

Edited by
Karl Esser

THE MYCOTA

A Comprehensive Treatise on Fungi
as Experimental Systems for Basic and Applied Research

Industrial Applications **X**

Second Edition

Martin Hofrichter
Volume Editor

 Springer

The Mycota

Edited by
K. Esser

The Mycota

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The Mycota

A Comprehensive Treatise
on Fungi as Experimental Systems
for Basic and Applied Research

Edited by K. Esser

X *Industrial Applications*
2nd Edition

Volume Editor:
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**Karl Esser**

(born 1924) is retired Professor of General Botany and Director of the Botanical Garden at the Ruhr-Universität Bochum (Germany). His scientific work focused on basic research in classical and molecular genetics in relation to practical application. His studies were carried out mostly on fungi. Together with his collaborators he was the first to detect plasmids in higher fungi. This has led to the integration of fungal genetics in biotechnology. His scientific work was distinguished by many national and international honors, especially three honorary doctoral degrees.

**Martin Hofrichter**

(born 1966) studied biology (with a focus on microbiology, bioorganic chemistry and ecology) at the Friedrich Schiller University of Jena, Germany. His doctoral thesis dealt with the degradation of aromatic compounds by the mold *Penicillium frequentans*. After completing his PhD in 1994 he worked on the catalytic system of manganese peroxidase produced by basidiomycetous fungi in Jena and Helsinki (Finland) and became a lecturer at the University of Jena in 1999. In the years 2000 and 2001 he worked at the University of Helsinki on the fungal decomposition of lignin, humic materials and organopollutants and became a Senior Research Fellow of the Academy of Finland. He became a Professor for Environmental Biotechnology at the International Graduate School of Zittau (Germany) in December 2001 and head of the Department of Bio- and Environmental Sciences in 2010. His research stays include the University of La Plata (Argentina), the Vietnamese Academy of Science and Technology and the Al-Farabi University of Almaty (Kazakhstan). His research examines various aspects of secreted fungal enzymes, with special emphasis on aromatic peroxygenases recently discovered in his lab.

Series Preface

Mycology, the study of fungi, originated as a sub discipline of botany and was a descriptive discipline, largely neglected as an experimental science until the early years of this century. A seminal paper by Blakeslee in 1904 provided evidence for self incompatibility, termed “heterothallism”, and stimulated interest in studies related to the control of sexual reproduction in fungi by mating-type specificities. Soon to follow was the demonstration that sexually reproducing fungi exhibit Mendelian inheritance and that it was possible to conduct formal genetic analysis with fungi. The names Burgeff, Kniep and Lindegren are all associated with this early period of fungal genetics research.

These studies and the discovery of penicillin by Fleming, who shared a Nobel Prize in 1945, provided further impetus for experimental research with fungi. Thus began a period of interest in mutation induction and analysis of mutants for biochemical traits. Such fundamental research, conducted largely with *Neurospora crassa*, led to the one gene: one enzyme hypothesis and to a second Nobel Prize for fungal research awarded to Beadle and Tatum in 1958. Fundamental research in biochemical genetics was extended to other fungi, especially to *Saccharomyces cerevisiae*, and by the mid-1960s fungal systems were much favored for studies in eukaryotic molecular biology and were soon able to compete with bacterial systems in the molecular arena.

The experimental achievements in research on the genetics and molecular biology of fungi have benefited more generally studies in the related fields of fungal biochemistry, plant pathology, medical mycology, and systematics. Today, there is much interest in the genetic manipulation of fungi for applied research. This current interest in biotechnical genetics has been augmented by the development of DNA-mediated transformation systems in fungi and by an understanding of gene expression and regulation at the molecular level. Applied research initiatives involving fungi extend broadly to areas of interest not only to industry but to agricultural and environmental sciences as well.

It is this burgeoning interest in fungi as experimental systems for applied as well as basic research that has prompted publication of this series of books under the title *The Mycota*. This title knowingly relegates fungi into a separate realm, distinct from that of either plants, animals, or protozoa. For consistency throughout this Series of Volumes the names adopted for major groups of fungi (representative genera in parentheses) areas follows:

Pseudomycota

Division: Oomycota (*Achlya*, *Phytophthora*, *Pythium*)
Division: Hyphochytriomycota

Eumycota

Division: Chytridiomycota (*Allomyces*)
Division: Zygomycota (*Mucor*, *Phycomyces*, *Blakeslea*)

Division:	Dikaryomycota
Subdivision:	Ascomycotina
Class:	Saccharomycetes (Saccharomyces, Schizosaccharomyces)
Class:	Ascomycetes (Neurospora, Podospora, Aspergillus)
Subdivision:	Basidiomycotina
Class:	Heterobasidiomycetes (Ustilago, Tremella)
Class:	Homobasidiomycetes (Schizophyllum, Coprinus)

We have made the decision to exclude from The Mycota the slime molds which, although they have traditional and strong ties to mycology, truly represent nonfungal forms insofar as they ingest nutrients by phagocytosis, lack a cell wall during the assimilative phase, and clearly show affinities with certain protozoan taxa.

The Series throughout will address three basic questions: what are the fungi, what do they do, and what is their relevance to human affairs? Such a focused and comprehensive treatment of the fungi is long overdue in the opinion of the editors.

A volume devoted to systematics would ordinarily have been the first to appear in this Series. However, the scope of such a volume, coupled with the need to give serious and sustained consideration to any reclassification of major fungal groups, has delayed early publication. We wish, however, to provide a preamble on the nature of fungi, to acquaint readers who are unfamiliar with fungi with certain characteristics that are representative of these organisms and which make them attractive subjects for experimentation.

The fungi represent a heterogeneous assemblage of eukaryotic microorganisms. Fungal metabolism is characteristically heterotrophic or assimilative for organic carbon and some nonelemental source of nitrogen. Fungal cells characteristically imbibe or absorb, rather than ingest, nutrients and they have rigid cellwalls. The vast majority of fungi are haploid organisms reproducing either sexually or asexually through spores. The spore forms and details on their method of production have been used to delineate most fungal taxa. Although there is a multitude of spore forms, fungal spores are basically only of two types: (i) asexual spores are formed following mitosis (mitospores) and culminate vegetative growth, and (ii) sexual spores are formed following meiosis (meiospores) and are borne in or upon specialized generative structures, the latter frequently clustered in a fruit body. The vegetative forms of fungi are either unicellular, yeasts are an example, or hyphal; the latter may be branched to form an extensive mycelium.

Regardless of these details, it is the accessibility of spores, especially the direct recovery of meiospores coupled with extended vegetative haploidy, that have made fungi especially attractive as objects for experimental research.

The ability of fungi, especially the saprobic fungi, to absorb and grow on rather simple and defined substrates and to convert these substances, not only into essential metabolites but into important secondary metabolites, is also noteworthy. The metabolic capacities of fungi have attracted much interest in natural products chemistry and in the production of antibiotics and other bioactive compounds. Fungi, especially yeasts, are important in fermentation processes. Other fungi are important in the production of enzymes, citric acid and other organic compounds as well as in the fermentation of foods.

Fungi have invaded every conceivable ecological niche. Saprobian forms abound, especially in the decay of organic debris. Pathogenic forms exist with both plant and animal hosts. Fungi even grow on other fungi. They are found in aquatic as well as soil environments, and their spores may pollute the air. Some are edible; others are

poisonous. Many are variously associated with plants as copartners in the formation of lichens and mycorrhizae, as symbiotic endophytes or as overt pathogens. Association with animal systems varies; examples include the predaceous fungi that trap nematodes, the micro fungi that grow in the anaerobic environment of the rumen, the many insect associated fungi and the medically important pathogens afflicting humans. Yes, fungi are ubiquitous and important.

There are many fungi, conservative estimates are in the order of 100,000 species, and there are many ways to study them, from descriptive accounts of organisms found in nature to laboratory experimentation at the cellular and molecular level. All such studies expand our knowledge of fungi and of fungal processes and improve our ability to utilize and to control fungi for the benefit of humankind.

We have invited leading research specialists in the field of mycology to contribute to this Series. We are especially indebted and grateful for the initiative and leadership shown by the Volume Editors in selecting topics and assembling the experts. We have all been a bit ambitious in producing these Volumes on a timely basis and there in lies the possibility of mistakes and oversights in this first edition. We encourage the readership to draw our attention to any error, omission or inconsistency in this Series in order that improvements can be made in any subsequent edition.

Finally, we wish to acknowledge the willingness of Springer-Verlag to host this project, which is envisioned to require more than 5 years of effort and the publication of at least nine Volumes.

Bochum, Germany
Auburn, AL, USA
April 1994

KARL ESSER
PAUL A. LEMKE
Series Editors

Addendum to the Series Preface

During the Fourth International Mycological Congress in Regensburg (1989) while relaxing in a beer garden with Paul Lemke (USA), Dr. Czeschlik (Springer-Verlag) discussed with us the possibility to publish a series about Fungi. We both were at first somewhat reserved, but after a comprehensive discussion this idea looked promising. We decided to name this new series *The Mycota*.

Then Paul Lemke and I created a program involving seven volumes covering a wide area of Mycology. The first volume was presented in 1994 at the Fifth International Mycological Congress in Vancouver (Canada). The other volumes followed step by step. After the early death of Paul Lemke (1995) I proceeded alone as Series Editor. However for Vols. X-XII I received support by Joan Bennett.

Since evidently the series was well accepted by the scientific community and since the broad area of Fungi was not completely covered, it was decided to proceed with eight more volumes. In addition, second editions of eight volumes were published.

I would like to thank Springer-Verlag, represented by Drs. Czeschlik and Schlitzberger for their support and cooperation.

Bochum, Germany
May 2010

KARL ESSER

Volume Preface to the Second Edition

For more than 5000 years mankind has been using fungi in the production of beverages and food, and the basics of these techniques are nowadays still the same as in ancient times. Thus already the Sumerians and Egyptians made use of yeasts to make beer, wine and bread. Fruiting bodies of basidiomycetes and ascomycetes, colloquially called mushrooms, have contributed to man's alimentation and medicine for centuries. While these mushrooms were initially still collected in forests, they were later cultivated on a larger scale in different regions of the world. Today, these traditional processes are performed on an industrial scale along with the manufacturing of the products of modern fungal biotechnology, such as organic acids, antibiotics, secondary metabolites and biotransformation products, as well as enzymes. Furthermore fungi and their enzymes can be applied for the processing of lignocelluloses and textiles or for bioremediation purposes. This issue of *The Mycota* deals with all these applications of fungi and tries to give an overview on recent achievements in this sector, not least against the background of rapidly developing white biotechnology and the growing field of modern molecular biology. In this context, the second edition of this book both tries to continue the excellent concept of the first edition by updating respective chapters and to introduce fresh ideas by supplementing new chapters.

The first section of the book (Chapters 1–4), describes the state of the art in the production and conservation of food and beverages using traditional processes of industrial mycology. In this context, both classic Oriental and European products such as beer, wine, cheese and bread as well as Asian fermented food like tofu, tempe or soy sauce are dealt with. These products are produced on a scale of millions of tonnes and represent the largest market for fungal biotechnology. Chapter 4 addresses the cultivation of edible mushrooms with focus on the production of fruiting bodies from lignocellulose-degrading basidiomycetes. So far, mushroom cultivation is the most economically successful method for bioprocessing lignocelluloses and many commercial systems operate throughout the world.

The second section (Chapters 5–13) is devoted to the production of fungal metabolites and enzymes representing value-added products used in medicine, agriculture or food and textile industry. In addition to metabolites such as β -lactams (Chapter 5) and other antibiotics (Chapter 6), ergot alkaloids (Chapter 9), citric and other organic acids (Chapter 10) as well as vitamins (Chapter 11), all of which have successfully been in use for decades, this section is also dedicated to promising fungal metabolites, such as new insecticidal and nematocidal metabolites (Chapter 7) and immunosuppressants (Chapter 8), which may be exploited in the near future. Chapter 12 deals with mushroom flavors and is probably the most comprehensive compilation on this topic published over the past ten years. I am particularly pleased about the contribution of the world's leading enzyme manufacturer, Novozymes A/S, to this book (Chapter 13), which deals with industrial enzymes from fungi, their large-scale production and application.

Section three (Chapters 14–18) presents the state of the art and recent developments in fungal biotransformation of small molecules, the bioconversion and treatment of lignocelluloses and the use of fungi in metal recovery. The use of fungi for whole-cell biotransformations is a well-developed field of fungal biotechnology and enables or facilitates access to complex bioactive compounds – both commercial drugs and candidates – due to their capacity to catalyze regio- and stereoselective reactions on small molecules. Numerous examples are presented in Chapter 14 and show that traditional biotransformation remains a viable and feasible option to derivatize natural products in a specific way. Lignocelluloses represent the major resource of renewable organic matter and their gentle, i.e. preferably biological disintegration will be a prerequisite for the development of suitable technologies converting them to chemical feedstock, for example, second-generation biofuels. To reach this ambitious goal, a profound knowledge of the underlying biochemical reactions is required. Therefore Chapter 15 focuses, in first place, on the basics of lignocellulose degradation by white-rot and brown-rot fungi. Though not all details of these radical mediated processes have been elucidated so far, important progress has been made towards the understanding of their enzymatic basis over the last years. Powerful hydrolytic enzymes (cellulases, xylanases) are produced by the model fungus *Aspergillus niger* and their production, optimization and application is described in Chapter 16. Not least, the removal of pitch deposits from pulp, disturbing the paper-making process, by lipases is a good example for the introduction of an innovative fungal product to the market (Chapter 17). The final chapter of this section shows that fungal biomass can be used to accumulate and remove different cations, which makes it promising for recovering valuable metals from dilute solutions and purify waste water.

The final section (Chapters 19–22) introduces some innovative recent developments in the field of applied mycology, among others, the preparation of fungal bioherbicides (Chapter 19), recent genomic approaches for the identification of biopolymer degrading enzymes (Chapter 20), new trends in using oxidative enzymes from fungi in chemical synthesis (Chapter 21) and new attempts to transfer fungal remediation technologies into practice (Chapter 22).

The new impetus given to fungal biotechnology in recent years has come from the rapid developments in genetic engineering which introduce the possibility of tailoring organisms: (i) to optimize the production of established or novel metabolites and enzymes of commercial importance and (ii) to transfer genetic material from one organism to another. In particular, Chapters 10, 16 and 20 take these developments in different fields into account.

From the onset of the conception of this book, it was obvious that the range of topics we wished to include virtually necessitated drawing on the expertise of a considerable number of scientists from different countries. I was aware that the adoption of such a multi-author format entailed risks that might impact negatively on the uniformity and timetable for completion of the book. However, when this book is viewed in its totality, I believe that the overall quality and usefulness of its contents vindicates the decision to ask colleagues from all over the world to contribute to this book.

I am very grateful to the many authors and experts in the field of applied mycology and fungal biotechnology who wrote chapters for this book. Their expertise, enthusiasm and the costly time which they devoted to their writing is highly appreciated.

Last but not least, I would like to express my gratitude to the series editor Karl Esser and Andrea Schlitzberger from the publisher Springer for their excellent support and the customary professionalism.

Volume Preface to the First Edition

The fungi, including yeast and mycelial species, are a very heterogeneous group of eukaryotic microorganisms with a broad potential of applications in various fields of industrial mycology. The use of yeast in food and beverage production dates back to ancient times. Thousands of years before Christ, the Sumerians, Babylonians and Egyptians used yeast to produce bread and alcoholic beverages. Today, yeast and other fungi are also the basic organisms used for the production, processing and conservation of food, both at the household and the industrial scale. The production of antibiotics and chemicals is another more recent field in which fungi play a paramount role. Today, from the economic point of view, fungi dominate biotechnology. It can be expected that this will not change in the near future since there is a growing interest to use these organisms for various novel applications.

In this issue of *The Mycota*, an overview is provided on both the state of the art in traditional fields of industrial mycology as well as on the evaluation of novel applications of fungi in agriculture, environmental biology and medicine. Chapters 1–3 are devoted to the traditional use of fungi in the production and processing of food and beverages. Different aspects dealing with the specific needs to control the quality of products, to improve production processes and to introduce novel products on the market are dealt with.

In the second section of the book, an overview of different fungal metabolites and enzymes is given. This is a huge field of applied mycology. Therefore, only some examples from different groups of compounds are provided, emphasizing the wide range of valuable substances produced by fungi. First, metabolites directed against bacteria as well as against insects and nematodes are introduced in Chapters 4–6. Whereas the first group of metabolites contains the classical antibiotics which have been so efficiently used in medicine over more than half a century, the latter substances have not developed into commercial products, but have important potential as plant protectants. Chapters 7 and 8 are devoted to the pharmaceutically important agents, immunosuppressants and the ergot alkaloids. In particular, the use of the former to prevent the rejection of transplanted organs is of prime relevance in the corresponding field of modern medicine. The search for improved substances and the development of improved strains following classical and modern strategies is currently the subject of intensive investigations. The same is true for the production of ergot alkaloids, which are produced as the result of the complex interaction between a fungus and a higher plant. Chapter 9 summarizes current knowledge about plant hormones produced by fungi. The potential applications of plant hormones in agriculture and plant protection are discussed. The production of organic acids is another “classical” field of applied mycology. It is introduced in Chapter 10, which focuses on citric acid, the major organic acid produced on an industrial scale. Chapters 11 and 12 deal with vitamins as essential micronutrients and with carotinoids, important food supplements. As in the production of other compounds,

these compounds will only be produced via fungal fermentation if the costs are competitive with the chemical production. A detailed knowledge of the biochemistry and molecular biology underlying the synthesis of these compounds is thus important. The last chapter of this section (Chapter 13) deals with enzymes, another group of compounds traditionally produced by fungi. In this chapter, emphasis is on plant cell degrading enzymes which have wide applications in the food and feed as well as in the paper and pulp industry.

In the next section of this book, Chapters 14–18 focus on the use of fungi for converting different substrates or for recovering substances from solutions, Chapter 14 deals with the use of fungi for catalyzing specific reactions, leading to the conversion of a certain substrate to a specific product. This field of applied mycology is well developed. However, there still remains a huge potential for improvement since many biocatalytic activities of fungi are unknown or are not used economically. Many possibilities to improve strains and processes can be envisaged, Chapters 15–17 summarize developments to utilize the ability of many fungi to disassemble complex polymers like lignin, cellulose and coal. These processes are very important for recycling organic material and are of particular industrial significance, e.g. in paper production, Biosolubilization of low-rank coal to a fluid form for transportation is another example. However, although in principle possible, the economic use of this approach remains to be demonstrated. Chapter 18 deals with the ability of fungi to remove different metals from dilute solutions. In principle, biosorption is a suitable approach to purify wastewater and recycle it efficiently.

The last three chapters of this Volume (Chapters 19–21) present examples of more recent concepts which, in the near future, may have an important impact on industrial mycology. Chapter 19 summarizes the strategies and developments in the field of weed control. The goal is the controlled damage of weed populations by fungal plant pathogens. From this chapter it becomes clear that currently there are a variety of problems and constraints that need to be overcome before bioherbicides will be able to compete with chemical herbicides. The genetic engineering of “unsuitable” pathogens into effective biocontrol agents seems to be one promising strategy. In addition to the construction of suitable bioherbicides, their conservation is another important practical issue. Chapter 20 deals with the problem of developing mycoherbicides. Finally, Chapter 21 provides an overview of the concept to use mycelial fungi as biological systems to produce heterologous proteins. The use of specific species, for which a huge body of experience exists and which are classified as being safe, appears to be very promising. Within the next decade this concept may open new avenues in industrial mycology.

I hope that, although additional topics could have been included in this issue, the selection of issues presented here provides a good overview of both traditional as well modern and future aspects of applied mycology. I also hope that this Volume will be of interest not only to researchers working in the immediate field of industrial mycology, but also to biologists and graduate students wishing to acquire an overview of the potential applications of fungi in the various fields of life sciences.

Finally, I would like to express my gratitude to all authors for their important contributions to this book.

Frankfurt, Germany
July 2001

H.D. OSIEWACZT
Volume Editor

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Traditional Food and Beverage Fermentation

1 Production of Bread, Cheese and Meat

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I. Introduction

Historic references to the fermentation of dough for baking and the fermentation of beer originate from the Sumerians and the Babylonians and, under the Pharaohs in ancient Egypt, the brewing of beer was a trade (Jørgensen 1948). At that time, the fermentation of bread was achieved by using a mixture of yeast and lactic acid bacteria maintained in a dough medium. After each fermentation, a portion of the dough was retained for starting the next batch or a close connection with beer brewing was established so that surplus yeast from breweries was used for production of bread. These same methods are still used in certain regions in Africa and probably other parts of the

world, where ancient technologies have survived and can be experienced today. In the industrialised part of the world, these methods remained in use and did not change until late in the eighteenth century when yeast was first propagated for direct use in bread making in the Netherlands by the so-called Dutch method, which had a very low efficiency. As a result of the work of Louis Pasteur and the Danish botanists Emil Christian Hansen and Alfred Jørgensen and others in the late nineteenth century, the role of oxygen in yeast propagation was realised, the anaerobic condition of fermentation ("life without oxygen") was understood, *Saccharomyces cerevisiae* was described and the use of pure cultures was introduced. This was a very significant breakthrough for the industrialised production of baker's yeast. A similar process improvement followed in 1920, with the introduction of the "fed-batch" process. In this process, sugar is fed incrementally during yeast propagation, avoiding repressions and leading to increased biomass production. It forms the basis of commercial processes used today for manufacturing baker's yeast and has developed into a highly centralised industry offering a cheap bulk commodity. This is contrary to the historical development of other industrial yeast cultures like brewer's yeast (Jørgensen 1948). For reviews on the history of baker's yeast, see Rose and Vijayalakshmi (1993) and Jenson (1998).

In cheese, the role of yeast is not yet fully understood, but the yeast seems to take part in several microbial interactions important for the fermentation and maturation process of several cheeses (Jakobsen and Narvhus 1995). The species of particular interest are *Debaryomyces hansenii* (anamorph: *Candida famata*), *Yarrowia lipolytica* (anamorph: *Candida lipolytica*), *Geotrichum candidum* (teleomorph: *Galactomyces candidus*) and *S. cerevisiae*. Furthermore, for the filamentous fungi, the use of *Penicillium roqueforti* and *P. camemberti* has a long history in cheese production. According

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to early records, names of blue and white mould cheeses are dated to the year 879 for Gorgonzola, 1070 for Roquefort, 1785 for Stilton and 1791 for Camembert (Robinson 1995). These cultures are important starter cultures, which are commercially available and widely used by the dairy industry. Their technological properties have been studied over a number of years and, although not fully understood, useful information has been collected as reviewed by Gripon (1993).

Compared with bread and cheese, meat is the least developed area concerning the use of yeast and filamentous fungi as starter cultures. Apart from a few examples of using *P. nalgiovense* for surface ripening of sausages and *D. hansenii* for fermentation of sausages and other meat products, limited information is available. The role of micro-organisms is unclear, but bacteria rather than fungi appear to be responsible for flavour development in fermented meat (Montel et al. 1998). However, several important meat products like Parma and Serrano hams are still spontaneously fermented and the possible role of yeasts and mycelial fungi has not been studied in detail. An increasing interest is seen in research work leading to the understanding of the role of micro-organisms in traditional spontaneously fermented meat products including the significance of yeast and mycelial fungi. For a review on fungal ripening of meat, see Cook (1995), Lücke (1998) and Sunesen and Stahnke (2003).

II. Bread

A. Baker's Yeast

Yeast-fermented breads in Europe and the United States are mostly based on wheat, although rye is commonly used for some popular bread types in Scandinavia and other northern European countries. A large variety of bread is produced, and the European tradition for consumption of bread seems to spread over the world, including regions like South-East Asia and Africa, as a result of the strong impact of European eating habits. Traditional bread making in Africa does not seem to be widely known, but strong traditions exist for fermentation of cereals, with yeast playing a significant role, especially in co-cultures with lactic acid bacteria, as was also the case in the past in Europe. For Sudan, 11 types of sorghum and millet bread

are described by Dirar (1993) and in other parts of Africa various cereal dough fermentations, like *kenkey* made from fermented maize dough in Ghana (Halm et al. 1993), play a substantial role in the daily food intake. A review on yeast in traditional African food is given by Jespersen (2003).

In most cases, the dominant yeast appears to be *S. cerevisiae* Meyen ex E.C. Hansen with the taxonomic delimitation given by Vaughan-Martini and Martini (1998). It is considered the principal species responsible for cereal fermentation and bread making as well as alcoholic fermentations, except for fermentation of lager beer.

Several methods based on molecular techniques have been reported for subspecies typing of *Saccharomyces* spp., one of the most popular methods has been determination of chromosome length polymorphism, e.g. by pulse field gel electrophoresis (PFGE) as done for *S. cerevisiae* isolates from spontaneously fermented maize dough in Ghana by Hayford and Jespersen (1999).

As chromosome length polymorphism is evident among the isolates, the technique clearly shows that several subspecies are involved in the fermentation process. Further, the observed chromosome profiles are quite similar to chromosome profiles observed for baker's yeast. According to general experience, the genomic stability of individual strains of baker's yeast appears to be high (Gasent-Ramirez et al. 1999).

Other molecular methods which seem to be well accepted for species recognition and clarification of phylogenetic relationships are based on sequencing of rDNA, such as the ribosomal internal transcribed spacer (ITS) region (Montrocher et al. 1998), or sequencing of the D1/D2 domain of the large subunit (26S) rDNA (Kurtzman and Robnett 1998). Functional genomics and proteomics are also valuable tools in clarification of strain differentiation and elucidation of the relationship between genotype and phenotypes (Garrels et al. 1997; Joubert et al. 2000).

Mixed cultures of yeast species may occur in bakeries depending on the type of flour used and other conditions employed (Jenson 1998; Hammes et al. 2005). Other than *S. cerevisiae* species are of interest, because they may be better suited for applications where *S. cerevisiae* cannot meet desired technological properties, like resistance to osmotic stress, freezing and thawing (Jenson 1998; Hammes et al. 2005).

An alternative baker's yeast could be *Kazachstania exigua* (previously *Saccharomyces exiguus*) Rees ex E.C. Hansen or its anamorph form *Candida holmii* (Jørgensen), according to several investigations as reviewed by Jenson

(1998). Another candidate is *Torulasporea delbrueckii* (Lindner) and its anamorph form *Candida colliculosa* (Hartmann) because of its high osmotolerance and resistance to freezing (Oda and Tonomura 1993a, b). *Candida krusei* and its teleomorph form *Issatchenkia orientalis* are often seen in microbial successions with *S. cerevisiae* in dough fermentations where it appears to take over when the conditions become too inhibitory, probably too acid, to *S. cerevisiae* (Hayford and Jakobsen 1999; Hayford and Jespersen 1999). Molecular typing has indicated that the strains observed are different from pathogenic strains of *C. krusei* (Hayford and Jakobsen 1999), a point which should also be considered when selecting new strains, although the yeast is supposed to be killed during baking. Other indigenous fermented cereals in Africa and in smaller traditional bakeries in Europe which still rely upon spontaneous dough fermentations may be considered as sources for alternative cultures or for cultures to be applied in mixed cultures with *S. cerevisiae*. The cultures may not only be selected to ensure efficient bread leavening, but also for the purpose of adding aroma characteristics to the bread. Tomer et al. (1992) specifically investigated the effect on volatile compounds in fermentation studies with *S. cerevisiae* and *Candida guilliermondii* without specifying which of the two species varieties (var. *guilliermondii* or var. *membranifaciens*) was used. They concluded that *S. cerevisiae* produced the highest number of aroma components and, in general, the yeast examined produced more flavour components than the lactic acid bacteria investigated.

It should be mentioned that lactic acid bacteria are often found in high levels during bread fermentations and the microbial interactions between baker's yeast and these bacteria can be very important, in particular in sourdough breads, as reviewed by Hammes and Ganzle (1998) and Hammes et al. (2005). Recent studies have made use of culture independent molecular methods for identification of the microbiota of bread fermentations (Zannini et al. 2009) and yeast population dynamics (Meroth et al. 2003).

B. Technological Properties of Baker's Yeast

Fermentation of Dough Carbohydrates. The main technological properties of baker's yeast can be summarised as the efficient fermentation of dough carbohydrates and formation of CO₂, influence on dough structure, shelf life, aroma formation, osmotolerance, acid tolerance and freeze-thaw resistance.

Baker's yeast does not ferment starch. The starch of the dough will be broken down by the action of α - and β -amylases from the dough, leading to the formation of mainly glucose, maltose and maltotriose, which can all be fermented by the

yeast, and higher carbohydrates (dextrins) which *S. cerevisiae* cannot ferment. Maltose is present at the highest level and the faster the yeast can ferment maltose, the faster the fermentation will occur. Fast fermentation is one of the most desired properties of baker's yeast.

Of the three major sugars, glucose is preferentially utilised by *S. cerevisiae* (Stewart et al. 1979), but efficient fermentation requires the rapid utilisation of both maltose and maltotriose. Gene dosage studies performed with laboratory strains of yeast have shown that the transport of maltose into the cell may be the rate-limiting step in the utilisation of this sugar (Goldenthal et al. 1987). Information on the maltose and maltotriose transporter genes present in brewer's yeast is therefore of some value in selecting suitable strains and in predicting fermentation performance.

Maltose utilisation in *S. cerevisiae* is conferred by anyone of five *MAL* loci, *MAL1* to *MAL4* and *MAL6* (Vanoni et al. 1989). Each locus consists of three genes: gene 1 encodes a maltose transporter, gene 2 encodes a maltase (α -glucosidase) and gene 3 encodes a transcriptional activator of the other two genes. Thus, for example, the maltose transporter gene at the *MAL6* locus is designated *MAL61*. The five *MAL* loci each map to a different yeast chromosome, as follows: *MAL1*, chromosome VII; *MAL2*, chromosome III; *MAL3*, chromosome II; *MAL4*, chromosome XI; *MAL6*, chromosome VIII. The *MAL* loci exhibit a very high degree of homology and are telomere linked, suggesting that they evolved by translocation from telomeric regions of different chromosomes (Michels et al. 1992). Since a fully functional or partial allele of the *MAL1* locus is found in all strains of *S. cerevisiae*, this locus has been proposed as the progenitor of the other *MAL* loci (Chow et al. 1983; Jespersen et al. 1999).

Based on information about the regulation of maltose utilisation, recombinant DNA technology has been used for constructing strains which are not repressed by glucose and are constitutive in their maltose uptake (Osinga et al. 1989). Such recombinant yeast show rapid gas production from maltose in plain dough. Recent studies (Higgins et al. 1999) suggest that varying constitutive maltase and maltose permease levels in strains of *S. cerevisiae* are important targets for selection of strains with improved maltose utilisation.

If the amylase activity of the dough is not sufficient to provide enough fermentable carbohydrates, external enzymes or fermentable sugars, e.g. glucose, fructose or sucrose, can be added. Baker's yeast normally has invertase in excess to

hydrolyse the extra sucrose added. Wheat flour contains about 2% (w/w) glucose, fructose, sucrose and maltose. The wheat amylases or added external enzymes can increase the amount of maltose to about 3% (w/w).

The optimum fermentation temperature is about 40°C. With regard to bread quality, a temperature of 25°C and a fermentation time of several hours should be applied. To decrease production time, higher temperatures, e.g. 35°C, are often used. During the initial phases of baking, fermentation continues, but it stops at around 50°C when *S. cerevisiae* and some relevant enzymes are inactivated.

Gas Production and Influence on Dough Structure. The main influence of fermentation on dough structure appears to be explained by mechanical stretching and modification of the dough protein (gluten) caused by the CO₂ evolution (Reed and Nagodawithana 1991). This is obviously linked directly to the fermentation capacity of the yeast as well as the amount of yeast present and hence the amount of CO₂ formed.

