan core accounts for only 10% of the total mannose and phosphate content, it is clear that the polysaccharide is highly branched. The core contains α-1,2-; α-1,3- and α-1,6-linkages, and is supposed to be composed of repeating units as shown in Fig. 29.1. The majority of the chains attached to the core phosphomannan is composed of at least ten repeating pentasaccharide phosphate residues. In other chains, pentasaccharide phosphate residues are replaced by a tetrasaccharide phosphate and to much lesser extent by hexa-, tri- and disaccharide phosphates (Parolis et al., 1998). After mild acid hydrolysis, the side chain delivers penta- or oligosaccharide monophosphates, which are called the low molecular weight fraction. High molecular weight and low molecular weight phosphomono-esters obtained after mild acid hydrolysis of the *P. holstii* polymer have become valuable tools in research on mammalian phosphomannosyl receptors (PMR). The pentasaccharide monophosphate has been coupled to proteins in order to target them to lysosomal receptors and to direct their uptake by cells which express phosphomannosyl receptors (Parolis et al., 1996). Preparations of both the pentasaccharide and the phosphomannan core have been used as an affinity ligand for isolating such receptors. The high molecular weight phosphomannose or an oligosaccharide derived from it, acts as antagonist or competitive inhibitor of the natural ligand of a D-mannose phosphate receptor. As a result of these investigations, it has been discovered that these phosphosugars display anti-inflammatory and immunosuppressive activity (Parish et al., 1996).

More recently, several patents have been filed on the preparation and use of sulfated oligosaccharides for their anticoagulant, antithrombotic and antiangiogenic activity. This phosphosulfomannan mixture, with the commercial name PI-88, is currently under multiple international Phase II clinical trails for its anticancer activity (www.progen.com.au). It inhibits angiogenesis in three ways: (1) heparan sulfate mimicry causes inhibition of heparanase, in this way preventing the release of angiogenic growth factors, (2) it is a competitive inhibitor of several angiogenic growth factors and (3) it stimulates the release of Tissue Factor Pathway Inhibitor. The inhibition of vascular smooth muscle cell proliferation, kinase signalling and arterial intimal thickening following balloon injury, gives PI-88 also a therapeutic potential in a variety of vascular disorders (Khachigian and Parish, 2004).

29.2.3 Aureobasidium pullulans

The best known yeast glucan, pullulan, is a linear polysaccharide produced by strains of the yeast-like fungus *Aureobasidium pullulans*; it is commercially produced and available from Hayashibara Co., Ltd., Japan. The main polymer consists of maltotriose units α-1,4-linked to one another by α-1,6-linkages (Fig. 29.2). The regular

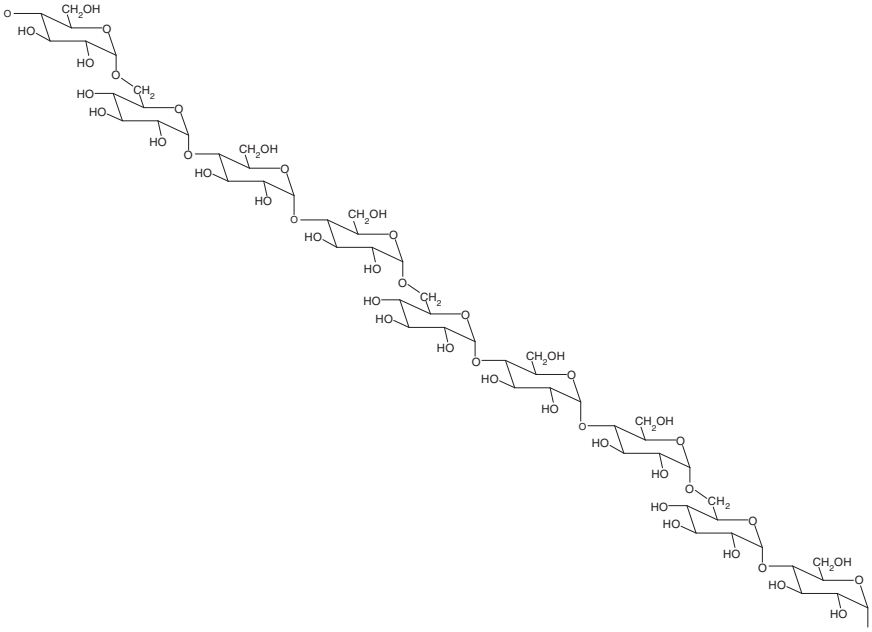

Fig. 29.2 The primary structure of repeating linkages of pullulan

introduction of α-1,6-linkages interrupts what otherwise would be a linear amylase chain, enhancing the flexibility, solubility and making pullulan resistant to amylases. Occasionally, maltotriosyl residues are replaced by maltotetraosyl residues in an apparently random fashion (Catley et al., 1986).

Many researchers reported other (non-pullulan) extracellular polysaccharides, depending on the used strain and/or medium. Yurlova and de Hoog (1997), for example, identified a distinct taxonomic group of *A. pullulans* strains that make "aubasidan", a homoglucan with with a β-13-linked backbone and α-14-linked side chains attached by β-16-linkages. Aubasidan can be used to immobilize cells for enzymatic conversion (Abelyan, 2000).

A. pullulans has a complex life cycle exhibiting a variety of forms ranging from yeast-like cells through multicellular fragments to chlamydospores. The transition from one form to another is influenced by several factors such as nutrient depletion, pH or presence of certain ions. Pullulan synthesis depends on the morphological state of the microorganisms, and according to Campbell et al. (2004) the swollen cells and chlamydospores (resting forms), and neither hyphae nor unicellular blastospores, often held responsible for pullulan formation, are responsible for the synthesis of the polysaccharide.

The mechanism of pullulan biosynthesis is far from being fully understood. When glucose is used as carbon source, the following pathway is proposed (Fig. 29.3).

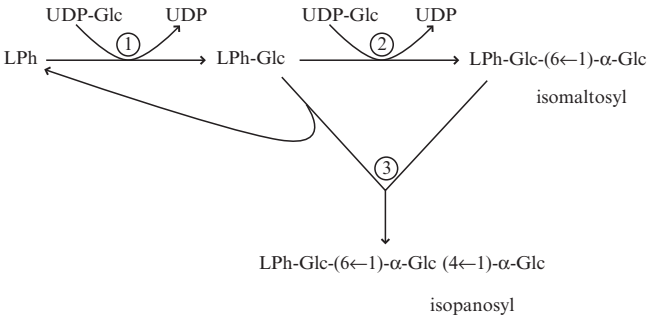

Fig. 29.3 Proposed pathway of pullulan biosynthesis via a phospholipid intermediate (LPh)

The first stage is the UDP-glucose mediated attachment of a glucose residue to a lipid molecule with a phosphoester bridge. A further transfer of the glucose residue from UDP-glucose (step 2) gives lipid-linked isomaltose. In the next step, isomaltosyl-residues participate in the reaction with lipid linked glucose to yield an isopanosyl residue. Further, isopanosyl residues are polymerized into the pullulan chain (Catley and McDowell, 1982).

To economize the production process, several agro-industrial waste streams (grape skin pulp extract, starch waste, olive oil waste effluents and molasses) were tested as substrates for the pullulan fermentation. Of these waste streams, grape skin pulp could be considered as the best substrate yielding the highest concentration of relatively pure pullulan (Israilides et al., 1999).

A. pullulans is considered a "black yeast"; this means it produces melanin which is sometimes difficult to separate from the pullulan. Culture conditions and strain selection are important issues in obtaining melanin-free pullulan. Another important issue during fermentation is the oxygen transfer. Pullulan itself is produced and deposited on the outside of the cells, and because of the low oxygen permeability of the pullulan, this layer acts as a barrier for the transfer of oxygen. This could be detrimental to the survival of the cells and, in turn, restrict the formation of pullulan. This feature can be overcome by e.g. good control of the dissolved oxygen, continuous removal of pullulan, fermentation in an air lift reactor, increasing the fermentor inner pressure or the use of a oscillatory baffled reactor (Gaidhani et al., 2005).

Pullulan has adhesive properties and can easily be modified chemically to provide reactive groups or better solubility. It has numerous applications in foods, pharmaceuticals, manufacturing and electronics.

An important application in the food industry is the use of pullulan films as (edible) food coating or packaging agent. It forms an oxygen impermeable film which is odorless, tasteless and colorless. Since it is a non-digestible fiber, it can also be added as a low caloric ingredient for dietary foods (Yuen, 1974). Studies suggest that dietary pullulan functions as a prebiotic, promoting the growth of beneficial bifidobacteria.

It finds application in the medicinal field as plasma extender and to maintain normal colloid-osmotic pressure in blood and tissues. In pharmacy it can be used as packaging material for drugs and in the cosmetic sector it can be applied as an emulsifying agent. It also seems to be a good bio-adhesive, and is consequently already being used on labels, stamps and envelopes (Lachke and Rale, 1995).

29.3 Basidiomycetous Yeasts

29.3.1 Tremella spp.

Tremella is one of the yeast genera belonging to the so called yeast-like fungi. These microorganisms are characterized by dimorphism: the haploid phase of *Tremella* is a yeast, but upon conjugation/fusion a dikaryotic mycelium is formed (Bandoni and Boekhout, 1998). Exopolysaccharides can be extracted from the fruiting bodies, but also the yeast stage can produce exopolysaccharides. All species of the genus *Tremella* live on other fungi but is not clear whether or not the extracellular polysaccharides take part in the attachment process.

29.3.1.1 Tremella fuciformis

The fruiting body of *Tremella fuciformis* Berk is a famous food and traditional drug used clinically as a tonic against weakness caused by illness and aging in China.

Hot water extracts of *T. fuciformis* Berk yield a crude polysaccharide, which can be separated in a neutral and acid fraction. The acidic fraction can then be further separated. All of the fractions were composed of a 1,3-linked D-mannose backbone, with side chains consisting of D-xylose, D-glucose, D-mannose, D-fucose and glucuronic acids in different rates (Gao et al., 1998). The structure of the acidic heteropolysaccharide is shown in Fig. 29.4.

Yui et al. (1995) further investigated the glucuronoxylomannan obtained from the fruiting body and found that the mannan backbone has the left-handed, three-fold helical symmetry. This helical structure is also found in the fungal exoglucans

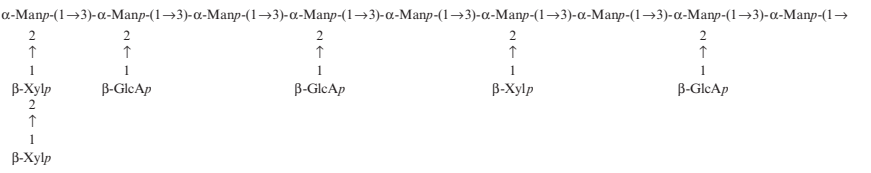

Fig. 29.4 Structure of the acidic heteropolysaccharide produced by *Tremella fuciformis*

curdlan and scleroglucan, known as antitumour and immunomodulating agents (Falch et al., 2000). The conformation of the polymer seems to influence considerably the biological activity of the polymer, such as affinity to acceptor molecules.

All the fractions and subfractions of the polysaccharide displayed the ability to induce human monocytes to produce interleukins (IL-1, IL-2, IL-6) and tumor necrosis factor in vitro (Gao et al., 1997, 1998). It was suggested that this immunostimulating effect might be caused by the common α-1,3-mannan in the fragments. Furthermore they have antitumour, hypoglycemic and cholesterol lowering activities (Kiho et al., 1994). Water soluble extracts of *T. fuciformis* fruiting bodies are also used in cosmetic products, exhibiting a highly moisturizing effect (Hasebe, 1988).

It is the acidic heteropolysaccharide produced by *T. fuciformis* that is held responsible for lowering the cholesterol level in blood serum (Yui et al., 1995). Cheng et al. (2002) studied this effect in rats, and found that next to a lowering of the total cholesterol level, the serum LDL-cholesterol and triacylglycerol level was lowered as well. The decrease in serum cholesterol was caused by a reduction in cholesterol absorption.

A patent has been granted to (Xiu, 1996) for the use of extracts of *T. fuciformis* Berk in the manufacture of a pharmaceutical agent for treatment of wounds and other skin injuries. One year later, this Chinese researcher obtained a patent for the use of these extracts for treatment of thrombophlebitis, atherosclerosis and senile degradation of microvessels. Agents containing *T. fuciformis* extracts have a stimulating effect on DNA synthesis in vascular endothelial cells. This effect is of major importance for growth, development, reproduction and regeneration of microvessels in the microcirculatory system (Xiu, 1997). It has also been found that this stimulating effect can be used in combination with known anti-HIV agents such as azidodideoxythymidine, in the treatment of AIDS patients to improve their condition.

29.3.1.2 Tremella mesenterica

The exopolysaccharides produced by *Tremella mesenterica* consist of two fractions: a neutral α-D-glucan and an acidic heteropolysaccharide. The α-D-glucan is composed of about 200 α-D-glucopyranose monomers, α-1,6 and α-1,4 bound in a ratio of 2 to 1. The structure of this polymer is comparable to pullulan, produced by the yeast-like fungus *Aureobasidium pullulans* (see 2.3). It does not stain with iodine and is not attacked by α- and β-amylases.

The acidic heteropolysaccharide produced by this strain is a strongly branched polymer with an α-1,3 bound mannose backbone. Its side chains are composed of D-xylose and glucuronic acid, as shown in Fig. 29.5 (Fraser et al., 1973). In addition to these two fractions, Slodki et al. (1966) also described the ability of *Tremella* species to synthesize starch at a pH of 5.0 and lower.

The glucuronoxylomannans display a hypoglycemic activity (Lo et al., 2006). Furthermore, *Tremella* glucuronoxylomannan is found to be useful to improve immunodeficiency, to protect against physical stress or aging and to prevent senile degradation of microvessels (Reshetnikov et al., 2000). Chen et al.

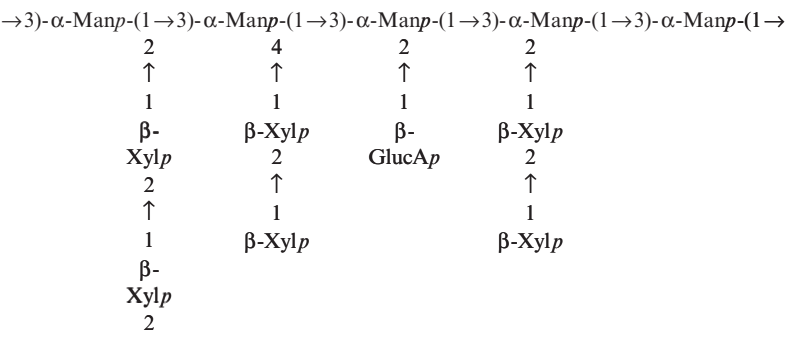

Fig. 29.5 Structure of the acidic heteropolysaccharide produced by *Tremella mesenterica* NRRL Y-6158. Variation on the xylose side chain arrangement is possible

(2006) stated that the carbon source used during fermentation affects the sugar and uronic acid composition of the exopolysaccharides, and by this way, also their cytokine stimulation activity. Xylose and glucose gave rise to exopolysaccharides with the highest immunomodulating activity due to their relatively high mannose content.

The microbial production process of the glucuronoxylomannans has been patented by an Israeli company (Wasser and Reshetnikov, 2001). In order to produce polysaccharides, *T. mesenterica* CBS 101939 was grown in aqueous media containing carbon (glucose, sucrose, starches such as wheat, corn meal and the like), nitrogen (yeast hydrolysates or extract, bacteriological peptone, corn steep liquor), inorganic salts containing potassium, ammonium, magnesium and trace elements such as iron, manganese, zinc and cupper. The fermentation is carried out at temperatures ranging from 20°C to 28°C (Wasser and Reshetnikov, 2001). A very useful medium for production of the polysaccharides by *T. mesenterica* NRRL Y-6158 is potato dextrose broth, but contrary to strain CBS 101939 this strain is not able to assimilate starch, which eventually leads to a mixture of starch and polymer upon recovery. A chemically defined medium developed at the authors' laboratory allowed for the synthesis and recovery of the pure polymer. Lowering the glucose source level by using a cyclic fed-batch fermentation resulted in higher production yields. The sugar possibly slowed down exopolysaccharide production by osmotic effects or catabolite regulation on the enzymes involved in the synthesis of extracellular polymer (De Baets et al., 2002a).

29.3.1.3 Tremella aurantia

The acidic polysaccharide isolated from *T. aurantia* shows an even higher antidiabetic activity than the one of *T. mesenterica* (Kiho et al., 2001). Analysis of the component sugars showed that the acidic polysaccharide was composed of D-mannose, D-xylose, glucuronic acid and D-glucose (molar ratio 4:2:1:0.3) and contained 2.2% *O*-acetyl groups. Since hypoglycemic activity might be related to the structure

of polysaccharides, studies of the chemical structure were performed. These analyses proved that the acidic polysaccharide consisted of an α-1,3-linked mannopyranosyl backbone in which some of the mannose sugars were substituted at position 2 with β-1,3-linked xylopyranose side chains and with β-D-glucopyranosyl-uronic acid at position 4 linked to terminal α-D-mannopyranose.

The non-reducing terminal α-D-mannopyranosyl residues may contribute to the potent hypoglycemic activity of the acidic polysaccharide in diabetic mouse models. In addition to the anti-diabetic activity displayed by this polymer, it also seemed to significantly increase the glucokinase, hexokinase and glucose-6-P-dehydrogenase activity, while it decreased the activity of glucose-6-phosphatase in normal and diabetic mouse liver after intraperitoneal administration. Also after oral administration, the acidic polysaccharide or its degradation products exhibit an anti-diabetic effect. Plasma cholesterol and triglyceride levels were lowered in normal and diabetic mice (Kiho et al., 2001).

29.3.2 Cryptococcus spp.

29.3.2.1 Cryptococcus laurentii

Cryptococcus laurentii is a non-pathogenic yeast, in contrast to *Cr. neoformans* (see 3.2.2). However, *Cr. laurentii* has been reported as a rare cause of pulmonary and cutaneous infection in humans. It may also be occasionally recovered as a saprophyte from skin (http://www.mycology.adelaide.edu.au/mycology).

Already in 1960, Abercrombie et al. investigated the production of extracellular polysaccharides by *Cr. laurentii* NRRL Y-1401. As in the case of *T. mesenterica*, the polysaccharides are composed of two different fractions: an acidic polysaccharide containing D-mannose, D-xylose and glucuronic acid, and a neutral glucan. This neutral glucan contains α-1,3 bonds, which are not found in the glucose polymer from *Tremella*, however (Abercrombie et al., 1960). The acidic polysaccharide synthesized by *Cryptococcus* shows high similarity to the one produced by *Tremella*, but polymers isolated from *Tremella* spp. generally contain much more D-xylose and have a more branched structure (Slodki et al., 1966).

In addition to the above-mentioned similarities, both yeast species have the ability to synthesize starch at a pH of 5.0 and lower. Based on the similarities in polymer production, (Slodki et al., 1966) suggested the existence of a taxonomic relationship between both species. According to these authors, there exists a reasonable possibility that some species of *Cryptococcus* represent one or more stages of the life cycle of some species of *Tremella*. Although Baharaeen and Vishniac (1984) suggested that 25S rRNA homology did not support a close relationship, studies by Guého et al. (1993), Fell et al. (1995) and Swann and Taylor (1993) have found otherwise. Bandoni and Boekhout (1998) describe that yeast stages of *Tremella* are similar to *Cryptococcus*.

Comparison between the extracellular heteroglycan of *Cr. laurentii* and its capsular polymers provided evidence of the fact that the exopolysaccharide has a capsular

origin (Tikhomirova et al., 1998). The molecular mass and the degree of branching of the exopolysacharides increase when the organism is grown under salt stress. This suggests that the exopolysaccharide has a function in the protection of the yeast cells from water loss and maintaining the growth conditions under salt stress (Breierova et al., 2005).

Yeast exoglycans from *Cr. laurentii* and also *Cr. luteolus* have been shown to absorb copper and lead ion (Elinov et al., 1999). Lutelan is the most active exoglycan in this respect. A highly viscous heteroglycan, crylan, secreted by a *Cr. laurentii* strain, seems to be a promising stabilizer of dispersed systems and can find wide application, particularly in the production of cosmetics (Tikhomirova et al., 1998).

A Russian research group from Saint Petersburg patented the use of *Cr. laurentii* and its exopolysaccharides as a food additive. They claim a reduction of increased cholesterol and triglyceride levels in blood serum and liver, and sorption of heavy metals (Anan'eva et al., 2002). A few years earlier, they already patented the production process for the heteropolysaccharide: they cultured the yeast in a nutrient medium containing glucose, peptone, yeast autolyzate, $MgSO_4$, $FeSO_4$, NaCl, $MnCl_2$ and $CaCO_3$ as a pH regulating agent (Anan'eva and Vitovskaja, 2000).

29.3.2.2 Cryptococcus neoformans

Cryptococcus neoformans is a very important opportunistic pathogen in AIDS patients and is the most common cause of fungal meningitis in the world. Three different varieties have been described: *Cr. neoformans* var. *grubiis* (serotype A), *Cr. neoformans* var. *neoformans* (serotypes D and A/D) and *Cr. neoformans* var. *gattii* (serotypes B and C) (Franzot et al., 1999). Strains of serotype A are the most common cause of cryptococcosis in all patients, with or without AIDS. It was determined that melanin synthesis and capsule formation are two well established virulence factors (McFadden and Casadevall, 2001). *Cr. neoformans* is prevalent worldwide in association with soil and avian excreta (Doering, 2000).

The cryptococcal capsule is composed of two polysaccharide components: 90% of the capsule consists of an acidic glucuronoxylomannan (GXM) with a molecular mass estimated at several million Daltons (Doering, 2000), while another 7% consists of a galactoxylomannan (GalXM) that has a molecular weight of 275 000 ± 25 000 Da. A third component of the cryptococcal capsule is a mannoprotein (MP). The cryptococcal capsule is easily visualized by light microscopy by using an Indian ink stain (Doering, 2000).