Yeast excretions may also affect the structure of the dough. It has been reported that excreted glutathione and cysteine could affect protein disulphide bonds in gluten (Stear 1990). Yeast quality and hydrolysis of yeast with release of proteolytic enzymes may also influence dough structure. However, regardless of the possible influence of the yeast, the mechanical treatment of the dough, in particular in modern bakeries, has a major, and more likely dominant, effect.

Influence on Bread Flavour. In comparison with the role of the yeast strain used for the fermentation and formation of flavour compounds in beer and wine, the influence of baker's yeast on bread flavour appears to be limited (Rose and Vijayalakshmi 1993; Jenson 1998). Few studies on the flavour characteristics of baker's yeast have been published, and the search for strains with special flavour properties does not seem to be given much attention.

The flavour components produced by the yeast in bread making include organic acids, aldehydes, ethanol, higher alcohols, esters and ketones. In addition, the less well defined yeasty flavour is often noticeable. This may occur in particular in the case of active dry yeast where a relatively higher concentration of yeast dry matter may be applied. Trials have been reported showing that *S. cerevisiae* and *C. guilliermondii* produced larger amounts of volatile flavour components than the lactic acid bacteria investi-

gated, *Lactobacillus brevis* and *Lactobacillus plantarum* (Tomer et al. 1992). The effects of stress exposure and interactions between yeast and lactobacilli on generation of aroma compounds in sourdough has been reported (Guerzoni et al. 2007)

C. Manufacture of Baker's Yeast

Baker's yeast is the oldest and still one of the most important products within biotechnology. Globally, the yearly production amounts to more than 2×10^6 t. The process has been thoroughly investigated and well described (White 1954; Reed and Nagodawithana 1991). It has also been reviewed in several publications, e.g. by Rose and Vijayalakshmi (1993) and Jenson (1998).

Traditionally, yeast biomass production occurs using a molasses-based heat-treated substrate, added nutrients, like urea or ammonium sulphate, vitamins and minerals, in fermenters equipped with aeration and agitation. Growth conditions are important for producing the maximum amount of yeast and for obtaining the desired yeast quality including a rapid dough fermentation and a high resistance to oxidative stress, drying, freezing and other forms of stress (Attfield 1997).

The optimal growth conditions during fermentation are primarily defined by nutrient feed rates, aeration, temperature, pH, ethanol level and the respiratory quotient (CO₂: O₂). For control purposes, fermenters are equipped with sensors for measurement of ethanol, CO₂ and O₂ in the exit gas. High aeration rates and fed-batch fermentation ensure that the carbohydrate level is kept low, allowing the yeast to produce biomass using respiratory metabolism. At the end of the fermentation, nutrient feed is stopped, but aeration is continued for 30–60 min, during which time a significant synthesis of trehalose takes place. High aeration, removal of nitrogen and carbohydrate derepression favour the gluconeogenic pathway and synthesis of trehalose. Trehalose is the typical storage carbohydrate in yeast. It plays an important protective role for the yeast cell when exposed to stresses like drying and freezing. The protective role of trehalose has also been demonstrated by Shima et al. (1999), by constructing trehalase mutants derived from *S. cerevisiae*. During fermentation, degradation of intracellular trehalose was inhibited in the trehalase mutants, which all showed improved freezing tolerance which may make the strains useful in frozen dough.

After fermentation, the yeast is recovered by filtration or centrifugation and processed into a commercial product. It can be in the form of cream yeast, compressed yeast or viable dry yeast.

Cream yeast is a liquid yeast obtained after concentration, washing and, if desired, stabilisation of the yeast at the end of fermentation (Cees 1991; Jenson 1998). The solids level is about 16–20% and the shelf life is approximately 14 days at 4°C. The cream yeast offers better opportunities for control over yeast activity and ease the use in large plant bakeries that can pump the yeast to several points and dose it accurately. For viable dry yeast, the yeast slurry is mixed with additives, e.g. sorbitan esters, carboxy methyl-cellulose and antioxidants. These additives protect the yeast against drying and reconstitution at use. Following mixing with additives, the yeast is concentrated by filtration then extruded mildly through a screen to form threads which are cut up and dried in a fluidised-bed dryer at room temperature. To maintain viability, the dry yeast needs to be protected against oxygen and is packaged accordingly (Jenson 1998). Compressed yeast has a solids level of about 30–35% and a shelf life of approximately 4 weeks at 4°C.

Evaluation of thermally dried *Kluyveromyces marxianus* as baker's yeast has recently been published. In comparison with commercial baker's yeast, no significant differences in the volatile aroma compounds and overall quality were observed (Dimitrellou et al. 2009).

III. Cheese

A. Yeasts

Yeasts are found in all sorts of cheeses. *Debaryomyces hansenii* (anamorph form: *C. famata*) is the predominant yeast species in semi-soft cheeses, whereas soft cheese are characterised by the additional presence of *G. geotrichum* (teleomorph form: *Galactomyces candidus*). Other predominant yeast species include *Yarrowia lipolytica*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis* and *K. marxianus*. The number of yeasts can be in the range 10⁶ colony-forming units (cfu)/g or even higher. The development of yeasts in cheeses occurs spontaneously, while the controlled use of yeasts as starter cultures in cheeses primarily is used for the production of some kinds of mould-ripened cheeses and smear-ripened cheeses.

The positive role of yeasts in the maturation and aroma formation in Camembert has been suggested in several investigations (Anderson and Day 1966; Schmidt and Lenoir 1978, 1980a, b; Schmidt and Daudin 1983; Rousseau 1984; Siewert 1986; Baroiller and Schmidt 1990; Gripon 1993). Baroiller and Schmidt (1990) concluded that the following five yeasts are predominant in

white mould cheeses: *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *D. hansenii*, *S. cerevisiae* and *Zygosaccharomyces rouxii*. A positive role for yeasts has been proposed for blue mould cheeses, and the yeasts in the interior of the cheese reach significantly higher numbers than observed for white mould cheeses (Hartley and Jezeski 1954; Galzin et al. 1970; Nunez et al. 1981; Kaminarides and Anifantakis 1989; Besancon et al. 1992; Hostin and Palo 1992; van den Tempel and Jakobsen 1998). According to the investigations mentioned, the predominant yeast populations are rather similar for the white and blue mould cheeses. It is characteristic of the ecosystem of the cheese, the cheese brine and the environmental conditions prevailing in the dairy that they select towards a uniform and well defined yeast population (Baroiller and Schmidt 1990; van den Tempel and Jakobsen 1998). Thus, a situation may exist which is very similar to a deliberate use of yeasts as starter cultures, which is still the exception rather than the rule in the dairy industry.

1. *Debaryomyces hansenii*

Originally two species were described: *Debaryomyces hansenii* (Zopf) Lodder et Kreger van Rij and *Debaryomyces fabryi* Ota. It was proposed to subdivide the species *D. hansenii* (Zopf) Lodder et Kreger van Rij into varieties: *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi* and their respective anamorphs, *Candida famata* var. *famata* and *C. famata* var. *flareri* (Nakase and Suzuki 1985). Physiologically, the two varieties can be distinguished by maximum growth temperatures and the enzyme glucose-6-phosphate dehydrogenase. The maximum growth temperature of the variety *hansenii* is 31–35°C, whereas that of the variety *fabryi* is 36–39°C.

However, based on molecular techniques, several authors have proposed to reinstate the two original species (Prillinger et al. 1999; Corredor et al. 2003; Romero et al. 2005; Quiros et al. 2006; Jacques et al. 2009; Nguyen et al. 2009). Furthermore, a number of strains within the variety *fabryi* have been proposed to form a new species *Debaryomyces subglobosus* (= *Candida flareri*). Finally, growth at 37°C may no longer be used to differentiate *D. hansenii* from *D. fabryi*. In contrast, riboflavin production seems more specific for *D. fabryi* and *D. subglobosus* strains compared to *D. hansenii* strains. In cheese *D. hansenii* appears to be the dominant form (Petersen et al. 2001). *D. hansenii* occurs in high numbers (10⁶–10⁸ cfu/g) in surface-ripened cheeses as well as in blue and white mould cheeses.

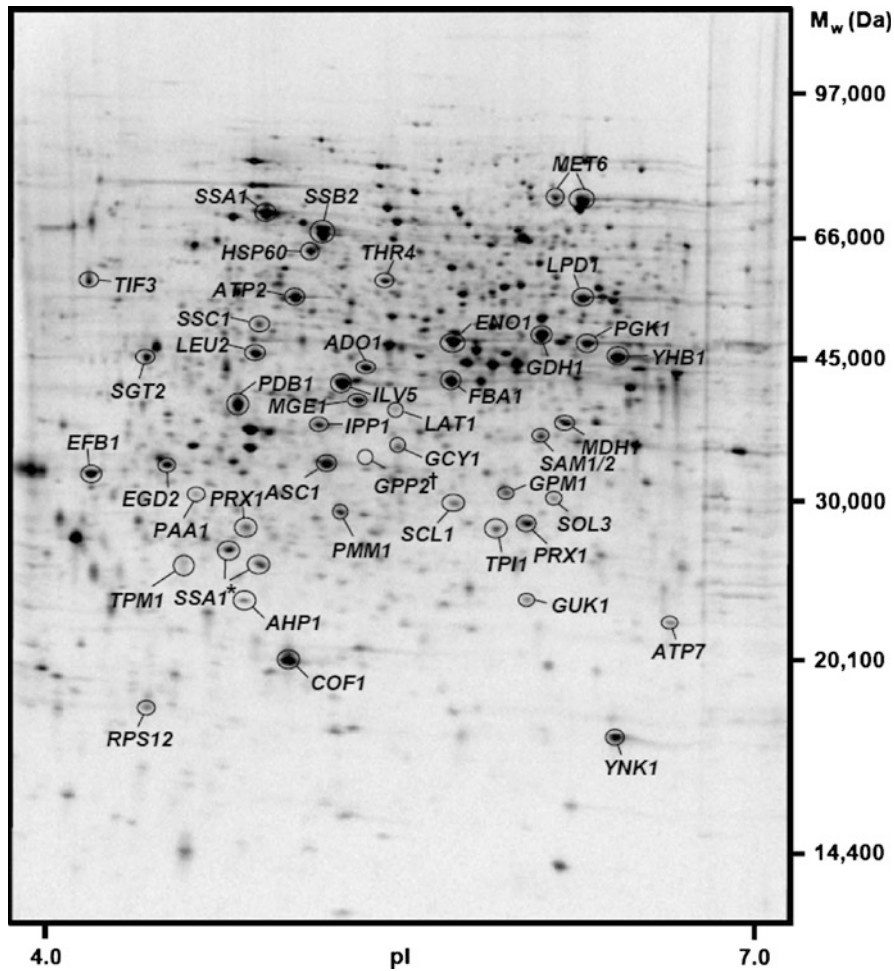


Fig. 1.2. Two-dimensional PAGE gel of *Debaryomyces hansenii* (type strain CBS 767), modified from Gori et al. (2007a). Protein spots were identified by MALDI-TOF MS. Identified proteins are labelled with their corresponding gene names. Examples of protein fragments and proteins strongly synthesised after NaCl addition are labelled with * and †, respectively

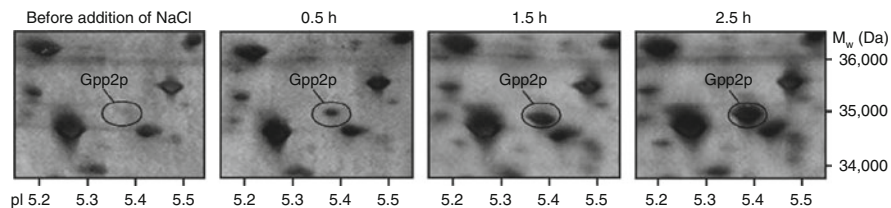


Fig. 1.3. Induction of glycerol-3-phosphatase (Gpp2p) in *Debaryomyces hansenii* (type strain CBS 767) during adaptation to 8% (w/v) NaCl. The figure is modified from Gori et al. (2007a)

showing *D. hansenii* from other habitats to be weakly proteolytic at 10°C (Lagace and Bisson 1990; Kobatake et al. 1992). Cheese isolates of *D. hansenii* showed extracellular proteinase activity after 3 weeks of incubation at 25°C, with degradation of all components of casein measured by capillary zone electrophoresis (van den

Tempel and Jakobsen 2000). These results seem to indicate that *D. hansenii* is proteolytic, but not contributing in any significant way to proteolysis during ripening of Danablu at 10–12°C. High intracellular protease and peptidase activities occur in *D. hansenii* (Nunez et al. 1981; Lenoir 1984; Baroiller and Schmidt 1990) with an optimum pH of

5.8 (Szumski and Cone 1962). These enzymes could contribute to cheese maturation after release by cell autolysis, provided that they are active at the pH and salt content of the cheese (Choisy et al. 1987). This remains to be investigated in detail.

D. hansenii is important for surface ripened cheeses like Munster, Limburger, Port Salut etc. (Eliskases-Lechner and Ginzing 1995; Bockelmann et al. 1997; Leclercq-Perlat et al. 1999; Wyder et al. 1999). Yeasts occur early in the ripening period on the cheese surface because of their ability to grow at low temperature, high humidity, relative low pH and high salt concentrations. They metabolise lactic acid and produce alkaline metabolites such ammonia, which can lead to a considerable increase of the surface pH (Eliskases-Lechner and Ginzing 1995).

The surface condition of the cheese is thereby modified to provide a suitable environment for the growth of the bacterial population, which typically is dominated by two groups of Gram-positive bacteria, coryneforms (*Arthrobacter* spp., *Brevibacterium* spp., *Corynebacterium* spp., *Microbacterium* spp.) and coagulase-negative staphylococci (Bockelmann and Hoppe-Seyler 2001; Bockelmann 2002; Bockelmann et al. 2005). More recently, a bacterial population has been reported consisting of Gram-negative bacteria of the genera *Pseudomonas*, *Xanthomonas*, *Enterobacter*, *Hafnia* and *Proteus* (Maoz et al. 2003; Feurer et al. 2004a, b; Mounier et al. 2005; Ishikawa et al. 2007; Rea et al. 2007). Though, they are very common, the latter are often considered as undesirable contaminants, but very little is known about the role of these bacteria in cheese ripening including their synthesis of volatile aromatic compounds. Generally, the bacterial population does not grow readily at a pH lower than 5.5 and are stimulated by growth factors synthesised by the yeasts. Cheese-making trials have demonstrated that the growth and survival of *D. hansenii* on bacterial surface ripened soft cheese is strongly correlated with the lactate concentration (Leclercq-Perlat et al. 1999).

The use of *D. hansenii* as a starter culture for surface ripening of white and blue mould cheeses has been proposed. Investigations have demonstrated the important role of *D. hansenii* in the ripening of cheese by its contribution to the formation of the “morge” (Purko et al. 1951; Hartley and Jezeski 1954), a slimy material partly composed of micro-organisms such as micrococci and other Gram-positive bacteria (Hammond 1976; de Boer and Kuik 1987). It was suggested by Besancon et al. (1992) that the lipolytic activity of yeasts, including *D. hansenii*, favours the liberation of fatty acids to the morge, contributing to its typical

properties. The morge allows foil to stick well to the surface of the cheese during the “plombage” phase of Roquefort. *D. hansenii* in combination with *Y. lipolytica*, *L. plantarum* and *Enterococcus faecium* has been suggested as a mixed starter culture for Picante cheese (Freitas et al. 1999).

2. *Yarrowia lipolytica*

Yarrowia lipolytica is the only species of the genus *Yarrowia* van der Walt and von Arx (Kurtzman and Fell 1998). Its anamorph form is *Candida lipolytica* and it is only distantly related to most other known ascomycetous yeasts (Kurtzman and Fell 1998).

Typical characteristics of *Y. lipolytica* include its inability to ferment sugars and very strong lipase and protease activity. It is a well defined species with minor strain variations. The assimilation pattern examined by API 32C (BioMerieux, Marcy l'Etoile, France) is characterised by assimilation of very few of the compounds (11 out of 32), in contrast to strains of *D. hansenii*, which assimilate 26 out of 32 compounds. Strains of *Y. lipolytica* grow at pH values of 4.0–6.0 at 10°C, the ripening temperature of cheeses like Danablu. They are, however, sensitive to NaCl and no growth was observed at a water activity (a_w) of 0.94 at 10°C (van den Tempel and Jakobsen 2000). The a_w is defined in food microbiology as the ratio between the equilibrium vapour pressure over a given food and the vapour pressure of pure water at the same temperature. This is a limiting factor for *Y. lipolytica* growth in Danablu and other blue mould cheeses. It has also been demonstrated that the assimilation of lactate and citrate by *Y. lipolytica* is strongly inhibited by NaCl concentrations higher than 4% (w/v), indicating that the growth as well as biochemical activity of *Y. lipolytica* will take place mainly in the centre of cheeses like Danablu during the early stages of ripening.

The pronounced lipolytic activity of *Y. lipolytica* has been reported in several studies (Alifax 1979; Hou and Johnston 1992; Rapp and Backhaus 1992; Roostita and Fleet 1996; van den Tempel and Jakobsen 2000). The lipolytic activity of strains of *Y. lipolytica* investigated by titration of FFA released from butterfat after 5 days of incubation at 25°C seems to be higher than the activity of strains of *P. roqueforti* under the same conditions (Larsen and Jensen 1999). Such results emphasise the potential role of *Y. lipolytica* as a starter culture in blue mould cheeses.

The strong proteolytic activity by strains of *Y. lipolytica* is well documented (Guerzoni et al. 1993). Proteolysis is seen in a broad temperature range with optimum activity at pH

5.8–6.2 (Szumski and Cone 1962; Kobatake et al. 1992). Various studies have proposed the use of *Y. lipolytica* for production of cheese flavour (Kalle et al. 1976). Patents describe the use of *Y. lipolytica* as a ripeness-accelerating agent in cheese, producing an authentic cheese flavour normally obtained after months of ripening (Unilever 1970; Lashkari 1971; Boudreaux 1985, 1987). Studies by Wyder et al. (1999) have confirmed the positive effect on cheese flavour by *Y. lipolytica* for Raclette cheese.

The potential of *Y. lipolytica* as a ripening agent in cheese has also been supported by cheese-making trials by Guerzoni et al. (1998). It was demonstrated that *Y. lipolytica* possesses some of the essential properties for use as a cheese starter: (1) the ability to grow and compete with other naturally occurring yeasts such as *D. hansenii* and *S. cerevisiae*, (2) compatibility with lactic acid starter culture and (3) the desired proteolytic activity. Cheese trials using *Y. lipolytica* as a starter obtained the highest score for flavour, body and texture, suggesting the use of this species in cheese production, especially in low-fat cheese (Guerzoni et al. 1998).

3. *Geotrichum candidum* (perfect: *Galactomyces candidus*)

A recent taxonomic revision concluded that the *Galactomyces geotrichum/Geotrichum candidum* complex contains four different species, of which *Geotrichum candidum* and its anamorph form *Galactomyces candidus* is one (de Hoog and Smith 2004). Synonyms include former names like *Oidium lactis* and *Oospora lactis*, indicating the link to milk and dairy products. It is a so-called filamentous yeast-like fungus, which is used as a starter culture for production of soft, semi soft cheeses and fermented milks. It is often used in co-cultures with other starter cultures, most typically *Penicillium camemberti* (Molimard et al. 1995).

Apart from cheese and milk, it is common in all kinds of moist substrates, particularly in citrus fruits and tomatoes and mainly as a cause of rot in ripe fruit. It has been assumed to be a plant pathogen, and it is occasionally associated with human diseases (Kurtzman and Fell 1998). The occurrence in such different habitats of *G. candidum* seems not to have been investigated at the molecular level to distinguish strains according to their origin and undesired properties in relation to their use in foods. Such investigations, including detailed pheno- and genotyping methods, are needed when looking for new strains to be applied in the dairy industry.

As reviewed by Boutrou and Guéguen (2005), *G. candidum* is common on the surfaces of cheeses, where it seem not exceed a final count of 10^6 cfu/g. Its low number could be due to its ability to form a pseudo-mycelium with long hyphae that consist of different numbers of cells nevertheless yielding only 1 cfu/plate. It is worth noting that *G. candidum* can grow under micro-aerophilic conditions and thus is able to grow not only on the cheese surface but also in the interior of the cheeses (Haasum and Nielsen 1998). However, lower numbers are typically seen for the interior compared to the surface. It grows in the temperature range 5–38°C with an optimum around 25°C at pH 5.0–5.5. It can grow within a large pH range: 3–11. It is lipolytic, depending on the presence of oxygen, and the pH optimum for the lipases is about 6.0. It is also reported to be proteolytic with extracellular proteinases capable of degrading α - and β -caseins. The optimum pH for casein hydrolysis is 5.5–6.0. Some strains may be too proteolytic, leading to unacceptable bitterness in cheeses.

The occurrence of *G. candidum* on surface-ripened cheeses seems to be accidental, but some strains contribute to the maturation of mould-ripened cheeses, and they seem to grow faster than the mould starters like *P. camemberti* (Molimard et al. 1995). Some strains of *G. candidum* have specific anti-microbial activities and, based upon their rapid growth, they can effectively compete with other yeasts and mycelial fungi in cheeses. This gives *G. candidum* a particular role in microbial interactions in cheeses, as discussed later.

In all aspects, the strain variations mentioned are very pronounced. This underlines the need for detailed geno- and phenotyping studies to define the strains applicable to the dairy industry.

4. *Saccharomyces cerevisiae*

According to the most recent taxonomic key (Barnett et al. 2000), the genus *Saccharomyces* Meyen ex Reess consists of 16 species, of which *Saccharomyces cerevisiae* originally was defined as the type species by Reess in 1870 (Ando et al. 1996). Compared to *D. hansenii*, *Y. lipolytica* and *G. candidum*, *S. cerevisiae* has been found less frequently in cheeses (Hansen and Jakobsen 2001). However, *S. cerevisiae* is available as a starter culture for Gorgonzola production (Hansen and Jakobsen 2001), and the evaluation of such

a strain as an additional starter culture in a Danish Gorgonzola like cheese has provided promising results (Hansen et al. 2001).

S. cerevisiae contributed to a softer texture, improved growth of *Penicillium roqueforti* and an increase in aroma compound formation and casein degradation (Hansen et al. 2001). *S. cerevisiae* has also been found present in Water Buffalo Mozzarella, in which it possibly multiplies by the availability of galactose released from lactose fermentation by lactic acid bacteria not capable of metabolising galactose themselves (Romano et al. 2001). Together with lactose fermenting yeasts, *S. cerevisiae* was thought to contribute with flavour compounds or their precursors, such as ethanol, acetaldehyde and ethyl acetate (Romano et al. 2001).

B. Filamentous Fungi

1. Blue Mould Cheeses

A flow diagram for production of the blue mould cheese Danablu is shown in Fig. 1.4.

a) *Penicillium roqueforti*

P. roqueforti Thom is used as secondary starter culture in the production of blue-veined cheeses, but is also found in a range of other products like bread, meat products and as a contaminant on cheeses (Lund et al. 1995). *P. roqueforti* has been known as *P. aromaticum*, *P. gorgonzola*, *P. stilton*, *P. suaveolens* and *P. biourgei*, but these names are not used anymore, as they cover the same species.

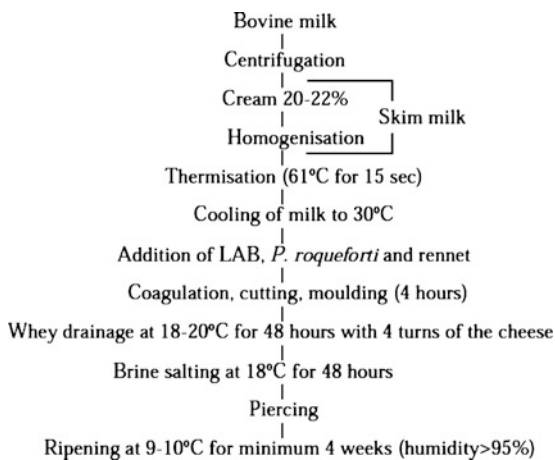


Fig. 1.4. Flow diagram for the production of Danablu

The taxonomy of penicillia is primarily based on their micro- and macro-morphology and growth characteristics on different media (Pitt 1991). However, with these methods it can be difficult to distinguish between species that are closely related (Bridge et al. 1989a; Pitt 1991). A number of chemical methods have therefore been introduced to be used in combination with the classical identification methods. A simple method for differentiating *P. roqueforti* from other penicillia is by its ability to grow on media with 0.5% acetic acid (Engel and Teuber 1978; Bridge et al. 1989b) combined with colony diameter (Bridge et al. 1989a). The production of secondary metabolites for differentiating *Penicillium* species has been used by several researchers (Frisvad 1981; Lund 1995; Boysen et al. 1996), and gas chromatographic analysis of either the fatty acids of the spores (Blomquist et al. 1992) or the profiles of volatile metabolites (Larsen and Frisvad 1995; Karlshøj and Larsen, 2005) has been described.

Various molecular genetic techniques like polymerase chain reaction (PCR), randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and analysis of internal transcribed spacers (ITS) in combination with biochemical characteristics are also gaining interest for identification and differentiation of penicillia and other fungi (Bidouchka et al. 1994; Boysen et al. 1996; Geisen et al. 2001; Flórez et al. 2007). RAPD has also been used as a rapid method for screening fungi for the presence of genes involved in biosynthesis of mycotoxins and thereby their possible ability to produce these toxins (Geisen and Holzappel 1995; Geisen 1996). The novel approach applied by Geisen et al. (2006) and Schmidt-Heydt and Geisen (2007a, b) based upon gene expression studies in meat products could be transferred to cheese matrices to predict not only mycotoxin formation but also cheese ripening. Furthermore, the molecular techniques offer the possibility of investigating microbial diversity, succession and location directly in the cheeses (Ercolini et al. 2003; Flórez and Mayo 2006).

b) Technological Characteristics of *Penicillium roqueforti*

Conditions for Growth. The growth of *P. roqueforti* is optimal between pH 4 and 5, but it grows without difficulty in the interval between pH 3 and 10 (Cerning et al. 1987). The NaCl content of the cheese affects the germination and growth of *P. roqueforti* to various degrees depending on the strain (Godinho and Fox 1981a).

The germination of conidia was stimulated by 1% NaCl for all the strains investigated by Godinho and Fox (1981a),

but differences in NaCl tolerance were observed. For some strains, germination was clearly inhibited by 3% (w/v) NaCl, while others were still able to germinate at 6% (w/v) NaCl. Similar results were seen for the effect of NaCl on growth (Godinho and Fox 1981a). It was proposed that the inhibitory effect of NaCl is primarily on the germination phase, as it was shown that after germination of the conidia up to 6% (w/v) NaCl had little effect on subsequent growth (Godinho and Fox 1981a).

In terms of water activity, the a_w value influences germination, growth and sporulation of *P. roqueforti*. The germination of the conidia is optimal at an a_w of 0.98–0.99 on laboratory media, but germination was observed even at 0.88 (Gervais et al. 1988b). The optimum a_w reported for growth is 0.99 (Gervais et al. 1988a), while sporulation is most intense at an a_w value of 0.96 (Gervais et al. 1988c). As regards germination, growth and sporulation of *P. roqueforti*, optimum conditions can be found in the interior part of blue-veined cheeses, which corresponds with the observation that the first visible growth is seen in the interior of the cheeses after 2–3 weeks of ripening.

Proteolysis. Proteolysis in blue veined cheeses is very extensive compared with other types of cheeses (Marcos et al. 1979) and is caused by complex interactions between rennet, plasmin, enzymes from the primary starter culture and from non-starter lactic acid bacteria, but with the main contribution from the proteinases and peptidases produced by *P. roqueforti* (Gripon et al. 1977; Trieu-Cuot and Gripon 1983). Properties of extracellular proteinases and peptidases produced by *P. roqueforti* are given in Tables 1.1, 1.2.

A significant increase in proteolysis is observed after growth of the mould has become

visible (Trieu-Cuot and Gripon 1983; Zarmoutis et al. 1996). The proteolytic activity of strains of *P. roqueforti* is very important for the development of the texture and water-binding capacity of the cheeses (Gripon 1993; Lawlor et al. 2003). Large differences in proteolytic activity, both quantitative and qualitative, have been observed between commercial strains of *P. roqueforti* (Stepaniak et al. 1980; Engel and Teuber 1988; Larsen et al. 1998) and this knowledge has been used to produce cheeses with different characteristics (Farahat et al. 1990).

P. roqueforti produces both extra- and intracellular proteinases and peptidases (Cerning et al. 1987) but the extracellular enzymes, especially the aspartic proteinase (Table 1.1), are thought to be the most important for ripening (le Bars and Gripon 1981). However, diffusion of the enzymes away from the mycelium does not seem to happen (Gobbetti et al. 1997). As the proteolytic system of *P. roqueforti* contains both proteinases and peptidases (Table 1.2), it is possible to degrade the caseins, leading to various-sized peptides and amino acids (Gripon 1993). The proteolysis can be so pronounced that α_{s1} - and β -casein almost disappear at the end of ripening (Marcos et al. 1979; Fernandez-Salguero et al. 1989; Zarmoutis et al., 1997). The micro-environment of the cheese, mainly the NaCl and pH, influences the proteolytic activity of the *P. roqueforti* strain and thereby proteolysis, which in general is less pronounced in the heavily salted outer part (Hewedi and Fox 1984; Gobbetti et al. 1997).

Little information is available on the genome of *P. roqueforti*, but the gene (*aspA*) encoding the extracellular

Table 1.1. Properties of extracellular proteinases produced by *Penicillium roquefortii*

Type	Molecular weight (Da)	pH optimum	Maximum pH stability	Reference
Aspartic proteinase	33 400	3.5 (casein and haemoglobin)	3.5–5.5	Zevaco et al. (1973)
	45 000 ^a to 49 000 ^b	3.0 (BSA) ^c 5.5 (casein)	3.0–6.0	Modler et al. (1974)
Metallo-proteinase	20 000	5.5 (casein)	5.0–9.5 (casein)	Gripon and Hermier (1974)
		4.2 (haemoglobin)	4.0–6.0 (haemoglobin)	

^aDetermined by gel electrophoresis

^bDetermined by gel filtration

^cBSA = bovine serum albumin

Table 1.2. Properties of peptides produced by *Penicillium roquefortii*

Type	Position	Molecular weight (Da)	pH optimum	Maximum pH stability	Reference
Carboxypeptidase ^a	Extracellular	110 000	3.5	5.0–5.5	Gripon (1977a)
		nd ^b	4.0 and 8.5	nd	Gripon and Debest (1976)
	Intracellular	155 000	3.6	2.0–6.0	Ichishima et al. (1978)
Aminopeptidase ^c	Extracellular	58 000–63 000	4.0 and 8.5	nd	Paquet and Gripon (1980)
		35 000	7.5–8.0	6.0–7.0	Gripon (1977b)
	Intracellular	nd	8.5	nd	Gripon and Debest (1976)
		53 000–58 000	7.5 and 8.5	nd	Paquet and Gripon (1980)

^apH optimum and stability range is determined against Z-Glu-Tyr

^bNot determined

^cpH optimum and stability range is determined against LNA (leucine *p*-nitroanilide)

aspartyl protease has been cloned and characterised (Gente et al., 1997). In the same investigation, gene expression studies showed that *aspA* was strongly induced by casein in the medium and efficiently repressed by ammonia. It was later shown that peptides from casein degraded by the mature enzyme can induce expression of *aspA* at both acid and alkaline pH (Gente et al., 2001). It was speculated that the promoter of *aspA* could be an attractive candidate for the development of a controllable gene expression system in *P. roquefortii*. Additionally, the *nmc* gene encoding a global nitrogen regulator in *P. roquefortii* has been cloned and characterised (Gente et al. 1999) and more recently investigations on the genetic mechanisms behind growth phases and roquefortin C biosynthesis of *P. roquefortii* has been initiated (Garcia-Rico et al. 2007, 2009). These investigations seem to represent the start of the genomic studies of mycelial fungal starter cultures applied in cheese maturation.

Lipolysis and Aroma. Lipolysis in blue cheeses, like proteolysis, is very intense compared with other cheeses (Woo et al. 1984). High amounts of free fatty acids (FFA) are found during the ripening of various kinds of blue cheeses (Anderson and Day 1965; Madkor et al. 1987; Contarini and Toppino 1995).

Degradation of lipids in blue veined cheeses is mainly caused by *P. roquefortii* (Kinsella and Hwang 1976; Coghill 1979; Gripon 1993), but the native milk lipase will contribute significantly at the beginning of the ripening, especially in cheeses produced from raw milk (Gripon 1993). Non-starter micro-organisms, like yeasts, are present in high numbers both on the surface and in the interior of the cheese (Gobbetti et al. 1997; van den Tempel and Jakobsen 1998) and could also have an effect as well.

Differences in lipolytic activity between commercial strains of *P. roquefortii* have been reported (Farahat et al

1990; Larsen and Jensen 1999) leading to different flavour profiles of the cheeses, as described by Farahat et al. (1990) and Gallois and Langlois (1990). *P. roquefortii* produces at least two extracellular lipases, an acidic and an alkaline lipase (Menassa and Lamberet 1982). The relative importance of the acidic and the alkaline lipase in cheese has not been fully determined. However, acid lipase activity dominated in six out of seven samples of French blue cheeses (Lamberet and Menassa 1983), indicating the importance of this lipase in cheese ripening. Several authors have reported a preference of the lipases for hydrolysis of short-chain fatty acids (Menassa and Lamberet 1982), but a significant hydrolysis of long-chain fatty acids and butterfat has also been observed (Menassa and Lamberet 1982; Larsen and Jensen 1999). The NaCl in the cheese influences lipolysis; low NaCl concentrations (2% w/v) lead to higher amounts of released FFA released, while at high NaCl concentrations (7% w/v) FFA seem to accumulate in the cheese (Godinho and Fox 1981b; Larsen and Jensen 1999).

The characteristic flavour and taste of blue-veined cheeses stems mainly from lipid degradation: the FFA themselves contribute both to the taste and aroma, but even more important are the compounds produced from the FFA, e.g. the methyl ketones (Kinsella and Hwang 1976; Gallois and Langlois 1990). The methyl ketones, and especially 2-heptanone and 2-nonanone, are the major aroma compounds in blue-veined cheeses (Gallais and Langlois 1990; Gonzalez de Llano et al. 1990; de Frutos et al. 1991). They have been reported to constitute 50–75% of the total aroma profile (Gallois and Langlois 1990; de Frutos et al. 1991) and their amount in the cheese can be correlated to the intensity of a “blue cheese” note (Rothe et al. 1982, 1986). Peptides and amino acids from the protein degradation are important for the flavour of the cheese and are also degraded further, e.g. to aldehydes, alcohols and various sulfur compounds (Hemme et al. 1982). In general, the

lipid-derived compounds dominate in the flavour of blue cheeses, but many other compounds are also found. The varying amounts detected of these compounds are thought to give a picture of the specific flavour profiles obtained for particular blue cheeses (Gallois and Langlois 1990).

2. White Mould Cheeses

a) *Penicillium camemberti*

P. camemberti is a unique culture in the way that it is strongly associated with cheeses like Brie and Camembert and very seldom observed in other foods and environments. Like *P. roqueforti* it only exists in the anamorph form. Taxonomic studies and identification of *P. camemberti* are based upon micro- and macromorphology, production of secondary metabolites and molecular techniques, as mentioned for *P. roqueforti*.

The genetics of *P. camemberti*, like for *P. roqueforti*, is not well investigated, but recently a few articles have been published, dealing with cloning and expression of genes involved in conidiation (Boualem et al. 2008) and a molecular characterization of the genes *niaD* and *pyrG*, which could be used as transformation markers (Navarrete et al. 2009).

A flow diagram for the production of Camembert and Brie is shown in Fig. 1.5.

b) Technological Characteristics of *Penicillium camemberti*

Conditions for Growth and Development in the Cheese. The optimum temperature range for growth is 20–25°C. Growth has been recorded at 5°C, but not at 37°C. With regard to pH, growth of *P. camemberti* can take place in the interval 3.5–6.5; a_w limits are similar to *P. roqueforti*, as



Fig. 1.5. Flow diagram for the production of Camembert/Brie

described above. In the cheese, the pH is reduced to about 4.6 during the first 24 h. During maturation, pH increases to about 7.0 in the outer part of the cheese and about 5.5 in the centre. In the final cheese, the a_w has been recorded as 0.93 and 0.97 in the surface and centre, respectively.

P. camemberti can grow on the surface of the cheese because of its salt tolerance, and salting of the cheese is important for the development of *P. camemberti*. In the process of maturation and texture development, an integrated role is played by lactate, ammonia and calcium (Le Graet et al. 1983; Vassal et al. 1986; Karahadian and Lindsay 1987; Spinnler and Gripon 2004). In the cheese, *P. camemberti* metabolises lactate to CO₂ and water, resulting in a higher pH. The change of pH is most pronounced on the surface of the cheese, which is covered by the white mycelium after a week of maturation (Von Seeler 1968). A pH gradient of decreasing values is established towards the centre of the cheese, causing the lactate to migrate towards the surface, where it is used as a carbon source by *P. camemberti*. When lactate becomes depleted, casein is metabolised, resulting in the formation of ammonia from amino acids. This leads to a further increase in pH and a stronger gradient, still with acid conditions in the centre. Calcium phosphate is soluble at acid pH, but in the regions of high pH it precipitates, resulting in a migration towards the surface. The depletion of calcium phosphate in the centre of the cheese assists in the development of the desired soft texture of the cheese.

The proteinases from *P. camemberti* are activated by the increasing pH, but the enzymes migrate very slowly into the cheese, and they only reach a depth of about 6 mm from the surface (Noomen 1983). This means that their direct participation in the enzymatic reactions in the cheese is limited. The important enzymatic activities in the interior of the cheese are caused by the enzymes from the rennet, the plasmin from the milk and enzymes from the lactic acid starter cultures. However, the pH change, or the establishment of a pH gradient caused by *P. camemberti*, is the key factor in cheese maturation (Lawrence et al. 1987).

Proteolysis. The proteolytic system of *P. camemberti* is very similar to that of *P. roqueforti* (Gripon et al. 1977, 1980; Lenoir and Auberger 1977a, b; Lenoir et al. 1979; Fuke and Matsuoka 1993; Spinnler and Gripon, 2004). *P. camemberti* produces two extracellular endopeptidases, an aspartic protease, a metallo-protease, five to six extracellular exopeptidases, an acid and an alkaline carboxypeptidase and three aminopeptidases. Pronounced strain variations are seen for the intracellular proteolytic

system. Several strains produce two different alkaline aminopeptidases. Furthermore, an acid and an alkaline carboxypeptidase as well as acid, neutral and alkaline endoproteases are produced. For more detailed information on the proteases and peptidases, refer to the description of *P. roqueforti*.

Lipolysis and Aroma. *P. camemberti* only produces one lipase (Lamberet and Lenoir 1976), unlike *P. roqueforti*, which produces two, and the enzyme is very similar to the alkaline lipase of the latter. Significant differences are seen among strains of *P. camemberti* in lipase formation and activity, and it seems to have a preference for short-chain fatty acids. The aroma formation is linked to lipolysis and to proteolysis. From lipids, the main components are methyl ketones and secondary alcohols (Karahadian et al. 1985a, b; Okumura and Kinsella 1985; Jollivet and Belin 1993) with a high degree of similarity to the aroma components produced by *P. roqueforti*.

The dominant compounds are the methyl ketones 2-nonanone, 2-undecanone, 2-heptanone and 2-pentanone. The corresponding secondary alcohols together with other compounds seem to contribute to the cheese aroma with an earthy, musty flavour, while especially 1-octen-3-ol gives the characteristic mushroom flavour (Karahadian et al. 1985a, b; see also Chapter 12). However, the conversion rate from ketones to secondary alcohols is much slower than in blue mould cheeses. The level of free fatty acids is low in white mould cheeses and normally below the sensoric threshold level.

Proteolysis leads to the formation of ammonia as mentioned above and amino acids important for the aroma profile of white mould cheeses. The further breakdown of amino acids leads to alcohols like 2-methylpropanol and 3-methylbutanol with fruitiness and fragrant aroma impressions. The formation of phenyl ethyl alcohol and its esters during ripening of Camembert cheese is reported (Roger et al. 1988). Volatile and non-volatile amines from decarboxylation of amino acids are also present. Bitter peptides may be produced, linked to certain strains of *P. camemberti* and their combined use with *G. candidum* as described above.

C. Microbial Interactions in Cheeses Involving Yeast and Filamentous Fungi

Dairy fermentations involve microbial interactions at several levels. The interactions lead to

different microbiota playing their role in a succession important to the progress of the fermentation and the quality of the final product. They take place in the form of complex interactions between the primary starter culture, yeast and *Penicillium* spp. in mould-ripened cheeses and between the primary starter culture, yeast and *Brevibacterium linens* as well as other bacteria in surface-ripened cheeses.

The microbial interactions not only serve the development of the desired microbiota. Seen from a technological point of view, they also assist in the elimination and control of undesired microorganisms, including specific spoilage organisms and possibly food-borne pathogens.

Mechanisms of Microbial Interactions. The mechanisms behind microbial interactions are numerous and are expressed as synergism or antagonism between micro-organisms. Synergism as seen in foods can rely upon mutual use and production of nutrients, changes of the micro-environment [for example pH, redox potential, water activity (a_w), atmosphere composition], breakdown of specific antimicrobial compounds, physical attachments between microbes, colonisation on food structures and changes of microstructures. Antagonism, which so far represents the most extensively studied interactions, includes formation of antimicrobial metabolites like organic acids, ethanol and other alcohols, carbon dioxide, hydrogen peroxide, diacetyl, production of bacteriocins, reuterin and “killer toxins”, competition for nutrients and unfavourable changes of the micro-environment.

Microbial signalling and sensing are new research areas of interest to food fermentations, and in particular solid state fermentations, where individual cells attach to and colonise food structures. For several food-relevant yeast species, it has been described how cell to cell communication can take place (e.g. during colonisation) and also how ammonia can mediate long-distance communication between colonies (Palkov 1998; Palkov and Vachova 2003; Gori et al. 2007b). This type of signalling could easily take place in a structured food like cheese, where yeasts will multiply as microcolonies attached to a solid support.

During ripening, interactions between rennet enzymes, proteinases and lipases from milk, the lactic acid starter culture and fungal starter cultures like *P. roqueforti* determine the maturation time, aroma, texture and appearance of the final

cheese. The amount of experimental work carried out within this field of complex interactions seems to be limited. However, microbial interactions between cultures of relevance to cheese maturation have been described (Devoyod and Muller 1969; Gripon et al. 1977; Hansen and Jakobsen 1997).

Between strains of *P. roqueforti* and primary starter cultures, like *Leuconostoc* spp., *Lactobacillus* spp. and *Lactococcus* spp., negative and positive interactions (i.e. inhibition or stimulation of *P. roqueforti*) have been demonstrated in laboratory media and a cheese-based model system (Hansen and Jakobsen 1997). It was observed that the interactions were highly affected by the medium used. In general, positive interactions were stronger and seen more frequently in the cheese model system than in laboratory media. For *P. roqueforti*, as well as for the lactic acid starter cultures, the interactions were strain specific. The positive interactions were expressed as faster growth rate, increase in sporulation, a more intense blue/green colour and a thicker, more velvet mycelial growth. Analysis of the amino acid composition of the cheese-based model systems inoculated with lactic acid starter cultures, *P. roqueforti* or the two together, indicated that the lactic acid bacteria stimulated *P. roqueforti* by releasing amino acids like arginine and leucine (Hansen and Jakobsen 1997).

Yeasts in dairy products may interact with other micro-organisms in three different situations: (1) they may inhibit or eliminate micro-organisms, which are undesired, because they cause quality defects, (2) they may inhibit the starter culture or (3) they may contribute positively to the fermentation or maturation process by supporting the function of the starter culture. Concerning inhibition or elimination of undesired micro-organisms, it has been indicated, but not finally proved, in studies with *Debaryomyces hansenii* that it inhibits the germination of *Clostridium butyricum* and *C. tyrobutyricum*, possibly by assimilation of organic acids in the cheese (Deiana et al. 1984). The “killer factor” has been demonstrated in yeasts isolated from cheese brines (Seiler and Busse 1990), and with its possible broad antimicrobial spectrum it may also affect starter cultures. Inhibition of the growth of *Mucor* spp. on the surface of Camembert by the dominant yeast flora has also been reported (Siewert 1986).

The possible role of interactions between commercial strains of *P. roqueforti* and yeasts isolated from blue veined cheese has been described by Hansen and Jakobsen (1998). Based

upon experiments including *P. roqueforti*, *D. hansenii*, *S. cerevisiae* and *Kluyveromyces lactis*, significant negative and positive interactions were demonstrated in laboratory media and in a cheese-based model system. The negative interactions were seen as inhibition of growth and sporulation of *P. roqueforti*, whereas positive interactions appeared as faster growth, a thicker, more velvet mycelium and a more intense blue colour of the conidia, as mentioned above.

The influence of yeasts on growth and sporulation of *P. roqueforti* varied significantly between the three yeast species, but the pattern of interaction was similar for all strains of *P. roqueforti* (Hansen and Jakobsen 1998). The least effect was seen for *D. hansenii*, showing very little interaction if any. Similar results were obtained by van den Tempel and Jakobsen (2000), who concluded that the main role of *D. hansenii* in blue mould cheeses is to create a stable micro-environment, which prevent undesired microbial growth by assimilation of the residual carbohydrates and acids. Depending on the strain, it might also contribute to the aroma profile but not change it significantly. Though, *D. hansenii* can produce undesired pigment on the surface of the cheese.

As mentioned earlier, Hansen and Jakobsen (2001) found one *S. cerevisiae* strain to interact positively with *P. roqueforti*. Whole-cell inocula of *S. cerevisiae* promoted growth and sporulation of *P. roqueforti*, but no interactions were seen when examining the supernatant or the disrupted cells of *S. cerevisiae*. The positive interaction was assumed to be based on the proteolytic system as the *S. cerevisiae* strain showing positive interactions with *P. roqueforti* was the only strain degrading casein. The stimulation of *P. roqueforti* was most pronounced for the least proteolytic strain of *P. roqueforti* examined. The effect on growth and sporulation of *P. roqueforti* was confirmed by using the *S. cerevisiae* strain as starter culture in experimental cheeses (Hansen et al. 2001).

K. lactis showed pronounced inhibition of growth and sporulation of *P. roqueforti*. The inhibition by *K. lactis* was observed for both whole-cell inocula and cell-free extract, indicating that nutrient competition was not the cause of inhibition (Hansen and Jakobsen 1998). van den Tempel and Jakobsen (2000) found that *Yarrowia lipolytica* were inhibitory towards mycelial growth and sporulation of *P. roqueforti*. As inhibition of *P. roqueforti* was not seen when using culture supernatants and cell-free extract of *Y. lipolytica*, the mechanism of inhibition seems to be explained by competition for nutrients.

Concerning positive interactions, yeasts are also reported to assist the development of *P. roqueforti* by gas production, leading to curd openness (Coghill 1979). Further, in the study mentioned above (Hansen and Jakobsen 1998) it was found by capillary electrophoresis that the *S. cerevisiae* strain examined was able to break down some of the α_{s1} casein and the $\beta A1$ and $\beta A2$ casein. Synergism in proteolysis has also been shown between *G. candidum* and *P. camemberti* (Boutrou et al. 2006).

The majority of yeasts isolated from mould-ripened cheese show lipolytic activities (van den Tempel and Jakobsen 1998). Further, they assimilate lactose, glucose, galactose and citrate, are osmotolerant and grow under the environmental conditions (pH, temperature, a_w) prevailing in Danablu, suggesting a positive contribution to the maturation of Danablu and a potential use as an adjunct starter culture.

In a study by Nielsen et al. (1998), designed to determine the antagonistic ability of fungal starters towards fungal contaminants on cheese, it was found that the cultures mainly showed mutual inhibition on contact. However, *G. candidum* grew almost unaffected by the presence of the contaminants *Penicillium commune*, *P. caseifulvum*, *P. verrucosum*, *P. solitum* and *Aspergillus versicolor* at 25°C (Jakobsen and Nielsen 1998). Known mycotoxins produced by the contaminants were detected in significantly lower quantity in dual cultures with *G. candidum*, compared with the single cultures. *G. candidum* caused a significant inhibition of the fungal contaminants on Camembert cheese. The results indicate that *G. candidum* plays an important role in competition with undesirable microorganisms in mould-fermented cheeses. Dieuleveux et al. (1998) have shown that *G. candidum* produces and excretes 2-hydroxy-3-phenylpropanoic acid with a broad-spectrum antibacterial effect and its potential use in cheeses was mentioned. The effect of this compound against moulds and yeasts seems not to have been investigated. However, inhibition of *P. roqueforti* in blue mould cheeses by *G. candidum* is a problem observed in dairies (unpublished data).

IV. Meat

The micro-environments of cheeses and meats are similar in several aspects. In both types of foods, the activity of fungi will depend on their ability to grow in an environment rich in protein and lipids, at low temperatures and at low water activities caused by high salt concentrations. This explains that *D. hansenii* is not the only dominant yeast in cheeses (Fleet 1990; Petersen et al. 2002) but also in fermented meat products (Encinas et al. 2000; Gardini et al. 2001; Aquilanti et al. 2007; Nielsen et al. 2008; Asefa et al. 2009) and that several filamentous fungi (*Penicillium nalgiovense*, *P. commune*, *P. solitum*) occur on both products (Filtenborg 1996, Asefa 2009).

The application of fungal starter cultures in the meat industry is described in reviews by Hammes and Hertel (1998), Sunesen and Stanhke (2003) and

Leroy et al. (2006). The suitability of mycelial fungal starter cultures for meat fermentation is based upon their degradation of lactic acid, their lipolytic and proteolytic activities and their contribution to aroma formation (Grazia et al. 1986; Leistner 1990). Studies have shown that addition of *Penicillium* or *Mucor* spp. to sausages can increase the degree of proteolysis and degradation of amino acids (Toledo et al. 1997) as well as the lipolysis (Selgas et al. 1999) and sensoric characteristics (Garcia et al. 2001), although there was a great variation between the fungi tested.

The levels of flavour compounds as branched-chain aldehydes and alcohols have been shown to be higher in sausages inoculated with filamentous fungi (*P. aurantiogriseum*, *P. camemberti*, *M. racemosus*) or treated with cell-free extracts (Bruna et al. 2000, 2001, 2003). Inoculation with *Penicillium* spp. (*P. aurantiogriseum*, *P. camemberti*, *P. nalgiovense*) could also lower the formation of oxidation products (Bruna et al. 2001, 2003, Sunesen et al. 2004) and give a more uniform drying of sausages (Grazia et al. 1986).

It is assumed that aroma formation and other technological properties described for cheese fermentations also apply for meat fermentations to a large extent. However, compared with the dairy industry, the application of fungal starter cultures in the meat industry is less common.

Spontaneous fermentations of sausages and hams are common in certain geographical regions, as described for sausages by Andersen (1995), and are well known for the Mediterranean-style hams. Spontaneous fermentations involve the risk of formation of mycotoxins and other quality defects. This may be controlled by the use of well defined fungal starter cultures which can be used as protective cultures that are able to prevent growth of undesired fungi (Geisen 1993; Berwal and Dincho 1995). However, contaminating moulds may be camouflaged by the starter culture and mycotoxins may be produced despite the use of starter culture (Iacumin et al. 2009). Recently, an approach based upon gene expression in meat was developed for detection of ochratoxin A produced by *Penicillium* spp. (Bogs et al. 2006; Geisen et al. 2006; Schmidt-Heydt and Geisen 2007a, b; Schmidt-Heydt et al. 2007). Examples of starter cultures used for maturation of meat products are *P. nalgiovense*, *P. camemberti* and *P. chrysogenum* (Cook 1995; Sunesen and Stahnke 2003; Ludemann 2010).

The role of yeasts in meat fermentation is still poorly defined. Some studies have shown overall better sensory properties after the addition of yeasts to meat products (Flores et al. 2004; Iucci et al. 2007), while others did not detect significant differences (Olesen and Stahnke 2000; Martin et al. 2006) or the development of off-flavours (Sanchez-Moliner and Arnau 2008). The dominant yeast in meat products is *D. hansenii*, which is also commercially available as a starter culture for meat fermentations, mainly sausages. It has both lipolytic and proteolytic activity (Patrignani et al. 2007), but the lipolytic activity is apparently very limited under the environmental conditions prevailing in fermented sausages (Sørensen and Samuelsen 1996; 1997). *D. hansenii* is aerobic and therefore mainly found in the outer part of fermented meats. Its growth results in depletion of oxygen, which is likely to have a positive effect on colour stabilisation, although *D. hansenii* does not reduce nitrate.

Manufacture of sausages with *Debaryomyces* spp. had a positive influence of flavour due to increased formation of ethyl esters and lower formation of volatile compounds derived from lipid oxidation (Flores et al. 2004). *Debaryomyces* spp. also produced branched-chain aldehydes, alcohols and acids that may be important for the sausage flavour, but production was influenced by inoculum size, growth phase, pH and salt content (Flores et al. 2004; Dur et al. 2004). Marked differences in volatile compound production between biotypes of *D. hansenii* differentiated by mitochondrial DNA patterns and RAPD-PCR have been observed (Andrade et al. 2009a, b).

Another yeast that may contribute positively to meat fermentations is *Candida utilis* (the anamorph form of *Pichia jadinii*). It is lipolytic and reported to improve the aroma formation in fermented meats (Mitva et al. 1986). *C. utilis* produced several volatile compounds in a sausage model, including branched-chain acids, aldehydes, alcohols and esters that were probably derived from breakdown of the amino acids valine, isoleucine and leucine (Olesen and Stahnke 2000). *C. utilis* can reduce nitrate to nitrite that forms a red-coloured complex with myoglobin characteristic for cured meat products.

V. Conclusions

The use of yeasts and filamentous fungi in the production of bread, cheese and meat products

has a long tradition and conservatism is still a strong element in most industrial applications, although developments are seen for three types of fermented food.

The consumption of yeast is increasing slightly as a result of increasing population, dietary changes and the introduction of bread to new geographical regions such as Asia and the developing countries. Further, the world market for yeast with extended shelf life and convenience products in the form of frozen dough is growing. This should place stress resistance in the centre of current research and development.

For the improvement of yeast strains and the development of novel strains, recombinant approaches should be continued. However, alternative cultures should also be sought in traditional fermentations to add new characteristics, e.g. aroma formation, to the standard yeast.

Within the dairy industry, the filamentous fungal cultures *P. roqueforti* and *P. camemberti* have been intensely studied with regard their proteolytic and lipolytic activities. The complexity was evident and new, advanced studies concentrating on the actual protein chemistry of the cheeses should be given a high priority. Novel techniques based upon mass spectrometry and other principles as applied for other cheeses offer good possibilities for such work.

Priority should also be given to studies of the genome of the filamentous fungi starter cultures. This should lead to the identification and understanding of the regulation of genes encoding technological properties and undesired characteristics, such as the production of mycotoxins.

The role of yeast in cheeses should be further explored and detailed information acquired to design more precisely their application as starter cultures. The same applies for filamentous fungi as well as yeasts for their use as starter cultures in meat products. Use should be made of the similarities seen between these two categories of food with transfer of the experiences gained in cheeses to meat fermentations.

Instead of investigating single cultures of filamentous fungi and yeasts, further focus on microbial interactions in food matrices should take place. Indeed, the addition of micro-organisms that express a specific function in pure culture does not lead necessarily to a similar phenotype in complex microbial environments. Further research in the role of cell-cell communication

known as quorum sensing in these microbial interactions could be of interest.

The general trend seen in a wider use of starter cultures with probiotic and other functional properties may also, in time, become relevant for fungi used in the production of bread, cheese and meat.

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2 Asian Fungal Fermented Food

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I. Introduction

A. Food Fermentation

The fermentation of food is often defined as the manufacture of foods employing the action of micro-organisms and their enzymes. This would ideally result in changes in the flavour, texture, colour and other quality attributes that are considered desirable by the consumer, all within the context of socio-cultural patterns of food preferences. The origin and attractiveness of several fermented foods is due to their prolonged shelf life, reduced volume, shorter cooking times and superior nutritive value as compared to the non-fermented ingredients. Fermented foods are encountered worldwide, and they are prepared from a wide variety of foods of animal and plant origin and micro-organisms.

Traditionally, food fermentation is carried out at a household scale. Whereas a considerable number of fermentation processes have been scaled-up for commercial purposes, it may be safely stated that most types of fermented foods are still manufactured at home-scale under conditions of variable hygiene, using relatively simple processing facilities. Such products often contain mixed microbial populations because of the lack of sterility and the use of natural (spontaneous) fermentation or mixed-culture fermentation starters.

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For the purpose of this chapter, fungal fermented foods are defined as those foods in which fungi (yeasts and mycelial fungi) predominate and play a functional role, i.e., they contribute to the desirable attributes of the fermented product.

B. Useful Fungi

The number of fungal species encountered in fermented foods is relatively limited, but they belong to various orders. Table 2.1 shows some major examples and the products in which they are predominant.

C. Fungal Fermented Foods World-Wide

Fungal fermentation is practised in nearly all continents. In African traditional culture, cherished fermented cereal products include beers and fermented porridges, most of which are fermented by a natural mixed culture of lactic acid bacteria and yeasts. The use of mycelial fungi for fermentation

in Africa is less common, but is practised in the fermentative detoxification of bitter cassava roots in, e.g., Mozambique and Uganda.

In the European region, the major traditional uses of yeasts include the production of alcoholic beverages and the fermentation of leavened bread whereas specific uses of mycelial fungi are mould-ripened cheeses and meat products. In several other products, such as in fermented olives, yeasts belong to the functional flora in combination with lactic acid bacteria.

The Asian continent provides the greatest variety of fungal fermented foods. The potential of yeasts to contribute flavour, nutritive value, alcohol and gas is evident in both starchy and leguminous foods. Mycelial fungi are used for their enzymatic ability to degrade polymeric substances, as well as for texture-forming properties.

There is an increasing international interest in the contribution to health by mould-fermented foods. In particular, fermented soybean foods attract world-wide attention for their content of bio-active peptides derived from glycinin (Gibbs et al. 2004); fermentation strongly improves digestibility, protection against diarrhoea and chronic

Table 2.1. Major functional fungal species in Asian fermented foods; adapted and enlarged from Samson (1993a)

Zygomycetes	
<i>Actinomucor</i>	<i>A. elegans</i> , <i>A. taiwanensis</i> (sufu, tou-fu-ru)
<i>Amylomyces</i>	<i>A. rouxii</i> (ragi)
<i>Mucor</i>	<i>M. circinelloides</i> , <i>M. rouxii</i> , <i>M. indicus</i> (ragi, murcha, tempe, pehtze)
<i>Rhizopus</i>	<i>R. microsporus</i> (tempe), <i>R. oligosporus</i> (tempe), <i>R. oryzae</i> (koji, nuruk, chu, murcha, tempe)
Ascomycetes	
<i>Monascus</i>	<i>M. purpureus</i> , <i>M. ruber</i> (angkak)
<i>Neurospora</i>	<i>N. sitophila</i> , <i>N. intermedia</i> (oncom)
Deuteromycetes	
<i>Aspergillus</i>	<i>A. oryzae</i> (koji), <i>A. sojae</i> (koji), <i>A. glaucus</i> , <i>A. melleus</i> , <i>A. repens</i> , <i>A. candidus</i> (katsuobushi), <i>A. niger</i> (koji)
<i>Penicillium</i>	<i>P. glaucum</i> (katsuobushi)
Yeasts	
<i>Brettanomyces</i>	<i>B. anomalus</i> (kumiss)
<i>Candida</i>	<i>C. javanica</i> (idli, kombucha, murcha)
<i>Endomyces</i>	<i>E. fibuliger</i> (murcha, ragi)
<i>Hansenula</i>	<i>H. anomala</i> (saké, koji)
<i>Hyphopichia</i>	<i>H. burtonii</i> (ragi)
<i>Saccharomyces</i>	<i>S. cerevisiae</i> (nan, toddy, murcha, kombucha), <i>S. dairensis</i> (tempe), <i>S. globosus</i> (kumiss), <i>S. kluyveri</i> (nan), <i>S. saké</i> (saké)
<i>Torulopsis</i>	<i>T. versatilis</i> (idli, kombucha, soy sauces, pastes)
<i>Trichosporon</i>	<i>T. pullulans</i> (idli), <i>T. beigelii</i> (tempe)
<i>Zygosaccharomyces</i>	<i>Z. rouxii</i> , <i>Z. sojae</i> (soy sauces, soy pastes)

degenerative diseases (Nout 2005). Obviously, as a result of today's intensive international commerce, an increasing number of formerly regional products are finding their way to foreign markets, making it even more important to educate the public about the properties and values of exotic foods.

D. Categories of Asian Fungal Fermented Foods

Considering the numerous regional names of fungal fermented foods, some attempt of classification is worthwhile. According to the type of products obtained, a distinction can be made of beverages (alcoholic and non-alcoholic), condiments or flavourings (including, e.g., soy sauces and pastes), protein-rich meat substitutes (tempe-like products) and bread- or cake-like products (e.g., idli; Lim 1991). Ko (1986) and Nout et al. (2007) distinguished various products on the basis of the functional micro-organisms during the fermentation such as chiefly by mycelial fungi, mycelial fungi and yeasts and a sequence of mycelial fungi followed by yeasts and bacteria. We will approach the issue from a technological angle, and distinguish three principally different fermentation processes, as follows:

1. Natural fermentation: the simplest type of process, whereby uncooked ingredients are mixed and are allowed to undergo an uncontrolled, "spontaneous" fermentation, i.e., without added starter. Usually the functional micro-organisms are present in the substrate or are provided by the environment (the utensils, the house atmosphere, etc.). In many cases these fermented products are cooked after fermentation. Examples are idli and nan (Table 2.2).
2. Starter-mediated single-stage fermentation: ingredients to be fermented are cooked first, followed by the addition of a specific starter (concentrate of viable spores or mycelium). During incubation the starter micro-organism (s) multiplies and modifies the food. After this stage, the fermentation is completed. Usually, the fermented product is cooked again prior to consumption. Examples include kumiss and oncom (Table 2.2).
3. Multiple-stage fermentation: this type of fermentation can be characterized by two or

more fermentation stages, for example a first stage of solid-substrate fermentation followed by a liquid fermentation. The objective of the first stage is to produce a high concentration of polymer-degrading fungal enzymes. During the second stage these enzymes degrade polymers such as starch (for beer or wine-making) and proteins (soy sauces and pastes). Examples are rice wines, soy sauces and pastes and vinegar (Table 2.2).

II. Tempe

A. The Product

Tempe or tempeh is a collective name for fungally fermented beans, cereals or some other food processing by-products. Tempe most probably originates from the island of Java, Indonesia. Yellow-seeded soy beans are the most common and preferred raw material to make tempe. Figure 2.1 shows soy bean tempe (in full: tempe kedede) being sold at a market of Malang, East Java, Indonesia. Tempe is a highly nutritious, easily digestible and delicious product, and as such it meets an increasing demand from consumers looking for high-quality meat replacers. Scientific reports were published already during the 1800s, and this early and subsequent literature has been covered in the excellent review by Ko and Hesseltine (1979). The overview of the major scientific literature was complemented in the review by Nout and Rombouts (1990). The review by Hachmeister and Fung (1993) is of interest because of its coverage of the use of various leguminous seeds and cereals for tempe making. More recently, the functionality of tempe was reviewed (Nout and Kiers 2005). The present section on tempe addresses the aspects of relevance for the functionality of the product and the control of the fermentation process.