The most abundant polymer, GXM, is composed of a linear (1→3)-α-D-mannopyranose chain substituted with β-D-xylopyranosyl, β-D-glucopyranosyluronic acid and 6-*O*-acetyl (3–10% of the residues). The ratio of D-xylose is variable and determines together with the disposition of the *O*-acetyl groups the antigenic activity of the GXM (Kozel et al., 2003). Six structural motifs (structure reporter groups or SRG, M1 to M6) were identified, composed of (1→3)-α-D-mannotriose units to which one (1→2)-β-D-Glc*p*A and several (1→2)-β-D-Xyl*p* (1→4)-β-D-Xyl*p* are attached in different amounts depending on the serotype (Fig. 29.6). The six mannosyl

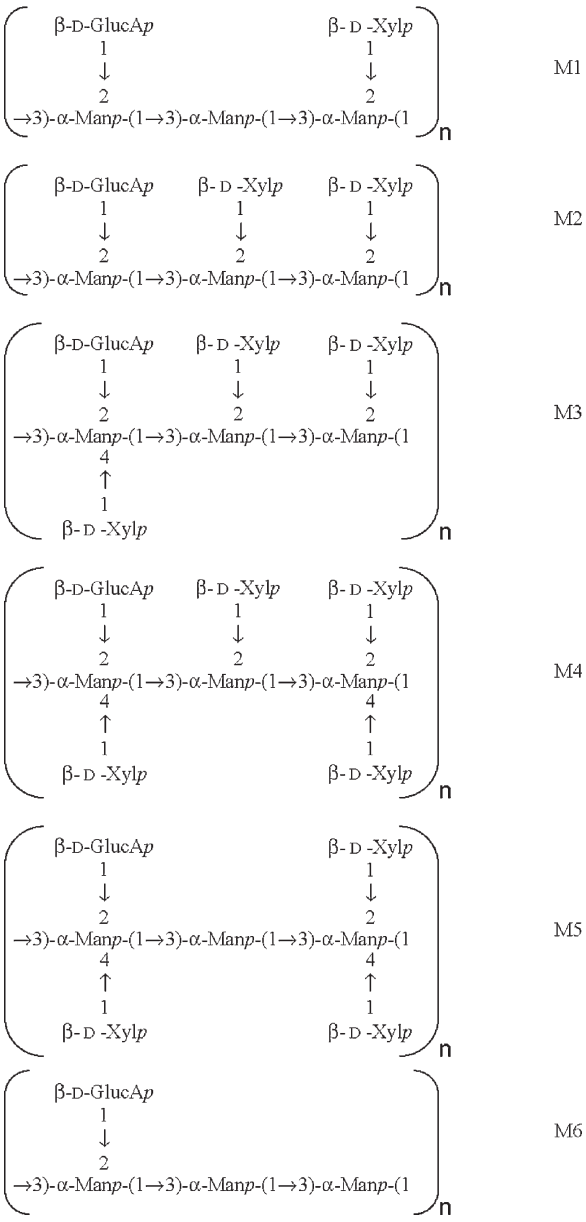

Fig. 29.6 The six mannosyl triads found in GXMs of *Cryptococcus neoformans* (by Cherniak et al., 1998)

triads occurred singularly or in combination with one or more of the other triads (Cherniak et al., 1998). The structure of the galactoxylomannan seemed to be quite complex with an a-(1→6)-galactan backbone being branched at the C-3 position of

alternate d-galactose units. The oligosaccharide side chains are composed of α-D-Man-(1→3)-α-D-Man-(1→4)-β-D-Gal, substituted with zero to three terminal β-D-Xyl residues (Vaishnav et al., 1998). However, (James and Cherniak, 1992) found the main chain to consist of D-mannose or D-galactose or alternating D-mannose and D-galactose residues.

Because of the importance of *Cr. neoformans* as a pathogen, and its capsule being the major virulence factor, elucidation of the biosynthesis pathway of this polysaccharide would open possibilities for treating this infection. For the same reason, *Cr. neoformans* is so far the only yeast species where research has been performed with regard to the regulation and molecular genetics of the capsular polysaccharide.

The major component of the GMX, mannan, is a common component in many yeast polysaccharides, and D-mannose is formed from D-glucose by isomerization (Spencer and Gorin, 1973). The pathway for biosynthesis of nucleotide sugar donors for D-xylose and d-glucuronic acid has been examined by Bar-Peled et al. (2001) and Griffith et al. (2004) and is identical for the non-pathogenic *Cr. laurentii.* UDP-D-glucuronic acid is formed by dehydrogenation of UDP-D-glucose by UDP-glucose dehydrogenase (UGD1). Nearly half of the sugar residues in the capsule are derived from UDP-D-glucuronic acid. UDP-xylose, for example, is synthesised from UDP-D-glucuronic acid by UDP-D-glucuronic acid decarboxylase (UXS1). Deletion of *ugd1* led among others to loss of the capsule. These results suggest that this enzyme represents the major, and maybe only, biosynthetic step for UDP-glucuronic acid in *Cr. neoformans*. Consequently, deletion of UDP-glucose dehydrogenase blocked not only the synthesis of UDP-glucuronic acid but also that of UDP-xylose. *Uxs1* deletion strains produce capsules that lack xylose, rendering them completely avirulent (Moyrand et al., 2002).

Complementation studies with capsule-deficient phenotypes enabled investigators to identify four genes being responsible for capsule synthesis: *cap59*, *cap64*, *cap60* and *cap10* (Chang and Kwon-Chung, 1999). The four genes are not essential for viability, but deletion results in acapsular mutants unable to cause infection. The exact function of the genes remains however unclear.

The assembly of D-mannose into the polysaccharide is catalyzed by an α-1,3 mannosyltransferase (Sommer et al., 2003). The protein was termed CMT1, for cryptococcal mannosyltransferase 1. CMT1 turned out to be homologous to the unidentified gene product from the above mentioned capsule synthesis *cap59* gene. CMT1 also co-purifies with a homolog of another capsule synthesis gene: *cap64*. Moyrand et al. (2004) identified six CAP64 homologous genes and the gene product of one of them, Cas31p, was identified as the CMT1 co-purified protein, suggesting the involvement of a protein complex in capsule biosynthesis. All six CAP64 homologous take part in capsule *O*-acetylation and/or xylose branching, making them good candidates for the source of *Cr. neoformans* serospecificity.

Klutts et al. (2006) characterized a cryptococcal xylosyltransferase (CXT1). The enzyme adds xylose in a β-(1,2) linkage to the reducing mannose, building blocks that are found either in GXM and GalXM. Upon disruption of the *cxt1* gene, two additional xylosyltransferase activities were detected. Also for the *cmt1* gene, no

differences in capsule structure were detected among the deletion and wild type strain (Sommer et al., 2003). Those results suggest the presence of multiple parallel glycosyltransferase systems.

Infection by *Cr. neoformans* begins most likely by inhalation of desiccated cells. Barbosa et al. (2006) suggest that infection of the alveolar cells is mediated by GMX-surface receptors. Contact with serum induces capsule formation and capsule release. Cryptococcal cells, as well as cell envelope components (GXM, galactoxylomannan and mannoprotein) display various immunomodulatory effects on the host response, such as the inhibition of phagocytosis, suppression of T-cell mediated immunity, and induction of immunogenic tolerance. Moreover, these capsular polysaccharides are able to interfere with the migration of phagocytes despite adequate stimulation of chemokine production. Their concerted action accounts for the mild inflammatory response often observed in cryptococcosis cells (Chaka et al., 1997).

A remarkable feature of *Cr. neoformans* is the ability to switch phenotype. A hypovirulent strain, which usually produces smooth colonies, developes wrinkled and pseudohyphal colonies after phenotypic switching. The GXM of the wrinkled colonies differs in the proportion of structural reporter groups (Fries et al., 1999). Variation in the structure of GXM was also found when initial and relapse isolates were studied from patients with recurrent cryptococcal meningitis (Cherniak et al., 1995). Charlier et al. (2005) studied the changes in cryptococcal capsule structure associated with crossing of the blood-brain barrier. The rapid changes in capsule structure could contribute to inability of the host immune response to control cryptococcal infection. The mechanism by which the yeast cells vary the structure of the GXM is not understood.

The capacity to reduce neutrophil influx makes cryptococcal polysaccharides interesting compounds to study in clinical models of inflammation (e.g. sepsis, auto-immune disorders) in which leukocyte influx can be potentially damaging to host tissues (Ellerbroek et al., 2004). Tissi et al. (2004) proved that GXM can act as an anti-inflammatory compound against septic arthritis in mice. They suggest a potential therapeutic use in pathologies involving inflammatory processes. Percollini et al. (2006) investigated the interactions of GalXM with the immune system and concluded that this molecule can be useful in the induction of apoptosis in cancer cells and the suppression of T-cell response in transplant recipients.

29.3.3 Rhodotorula spp.

Rhodotorula rubra (*R. mucilaginosa*) produces linear mannans (Pavlov et al., 1992). The structural features and physicochemical properties of this mannan have been investigated by Elinov et al. (1979). This study indicated that the mannan alternating contains β-1,3- and β-1,4-linkages. The yeast mannan increases serum levels of cholinesterase, α-amylase, aldolase and liver and erythrocytic catalase. Moreover, the mannan inhibits the development of experimental tumors in animals (Elinov et al., 1979). The sulfated form of this polymer (ronasan) has been shown to stimulate antibody formation cells and macrophages (Elinov et al., 1995).

According to Ulyanova et al. (1992), ronasan exhibited a clearly pronounced radioprotective effect.

Simova et al. (2004) cocultured *R. rubra* and a yoghurt starter culture on a medium containing cheese whey, in this way developing a low-cost production process for the exopolysaccharide.

R. rubra is the only *Rhodotorula* species that is frequently isolated from human sources, and that in contrast to the beneficial properties of the extracellular polysaccharides, possibly is one of the only species of *Rhodotorula* that can cause human infections (Fell and Statzell-Tallman, 1998).

Frengova et al. (1997) studied exopolysaccharide production by *R. glutinis* and analyzed the monosaccharide composition. This learned that D-mannose, D-glucose, D-galactose and D-xylose are structural elements of the polysaccharide molecule. Mannose dominated in the biopolymer (57.5%) accounting for the biological activity of the exopolysaccharides produced by *R. glutinis*. According to certain authors, a mannose content of more than 50% in the polysaccharide macromolecule of *Rhodotorula* sp. is a premise for bioactivity (Elinov et al., 1988). *R. glutinis* KCTC 7989 is capable of producing a novel heteropolysaccharide composed of neutral sugars (85%) and uronic acid (15%). The neutral sugar composition was identified as D-mannose, L-fucose, D-glucose, and D-galactose in a 6.7:0.2:0.1:0.1 ratio. So again, the mannose content is higher than 50% (Cho et al., 2001).

The exocellular mannans of *R. glutinis* have been used in serological diagnosis of leptospirosis. This disease is caused by bacteria of the genus *Leptospira*, and the antigens of these pathogens were shown to have a common backbone structure: →3)-β-d-Manp-(1→4)-β-d-Manp-(1→. Due to the similarity of the repeating units, *Rhodotorula* mannan was found to be useful in diagnosis of leptospirosis because it can cross-react to IgG and/or IgM specific for *Leptospira* (Matsuo et al., 2000).

R. minuta has been described to produce galacto-oligosaccharides from lactose and gluco-oligosaccharides from cellobiose (Onishi and Yokozeki, 1996). Oral administration of these oligosaccharides promotes the proliferation of intestinal bifidobacteria.

Other members of the *Rhodotorula* genus, such as *R. aurantiaca*, and *R. acheniorum*, have also been described as producers of extracellular polysaccharides. The exopolysaccharide of *R. acheniorum* can contain up to 92.8% mannan. The mannans are further tested in food applications because of their good water-binding capacity and intrinsic and dynamic viscosity (Pavlova et al., 2005). *R. bacarum* is not only capable to produce mannans, but certain strains can also produce pullulan (see 2.3 *Aureobasidium pullulans*), even at high yields and without melanin synthesis (Chi and Zhao, 2003).

29.3.4 Sporobolomyces spp.

Analogous to the phosphorylated mannans produced by *Pichia holstii*, *Sporobolomyces* spp. Y-6493 and Y-6493 synthesize phosphorylated galactans when grown on d-glucose (Slodki, 1966). These phosphogalactans are composed of α-1,3- and α-1,6-linked galactosyl units in nearly equal amounts.

Studies on exopolysaccharide synthesis by *Sp. albo-rubescens* indicated that the nitrogen source used for growth of the yeast cells determines the composition of the polymer (Elinov et al., 1997; Anan'eva and Vitovskaya, 1998). In a synthetic medium containing ammonium sulfate as the nitrogen source, a β-mannan is synthesized. According to Elinov et al. (1997), a medium with peptone and yeast autolysate as nitrogen sources stimulated the synthesis of a high molecular weight acidic heteroglycan composed of D-mannose, D-glucose and D-glucuronic acid (1:1:2) and trace elements of D-galactose. In a slightly modified medium containing the same nitrogen sources, Ananeva and Vitovskaya (1998) reported the production of two polymer fractions: a heteroglycan consisting of D-mannose, D-glucose and D-glucuronic acid (1:2:2) and a neutral polymer mainly composed of mannose.

Studies on capsule formation by *Sp. albo-rubescens* CBS 482, performed in the authors' laboratory, showed that ammonium plays an important role in both exopolysaccharide production and recovery (De Baets et al., 1998). During fermentation a drastic drop in pH was measured. This pH drop occurred with $(NH_4)_2SO_4$ present in the medium and seemed to be indispensable for production of the polysaccharides. Similar observations were made by Elinov et al. (1992) and Pavlova et al. (2004) upon studying exoglycan biosynthesis by respectively *Sp. holsaticus* and *Sp. salmonicolor*.

Several publications report the immunomodulating action of polysaccharides produced by *Sp. albo-rubescens*. According to Elinov et al. (1995) the polymer is capable of stimulating the functional status of macrophages and antibody forming cells. The function of immunocompetent cells, depressed by cytostatics, can be normalized by injection of preparations of the yeast glucan from *Sp. albo-rubescens*. Ulyanova et al. (1992) found that the heteroglycan synthesized by *Sp. albo-rubescens* exhibited a clearly pronounced radioprotective effect. Related to this characteristic, the exopolysaccharide is also capable of absorbing copper and lead ions (Elinov et al., 1999).

29.4 Analysis

We have illustrated here that a large diversity can be found among yeast exopolysaccharides. Also for the same species, the strain involved, medium composition and fermentation conditions can alter the composition and even induce synthesis of different exopolysaccharides. Despite those variations in sugar composition, the linkage pattern of a specific exopolysaccharide remains unchanged and most of the specific biological activity of the exopolysaccharides is probably due to its linkage pattern and consequent structure. The β-(1,3) linkage in *C. albicans* CAWS, for example, makes the molecule inert to metabolisation in the human body, resulting in accumulation in the blood and activation of the factor G cascade. Another example is pullulan. The presence of an α-(1,6) linkage between the α-(1,4) coupled maltotriose units gives the molecule its unique physical properties.

Study and survey of the extracellular polysaccharides synthesized by yeasts or yeast-like fungi revealed that these polymers certainly have a potential value in fields such as cosmetics and nutrition, but also in medicine and pharmacy. It is thought that especially the large portion of mannan contributes to the biological activity, often associated with yeast extracellular polysaccharides. It also turns out that certain chemically sulfonated exopolysaccharides exhibit a specific antitumour activity.

A general process for the production of biopolymers by aerobic culturing of microorganisms in an aqueous nutrient medium consisting of a water-oil emulsion, was patented by Henkel KGaA (Schindler et al., 1984). Several extrapolymer forming bacteria were successfully grown in this medium, in addition to the following yeast strains: *Rhodotorula* spp., *Pichia* spp., *Pachysolen tannophilus*, *Lipomyces* spp., *Hansenula capsulata*, *H. holstii*, *Cryptococcus* spp., making the commercial production of yeast exopolysaccharides possible.

There are quite a number of opinions regarding the physiological functions of yeast capsules. According to Golubev (1991), different functions can be assigned to yeast capsules: adhesion of cells to solid surfaces to prevent washing off, barrier protecting the cells from radiation or amoebic attack, virulence factor and increased resistance to desiccation. In the author's opinion, the main functions of yeast capsules relate to those environmental factors which determine survival and propagation in quite diverse habitats.

29.5 Future Perspectives

Some yeast exopolysaccharides show unique physico-chemical properties, making them useful for technical applications. However, he only "high volume" yeast exopolysaccharide on the market is pullulan, although several others also expose interesting properties. In order to be able to compete with their chemical counterparts, a sufficient high production and an efficient purification process is required. The growing environmental awareness and government policy has caused recent interest in products based on renewable resources. Yeast exopolysaccharides are fermentation products and consequently belong to this category.

For yeast exopolysaccharides with pharmaceutical or cosmetical applications, high production rates are less important. However, in both cases biosynthesis pathways and genetic regulation is poorly understood (with exception of the pathogenic *Cr. neoformans*), and further study is certainly required in order to improve the production process for polysaccharides with commercial application potential. The number of patents that have been granted show the growing interest in these bio-polymers and reflect that this neglected field of research will certainly expand in the following years.

29.6 Conclusions

Several yeasts and yeast-like fungi are known to produce extracellular polysaccharides. Most of these contain D-mannose, either alone or in combination with other sugars or phosphate. A large chemical and structural variability is found between yeast

species and among different strains. Even for the same strain, differences in medium composition and fermentation conditions can cause variation in sugar composition.

The types of polymers which are synthesized can be chemically characterized as mannans, glucans, phosphomannans, galactomannans, glucomannans and glucuronoxylomannans. Despite these differences, almost all of the yeast exopolysaccharides display some sort of biological activity, either malign or beneficial. The latter ones already find application in chemistry, pharmacy, cosmetics or as probiotic. Furthermore, some yeast exopolysaccharides, such as pullulan, exhibit specific physico-chemical and rheological properties, making them useful in a wide range of technical applications. However, there is still research to be done on biosynthesis, production and recovery in order to further open perspectives for commercialisation of the exopolysaccharides mentioned in this chapter.

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Chapter 30
Industrially Important Carbohydrate Degrading Enzymes from Yeasts: Pectinases, Chitinases, and β-1,3-Glucanases

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Contents

Abstract Polysaccharide degrading enzymes are hydrolytic enzymes, which have a lot of industrial potential and also play a crucial role in carbon recycling. Pectinases, chitinases and glucanases are the three major polysaccharide degrading enzymes found abundantly in nature and these enzymes are mainly produced by fungal strains. Production of these enzymes by yeasts is advantageous over fungi, because the former are easily amenable to genetic manipulations and time required for growth and production is less than that of the latter. Several yeasts belonging to *Saccharomyces, Pichia, Rhodotorula and Cryptococcus* produce extracellular pectinases, glucanases and chitinases. This chapter emphasizes on the biological significance of these enzymes, their production and their industrial applications.

Keywords Yeast, glucans, hydrolytic enzymes, pectinase, chitinase, glucanases

30.1 Introduction

Pectin (polymer of α-D-galacturonic acid), chitin (polymer of N-acetyl D-glucosamine) and glucan (polymer of D-glucose) are three most important groups of polysaccharides available in biosphere. The widely prevalent form of glucan is cellulose,

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which is also the most abundant biopolymer in the biosphere followed by chitin. Pectin and glucans are major plant polymers forming most of its dry weight, whereas chitin is mostly widespread in animal kingdom as it forms the hard and lustrous exoskeleton of arthropods. Pectin forms a matrix in which the cellulose and hemicellulose polysaccharides of the plant cell are embedded. This acts as a cementing agent and is mainly involved in providing structural and mechanical support to the plant parts, maintaining and determining the cell shape, helps to resist the internal turgor pressure of cell, controls the rate and direction of growth, responsible for plant architecture and forms, regulate diffusion of material through the apoplast and also provides protection against pathogens. Pectins account for about 30% of the primary walls of dicotyledonous and non-graminaceous monocotyledonous plants and between 5 to 10% of the walls of grasses. Pectic polysaccharides are mainly of three types; homogalacturonan, rhamnogalacturonan-I, and substituted galacturonans (Rhamnogalacturonan-II) (Ridley et al., 2001). Homogalacturonan is a linear chain of 1, 4-linked α-D-galactouronic acid residues in which some of the carboxyl groups are methyl esterified. Homogalacturonan are again differentiated into protopectin, pectin, pectinic acid and pectic acid depending on the degree of esterification. Protopectin is highly esterified insoluble parent pectic substance (degree of esterification > 90%) and pectin is the soluble pectic substance with esterification in the range of 40–90%. Pectinic acid has an esterification level of 0 to 40% whereas pectic acid is the unesterified form of pectin (Whitaker, 1991). The galacturonic acid residues may also be acetylated with acetic acid at C2 and C3 position (Ishii, 1995, 1997). Pectinases are a complex group of enzymes that act on the pectic substances and cause its breakdown into galacturonates. Pectinases are produced by microorganisms as well as plants. Pectin esterase (PE), polygalacturonases (PG) and pectin lyase (PL) are the three important pectinases that are reported in yeasts where as the presence of pectate lyase (PGL) and polymethylgalacturonase (PMG) in yeasts has not been reported so far. Most of the literature describes the production of pectolytic enzymes by fungal species. More research has to be focused on development of economical production of pectinases by yeast.

The other important plant polysaccharides, glucans, constitute a large group of polymers including cellulose, starch, laminarin, glycogen and dextrans. Cellulose forms the plant body and starch is the major storage form of plant carbohydrate. Glycogen is a store house of instant energy in animals and releases glucose units whenever required by the cell. β-glucans constitute the most abundant class of naturally occurring polysaccharides because of the wide occurrence of the β -1, 4- glucan, cellulose. However, many other β-glucans are produced by both microbial and nonmicrobial (plant) sources. β-glucans are homopolymers of D-glucose linked by β-glycosidic linkage. Some are relatively simple molecules consisting of linear chains of glucose residues where as some are highly branched polymers forming helical polymers of high molecular weight e.g. starch and glycogen. Unlike straight chain polymers, these branched polymers form granules in cell cytoplasm and the most effective way to store cellular glucose. Glucanases are enzymes that break the glycosidic bonds between the glucose monomers of glucans. β-1,3-glucanases

occur in a wide variety of organisms including plants and microbes (Fincher and Stone, 1986; Pitson et al., 1993). These enzymes are glycoproteins and the carbohydrate moiety is primarily made of D-mannose, variable quantities of D-glucose and small amounts of D-glucosamine. The relative percentage of each sugar varies according to the micro-organism producing the enzyme. The linkage established between the polypeptide and carbohydrate moieties has been characterized as belonging to the N-acetylglucosaminoyl-asparagine type (Sanchez et al., 1981). Further details can be seen in reviews by Fincher and Stone (1986) and Pitson et al., (1993).