B. Traditional Manufacturing Process

Figure 2.2 summarizes the major process unit operations involved in making tempe. In most small-scale Indonesian tempe workshops, the soy beans are dehulled in a wet process. If sufficient water and cheap labour is available, wet dehulling has the advantage that no major equipment is essential and the beans suffer very little mechanical

Table 2.2. Selected Asian fungal fermented foods

Product	Main ingredients		Functional microflora	References
Natural fermentation				
Idli (India): breakfast steamed cakes	Rice and black gram dal		<i>Torulopsis</i> , <i>Candida</i> , <i>Trichosporon pullulans</i> , lactic acid bacteria	Batra (1986)
Nan (India): leavened bread	Wheat flour		<i>Saccharomyces cerevisiae</i> , <i>S. kluyveri</i> , <i>Lactobacillus</i>	Batra (1986)
Tou-shi, hamanatto (China): condiment	Soy beans		<i>Asp. oryzae</i> , <i>Mucor</i>	Wang and Fang (1986)
Red kojic rice, angkak (China): colourant	Rice		<i>Monascus purpureus</i>	Lim (1991)
Toddy (Malaysia): palm wine	Palm sap		<i>Saccharomyces</i> spp.	Lim (1991)
Starter-mediated, single-stage fermentation				
Tapé ketan (Indonesia): sweet snack	Glutinous rice	Ragi tapé (rice)	<i>Amylomyces rouxii</i> , <i>Endomyces fibuliger</i> , <i>Hyphopichia burtonii</i>	Ko (1986), Nout (1995)
Oncom (Indonesia): side dish	Groundnut press cake	Mycelium grown on cassava fibre	<i>Neurospora sitophila</i>	Ko (1986)
Tempe bongkrek (Indonesia): side dish	Coconut press cake	Usar	<i>Rhizopus</i> spp.	Ko (1986)
Katsuobushi (Japan): fish	Bonito fish	Fermentation vessel, or pure cultures	<i>Asp. glaucus</i> , <i>Pen. glaucum</i> , <i>Asp. melleus</i> , <i>Asp. repens</i> , <i>Asp. candidus</i>	Cook and Campbell-Platt (1994)
Airag (kumiss)	Milk	Starter cultures pre-grown in milk	<i>Saccharomyces globosus</i> , <i>Brettanomyces anomalus</i> , lactic acid bacteria	Naersong et al. (1996)
Kombucha (tea)	Tea, sugar	Cellulosic microbial film	<i>Saccharomyces</i> spp., <i>Candida</i> spp., <i>Torulopsis</i> spp., acetic acid bacteria	Nout (1992)
Multiple-stage fermentation				
Tempe kedele (Indonesia): side dish	Soya	Usar	<i>Rhizopus oligosporus</i> , <i>R. oryzae</i> , <i>Mucor indicus</i> , various yeasts and bacteria	Ko (1986), Nout et al. (1992), Nout (1995), data in this chapter
Yakju and takju (Korea): wines	Rice	Nuruk (wheat)	<i>Asp. oryzae</i> , <i>Asp. sojae</i> , <i>Rhizopus</i>	Mheen et al. (1986), data in this chapter
Huan-jiu (China): wine	Rice	Chu (wheat)	<i>Asp. oryzae</i> , <i>Asp. sojae</i> , <i>Rhizopus</i>	Mheen et al. (1986), Fukushima (1998), data in this chapter
Sake (Japan): wine	Rice	Koji (rice)	<i>Asp. oryzae</i> , <i>Hansenula anomala</i> , <i>Sacch. sake</i>	Nout (1995), Fukushima (1998)
Schochu (Japan): spirit	Barley, rice or sweet potato	Koji (barley or rice)	Black Aspergilli	Fukushima (1998)
Bai-jiu (China): spirit	Kaoliang, wheat, rice or other cereals	Chu (wheat)	<i>Rhizopus</i> spp.	Fukushima (1998)
Chiang, jnard (India, Nepal): beer	Barley, millet	Murcha (rice)	<i>Mucor circinelloides</i> , <i>Mucor rouxii</i> , <i>Rhizopus oryzae</i> , <i>Candida javanica</i> , <i>Endomyces fibuliger</i> , <i>Saccharomyces cerevisiae</i>	Batra (1986), Nout (1992)
Kochujang (Korea): spicy catsup	Rice, wheat flour and meju	Meju (soy beans)	<i>Asp. oryzae</i> , <i>Asp. sojae</i> , <i>Bacillus subtilis</i>	Mheen et al. (1986)

continued

Table 2.2. continued

Product	Main ingredients	Functional microflora	References	
Chiang-yu, shi-tche (China), kecap (Indonesia), shoyu (Japan): soy sauces	Soy beans, wheat	Koji (soy beans and wheat)	<i>Asp. oryzae</i> , <i>Asp. sojae</i> , <i>Rhiz. oryzae</i> , <i>Zygosaccharomyces rouxii</i> , <i>Z. sojae</i> , <i>Hansenula</i> spp., <i>Torulopsis</i> spp., lactic acid bacteria	Ko (1986), Wang and Fang (1986), Nout (1995), Fukushima (1998), data in this chapter
Chiang (China), taoco (Indonesia), miso (Japan): soy paste	Soy beans, wheat, rice, barley	Koji (rice, barley or soy beans)	<i>Asp. oryzae</i> , <i>Asp. sojae</i> , <i>Rhiz. oryzae</i> , <i>Z. rouxii</i> , <i>Torulopsis versatilis</i> , lactic acid bacteria	Ko (1986), Wang and Fang (1986), Nout (1995), Fukushima (1998)
Tou-fu-ru (= sufu, furu) (China): soy paste	Soy bean curd (tofu)	Pehtze (tofu)	<i>Actinomucor</i> , <i>Mucor</i>	Wang and Fang (1986), data in this chapter
Tsu (China): vinegar	Millet, rice, sorghum		Mycelial fungi, yeasts, acetic acid bacteria	Wang and Fang (1986)
Jiu (China): Chinese liquor	Sorghum	Daqu (wheat, barley, peas)	<i>Aspergillus</i> , <i>Mucor</i> , <i>Rhizopus</i> , <i>Monascus</i> , <i>Trichoderma</i> , <i>Absidia</i> , <i>Rhizomucor</i> , <i>Emericella</i> ; <i>Saccharomyces</i> , <i>Candida</i> , <i>Pichia</i> , <i>Issatchenkia</i>	Xie et al. (2007), Zhang et al. (2007), Wang et al. (2008a)



Fig. 2.1. Soy bean tempe (tempe kedele) sold at a market of Malang, East Java, Indonesia

damage. At a larger scale or when labour costs are high, dry dehulling is more economic, despite the disadvantage of higher losses of soy beans resulting from the abrasion of the soy bean hulls.

In all cases, soaking is an essential step to increase the moisture content of the beans as to render them edible and enable microbial activity during the fermentation, but also to extract naturally occurring antimicrobial substances (saponins) and bitter principals. For this reason, the soaking water must be discarded and the beans cooked in fresh water. Cooking times vary according to custom and depend on the equipment used. The warming-up and cooling-down may take considerable time; of essence is the actual cooking at approximately 100 °C, which should last 20–30 min. After cooking the hot water is discarded as soon as possible, and the very hot beans are spread out on trays to enable steaming-off of the beans. This evaporation

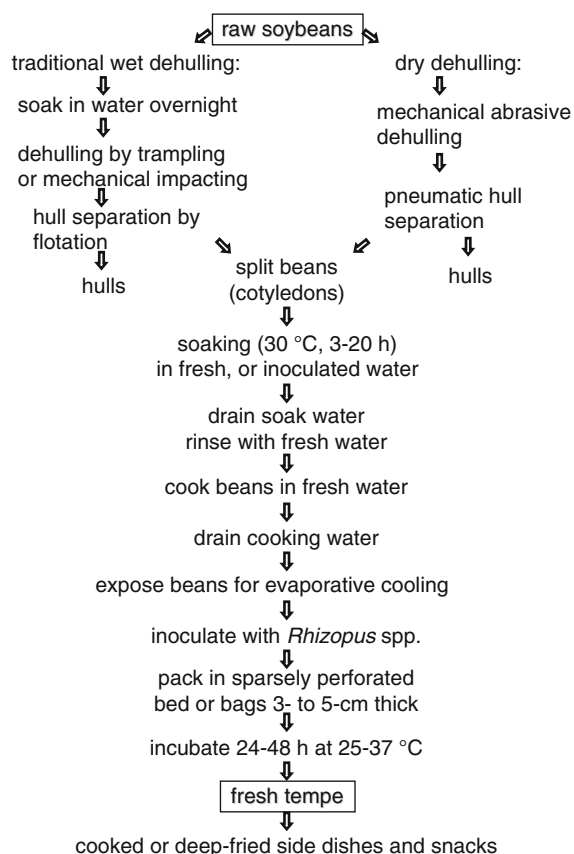


Fig. 2.2. Traditional manufacture of tempe

has two major effects: the removal of free water that could otherwise enhance microbial spoilage during the later stages of the process and a rapid cooling to about 20–25 °C within 10–15 min. The cooled beans are inoculated using tempe starter containing mainly sporangiospores of *Rhizopus oligosporus*, *R. oryzae* and sometimes *Mucor* spp. These are mixed homogeneously, followed by preparing layered beds or packages of approximately 3–5 cm thickness. An essential condition is that a limited supply of air can reach the beans. For this purpose, packaging materials or bed covers are perforated in a more or less evenly distributed manner. Polythene sheet or bags are commonly used for commercial purposes; traditionally banana leaves or other plant material is used. Both give good quality products. Incubation for 1–2 days at ambient temperature (25–30 °C) is enough to allow spore germination and outgrowth of luxuriant mycelium binding the beans together to form a solid, sliceable cake of fresh tempe. Due to the restricted air supply, fungal spores are not or hardly produced and this results in an attractive creamy-white tempe colour with very little grey or black discolouration due to the pigmented sporangiospores. Fresh tempe is not eaten raw, but first cooked, e.g., in stews or fried in oil to give delicious crisps (tempe kripiik). There are numerous ways to enjoy tempe (Shurtleff and Aoyagi 2001).

C. Fungi Involved and Their Relevant Properties

The major genus of importance for tempe making is *Rhizopus microsporus*, with varieties *microsporus*, *oligosporus*, *rhizopodiformis* and *chinensis* (Liou et al. 1990; Nout and Rombouts 1990). Zheng and Chen (1998) describe an additional variety *tuberosus*. Nout et al. (1992) investigated the leaves of the Indonesian Waru tree (*Hibiscus tiliaceus*) of which the leaves are used as a carrier for tempe mould starter locally known as “usar”. On leaves harvested in Indonesia, *R. oryzae* and *R. microsporus* var. *oligosporus* (further referred to as *R. oligosporus*) were found frequently besides a mixed flora of soil fungi; on leaves of the same *Hibiscus* spp. harvested in Africa and Europe the same soil fungi were found but no *Rhizopus* spp. This suggests that the widespread use of *Rhizopus* spp. in the manufacture of tempe results in its preponderance in the air spora. Most likely, *Hibiscus* leaves act as one of its natural reservoirs.

Of all sporangiospores present in starter concentrates used to initiate the fermentation, only a minor part may be viable, whereas significant numbers of spores have been sub-lethally injured. It was observed that well dried sporangiospores remain viable at ambient tropical temperatures for about three months, after which period they

need to be activated. The germination of sporangiospores is strongly activated by amino acids such as L-alanine (Thanh et al. 2007).

The germination of *R. oligosporus* sporangiospores is influenced by temperature, presence of organic acids and fungal “self-inhibitors”. De Reu et al. (1995) observed that 37 °C was optimum for germination and that acetic acid was inhibitory. The latter is of relevance as during the first soaking period, heterofermentative lactic acid bacteria may produce lactic and acetic acids.

Whereas it was shown earlier that *Rhizopus* spp. can grow at low (0.2%) oxygen concentrations, Lin and Wang (1991) observed that of 18 tested strains none was able to grow under absolute anaerobic conditions. They also observed that several strains were sensitive to oxygen toxicity (H_2O_2 or O_2^- or OH^\bullet), and that their growth was restored in the presence of catalase. Other environmental conditions affecting the formation of biomass are the temperature, CO_2 concentration and water activity (Sparringa et al. 2002). Monitoring of fungal growth and biomass formation on natural substrates can be achieved only by indirect means. Nout et al. (1997) provide an overview of techniques of indirect estimation of biomass. In tempe, Peñaloza et al. (1992) reported the use of on-line capacitance measurement; alternatively glucosamine is measured as a measure of cell wall chitin production. Sparringa and Owens (1999) proposed a conversion factor of 12 g dry biomass per gram glucosamine for *R. oligosporus*.

Rehms and Barz (1995) found that several tempe-forming *Rhizopus* spp. (*R. oligosporus*, *R. microsporus* var. *chinensis*, *R. oryzae*, *R. stolonifer*) were able to utilize the flatulence-associated oligosaccharide raffinose as the sole carbon and energy source. However, Graffham et al. (1995) also studied the nutritional requirements of mucoraceous mycelial fungi and observed that *Rhizopus* spp. could not use raffinose and stachyose or the mineral-complexing phytic acid as sole carbon and energy source. This was underlined by the finding of Ruiz-Téran and Owens (1999) who observed that, in bacteria-free tempe, levels of stachyose and raffinose were reduced during the tempe process as a result of leaching during soaking and cooking, but that the residual levels were not reduced further by the action of a strain of *R. oligosporus*. The fact that these substances are degraded nevertheless during the fermentation of “ordinary tempe” underlines the importance of mixed cultures of fungi as well as some of the accompanying bacterial species during the fungal fermentation.

The growth of *R. oligosporus* on a starchy model substrate was described by Mitchell et al. (1990) as a multiple-stage

process as follows: release of amylolytic enzymes by the mycelium, enzyme diffusion, starch hydrolysis by the enzymes, diffusion of glucose, uptake of glucose by mycelium. The mycelium penetrates into several layers of soy bean cells to approx 25% of the width of the cotyledon (Ko and Hesselstine 1979). This was recently confirmed by the finding that the penetration in 40 h was approx 2 mm; the mycelium moved in intercellular spaces and did not penetrate intact soy bean cells (Varzakas 1998). Several empirical methods were developed to measure the strength of the mycelial binding of the soy beans. Ariffin et al. (1994) developed an improved technique and reported that tempe incubated at 30 °C developed maximum strength after 30 h, after which the mycelium degenerated gradually.

Limitation of heat and mass transfer easily results in overheating of substrate beds in solid-substrate fermentations. Mixing during fermentation can be applied to improve heat and mass transfer and to obtain a more homogenous fermentation. Han et al. (1999) studied the effect of mechanical stress caused by intermittent rotation on the behaviour of *R. oligosporus* and *R. microsporoides* on soy beans. They observed that biomass formation and enzyme production are negatively affected by mechanical stress, and that the sensitivity towards mechanical stress of the two strains tested was quite different.

As mentioned above, bacteria and yeasts accompany the mould fermentation. If lactic acid bacteria can dominate during soy bean soaking, the cooked beans will be slightly acid and this was shown to have important implications for the microbial composition of the final product (Mulyowidarso et al. 1990; Nout and Rombouts 1990). Wiesel et al. (1997) reported that mixed inocula of *R. oligosporus*, *R. oryzae*, *Citrobacter freundii* and *Brevibacterium epidermis* resulted in tempe covering the daily requirements of niacin, vitamin K, ergosterol, tocopherol, pyridoxine, riboflavin and biotin.

D. Biochemical Modifications and Implications for Health

Rhizopus spp. and the accompanying microflora consisting of bacteria and yeasts produce a range of enzymes that degrade proteins, lipids and carbohydrates of cooked soy beans. Proteases of *Rhizopus* were found to be mainly cell wall bound (Baumann and Bisping 1995). Heskamp and Barz (1998) studied proteases of nine strains of *R. oryzae*, *R. microsporoides* var. *chinensis*, *R. stolonifer* and

R. oligosporus and obtained various isoforms of aspartic (35 kDa) and serine (33 kDa) proteases. The proteolytic activity of *R. oligosporus* proved to be functional in cereals such as buckwheat, resulting in a lowering of allergenic proteins and an improvement of rheological behavior in noodle making (Handoyo et al. 2006).

Hering et al. (1990) found that during laboratory tempe fermentation the total level of crude lipids did not change much, but that the relative level of unsaturated fatty acids (especially oleic acid) increased. Sarrette et al. (1992) observed that as a function of time a range of polysaccharide degrading enzymes are formed by *R. oligosporus* growing in soy beans. The highest activities were measured after 20–30 h of fermentation; polygalacturonase, endocellulase, xylanase, arabinase, β -D-glucosidase and α -D-galactosidase were most prominent. De Reu et al. (1997) were able to correlate these polysaccharidase activities with softening of the soy beans and decreasing levels of non-starch polysaccharides.

Whereas some of the low-molecular-mass breakdown products will be metabolized by the microflora, the overall result of the microbial enzyme activity is a considerable degradation of polymeric substances in oligomeric and smaller units improving tempe digestibility process (Matsuo 1996; Kiers et al. 2000). This degradation is reflected in the spectacular increase of water-soluble dry matter and the in vitro accessibility (soluble matter that passes through dialysis tubing mimicking intestinal absorption). Table 2.3 shows the effect of *R. oligosporus* LU575 on absorptability with and without enzymic digestion. These

Table 2.3. In vitro digestibility of soy bean tempe fermented with a pure culture of *Rhizopus oligosporus* LU 575 (Kiers et al. 2000)

Fermentation period at 30 °C (h)	Accessibility (% dry matter) ^a		Fermentability (% dry matter) ^b
	Without digestion	With digestion ^c	
0	2	21	8
24	18	22	1
48	22	27	1

^aWater-soluble matter passing through dialysis membrane (14 kDa cut-off).

^bBased on production of gas and acetic acid by *Clostridium perfringens* ATCC12916 at 37 °C during 24 h.

^cAll at 37 °C in artificial saliva (pH 7) for 30 min, artificial gastric juice (pH 4) for 60 min, pancreatic solution (pH 6) for 30 min.

limited data indicate that tempe is of particular interest in patients suffering intestinal digestive deficiencies. The same table shows the fermentability (by anaerobic colon microflora) of undigested residues that would enter the colon in vivo. Residues of tempe reduced fermentability in the human gut and therefore tempe is more desirable to eat than cooked soy beans.

Other biochemical changes include the formation of vitamins (riboflavin, nicotinic acid, pantothenic acid, pyridoxin, folates, some biotin). It was observed that during the fungal fermentation, a fivefold increase of folates (mainly 5-formyl tetrahydrofolate and 10-formyl tetrahydrofolate) takes place in tempe probably as a result of de novo synthesis (Ginting and Arcot 2004). Cyanocobalamin is formed by bacteria in tempe, but it has not yet been established whether this can be utilized by humans. Several antinutritional factors are degraded during the process of tempe manufacture, mostly by leaching and thermal inactivation during soaking and cooking. However, phytic acid is degraded by fermentation (Eklund Jonsson et al. 2006) and this results in improved mineral bio-availability. For instance, Kasaoka et al. (1997) found in studies with iron-deficient rats that consumption of tempe achieved higher liver iron levels than unfermented cooked soy beans.

Of much interest is the modification of soy bean isoflavones by microbial activity, into substances with antioxidant and radical-scavenging activity that could have health-promoting effects. For instance, Klus and Barz (1998) demonstrated the formation of polyhydroxylated isoflavones from biochanin A and genistein by *Micrococcus* and *Arthrobacter* spp. isolated from tempe, and Matsuo et al. (1997) showed that 3-hydroxyanthranilic acid (HAA) is formed by fungal transformation of soy bean flavonoids. GABA-enriched tempe-like fermented soybean was produced by anaerobic fermentation with selected strains of *Rhizopus microsporus* (Aoki et al. 2003b); GABA (gamma-amino butyric acid) reduced hypertension in rat models over two months (Aoki et al. 2003a).

The anti-diarrhoeal effect of tempe that had been observed in child feeding in Indonesia, could also be established in piglets (Kiers et al. 2003). Piglets suffered less frequent and less severe weaning diarrhoea and had more efficient feed conversion when fed mixed feed containing soy bean tempe (Kiers et al. 2003). This could be ascribed to a reduction of fluid loss from the upper gut (Kiers et al. 2007), as well as a reduced adhesion of pathogenic *Escherichia coli* (ETEC) to the pig intestinal mucosa (Kiers et al. 2006). The bioactive component is water-soluble and has a molecular mass of >5 kDa (Kiers et al. 2007). It was

established recently that this tempe related bio-activity also reduced the adhesion of ETEC to human intestinal cells of the Caco-2 type (Roubos-van den Hil et al. 2009).

Within the genus *Rhizopus*, the formation of anticancer drugs rhizoxins and toxic rhizonins have been described. From a range of *Rhizopus* species and strains grown on semi-synthetic and natural substrates, *R. oligosporus* and *R. chinensis* did not produce the secondary metabolites mentioned above. In contrast, *R. microsporus* produced rhizoxins and one strain produced rhizonins (Jennessen et al. 2005). It was found recently, however, that the production of rhizonin is not caused by the fungus (Partida Martinez et al. 2007) but by symbiotic bacteria of the genus *Burkholderia* that are localized in the fungal cytosol.

E. Industrial Aspects

Whereas small-scale home production uses the traditional equipment and starters, several innovations should be mentioned because they have changed the scene of tempe making. For instance, wet bean dehulling is carried out mechanically using simple motor-driven concrete-disc impactors. For packaging fermentation beds, polythene sheet has displaced the banana leaves of old. Powdered starter concentrates are commercially available.

In Indonesia, the annual production of tempe was estimated at about 80000 t with 14% of the soy beans produced in Indonesia being used for tempe production (Yokotsuka and Sasaki 1998).

Recently, some interesting process lines and novel products were developed (Nout and Kiers 2005). Semi-continuous process lines of a capacity of 600 kg/day tempe are commercially used. Dry dehulled beans are soaked overnight in a hopper vessel and transported by belt conveyor through a boiling water bath in a period of 20 min. Next they are drained and dried and cooled under a fan; if needed the bean temperature can be adjusted using heating section. Inoculation takes place using a mechanical dispenser, and the inoculated beans are filled into perforated polyethylene tubing of 5 cm diameter. The incubation of these sausages takes place at 32 °C for 24 h, in a carefully designed room with forced ventilation. These products are pasteurized for improved shelf-life and microbiological hygiene (Fig. 2.3). In addition to the standard traditional tempe in sausage shape, a series of products are commercially available that can be easily recognized by west European consumers, for instance smoked tempe with sausage taste, various salads, burgers and meat loafs.

New products can be developed for dedicated markets such as the elderly and the diseased. For example, a trebling of the isoflavone level of tempe could be achieved by inclusion of 20% isoflavone-rich soy bean germ (Nakajima et al. 2005). Dopamine is used in the management of Parkinsons' disease. Faba beans are a good source of its precursor, laevo-dihydroxy phenylalanine (L-DOPA). It was observed that tempe-like fermentation of faba beans with *R. oligosporus* resulted in a doubling of the L-DOPA



Fig. 2.3. Packaged, pasteurized tempe (De Hobbit, Belgium)



Fig. 2.4. Red kojic rice (Angkak)

content in addition to a favourable increase of antioxidant polyphenols (Randhir et al. 2004).

III. Red Kojic Rice (Angkak)

A. The Product

Red kojic rice, also referred to as Angkak, Anka, Red Qu, Chinese red rice, or *Monascus* fermented rice (Fig. 2.4) is traditionally obtained by fermentation of cooked rice with the mycelial fungi *Monascus* spp., such as *M. purpureus*, *M. anka* or *M. ruber*. It has a specific aroma and purple-red colour and is used as a natural colorant in red spirit, red furu and red rice (Chiao 1986). Excellent reviews of the secondary metabolites of *Monascus* are given by Blanc et al. (1994), Juzlova et al. (1996) and Lin et al. (2008).

B. Traditional Manufacturing Process

Traditionally, polished rice is soaked overnight, cooked or steamed, cooled and inoculated with spores of *Monascus* spp. (Fig. 2.5). Solid-substrate fermentation

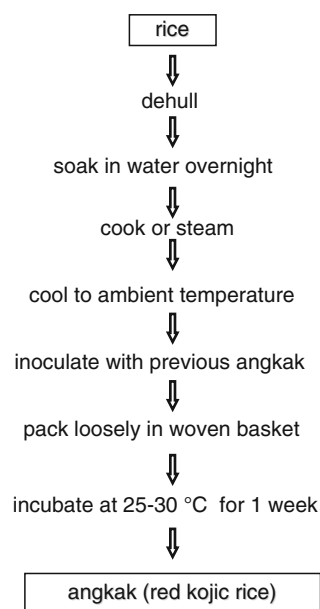


Fig. 2.5. Traditional manufacture of Angkak (red kojic rice)

during approximately one or two weeks allows the mould to grow and produce its secondary metabolites. Major pigments (Fig. 2.6) include the orange pigments rubropunctatin and monascorubrin, purple pigments rubropunctamin and monascorubramin and the yellow pigments ankaflavin and monascin (Pastrana et al. 1995). They are heat-stable and stable over a wide pH range, and thus they are of interest as “bio-colorants” in foods. Recently, using TOFMS, the additional yellow pigments xanthomonascin A, xanthomonascin B, monascopyridine A, monascopyridine B and yellow II were recorded. The nature and quantity of individual pigments produced are strain- and environment-dependent (Miyake et al. 2008).

C. Fungi Involved and Their Relevant Properties

Growth and pigment production in a liquid culture of *Monascus anka* MF 107 was distinguished into three phases by Fu et al. (1996), viz. a first phase dominated by mycelial growth and pH decrease from 5.5 to 4.6, a second phase of steady pigment production and pH increase from 4.6 to 8.4 and a third phase of gradual deterioration.

The role of the key chain elongation process by which the poly-beta-ketide carbon skeleton of the pigments is assembled was studied by Hong et al. (1995). Of several precursors tested, crotonic acid and sorbic acid enhanced

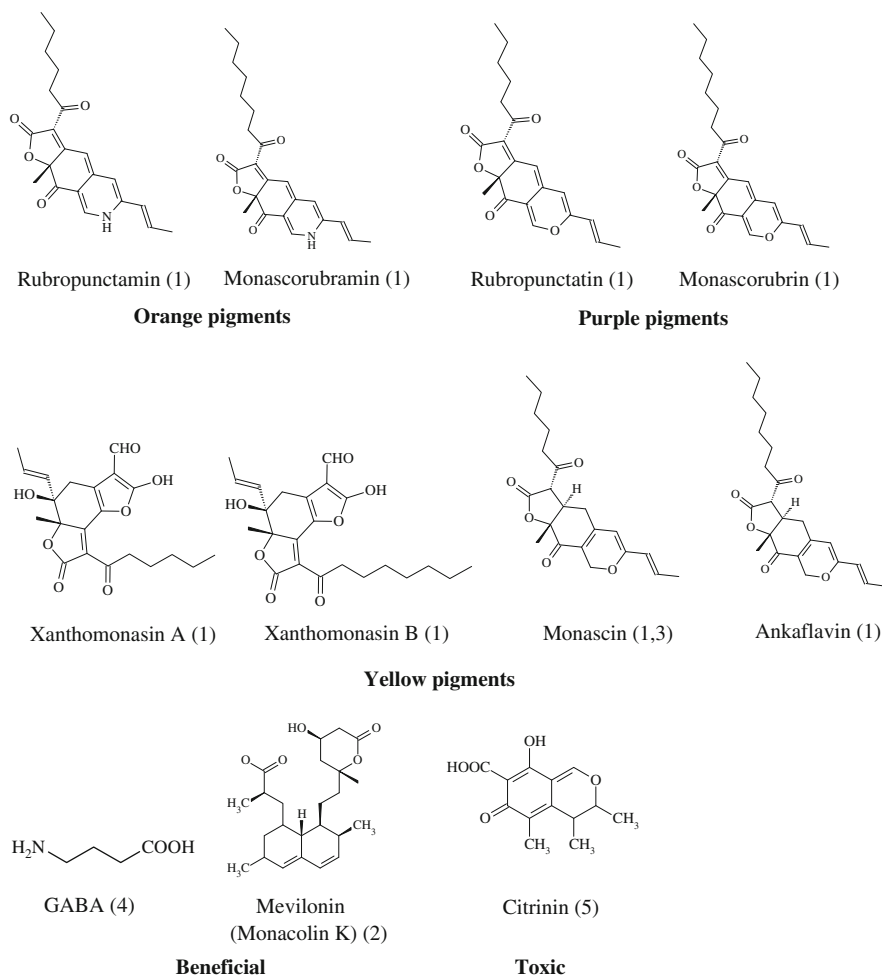


Fig. 2.6. Selected secondary metabolites of *Monascus* spp. in red koji rice. Compiled from literature references. (1) Akihisa et al. (2005), (2) Juzlova et al. (1996), (3) Jongrun-

gruangchok et al. (2004), (4) Wang et al. (2003), (5) Liu et al. (2005)

pigment productivity, with sorbic acid being incorporated more efficiently. Ethyl esters of these acids as well as cinnamic acid had a stimulatory impact on the biosynthesis. Apparently, the biosynthesis routes are not highly specific, as new red pigments were produced when these poly-beta-ketide intermediates were used as precursors. The red/yellow ratio could be influenced by the ratio of carbon (glucose) and nitrogen (mono-sodium glutamate) sources (Pastrana et al. 1995).

The physiology of a strain of *Monascus anka* KCCM 11832 was studied in liquid culture by Kang and Jung (1995). Ohantaek and Mudgett (1992) studied *M. purpureus* ATCC 16365. Both groups observed that optimum environmental conditions for pigment production are different from those for mycelial growth. For pigment production, shaking of submerged cultures strongly stimulated pigment formation. In solid-state fermentation, 50% O₂ was optimum for pigment production. Optimum conditions for pigment production were 25 °C at pH 6 in a medium

containing 2% rice, 0.05% peptone and 0.1% MgSO₄ during 7 days. Less peptone and higher temperatures resulted in more mycelium.

In addition to the pigments, *Monascus purpureus* also produces a typical angkak flavour. The volatile metabolites as reported by Juzlova et al. (1998) included alcohols, aldehydes, ketones, esters and terpenoid compounds. The highest flavour activity (concentration: threshold value) was ascribed to 2-heptanone, 2-nonanone, ethyl acetate, ethanol, 2-methyl-1-propanol and 3-methyl-1-butanol (Chung et al. 2004). In an earlier study, Peters et al. (1993) reported that in media containing saccharides (glucose) and fatty acids (octanoic acid), the relative toxicity of the fatty acid forced the mould into a detoxification process, oxidising octanoic acid to methyl ketones and secondary alcohols. Only after complete detoxification, saccharides were assimilated for fungal metabolism. These properties are of importance for controlled production of singular flavour components.

During recent years, much interest has been shown in the potential health benefit but also in the possible risks of angkak. Several studies investigated the effects of *Monascus* metabolites, such as in vitro and in vivo anti-inflammatory and hypo-allergenic (Akihisa et al. 2005), stimulation of bone formation (Gutierrez et al. 2006), colon cancer prevention (Hong et al. 2008), down-regulation of adipogenic transcription factors (Jeon et al. 2004) and prophylaxis of Alzheimer disease pathogenesis (Lee et al. 2008a). In particular, monacolin K (lovastatin) and gamma-amino butyric acid (GABA) Fig. 2.6 are of medical interest (Lin et al. 2008). It was reported by Wang et al. (1997) that during an 8-week trial in a group of 324 hyperlipidemia patients, a daily dose of 1.2 g angkak resulted in significant reductions of serum total cholesterol and low-density cholesterol.

D. Industrial Aspects

Traditional angkak is produced commercially at an industrial scale in China. Most angkak is produced as intermediate product, to be used for colouring sufu and wines. For example, the largest plant in Beijing produces 16000 t/year furu and 200 t/year angkak. The annual production of angkak for the whole of China is estimated at over 15000 t (B-Z. Han, personal communication). Because of the solid texture of the product, its manufacture is by solid-substrate fermentation. Lucas et al. (1993) described a swing bioreactor providing mild agitation. In this reactor *Monascus purpureus*-inoculated rice was fermented into angkak. The advantage of controlled reactors is that environmental conditions can be controlled. Optimum conditions for the angkak fermentation included 34% moisture content of the substrate (cooked rice) and a fermentation period of 7 days at 28.8 °C.

In addition, the use of angkak extracts or purified *Monascus* pigments as colouring agents has been patented for a variety of foods (Samson 1993a), including salami and sausages (Fabre et al. 1993). In the late 1970s it was observed that submerged fermentation was considerably faster and gave higher yields of pigments than solid-substrate fermentation. Consequently, for industrial pigment production, submerged fermentations appeared to be more appropriate (Chiao 1986). More recently, solid-liquid fed-batch cultures gave even higher pigment yields (Lee et al. 1995). Further, the use of liquid cultures with inorganic nitrogen sources was associated with the formation of bioactive orange pigments (monascorubrin and rubropunctatin) that had some embryotoxicity and teratogenicity (Martinkova et al. 1995). In solid-substrate rice fermentations or in liquid cultures with organic nitrogen sources, these orange pigments were converted into inactive compounds (amines).

Certain strains of *Monascus* spp. can produce the mycotoxin citrinin (Fig. 2.6). The levels of citrinin in

controlled fermentations can be maintained at safe levels by optimizing culturing conditions and by using selected non-toxic strains. However, in many industrial operations, uncontrolled starter cultures are used for the fermentation. Some investigations of citrinin levels in industrially produced angkak revealed that 20% of Korean angkak exceeded the tolerated level of 50 g/kg (Kim et al. 2007); in retail samples from Taiwan lipid extracts had citrinin levels ranging from 280–6290 g/kg, and some sample extracts exerted cytotoxic effects on the human cell line HEK293 (Liu et al. 2005). Newly discovered monascopyridines C and D were associated with tumor formation (Knecht et al. 2006). These new findings indicate the need to control the quality of starters and process conditions in order to minimize the health risk due to toxic secondary metabolites (Trucksess and Scott 2008).

IV. Amylolytic Starters

A. The Product

Amylolytic starters are used to saccharify starchy and other high-molecular-weight carbohydrate material for fermentation processes. Ragi means inoculum (Cronk et al. 1977) and the name following ragi indicates the use of starter, thus ragi-tempe is an inoculum for tempe. There are several amylolytic ragi-type starter cultures available in the markets in most Asian countries and these include Indonesian ragi-tapé, ragi *peuyeum* (for cassava), Malaysian ragi-tapai, *juj-paing*, Thai *loopang* (grown on bran), Philippine *bubod levadura*, Chinese *levure chinoise*, Indian *bakhar* and Nepalese *murcha*. Figure 2.7 shows *murcha* sold at a market in Darjeeling, India. Amylolytic starter cakes have been used to substitute diastatic malt



Fig. 2.7. *Murcha* amylolytic starters sold at a market of Darjeeling, India

extract as a saccharifying agent in producing rice-based weaning food (Yusof et al. 1995). Takeuchi et al. (2006) purified and characterized an amylolytic enzyme from yeast, *Pichia burtonii*, which was isolated from murcha. The extracellular enzyme, a glycoprotein, showed strong amylolytic properties in presence of starch but its activity was inhibited by metal ions such as Cd^{2+} , Cu^{2+} , Hg^{2+} , Al^{3+} and Zn^{2+} .

Tane koji, an essential enzyme starter in the production of soy sauce, miso, saké and amazake (sweetened rice), is described in Section VI. Lin et al. (2006) reported on an enhanced antioxidative activity of soybeans fermented with GRAS filamentous fungi (*Aspergillus oryzae*, *A. sojae*, *A. awamori*, *Actinomyces taiwanensis*, *Rhizopus* spp.). They found that α -diphenyl-2-picryl-hydroxyl (DPPH) scavenging effects and the Fe^{2+} -chelating ability and reducing power of the fermented soybean were high. Soybeans fermented with *A. awamori* had at least a sixfold higher antioxidant capacity than the control, thus indicating a potential for developing a healthy food supplement. Lee et al. (2008b) reported similar results with blackbean koji.

B. Traditional Manufacturing Process

In the preparation of starter cake shown in Fig. 2.8, rice or wheat is ground and thoroughly mixed with spices. A mixture of garlic, pepper, rhizomes, onion and root is used in the preparation of the starter and producers regard their recipes as secret passed from generation to generation. The ratio of ground rice to mixed spices is about 14:1. Water is added to make a dough-like material which is shaped into small balls or cakes of about 4 cm in diameter and 1cm thick. Dry powdered ragi from previous batches is sprinkled over the cakes. The latter are then placed on a wooden bamboo tray, covered with a cloth and incubated at ambient temperature for 2–5 days, during which the dough is slightly raised and covered with fungal mycelia. Spices in the ingredients play a major role in preventing growth of undesirable micro-organisms. The cakes are air- or sun-dried and have a shelf-life of several months (Ko 1986; Cook and Campbell-Platt 1994; Saono et al. 1996).

In the Philippines, *bubod levadura* is made by several processes depending on the locality or region. In the Benquet process, glutinous rice is soaked, drained, ground and mixed with pureed ginger and wild roots and then inoculated with powdered bubod containing *Mucor* spp. and other amylase producers which are responsible for the early fermentation and then with yeasts which are responsible for the remainder of the fermentation. In the Bontoc process, no spices are added and the cakes are not usually inoculated, but in the Ifugao process, rice grains are

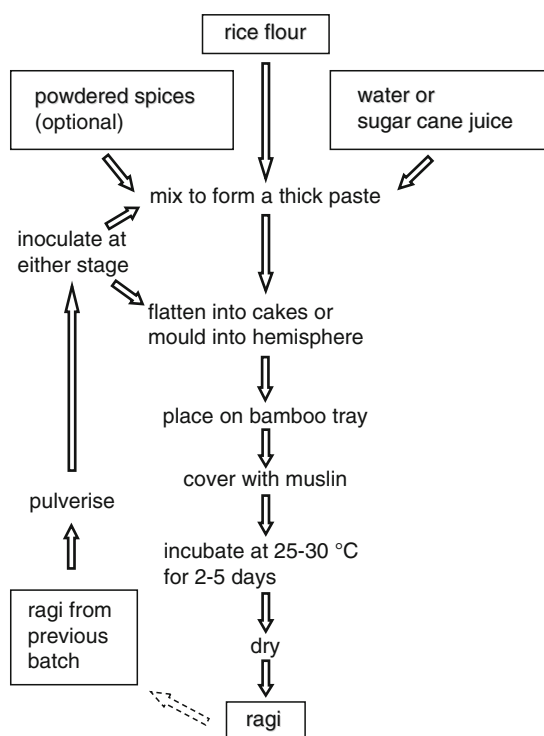


Fig. 2.8. Traditional manufacture of Ragi

roasted before soaking and spices are added and then inoculated with powdered bubod (Tanimura et al. 1978).

C. Fungi Involved, Their Relevant Properties and Implications for Health

Studies on the microflora of ragi and Chinese yeast cake date from Went and Prinsen Geerligs (1895). Several mycelial fungi and yeasts, with amylolytic activities, have been isolated from ragi cakes (Dwidjoseputro and Wolf 1970; Hesseltine et al. 1985). The principal moulds are *Amylomyces rouxii*, *Rhizopus* spp., *Mucor* spp., *Aspergillus* spp. and *Fusarium* spp. (Table 2.4). *Hansenula* spp., *Endomycopsis* (*Saccharomycopsis*) *fibuligera*, *Candida* spp. and *Saccharomyces cerevisiae* are the common yeasts in many starter cakes. Murcha or marcha is a traditional amylolytic starter commonly used in the Himalayan regions of India, Nepal and China. Tsuyoshi et al. (2005) isolated several yeast strains from murcha and the main species were *Saccharomyces bayanus*, *Candida glabrata*, *Pichia anomala*, *Saccharomycopsis fibuligera*, *Saccharomycopsis capsularis* and *Pichia burtonii*. Some of the species

Table 2.4. Amylolytic moulds isolated from Malaysian and Indonesian tempe, tempe-ragi, tapé and ragi-tapé. Functional moulds are indicated in **bold**. Adapted from Merican and Yeoh (1989) and Steinkraus (1996)

Product	Country/origin	Fungal species
Tempe	Malaysia/Kajang, Selangor	Rhizopus spp., <i>Aspergillus</i> spp., <i>Penicillium</i> spp.
Tempe	Malaysia/Kuala Lumpur, Selangor	Rhizopus spp., Mucor spp.
Tempe	Malaysia/Bangi, Selangor	Rhizopus spp., <i>Aspergillus</i> spp.
Tempe	Indonesia/jakarta	Mucor javanicus , <i>A. niger</i> , <i>Fusarium</i> spp.
Ragi-tempe	Indonesia/Surakarta	R. oryzae , R. stolonifer
Ragi-tempe	Indonesia/Malang	R. oryzae , R. arrhizus , R. oligosporus , Mucor rouxii
Tapé and ragi-tapé	Indonesia	Rhizopus spp., R. oryzae , Mucor rouxii , M. javanicus , M. circinelloides , <i>Fusarium</i> spp., <i>Aspergillus</i> oryzae , Amylomyces rouxii

showed high amylolytic activity. Sujaya et al. (2004) used five different types of ragi tapé starters for the production of Balinese rice wine, brem, and isolated 51 yeast strains, with *Saccharomyces cerevisiae* accounting for 69% of the total yeast isolates.

Hesseltine et al. (1988) studied 41 amylolytic oriental fermentation starters from seven Asian countries and found that they contained three genera of *Mucorales* (*Rhizopus*, *Mucor*, *Amylomyces*). Every sample contained at least one Mucoraceous mould and one yeast. Later, they studied nearly 100 amylolytic yeast strains isolated from ragi and other starters and found the predominant yeasts were *Endomycopsis* (*Saccharomycopsis*) *fibuligera* and, to a lesser extent, *Saccharomycopsis malanga* (Hesseltine and Kurtzman 1990). Kozaki and Uchimura (1990) also isolated three types of moulds (two *Mucor* spp., one *Rhizopus* spp), two types of yeast (*Saccharomyces cerevisiae*) and a mycelial yeast, *Saccharomycopsis fibuligera* from Philippine bubod and tapuy. Of 41 yeast strains isolated from Indonesian ragi and tapé (Indonesian sweetened rice), 19 had amylolytic activity, none were proteolytic and all the moulds were amylolytic (Saono et al. 1996). The exact mycoflora of ragi varies with location and the particular food for which the starter is to be used.

Certain genera of yeasts and moulds are known to play a leading role in the fermentation of rice wine and sweetened rice. Based on their abilities to produce high amylolytic activities (α -amylase and/or amyloglucosidase), a number of strains of *A. rouxii*, *Rhizopus* spp. and *Endomycopsis* spp. were selected and a combination of *A. rouxii* and *E. fibuligera* for instance was shown to produce a good quality tapé. Fungal amylases are in demand in industrial food processes and are preferred over other microbial sources due to more accepted GRAS status (Gupta et al. 2003). However amylolytic lactic acid bacteria have also

been used in a simple novel fermentation process for the preparation of high-energy-density fermented gruels for young children (Nguyen et al. 2007).

Amylomyces rouxii is an important constituent of starter cultures used in the production of fermented foods in the Far East, Southeast Asia and the Indian subcontinent. The filamentous fungus is closely related to certain strains of *R. oryzae* producing lactic acid. According to Ribes et al. (2000), *R. oryzae* occasionally causes the human disease, mucormycosis; it is also a possible pathogen in industrial crops (Phytopathological Society of Japan 2000). Saito et al. (2004) compared *A. rouxii* and *R. oryzae* in lactic fermentation of potato pulp and other agricultural by-products into food materials. They concluded that *A. rouxii* was preferable to *R. oryzae* for use in fermenting those substrates as food materials.

D. Industrial Aspects

Starter inocula are generally produced by household or village manufacturers who use closely guarded recipes. Because of the difficulty in controlling microbial activities of mixed cultures contained in starter cakes, pure cultures of the selected strains of *Rhizopus* spp. (mainly *R. oryzae*) have been used for industrial production of enzyme starters, in particular, for the distillery industry (Lotong 1998).

Mass production of spores of *Rhizopus* spp. on rice adhering to inside surfaces of flasks is applied in the fermentation industry in Asian countries, in particular, Taiwan and Thailand. The process involves steeping ordinary or glutinous rice for 3–6 h and transferring to a flask or bottle. The container is swirled to spread the grains inside its surface and it is plugged with cotton and steamed. After cooling it is inoculated with spore suspension and incubated at 35 °C for 4–5 days after which large quantities of spores are produced.

In commercial runs in Thailand, enzyme preparations are made by grinding glutinous rice into coarse powder, followed by mixing with rice bran. The mixture is steamed and after cooling, mixed with spore suspension and spread onto wooden bamboo trays. The inoculated material is incubated at ambient temperature for 36–40 h after which the koji cakes are sun-dried and ground into a powder. In some distilleries, glutinous rice may not be used as it has been reported that less amylolytic enzyme, amyloglucosidase, is produced in substrate containing glutinous rice (Lotong 1998).

V. Furu (Sufu)

A. The Product

Sufu, or tou-fu-ru (Wang and Fang 1986) is an ancient Chinese cheese-like product with a creamy consistency (Fig. 2.9). Sufu is made from soy beans and is an easily digested and nutritious protein food (Su 1986). It is a popular side-dish, e.g., with breakfast rice. There are several varieties of colours ranging from grey to dark red (obtained with angkak) and flavours ranging from sweet and bland to strong and offensive. A comprehensive overview of the different categories of furu, their flavor characteristics and industrial manufacture was given by Han et al. (2001b).

B. Traditional Manufacturing Process

Furu or sufu is obtained by a three-stage process (Fig. 2.10). During the first stage, soy bean curd (tou-fu or tofu) is obtained by extraction of soymilk from soy beans followed by precipitation of the soy protein by acid or by added calcium salts. The precipitate is pressed



Fig. 2.9. Furu (Sufu)

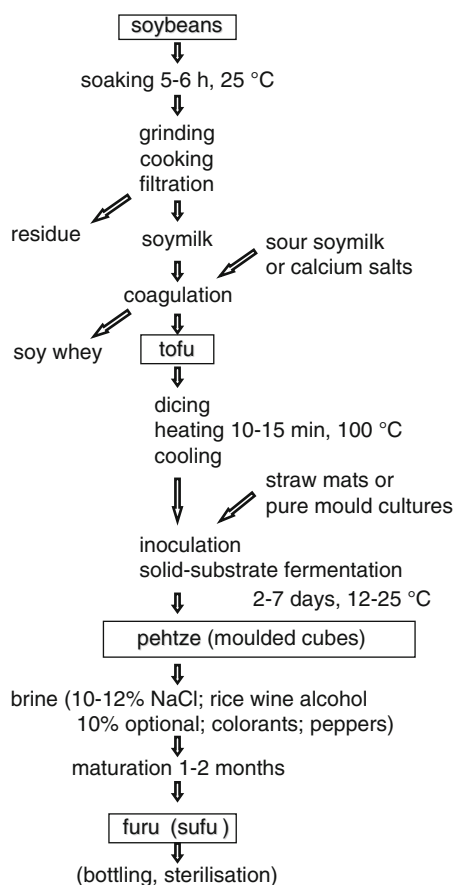


Fig. 2.10. Traditional manufacture of Furu (Sufu)

to blocks of tofu. The second stage is a fungal solid-substrate fermentation. Cubes of tofu are heated at 100 °C for 10–15 min to reduce the moisture content to about 70%, firming-up its consistency and pasteurizing the cubes before they are inoculated (naturally by covering them with straw, or by using pure culture starters) with mycelial fungi such as *Actinomucor elegans*, *A. taiwanensis*, *Mucor hiemalis*, *M. silvaticus*, *M. praini*, *M. subullissimus*, or *Rhizopus chinensis*. After 2–7 days at 12–25 °C depending on strains used (Fukushima 1985) the mycelium has covered the tofu cubes. The product is now referred to as pehtze. During the third stage, an enzymatic ripening takes place. The pehtze is submerged in brine and kept in closed jars for 2–4 months. During this period the fungal (mainly proteolytic) mycelia-bound enzymes are released (Wang and Hesseltine 1970) and, while diffusing into the pehtze, the texture softens and flavour is developed. The composition of the brine strongly influences the ripening process, as well as the flavour of the final sufu. Brines exclusively containing approx. 12% NaCl combined with the use of mixed fungal–bacterial pehtze inocula result in the most offensive flavours. Mixed brines consisting of approx. 10% NaCl and 10% rice wine ethanol give more neutral flavours. In Japan, very bland flavours are preferred. The Japanese product “tofuyo” shows a similarity

to sufu because it is obtained by enzymatic ripening of tofu (Yasuda and Kobayashi 1989). The difference however, is that the required enzymes are obtained from angkak rather than from the typical sufu mycelial fungi mentioned earlier. Using various additive ingredients (angkak, soy mash, rice wine, hot peppers) a variety of sufu colours and fragrances can be achieved (Wang and Hesseltine 1970). Finally, sufu is bottled with brine and heated to achieve commercial sterility. The predominant groups of micro-organisms in furu are Gram-positive spore-forming bacteria and lactic acid bacteria in low-salt sufu, whereas some sufu contained significant levels of *Bacillus cereus* and *Clostridium perfringens* (Han et al. 2004b). From a range of commercial sufu from all over China, it was observed that the most likely spoilage microflora are lactic acid bacteria such as *Lactobacillus casei* (Han et al. 2001a). Although most products were safe from the microbiological point of view and no viable *Staphylococcus aureus* were encountered, some contained detectable levels of staphylococcal enterotoxin A (Han et al. 2001a). A sampling plan was proposed to monitor the safety of this type of product. Criteria included tolerable levels of Enterobacteriaceae, *B. cereus*, *C. perfringens*, and *S. aureus* (Han et al. 2001a).

C. Fungi Involved and Their Relevant Properties

The fungal genera involved (*Actinomucor*, *Mucor*, *Rhizopus*) all belong to the Mucoraceae. Predominating mycelia fungi from sufu of China and neighbouring Vietnam are *Actinomucor repens*, *Actinomucor taiwanensis*, *Mucor circinelloides*, *Mucor hiemalis*, *Mucor racemosus*, and *Rhizopus microsporus* var. *microsporus* (Han et al. 2004a).

The temperature range for growth of these species is 20–30 °C. During the summer season in Beijing, the ambient temperatures exceed this range. It was found that the temper fungus, *Rhizopus oligosporus*, is better suited to

higher temperatures and provides a very similar modification of the pehtze. Optimum relative humidity for pehtze development was found to range over 95–97% (Han et al. 2003a). Incubation temperatures also influence the modification of isoflavones and the activity of β -glucosidase by *Actinomucor elegans* (Yin et al. 2005). At 26 °C, more beneficial isoflavone aglycons were formed than at 32 °C. It would be worthwhile to investigate the temperature-dependent isoflavone modification by *R. oligosporus*. Optimum conditions for growth of *A. taiwanensis* on tofu were 25–30 °C at 97% relative humidity when inoculated on tofu of 65% moisture content. Under these conditions, a maximum production of protease, α -amylase, α -galactosidase and lipase enzymes was reported by Chou et al. (1988). A glutaminase from *A. taiwanensis* was purified and studied (Lu et al. 1996). Its pH and temperature optima were 8.0 and 45 °C, and it was stable at ≤ 35 °C and pH 6.0–8.0. The enzyme still exhibited 50% of its maximum activity in the presence of 10% w/v NaCl, demonstrating its potential impact during the ripening stage.

The fungal proteases are not extracellular, but mycelium-bound. The salty brine provides the ionic strength needed to release them into the solution, enabling their diffusion into the soy bean curd (tofu). Their effect is the degradation of the soy bean protein into peptides, free amino acids and other non-protein nitrogenous substances. Table 2.5 illustrates the gradual degradation as a function of brine composition and mould species involved in pehtze fermentation.

The degradation of soy bean protein was enhanced in the absence of ethanol during the ripening (Chou and Hwan 1994). No intact proteins could be detected in ripe tofuru (sufu), and the resulting peptides had molecular mass <10 kDa. Free amino acids included tyrosine and considerable levels of hydrophobic amino acids (Rao et al. 1996). High amounts of glutamic acid and leucine were reported by Chou and Hwan (1994). In view of the bitter-tasting

Table 2.5. Biochemical changes taking place during Sufu maturation (data selected from Chou and Hwan (1994))

Maturation time at 25 °C (days)	<i>Actinomucor taiwanensis</i> ^a				<i>Actinomucor elegans</i>			
	S ^b		S+A ^c		S		S+A	
	ANR ^d	FFA ^e	ANR	FFA	ANR	FFA	ANR	FFA
0	5	45	5	45	5	30	5	30
15	9	70	6	80	11	110	9	80
30	15	120	8	110	17	190	8	190
45	18	180	9	80	18	220	9	180
60	20	160	11	70	23	240	15	170
75	27	170	13	75	23	300	18	100

^aMycelial fungi used for pehtze fermentation.

^bBrine: 12% w/v NaCl.

^cbrine: 12% w/v NaCl + 10% w/v ethanol.

^dAmino nitrogen ratio = (amino N/total N) \times 100%.

^eFree fatty acids (mg/g lipid).

hydrophobic amino acids, it will be of interest to investigate the significance of their presence in sufu for its taste. The modification of pehtze by degradation of protein and lipids is affected by the salt levels in the maturation brine. Lowering the salt level from the regular 14% to 8% or even 5%, shows more rapid and complete degradation of protein and lipid fractions (Han et al. 2003b) and this also includes the softening of the pehtze texture. The major amino acids present in the fraction of water-soluble nitrogen compounds are, in descending order: glutamic acid, leucine, aspartic acid, alanine, phenylalanine and lysine (Han et al. 2004c). Considering the flavor-enhancing activity of glutamic acid and glutamate, the addition of furu to dishes obviously strengthens the meaty flavour.

The degradation of soy protein during furu maturation results in the formation of bio-active peptides that have a beneficial effect by inhibiting angiotensin converting enzyme (ACE), being anti-thrombotic and having surface tension and anti-oxidant properties (Gibbs et al. 2004). It was reported that peptidases of the sufu mould *Actinomyces elegans* result in a debittering of certain hydrophobic peptides in soy protein hydrolysates (Li et al. 2008). It may be inferred that such beneficial effects also take place in the maturation of furu. Some lipase activity is produced that releases fatty acids from the soy bean oil (Table 2.5). The presence of ethanol during maturation was associated with lower levels of free fatty acids in matured sufu (Chou and Hwan 1994). The fatty acids react with the wine alcohol to produce esters that add to the fruity flavour (Campbell-Platt 1987). The complex flavour of sufu was reported to contain 22 esters, 18 alcohols, seven ketones, three aldehydes, pyrazines, two phenols and other volatile compounds by Hwan and Chou (1999). Maturation in the presence of ethanol (rice wine) resulted in higher levels of volatiles.

D. Industrial Aspects

Sufu is produced at an industrial scale. The production of tou-fu is highly mechanized. In China alone, annual production is estimated at about 300000 t (B.-Z. Han, personal communication). Pehtze fermentations are carried out using tray fermentations in incubation chambers, and maturation in large vessels.

The control of maturation, flavour development and consistency are closely guarded secrets. Obviously, combinations of salt, wine and additives as well as humidity and temperature control are key factors.

From the microbiological point of view, it may be expected that yeasts and bacteria play a role in the flavour and texture of sufu. Little published data are available on these issues, except a study on the halophilic flora of sufu.

During the brining stage, halophilic lactic acid bacteria, notably *Tetragenococcus halophila* (previously *Pediococcus halophilus*) are found in a majority of brands of sufu (Pao 1995). In view of the microbiological safety of sufu, mixed brines containing 10% NaCl and 10% ethanol were preferred for the protection against disease-associated bacteria such as *Staphylococcus aureus*. Salt concentrations used traditionally in the maturation brine (about 20%) are high, and have the function to safeguard the product against spoilage. It is of commercial interest to reduce salt levels because maturation can proceed faster as mentioned earlier. In addition, there is no harm in reducing salt intake by the average consumer. How far could salt levels be lowered, while still maintaining a wholesome and safe product? It was observed that lactic acid bacteria spoilage may occur when 10% salt (in brine) is used. At levels $\leq 18\%$ in the brine, staphylococcal enterotoxins were produced during challenge experiments with *Staphylococcus aureus* (Han et al. 2005). Micro-organisms possessing lysine decarboxylase such as *Bacillus subtilis* can form mildly toxic biogenic amines, e.g., histamine (Kung et al. 2007). Although most tested sufu had safe levels of biogenic amines, some contained 160 ppm histamine which is three times the tolerable limit. The salt levels of the investigated sufu's ranged from 6% to 12% (in the product). It is not known which salt level was present in the histamine-containing sample, but considering the above data a suggested safe minimum level would be $\geq 8\%$ salt in the product.

VI. Soy Sauce

A. The Product

Soy sauce is a light to dark brown liquid with a meat-like salty flavour used in cooking and as a table condiment (Fig. 2.11). Traditionally made in



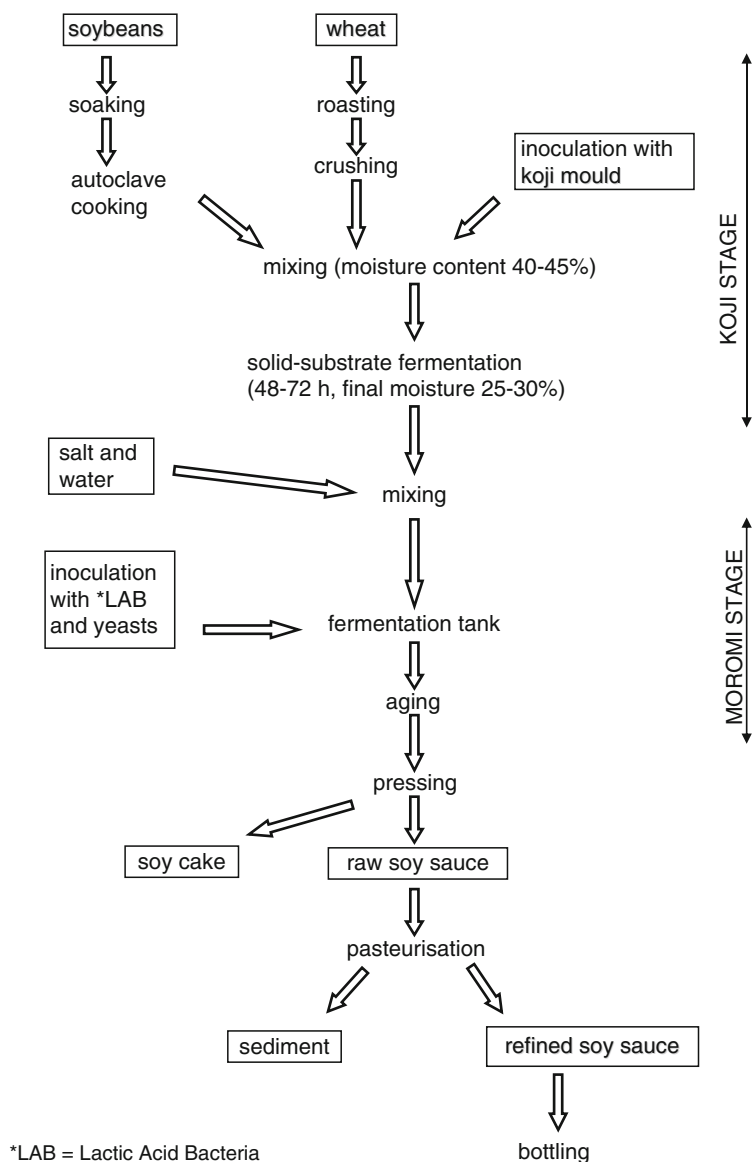
Fig. 2.11. Soy sauce

China, Japan, Korea, Thailand, Philippines, Indonesia, Malaysia and Singapore, soy sauce is now also produced in Europe and both North and South America. It is of very ancient lineage and today the production of soy sauce includes every level of sophistication from domestic or village-scale, to advanced controlled production systems of a very high quality product. There are two specific fermentation procedures involved in soy sauce production, namely aerobic koji fermentation involving the use of *Aspergillus oryzae* or *A. sojae* and an anaerobic moromi or salt mash which undergoes lactic acid bacteria and yeast fermentations.

B. Traditional Manufacturing Process

Traditionally soy beans, *Glycine max*, are soaked in water, boiled and drained and mixed with ground or crushed roasted wheat. The mixture is placed on trays and mixed with *Aspergillus oryzae* or *A. sojae* (tane-koji) and allowed to ferment at about 30 °C for 5 days to form koji. The principal function of the mould is the elaboration and release of a range of hydrolytic enzymes, including amylases, proteases, cellulases, invertases, as well as lipolytic enzymes. The koji is mixed with salt brine (23% w/v) in a ratio of 1.0:1.5 to make the salt mash or moromi, which undergoes lactic acid bacteria and yeast fermentations for at least one year at ambient temperatures during which colour and flavour develop resulting in quality soy sauce (Fig. 2.12). Under moromi fermentation conditions, the

Fig. 2.12. Traditional manufacture of soy sauce



koji mould is rapidly destroyed but its extracellular enzymes continue to hydrolyse substrates, albeit slowly, in the saline environment. The strong brine creates a favourable condition for a few desirable organisms. At first *Tetragenococcus halophila* grows and produces lactic acid which lowers the pH to 5.5 or less and then acid-tolerant dominant yeast, notably *Zygosaccharomyces rouxii* grows and produces about 3% alcohol and several compounds which add characteristic aroma to soy sauce. The fermented moromi is then filtered, siphoned, drained or decanted. The raw soy sauce is then boiled or pasteurized – a process which produces not only stability, flavour and colour in the final product but also clarity, inactivation of residual enzymes and resistance to spoilage yeasts.

C. Fungi Involved, Their Relevant Properties and Implications for Health

The most important function of the koji mould in soy sauce fermentation is the production of extracellular and exocellular enzymes, in particular, carbohydrase and protease complexes. The yellow-green *Aspergilli* (koji moulds) used in Asian soy bean fermentations have long been a subject of much debate amongst fungal taxonomists. In *Aspergillus* classification (Table 2.6), the koji moulds belong to the Section Flavi Gains (= *Aspergillus flavus* group Raper and Fennell; Samson 1993b; Samson et al. 2004). The true koji moulds comprise *Aspergillus oryzae* (Raper and Fennell 1965), *Aspergillus sojae* (Murakami et al. 1982) and *Aspergillus tamaris* Kita (Raper and Fennell 1965), the distinction between them being based on conidial head colour, growth at 37 °C and dimensions of conidiophores, vesicles and conidia. The selection of strains of *Aspergillus* used in soy sauce fermentation in Japan and the Far East

Table 2.6. Accepted *Aspergillus* species Section Flavi (Samson 1993b)

Species	Author
<i>A. avenaceus</i>	Smith
<i>A. clavato-flavus</i>	Raper and Fennell
<i>A. flavus</i>	Link
<i>A. leporis</i>	
<i>A. nomius</i>	Kurtzman et al.
<i>A. oryzae</i> (Ah16)	Cohn
<i>A. parasiticus</i>	Speare
<i>A. sojae</i>	Sakaguchi and Yamada
<i>A. subolivaceus</i>	Raper and Fennell
<i>A. tamaris</i>	Kita (= <i>A. flavo-furcatis</i> Batista and Maia)
<i>A. zonatus</i>	(Kwon and Fennell) Raper and Fennell

is based, among others, on ability to sporulate for the preparation of seed starter, colour and flavour of the final product, enzyme production, inability to produce toxins and length of stalk.