Likewise, chitin and its close relative chitosan occur as structural polysaccharide standing next to cellulose in abundancy and occur in the exoskeleton of arthropods mostly belonging to class insecta (Merzendorfer and Zimoch, 2003) and also in the cell walls of some plants and fungi including yeasts (Wang and Chang, 1997; Riccardo and Muzzarelli, 1999). In yeasts, the rigid cell wall contains chitin, glucans and other polysaccharides (Park et al., 1997). The amount of chitin in the cell wall of yeast is much lower, but bud scars have been shown to be largely composed of chitin (Kuranda and Robbins, 1991). The outer shell of crustaceans and nematodes also contain this polysaccharide in abundant amount. Chitin is a straight chain polymer of N-acetyl D-glucosamine (GlcNAc), linked to each other by β-1,4-glycosidic linkage (Berkely et al., 1979). The near relative of chitin is chitosan, a polymer of glucosamine units which is also found to coexist with chitin as structural polysaccharide. Approximately 75% of the total weight of shellfish such as shrimp, crab, and krill, is considered waste and 20 to 58% of the dry weight of the said waste is chitin. Chitinases are the enzymes, which degrade chitin and chitosan in the animal waste resulting in the formation of oligosaccharides, which in turn reused by plants as organic compounds (Patil et al., 2000). It is estimated that the worldwide annual recovery of chitin from the processing of marine invertebrates is about 37,300 metric tons. Effective recycling of this chitinous material needs chitinases in large scale. Chitinases are chitin degrading enzymes which occur in a wide range of organisms including viruses, bacteria, fungi, nematodes, insects, fishes as well as higher plants and animals (Jeuniaux, 1966). Apart from its ecological role in recycling the organic wastes, it has significant biological roles. Because chitin resists chemical and physical breakdown, microorganisms must play a major role in its degradation. Surprisingly, almost no free chitin is found in marine sediments which are considered to be the richest deposit of chitin, demonstrating the efficiency of these microbial systems.

Production of these carbohydrate degrading enzymes using genetic and biological techniques is a challenging task. However there are very few reports on the production of these enzymes from yeasts. As the growth of yeasts is faster than fungi, fermentation time can be shortened to a large extent which in turn reduces the cost of production. Yeasts have been well studied for the industrial production of alcohol and other important metabolites. However the production of these enzymes from yeasts has received less attention. Since the genome sequence is available for many yeast species, recombinant production of the protein can be

achieved easily by applying the genetic engineering methods. Further more, the yeast, being a eukaryotic organism can be used to produce all above eukaryotic proteins without any complications of their expression and post-translational modification as observed in prokaryotic organisms. In this chapter we focus mainly on the biological significance and production of these enzymes from yeasts on an industrial scale and their diverse applications.

30.2 Biological Significance of Carbohydrate Degrading Enzymes

As described above, pectinases, chitinases and glucanases are produced by a wide range of organisms. Yeasts are a group of unicellular fungi, bound by rigid cell walls made up of glucans, pectins, and chitin. Therefore, they have developed mechanism to synthesize and secrete an array of enzymes capable of hydrolysing these polysaccharides.

30.2.1 Biological Significance of Pectinases

Pectinases play an important role in fruit ripening, plant pathogenesis and pectin carbon cycle. An important part of fruit walls, pectin is broken down by pectinase to pectinic acid and finally to pectic acid. Fruit ripening involves modification in cell wall resulting in fruit softening, tissue deterioration and pathogen susceptibility. Factors controlling in vivo hydrolysis have not been fully explored but might include apoplastic pH, cell wall inorganic ion levels, non-enzymic proteins including the noncatalytic β-subunit and expansins, wall porosity, and steric hindrances. Recent studies of cell wall metabolism during ripening have demonstrated an orderly process involving, in the early stages, cell wall relaxation and hemicellulose degradation followed, in the later stages, by pectin depolymerization. A limited number of studies have indicated that radical oxygen species generated either enzymatically or non-enzymatically might participate in scission of pectins and other polysaccharides during ripening and other developmental processes (Donald et al., 2001). Pectinases especially PG was also known to play a major role in tissue breakdown during the final stages of fruit ripening. Increase in pectinases activity (PE and PG) and decrease in molecular weight of pectin was observed during ripening of melons and carambola fruits suggesting that pectinases are not directly involved in fruit ripening but are involves in fruit ripening associated pectin disassembly (Chin et al., 1999).

Pectinases from phytopathogenic fungi such as *Aspergillus flavus*, *Fusarium oxysporum* and *Botrytis cinerea* are also known to play a vital role in plant pathogenicity or virulence by degrading the pectic compounds present in cell wall (Cleveland and Cotty, 1991; Di Pietro and Roncero, 1996). Plant pathogens deliver

a variety of virulence factors to host cells to suppress basal defence responses and create suitable environments for their propagation. Plants have in turn evolved disease resistance genes whose products detect the virulence factors as a signal of invasion and activate effective defence responses. Plant pathogens release a set of enzymes like PL, PG, PE, cellulases and proteases, the concerted action of all these enzymes determines the virulence of the pathogen. The products of the action of these enzymes negatively regulate the release of pectinases from plants as well as it helps in producing certain pathogenesis related proteins which affects the pathogens negatively. A lot of genetic studies support that PG serve as a virulence factor; however there exists controversy in explaining the role of PG in fungal virulence and infection process (Lang and Dorenberg, 2000). Pectinases are also known to trigger the defensive genes in plants. For example, PG was expressed in tomato leaves in response to wounding (Bergey et al., 1999).

Microorganisms in soil degrade pectic substances present in plant waste by secreting pectinases in an extra cellular fashion thus make the pectin rich organic materials to be recycled in the biosphere. PE and PGs act by hydrolysis mechanism to form pectic acid and saturated oligogalacturonates. Pectin lyase degrades pectic substance by transelimination mechanism yielding 4,5-unsaturated oligogalacturonates. The initial steps in degradation of pectic substances are catalyzed by pectinesterases, polygalacturonases, and pectic lyases, which result in the formation of saturated and unsaturated galacturonan. These glacturonans are further catabolized to 5-keto-4-deoxy-uronate and finally to pyruvate and 3-phosphoglyceraldehyde (Vincent-Sealy et al., 1999). The schematic representation of action of PE, PG, and PL is represented below (Fig. 30.1A). The pyruvate and 3-phosphoglyceraldehyde are metabolized by the normal process of glycolysis and citric acid cycle to form carbon dioxide and water.

30.2.2 Biological Significance of Chitinases

Chitinases are biologically potential hydrolytic enzymes which perform a multitude of physiological functions in different groups of organisms starting from unicellular prokaryotes to most complex eukaryotes like plants and animals. In bacteria, chitinase are involved in mineralization of chitinous wastes in soil and helps in nutrition and parasitism (Flach et al., 1992; Connell et al., 1998). In fungi, chitinases seem to play a role in cell division and differentiation (Kuranda and Robbins, 1991; Gooday et al., 1992; Mellor et al., 1994). It also plays a nutritional role related to mycoparasitic activity displayed by several species of fungi related to genus *Trichoderma.* A number of *Trichoderma* isolates are able to excrete hydrolytic enzymes such as chitinases, proteases and β-glucanases in to the medium when grown in the presence of laminarin, chitin or the cell walls of phytopathogenic fungi (Lorito et al., 1993). Malarial parasites produce sufficient quantities of chitinase to penetrate the chitin containing peritrophic matrix of the mosquito midgut (Langer et al., 2002). In plants, these enzymes provide resistance against fungal pathogens because of their inducible nature and antifungal activities in vitro. Plant chitinases degrade the chitin of isolated cell walls from fungi and some also degrade bacterial cell walls due to their lysozymal activity (Carstens

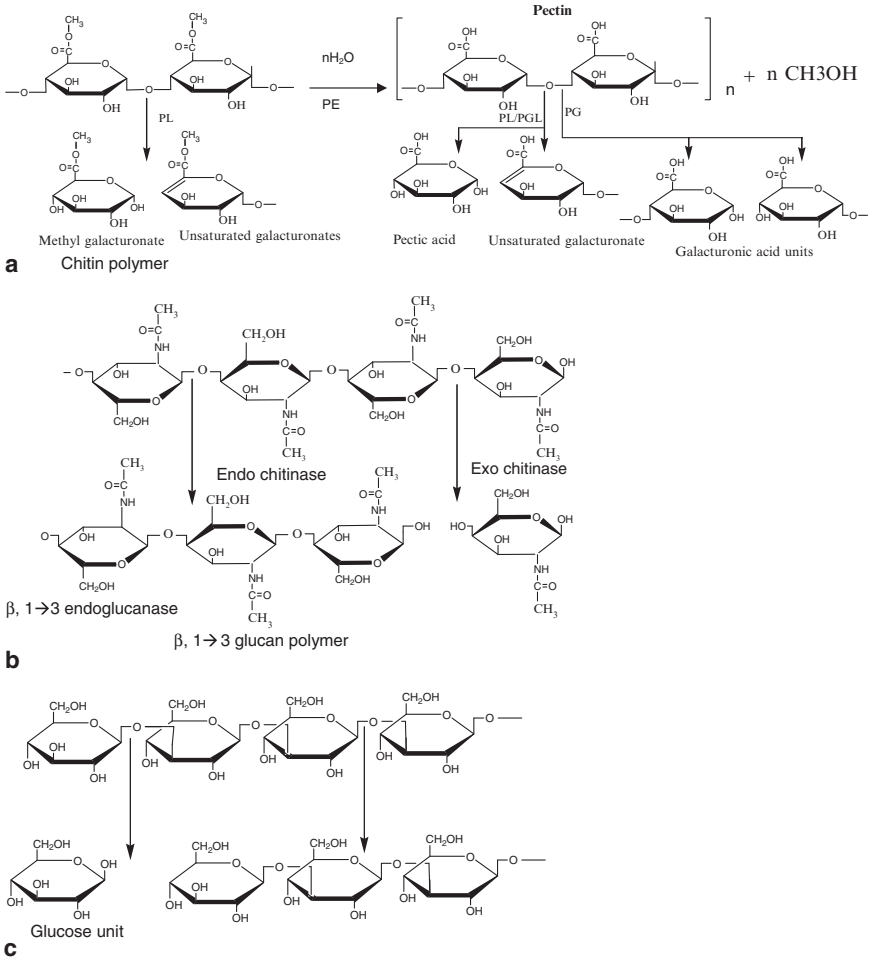

Fig. 30.1 Mode of action of three different carbohydrate degrading enzymes from yeast. All three enzymes are hydrolases that carryout the hydrolysis of glycosidic linkages between the monomers. (**a**) The schematic representation of action of pectinases i.e. PE (pectin esterases), PG (polygalacturonase), PL (pectin\pectate lyase) and PGL (polygalacturonic acid lyase) is represented. PE acts by hydrolysis mechanism and breaks the pectin into methanol and pectic acid. PL and PGL act by transelimination mechanism yielding saturated and unsaturated oligogalacturonates where as PG acts by hydrolysis mechanism and the products of hydrolysis are saturated oligogalacturonates. (**b**) Homopolymer chitin is acted upon by chitinases to hydrolyze the β-1,4-linkages. Exochitinase releases N-acetyl glucosamine units from the end i.e. hydrolyses only the most outer glycosidic linkage on the polymer, where as endochitinase acts on more internal sites to degrade it into short chain chitin polymers named chitooligomers. These chito oligomers are further acted upon by endo or exo chitinases till the monomers are released for further metabolism (C) β-1,3-glucanase acts on the most abundant form of glucans named β-1,3-glucan to hydrolyze the β-1, 3-glycosidic linkage. Exo-β-1,3-glucanase releases glucose units from the end of the polymer by acting upon the outermost β glycosidic linkage, where as Endo-β-1,3-glucanase are more non-specific and releases oligomers of various size by acting on various internal bonds. These gluco oligomers are further acted upon by endo or exo glucanase till the free glucose molecules are released for further metabolism

et al., 2003; Itoh et al., 2003; Schlumbaum et al., 1986). In plants, chitinases are known as pathogen related proteins (PRPs) because, chitinases as well as β-1,3-glucanases are induced in them by pathogens (Abeles et al., 1970; Wyatt et al., 1991). They also inhibit the mycelial growth of many pathogenic fungi in vitro. These observations together with the fact that chitin and glucans are the main structural component of fungal cell wall suggest that the plants producing these enzymes effectively control the harmful pathogens. Recently, many transgenic plants such as broccoli, cucumber, rice, potato and wheat have been produced by incorporating the chitin degrading genes from other plants; those provide resistance to these plants against many bacterial as well as fungal pathogens (Lorito et al., 1998; Tabei et al., 1998; Mora and Earle, 2000; Datta et al., 2001). This area of research is particularly interesting to produce many disease resistant plant species and thus is an important step towards the global food problem. Specific isoforms have also been reported to play a role in embryo development (Kim and Chung, 2002), pollination and sexual reproduction (Leung, 1992). During ecdysis in insects and crustaceans, chitinases are associated with the partial degradation of old cuticle, a chitinous coat, providing protection to the organism but slowing down the growth rate. Thus the chitinolytic enzymes help the growth of insect larvae by degrading the hard chitinous cuticle, there by making the larva to grow into an imago (adult stage of insect) (Kramer and Fukamiso, 1985; Merzendorfer and Zimoch, 2003). The chitinase gene is shown to be conserved in rodents and primates (Gianfrancesco and Musumeci, 2004). In vertebrates, these enzymes are a part of digestive tract helping the digestion of chitinous substances taken by them. In human, chitotriosidase activity helps in defence against nematode infections (Choi et al., 2001). Moreover, its enzymatic activity is markedly elevated in serum of patients suffering from lysosomal lipid storage disorders, sarcoidosis and thalassemia (Aguilera et al., 2003).

Chitinase level is shown to be high in goat and bovine blood serum. This might be a function of slow renal secretion which keeps the enzyme level comparatively low in case of abnormal lysozyme production (monocyticmyelomonocytic leukemias and renal diseases) (Lundblad et al., 1974).

In yeasts, cell walls are dynamic structures which constantly change during cell division, growth and morphogenesis. These dynamic changes are brought about by a group of polymer degrading enzymes which are associated with the cell wall through out the lifecycle of the organism and categorized into the cell wall synthesizing and cell wall hydrolyzing enzymes. The cell wall hydrolyzing enzymes in yeasts, characterized to date have chitinase or glucanase activity which also exhibit transglycosylase activity (David, 2004). They may therefore contribute to breakage and re-forming of bonds within and between polymers, leading to re-modeling of the cell wall during growth and morphogenesis of yeasts (Kuranda and Robbins, 1991). This enzyme plays a major role in separating the daughter cells from the parent cells by degrading the chitin ring deposited at the bud site. This was found to be an endochitinase with an approximate molecular weight 130 kDa and glycosylated extensively. Mutations involving a disruption of the open reading frame of the gene (CST1) synthesizing this protein leads to a defect in bud cell separation and later it was supported by another experiment involving the inhibition of the protein by demethylallosamidin. Chitinases catalyze hydrolysis between two monomers of chitin chain. Depending upon the site

of their action, chitinases can be classified into two major categories: endochitinases (EC 3.2.1.14), which bring about random hydrolysis of *N*-acetyl-b-D-glucosaminide1, 4-β-linkages in chitin and chitodextrins and exochitinase (EC 3.2.1.52), which catalyse the successive cleavage of external bonds (Fig. 30.1B) (Fischer and Stein, 1960; Cabezas, 1989). Exochitinases can be divided into two subcategories: chitobiosidases (EC 3.2.1.29), which catalyze the progressive release of diacetylchitobiose starting at the nonreducing end of chitin microfibril, and β-(1, 4)-N-acetyl glucosaminidases (EC 3.2.1.30), which cleave the oligomeric products of endochitinases and chitobiosidases, generating monomers of GlcNAc (Vyas and Deshpande, 1989; Sahai and Manocha, 1993).

30.2.3 Biological Significance of β-1,3-glucanases

The biological significance of β-1,3-glucanase is varied. This enzyme plays a nutritional role as it can bring about the conversion of β-1,3 linked complex carbohydrate polymers to simpler glucose molecules that can be assimilated by the microorganism for growth and metabolism. Apart from that many fungi, particularly yeasts have β-1,3-glucan as a structural component of the cell wall. Glucan degrading enzymes in them come into play at points in the life cycle of yeast which involve glucan metabolism. The ability of these enzymes to bring about breakage and rearrangement of bonds between the linked molecules helps in structurally modifying the cell wall in various life processes in yeast (Fleet and Phaff, 1981). β-1,3-glucanases have been implicated in many metabolic processes such as apical growth, branching, bud formation, mating and formation and release of ascospores (Cortat et al., 1972; Crandall et al., 1977; Rey et al., 1980; Larriba et al., 1995).

β-1,3-glucanases hydrolyze the *O*-glycosidic linkages of β-1,3 linked glucans (Fig. 30.1C) and are classified according to their mode of action into two specific types (Kulminskya et al., 2001): (i) The exo-β-1,3-glucanases (EC 3.2.1.58) which sequentially release glucose residues from the non reducing terminus of a substrate; and (ii) The endo-β-1,3-glucanases (EC 3.2.1.39) that are capable of cleaving internal β-1,3-linkages at random sites along the polysaccharide chain, releasing short oligosaccharides. Both the enzymes are known to act synergistically, bringing about the breakdown of glucan polymer (Johnson, 1968).

30.3 Production of Carbohydrate Degrading Enzymes from Yeasts

Enzyme technology is an interdisciplinary field and enzymes are routinely used in many environmental-friendly industrial sectors. With the advancement in biotechnology especially in the area of genetics, protein engineering, developments in bioinformatics, and the availability of sequence data have opened a new era of enzyme

applications in many industrial processes. Carbohydrate degrading enzymes have attracted the attention of many scientists working in this field as invaluable components of food, medicine and biocontrol industries. These carbohydrate degrading enzymes have been produced naturally from microorganisms in controlled conditions as well as using the recombinant DNA technology.

30.3.1 Production of Yeast Pectinases

The pectinase production in yeasts has received less attention and a few yeast species show this ability. Luh and Phaff were the first to report pectic enzymes in *Saccharomyces fragilis* (Luh and Phaff, 1951) in the year 1951 later in the year 1954 they reported the properties of the pectic enzymes produced by *Saccharomyces fragilis* (Luh and Phaff, 1954). Roelofsen reported certain yeasts belonging to the genera *Candida, Pichia, Saccharomyces* and *Zygosaccharomyces* have the ability to attack cell wall pectin, suggesting that the yeasts produced polygalacturonases (Roelofsen, 1953). Pectinases have also been reported in *Kluyveromyces marxianus*, *K. fragilis* (Schwan et al., 1997), *K. lactis* (Sakai et al., 1984) and *Geotrichum lactis* (Murad and Foda, 1992), which produces an endo-PG assumed to be important in the breakdown of the pectinaceous layer surrounding coffee and cocoa beans during processing (Pardo et al., 1991).

It has been found that production of pectolytic enzymes in yeasts is a constitutive property and does not require pectin and polygalacturonic acid to induce the synthesis of these enzymes, whereas certain species like *C. albidus* (Federici, 1985), *G. lactis* (Pardo et al., 1991) and *K. fragilis* (Garcia-Garibay et al., 1987) are inducible. It has been reported in *K. marxianus* and *S. cerevisiae* that presence of inducer (pectin) does not influence the production of pectolytic enzymes (Schwan and Rose, 1994; Blanco et al., 1994). Production of PL and PGL has been found to be partially constitutive in the yeast strain *Debaryomyces nepalensis* (Gummadi and Kumar, 2006a). It has been reported that *D. nepalensis* produces more PL and PGL in media containing carbon source and inducer than in medium containing only carbon source. Similar reports have also been obtained for production of polygalacturonase by *S. fragilis*, where the PG activity was found to be higher in media containing both carbon source and inducer (2% glucose + 1.8% pectin) than when the cells are grown in media containing only 1% glucose (Lim et al., 1980).

It has also been reported in literature that the production of pectic enzymes from yeasts are subjected to catabolite repression. It has been reported in *S. cerevisiae* (Blanco et al., 1994; Blanco et al., 1997) and *C. albidus* (Federici, 1985) that production of pectinase enzymes was repressed by presence of carbon source. The production of PG was found to be repressed in *K. marxianus* when the glucose concentration in the medium was around 10% (w/v) (Schwan and Rose, 1994). Addition of sucrose also had the same repressing effect on production of PG but the addition of other carbon sources, such as galactose or fructose, resulted in reduction

of PG production. By contrast in *D. nepalensis* and *S. cerevisiae* strain 1389, galactose was found to be the best carbon source for pectic lyases and PG production respectively (Gummadi and Kumar, 2006b; Blanco et al., 1997). Dissolved oxygen also plays an important role in production of pectic enzymes from yeasts. The pectolytic activities in strains like *K. marxianus* (Barnby et al., 1990), *K. fragilis* (Garcia-Garibay et al., 1987) and *K. lactis* (Murad and Foda, 1992) were detected when they are grown without shaking and also in anaerobic conditions and the activity was abolished at high aeration rates. It has also reported that decrease in dissolved oxygen concentration in culture medium increases the rate of pectin decomposition by *Saccharomycopsis fibuligera* (Fellows and Worgan, 1984).

The influence of other parameters such as the pH of the medium, inoculum size, incubation time or the addition of nitrogen sources, metal ions has received less attention. It has been reported that highest pectolytic activities were obtained in media containing yeast extract by *Debaryomyces nepalensis* (Gummadi and Kumar, 2006b). Similarly Murad and Foda (1992) found that the highest value of enzymatic activity when media contained yeast extract or peptone. The effect of medium components and various operating parameters such as pH, agitation and aeration has also been studied for maximum production of pectin lyase (PL) and pectate lyase (PGL) by *D. nepalensis* (Gummadi and Kumar, 2006d, 2007). The optimal levels of pH, aeration and agitation rate was found to be 7.0, 300 rpm and 1 vvm, respectively. Combined feeding of inducer and carbon source at 12 h was found to be the best strategy for enhanced production of PL and PGL. Schwan et al. (1997) isolated 12 yeast strains from cocoa fermentations viz., *K. marxianus, S. cerevisiae var. chevalieri, C. rugopelliculosa,* and *K. thermotolerans* produced extracellular, constitutive PG but no PME, PL, or PGL. Of these *K. marxianus* produced the maximum amount of PG and the optimum pH for production was 5.0. It is believed that *K. marxianus* is the key yeast involved in the hydrolysis of cocoa pulp pectin in initial cocoa fermentations. PG secreted by *K. marxianus* CCT 3172 had a strong activity in reducing the viscosity of cocoa pulp. This strain was found during the first 36 h of cocoa fermentation when the pulp is degraded.