The two main groups of enzymes produced by *A. oryzae* during koji fermentation are carbohydrases (α -amylases, amyloglucosidase, maltase, sucrase, pectinase, β -galactosidase, cellulase, hemicellulase, pentosan-degrading enzymes) and proteinases, although lipase activity has also been reported (Aidoo et al. 1994; Chou and Rwan 1995). These major enzymes hydrolyse carbohydrates and proteins to sugars and amino acids and low-molecular-weight peptides respectively. These soluble products are essential for the moromi fermentation.

Zygosaccharomyces rouxii is the dominant moromi yeast which grows to produce 3% alcohol and several compounds which add characteristic aromas to soy sauce, although other yeasts such as *Candida versatilis* and *C. etchellsii* produce phenolic compounds, 4-ethylguaiacol and 4-ethylphenol, which contribute to the soy sauce aroma. A review on the diversity and functionality of yeasts in soy sauce and other yeast fermented Asian foods has been published (Aidoo et al. 2006).

The discovery of aflatoxins in the 1960s led to an extensive examination of koji moulds for toxin production. Although no aflatoxins have been demonstrated in *A. oryzae*, *A. sojae* or *A. tamaris*, it has been reported that all can produce other mycotoxins such as aspergillilic acid, cyclopiazonic acid, kojic acid under specific environmental conditions (Table 2.7; Frisvad 1986; Trucksess et al. 1987). Aflatoxigenic fungi do not appear to occur in regions with a mean temperature below 16 °C and this therefore may explain why traditional Japanese fermented foods such as soy sauce, miso (fermented soybean paste), sake, katsuo-bushi (dried bonito) and others do not contain aflatoxins (Tanaka 2002). Although other mycotoxins including sterigmatocystin (precursor of aflatoxins) and ochratoxin A could not be detected, the effects of other mycotoxins associated with fermented foods and herbal plant foods on human epithelial cell lines have been reported (Manabe 2001; Calvert et al. 2005; Mohd Fuat et al. 2006).

Table 2.7. Toxins reported to be produced by koji moulds (Frisvad 1986; Trucksess et al. 1987)

Mould	Mycotoxin
<i>Aspergillus oryzae</i>	Cyclopiazonic acid, kojic acid, maltoryzine, β -nitropropionic acid
<i>A. sojae</i>	Aspergillilic acid, kojic acid
<i>A. tamaris</i>	Kojic acid

Kataoka (2005) reported that flavones from Japanese-style soy sauce had beneficial health properties and also antibacterial activity against the most common pathogenic organisms. In spite of its high salt content, soy sauce in the diet could protect against cardiovascular diseases (CVD) because of the levels of antioxidants. McVeigh et al. (2006) reported that soy proteins, regardless of isoflavone content, were responsible for cardiovascular benefits. Ørgaard and Jensen (2008) have published a review of the most recent research findings on the potential benefits of soy isoflavones on obesity in humans. Murooka and Yamshita (2008) also reviewed the health benefits of traditional fermented products, particularly shoyu, miso, tempe, natto and black rice vinegar, produced and sold in Japan.

D. Industrial Aspects

About 4×10^6 t of soybeans are used annually in Japan and about 1×10^6 t go into food consumption. In 1964 there were over 4000 producers of soy sauce or shoyu as local industry in Japan. Now, there are about 1600 soy sauce producers, the majority belonging to the Japan Federation of Soy Sauce Manufacturers Cooperatives (JFSSMC). Annual soy sauce consumption in Japan is 920 000 kl and it is estimated that United States consumption of Japanese-style soy sauce is about 130 000 kl. There are four Japanese-style soy sauce breweries in the US and 13 in Asia, Europe and elsewhere, with a cumulative annual production

except Japan of 210 000 kl, which is increasing yearly (JFSSMC 2009). According to JFSSMC, the consumption of soy sauce in Japan has dropped in recent years due mainly to decreasing population and attendant ageing society. In spite of this, Japan's exports of soy sauce to 60 countries have increased in recent years. One of the major producers of shoyu, Kikkoman, started producing genuine fermented shoyu in the United States. Kikkoman also produces shoyu in Singapore, Brazil, Taiwan and the Netherlands, and further shoyu plants are planned in, e.g., California (Yokotsuka and Sasaki 1998).

The large-scale industrial production of soy sauce involves five main unit operations, viz. the preparation of the raw materials, the koji process, mash or moromi production, pressing and finally refining (Fig. 2.12). The koji process for making enzymes needed in soy bean fermentation traditionally uses wooden trays which are stacked vertically with about 10 cm gaps separating them, and each tray is filled to a depth of about 5–8 cm (Yokotsuka 1983; Aidoo et al. 1984).

In the koji fermentation, it is important to: (1) maximise enzyme production, (2) prevent denaturation of the enzymes, (3) avoid presence of undesirable microorganisms and (4) minimise the utilisation of nutrients by the koji moulds. The mechanical bioreactor types of koji manufacture have been almost exclusively developed in Japan and the three major features of these bioreactors are the batch and continuous types, the rotary drum and the surface-flow system of aeration (Yokotsuka 1983; Fukushima 1998; Yokotsuka and Sasaki 1998). Package-type rotary koji-making equipment is shown in Figs. 2.13 and 2.14. These koji bioreactors now involve automated inoculation of substrate, controlled mass transfer, automated heaping and turning of the fermenting mass and automated harvest of the finished koji. These industrial koji bioreactors enable excellent control of temperature and humidity (two

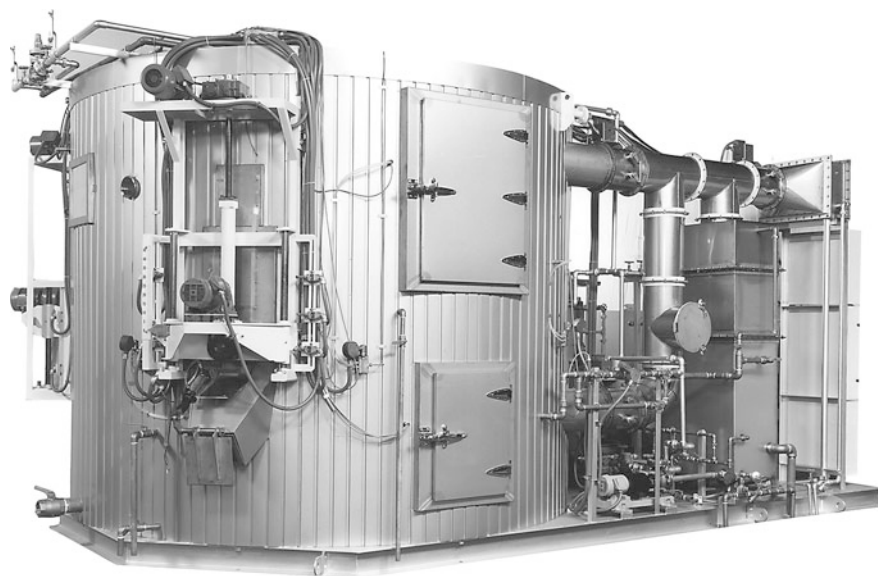


Fig. 2.13. Rotary type automatic koji making equipment. Reproduced with permission from Fujiwara Techno-Art Co. Ltd., Japan

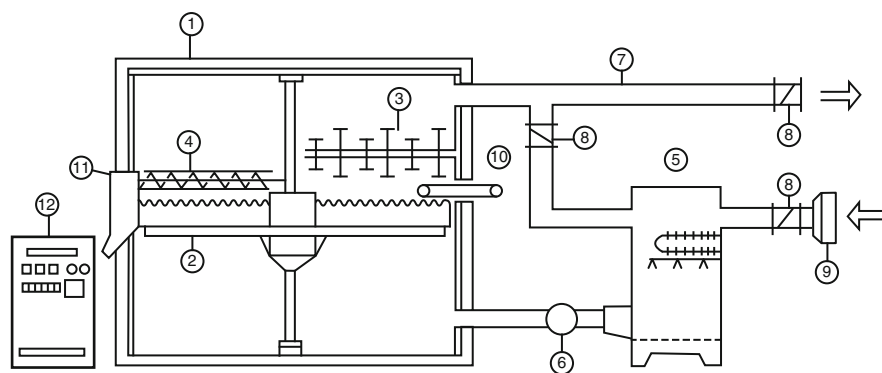


Fig. 2.14. Cross section, rotary type automatic koji making equipment. Reproduced with permission from Fujiwara Techno-Art Co. Ltd., Japan. 1. Koji room, 2. Culture bed, 3. Turning machine, 4. Heaping and dischar-

ging machine, 5. Air conditioner, 6. Blower, 7. Air duct, 8. Air damper, 9. Plate fin heater, 10. Koji material inlet, 11. Koji product outlet, 12. Control panel

important environmental conditions for mould growth), effective mass transfer and removal of inhibitory gases and volatiles and further ensure regular and complete mixing. In essence, this is the most advanced example of a solid-substrate fermentation worldwide.

The submerged moromi fermentation is carried out in wood, concrete or steel tanks with a capacity of 10–30 m³ and, in Taiwan, the traditional ceramic pots and wooden tanks are now replaced with epoxy resin-coated cement tanks with a capacity of over 30 t. The moromi is separated by a pressing machine into raw soy sauce and cake. This process takes 2–3 days and although such mechanised systems add extra cost, the lower pressures and longer filtration times result in a loss of flavour. The raw soy sauce is then cooled, blended according to user demand, pasteurised at 70–80 °C and finally bottled.

In 1991 Kikkoman reported on the application of new biotechnology to soy sauce fermentation whereby immobilised cells in a bioreactor were used for continuous production of soy sauce of good quality. The technique involved the use of a broth culture of *A. oryzae* to hydrolyse a liquified soy bean–wheat mixture and the liquid was passed through a series of bioreactors containing immobilised enzyme, glutaminase, from *Candida famata*, immobilised cells of *Tetragenococcus halophilus*, *Zygosaccharomyces rouxii* and *C. versatilis* (Hamada et al. 1991).

The use of automated machinery for soy sauce production was initiated by major Japanese shoyu manufacturers. Now the equipment has wider industrial application in the production of miso, saké, amazake, beer malt, antibiotics and other enzyme preparations.

VII. Wines

A. The Products

In principle, the term “wine” is used for products of alcoholic fermentation of fruit juices containing readily fermentable mono- and disaccharides. In



Fig. 2.15. Saké

this chapter however, we follow the common usage of the term wine to include Oriental rice wines and derived products. These are produced from the hydrolytic breakdown of cereal starches and other polysaccharides. In all cases, the breakdown of the carbohydrate source is primarily due to amylolytic enzymes elaborated by mycelial fungi. The wine ranges from simple Thai rice wine to highly sophisticated Japanese saké (Fig. 2.15). Rice and/or cereal wines are produced at both cottage and commercial scale in Japan,

China, Korea, Thailand, Philippines and Burma (Campbell-Platt 1987; Cook and Campbell-Platt 1994). Wines may be distilled to obtain a liquor or spirit, for instance the famous Indonesian brem bali, an alcoholic liquor produced in Bali from the liquid portions of tapé ketan.

B. Traditional Manufacturing Process

Saké is a pale yellow rice wine of Japanese origin with an alcohol content of 15–16% or higher (Fig. 2.16). Rice, *Oryza sativa*, is polished to remove protein, lipids and minerals which are in excess in the bran and germ, washed, steeped in water and steamed for 30–60 min, then cooled. A starter or *tane-koji* is then made by culturing *Aspergillus oryzae* on rice at 28–30 °C for 5–6 days. The rice is mixed with starter and yeast *moto* or *ragi* starter and water to form the main mash or moromi. The main fermentation is carried out in open tanks starting at a temperature of about 12 °C and increasing to temperatures not exceeding 18 °C. After 3 weeks of fermentation, the mash is pressed out, allowed to settle, filtered, pasteurised at 55–65 °C, blended, diluted with water and bottled.

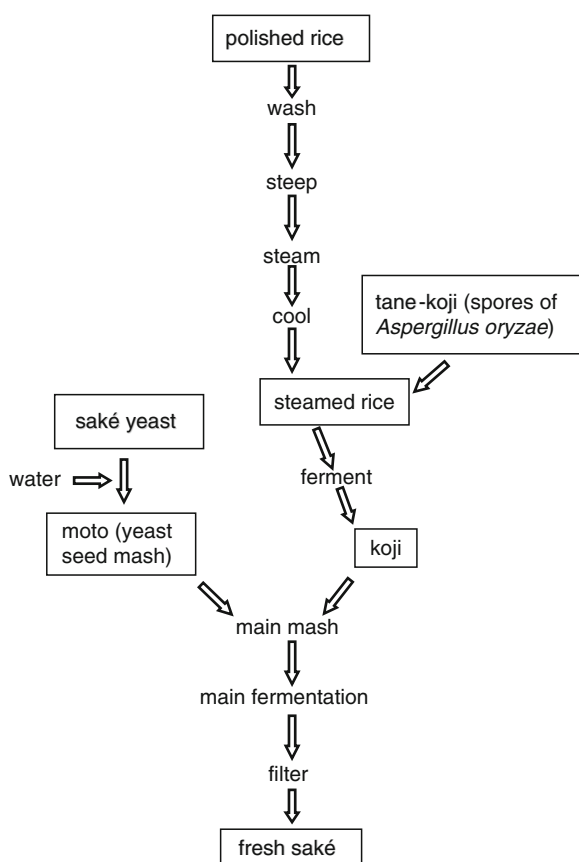


Fig. 2.16. Traditional manufacture of Saké

Malaysian rice wine or *tapai* is lighter in colour ranging from red to pink. It is made from cooked gelatinised rice and red pulverised *ragi* (yeast cake or *jui-piang*) and fermented for up to 30 days at 25 °C.

Yakju and *takju* are Korean alcoholic beverages originally made from rice, but are now made from wheat, barley, corn or millet. The starter or *nuruk* is prepared by inoculating *Aspergillus usamii* on moist wheat for up to two months. The mycoflora of *nuruk* includes *Rhizopus*, *A. niger* and yeasts. *Nuruk* serves as a source of amylolytic enzymes to saccharify starch, followed by conversion of the sugars to ethanol. In the traditional *yakju* process, steamed, cooled rice is mixed with *nuruk* and yeast inoculum is added. *Takju* is made by diluting fresh *yakju* liquor prior to filtration. Kim et al. (2004) studied the effects of *yakju* on human and mouse cancer cell lines and concluded that the Korean rice wine had strong anti-cancer effects as a result of certain constituents in the wine. In the Philippines, *tapuy* (Igorot ethnic group) is an acidic, but sweet alcoholic rice wine and is known by other names such as *binubudan* (Ifugao), *binuburan* (Ilocano), or *purad* (Tagalog). The Thai rice wine is a cloudy yellow liquid made from glutinous rice and, in India, *madhu*, *jnard* and *ruhi* are social drinks made from rice and produced in the Nagaland and in the eastern hill regions.

C. Fungi Involved and Their Relevant Properties

Although the starters used for hydrolysis of rice and other cereals in the production of wine are generally complex mixtures of essential and non-essential micro-organisms, the major amylolytic enzyme producers range from the mycelial fungi *Aspergillus*, *Mucor* and *Rhizopus* to *Amylomyces rouxii* (Vo et al. 1993; Park et al. 1995; Basuki et al. 1996; Steinkraus 1996).

The fungi which are involved in the production of rice wine are given in Table 2.8. Selected strains of *A. oryzae* are used in the preparation of *tane-koji* in the manufacture of saké. The mould produces α -amylase (liquefying amylase) and amyloglucosidase (saccharifying amylase) to hydrolyse starches to dextrin, maltose and glucose and produces acid and alkaline proteases to hydrolyse proteins to peptides and amino acids. Other essential moulds in the production of rice wine include *A. usamii* and *Amylomyces rouxii*. Moulds belonging to the genera *Mucor* and *Rhizopus* are usually the main enzyme producers for the production of rice wines in India. The main yeasts which ferment saccharified rice starch to alcohol are *Endomycopsis burtonii*, *E. fibuliger*, *Saccharomyces cerevisiae* and *Candida lactosa*, although *Endomycopsis* (*Saccharomyces*) *fibuligera* produces amylolytic enzymes

Table 2.8. Fungi used in the production of Asian rice wines

Country	Wine	Yeasts and moulds
China	Shaoxing	<i>Aspergillus oryzae</i> , <i>Rhizopus</i> spp., <i>Saccharomyces cerevisiae</i>
Japan	Saké	<i>A. oryzae</i> , <i>S. saké</i> , <i>Hansenula anomala</i>
Korea	Yakju, Takju	<i>A. oryzae</i> , <i>A. sojae</i> , <i>Rhizopus</i> spp., <i>S. cerevisiae</i> , <i>H. anomala</i> , <i>H. subpelliculosa</i> , <i>Torulopsis saké</i> , <i>T. inconspicua</i> , <i>Pichia polymorpha</i>
Thailand	Sato, Ou	<i>Mucor</i> spp., <i>Rhizopus</i> spp., <i>Candida</i> spp., <i>Saccharomyces</i> spp.
Indonesia	Arak, Brem Bali (distilled liquors)	<i>Mucor</i> spp., <i>Rhizopus</i> spp., <i>Candida</i> spp., <i>Saccharomyces</i> spp.
Philippines	Tapuy	<i>Endomycopsis fibuliger</i> , <i>Rhodotorula glutinis</i> , <i>Debaromyces hansenii</i> , <i>Candida parapsilosis</i> , <i>Trichosporon fennicum</i>
Malaysia	Tapai	<i>Amylomyces rouxii</i> , <i>Rhizopus</i> spp., <i>Endomycopsis</i> spp.
India	Ruhi, Madhu, Jnard	<i>Mucor</i> , <i>Rhizopus</i>

as well (Reiser and Gasperik 1995; Yip et al. 1997; Brimer et al. 1998).

Other yeast species, *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. Isoamyl acetate is an important flavour component in sake brewing. However various yeast mutants with improved fermentative activity and precursor of inhibitors of genes responsible for synthesis of flavour components have been reported. Studies showed that production of flavour compounds in saké brewing could be improved with the mutant strain resulting in 1.4-fold increase in isoamyl acetate. Mutant strains with enhanced fermentative activity could improve occasional 'stuck' or high ethanol fermentations which often result in low-quality saké (Watanabe 2002; Hirooka et al. 2005).

D. Industrial Aspects

The commercial production of saké in Japan employs a highly sophisticated technology in contrast to the indigenous production of rice wine in other parts of Asia. In the manufacture of saké, starch saccharification is achieved during production of koji with highly automated koji bioreactors (see Soy sauce koji). Fugiwara Techno-Art Company (Japan), a leading manufacturer of koji making and brewing equipment, has developed the sky-type continuous rice steamers which have become popular because of the quality of rice produced, low operating cost and ease of operation.

On a commercial scale, the main fermentation mash or moromi containing koji, steamed rice and water is fermented with moto in 10–20 m³ capacity tanks each containing from 1.5–10.0 t of rice. After fermentation, which usually takes about three weeks, the mash is pressed and the saké is allowed to settle for up to 10 days. One tonne of polished rice yields about 3000 l of sake (20% ethanol, v/v) and 200–250 kg of moromi filter cake, *sakekasu* which is used to make pickles and soups. In Korea,

there are over 3000 breweries for the production of yakju and takju, with annual production in excess of 8700 t and 1 600000 t, respectively.

Saké yeast is known for its high alcohol yielding capacity. In Japan, one of the leading research institutes in saké brewing, Gekkeikan, has produced a 'super yeast', capable of fermenting pretreated cellulosic material to alcohol (Gekkeikan 2008). Super yeast was created by integrating koji mould genes that produce cellulolytic enzymes into saké yeast using cell surface engineering with the enzymes being densely displayed on the surface of the yeast. Gekkeikan hopes that such technology developed for the production of bioethanol in the Japanese sake brewing would contribute to the resolution of environmental issues.

VIII. Chinese Liquor

A. The Product

Chinese liquor or "jiu" is a collective name for a wide variety of strong alcoholic liquors, obtained by distillation of cooked sorghum that has undergone alcoholic fermentation. The starter used for the alcoholic fermentation is called "qu". In many cases, the qu is brick-shaped and because of the large size of the bricks (they can weigh 1–2 kg each), the starter is called "big qu", in Chinese "daqu". Chinese liquor is very important in Chinese culture and has been described since historic times. It plays an important role during festivities, hospitality and business events. Its strength can be around 50% v/v alcohol, although nowadays the standard tends towards the internationally usual 38–40% v/v alcohol. The flavor of Chinese liquor varies according to manufacturer, from very mild to sauce- and strong-flavoured.

B. Traditional Manufacturing Process

Figure 2.17 shows the principle of the traditional manufacturing process. There are many specific variations that are applied in commercial production; many specific details are kept confidential for competitive reasons. In principle, the process consists of the following stages.

1. Production of “daqu” starter. The ingredients wheat, barley and peas are ground, mixed and the mixture made into a stiff dough with water. The dough is filled into brick-shaped molds and pressed to obtain enough cohesion to allow the bricks to retain their shape. The bricks are stacked a few layers high in an incubator room where temperature and humidity can be controlled. During a period of several weeks, the bricks are incubated according to a defined time-temperature profile. During this period, the bricks are colonised by successive populations of fungi and bacteria. The microflora is complex and undefined. However, the strict control of ingredients formulation and processing conditions results in “typical” daqu types, which are distinguished by their colour (exterior as well as interior) and flavor. After completion of the incubation stage, the now hardened daqu bricks are stored under protective roofs for periods up to 12

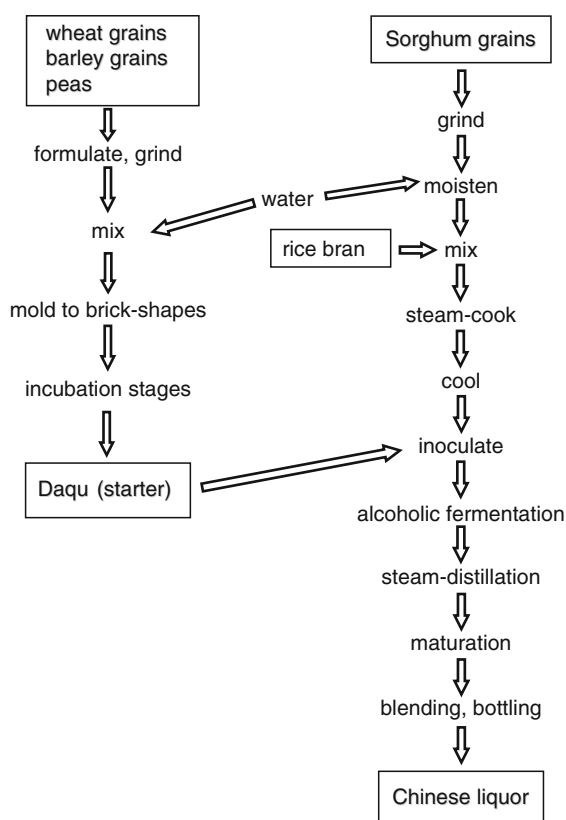


Fig. 2.17. Traditional manufacture of Chinese liquor. Outline of principle only.

months so that enough starter is available throughout the year.

2. Alcoholic fermentation. *Sorghum* grains (mostly red sorghum) are ground, moistened with some water and left overnight to allow a homogeneous distribution of moisture. The moist sorghum meal is then steam-cooked in order to gelatinize the sorghum starch. Prior to cooking the sorghum had been mixed with rice bran in order to avoid excessive stickiness of the steamed mixture. The steamed mixture is cooled to inoculation temperature and a considerable quantity (10–20% of total weight) of powdered daqu is mixed into the mixture. The inoculated sorghum is transported to the incubation room where it is filled into airtight vessels and allowed to ferment during a period of 2–3 weeks, depending on local conditions and preferences. During this period the daqu micro-organisms and their enzymes will act upon the compounds from sorghum, daqu and rice bran. Because of the high inoculation level, it is fair to state that the daqu itself also forms part of the substrate.
3. Distillation and maturation. After fermentation the vessel is emptied and its contents are transferred to a steam distiller. Here, all volatiles are steam-distilled and collected in a water-cooled condenser. The raw distillate is standardized to the desired alcoholic strength and transferred to earthenware maturation jars of 500–1000 l, and will remain there for maturation. This may take several months to years, and should result in a harmonious and stable flavor. Finally, the single or blended matured liquor is bottled in glass or traditional earthenware jars.

C. Fungi Involved and Their Relevant Properties

The microbiology of this process should be distinguished in the microbial succession during the production of daqu and the process of alcoholic fermentation. Daqu is colonized by a complex mixed microflora of fungi and bacteria. Among the filamentous fungi, *Aspergillus*, *Mucor*, *Rhizopus*, *Monascus*, and *Trichoderma* spp. were encountered in Moutai daqu (Wang et al. 2008a), and *Absidia*, *Rhizopus*, *Rhizomucor*, *Aspergillus* and *Emericella* spp. in Shaoxing qu (Xie et al. 2007). Predominating yeasts were identified as *Saccharomyces*, *Hansenula*, *Candida*, *Pichia*, and *Torulasporea* spp. in Moutai daqu (Wang et al. 2008a) and *Saccharomyces*, *Candida*, *Clavispora*, and *Pichia* in Shaoxing qu (Xie et al. 2007). Predominating bacteria were described as *Bacillus*, *Acetobacter*, *Lactobacillus* and *Clostridium* spp. (Wang et al. 2008b). There is a recent research development in daqu microbiology because scientific data on its composition are still quite scarce. Nevertheless, such data are essential to further development of well controlled and

defined products. Concerning the functionality of the groups of micro-organisms, it has been proposed that bacteria are important sources of proteases and amylases to digest starch and to provide butyric acid for fermentation and flavour development; yeasts are responsible for the alcoholic fermentation, and production of volatile ester flavours; and moulds would contribute to degradation of polymeric substrates, formation of esters and other volatiles.

The alcoholic fermentation could be expected to be dominated by yeasts. Indeed, *Issatchenkia* and *Saccharomyces* spp. have been encountered (Zhang et al. 2007). After one week of fermentation, *Lactobacillus acetotolerans* (Zhang et al. 2005; Wang et al. 2008b) was reported as a predominant organism. Other bacterial species were reported and these may play a distinctive role in typical flavour formation. It was suggested that *Bacillus*, *Bacteroides* and *Clostridia* may contribute to strong aromas whereas *Bacillus*, Flavobacteria and Gammaprotobacteria would give rise to sauce-flavoured liquor (Wang et al. 2008b).

The flavours in Chinese liquors comprise a wide range of volatiles such as the fruity and floral ethyl and butyl esters of butanoic, pentanoic, hexanoic and octanoic acids, the sweaty hexanoic acid and the nutty or roasted notes of pyrazines such as 2,5-di-methyl-3-ethylpyrazine (Fan and Qian 2005, 2006a). A range of 27 alkyl and acetylpyrazines was identified (Fan et al. 2007); clove, smoky and goatly flavour notes were ascribed to 4-ethylguaiacol, 4-methylguaiacol and 4-ethylphenol (Fan and Qian 2006b).

D. Industrial Aspects

The annual production of Chinese liquors was estimated at 5×10^6 t in 2007. This quantity is based on data from some large-scale companies; in addition, there is an unknown number of smaller-scale semi-artisanal workshops where liquors are produced. Very little is known about the processes carried out, the micro-organisms involved and the composition of the liquors produced. In view of the importance of these products, it may be expected that further research will yield meaningful data to enable industrial development of safe and unique products.

IX. Conclusions

Fungal food fermentations are practised in nearly all the continents, but those originating in Asia are of very ancient lineage and also present the

greatest variety of products. Some of the traditional production processes developed from low and intermediate biotechnology into highly sophisticated and automated systems.

Examples of some well known fungal fermented foods are presented in this chapter and traditional manufacturing processes, biochemical changes, the essential fungi involved in the fermentation process and industrialisation, process control and innovations are also presented. However, it is recognised that there are still numerous lesser-known or less developed fermented products in the continent that are not covered. One of the biggest hurdles in transfer of technology is the transfer and utilisation of information from the laboratory research base to pilot and commercial scale and the Oriental fermented food industry has been reasonably (or highly!) successful in this respect.

Problems, advantages and future developments associated with fungal fermented foods may be summarised as follows:

- Some of the constraints of fungal food fermentations are: (1) optimisation of the fermentation process; the majority of these processes are based on solid substrate fermentations (SSF) and one of the problems with SSF is limited heat and mass transfer, (2) the types of organisms are usually limited to those that can grow at reduced moisture levels, (3) using monitoring devices to determine moisture, pH, etc. becomes a problem and (4) the spore inoculum needed may be quite large.
- There is a greater need to monitor possible production of toxic fungal metabolites, in particular, mycotoxins which may be formed during fermentation.
- The advantages of fungal food fermentations include: (1) bioenrichment of food through a diversity of aromas, flavours and texture, (2) bioenrichment of foods with vitamins, proteins, amino acids and essential fatty acids, (3) preservation of food through production of alcohols, acids and esters, (4) production of food colours, (5) improved digestibility of food, (6) production of edible fungal biomass and single-cell protein.
- The fermentation systems are usually simple, requiring less space in relation to yield of product because less water is used and the substrate is concentrated. The desired product may be

readily extracted by addition of solvent. Thus, neutraceuticals and novel compounds may be produced from fermented foods by commercial companies. Also the ingredients are relatively simple, for instance whole grain with water sufficient to moisten the substrate.

Future developments should focus on improving process control, the use of immobilised systems and/or enzymes and use of genetically modified organisms to maximise productivity without health risks.

Extensive research and developmental studies in isolation and/or selection of desirable fungi and carefully controlled fermentation processes of some Asian fungal fermented foods have led to products of high quality, improved digestibility, extended shelf life, maximum utilisation of raw materials, exclusion and/or reduction of fungal toxins and improved nutritional values. A plethora of published scientific data on Asian fungal food fermentations, viz. natural or spontaneous fermentation (e.g., idli), starter-mediated single-stage fermentation (e.g., tempe kedele) and multi-stage fermentation (e.g., soy sauce), is a testimony of the extent to which research and development have been devoted to such industry. It is noteworthy that industrialisation of the traditional methods used in the production of fungal fermented foods has led to improved and highly acceptable products without change in flavour, texture, colour, aroma or fragrance of the products. However there are still many fungal fermentation processes in Asia that require improvement in substrate preparation and utilisation, process control, product yield and hygiene standards, particularly at a small to medium or cottage scale.

We are of the opinion that the introduction of some of these fungal fermented foods (tempe, soy sauce, saké) into foreign markets, particularly in Europe, and in both North and South America is essential as demand for healthy foods, naturally fermented products, protein-rich meat substitutes, exotic foods of plant origin, is increasing world-wide.

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3 Production of Beer and Wine

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I. Introduction

Beer and wine both result from the metabolic action of yeast cells. Thus, these beverages are early products of biotechnology, since biotechnology can be defined as the technical use of organisms or parts thereof for productions or services. However, traditional alcoholic drinks were already enjoyed long before fermenting yeasts became known (after the discovery of the

microscope) as micro-organisms. We can assume that wine and beer as esteemed alcoholic beverages are nearly as old as the history of human cultures. We have archeological evidence from Babylonia, Mesopotamia, Egypt and other regions that wine and beer-like beverages were already well known and commonly used thousands of years before Christ.

With Louis Pasteur (1822–1895), Emil Christian Hansen (1842–1909) and other scientists a more rational basis for alcoholic fermentation was introduced after 1850 and further developed so that the biochemistry of the process – from active transport of fermentable sugar into the cells, intracellular conversion to ethanol up to its excretion – is well understood today (Fig. 3.1). Techniques of DNA recombination, as carried out for the first time in 1973 (Cohen et al. 1973), even offer the opportunity for genetic engineering of the cereal and yeast genomes and therefore for targeted improvements in technological and other properties (Dequin et al. 2001).

Wine results from fruit juice submitted to an alcoholic fermentation. Most wines are made from grapes belonging to the botanical genus *Vitis*, which includes nearly 60 wild species. Techniques for the identification of grape varieties include the use of artificial neuronal networks (Manusco et al. 1998) or the genotyping of different SSRs loci (Almadanim et al. 2007) for the *Saccharomyces cerevisiae* typing, as well as the screening of single-nucleotide polymorphisms in genes involved in wine production (Vigentini et al. 2009). For neuronal networks, the phyllogenic data of 450 leaves are submitted to a computer to build up a network which is typically organized in layers for data reception, processing and output. Beside grapes, also other fruits can be used for the production of wines, e.g. apples, strawberries, currants, blackthorn or more special fruits like gabiropa (Duarte et al. 2009).

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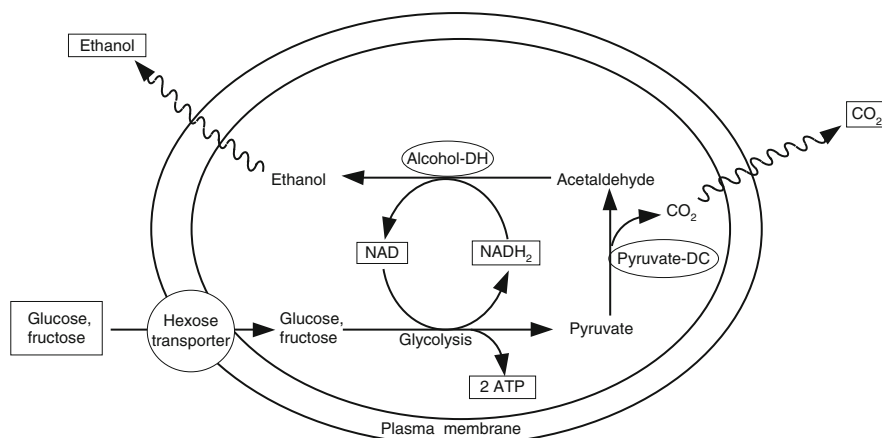


Fig. 3.1. Simplified scheme of alcoholic fermentation in a yeast cell

For the large-scale production of wine, only the grape species *Vitis vinifera* L. is of importance. This species is the result of a selection process which has been running for thousands of years with the aim of producing good wines. For many years, yeast strain and grape improvement have been also possible by recombinant genetic techniques (Pretorius and van der Westhuizen 1991). Resistance to fungal pathogens is desirable, therefore *V. vinifera* was transformed with a *Trichoderma* endochitinase gene (Kikkert et al. 2000). Wine quality results from the grape properties on the one hand and the winemaking process on the other. The science and technology of dealing with the grapes lies in the viticulture, an important part of enology, where winemaking with cellar techniques contribute to other important parts. Differences between grape varieties have led to the largest and most simply recognised and controllable differences influencing wine composition and quality. When selecting a grape variety, requirements for cultivation, influences on wine flavour and economical aspects have to be taken into consideration.

Beer is made from barley and/or other starch-containing cereals, hops and water. In order to enable yeast fermentation, the starch of the cereals must first be converted into fermentable sugars, e.g. maltose, by germinating the grains (malting) and by a subsequent enzymatic process (mashing). Thus, in brewing, an enzymatic breakdown of oligo- and poly- to monosaccharides is, in contrast to wine making, additionally necessary. This makes brewing somewhat more difficult than wine

making and we must assume that brewing is the younger technology. However, also beer brewing can be traced back for more than 4000 years.

In countries like France or Italy, wine consumption often amounts to more than 90 l person⁻¹ year⁻¹, and more than 100 l person⁻¹ year⁻¹ beer are consumed in beer-preferring populations, e.g. Czech Republic, Germany, Belgium or Britain. Thus, wine and beer are of enormous commercial importance, both together with a world-wide annual production volume of more than 10⁹ hl corresponding to a value ranging near US \$100 × 10⁹. That is much more than the value of insulin, monoclonal antibodies, mammalian proteins or other typical products of modern biotechnology.

II. Beer Brewing

Details of the brewing process depend very much on the type of beer to be produced and can also differ considerably from country to country and even from brewery to brewery (Briggs et al. 2004; Kunze 2004). In the following, emphasis is given to the major production routes of top- and bottom-fermented full beers. For further details and to get a better insight in other brewing techniques, it is recommended to consult one of the above-mentioned reviews or of the comprehensive textbooks of Back (2008), Bamforth (2006), Boulton and Quain (2006), Eßlinger (2009), Narziß (2004) or Priest and Stewart (2006).

A. Raw Materials

In Germany, the so-called *Reinheitsgebot* (purity law, existing since 1516) prescribes that only barley in malted form, hops, water and yeast as a processing aid are allowed to make beer. Other materials coming into contact with beer, e.g. filter aids, must be harmless and insoluble, and they are not tolerated in the finished beer. Those processing aids must not be declared (O'Rourke 1999). For beers exported from or imported to Germany, as well as for special beer types, e.g. wheat beer or sorghum beer, also ingredients other than barley malt, e.g. various unmalted cereals, adjuncts, enzymes (Bendler and Lemaire 1999; Glatthar et al. 2005; Ratnavathi et al. 2000), sugar, etc., may be allowed. Also for English-style ales, cereals other than barley are used (Taylor et al. 1998).

Barley is the most expensive raw material per litre beer brewed. It is the typical but by far not the only cereal for making beer (Zamkow et al. 2004). In preparation for brewing, barley must be submitted to a malting process, where the grains germinate and synthesise enzymes, which are necessary for the subsequent steps of mashing and fermentation. Preferably, two-row spring barley (*Hordeum distichum*) with large grains is used to make malt and beer. It is richer in starch and relatively poor in protein as compared with the six-row winter barley (*Hordeum hexastichum*), which has small grains. In comparison to other cereals, barley, even after threshing and malting, retains the husk still adhering to the grain. After malt milling and mashing in the brew-house, the husk particles are used as a filter material for mash separation in the lauter tun.

Hops (*Humulus lupulus*) gives the beer a refreshing bitterness and a slightly sedative effect; it suppresses the growth of Gram-negative bacteria and improves foam stability. Hops variety and quality and treatment considerably influence the beer taste (Srecec et al. 2008; Weyh and Hagen 1998) and foam quality (Evans and Surrel 2008). Only the female hop cones are desired, since every cone contains more than 10 000 microscopic lupulin glands with the bitter hop resins. The soft resins contribute most to the bitterness of a beer with their α -acids and to a lesser extent with their β -acids (Techakriengkrai et al. 2004), and hop oils are responsible for the characteristic aroma.

Male hop flowers contain only 10–100 lupulin glands. Thus, the male plants are unwanted and even strictly kept away from the hop cultivation areas, because fertilisation would lead to reduced quality of the female hop cones for brewing. Since hops can only be harvested during a limited period of the year, several methods to make the hop cones stable, until used in the brewery, are practised. Hops can be sulfured and compressed into bales, but also then, hops remain bulky and can be stored only for a limited time. Further processing to hop pellets and various hop extracts (Held 1998; Weiss et al. 2002) leads to more stable and concentrated forms. For further details on hop chemistry and technology see also De Keukelaire (2000), Peacock (1998) or Barth et al. (1994).

Water is quantitatively the most important raw material. To make 1 hl of beer, between 3 hl and 10 hl water are needed in a brewery, most of it as processing water for various purposes and about 1.3 hl going into the wort and therewith into beer. The character of the beer is very much dependent on the quality of the brewing water and, in the past, the water of traditional brewing places, e.g. Pilsen, Burton-on-Trent, Munich, etc., considerably influenced the beer type. However, with the present-day methods of water treatment, local water characteristics have become less important.

Yeast is necessary to make beer from wort, and it considerably determines the type of the product (Lodolo et al. 2008). Yeast catalyses the conversion of fermentable sugars to ethanol by glycolysis followed by decarboxylation of the pyruvate to acetaldehyde which is finally reduced to ethanol (Fig. 3.1). Selected top- and bottom-fermenting strains of the species *Saccharomyces cerevisiae* are used for beer brewing. Within this species a lot of types and strains exist (Hampsey 1997).

In the past, the bottom-fermenting yeasts were classified as *S. carlsbergensis*, later as *S. uvarum*. The major difference of total breakdown of the trisaccharide raffinose by bottom-fermenting yeasts instead of one-third raffinose breakdown by the top-fermenting yeasts, according to the recent understanding of many taxonomists, does not justify the classification of both types as separate species but as different subspecies of *S. cerevisiae* (i.e. *S. cerevisiae carlsbergensis*, *S. cerevisiae cerevisiae*). Other taxonomists (Spencer and Spencer 1997) and culture collections, e.g. the American Type Culture Collection (ATCC) and the Deutsche Stammsammlung für Mikroorganismen und Zellkulturen (DSMZ), name the bottom-fermenting brewer's yeast *S. pastorianus*.

Besides its main function to form ethanol from sugar, brewer's yeast also considerably

contributes to the taste and character of a beer via a multitude of metabolic activities. Principally, yeast can live both in the absence of oxygen (anaerobically) and when oxygen is available (aerobically). In breweries, increased growth under aerobic conditions is sometimes used for a short period at the beginning of the fermentation, when brewers bubble air into the wort. Oxygen is then removed quickly by cell respiration, and in the subsequent phases of the main and after fermentations, conditions are kept anaerobic. It is of general importance that the production strains maintain their fermentation fitness over a long time and adapt to the many handling stresses occurring in the industrial brewery (Gibson et al. 2008).

B. Malt Production

Beer brewing begins in the malthouse, even if malt production is not carried out in the brewery but elsewhere, often far from the brewery. Malt, as a raw material, considerably influences the brew house work (Litzenburger 1997). The main purpose of malting is to produce enzymes in the barley kernel which can catalyse a number of changes in the barley components necessary for making beer. Dissolution of the cell walls of the endosperm and partial breakdown of the starch and proteins are important enzymatic processes in the kernel with regard to the subsequent brewing.

In the course of malting, the barley is made to germinate by raising its humidity above 40% and by subsequent incubation at temperatures up to 25°C. The germinating process is stopped at a time appropriate to guarantee sufficient enzyme production and enzymatic conversion on the one hand and to avoid too much loss of substances during germination on the other hand. Fig. 3.2 shows the typical procedures of malt production.

Cleaning of the barley is carried out to remove any unwanted material from non-barley grains, broken kernels, stones, etc. After that, the barley is submerged and aerated until a water content of 42–47% is reached. This process is called **steeping**. For pale malt, e.g. Pilsener, the water content of the steeped barley is kept a little lower (42–44%) than for dark malt, e.g. Munich type, where 45–47% is preferred. The steeping time depends very much on the steeping temperature, but also

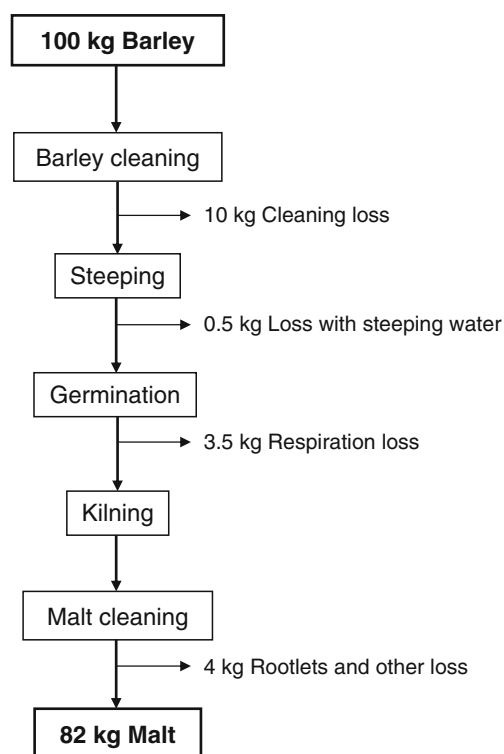


Fig. 3.2. Scheme of malt production

on kernel size and other factors. Water temperatures of 10–15°C and steeping times of 50–80 h are usual.

Germination already starts during steeping. However, most of the germination takes place during the following 6–10 days, when the steeped barley is kept at 17–25°C in a drum, a box or as a moving batch. Malt for pale beers (Pilsener type) is kept at a lower temperature and for a shorter time than malt for dark beers (Munich type). During the germination process, the barley must be turned and aerated in order to remove the CO₂ and heat produced by respiration. In traditional floor malting the turning was done with malt shovels twice a day. At the present day, the process is carried out with less hand-work and with a very much better control of temperature and humidity in pneumatically operated malting systems.

Kilning removes water by passing large amounts of hot air through the green malt on single- or two-floor kilns. Thus, the malt becomes stable and storable and colour and flavour components are formed from sugars and amino acids

by complex Maillard reactions leading to melanoindins. To protect the enzymes from inactivation, first a pre-drying phase, called withering, is practised at a lower temperature (e.g. about 40°C of the drying air) within the first hours. Then, in the curing phase, the temperature of the influent air is increased to nearly or somewhat above 100°C. The malt temperature remains below these temperatures due to the evaporative cooling. The kilning process takes 20–50 h and depends on the equipment and type of malt.

Malt cleaning is necessary to remove the rootlets still attaching to the kilned product. For the brewing process these rootlets are of no use, but they are valuable by-products which can be sold as fodder additives or for other fermentation industries. The malt kernels then resemble unmalted barley, however their taste is much more aromatic. Storage of the malt in silos for at least 1 month before use in a brewery is advisable, because fresh malt can lead to difficulties in lautering and fermentation. During this storage, the water content slowly increases to about 5%. Before use in the brewery, a polishing to remove split-off husks and other particles from the malt is usually applied.

C. Steps in the Brew House

In the brew house, malt is grinded, mixed with water and processed to wort, which is the substrate for the subsequent fermentation. To produce 1 hl wort with 12% extract requires about 17 kg malt. Fig. 3.3 gives a survey on the usual steps from malt to wort.

1. Malt Grinding and Mashing

By grinding with dry, wet or hammer mills, the malt is broken into small fragments, called malt grist. Since the barley husks have to serve in the further process as filter aids for lautering, they are protected as far as possible against destruction during the milling procedure. Malt grist and water are mixed together to form the mash. Following, this mixture is submitted to various temperatures, where different enzymatic conversions are supported. Namely, the breakdown products of starch, proteins and β -glucans are of major importance. All substances going into solution during the mashing procedures form the actual extract. It is intended to obtain as much and as

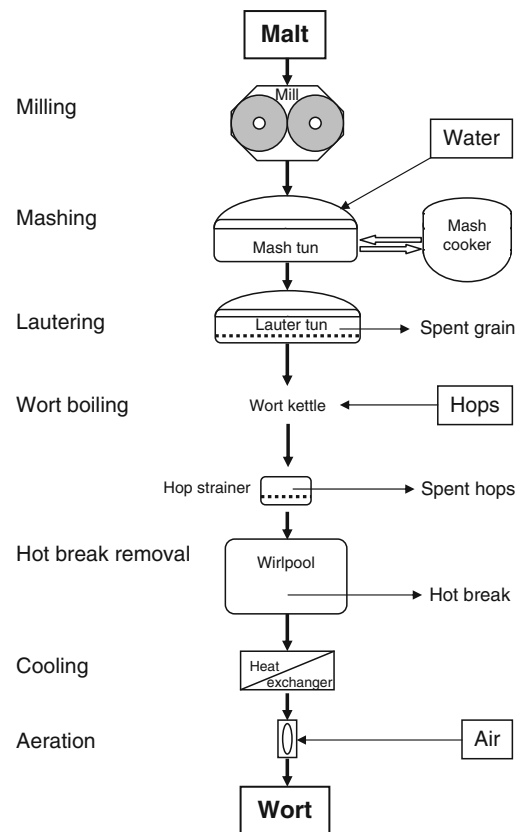


Fig. 3.3. Wort production line in the brew house

Table 3.1. Example of a three-mash decoction process

Stage name	Main mash	Boiled mash
Mashing-in	37°C	
Protein rest	51°C	First boiling
Maltose production rest	65°C	Second boiling
Final saccharification rest	77°C	Third boiling

good extract by mashing as possible from the given malt. Mashing is either carried out as a so-called **infusion process** in the mash converter or as a **decoction process**, where a part of the main mash is removed from the converter, made boiling in the mash cooker and brought back to the mash converter (Fig. 3.3). According to the number of boiling actions brewers speak of a single mash, two mash or three mash process. Table 3.1 summarises a typical three-mash process as an example.

There is an enormous variety of mashing programs and special procedures in the different breweries (Schwill-Miedamer et al. 1998). In general, the total mashing time is around 3 h. The draw-off parts in a decoction mashing (Table 3.1) are carefully calculated, so that the desired temperature raisings are obtained in the residual mash after bringing back the boiled mash. There is a tendency in modern brewing to reduce the number of stages in mashing and even mashing at only one temperature (e.g. 2 h at 62°C is possible with good malt qualities).

2. Lautering

In order to make wort from the mash, the undissolved substances must be removed from the mash while as much as possible of the extract should be recovered in the wort. The necessary separation is usually carried out in a lauter tun; only very few breweries use mash filters.

The lauter tun is a vessel having a “false” bottom with a multitude of gaps, each about 1 mm wide. The mash is distributed over this false bottom, the husks and other insoluble material of the malt kernels are retained and act as a filtering aid, so that with the time only turbidity-free wort passes through. Turbid wort from the beginning of lautering is brought into the tun once again. Towards the end of the lautering process, extract retained by the husks and spent grains is washed out to a certain extent with hot water. The remaining spent grains are removed and used as fodder. In order to get the final wort with a definite extract concentration, the wort must have about 5% more extract in the first wort at the beginning of lautering. The subsequent spargings dilute the wort (“make it thinner”). In general, the lautering takes about 2 h.

3. Wort Cooking

The wort is boiled in the wort kettle (also named copper), mostly for 1–2 h, with the addition of 150–500 g l⁻¹ pressed hops, depending on the beer bitterness wanted. In case of hop concentrates, the amounts of preparation added can be reduced. In some breweries, high-temperature wort boiling under pressure and other new developments have been introduced (Fohr and Meyer-Pittroff 1998). In those systems, only 3–5 min at 140–145°C can be sufficient to reach effects similar to those from 1–2 h normal boiling.

During wort boiling, hop components are dissolved into the wort and *iso*-components are formed from the α -acids, which, without isomerisation, would be insoluble in cold wort. Many proteins are precipitated as protein–protein or protein–polyphenol complexes, enzymes are inactivated and the wort gets disinfected. Furthermore, aroma and colouring components are formed during wort cooking.

Table 3.2. Typical composition of wort extract

Component	Content (%)
Glucose	8
Maltose	55
Maltotriose	10
Higher dextrins	20
N-containing substances	5
Minerals	2

Last but not least, boiling leads to evaporation of water and, thus, the extract concentration of the wort can be brought into the right range desired for the beer to be produced. The wort extract then has a composition as given in Table 3.2.

4. Cooling and Wort Treatment

After cooking, the wort is turbid; it contains up to 8 g l⁻¹ hot break, also called coarse break. A hop strainer, a vessel with sieve bottom, then removes the spent hop particles, most of them much larger than 100 μ m. The remaining turbidity with typical particle sizes below 100 μ m can be removed by filtration or by centrifugation, but also by the following means, being most typical for breweries:

- **coolship**, the traditional apparatus for break removal;
- **settling tank**, needing less space than a coolship;
- **whirlpool**, a vessel applying the “teacup effect”.

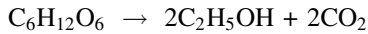
Instead of coolships and settling tanks, more and more whirlpools have been installed in breweries over the past three decades. A whirlpool is a cylindrical vessel working according to the same principle to be observed when a cup of tea with leaves in it is stirred. Due to the currents and forces occurring, the leaves assemble in the middle of the stirred tea. In case of the whirlpool, the turbid wort is pumped tangentially into the vessel. The rotation then brings the hot break to settle in the middle of the tank and clarified wort can be drawn off at the side.

After removal of the hot break, quick wort cooling is carried out in a heat exchanger. Some breweries then remove the cold break by wort flotation or by filtration over perlite. The cold wort is aerated in order to improve the starting conditions for the yeast cells. The oxygen dissolved in wort then amounts often to more than 8 mg l⁻¹; this oxygen is quickly used after

addition of yeast, and it considerably improves the subsequent yeast growth and fermentation.

D. Fermentation

As shown in Fig. 3.1, brewer's yeast converts glucose and other hexoses to ethanol and carbon dioxide following the overall equation according to Gay-Lussac:



The disaccharide maltose, the typical sugar in beer wort (see Table 3.2), is easily hydrolysed by α -glucosidase (maltase), inside the yeast cell, into two glucose molecules and thus makes the wort fermentable. Depending on the properties of the yeast strain, some oligosaccharides with more than two glucose molecules, e.g. maltotriose or maltotetraose, can also be hydrolysed and fermented to ethanol.

About 0.5 l thick yeast slurry are added to 1 hl wort to start the fermentation process. This yeast addition roughly corresponds to 20×10^6 yeast cells ml^{-1} wort. The yeast is mostly taken from one fermentation to the next. However, problems can occur when a long series of fermentations is carried out, each pitched with the crop of the preceding batch (Wackerbauer et al. 1997). Propagation from laboratory cultures is practised, at least from time to time. Yeast propagation can be carried out in open growth vessels or under sterile conditions in propagation tanks (Jones 1997; Munday and Dymond 1998). The yeast pitching rate can considerably influence the fermentation performance and also the beer flavour (Verbelen et al. 2009). Open yeast propagation is only usual in small breweries. The possible use of dried yeast for brewing is sometimes discussed but not commonly practised (Fels et al. 1998).

As a rule, for bottom-fermentation, as applied for Pilsener, Dortmunder and other lager beers, the temperatures are kept considerably lower than for top-fermentation, typical for alt, porter stout and other ale types. There are often considerable deviations from the data given in Table 3.3.

Most breweries still prefer open tanks in the fermentation cellar, only some are using closed vessels. In those cases, the CO_2 formed during fermentation is mostly collected and further processed to dry ice. As a result of the heat released by the metabolic activity of the yeast, the temperature tends to rise. Although higher temperatures lead to an increased fermentation rate, much care is taken to keep the temperatures low by cooling. Cold fermented beer is said to be of better quality due to less formation of

Table 3.3. Data of top- and bottom-fermentation

Parameter	Top fermentation	Bottom fermentation
Initial temperature ($^{\circ}\text{C}$)	14–16	5–7
Highest temperature ($^{\circ}\text{C}$)	20–24	8–11
Duration of fermentation (days)	3–5	6–8

Table 3.4. Phases of beer fermentation

Fermentation stage	Appearance of the surface
Initial stage	Beginning of covering with white foam
Low krausen	Increase of the fine bubble foam to a creamy cover
High krausen	High foam with coarser bubbles, most intensive phase
Collapsing krausen	Foam looks browner and decreases
Collapsed foam	Dirty brown layer of collapsed foam

by-products, e.g. esters and higher alcohols. Yeast metabolism considerably influences the beer flavour (Hammond 1986; Krüger 1998).

When brewers describe the phases of fermentation, they mostly refer to the appearance of the surface of the fermentation broth (Table 3.4). During fermentation the degree of attenuation is carefully analysed. It shows (as a percentage) how much of the original wort extract has been fermented. Normally, a 12% extract of the wort is reduced to 3–4%. Thus an 8–9% extract has been fermented. As can be derived from the wort composition (Table 3.2), a considerable part of the extract, e.g. high molecular dextrin, is not fermentable. Transfer of the young beer from the fermentation to the lager cellar is carried out as soon as the desired degree of attenuation is reached. The settled yeast remains in the fermentation vessel and, after removing of undesired top and bottom parts of it, the core yeast can be used as thick liquid seed yeast for another fermentation process (O'Connor-Cox 1997). In some cases, topping up is carried out (i.e. the addition of fresh wort to the yeast in the same tank). By that means, much time normally required for the initial fermentation stage is saved.

E. Maturation

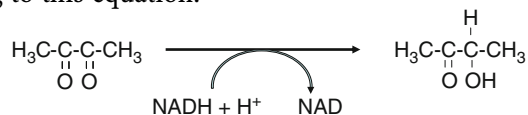
For a premium lager beer, the young beer must be submitted to an extended maturation of 2–5 weeks in the lager cellar. Lagering, storage and ageing

after fermentation and maturation are terms used more or less as synonyms for this process. Some breweries practise fermentation and maturation simultaneously in cylindroconical tanks. However, in the following, maturation is treated as a separated process having the following main purposes:

- accumulating CO₂ in the beer,
- degrading unwanted components,
- approaching the final attenuation degree.

An overpressure of about 0.5 bar (50 kPa) is built up in the closed lagering vessels and kept until filled with beer. Thus, CO₂ can enrich the beer during the after fermentation. As a further means to get sufficient CO₂ dissolved in the beer, the temperature is lowered subsequently during ageing down to between 0°C and -2°C. Thus, a CO₂ concentration of 0.35–0.55% is typically reached in the finished beer.

Among the many conversions and degradation processes during ageing, the removal of diacetyl is of particular interest, since the buttery taste of diacetyl is not generally accepted in beer. Depending on the beer type and sensitivity of the beer-drinking persons, the diacetyl content should be decreased to less than 0.1 mg l⁻¹. This happens preferably by reduction of diacetyl to acetoin, having a less disturbing flavour, according to this equation:



The degree of attenuation should finally become as high as possible (i.e. it should approach the attenuation limit), which results from the conversion of all fermentable wort sugars into ethanol. The degree of attenuation is mostly given as an apparent value, where the extract contents are measured as indicated by the densimeters in wort and beer as it is, without removing the alcohol and replacing it by water. The real attenuation is always lower than the apparent attenuation. The real attenuation degree (V_{real}) can be roughly calculated from the apparent attenuation (V_{app}) according to:

$$V_{\text{real}} = V_{\text{app}} \times 0.81$$

Brewers often prefer the apparent attenuation that is easier to be determined. Depending on the beer type and the mashing conditions, the

apparent final attenuation, obtained after ageing, is mostly between 70% and 85%. For dietetic beer it is regularly above 100%.

F. Filtration, Stabilisation and Packaging

Coming from the lagering cellar, the beer is submitted to several procedures to make, normally, a clear and stable end-product in the filter cellar. Mostly filtrations of increasing sharpness are carried out with different filter sheets for coarse, clarifying polishing and sterile filtration. Therefore, a number of powder filters and different filter aids like perlite, kieselguhr, cellulose or others are used (Braun et al. 2009; Villar et al. 2004; Weigand and Zuber 2006). Sometimes clarification by microfiltration is taken into consideration (Gan et al. 2001). Beer stabilisation with adsorbent materials, e.g. polyvinyl polypyrrolidone (PVPP) and silica gels, is common practice in most breweries (Leiper et al. 2005; Rehmanji et al. 2000; Schlenker 1999) to get rid of parts of the proteins and polyphenols. In consequence, the tendency to form protein-polyphenol turbidities in the finished beer is reduced (O'Rourke 2002).

In countries like the United States, where beer is preferably consumed very cold, enzymatic **chill-proofing** is practised to make the beer stable against chill haze occurring in the cold. Papain, ficin and bromelain are proteolytic enzymes of plant origin mostly used for this purpose (Hartmeier 1978, 1979a). In some cases, also oxygen removal by native or immobilized glucose oxidase is envisaged to stabilise beer (Hartmeier 1978, 1979b; Hartmeier and Willox 1981).

Before filling, the filtered beer is stored in pressurised storage tanks. To improve the microbiological stability of the beer, pasteurisation (10 min at 62°C) and hot filling of the beer can be carried out. Cold sterile filling is another method practised. The finished beer is preferably filled in steel kegs, aluminium containers, glass bottles or metal cans. High-speed fillers in use today are able to fill more than 2000 bottles/cans min⁻¹ without introducing significant amounts of oxygen into the beer in a pre-purged filling system.

G. Final Beer

Finished beers can have a quite different composition according to the beer type produced.

Table 3.5. Typical analytical data of some beers

Parameter	Units	Pilsener	Märzen	Bock (dark)
Original wort gravity	[%]	11.8	13.5	17.0
Apparent final attenuation	[%]	80.0	75.0	72.0
Alcohol by weight	[%]	4.1	4.3	5.2
Colour	[EBC units]	8	25	60
Bitterness	[Units]	33	24	22

Table 3.5 gives three examples of bottom-fermented beers. Besides the few data given there, a multitude of further ingredients and characteristics of the beers are of importance. A major goal of careful quality control is to prevent beer off-flavours which can have their origin in a number of reasons in the brewing process (McGarrrity et al. 2003; Ragazzo-Sanchez et al. 2009; Shelton 1998) and in ageing (Vanderhaegen et al. 2006).

Beer types can be roughly classified as top-fermentation and bottom-fermentation beers. Or, with regard to colour, pale (Pilsener, Export), dark (Kulmbacher, dark Munich beers) and middle-coloured beers (Märzen, Vienna beer) can be distinguished. Most beers result from bottom-fermentation, e.g. pilsener, export, bock, märzen and lager beer. However, many top-fermented specialities exist with wheat beers: alt and kölsch in Germany, ale, porter and stout in Great Britain or lambic, gueuze and trappist beers in Belgium.

Alcohol-free beer is in reality not totally free of alcohol. Legislation in Germany, for example, allows up to 0.5% alcohol in the final beer to be declared “alcohol-free”. In order to avoid alcohol formation, the original wort gravity is kept lower at 5–6% instead of 11–12% in normal beer production. The mashing programs for low-alcohol beers are carried out in a way that prevents good saccharification. Yeasts with low fermentative power are sometimes chosen and, last but not least, alcohol can be removed by vacuum distillation or reversed osmosis from the beer.

Dietetic beer, made for persons wanting or needing a low carbohydrate diet, has a carbohydrate content of less than 7.5 g l⁻¹. Normal (full) beer has about 30 g l⁻¹ carbohydrates, resulting from the non-fermentable oligo- and polysaccharides remaining in the original wort. Since cooking in the brew house destroys all enzymes, no further breakdown of these carbohydrates during fermentation and storage is possible in the classic process. For the production of dietetic beer, uncooked wort or microbial enzymes are added before finishing the fermentation. Furthermore, some alcohol is removed to make the dietetic beer no richer in alcohol than normals beers.

Table 3.6. Examples of beer-like beverages

Beverage	Cereal(s)	Region	Organism(s)
Bouza	Wheat, maize	Egypt	Yeast
Kvass	Barley, rye	Russia	Yeast
Pombe	Millet	Africa	Yeast
Sake	Rice	Japan	Mould, yeast
Sorghum beer	Sorghum, maize	Africa	Yeast, bacteria
Tape	Cassava	Malaysia	Mould, yeast

H. Beer-Like Beverages

The definition of beer is different according to regional traditions and legislation. Beverages called beer in several countries are under a more restrictive definition, like in Germany, at the most, beer-like beverages. From the enormous number of fermented beverages having beer-like character (Hardwick et al. 1995), a few examples are listed in Table 3.6.