Attempts were also made to increase the activity and production of pectolytic enzymes by using statistical optimization techniques. These optimization techniques are advantageous to the conventional one variable at a time approach as it takes into account the interaction effects between the different factors under study. The authors have reported the optimization of medium components and fermentation variables by using Plackett-Burman design and CCD. The optimum activities of PL and PGL were found to be 10.7 U ml^{-1} and 8.7 U ml^{-1} after optimization which shows a 2.5 and 2.9 fold increase in PL and PGL production respectively (Gummadi and Kumar, 2006c). To further investigate the production of PL and PGL, microbiological parameters (plate age, inoculum age and amount of inoculum) were optimized to develop an effective seed culture for maximizing production and found the activities increased to 12.7 and 10.5 U ml^{-1} of PL and PGL respectively (Gummadi and Kumar, 2006c). Recently, it has been shown that the pectinase producing yeast *Debaryomyces nepalensis* (NCYC 3413) is halotolerant and is capable of producing pectinase under high salt conditions. The effect of different

salt such as NaCl, KCl and LiCl on its growth and production of pectinase has been studied (Gummadi et al., 2007). This strain was shown to produce pectinase at all high salt concentrations, namely, 2 M NaCl, 2 M KCl, and 0.5 M LiCl, and the maximum specific activity was observed when the strain was grown in 2 M NaCl. This study is of utmost importance as this enzyme produced is functional at higher salt concentrations and can be utilized for the degradation of pectin wastes where, most of the other pectinolytic enzymes is denatured and non functional (Gummadi and Kumar, 2006c).

30.3.2 Production of Yeast Chitinases

Like the production of pectinases, production of chitinase on industrial basis has been an exciting area in bioprocess engineering due to the multitude of roles played by the enzyme in food, medicine and pest control industries. Microbial chitinase has been produced by liquid batch fermentation, continuous fermentation and fed-batch fermentation. In addition to these, solid-state fermentation and biphasic cell systems are some emerging techniques for the production of chitinases on industrial scale. Generally, chitinase produced from microorganisms is inducible in nature. Extra cellular chitinase production is reported to be influenced by media components such as carbon sources, nitrogen sources, and agricultural residues such as rice bran, wheat bran, etc. (Bhushan, 1998; Dahiya et al., 2005). An enhancing effect of glucose on chitinase production was reported by Bhushan (1998) when glucose was used with chitin in production medium. However, a suppressing effect of glucose on chitinase production was reported by Miyashita et al. (1991). Report for the production of chitinase from yeast species in industrial basis is scant. Recombinant DNA technology is now being applied in yeasts to increase the production of the enzyme.

Microbial production of chitinase by using recombinant DNA technology has been exciting and is worth mentioning in details. The chitinase gene has been cloned and characterized from many species of micro organisms. Most of the cloning and over expression experiment have been performed in bacteria. In 1998, Sondheim and coworkers cloned two chitinase genes from *Serratia marcescens* and successfully expressed in *Pseudomonas* sp. (Sondheim et al., 1998). Robbins et al. cloned and expressed a chitinase encoding gene from *Streptomyces plicatus* and expressed it in high level in *E. coli* and *Streptomyces lividans* (Robbins et al., 1992). Sowka et al. identified and cloned a 32 kDa endochitinase from Avocado, the fruit of the tropical tree *Persea Americana* and expressed it in yeast *Pichia pastoris* (Sowka et al., 1998). The *Candida albicans* genome contains three chitinase genes, *CHT1*, *CHT2*, and *CHT3*, which are homologous to the *Saccharomyces cerevisiae CTS1* gene and *C. albicans CHT4*, which is homologous to *S. cerevisiae CTS2* (Dunkler et al., 2005). Recently a nucleus encoded chitinase (KlCts1p) has been characterized from yeast *Kluyveromyces lactis* (Colussi et al., 2005). This species is an industrially important yeast species due to its ability to grow to a high cell density and abundantly

secrete heterologous protein (Rocha et al., 1996). Non catalytic chitin binding domains (ChBD) are used to affinity purify the chitinase. The ability of a ChBD to selectively bind chitin has led to the use of ChBDs as affinity tags to facilitate attachment of recombinant proteins to chitin coated surfaces. KlCts1p is a nucleus-encoded endochitinase containing a family 18 catalytic domain and a type 2 ChBD. Additionally, *K. lactis cts1* cells produce no detectable chitinase activity, secrete no chitin-binding proteins and are still able grow to a high density. This makes the strain background suitable for production of recombinant ChBD-tagged proteins. *Saccharomyces cerevisiae* conditional mutants, which fail to generate an osmotically stable cell wall has been shown to release expressed proteins in batch and continuous culture. Hence the production of chitinase can be enhanced by applying these recently developing techniques (de la Fuente et al., 1993).

30.3.3 Production of β-1,3-Glucanase from Yeasts

The production of β-glucan-degrading enzymes is a characteristic attributable to a wide variety of organisms, although the fungi are the most common producers of this enzyme. The production of β-1,3-glucanases by many species of yeast is well established. β-1,3-glucanases are produced by microorganisms such as bacteria and yeasts as a part of extracellular complex of enzymes that lyse yeast cell wall (Obata et al., 1977). In yeasts β-1,3-glucanases are produced as an antagonistic effect against other yeasts. These enzymes appear to have antifungal activity (Lorito et al., 1993, 1994). In *Saccharomyces cerevisiae* the production of exo-β-1,3-glucanases is growth-associated and cell-cycle regulated, suggesting that their activities are required at specific stages during morphogenesis (Rey et al., 1980). In recent times, keeping this property in mind, these lytic enzymes have been purified from a variety of yeasts and the have been harnessed for various industrial application.

Glucanases have been described in many yeasts of both ascomycetous and basidiomycetous origin (Brock, 1965; Notario et al., 1976; Villa et al., 1978). The yeast species widely used for the production of β-1,3-glucanases is *Saccharomyces cerevisiae* (Farkas et al., 1973). Most strains of *Saccharomyces cerevisiae* produce a cell wall bound endo-β-1,3-glucanase (Mrsa et al., 1993) and extracellular exo-β-1,3-glucanase (Santos et al., 1979; Nebrada et al., 1986; Kuranda and Robbins, 1987), which is localized in the periplasmic space and is reported to be secreted to the extracellular medium. Rey et al. (1982) have reported the presence of two different β-1,3-glucanases in *Saccharomyces*, one during the vegetative cycle and another during the sporulation cycle. It has also been observed in batch and continuous culture studies that there is a strong correlation between synthesis of β-exoglucan activity and specific growth rate. This indicates that β-1,3-glucanase play an important role during the budding cycle of yeast (Olivero et al., 1985). *Saccharomyces* β-1,3-glucanase system is considered to be very complex and as many as six different types of β-1,3-glucanases have been reported which are different in terms of properties and substrate specificities (Hein and Fleet, 1983). Apart from *Saccharomyces*,

the yeast genera *Pichia* and *Candida* has also been implicated in many cases as major producers of β-1,3-glucanase. Other yeast species such as *Fabospora fragilis* and *Hansenula anomala* have also been reported in the production of β-1,3-glucanases (Abd-el-al and Phaff, 1968). However studies on the production of β-1,3-glucanase in fermenters and reports on optimization of parameters for production of this enzyme by yeasts is lacking.

Recombinant DNA technology has been variously used for the production of β-1,3-glucanases. Since yeast strains like *Candida* which produce this enzyme raise the issue of being pathogenic, the safer technique would be to isolate the gene coding for this protein from yeast, insert the gene fragment in a common bacterial vector and over express the enzyme in bacterial strains like *E. coli* which are considered safe. Recombinant DNA technology also offers a scope for genetically modifying the enzyme to suit specific needs. The genes coding for β-1,3-glucanase in *Saccharomyces* are designated as *EXG1, EXG2* and *SSG* whereas the similar gene in *Candida* is designated as *XOG1* (Larriba et al., 1995) and these genes have been used for cloning and overexpression in bacterial systems. β-1,3-glucanase genes from *Pichia anomala* (Grevesse et al., 2003) and *Pichia pastoris* (Xu et al., 2006) have been also identified and over expressed.

30.4 Applications of Carbohydrate Degrading Enzymes

The biological significance of pectinases, chitinases and glucanases has already been described in section 2.1, 2.2 and 2.3 respectively. In this section, we briefly describe the application of these enzymes in various food, medicine and bioprocess industries.

Microbial pectinases are of commercial interest as they are used in many industrial applications ranging from food to textile processing. Yeast strains like *S. cerevisiae, K. marxianus* and *G. lactis* are able to produce only one type of pectolytic enzyme and do not produce PE (Fernandez-Gonzalez et al., 2004). PE is undesirable mainly in wine fermentation where there will be chances of release of methanol from pectins. Yeasts have been found to produce mainly one type of enzyme hence they can be used with other specific pectolytic enzymes from fungal origin in standardized quantities for many industrial applications. These applications include extraction and clarification of fruit juices, cotton scouring (Hoondal et al., 2002), degumming of fibers, and improvement of the quality of fibers (Beg et al., 2001). In fruit juices, pectic substances remain as suspended insoluble particles affecting the clarity of the extracted juice by increasing the turbidity. These pectic substances need to be removed in order to remove turbidity and prevent cloud-forming spoilage. Until now, the pectic enzymes produced by the mould *Aspergillus niger* (GRAS certified organism). Commercial preparations of pectic enzymes of fungal origin contain a complex mixture of enzymes including pectinases, cellulases and amylases. Gainvors et al. (1994) reported the enzymes PG, PE, PL from *S. cerevisiae* (SCPP 2180) had the same effect on the turbidity as the same quantity of the commercial

preparation endozyme (Pascal Biotech SARL-Paris). It has been reported that when wine fermentations are carried out using PG producing strains of *S. cerevisiae*, the clarification process is greatly facilitated thus reducing the filtration time by almost 50% (Blanco et al., 1997). Pectic enzymes from *S. cerevisiae* when subjected to apple juice clarification showed a decrease in viscosity by 90% but have little effect on cloud strength (Hassan et al., 1995). Blanco et al. (1997) reported grape juice degradation by PG produced from *S. cerevisiae.* The viscosity of the grape juice was not appreciably reduced but had an effect on the filtration time there by causing a reduction of 50% time on filtration. Most of the literatures describe the production of pectolytic enzymes by fungal species. Extensive investigation has to be carried out on development of economical production of pectinases by yeast.

Chitinases also have multiple potential applications in food industries, pharmaceuticals, and antibiotics. Chitin and its derivatives are of interest because they have various biological activities, such as those of an immunoadjuvant and a flocculant of wastewater sludge, and agrochemical uses. Chitinases are the enzymes involved in the processing of chitin and its derivatives. These enzymes have become immensely important tools in basic scientific research. They are used as molecular tracers to detect the presence and location of chitin and chitosan, the most abundant biopolymers of fungal cell walls by cytochemical techniques (Grenier et al., 1991). By conjugating gold particles to chitinase and chitosanase, various workers have been able to detect the chitin, GlcNAc residues and chitosan in the secondary cell walls of plants and in pathogenic fungi (Benhamou and Asselin, 1989). Chitinases have been used along with other enzymes for the production of fungal protoplast (Mizuno et al., 1997). This enzyme has also been used to detect the presence fungus in soil sample (Miller et al., 1998). This raises the possibility of detection of infectious fungal strains in human. Apart from the physiological functions and application in basic research, chitinases have incredible potential in biotechnological and bioprocess engineering industries. A wide range of industrially important products have been synthesized and still a large number wait to be produced industrially. There are several reports describing the use of this enzyme to produce single cell proteins from chitinous waste. In Cody et al. (1990) suggested the conversion of chitin to ethanol using this enzyme. Chitooligosaccharides, glucosamine, and GlcNAc are the industrially important products those are produced from chitin by the use of chitinase (Cody et al., 1990). Chitooligosaccharides are potentially useful in human as medicines. For example, chitohexoses and chitoheptaoses showed antitumor activity (Pope and Davis, 1979). This enzyme has potential applications in antifungal drugs and creams for tropical application. Mosquito control is also another important application of chitinolytic enzymes worth mentioning. *Aedes aegypti*, a vector of yellow fever and dengue, has been shown to be killed by this enzyme (Ferron, 1985; Mendonsa et al., 1996). As the chitinase produced by one fungal species is inhibitory to another species, the design of antifungal drugs is one of the most important targets of the future research particularly; chitinases are used in agriculture to control plant pathogens (Wang et al., 2002).

β-1,3-glucanase is another important lytic enzyme and has many applications industrially. One of the most important application of β-1,3-glucanase is its use as

a biocontrol agent against plant pathogenic yeasts. Since it can depolymerize and hydrolyze yeast cell wall that is made up of glucans (Castoria et al., 1997). This enzyme has been a tool for basic scientific research. It has been used for the elucidation of structure of fungal cell wall and structure of β-1,3-glucan. It used for determining taxonomic and evolutionary relationship among yeasts. Another important application of this enzyme is its use for controlled yeast lysis, which resulted in the selective release of cloned intracellular protein particles from recombinant yeast strains (Asenjo et al., 1993). This method of yeast lysis is superior to mechanical homogenization, which are nonspecific and result in release of many other cellular components that hamper purification of target protein. Use of β-1,3-glucanase for lysing yeast cell wall preserves the structure and activity of the protein unlike mechanical disruption which may result in partial destruction of the product (Salazar et al., 1999). This makes β-1,3-glucanase a useful tool in industrial application for extraction of recombinant proteins from yeasts. This enzyme also finds application in the brewing industry (Manners and Wilson, 1974) and in the preparation of protoplasts (Sietsma et al., 1968). Although glucanases with little or no cellulase activity are not suitable for efficient hydrolysis they have been shown to be suitable for other applications like the biopolishing of cotton fabrics (Klahorst et al., 1994), the improvement of drainabilty and the beatability of pulp (Stork et al., 1995; Kamaya, 1996). β-1,3-glucanase can be used for deinking of paper (Guebtiz et al., 1998), enhancing the efficiency of laundry detergent (Kang and Rhee, 1995) and the efficient utilization of plant materials in animal feed (Campbell and Bedford, 1992). Apart from that, yeast β-1, 3-glucanases also find application in molecular biology. Exo β-1, 3-glucanase was used as an efficient and readily detectable reporter gene system for identification of promoter regions in *Saccharomyces cerevisiae* (Cid et al., 1994). Bioprocess development for the production of β-1,3-glucanase therefore seems to be essential from an industrial point of view.

30.5 Conclusions and Future Perspectives

Keeping in view the multitude of applications in various fields, research on the above three important carbohydrate degrading enzymes will continue to go on for producing them in good quantities on industrial scale. Yeast cells are considered as a better expression system for eukaryotic proteins. Although research is going on to produce pectinases, chitinases and glucanases in various yeast species, it needs to go at a much faster rate, keeping pace with the ever increasing requirement of the enzymes. Several aspects of the carbohydrate degrading enzymes from yeasts have stimulated research in the study of biochemical, regulatory, and molecular aspects of these enzyme systems. Researchers are now directing their studies toward the discovery and engineering of novel enzymes that are more robust with respect to their kinetics and the identification of active site residues. In the future, protein engineering may offer the possibility of generating novel forms of

pectinases, chitinases and glucanases with entirely new functions. Molecular methods should be developed to study the production of chitinases, glucanases and pectinases without the isolation of yeast species in pure cultures. An alternative approach has been the construction of genomic libraries to retrieve genes from natural bacterial communities without cultivation (Schmidt et al., 1991). This method should also be developed for the synthesis of recombinant chitinases in yeasts, as it represents the simplest model system for the over expression of eukaryotic genes and proteins with the mechanisms of post translational modification(s). Advanced tools of bioinformatics can be applied to search for homologous sequences in diverse range of organisms and best strain producing these enzymes should be found out and the quality should be improved to equip this strain for producing these enzymes in a wide range of controlling factors like temperature, pH and aeration. Process optimization for the production of these enzymes should be carried out to improve the production rate. Development of several products of pectin, glucan, and chitin depend upon the improved production of their degrading enzymes.

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Chapter 31
Yeast Acid Phosphatases and Phytases: Production, Characterization and Commercial Prospects

Parvinder Kaur and T. Satyanarayana

Contents

Abstract The element phosphorus is critical to all life forms as it forms the basic component of nucleic acids and ATP and has a number of indispensable biochemical roles. Unlike C or N, the biogeochemical cycling of phosphorus is very slow, and thus making it the growth-limiting element in most soils and aquatic systems. Phosphohydrolases (e.g. acid phosphatases and phytases) are enzymes that break the C-O-P ester bonds and provide available inorganic phosphorus from various inassimilable organic forms of phosphorus like phytates. These enzymes are of significant value in effectively combating phosphorus pollution. Although phytases and acid phosphatases are produced by various plants, animals and microorganisms, microbial sources are more promising for the production on a commercial scale. Yeasts being the simplest eukaryotes are ideal candidates for phytase and phosphatase research due to their mostly non-pathogenic and GRAS status. They have not, however, been utilized to their full potential. This chapter focuses attention on the present state of knowledge on the production, characterization and potential commercial prospects of yeast phytases and acid phosphatases.

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31.1 Introduction

Phosphorus (P) is a naturally occurring element with a number of indispensable biochemical roles. It is a vital component of the genetic material (DNA or RNA) found in all cells, involved in energy transfer reactions in the form of ATP and ADP, and it is also present in phospholipids. Though P is absent from amino acids, the building blocks of proteins and from carbohydrates, it is essential for their synthesis as ATP, the biospheric currency of metabolism. Similarly, it plays an important role in photosynthesis, respiration, redox reactions, signaling, carbohydrate metabolism and nitrogen fixation. Thus no life is possible without P (Deevey, 1970). However, P lacks a rapid global cycle like that of carbon or nitrogen and thus in nature, mobilization of P is slow. Furthermore, the low solubility of phosphates and their rapid transformation to insoluble forms make the element a growth-limiting nutrient, particularly in aquatic ecosystems. Around 20–85% of the total phosphorous in all agricultural soils is present in organic form, which includes a range of inositol phosphate esters, phospholipids, nucleic acids, phosphate linked to sugars, derivatives of phosphoric acid as well as phytates. Phosphorus tends to be the growth-limiting element in most soils and aquatic systems. Throughout the globe, 70% of the cultivable land has either acid or alkaline soils (Lopez-Bucio et al., 2000), and in these soils, P tends to form compounds that are not readily available for plants (Marschner, 1995). As P can form a component of organic compounds and can interact with a number of divalent and trivalent cations in the soil, it becomes the least readily available nutrient in the rhizosphere (Raghothama, 2000). Though P is abundant in the soil, it is not in a readily available form for uptake by plants. To compensate for the low availability of P in agricultural soils, fertilizers are often used and the excess P is washed off to water bodies. In rivers, streams and lakes, phosphorus can cause problems by stimulating excess plant and algal growth leading to deterioration in the quality of the water (Bali and Satyanarayana, 2001; Kaur et al., 2003). There are other problems associated with the use of P fertilizers: firstly they are prepared from rapidly depleting non-renewable resources and secondly, a considerable proportion of the applied P fertilizer gets lost due to the interactions in soil (Vance, 2001).

Plants can use P from the organic sources only after hydrolysis of the C-O-P ester bond by phosphatases, resulting in the release of P as inorganic phosphate. Therefore, phosphatases are important for P nutrition of plants especially when there is a shortage of available inorganic P in soil. Phosphatase activity in the rhizosphere or soil solution may originate from plant roots (Tarafdar and Jungk, 1987), from mycorrhizas (Tarafdar, 1995) or from microbes (Tarafdar and Chhonker, 1979; Tarafdar et al., 1988). Microorganisms may produce both alkaline and acid phosphatases (Tarafdar, 1989). The largest fraction of organic phosphorous in the soil (approximately 50%) appears to be in the form of phytin and its derivatives,

which require a special class of phosphatases i.e. phytases for hydrolysis. Most plants contain 50–80% of their phosphorus content in the form of phytate. This is largely unavailable to monogastric animals as well as humans, due to the lack of adequate levels of phytases (phytate-hydrolyzing enzymes). This necessitates the supplementation of animal with inorganic P. The release of phytates as well as the excess P into the environment in turn leads to phosphorus pollution problem, eutrophication.

Various microbes including fungi, yeasts and bacteria are known to produce acid phosphatases and phytases. As long-term prospects of inorganic-P supply and its environmental consequences remain a matter of concern, concerted efforts to exploit microbial enzymes for the management of P in animal feeds, human foods and agriculture are in progress. In view of these concerns, in this chapter, an attempt has been made to describe the production, characteristics and potential biotechnological applications of yeast acid phosphatases and phytases.

31.2 Phosphatases

The term phosphatase is commonly used for the enzymes which catalyze the hydrolysis of a variety of phosphomonoesters, and thus degrade complex organic phosphorus compounds releasing orthophosphate. Hence, phosphatases are orthophosphoric monoester phosphohydrolases and their reaction mechanism can be divided into four key steps: a) non-covalent binding of the substrate to the enzyme, b) release of alcoholic group from the complex and covalent binding of the orthophosphate to the enzyme forming a phosphoryl-enzyme compound, c) uptake of water by the phosphoryl-enzyme compound to form a non-covalent complex, and d) release of orthophosphate and regeneration of free enzyme (McComb et al., 1979) (Fig. 31.1). Phosphatases are widely distributed in nature and generally they do not show high substrate specificity and thus, are able to hydrolyze a wide variety of esters and anhydride phosphoric acids, releasing phosphate. They are able to perform transphosphorylation reactions from phosphoesters of phenol, *p*-nitrophenylphosphate and others to various receptors such as glucose and pyridoxine (Guimaraes et al., 2004). They have been implicated in translocation mechanisms and regulatory processes involved in sugar metabolism, which may enhance growth (Famurewa and Olutiola, 1994). Acid phosphatases are also reported to be involved in signal transduction mediated by phospholipase-D and cell regulation such as the de novo synthesis of glycerolipids, in which a phosphatidic acid phosphatase converts phosphatidic acid into diacylglycerol (Guimaraes et al., 2004).

The phosphatases are a diverse class of enzymes and can act on a variety of phosphate esters including *p*-NPP, phytic acid, AMP, ADP, ATP, glucose-6-phosphate, glycerol-3-phosphate and lecithins. The acid phosphatases with the ability to hydrolyze phytic acid are particularly important because of their immense biotechnological potential in food and feed industry as well as in preventing environmental pollution (Vohra and Satayanarayana, 2003). The phytase and acid phos-

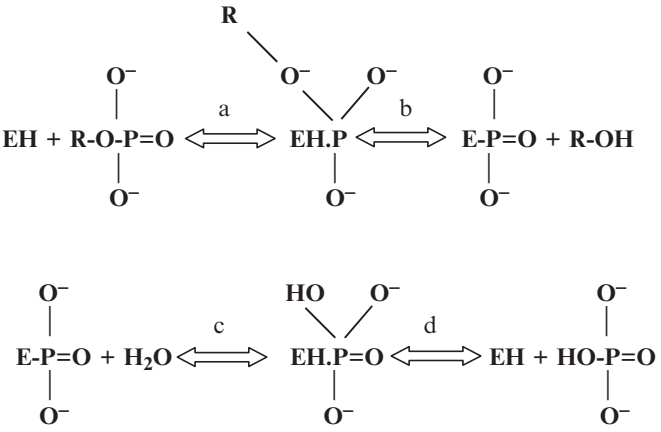

Fig. 31.1 Reaction mechanism for the phosphatase-catalyzed hydrolysis of phosphate esters in four steps (a to d). (McComb et al., 1979)

phatase enzymes can work in coordination, where phytase can split the molecule of phytate in a selective manner, while, acid phosphatase can attack the inositol phosphate intermediates independently, and as a result accelerate the total dephosphorylation process (Zyta, 1993).

31.2.1 Classification of Phosphatases

Phosphatases have been conventionally classified into:

A) Alkaline Phosphatases (EC 3.1.3.1) with pH optima well above 7
B) Acid Phosphatases (EC 3.1.3.2) with pH optima below 7

The revised classification categorizes orthophosphoric monoester phosphohydrolase activities into five distinct families:

a) Alkaline phosphatases
b) Purple acid phosphatases
c) Low molecular mass acid phosphatases
d) High molecular mass acid phosphatases
e) Protein phosphatases

Acid phosphatase is a ubiquitous enzyme found in various organisms and its primary role is the hydrolysis of external phosphate esters, which do not penetrate the plasma membrane. Low and high molecular mass acid phosphatases generally hydrolyze phosphomonoesters according to a two step mechanism in which the

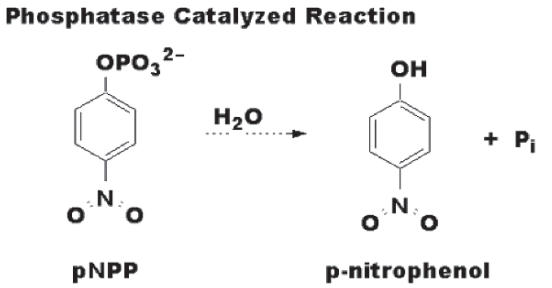

Fig. 31.2 Reaction catalyzed by acid phosphatase on *p*-nitrophenylphosphate (*p*-NPP)

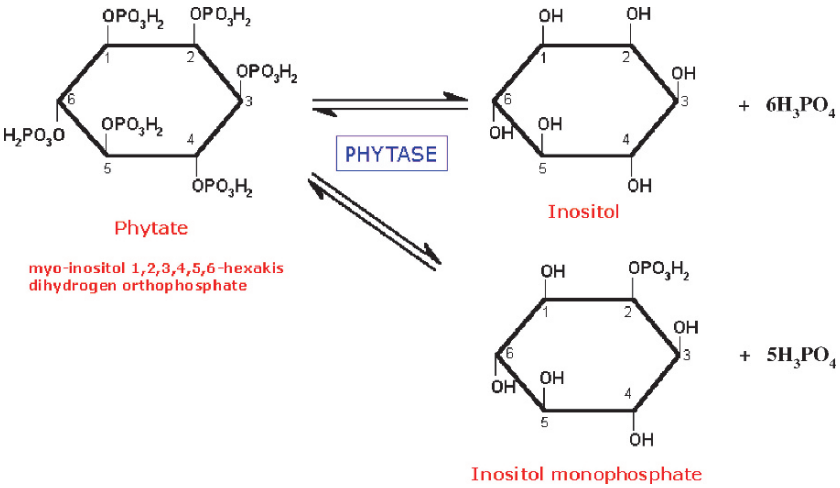

Fig. 31.3 Reaction catalyzed by phytase on phytic acid

enzyme bound substrate produces a covalent phospho-enzyme intermediate and an alcohol; hydrolysis of the intermediate results in the formation of P_i and can be rate limiting. The reaction catalyzed by acid phosphatase on *p*-nitrophenylphosphate (*p*NPP) is depicted in Fig. 31.2.

31.3 Phytases

Phytases (*myo*-inositol hexaphosphate phosphohydrolases) are a subgroup of acid phosphatases which hydrolyze phytic acid to *myo*-inositol and inorganic phosphates through a series of *myo*-inositol phosphate intermediates. There are two phytases as classified by Nomenclature Committee of the International Union of Biochemistry

and Molecular Biology (NC-IUBMB) in consultation with the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (JCBN):

3-phytase (EC 3.1.3.8), which first hydrolyses the ester bond at the 3 position of *myo*-inositol hexakisphosphate (Johnson and Tate, 1969). It is characteristic for microorganisms.

6-phytase (EC 3.1.3.26), which first hydrolyses the ester bond at the 6 position of *myo*-inositol hexakisphosphate (Cosgrove, 1969, 1970). It is typical for plants.

Recently phytases have also been classified as HAP (Histidine acid phosphatase), BPP (β-Propeller phytase) and PAP (purple acid phosphatase) depending upon their catalytic properties (Mullaney and Ullah, 2003). The reaction catalyzed by phytase on phytic acid is depicted in Fig. 31.3.

31.4 Acid Phosphatases and Phytases from Yeasts

Inorganic phosphate (Pi) is the preferred extracellular phosphate source of yeasts. Upon its depletion, phosphate is liberated from organic substrates by extracellular phosphatases (Johnston and Carlson, 1992). Phosphorus metabolism relies on several constitutive and phosphate-repressible acid and alkaline phosphatases in various yeasts using the *pho* regulon. Acid phosphatases have been reported from several yeast species (Table 31.1).

The enzyme and its regulation mechanism are best studied in the baker's and brewer's yeast, *Saccharomyces cerevisiae* (Suomalainen et al., 1960; Schmidt et al., 1963; Oshima et al., 1996; Oshima, 1997). Two forms of acid phosphatases are known in the yeast *S. cerevisiae* (Kozulic et al., 1984): a constitutive form encoded by *PHO 3* gene, and a repressible form encoded by three genes- *PHO 5*, *PHO 10*, and *PHO 11*. Both the acid phosphatases are glycoproteins with carbohydrate content of 50% of the molecular weight. The expression of the repressible or the constitutive acid phosphatases could be switched on by regulation of the concentration of the inorganic phosphorous in the medium in strains which possess corresponding

Table 31.1 A list of acid phosphatase producing yeasts

Yeast	Reference
Arxula adeninivorans	Buttner et al., 1991
Candida albicans	Odds and Hierholzer, 1973
Candida lipolytica	Vasileva-Tonkova et al., 1993
Hansenula polymorpha	Phongdara et al., 1998
Pichia guilliermondii	Strugovschikova et al., 1976
Pichia pastoris	Payne et al., 1995
Rhodotorula rubra	Watorek et al., 1977
Saccharomyces cerevisiae	Suomalainen et al., 1960; Toh-e et al., 1973; Nosaka, 1990
Saccharomyces mellis	Weimberg and Orton, 1964
Schizosaccharomyces pombe	Dibenedetto and Cozzani, 1975; Schweingruber et al., 1986
Torulopsis rubra	Rautanin and Kaerkkaeinen, 1951
Yarrowia lipolytica	Lopez and Dominguez, 1988

structural genes. The brewer's bottom yeast possesses scarcely half of the acid phosphatase activity found in commercial baker's yeast (Suomalainen et al., 1960). Acid phosphatases have also been studied in the other conventional yeast *Schizosaccharomyces pombe* (Dibenedetto and Cozzani, 1975; Schweingruber et al., 1986); in several non-conventional yeasts like *Arxula adeninivorans* (Buttner et al., 1991; Minocha et al., 2007), *Yarrowia lipolytica* (Lopez and Dominguez, 1988), *Kluyveromyces lactis* (Altikrete et al., 1984), *Candida albicans* (Odds and Hierholzer, 1973), as well as in the methylotrophic yeasts, *Hansenula polymorpha* (Phongdara et al., 1998) and *Pichia pastoris* (Payne et al., 1995).

Several investigations have also been carried out on the role of yeasts (conventional like *S. cerevisiae* as well as various non-conventional yeasts) in phytate degradation (Kaur and Satyanarayana, 2007) [Table 31.2]. Lambrechts and coworkers (1992) screened 21 yeast strains for their ability to hydrolyze phytates, and among these, *Schwanniomyces castellii* CBS 2863 exhibited highest phytase activity (Segueilha et al., 1992). Among several hundred yeast species investigated for extracellular phytase, *Pichia spartinae* and *P. rhodanensis* exhibited highest levels of phytase with the optimal reaction temperature at 75–80°C and 70–75°C, at pH 3.6–5.5 and 4.5–5.0, respectively (Nakamura et al., 2000). The yeasts have been reported to be a rich genetic resource for heat-resistant phytases; the possibility of using these phytases in industry has not, however, been extensively investigated. Among others, the red yeast *Rhodotorula gracilis* (Bindu et al., 1998), *Arxula adeninivorans* (Sano et al., 1999), *Pichia anomala* (Vohra and Satyanarayana, 2001) and *Candida krusei* WZ-001 (Quan et al., 2001) have also been shown to produce phytase. Recently, there was a report on phytase-producing marine yeasts, where ten yeasts with high phytase activity were obtained and identified to be closely related to species of *Hanseniaspora uvarum*, *Yarrowia lipolytica*, *Kadamaea ohmeri*, *Issatchenkia orientalis*, *Candida tropicalis* and *C. carpophila*. (Hirimuthugoda et al., 2006).

Table 31.2 A list of phytase producing yeasts

Yeast	Reference
Arxula adeninivorans	Sano et al., 1999
Candida spp.	Nakamura et al., 2000
Candida krusei	Quan et al., 2001
Cryptococcus sp.	Greenwood and Lewis, 1977
Kluyveromyces lactis	Nakamura et al., 2000
Pichia anomala	Nakamura et al., 2000
P. anomala	Vohra and Satyanarayana, 2001
P. rhodanensis	Nakamura et al., 2000
P. spartinae	Nakamura et al., 2000
Rhodotorula gracilis	Bindu et al., 1998
Saccharomyces cerevisiae	Howson and Davis, 1983
S. cerevisiae	Nayini and Markakis, 1984
S. kluyveri	Nakamura et al., 2000
Schwanniomyces occidentalis	Segueilha et al., 1992
S. castellii	Lambrecht et al., 1992
Torulaspora delbrueckii	Nakamura et al., 2000

31.4.1 Production

Various nutritional and physical parameters are known to affect the production of enzymes. The most important physical parameters that exert a profound influence on the growth of organisms and production of metabolites from microorganisms are pH, temperature, agitation, dissolved oxygen and osmotic pressure. Nutritional parameters include carbon, nitrogen and phosphorous sources, trace elements and vitamins. Other factors like the age and level of inoculum and additives like surfactants also influence the production.

There are hardly any reports on production aspects of acid phosphatases from yeasts, though phytase production from yeasts has been fairly well investigated. The constitutive or inducible nature of phosphatase production has been widely investigated. In *S. cerevisiae*, the expression of *PHO 3* gene occurs at high concentration of inorganic phosphate in the medium, while the expression of the repressible acid phosphatase occurs only during phosphorous starvation. When the expression of the repressible acid phosphatase is stimulated, the synthesis of the constitutive form is repressed. Consequently, the expression of the repressible or the constitutive acid phosphatases could be switched on by regulation of the concentration of inorganic phosphate in the medium (Tait-Kamradt et al., 1986). During the industrial cultivation of baker's yeast, the following observations were made: the acid phosphatase activity first increases, then falls to one tenth during the growth period of the pre-commercial stage when the cultivation conditions become more aerobic. However, the enzyme activity intensifies during the cultivation of the commercial yeast stage when the aeration increases and the P content decreases and during this P starvation condition, the acid phosphatase increases sharply, becoming 8-fold in 8 h as compared to the seed yeast (Suomalainen et al., 1960). An analogous behavior in phosphate-starved baker's yeast has been reported by other researchers (Schmidt et al., 1956). Similarly, Rautanin and Kaerkkaeinen (1951) discovered strong increase in acid phosphatase during incubation of *Torulopsis utilis* in a phosphate-deficient medium.

Several species of the soil yeast, *Cryptococcus* were found to produce acid and alkaline phosphatases and could hydrolyze *p*-nitrophenylphosphate (*p*NPP), glycerophosphate, pyrophosphate and inositol hexaphosphate (phytate) [Greenwood and Lewis, 1977]. The methylotrophic yeast, *Pichia pastoris* produces an inducible secretory acid phosphatase (Pho1p), which can be induced 100-fold by phosphate-starvation, thus it was studied as a model system of protein secretion (Payne et al., 1995). Recently, the extracellular acid phosphatase-encoding *Arxula adeninivorans APHO1* gene was cloned and characterized (Kaur et al., 2007) and a recombinant *A. adeninivorans APHY SWARS* strain expressing the cloned *APHO1* was generated The cultural conditions for maximizing the production of acid phosphatase from the recombinant *A. adeninivorans* strain were optimized using statistical approach with a resultant 3.86 and 4.19- fold increase in enzyme production in shake flasks and lab fermenter, respectively (Minocha et al., 2007).

Submerged fermentation seems to be the method of choice in the production of yeast phytases. In *Schwanniomyces castellii*, phytase production was carried out

continuously in a fermenter aerated at 1 vvm and agitated at 600 rev/ min. Galactose (1%) was found to be the preferred carbon source and sodium phytate (0.06%) was also required for phytase production (Segueilha et al., 1992). The yeast *A. adeninivorans* secreted high levels of phytase during active growing phase at 44°C and galactose was found to be a better carbon source than glucose, while yeast extract (1%) and peptone (1%) served as good nitrogen sources. Phytate did not show an inducible effect on phytase production (Sano et al., 1999). The red yeast *R. gracilis* produced phytase constitutively, with maximum production being in a medium lacking phytate in 18 h (Bindu et al., 1998). A high phytase yield by *C. krusei* WZ-001 was attained in 48 h at 30°C and pH 7.0. Glucose (5%) and polypeptone (0.7%) were found to be suitable carbon and nitrogen sources, respectively; phosphate repressed phytase synthesis in the medium (Quan et al., 2001).

The phytase production from *P. anomala* has been extensively studied. A high phytase titre was attained in the synthetic medium containing glucose (4%) and beef extract (1%), supplemented with Fe^{2+} (0.15 mM) at 20°C in 24 h (Vohra and Satyanarayana, 2001). Using Response Surface Methodology (RSM), further enhancement in the titres was achieved, whereby the interaction of 3 variables (glucose, beef extract and inoculum density) was studied. Thus by using the statistical approach, concentrations of glucose (2%) and beef extract (0.5%) became half of those previously optimized, thus reducing the production cost (Vohra and Satyanarayana, 2002b). A medium consisting of cane molasses, a by-product of sugarcane industry was formulated and an improved phytase yield with reduced production cost was attained in comparison to the synthetic glucose-beef extract medium (Vohra and Satyanarayana, 2004). This medium was also optimized using statistical approaches (Plackett-Burman and RSM), that resulted in higher phytase yields (Kaur and Satyanarayana, 2005).

31.4.2 Purification

A combination of various methods has been usually employed for purifying phosphatases and phytases. A typical salt or solvent precipitation for concentrating the enzyme followed by chromatography such as ion exchange or gel filtration is the general methodology employed.

The acid phosphatase from *R. rubra* was purified 44-fold by precipitation with ethanol followed by chromatography on DEAE- and CM-cellulose (Watorek et al., 1977), while that from derepressed cells of *Y. lipolytica* was purified 176-fold by ammonium sulfate precipitation, chromatography on DEAE-Sephacel and gel filtration in Biogel A 5-M (Lopez and Dominguez, 1988). Similarly, the non-specific acid phosphatase from *C. lipolytica* was purified 111-fold by chromatography on DEAE-cellulose and gel filtration on Sephadex G-100 and Sepharose 4B (Vasileva-Tonkova et al., 1993). The two forms of acid phosphatases from *A. adeninivorans* were purified by acetone precipitation followed by separation on cellulose phosphate column (Buttner et al., 1991). The acid phosphatase from the pathogenic

C. albicans was purified 87-fold by four stages of column chromatography viz., DEAE-cellulose, CM-Sephadex, Sephadex G-200 and Sepharose 2B (Odds and Hierholzer, 1973).

Similar methods have been employed for purification of phytases. A three-stage purification method including anion and gel filtration chromatography was used to purify the phytase from *S. castellii* (Segueilha et al., 1992). The phytase from *A. adeninivorans* was partially purified using Sephadex G50 filtration and DEAE chromatography (Sano et al., 1999), while that from *C. krusei* WZ-001 was purified to homogeneity by ion-exchange chromatography, hydrophobic interaction chromatography and gel filtration (Quan et al., 2002). The phytase of *P. anomala* was purified to homogeneity by a two-step process of acetone precipitation followed by anion exchange chromatography using DEAE-Sephadex (Vohra and Satyanarayana, 2002a).

31.4.3 Characterization

The properties of enzymes are important in determining their potential applications in various industries. The physico-chemical properties of some yeast acid phosphatases and phytases are enlisted in Tables 31.3 and 31.4, respectively.

31.4.3.1 Temperature

Phosphatases and phytases with high temperature optima are desirable in the animal feed industry because feed pelleting involves a step of 80–85°C for a few seconds. The acid phosphatase and phytase from *A. adeninivorans* exhibited temperature optima at 50–55°C (Buttner et al., 1991) and 75°C (Sano et al., 1999), respectively. The acid phosphatase of *C. lipolytica* has temperature optima of 55°C and is stable upto 60°C (Vasileva-Tonkova et al., 1993), while that of *Y. lipolytica* was highly thermolabile as it got destroyed by exposure to 40°C for 30 min (Lopez and Dominguez, 1988). The acid phosphatase of *R. rubra* retained activity after 1 h incubation at 50°C, but lost half of its activity after 1 h incubation at 65°C (Watorek et al., 1977). The acid phosphatase II (AP II) of *Pichia guilliermondii* was optimally active at 40°C (Strugovschikova et al., 1976). The acid phosphatase from *C. albicans* was completely inactivated after 12 min incubation at 70°C, though no loss of activity was detected even after 40 h of exposure at 37°C (Odds and Hierholzer, 1973).

The phytase of *Schwanniomyces castellii* was found to be optimally active at 77°C (Segueilha et al., 1992), while that of *C. krusei* WZ-001 was optimally active at 40°C (Quan et al., 2002). In *Pichia rhodanensis, P spartinae* and *P. anomala*, the phytases exhibited optimal activity at 70–75, 75–80 (Nakamura et al., 2000) and 60°C (Vohra and Satyanarayana, 2002a), respectively. Thermostability of phytase is also considered to be an important and useful criterion for its industrial application due to the requirement of withstanding pelleting temperatures. The *S. castellii*

Table 31.3 Kinetic properties of yeast acid phosphatases

Yeast	Optimal temp. (°C)	Optimal pH	Molecular weight (kDa)	K_m (mM)	Inhibitors	Reference
Arxula adeninivorans	50–55	5.2–5.5	250–320	3.5	Cu^{2+}, Hg^{2+}	Buttner et al., 1991
Candida albicans	-	4.5	115–131	0.33	Hg^{2+}, Cd^{2+}, Cu^{2+}	Odds and Hierholzer, 1973
Candida lipolytica	55	5.8	95	3.64	-	Vasileva-Tonkova et al., 1993
Pichia guilliermondii	40	5.5		1.4	MoO_4^{2-}, F^-, Cu^{2+}, Be^{2+}	Strugovschikova et al., 1976
Rhodotorula rubra					Molybdate, Arsenta, phosphate and F^-	
	-	4.0–4.6	-	0.1		Watorek et al., 1977
Saccharomyces cerevisiae	-	3–4	100–200	1.3	-	Boer and Steyn-Parve, 1966
Schizosaccharomyces pombe	-	-	381–383	0.17	-	Dibenedetto and Cozzani, 1975
Yarrowia lipolytica	-	6.2	90–200	0.55	-	Lopez and Dominguez, 1988

phytase was stable at 74°C for 1 h (Segueilha et al., 1992), whereas that of *C. krusei* was easily inactivated at temperatures above 50°C (Quan et al., 2002). The phytase of *P. anomala* was also found to be thermostable with a half-life of 5 min at 80°C (Vohra and Satyanarayana, 2002a).

31.4.3.2 pH

Most acid phosphatases and phytases are active within the pH range 4.0–6.0 and the enzyme activity decreases rapidly at pH values lower than 3.0 and higher than 7.5. The optimum pH values for acid phosphatases and phytases of *A. adeninivorans* were 5.2–5.5 (Buttner et al., 1991) and 4.5 (Sano et al., 1999), respectively. The pH optimum of acid phosphatases from *S. cerevisiae,* which are located on the outer cell surface (McLellan and Lampen, 1963), was estimated to be 3.6 (Schurr and Yagh, 1971) or 4.4 (Suomalainen et al., 1960), and the enzyme was completely inactivated at 45°C (Schurr and Yagh, 1971). The acid phosphatase of *C. lipolytica* exhibited pH optima of 5.8 and was stable at pH values between 3.5 and 5.5

Table 31.4 Kinetic properties of yeast phytases

Yeast	Optimal temp. (°C)	Optimal pH	Molecular weight (kDa)	K_m (mM)	Inhibitors	Reference
Arxula adeninivorans	75	4.5	-	0.25	Mg^{2+}, Ca^{2+}, Zn^{2+}	Sano et al., 1999
Candida intermedia	65	4.5	-	-	-	Nakamura et al., 2000
C. krusei WZ-001	40	4.6	330	0.03	Zn^{2+}, Mg^{2+}, pCMB, PMSF, iodoacetate	Quan et al., 2002
C. tropicalis	65	4.5	-	-	-	Nakamura et al., 2000
Kluyveromyces thermotolerans	60–65	4–5	-	-	-	Nakamura et al., 2000
Pichia anomala	60	4.0	64	0.20	Fe^{3+}, Hg^{2+}, Zn^{2+}, 2,3-butanedione, Gu-HCl, urea	Vohra and Satyanarayana, 2002
P. rhodanensis	70–75	4.0–4.5	-	0.25	-	Nakamura et al., 2000
P. spartinae	75–80	4.5–5.5	-	0.33	-	Nakamura et al., 2000
Schwanniomyces castellii	77	4.4	490	0.038	Zn^{2+}, Cu^{2+}, pCMB	Segueilha et al., 1992

(Vasileva-Tonkova et al., 1993), whereas that from *Y. lipolytica* had an optimum pH of 6.2 and was destroyed by exposure to pH 3 for 30 min (Lopez and Dominguez, 1988). The optimum pH for acid phosphatase of *R. rubra* is 4.0–4.6 and it is stable over pH range between 2.6 and 6.0 (Watorek et al., 1977). The AP II of *P. guilliermondii* was optimally active at pH 5.5 (Strugovschikova et al., 1976), while the enzyme of *C. albicans* was optimally active at 4.5, and was stable at room temperature and at 4°C in the pH range of 4–8 (Odds and Hierholzer, 1973).

The phytase of *S. castellii* was optimally active at pH 4.4 (Segueilha et al., 1992). Nakamura and coworkers (2000) studied a number of yeast strains for extracellular phytases. All the yeast phytases were optimally active in the pH range between 4.0 and 5.0 when assayed at 50–60°C. Many strains produced another phytase at 37°C with pH optimum between 3.0 and 4.0. This suggests that there are either more than one phytase or the same protein exhibits different pH optima at different temperatures. Phytases from *C. krusei* (Quan et al., 2002) and *P. anomala* (Vohra and Satyanarayana, 2002a) exhibited pH optima at 4.6 and 4.0, respectively.

31.4.3.3 Effect of Metal Ions

The effect of metal ions on enzyme activity varies. The acid phosphatase of *A. adeninivorans* was inhibited by Cu^{2+} and Hg^{2+} (Buttner et al., 1991), while that of *P. guilliermondii* was inhibited by MoO_4^{2-}, F^-, Cu^{2+} and Be^{2+} (Strugovschikova et al., 1976). The acid phosphatase of *C. albicans* was inhibited by Hg^{2+}, Cd^{2+} and Cu^{2+} (Odds and Hierholzer, 1973). The phytase of *S. castellii* was slightly inhibited in the presence of 5 mM Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+}. The cations Zn^{2+} and Cu^{2+} (0.5 mM) caused around 50% inhibition of enzyme activity, while 5mM Zn^{2+} and Cu^{2+} strongly inhibited (Segueilha et al., 1992). The phytase of *C. krusei* WZ-001 was strongly inhibited by Zn^{2+} and Mg^{2+} (1 and 5 mM), but it was not significantly inhibited in the presence of Ba^{2+} and Pb^{2+} (Quan et al., 2002). The phytase of *P. anomala* did not exhibit any requirement of metal ions for activity and it was strongly inhibited by Fe^{3+}, Cu^{2+}, Zn^{2+} and Hg^{2+} (Vohra and Satyanarayana, 2002a).

31.4.3.4 Substrate Specificity

Acid phosphatases are generally identified by their reaction with the substrate *p*NPP, though they hydrolyze several other phosphoesters. Phytases usually show broad substrate specificity, with a high affinity for phytates. Broad substrate specificity was reported for phytases of *S. castellii*. The K_m value for phytate was the lowest, while for glucose-1-phosphate, glucose-6-phosphate, *p*-nitrophenyl phosphate and ATP, the values were high (Segueilha et al., 1992).

The acid phosphatases of *A. adeninivorans* exhibited a K_m value of 3.5 mM for *p*NPP (Buttner et al., 1991), which was similar to that of *S. cerevisiae* acid phosphatase (Nosaka, 1990). The *S. cerevisiae* strain H-42 was reported to have 2 kinds of acid phosphatases: a constitutive and a Pi repressible one (Toh-e et al., 1973). The constitutive one had a K_m of 9.1×10^{-4} M for *p*NPP, which was higher than that of the repressible enzyme i.e. 2.4×10^{-4} M. The constitutive and repressible acid phosphatases are encoded by different genes, viz. *pho*C and *pho*B, *pho*D or *pho*E gene, respectively. These enzymes are under the same regulatory mechanism or share a common polypeptide. Among others, a thiamin-repressible acid phosphatase encoded by *PHO3* gene has also been studied in *S. cerevisiae* (Nosaka, 1990). This enzyme showed K_m values of 1.6 and 1.7 μM at pH 5 for thiamin monophosphate and thiamin pyrophosphate, respectively, which were 2–3 orders of magnitude lower than those for *p*NPP. The high affinity of this enzyme for thiamin phosphates was due to its thiamin-binding properties. In *Schizosaccharomyces pombe* also a thiamin repressible acid phosphatase is present, which is encoded by *pho4* (Schweingruber et al., 1986). The nonspecific acid phosphatase from *S. pombe* exhibited a broad substrate specificity (Dibenedetto and Cozzani, 1975) and so did the purified phosphatase of *C. lipolytica* (Vasileva-Tonkova et al., 1993). The derepressible acid phosphatase from *Y. lipolytica* was found to be an unspecific phosphohydrolase with

similar activity on several different phosphate esters (Moran et al., 1989). Moreover, partial inhibition and non-Michaelian inhibition of 'non-competitive' nature was observed (Gonzalez et al., 1993), although acid phosphatase from the same yeast exhibited a Michaelis constant of 5.5×10^{-4} M for *p*NPP (Lopez and Dominguez, 1988). The substrate specificity of *R. rubra* acid phosphatase is fairly broad; it can use monoesters of carbohydrates, nucleosides and inorganic pyrophosphate as substrates (Watorek et al., 1977). The non-specific phosphomonoesterase from *P. guilliermondii* could also hydrolyze several substrates (Strugovschikova et al., 1976).

The phytase of *P. anomala* also exhibited broad substrate specificity, with comparable efficiency on phytate, ATP and *p*-nitrophenyl phosphate (Vohra and Satyanarayana, 2002a). Similarly, the phytase of *C. krusei* had a broad substrate specificity with maximum activity on phytate (Quan et al., 2002). The rate of enzymatic hydrolysis based on Michaelis-Menten kinetics is generally expressed as the Michaelis constant (K_m). The K_m values of some yeast acid phosphatases and phytases are listed in Tables 31.3 and 31.4 respectively.

31.4.3.5 Molecular Characteristics

The acid phosphatases are high molecular weight proteins with high glycosylation levels. The molecular weights of the two acid phosphatases from *A. adeninivorans* were 320 and 250 kDa for enzyme I and II, respectively, while after deglycosylation, it reduced to 75 and 46 kDa, respectively (Buttner et al., 1991). The acid phosphatase from *C. lipolytica* is a glycoprotein with 67% neutral sugars and a molecular weight of 95 kDa (Vasileva-Tonkova et al., 1993). The glycoprotein acid phosphatase from *C. albicans* is a nanoprotein with hexose to protein ratio of 7:1 and molecular weight between115–131 kDa (Odds and Hierholzer, 1973).

The phytases are also high molecular weight proteins ranging from 40–500 kDa. The phytase of *P. anomala* had a molecular mass of 64 kDa (Vohra and Satyanarayana, 2002a), while phytase of *C. krusei* had a molecular mass of 330 kDa, and was composed of 2 different subunits (Quan et al., 2002). The phytase from *S. castellii* had a molecular weight of 490 kDa with around 31% glycosylation. The glycosylated protein was tetrameric, with one large subunit (M.W. 125000) and three identical small subunits (M.W. 70,000) [Segueilha et al., 1992].

There are very few reports on the active sites of yeast phytases. In *P. anomala* phytase, arginine residues were found to be involved in catalysis (Vohra and Satyanarayana, 2002a). The amino acid sequence analysis of some yeast phytases revealed the presence of a conserved eight-cysteine motif. These were not directly associated with catalysis, but appeared to have a role in the formation of disulfide bridges, and thus contribute to stability and heat tolerance in phytases (Mullaney and Ullah, 2005).

31.5 Commercial Prospects

The acid phosphatases and phytate-hydrolyzing enzymes find several applications in various fields, the most important ones being phytate elimination in feed and food industries, and preparation of *myo*-inositol phosphates (degradation products of phytate).

31.5.1 Soil Amendment

The organic form of phosphorus constitutes 4–90% of the total soil P, and of this, inositol hexaphosphate (phytic acid) in the form of adsorbed or insoluble phytate salts can constitute in certain locations upto 50% of the total organic phosphorous in the soil (Dalal, 1978). As these phosphate ester containing compounds immobilize phosphorous in the soil, making phosphorous unavailable, the addition of phosphatases and specifically phytases becomes important. The species of soil yeast *Cryptococcus* have been studied for their phosphatases and utilization of inositol hexaphosphate as sole P source (Greenwood and Lewis, 1977).

31.5.2 Applications in Feed

Animal feeds mainly comprise plant feedstuffs and about two thirds of phosphorus of feedstuffs of plant origin is in the form of phytate P. Under most dietary conditions, phytate P is unavailable to these animals. Incorporation of phytases into feeds reduces the need for phosphorus supplementation. This improves the digestibility, and therefore, the nutritive value of the feed, thereby enhancing the growth rate of monogastric animals, lowering the level of phosphorus excretion, and thus reducing chances of environmental pollution.

There are two basic ways of using phytase in feeds. The first possibility is replacement of Pi supplementation with phytase. As the reaction conditions (pH, temperature, moisture, incubation time) in the animal stomach or intestine are not optimal, the second method of phytase use i.e. feed pretreatment to reduce the phytate content in the feed ingredients, becomes more feasible (Simell et al., 1989). Numerous lab experiments have confirmed that 500–1000 units of phytase can substitute approximately 1 g Pi supplementation and reduce the total phosphorus excretion by 30–50% (Kemme et al., 1997; Liu et al., 1997; Yi et al., 1996).

There are several reports on the use of yeast phytases for animal feed pretreatment or supplementation. Segueilha and coworkers (1993) showed that phytase from *S. castellii* could be successfully used for removing phytic acid from wheat bran and glandless cotton flour, thus proving the strain's efficacy in pretreatment of animal feeds. Some researchers attempted dietary supplementation of pigs with

phytase-containing yeast culture, and reported that it failed to improve phytate P availability (Cromwell and Stahly, 1978; Chapple et al., 1979). However, Matsui et al. (2000) showed that dietary yeast phytase improved bioavailability of P in growing pigs, but its efficacy was less than that of *Aspergillus niger* phytase. The lower efficacy of yeast phytase was the result of low stability of yeast phytase in the presence of pepsin.

An *E. coli* (isolated from pig colon) derived phytase [ECP] was expressed in *P. pastoris* system, then compared with 2 commercial feed phytases with respect to the phosphorus-releasing efficacy in young chicks and pigs, and the former was found to be more advantageous than the latter in chicks. When these were added to corn-soybean meal diets with no supplemental Pi, ECP supplementation resulted in superior weight gain and tibia ash values, more so in young chicks than in young pigs, thus proving the greater efficacy of ECP (Augspurger et al., 2003). The safety evaluation of an *E. coli* phytase expressed in *Schizosaccharomyces pombe* was done using various in vitro and in vivo toxicity tests, with the aim of its intended use in animal feed. The product BD006 phytase was found to be devoid of any relevant toxic effect, thus attesting its safety for use in swine and poultry (Ciofalo et al., 2003). The potential of using *S. cerevisiae* as a phytase carrier in the gastrointestinal tract was also studied, whereby the hydrolysis of phytic acid by a high-phytase recombinant yeast strain was studied in simulated digestive conditions. The high-phytase yeast brought about 60% reduction in IP_6 in the early gastric phase, as compared to no reduction by wild type strain. However, the degradation in late gastric and early intestinal phases was insignificant. Thus dependency on pH seemed to be an important limiting factor for phytase expression or activity in the high-phytase yielding yeast strains (Haraldsson et al., 2005).

The supplementation of feed of broiler chicks with cell-bound phytase of *P. anomala* resulted in improved growth (77.7% gain in control over 90.2% in the enzyme fed), better phosphorus retention in the body (29% in control over 73.6% in enzyme fed) and reduced excretion of phosphorus in the faeces (Satyanarayana and Vohra, 2003). Thus the FCR (feed conversion ratio: defined as the ratio of feed consumed per unit gain in body weight) of the enzyme fed chicks was lower than the control, proving the economic viability of the *P. anomala* phytase for application as chick feed supplement (Vohra et al., 2006).

After first commercialization attempts by Gist-Brocades Europe (1993–1994), several companies like Alko Co. (Finland), Altech (USA) and BASF (USA) started the industrial scale production of phytase marketed under the names Finase, Allzyme and Natuphos, respectively, and successfully utilized it in feed applications. But all these are obtained from filamentous fungi. However, Danisco animal nutrition has recently launched a phytase by the name of Phyzyme™ XP, which is a 6-phytase from *E. coli* expressed in the yeast *Schizosaccaromyces pombe*. Two trials were conducted to evaluate the efficacy of this new microbial phytase (Phyzyme XP) for broiler chicks and as compared to the control diets, phytase supplemented diets increased weight gain and feed intake as well as feed efficiency during the starter period (Dilger et al., 2004). Overall, tibia and toe ash were also improved due to phytase addition. In conclusion, the microbial phytase

elicited improved growth performance, bone mineralization, and P utilization in broiler chicks.

31.5.3 Applications in Foods

The presence of phytates in plant foodstuffs is well known. A large portion of global population tends to suffer from iron and zinc deficiencies due to the ingestion of phytate rich plant diet (Bentley et al., 1997; Tatala et al., 1998), and studies in humans indicate that absorption of zinc and iron from a meal corresponds directly to its phytate content (Brune et al., 1992; Navert et al., 1985). The dietary phytase is inactivated during cooking, and therefore, phytate digestion is very poor, and thus affects mineral absorption in the small intestines. The supplemental phytases can play an important role in human nutrition both for phytate degradation during food processing and in the gastrointestinal tracts. The known safe use over the decades of yeasts as well as their probiotic role makes them a better source over other microbes.

An important application of phytase is in bread making, wherein the use of phytase was suggested for producing low phytin bread (Simell et al., 1989). There are some studies on acid phosphatase and phytase activity in baker's yeast (Greiner et al., 2001; Haraldsson et al., 2005) and several researchers have emphasized the role of these yeast enzymes in bread making. During whole meal bread making, reduction in phytate levels were found to be the result of both wheat and yeast phytase activities (Turk et al., 1996). The use of high yeast levels and fermentation times in conventional long fermentation process to obtain high-fibre bread with low phytate content has been suggested (Harland and Harland, 1980).

Turk and coworkers (2000) had also studied 2 commercial strains of Baker's yeast (*S. cerevisiae*) with the ability to express phytase activity and they found that they could rapidly degrade all the IP_6 within 24 hrs, forming mainly DL-Ins(1,2,4,5,6)P_5, DL-Ins(1,2,5,6)P_4 and DL-Ins(1,2,6)P_3. Degradation of phytate by lactic acid bacteria and yeasts (*S. cerevisiae*) during whole meal dough fermentation has been studied using NMR technique, and fermentation using lactic acid bacteria was found to be good (Reale et al., 2004). Very recently it has been shown that a high-phytase S. *cerevisiae* strain, without the use of any heterologous DNA, may be suitable for the production of food-grade phytase and for the direct use in food production (Veide and Andlid, 2006). The role of yeast in sake (alcoholic drink from rice) fermentation was studied and it was found that the yeast repressible acid phosphatase liberates inositol from phytate and subsequently they utilize it for their growth (Furukawa et al., 2001).

Since certain *myo*-inositol phosphates have been suggested to have beneficial health effects, such as reducing the risk of heart disease (Potter, 1995), renal stone formation (Modlin, 1980) and certain cancers (Graf and Eaton, 1993; Shamsuddin and Vucenik, 1999), yeast phytases may find application in food processing to produce functional foods (Konietzny and Greiner, 2003).

31.5.4 Potential in Aquaculture

In aquaculture, feed costs constitute up to 70% of total fish production costs (Rumsey, 1993). Several studies have been conducted on the use of soybean meal or other plant meals in aquaculture by substituting low cost plant protein for a more expensive protein source such as menhaden fish meal, and a significant cost reduction could be achieved (Mullaney et al., 2000). Thus, phytase has been evaluated as a means to promote the use of low-cost plant meals in the aquaculture industry, as well as to maintain acceptable phosphorus levels in water. Yeast and yeast phytases may provide the added advantage in fresh water and marine aquaculture due to their probiotic role.

31.5.5 Preparation of Myo-Inositol Phosphates

There is an increasing interest in inositol phosphates and phospholipids as they play an important role in transmembrane signaling and mobilization of calcium from intracellular reserves; thus culminating in the need for various inositol phosphate preparations (Billington, 1993). The inositol phosphate derivatives find uses as enzyme stabilizers (Siren, 1986a), enzyme substrates for various metabolic studies, inhibitors of enzymes and thus as potential drugs, and as chiral building blocks (Laumen and Ghisalba, 1994). Certain *myo*-inositol phosphates also have beneficial health effects as mentioned before. The number and position of the phosphate groups on the *myo*-inositol ring is of great significance for their physiological functions.

The sequential hydrolysis of *myo*-inositol hexakisphosphate (IP_6) by phytases releases various lower inositol phosphates (IPs), which are useful for studying their physiological effects. An efficient immobilized bioreactor comprising phytase would be advantageous for producing various lower isomers of phytate. Quan et al. (2003) had immobilized cells of *C. krusei* in Ca-alginate gel beads for the preparation of various IPs and the pure isomers were then isolated by ion-exchange chromatography.

Phosphatases and phytases can also be used to prepare novel specific phytate derivatives (Greiner and Konietzny, 1996). The phytase from *S. cerevisiae* was used for the preparation of D-*myo*-inositol 1,2,6-triphosphate, D-*myo*-inositol 1,2,5-triphosphate, L-*myo*-inositol 1,3,4-triphosphate and *myo*-inositol 1,2,3-triphosphate by Siren (1986b). Other investigators have also used phytases from various sources to produce specific *myo*-inositol derivatives. Among the advantages of enzymatic hydrolysis over chemical synthesis are the stereospecificity and mild reaction conditions.

31.6 Future Perspectives and Conclusions

The study of yeast acid phosphatases and phytases can be important for numerous reasons. The yeast *S. cerevisiae* has several phosphatases and its phosphatase (*PHO*) system has been extensively studied due to various technical advantages.

The phosphatase system needs to be explored in other yeasts. The commercial potential of acid phosphatases is mostly limited to their role in soil amendment and phytate degrading capability.

Due to the non-pathogenic status of most yeasts, they are preferable enzyme sources for exploring their applicability in foods. Moreover, IP_6 being a common compound in nature, yeasts will be frequently exposed to it, as well as during brewing, bread and wine-making. Thus, phytate-degrading activity should be important for the yeast to metabolize phosphorus and inositol. Neither the capability to produce phytases cost-effectively nor the genes encoding yeast phytases have been investigated in depth. Therefore, further research efforts are called for discovering new phytases from yeast sources as very few are presently known, and to develop economical process for their large scale production. Engineering of phytases to introduce desired characteristics of pH and temperature stability, broader substrate specificity and higher catalytic rates are other issues that need to be addressed. The primary sequence of few yeast phytases has been studied, and thus, there is a need to isolate and sequence other yeast phytase genes and to elucidate their secondary and 3-D molecular structures, which would also help in understanding the structure-function relationships. The potential applications of yeast phytases are immense, especially in animal feeds and human nutrition. The safe status of most yeasts and their well-known probiotic effects make them ideal candidates for use in feeds and foods. On the other hand, the gene encoding a secretory acid phosphatase in these simple eukaryotes provide an attractive alternative for heterologous gene expression as seen in *S. cerevisiae*, *P. pastoris* and *H. polymorpha*. More detailed and comprehensive understanding and insight into yeast acid phosphatases and phytases is expected the near future.

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Chapter 32
Nitrile Metabolizing Yeasts

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Contents

Abstract Nitriles and amides are widely distributed in the biotic and abiotic components of our ecosystem. Nitrile form an important group of organic compounds which find their applications in the synthesis of a large number of compounds used as/in pharmaceutical, cosmetics, plastics, dyes, *etc*. Nitriles are mainly hydrolyzed to corresponding amide/acid in organic chemistry. Industrial and agricultural activities have also lead to release of nitriles and amides into the environment and some of them pose threat to human health. Biocatalysis and biotransformations are increasingly replacing chemical routes of synthesis in organic chemistry as a part of 'green chemistry'. Nitrile metabolizing organisms or enzymes thus has assumed greater significance in all these years to convert nitriles to amides/ acids. The nitrile metabolizing enzymes are widely present in bacteria, fungi and yeasts. Yeasts metabolize nitriles through nitrilase and/or nitrile hydratase and amidase enzymes. Only few yeasts have been reported to possess aldoxime dehydratase. More than sixty nitrile metabolizing yeast strains have been hither to isolated from cyanide treatment bioreactor, fermented foods and soil. Most of the yeasts contain nitrile hydratase-amidase system for metabolizing nitriles. Transformations of nitriles to amides/acids have been carried out with free and immobilized yeast cells. The nitrilases of *Torulopsis candida* and *Exophiala oligosperma* R1 are enantioselective and regiospecific respectively. *Geotrichum* sp. JR1 grows in the presence of

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2M acetonitrile and may have potential for application in bioremediation of nitrile contaminated soil/water. The nitrilase of *E. oligosperma* R1 being active at low pH (3–6) has shown promise for the hydroxy acids. Immobilized yeast cells hydrolyze some additional nitriles in comparison to free cells. It is expected that more focus in future will be on purification, characterization, cloning, expression and immobilization of nitrile metabolizing enzymes of yeasts.

Keywords Nitrile, yeast, nitrilase, nitrile hydratase-amidase, *Exophiala oligosperma*, *Torulaopsis candida*.

32.1 Introduction

Nitriles and amides are important group of organic compounds containing cyano (-C≡N) and amido (-$CONH_2$) moiety respectively. A large number of nitriles and amides have been reported to be present in biotic and abiotic components of our ecosystem. These compounds have assumed greater significance over these years to organic chemists (as starting materials for the synthesis of a range of amides or acids) (Kobayashi et al., 1990) and environmental toxicologists (as many of the nitriles and amides are potentially carcinogenic and neurotoxic) (Johannsen et al., 1986).

Nitriles are easily synthesized and converted to corresponding acid/amide. A number of important amides (e.g. acrylamide, nicotinamide) and acids (e.g. amino acid, adipic acid, nicotinic acid and mandelic acid) are synthesized from the nitriles (Yamada and Kobayashi, 1996). Nitriles are also used as solvents, antibiotics, precursors in the synthesis of plastics, resins, dyes, cosmetics, etc. while amides find their application as pharmaceutical intermediates and commodity chemicals (Kobayashi et al., 1990; Hoyle et al., 1998).

The industries involved in the production or use of nitriles release significant amount of nitriles or amides in their effluents that contaminate soil, water and air (Pollak et al., 1991). It has been estimated that one ml of automobile exhaust gases contains 1 μg of hydrogen cyanide (HCN) and 100 μg of acetonitrile (Evan et al., 1985) which pollute air. A substantial proportion of organic nitrogen in shale oil is in the form of linear alkyl nitrile (Schuchmann and Laidler, 1972). Some widely used herbicides such as bromoxynil (3, 5 dibromo-4-hydroxybenzonitrile), ioxynil (3, 5 diiodo-4-hydroxybenzonitrile) and dichloro-benil (2, 6 dichlorobenzonitrile) are nitriles and their indiscriminate use pollutes soil and water system (Harper, 1977a, 1977b).

Many nitrile compounds are synthesized by plants, animals and microbes as defense against pathogens, predators or as chemical means to succeed in competition among diverse groups in different ecological niches. More than 2000 plant species have been reported to contain compounds with nitrile group (e.g. cyanoglycosides, cyanolipids, ricinine, indole-3-acetonitriles and cyanohydrins) (Conn, 1981). Cassava (*Manihot esculenta*) and limabeans (*Phaseolus lumatuas*) are used as food in many parts of the world especially in South America and Africa and these contain significant level of cyanogenic glycosides.

Sponges synthesize some compounds containing nitrile group, which have antimicrobial properties (Legras et al., 1989). Certain arthropods squirt cyanide and mandelonitrile as special chemical defense against their predators (Duffey, 1981). The presence of some cyanide related compounds have been demonstrated in algae, fungi, bacteria and bone oil (Legras et al., 1990).

The limitations of chemical technologies employed in the production or conversion of nitriles vis-à-vis emphasis on 'green chemistry' have lead to explore living systems especially microbes for synthesis, transformations and degradation of nitriles and amides to solve the problems encountered in large scale organic synthesis of amides/acids using nitriles/amides and remediation of soil, water and air systems contaminated with nitriles or related compounds. Microbes or their nitrile degrading enzymes have been looked for improving quality of food products containing cyanogenic glycosides, cyanolipids, cyanohydrins or other cyanide compounds. In the present chapter microbial nitrile metabolism with emphasis on nitrile metabolizing yeasts and their potential applications will be discussed.

32.2 Nitrile Metabolism in Microbes

The nitrile metabolism was first reported in plant system (Thimann and Mahadevan, 1964). These studies were then extended to microbes and the first microorganism reported to metabolize nitrile was *Pseudomonas* sp. (Hook and Robinson, 1964). This was followed by a series of reports on microbial systems involved in the utilization or conversion of nitriles. The nitrile metabolizing microbes have been extensively reviewed (O'Reilly and Tuner, 2003; Banerjee et al., 2002). Most of research in all these years confined to microbial enzymes involved in the degradation or conversion of nitriles to corresponding amide or acid and ammonia. However, the nitriles synthesis in microbes has attracted comparatively little attention of researchers. Syntheses of aminonitriles (Strobel, 1966, 1967) and hydroxynitriles (Legras et al., 1989) have been reported earlier.

Asano and Kato (1998) have reported a novel microbial enzyme aldoxime dehydratase involved in the synthesis of nitriles in *Bacillus* sp. strain OxB-1. Later it was shown that a large number of microbes (about 205 strains) have aldoxime dehydratase activity, out of which 188 strains also exhibited nitrile-hydrolyzing activity. It seems that nitrile synthesizing and degrading activities widely coexist in microbes. The metabolic gene organization of the "nitrile pathway" has been recently characterized in *Pseudomonas chlororaphis* B23 and *Pseudomonas* sp. K9 (Hashimoto et al., 2005; Kato and Asano, 2006). The 'nitrile pathway' consists of aldoxime → nitrile → amide → acid → acyl-CoA and the enzymes involved in this pathway are aldoxime dehydratase, amidase, nitrile hydratases and acyl-CoA synthetase. The existence of aldoxime dehydratase and nitrile hydrolyzing activity in *Candida guilliermondii* IFO 045, *C. methanolica* TPU and *Pichia miso* TPU 1306 (Kato et al., 2000b) suggests probability of similar nitrile pathway in yeasts. Some important nitrile metabolizing microbes have been listed in Table 32.1. Among microbes, bacteria, yeasts,

Table 32.1 Some selected well explored nitrile metabolizing microorganisms

Microorganisms	Nitrile metabolizing enzymes	Reference
A. Bacteria		
Rhodococcus rhodochrous J1	Nitrilase, nitrile hydratase-amidase	Kobayashi et al. (1989), Odaka et al. (1997), Chebrou et al. (1996)
Rhodococcus rhodochrous PA-34	Nitrilase, nitrile hydratase	Bhalla et al. (1992), Raj et al. (2006)
Pseudomonas chlororaphis B23	Aldoxime dehydratase, nitrile hydratase- amidase	Nagasawa et al. (1987), Ciskainik et al. (1995), Oinuma et al. (2003)
Klebsiella pneumoniae NCTR1	Amidase	Nawaz et al. (1996)
Rhodococcus sp. YH3-3 TPU 3453	Aldoxime dehydratase	Kato et al. (1998)
Bacillus sp. strain OxB-1	Aldoxime dehydratase	Asano and Kato (1998)
B. Yeasts		
Torulopsis candida	Nitrilase	Fukuda et al. (1973)
Exophiala oligosperma R1	Nitrilase	Rustler and Stolz (2007)
Candida fabianii	Nitrile hydratase- amidase	Brewis et al. (1995)
Kluyveromyces thermotolerans MGBY 37	Nitrile hydratase-amidase	Prasad et al. (2005)
C. Fungi		
Basidiomycetes	Nitrile synthetase	Strobel (1966)
Fusarium oxysporum f.sp. melonis	Nitrilase	Goldlust and Bohak (1989)
Aspergillus niger	Nitrilase	Šnajdrová et al. (2004)
Myrothecium verrucaria	Nitrile hydratase	Maier-Greiner et al. (1991)
Fusarium oxysporum f. sp. *nicotianae*	Aldoxime dehydratase	Kato et al. (2000b)

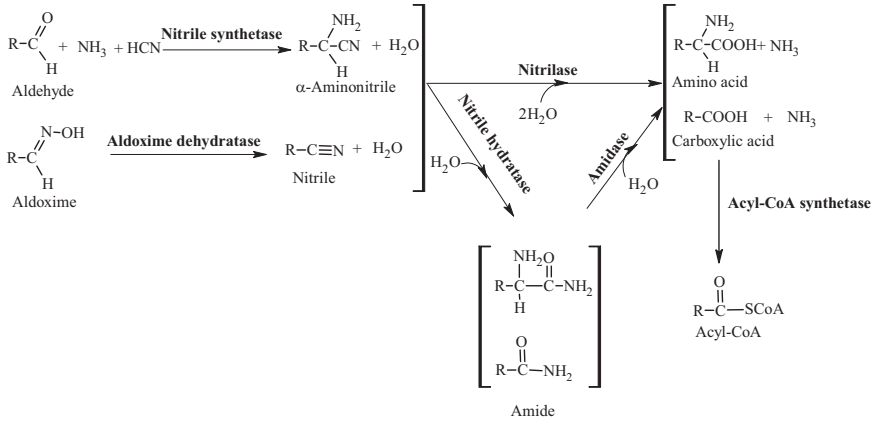


Fig. 32.1 Nitrile metabolism in microbes

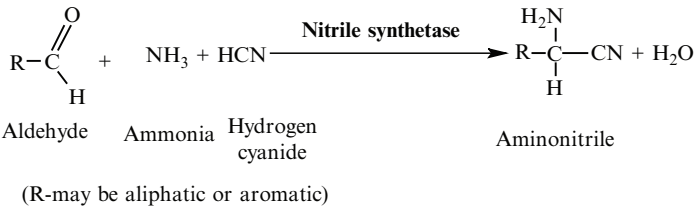
cyanobacteria and moulds have shown the potential for utilization of nitriles while only few bacteria and fungi have the capabilities to form nitriles.

The enzymes involved in nitrile metabolism have been shown in Fig. 32.1.

The natural substrates of these enzymes in the microbial system have not been precisely identified, however, the use of synthetic substrates immensely helped in characterization of various enzymes involved in nitrile metabolism and their industrial potential have been extensively studied (Yamada et al., 1986; Yamada, 1992; Zhou et al., 2005).

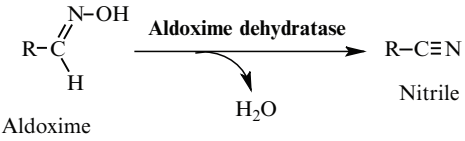
The enzymes implicated in nitrile metabolism are briefly described below:

1. *Nitrile synthetase*: This enzyme catalyses the formation of α-aminonitrile from aldehyde, ammonia and hydrogen cyanide as per following reaction:



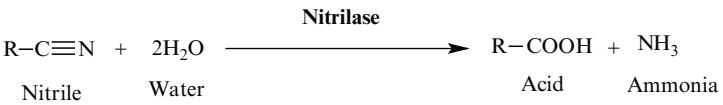
α-Aminonitriles are chemically synthesized by Strecker's method and used in the synthesis of both proteoinogenic and nonproteinogenic α-amino acids. This enzyme has been found in a psychrophilic basidiomycete (Strobel, 1966) and *Rhizoctonia soloni* and studied for the synthesis of α-aminopropionitrile and α-aminobutyronitrile respectively (Mundy and Liu, 1973).

2. *Aldoxime dehydratase (EC 4.99.1.7):* A novel heme-containing enzyme, aldoxime dehydratase has been reported recently (Asano and Kato, 1998; Kato et al., 2000a) which catalyses the conversion of aldoxime into nitrile as per reaction given below:



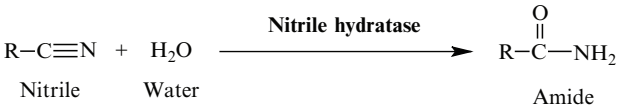
The discovery of this enzyme has lead to change the nomenclature of 'aldoxime-nitrile pathway' (Kato et al., 2005).

3. *Nitrilase (EC 3.5.5.1):* This is the first enzyme of nitrile metabolism reported from plants (barley leaves) (Thimann and Mahadeven, 1964) and is extensively studied in microbial systems. It hydrolyses nitriles to corresponding acid and ammonia:



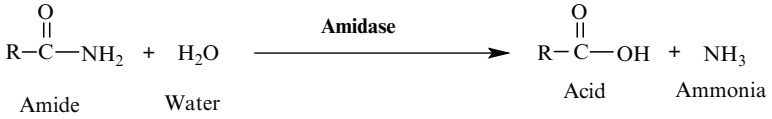
Initially it was thought that nitrilases act on aromatic nitriles only (Harper, 1977a, b; Bandopadhyay et al., 1986) but later on aliphatic nitrile specific nitrilases (Kobayashi et al., 1990; Gavagan et al., 1999; Levy-Schil et al., 1995) or nitrilases that catalyse the hydrolysis of both aliphatic and aromatic nitriles were discovered (Kobayashi et al., 1989; Dhillon et al., 1999; Bhalla et al., 1992). It has been widely explored for its potential to synthesize industrially important acids from nitriles (Godfredsen et al., 1985; Nishise et al., 1987; Yamamoto et al., 1990; Bhalla et al., 1992; Kobayashi and Shimizu, 1994; Wu and Li, 2003).

4. *Nitrile hydratase (EC 4.2.1.84)*: It is one of the very important enzyme of nitrile metabolism that converts nitriles to amide as per reaction given below:



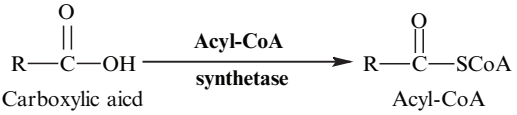
Nitrile hydratases have been reported from a number of microbes (Banerjee et al., 2002) and have been used for industrial production of acrylamide and nicotinamide (Nagasawa et al., 1993; Yamada and Kobayashi, 1996). Earlier it was assumed that the preferred substrates for nitrile hydratases are aliphatic nitriles but subsequently it has been shown that these enzymes can also hydrate aromatic and heterocyclic nitriles.

5. *Amidase (EC 3.5.1.4):* This enzyme hydrolyses amides to acid and ammonia:



In nitrile metabolism, amidase converts the amide formed by nitrile hydratase into acid and ammonia. Due to enantioselectivity, amidases have been studied for the production of optically active organic acids (Fournand and Arnaud, 2001; Banerjee et al., 2002).

6. *Acyl-CoA synthase.* This enzymes has been recently reported to be involved in the nitrile metabolism and converts carboxylic acid formed during hydrolysis of nitriles/amides to acyl-CoA (Hashimoto et al., 2005; Kato and Asano, 2006).



32.3 Nitrile Metabolizing Yeasts

Yeasts are single-celled (may form pseudohyphae or true hyphae) fungi mostly confining systematic position under ascomycetes and basidiomycetes and approximately 1,500 species of yeasts have been described (Kurtzman and Fell, 2006). They have been the integral part of food fermentation since the man learned that fermented foods (fruits, vegetables, milk, bread, beverages) have longer shelf life and better taste, flavor and nutritional value. Among the microbes, yeasts are considered to be safe (as these have been selected and continuously used for food processing since antiquity after prolonged hit and trial by man) and convenient systems (because of their unicellular structure, simple nutritional requirements, large public acceptability, easy genetic and molecular manipulations) (Stahl and Niederhaus, 2003). Yeasts are used in organic synthesis to catalyse the enantio- and regio- selective transformation of substrates to industrially and pharmaceutically important products (Kayser et al., 1999; Gervais et al., 2003).

As compared to bacteria, there are few yeasts so far reported to degrade nitriles. These have potential application and scope in the removal of cyano group from cyanoglucosides and cyanolipids in food materials (e.g. cassava and limabeans) or for carrying out biotransformation of nitriles and amides for the production of pharmaceutical (e.g. ibuprofen, lipitor) and food additives (e.g. nicotinamide, nicotinic acid). The nitrile metabolizing yeasts hitherto reported belong to basidiomycetes (e.g. *Aureobasidium pullulans, Cryptococcus* sp.) and ascomycetes (e.g. *Debaryomyces hanseii, Candida* sp.) groups (Table 32.2).

In the subsequent sections, isolation, culture conditions, characteristics of enzyme systems and biotransformation of nitrile and amide by nitrile metabolizing yeasts are described.

32.3.1 Isolation of Nitrile Metabolizing Yeasts

The nitrile metabolizing yeasts have been isolated from habitats as diverse as fermented foods (Prasad et al., 2005) and cyanide treatment bioreactor (Rezende et al., 1999; van der Walt et al., 1993; Brewis et al., 1995; Dias et al., 2000a). The nitrile degrading yeasts isolated from various habitats have been listed in Table 32.2. The enrichment culture techniques have been used to isolate yeasts from water samples collected from gold extraction circuits and cyanide treatment bioreactor (Rezende et al., 1999). These researchers have isolated around fifty two yeast strains belonging to *Candida, Debaryomyces, Aureobasidium, Geotrichum, Pichia, Rhodotorula, Tremella, Hanseniaspora and Cryptococcus* genera. van der Walt et al. (1993) have reported nitrile metabolizing activity in *Saccharomyces* and *Williopsis*. Most of the isolates used acetonitrile as the sole source of nitrogen and carbon (Rezende et al., 2004) or as nitrogen source (Dias et al., 2000a).

Table 32.2 List of nitrile metabolizing yeasts

Ascomycetes
i) Isolated from cyanide treatment bioreactor (Rezende et al., 1999; van der Walt et al., 1993; Brewis et al., 1995; Dias et al., 2000a)
Candida sp.
C. boidini
C. intermedia
C. pseudointermedia
C. vinaria
C. famata
C. guilliermondii
C. tropicalis
C. fabianii
Debaryomyces hanseii
Geotrichum sp.
Hanseniaspora sp.
Pichia kluyveri
Saccharomyces cerevisiae
Williopsis saturnus
ii) Isolated from soil (Fukuda et al., 1973; Rustler and Stolz, 2007)
Torulopsis candida
Exophiala oligosperma R1
iii) Isolated from traditional fermented foods and beverages (Thakur et al., 2004; Prasad et al., 2005)
Candida valida
Kluyveromyces thermotolerans
Saccharomyces sp.
Basidiomycetes
Isolated from cyanide treatment bioreactor (Rezende et al., 1999)
Aureobasidium pullulans
Cryptococcus sp.
Rhodotorula glutinis

A yeast (*Torulopsis candida* GH405) that catalysed the enantioselective conversion of DL-α hydroxynitrile to L-α hydroxyacids has been isolated from a field soil by enrichment culture using DL-α hydroxyvaleronitrile as the sole source of nitrogen. It also utilized acetonitrile, propionitrile, butyronitrile, DL- α- hydroxyisovaleronitrile and DL-α- hydroxyisocapronitrile as source of nitrogen (Fukuda et al., 1973). Prasad et al. (2005) screened forty yeast strains previously isolated from traditional fermented foods and beverages of Himachal Pradesh (Thakur et al., 2004) and detected nitrile hydrolyzing activity in seven isolates (five belonging to *Saccharomyces* genus, one each to *Kluyveromyces* and *Candida*).

Very recently a unique nitrile degrading acidotolerant black yeast (*Exophiala oligosperma* R1) has been isolated from the environment employing enrichment culture technique and it is considered to be of potential application in catalyzing the conversion of hydroxynitriles and aminonitriles (which are unstable at neutral pH) at low pH (Rustler and Stolz, 2007). In most of the isolations either acetonitrile or

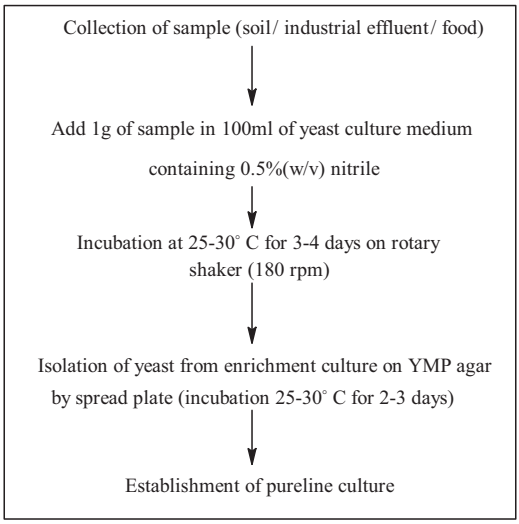


Fig. 32.2 Isolation of nitrile metabolizing yeasts

specific nitriles like α-hydroxyvaleronitrile (Fukuda et al., 1973) have been added in the isolation media to enrich the culture with nitrile metabolizing yeasts. A simple procedure for isolation of nitrile degrading yeasts from the environment is outlined in Fig. 32.2.

32.3.2 Culture Conditions for Cultivation of Nitrile Metabolizing Yeasts

The nitrile metabolizing yeasts have been isolated and reported from Japan, Brazil, India and Germany. The culture conditions employed by various groups working on nitrile utilizing yeasts have been summarized in Table 32.3. For isolation and cultivation of yeasts for exploring their nitrile metabolism, following parameters have been largely considered:

a) *Medium*: Most of the researchers have used different media for isolation and propagation of yeast for investigating nitrile metabolizing capabilities. For isolation of nitrile metabolizing yeasts, complex media like yeast nitrogen base (Difco) containing glucose (Linardi et al., 1996; Dias et al., 1996), YMPD (yeast extract, malt extract, peptone and dextrose) (Prasad et al., 2005) or complex media with inorganic salts have been employed. In order to isolate yeast with specific nitrile metabolizing activity for particular nitrile (e.g. hydrolysis

Table 32.3 Culture conditions for isolation and production of nitrile degrading enzymes of yeasts

S. No	Yeast	Isolation medium	Production Mmedium	pH	Temperature	Time/shaking condition
1.	*Candida famata*	Yeast nitrogen base (Difco) with 1% glucose and 10 mg % chloramphenicol	Yeast carbon base (Difco) with 12 mM acetonitrile	-	28°C	72h, gyratory 250 rpm
2.	*Torulopsis candida*	1% glucose, 0.01% yeast extract and inorganic salts, 0.1% DL-α-hydroxy-isovaleronitrile	1g test carbon compound, 0.15g $(NH_4)_2HPO_4$, 0.1g KH_2PO_4, 0.05g $MgSO_4$ $7H_2O$, 0.01g $CaCl_2.2H_2O$, 2 μg biotin per 100 ml	7.2	30°C	3 days, reciprocal
3.	*Kluyveromyces thermotolerans, Candida valida, Saccharomyces cerevisiae*	3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g dextrose, 15 g agar per liter	3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, 4 ml propionitrile	6.8	30°C	24h, gyratory 180 rpm
4.	*Exophiala oligosperma*	80% 500mg $MgSO_4$ $7H_2O$, 25 mg $CaCl_2$, 10mg Fe(III) citrate, 0.5 ml of trace element solution (SL6) (per liter) 1.6 % (w/v) agar, 20 % 500mM Na-citrate-phosphate buffer pH 4.0, containing 20 mM glucose, 0.2 % casamino acids and 2 mM phenylacetonitrile		4	30°C	72-96h, rotary 100 rpm

of the DL-α hydroxynitrile), the same nitrile had been added in the isolation medium (Fukuda et al., 1973). Some antibiotic like chloramphenicol can be added to inhibit the growth of prokaryotic microbe and to allow enrichment of culture with nitrile metabolizing yeasts. However, a more defined and buffered medium has been employed for the isolation of black yeast (*Exophiala oligosperma* R1) (Rustler and Stolz, 2007).

For cultivation of yeasts to study the nitrile metabolizing activities, nitriles like acetonitrile, propionitrile, phenylacetonitrile and DL-α hydroxynitriles have been invariably added in the medium (Table 32.3). The yeast cells cultured in medium containing 0.5% ammonium sulphate as the sole source of nitrogen could not hydrolyse DL-α- hydroxynitrile indicated that the enzymes involved in nitrile conversion in this organism were inducible (Fukuda et al., 1973). The addition of nitrile in culture medium is essential as in most of the microorganism hitherto reported, the nitrile metabolizing activities are inducible (Goldlust and Bohak, 1989; Bhalla et al., 1992) and very few organisms produce nitrile metabolizing enzymes constitutively (Kobayashi et al., 1989, 1990). The nitrile hydrolyzing system of *Kluyveromyces thermotolerans* MGBY 37 comprised constitutive nitrile hydratase-amidase enzymes. This organism exhibits two types of amidases i.e.

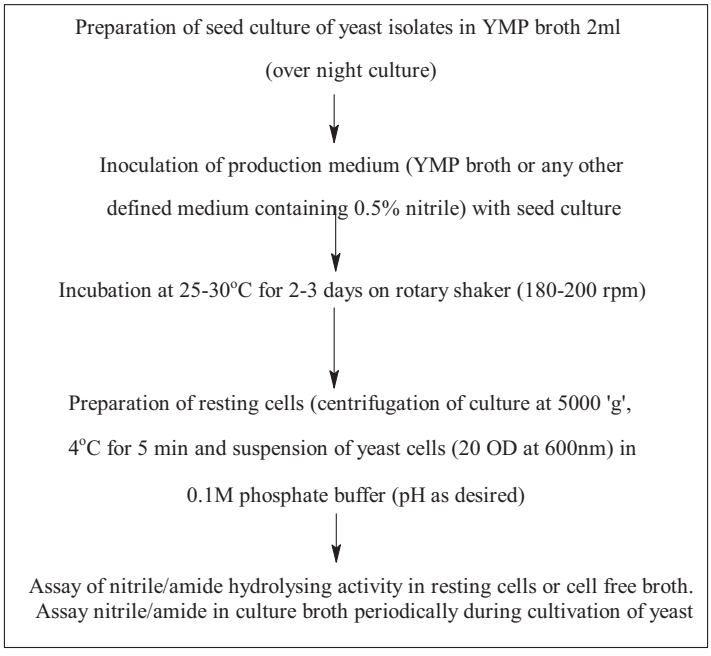


Fig. 32.3 Screening of yeast isolates for nitrile metabolizing activities

Table 32.4 Inducers supplemented in production medium for induction of nitrile metabolizing enzymes in microorganisms

Microorganisms	Inducer	Concentration	Reference
Alcaligenes faecalis	Isovaleronitrile	0.1% (v/v)	Mauger et al. (1990)
Fusarium oxysporum f sp. *melonis*	Benzonitrile	0.1% (v/v)	Goldlust and Bohak (1989)
Rhodococcus rhodochrous PA-34	Isobutyronitrile	0.5% (v/v)	Bhalla et al. (1992)
Nocardia globerula NHB2	Propionitrile	0.3% (v/v)	Sharma et al., (2006)
Rhodococcus equi A4	Methacrylamide	10 mM	Prepechalov et al. (2001)
Cryptococcus sp. UFMG-Y28	Benzonitrile	12 mM	Rezende et al. (1999)
Exophiala oligosperma R1	Phenylacetonitrile	2 mM	Rustler and Stolz (2007)
Kluyveromyces thermotolerans MGBY 37	Acetonitrile	0.4% (v/v)	Prasad et al. (2005)
Torulopsis candida	DL- -hydroxyisovaleronitrile	0.1% (v/v)	Fukuda et al. (1973)

\ constitutive amidase having affinity for N-heterocyclic aromatic, unsaturated and saturated aliphatic amides and inducible amidase with affinity for aromatic amide. Formamide is the best inducer for the latter type of amidase activity (Prasad et al., 2005). There is no one universal inducer of nitrile metabolizing enzymes and thus different organisms require different inducers (Table 32.4).

b) *pH, temperature and shaking conditions*

These parameters are as important for isolation and cultivation of nitrile metabolizing yeast as for the propagation of any other microorganism. The medium pH was 6.2 (Prasad et al., 2005), 7.2 (Fukuda et al., 1973) and 4.0 (Rustler and Stolz, 2007) and the selection of appropriate pH mostly depends on the pH of sample/habitat from which yeasts are to be isolated. The incubation temperature employed for isolation of yeasts and during production of nitrile degrading enzymes by yeasts varied from 25°C to 30°C (Rezende et al., 1999; Dias et al., 2000a; Rustler and Stolz et al., 2007). All the nitrile metabolizing yeasts reported so far are mesophilic in their temperature requirement and needed vigorous shaking for induction of nitrile degrading activities in the culture (Table 32.3). It appears that nitrile metabolism in yeast is operational under aerobic growth, though it needs further investigation.

The duration of incubation for the production of nitrile metabolizing activities in yeasts is 72–96 h which is comparatively longer as compared to well known nitrile hydrolyzing bacterial systems (Linardi et al., 1996; Ruslter and Stolz, 2007). 180–250 rpm gyratory or reciprocal shaking is required during cultivation of yeasts for the production of nitrile degrading enzymes. It has been proposed that oxygen

consumption is an estimate of enzyme activity (Nawaz et al., 1996) and a reliable parameter for assessing cell viability and physiology in the new environment i.e. utilizing nitriles. The results on oxygen consumption experiments conducted by Rezende et al. (1999) suggest that nitriles are biologically oxidizable substrates and yeasts may prove to be useful system for the detoxification or bioremediation of sites contaminated with nitriles and amides (Rezende et al., 1999).

32.3.3 Screening of Yeasts for Nitrile Metabolizing Activities

The yeasts isolated from specific habitats or procured from culture collection are usually screened to assess and confirm the type and level of nitrile metabolizing enzyme activities they have. Usually a preculture (seed culture) is first prepared and added to the production medium which may be any medium (defined or complex) that supports growth of yeasts and should contain low molecular weight aliphatic or aromatic nitrile (0.5–1.0% w/v acetonitrile/ benzonitrile or any other nitrile) that may act as inducer as well as sole source of carbon or nitrogen or both. Incubation is normally done in a rotary incubator shaker (180–200 rpm) for 2–3 days. The presence of nitrile in the medium may exert inhibitory effect on initial stage of growth of yeast possibly due to toxic effect of nitrile and to the fact that yeasts are not pre-exposed or adapted to this substrate (Rezende et al., 1999). However, *Candida famata* did not exhibit prolonged lag phase when grown in media containing high nitrile concentrations (Linardi et al., 1996).

The concentration of nitrile or its corresponding amide or acid in the culture is periodically determined that reveals nitrile metabolizing capacities of the yeast isolates. After the desired length of incubation, the yeast cells are sedimented by centrifugation and suspended in phosphate buffer and such a cell suspension is usually called as resting cell suspension. The activity of nitrile metabolizing enzymes (nitrilase/nitrile hydratase and amidase) is assayed in the resting cells either spectrophotometerically or using gas chromatography (GC) (Dias et al., 2001a) or high performance liquid chromatography (HPLC) (Rezende et al., 1999; Dias et al., 2000a). A simple strategy for screening of yeasts for exploring their nitrile metabolizing activities is summarized in Fig. 32.3. Methods for culture of yeasts and assay of nitrile hydrolyzing enzymes have been described by Rezende et al. (1999, 2004), Prasad et al. (2005) and Rustler and Stolz (2007).

32.3.4 Characteristics of Nitrile Metabolizing Activities of Yeast

The utilization of various nitriles and amides by yeast strains generally involves hydrolysis in two step reaction mediated by both inducible and intracellular nitrile hydratase and amidase. Whole cells and cell free extract have been used for conversion of nitrile or amide to amide or acid. Among the various enzymes of nitrile

metabolism (described above under section nitrile metabolism in microbes), the majority of the yeasts are endowed with nitrile hydratase and amidase activities while some of yeasts like *Torulopsis candida* (Fukuda et al., 1973) and *Exophiala oligosperma* R1 (Rustler and Stolz, 2007) harbor only nitrilase activity. The yeast *Cryptococcus* sp. UFMG-Y28 has nitrilase in addition to nitrile hydratase and amidase activities. Yeasts have specificity for aromatic nitriles while nitrilase of *Torulopsis candida* catalyzed the conversion of aliphatic hydroxynitriles to corresponding hydroxyacids.

Why yeasts have selected two enzyme system for metabolizing nitrile is rather difficult to answer. However, it appears that the amide (the product of nitrile hydratase activity) might be an important intermediate partly used in the synthesis of another metabolite through other pathway and the remaining amide enantioselectively converted to the desired acid as most of the amidases are enantioselective (Trott et al., 2001; Ciskanik et al., 1995) while majority of nitrile hydratases lack enantioselectivity.

The characteristics of nitrile metabolizing activities of yeast have been summarized in Table 32.5.

This information is based on transformation of nitrile or amide by whole cells and biochemical characteristics of purified nitrile metabolizing enzymes of yeasts are yet to be studied.

Table 32.5 Nitrile metabolizing enzyme systems in yeasts

Yeast	Enzyme system	Preferred substrate	Reference
Candida fabianii	Nitrile hydratase and amidase	3-cCyanopyridine	Brewis et al. (1995)
Candida famata	Nitrile hydratase and amidase	Saturated and unsaturated aliphatic nitriles and amides	Linardi et al. (1996)
Candida guilliermondii UFMG-Y65 (CCT7207)	Nitrile hydratase and amidase	Cycloalkylnitrile, arylnitrile and heterocyclic nitriles	Dias et al. (2000a, 2001a)
Cryptococcus flavus UFMG-Y61	Nitrile hydratase and amidase	Isobutyronitrile	Rezende et al. (1999)
Cryptococcus sp. UFMG-Y28	Nitrilase, nitrile hydratase and amidase	Benzonitrile (Nitrilase), propionitrile and acetonitrile	Rezende et al. (1999)
Exophiala oligosperma R1	Nitrilase	Phenylacetonitrile and 2-phenylpropionitrile	Rustler and Stolz (2007)
Geotrichum sp. JR1	Nitrile hydratase and amidase	Acetonitrile	Rezende et al. (2004)
Kluyveromyces thermotolerans MGBY 37	Nitrile hydratase and amidase	N-heterocyclic, aromatic nitriles and amides	Prasad et al. (2005)
Rhodotorula glutinis UFMG-Y5	Nitrile hydratase and amidase	Methacrylnitrile	Rezende et al. (1999)
Torulopsis candida	Nitrilase	DL-α-hydroxynitrile	Fukuda et al. (1973)

32.4 Transformation of Nitriles/Amides by Nitrile Metabolizing Yeasts

The nitrile metabolizing organisms have been investigated over these years mainly for two purposes: i) To find their applications in organic synthesis ii) To utilize their potential in bioremediation of nitrile/amide contaminated soil or water system or in management of effluent containing nitriles and amides. Comparatively more attention has been focused on the application of nitrile metabolizing organisms and their enzymes for substitution of chemical processes with enzymatic processes for conversion of nitriles and amides to corresponding amides and acids (Kobayashi et al., 1989, 1990).The transformation of acrylonitrile to acrylamide (Nagasawa et al., 1993; Yamada and Kobayashi, 1996) and 3-cyanopyridine to nicotinic acid (Nagasawa et al., 1988) is being carried out at industrial scale using *Rhodococcus rhodochrous* J1 nitrile hydratase and nitrilase respectively. Annually 95000 tonnes of acrylamide (Mitsubishi Rayon, Japan) and 6000 tonnes of nicotinamide (Lonza AG, Switzerland) in being produced through enzymatic processes. As compared to bacterial systems, less work has been done on the conversion of nitriles to amides or acids employing yeasts. However, studies carried out so far, have revealed fairly good potential of nitrile metabolizing yeasts for transformation of nitriles. In the following section, biotransformation of nitriles and amides employing free and immobilized cells of yeasts is described.

32.4.1 Biotransformation of Nitriles and Amides with Free Cells of Yeasts

The transformation of nitriles and amides by yeasts strains is mediated by the sequential action of nitrile hydratase and amidase. The yeast cells are mostly cultured in medium containing nitriles, so that significant level of the nitrile degrading enzymes are synthesized in the cells and the cells are sedimented and suspended in phosphate buffer. Since enzymes are intracellular and quite often involves two enzymes (nitrile hydratase and amidase), it is very convenient and economical to carry out transformation reactions using whole cells.

The studies pertaining to exploration of yeasts for transformation of nitriles have revealed that yeasts can convert a range of nitriles and amides to corresponding acids (Table 32.6).

Among the nitriles, aliphatic nitriles (acetonitrile, butyronitrile, propionitrile, methacrylamide, succinonitrile and isobutyronitrile) have been the preferred substrates for nitrile metabolizing enzymes of *C. guillermondii* CCT 7207 and out of various amides tested, only aliphatic amides (acetamide and isobutyramide) could be hydrolyzed by this yeasts (Dias et al., 2001a). Aromatic (benzonitrile) and heterocyclic (2-cyanopyridine, 3-cyanopyridine and 4-cyanopyridine) nitriles were not hydrolyzed by the free cells of *C. guilliermondii* CCT7207 (Dias et al., 2001a).

Table 32.6 List of some nitrile/amides transformed by yeasts

Substrate	Product*	Enzyme	Reference
DL-α-hydroxyisovaleronitrile	L-α-hydroxyisovaleric acid	Nitrilase	Fukuda et al. (1973)
DL-α-hydroxyisocapronitrile	L-α-hydroxyisocaproic acid	Nitrilase	
Acetonitrile	Acetamide, acetic acid	Nitrile hydratase-amidase	Prasad et al. (2005), Rezende et al. (2004), Linardi et al. (1996), Rezende et al. (1999)
Benzonitrile	Benzamide, benzoic acid	Nitrile hydratase-amidase	Dias et al. (2000a), Prasad et al. (2005)
3-Cyanopyridine	Nicotinamide, nicotinic acid	Nitrile hydratase-amidase	Dias et al. (2000a), Prasad et al, (2005)
Butyronitrile	Butyramide, butyric acid	Nitrile hydratase-amidase	Dias et al. (2000a)
Adiponitrile	Adipamide, adipic acid	Nitrile hydratase-amidase	
2-Cyanopyridine	Pyridine-2-carboxyamide, 2-picolinic acid	Nitrile hydratase-amidase	
4-Cyanopyridine	Isonicotinamide, isonicotinic acid	Nitrile hydratase-amidase	
Succinonitrile	Succinamide, succinic acid	Nitrile hydratase-amidase	
Glutaronitrile	Glutaramide, glutaric acid	Nitrile hydratase-amidase	
Glutaramide	Glutaric acid	Amidase	
Succinamide	Succinic acid	Amidase	
Adipamide	Adipic acid	Amidase	
Isobutyronitrile	Isobutyramide, isobutyric acid	Nitrile hydratase-amidase	Dias et al. (2000a), Rezende et al. (2004)
Methacrylonitrile	Methacrylamide, methacrylic acid	Nitrile hydratase-amidase	
Propionitrile	Propionamide, Propionic acid	Nitrile hydratase-amidase	
Phenylacetonitrile	Phenylacetic acid	Nitrilase	Rustler and Stolz (2007)
2-Chlorophenylacetonitrile	2-cChlorophenylacetic acid	Nitrilase	
3-Chlorophenylacetonitrile	3-cChlorophenylacetic acid	Nitrilase	
4-Chlorophenylacetonitrile	4-cChlorophenylacetic acid	Nitrilase	
4-hydroxyphenylacetonitrile	4-hHydroxyphenylacetic acid	Nitrilase	
Indole -3-acetonitrile	Indole-3-acetic acid	Nitrilase	
Acetamide	Acetic acid	Amidase	Prasad et al. (2005), Linardi et al. (1995), Rezende et al. (1999)
Acrylamide	Acrylic acid	Amidase	Prasad et al. (2005)
Nicotinamide	Nicotinic acid	Amidase	
Benzamide	Benzoic acid	Amidase	
Acrylonitrile	Acrylamide, acrylic acid	Nitrile hydratase-amidase	
Isobutyramide	Isobutyric acid	Amidase	Rezende et al. (1999)
Propionamide	Propionic acid	Amidase	
Methacrylamide	Methacrylic acid	Amidase	

*In many cases product listed has been deduced from the release of ammonia by the action of enzymes on nitrile

The free and resting cells of *Cryptococcus* sp. UFMG-Y28 effectively hydrolyzed acetonitrile (97 mM) and propionitrile (120 mM) and their corresponding amides while *Cryptococcus flavus* UFMG-Y61, *Rhodotorula glutinis* transformed isobutyronitrile (120 mM) and methacrylonitrile (48 mM) or their amides into organic acids respectively (Rezende et al., 1999). Most of the biocatalysis and biotransformation reactions have been carried out with free or immobilized whole cells. However, Rezende et al. (1999) used cell free extract of *Cryptococcus* sp. and *Rhodotorula glutinis* for the hydrolysis of nitriles and amides and reported that propionitrile and isobutyronitrile are better substrates than other nitriles.

The nitrile and amide hydrolyzing activity of *Kluyveromyces thermotolerans* MGBY 37 has exhibited more affinity for conversion of heterocyclic (3-cyanopyridine) and aromatic (benzonitrile) nitriles to respective organic acid as compared to saturated and unsaturated nitriles. The rate of its amidase catalysed transformation of heterocyclic amide (nicotinamide) to corresponding acid (nicotinic acid) was about five to ten times higher than that of benzamide, acrylamide and acetamide (Prasad et al., 2005).

The application of biocatalysts in organic synthesis or biotransformation depends on their extent of activity, stability and enantioselectivity. The information available in literature indicates that nitrile degrading enzymes of yeasts exhibit moderate activity, fairly high stability and substrate tolerance as compared to their counterparts from prokaryotic sources. However, not much work has been carried out on the enantioselectivity of nitrile metabolizing enzymes of yeasts. There is only one report in which resting cells of *Torulopsis candida* enantioselectivity converted 50% of DL-α- hydroxyvaleronitrile and 30% of DL-α- hydroxyisovaleronitrile to L- α- hydroxyisovaleric acid and L-α- isocaproic acid (Fukuda et al., 1973).

The nitrilase of acidotolerant black yeast-*Exophiala oligosperma* R1 exhibited regiospecificity. A number of aromatic nitriles were tested for their conversion with cell free extract of this yeast and maximum activity was observed with 3-chlorophenylacetonitrile which was about 4 and 3 times higher than the nitrilase activities recorded with 2-chlorophenylacetonitrile and 4-chlorophenylacetonitrile respectively (Rustler and Stolz, 2007). From biotransformation point of view, *Geotrichum* sp. JR1 has shown very good potential as this yeast strain can grow in the presence of acetonitrile concentration as high as 2M (Rezende et al., 2004) and thus may prove to be useful system for detoxification or bioremediation of sites contaminated with nitriles and amides.

32.4.2 Biotransformation of Nitriles and Amides with Immobilized Cells of Yeasts

Immobilization of enzymes and cells is increasingly gaining importance to make bioprocess more economical as biocatalysts can be recycled till the operational cost of the process remains much below the value of product recovered. Most of the

bioconversions of nitriles have been carried out using free cells and little information is available on the use of immobilized microbes for transformation of nitriles (Dias et al., 2000b; Takagi et al., 1994; Nawaz et al., 1993). Among the various methods available for immobilization of cells (Chibata, 1978), yeasts have been immobilized by entrapment in alginate, κ-carageenan and citric pectin (Dias et al., 2000b). Alginate immobilized cells of *Candida guilliermondii* UMFG-Y65, degraded acetonitrile more efficiently than κ-carrageenan or citric pectin immobilized cells (Dias et al., 2000b).Since calcium alginate beads are unstable in phosphate buffer (commonly used buffer in reactions comprising bioconversion of nitriles), barium chloride has been employed for cross-linking of alginate to provide better stability to the resultant beads during biotransformation reaction (Dias et al., 2001a). These researchers have studied the impact of alginate and barium chloride concentration on bead formation and worked out optimum concentration of alginate and barium chloride for immobilization of yeast cells. As expected, the oxygen uptake rate (OUR) of yeasts cells decreases upon immobilization which becomes more pronounced with increase in alginate concentration in beads (Dias et al., 2001b).

It is worth mentioning that upon immobilization, yeasts cells were able to hydrolyze some additional nitriles (acrylonitrile, Adiponitrile, benzonitrile, 2-cyanopyridine, 3-cyanopyridine, 4-cyanopyridine and glutaronitrile) and amides (adipamide, acrylamide, benzamide, glutaramide, succinamide) which free cells could not (Dias et al., 2001b). This shift in substrate affinity of yeast cells upon immobilization can be explained on the basis of changed microenvironment in the matrix around the yeast cells that favors transport of such nitriles and amides into the cells. The use of immobilized yeast cells thus seems to have better scope for application in biotransformation of toxic nitriles and for the production of organic amides and acids.

32.5 Conclusions

Nitrile metabolism in yeasts has been well studied but not as extensively as in prokaryotic systems. The nitrile metabolizing enzymes of yeasts need to be purified and characterized in terms of stability, substrate specificity and enantioselectivity. More efforts are required to clone the genes coding nitrile-degrading enzymes and decode their nucleotide and amino acid sequences vis-à-vis control elements involved in expression. For making yeast systems economical and viable alternatives for applications in organic synthesis or detoxification of nitrile and amide polluted soil, water or air, immobilization of whole cells or purified enzymes of yeasts in matrices which are stable in buffer used for biocatalysis is essentially needed to be explored further. Above all, yeasts are more friendly and acceptable to man than many other microbial systems, and therefore, more habitats ought to be screened to find yeasts for the desired applications. It is expected that in years ahead research on nitrile metabolizing yeasts will address some of these aspects.

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