Pulque is sometimes also listed among the beer-like beverages (Hardwick et al. 1995). However, it is more a kind of wine, since there is a fruit juice and not a starch-containing raw material being saccharified as is typical for beer production. Pulque is made in Mexico and some other countries from juice of the agave with *Zymomonas mobilis*, a fermenting bacterium (Tovar et al. 2008).

Sake, often named rice wine, is according to its production mode more a beer than a wine, since rice, as the starting material, is submitted to a saccharification and subsequent fermentation process (Yoshizawa and Ishikawa 1985). *Aspergillus oryzae* deals as a mould with amylolytic enzymes necessary for the breakdown of the rice starch. The yeast, sometimes named *Saccharomyces sake*, is now regarded and classified as *S. cerevisiae* by most taxonomists. More details and a figure on saké production are given in Chapter 2, dealing with Asian fungally fermented foods.

In Africa, millet and sorghum are predominant raw materials for beer-like beverages. (Grieff 1966; Taylor 1992). **Pombe**, is made from millet with *Schizosaccharomyces pombe* as fermenting organism. This fission yeast allows fermentation at a higher temperature than possible

with normal brewer's yeast. Not only cereals, but also cassava, a root with starch contents of 80% and more, can be used as main raw material or as adjunct to make beverages similar to beer (Rajagopal 1977). **Tape ub** is one of the Cassava-originating beers given in Table 3.6 (Tanaka et al. 1981); other cassava-based types of tape are tape ketella made in Indonesia and tape telo brewed on Java.

III. Wine Making

A. Grape Varieties and Composition

The decision of the viticulturist for a certain grape variety depends on many factors, including biology, climatology and the economics or traditions of the wine region. A few grape varieties are listed in Table 3.7. A very important factor for the development of the grapes is the temperature in the vineyard, which influences considerably the seasonal and regional differences in grape characteristics (Boulton et al. 1995). The rough content ranges of grape compounds are given in Table 3.8.

The contribution of grape skins to red wine is not restricted to colour; it is also important because often flavour seems to be associated with the skins. Negative contributions of the skins and also from seeds, damaged by heavy crushing or pressing, can be tannins which are undesired in higher amounts. The juice content is important for the winemaker because it determines the amount of juice which may be pressed from each tonne of grapes. In case of post-harvest drying,

grapes show metabolic changes (Constantini et al. 2006).

Juice sugar concentrations are often measured by densimeters, in which the specific gravity of the juice is determined (Boulton 1995). There are different densimetry scales, e.g. degrees Brix ($^{\circ}\text{Bx}$, introduced in the mid-1800s), degrees Baumé ($^{\circ}\text{Bé}$, commonly used in Australia and France) and degrees Oechsle ($^{\circ}\text{Oe}$, used in Germany): 1°Bx means 1% (w/w) sugar, 1°Bé is equivalent to 1.8°Bx and 1°Oe is equivalent to 0.25°Bx . Typical concentration ranges of some components of grape juice and dry table wine are given in Table 3.9. Several hundreds of compounds in grapes have already been identified, and the grapes often differ by variety.

The major soluble carbohydrates in grape berries are glucose and fructose. The sucrose content is seldom above 10% of the total sugars. Most *Vitis vinifera* varieties have a glucose/fructose ratio near 1.0 at ripeness but a higher fructose content when they are over-ripe. The concentration of pectin (polymethylgalacturonic acid) increases during ripening. Pectins considerably influence wine making due to their effects on pulp consistency, pressing, juice viscosity and clarification. To prevent this, pectinolytic enzymes are widely used to break down the pectins.

Ethanol is almost absent in musts before fermentation. Methanol can derive from the hydrolysis of polymethylgalacturonic acids (pectin) and is to be found in ppm concentrations in grape juice. Trace quantities of inositol and glycerol are always present in grapes and significant amounts of glycerol may be produced during fermentation,

Table 3.7. Grape varieties for different wines

White table wines	Red and rosé table wines	Dessert wines
Chardonnay	Cabernet Sauvignon	Carignane
Sauvignon blanc	Merlot	Grenache
Semillon	Shiraz	Grillo
Riesling	Malbec	Palomino
Gewürztraminer	Pinot noir	Rubired
Muscat blanc	Ruby cabernet	Mission
Pinot grigio	Zimfandel	Inzola

Table 3.8. Composition of grapes

Compound(s)	Content [%]
Juice and pulp	0–74
Skins	5–20
Seeds	0–6
Stems and pedicels	2–8

Table 3.9. Components of grape juice and dry table wine

Constituent	Content in juice [g/l]	Content in wine [g/l]
Water	700–850	800–900
Carbohydrates	150–270	1–10
Glucose	80–140	0.5–5.0
Fructose	80–140	0.5–5.0
Pectins	0.1–1.0	Traces
Ethanol	Traces	65–120
Glycerol	Traces	3–14
Acids	3–15	5–11
Tartaric acid	2–10	1–6
Malic acid	1–8	0–8
Lactic acid	Traces	1–5
Succinic acid	Traces	0.5–1.5
Acetic acid	Traces	0.2–1.5
Inorganic salts	3–5	1.5–4.0
Nitrogen compounds	0.3–1.7	0.1–1.0
Volatile compounds	Traces	Traces

increasing the sweetness and viscosity of wine. The low pH value of grape juice (pH 2.8–4.2) is related to its two main acids: tartaric and malic acid. The concentration of both acids is reduced during winemaking, tartaric acid because of precipitation of potassium bitartrate and malic because of malo-lactic fermentation (Buschkiel et al. 1994; Formisin et al. 1997). Phenolic substances are very important for the characterisation of wine style and quality (“wine character”). The anthocyanins and coloured tannins, the astringent and bitter flavours belong to this group. Inorganic salts are in general of low importance. Nitrogenous compounds include ammonium salts, amino acids, peptides, proteins and nucleic acid derivatives. They are important as nutrients for the yeast fermentation or as factors involved in haze formation. Urea in wine originates from arginine and is, in combination with ethanol, the main source for ethyl carbamate produced during storage. Ethyl carbamate is a well known carcinogen and it could be of interest to prevent the formation of ethyl carbamate by the use of wine yeasts metabolising L-arginine without producing higher amounts of urea (Valero et al. 1999). The list of volatiles in wines is rather long and, although present only in small amounts, they are almost entirely responsible for the characteristic flavours of wines (Coombe and McCarthy 1997).

B. Wine Types

Wine types can be defined because of their alcohol or sugar content, their colour or the presence or absence of dissolved carbon dioxide gas. Concerning the alcohol content, wine types are usually divided into two major classes; these are table wines (9–14% v/v ethanol) and fortified wines (15–22% v/v ethanol). Wines with small amounts of sugar are called dry, while the sugar-rich wines are sweet. Depending on the intensity of red colour wines can be divided into red, rosé or white wines.

Wines without excess carbon dioxide, the still wines, are the opposite to sparkling wines. Besides these major compositional features, also other features have to a minor extent an influence on the wine. These are grape-related factors, such as district, composition at harvest or *Botrytis* infection, and winemaking factors, such as acid addition or reduction, level of phenolic compounds, wood maturation, types of spirit for fortification or secondary microbial processes.

C. Wine Production

Many steps in the production of red and white wine are similar, but a major difference is that, in the case of red wine production, the extraction

of red pigments and phenolic materials from the grape skins is desired to a certain extent. As a result, red wine is mostly produced by fermentations on skins (sometimes also other colour extraction techniques are used). In the case of white wine, the extraction of phenolic materials from skins is generally not desired; therefore, yeast fermentation starts after the pressing process (Fig. 3.4).

1. Grape Harvest

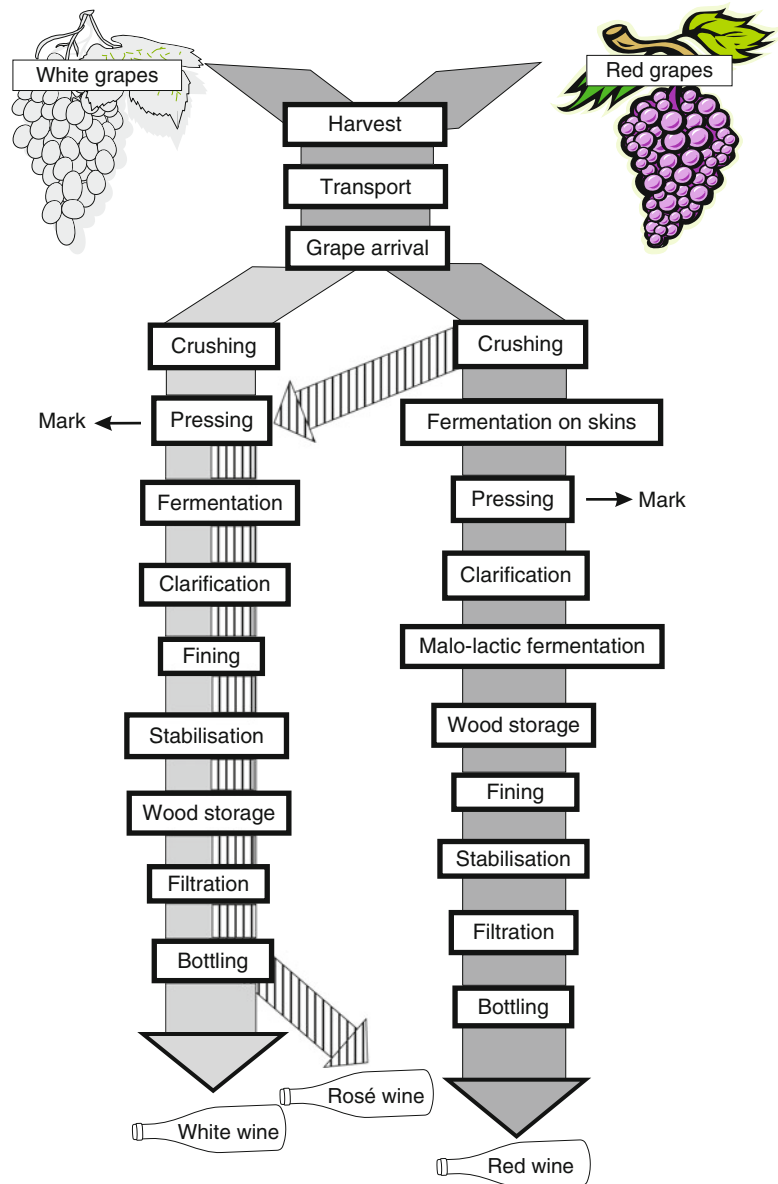
The progress of ripening can be derived from the analysis of maturity parameters of selected grapes, which should represent the whole vineyard. In this way, the optimal harvest time in the vineyard can be estimated. In many wine regions, the local authorities decide on the beginning of the grape harvest. Harvesting can be carried out by hand or by use of a harvesting machine, which naturally works faster and is more economical (Uhl 1998). Hand picking is less damaging to the grapes and the vineyard and is still in use for superior grape qualities and in vineyards inaccessible for machines. The percentage of mechanically harvested grapes has increased since improved harvesting machines became available.

Taste panels can distinguish between the wines of mechanically and the wines of manually harvested grapes, but there is no significant preference (Clary et al. 1990). The main difference in wine quality, after either manual or mechanical grape harvesting, results from the treatment and the time between harvest and wine production. It is easier to get hand-harvested grapes without damage to the winery than mechanically harvested grapes, which therefore are more sensitive to microbial attack and oxidation. The addition of sulfur dioxide in form of sodium or potassium *meta*-bisulfite can counteract these negative effects.

2. Crushing

The crushing process comprises berry breakage, juice release and its separation from stems and skins. According to the sequence of destemming and crushing, destemmer crushers and crusher destemmers are in use. In the first case, grapes are fed into the crusher hopper where they are first destemmed and then crushed into a must pump below the crusher. The stems are not in contact with the must and extraction of undesirable stemmy components is avoided. Crusher

Fig. 3.4. Basic processes for the production of white, rosé and red wine



destemmers break the berries with the stems, resulting in a possibly higher extraction of stem components and a higher risk of microbial contamination. Sometimes also the traditional crushing by treading the grapes in large stone tanks is still used, e.g. for the production of some ports.

Some defects and undesirable features of young wines can result from inappropriate treatment of the original must. Possible additions to the freshly crushed grapes are sulfur dioxide, which can be added either as solid, liquid or gas, selected yeasts with the aim to minimise the growth of wild

yeasts, pectolytic enzymes in order to facilitate the separation process and tartaric acid to increase the acidity.

3. Pressing Process

Red wine fermentations are carried out on the skins to allow sufficient extraction of colour and other compounds. This extraction is supported by an increasing alcohol content during the fermentation. The skins are removed after fermentation by pressing. In the case of white wine, a pressing

process precedes the fermentation, aiming to minimise the release of phenols from the skins and to reduce the microflora on the skins.

Presses can work batchwise or continuously; in both cases, oxygen contact to the must should be minimised (Castellari et al. 1998). Batch presses are filled, used for pressing, emptied and filled again. They can be categorised as basket presses, moving head presses, bladder presses or membrane presses. Screw presses, impulse presses and belt presses are applied for continuous pressing. The type of press used has an influence on the juice yield. Maximum yields of up to 800 l juice t⁻¹ grapes can be obtained.

4. Fermentation

Traditionally, wine is produced from the “wild” fermentation of grape juice by the yeast flora naturally occurring on the grape surface and within the winery environment. Besides *Saccharomyces cerevisiae*, there exist other micro-organisms on the grapes, e.g. other non-*Saccharomyces* yeasts with fermentative power (Lonvaud-Funel 1996). These wild yeasts include the genera *Kloeckera*, *Hanseniaspora*, *Debaryomyces*, *Hansenula*, *Pichia*, *Candida*, *Torulaspota* and *Metschnikowia* and contribute to the flavour and aroma of wines by the production of higher alcohols, esters, aldehydes and acids (Heard 1999; Urso et al. 2008).

In the 1960s, the practice of inoculation of musts with commercial yeast starter cultures was introduced with the aim to reduce the influence of the wild yeasts on the wine quality. The wine yeasts are produced under highly aerobic conditions on media with low glucose concentrations but abundant nutrients and are available in an active dried form or as liquid inoculum. *Saccharomyces* dominates the grape juice fermentations especially because of its ethanol production. *S. cerevisiae* strains differ in production of by-products, e.g. fusel alcohols, fatty acids or esters (Ooi et al. 2008). The non-*Saccharomyces* wild yeasts are, by far, less ethanol-tolerant than *Saccharomyces* (de Frenne 1998). Characteristics of importance for the selection of wine-yeast strains are the fermentation rate, sulfur dioxide tolerance, high sugar tolerance, flavour production and good wine quality. Improvement of the yeasts can be realised by selection and hybridisation. The standard level of yeast addition is 0.1–0.3 g active dry yeast l⁻¹ must. The use of selected yeasts contributes to improving the wine quality (Dorneles et al. 2005).

Wine fermentations are performed in open or closed bioreactors (fermenters). Open reactors often have wooden heading boards to keep the cap submerged whereby colour extraction is

improved and oxidation processes are avoided. Heat release during fermentation is necessary because the conversion of grape sugars to ethanol is an exothermic reaction and the temperature increase can lead to the death of added yeasts (Williams 1982). The colour extraction during fermentation is completed to about 80%, when the fermentation has progressed about half-way. Afterwards, the extraction time of colour slows down while that of undesirable tannins becomes faster. The fermentation progress is often measured by drawing samples from the fermenter once or twice a day combined with density measurement, which is favored over measurements of the refractive index.

5. Clarification

After fermentation, the wine is cloudy because of the presence of micro-organisms, cell debris or fining agents added during fermentation. The simplest but time-consuming clarification is the natural settling of suspended solids due to gravity. Traditionally, wine is clarified by repeated decantation or filtration with the help of fining agents (Reeves 1999). Cross-flow microfiltration can be used in the wine industry but is problematic due to membrane fouling (Güell 1999).

6. Stabilisation and Fining

Fining comprises the addition of adsorptive compounds to the wine with a subsequent settling or precipitation of partially soluble components from the wine, as well as hot filling, autoclaving or pasteurization of haze (Goessinger et al. 2006). The addition of casein, albumin, gelatine or polyvinyl polypyrrolidone (PVPP) is used to remove hardness, bitterness or astringency due to tannic acids or phenolics. Some white grape proteins are denatured and cause haze when the wine is exposed to higher temperatures. These proteins can be removed by fining with bentonite. At low temperatures, potassium *bi*-tartrate can crystallise, which is undesired in the customers' bottle and can be prevented by chilling the wine at or below 0°C, with subsequent filtration. By the fining of wines, the solute concentration is not reduced to zero but to a concentration which is considered acceptable.

7. Storage and Bottling

Between the fermentation process and the bottling of wine, ageing occurs in barrels, tanks or bottles and can take up to a decade or more (Gillespie 1998). The storage vessels most used are stainless steel tanks, wax-coated tanks, synthetic-lined vessels, small wood barrels and large wooden vats and casks. Stainless steel tanks and large wooden vats are used for normal storage, whereas small wooden barrels are used to impart wood flavour to the wines. During storage, air must be excluded to avoid oxidation and the wine should be regularly checked to ensure that undesirable changes do not occur.

The preparation of wines for bottling involves a final filtration with sterile-grade filter pads or membranes and a modification of the dissolved oxygen and carbon dioxide levels in the wine (Serrano et al. 1992). The filtration and all equipment must be sterilised by heat to prevent microbial contamination. After filtration, the wine is immediately filled into bottles by equipment for filling, cork-inserting, labelling and capsulating or foiling. Corks are exposed to SO_2 gas in sealed plastic bags for disinfection. However, bacteria and yeasts are still to be found within the cork lenticels and may have an influence on the aroma of the wine (Jäger et al. 1996).

D. Production of Sparkling Wine

Sparkling wine can be produced by carbonating wine under pressure prior to bottling and sealing. This simple carbonation is the cheapest way to make large quantities of sparkling wine. Another possibility is by secondary yeast fermentation with the sugared wine in a closed vessel to generate the CO_2 pressure necessary to keep it in solution. In closed steel vessels, base wine is sweetened by the addition of sugar and then yeast for the secondary fermentation is added (Fig. 3.5). After the fermentation has come to the end, the sparkling wine is clarified under pressure, sweetened to the desired degree and bottled.

Bottle fermentation is the most expensive method and sparkling wines of higher quality, e.g. champagnes, are produced by this method. A sweetened base wine is inoculated with yeast and kept in the bottle over months and even years. Clarification is carried out by riddling the bottles in an inverted position, thus depositing the yeast

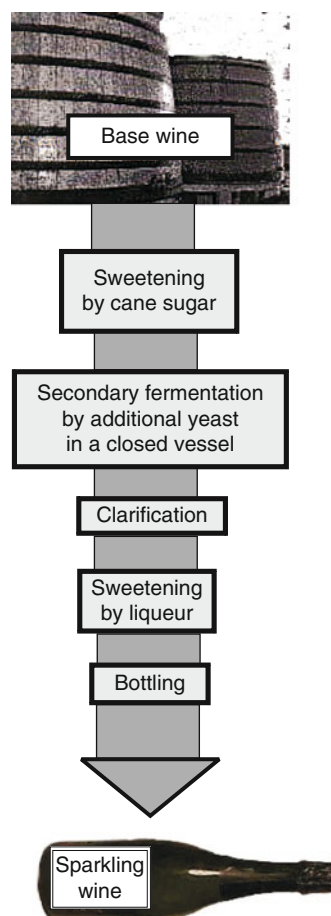


Fig. 3.5. Basic processes for the production of sparkling wine

and other turbidities more and more on the cork. Then, the necks of the bottles are frozen and, by removing the corks, the frozen yeast is removed (Champagne method). Sugar syrup or sweet must (liqueur) is added and the bottles are corked and wired. Secondary fermentation in bottles can also be followed by transferring the wine to a collecting tank under pressure, where the wine, in bulk, is clarified, filtered, sweetened and filled under aseptic conditions and under CO_2 pressure into bottles (transfer process).

E. Production of Fortified Wines

Fortified wines are produced starting from base wines having an original alcohol concentration of at least 12% v/v by fermentation. The alcohol content is then increased by the addition of high-strength distilled alcohol spirits to the range of 18–22% v/v. Many variations of processes for fortified wines have been developed in different

areas, leading to products such as sherries, muscats and ports.

Different types of sherries derive from the basic sherry process which was developed in Spain. Flor yeasts grow on the surface of wine fortified up to 15% v/v ethanol and produce acetaldehyde from ethanol which, in combination with other by-products, leads to the characteristic flavour of the sherry. **Muscats** are special fortified sweet wines where the grape variety, e.g. *Muscat Frontignan* or *Muscadelle*, has a great influence on the specific character. The aromatic fruit flavour becomes more complex by wood-ageing and blending with very old wine. **Port** wine is made from red grapes and fortification with brandy is carried out during the fermentation. Ports are relatively dry and astringent due to the combination of lower sugar concentration and increased tannin extraction from the red grape skins. Some port types age in small wooden barrels where colour and tannins precipitate and the flavour changes to a more woody and aged character.

IV. Conclusions

Brewing and wine making can be concluded as traditional processes with a lot of impact from modern developments. Although, in recent decades, there has been a considerable decline in the number of breweries and wineries world-wide, the amount of beer and wine produced in fewer and more industrial companies is still increasing and will further increase in the near future. Thus, beer and wine together will retain their prominent position among the products made by micro-organisms. In the foreseeable future of the next 10 years, no product of modern biotechnology, e.g. hormone, antibody or other pharmaceutical, will get a trade volume similar to beer and wine. Among modern developments in the field of alcoholic beverage fermentation, the following directions are to be seen as promising for further improvements:

- immobilised biocatalysts,
- genetically modified raw materials,
- genetically modified yeasts,
- modelling and control.

Immobilised biocatalysts, comprising enzymes and whole cells, have been intensively developed in the past four decades (Chibata 1978; Hartmeier 1988; Weetall and Suzuki 1975). Yeast immobilisation is known to influence the cellular metabolism, which can be positive or

negative with regard to beer production (Dragone et al. 2007; Masschelein 1994; Norton and D'Amore 1994; van Iersel 2000). For accelerated maturation both in secondary beer fermentation and in dietetic and alcohol-free beer, large-scale productions with carrier-fixed yeast cells are sporadically carried out (van Iersel et al. 1998; Virkajarvi and Linko 1999). Continuous primary beer fermentation using immobilised yeast cells is also far developed but only exceptionally practised on a large scale (Branyik et al. 2005; Pilkington et al. 1998; Verbelen et al. 2006). Alginate-entrapped yeast for the production of sparkling wines is already on the market and used by several companies (Divies 1994; Yokotsuka 1997). Immobilised *Saccharomyces cerevisiae* on carriers shows a high variety, e.g. watermelon pieces can be used for the immobilisation of yeast in order to perform repeated or continuous fermentations (Reddy et al. 2008). An increase in using immobilised enzymes and whole-cell systems in brewing and wine making can be predicted and integration of different biocatalytical properties in one integrated system will be used as well (Nedovic et al. 2005; Scott and O'Reilly 1996).

Genetic modifications by traditional mutagenising and modern recombination techniques (including genetic engineering) have successfully been carried out in many laboratories world-wide for the raw materials, e.g. hop, barley and grapes, and for the yeasts as well (Liu et al. 2008; Nedervele et al. 1997; Puig et al. 1998). The integration of desirable new properties, e.g. killing factors, flavour components or additional and increased enzyme activities, into *Saccharomyces cerevisiae* is under intensive investigation (Domingues et al. 2000; Hauf et al. 2000; Vanderhaegen 2003). Among others, brewing with genetically modified amyolytic (Zhang et al. 2008) and low-diacetyl-producing yeasts are promising (Wang et al. 2008). As a further goal, the reduction of ethanol in beer is envisaged (Nevoigt et al. 2002). However, to be performed on a large scale in the near future, existing legal restrictions will have to be redressed, not least against the background of considerable consumer fears (Hammond 1998), i.e. the question of acceptance by the consumer will have to be solved first.

Modelling and control of the complex biochemical and microbiological processes is increasingly used also in the traditional technologies of brewing and wine making (Defernez et al.

2007; Einsiedler et al. 1998; Logist et al. 2009; Trelea et al. 2004). Thus, brewing and wine making are on the way to modern bioprocessing. The trend to highly controlled processing will certainly continue; but against all these modern trends, the existence of small and highly specialised breweries and wineries making their characteristic high-quality products will never be in danger, because there will be always consumers who can and want to pay more for unique rather than bulk products.

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4 Production of Edible Mushrooms

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I. Introduction

Among all the edible commodities, mushrooms have attracted the attention of human beings, not only because of their fascinating shape, size, colours and structures, but also because of their edible nature, nutraceutical properties and their practical applications in various industrial products. Mushrooms are a source of food, nutrition and minerals, and nowadays they are also valued because of their bioactive molecules to fight against several diseases. Among the novel protein sources, mushrooms, yeasts and algal foods are frequently mentioned as alternative protein sources. Out of these, mushrooms, due to their unique flavour, palatability and direct utilization supersedes all non-conventional foods (Worgan 1968; Bano 1978). Mushrooms either belong to the Ascomycotina or the Basidiomycotina and may be hypogeous (below soil) or epigeous (above soil). They can be found growing in nature as saprophytes on any kind of organic matter or on fresh or dead wood or associated with plant roots as mycorrhizal fungi (ectomycorrhiza). They are found in all types of ecosystems in the tropical, sub-tropical and temperate regions of

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Table 4.1. World production of cultivated edible mushrooms in 1986 and 1997 (modified according to Chang 1999)

Species	1986		1997		Increase (%)
	Fresh weight ($\times 1000$ t)	Worldwide (%)	Fresh weight ($\times 1000$ t)	Worldwide (%)	
<i>Agaricus bisporus</i>	1227	56.7	1956	31.8	59.4
<i>Lentinula edodes</i>	314	14.4	1964	25.4	398.1
<i>Pleurotus</i> sp.	169	7.7	876	14.2	418.3
<i>Auricularia</i> spp.	119	5.5	485	7.9	307.6
<i>Volvariella volvacea</i>	178	8.2	181	3.0	1.7
<i>Flammulina velutipes</i>	100	4.6	285	4.6	130
<i>Tremella</i> spp.	40	1.8	130	2.1	225
<i>Hypsizygus</i> sp.	–	–	74	1.2	–
<i>Pholiota nameka</i>	25	1.1	56	0.9	124
<i>Grifola frondosa</i>	–	–	33	0.5	–
Others	10	0.5	518	8.4	5080
Total	2182	100	6158	100	182.2

the world. Many species produce a set of lignocellulolytic enzymes like peroxidases, laccases, aryl alcohol oxidase, endoglucanases, laminarinase and xylanases. These enzymes help the mycelium of white-rot and brown-rot mushrooms to recycle and degrade complex lignocellulosic materials including agricultural by-products. One has always to keep in mind that the “typical mushroom” just represents a temporarily formed sexual reproduction unit, the fruiting body, and that the actual fungus is growing, mostly invisible to us, as a hidden mycelium in the ground.

II. Present Scenario of Mushroom Production

Agaricus bisporus (white button mushroom) and *Pleurotus* spp. (oyster mushroom) are commercially cultivated world-wide due to their broad acceptability in the society, while other edible mushrooms like *Auricularia* spp. (black ear mushroom), *Volvariella* spp. (paddy straw mushroom) and *Tremella* spp. are in particular popular in China and *Grifola* and *Hypsizygus* spp. are cultured in Japan. The total world production of all mushrooms was around 6×10^6 t in 1997 and was estimated to have reached 22×10^6 t in 2009 (Chang 1999; <http://opaals.iitk.ac.in:9000/word-press/index.php/mushroom-cultivation>). Twenty years ago, *A. bisporus* was contributing 70% of the total world mushroom supply but, by the mid-1990s, this has decreased to 37%. In 2001–2002, the United States alone produced about 393000t of mushrooms. *A. bisporus* alone

contributed to 90% of total production, while *Pleurotus*, *Lentinula*, *Grifola*, *Hypsizygus*, *Flammulina* and *Hericium* were other mushrooms gaining popularity. The market share of mushroom trade in the world in 2001 was worth US \$ 40×10^9 , of which 70% was from the cultivation of edible mushrooms, while 20% was from medicinal mushrooms and 10% from wild edible mushrooms collected from nature (e.g. *Boletus* spp., *Cantharellus* spp.).

The seven most important cultivated mushrooms are: *Agaricus bisporus*, *Lentinula edodes*, *Pleurotus* spp., *Auricularia* spp., *Volvariella volvacea*, *Flammulina velutipes* and *Tremella fuciformis*. The total world production of individual species for 1986 and 1997 is given in Table 4.1 (Chang 1999). In China, the main thrust is on *Lentinula edodes* and *Pleurotus* spp., while *A. bisporus* ranks fourth among all the cultivated mushrooms. There has been diversification of cultivated edible mushroom species, which is responsible for increased world production during the 1980s and 1990s. Several new wild-type mushroom species, subspecies and strains have been collected over the past decade and are now subject to domestication.

III. Main Genera of Cultivated Mushrooms

In the literature there are about 15 000 macrofungi, and 5000 of them are considered edible while 2000 mushroom species are prime edibles, belonging to 31 genera. Researchers have experimentally grown about 100 species, 50 species are economically used and 20–30 species are

cultivated in different parts of the world. The main genera of edible basidiomycetous mushrooms are *Agaricus*, *Agrocybe*, *Albatrellus*, *Auricularia*, *Boletus*, *Cantharellus*, *Calvatia*, *Clavaria*, *Clitocybe*, *Coprinus*, *Dictyophora*, *Flammulina*, *Gloesterium*, *Hericium*, *Hydnum*, *Kuehneromyces*, *Lactarius*, *Lentinula*, *Lepista*, *Lyophyllum*, *Marasmius*, *Podaxis*, *Phellorina*, *Pleurotus*, *Pholiota*, *Polyporus*, *Ramaria*, *Rhizopogon*, *Russula*, *Scleroderma*, *Sparassis*, *Stropharia*, *Termitomyces*, *Tremella*, *Trappeinda*, *Tricholoma* and *Vovariella*. The edible/medicinal ascomycetes are species of *Cordyceps*, *Tuber*, *Morchella*, *Terfezia*, *Tirmania*, *Elaphomyces* and *Helvella*.

Most of the species of *Amanita* are poisonous but some species like *A. caesarea* and *A. hemibapha* make excellent eating. The majority of *Agaricus* species are edible but e.g. *A. xanthodermus* is poisonous. So, it is not necessarily the case that all species of a genus are edible and, vice versa, not all species of a poisonous group are poisonous. One has to be very careful while picking wild mushrooms for consumption because deaths due to mushroom poisoning are reported every year. For example, during 1996 in the Ukraine, 2860 people were poisoned due to consumption of wild mushrooms and 166 people died. Sometimes deaths are due to insect- and pest-infected mushrooms.

It is not possible to compile and discuss the cultivation methods of all edible mushroom species. However, a few of them, which are widely cultivated in Asian countries as well as a few promising species not paid much attention so far are described in detail here. This chapter does knowingly not deal with *Agaricus bisporus*, since a comprehensive literature exists about this mushroom (e.g. see Wood and Goodenough 1977; Whiteford and Thurston 2000), and likewise some other edible fungi, though commercially produced, are just touched in the margin (*Tremella ficiformis*, *Hypsizygus marmoratus*, *Pholiota nameko*, *Grifola fondosa*, etc.).

IV. Cultivation of *Calocybe indica* P. & C. (Milky Mushroom)

C. indica was first reported from India by Purkayastha and Chandra in 1974 (Fig. 4.1). This litter-decomposing agaric naturally grows on the humus-rich soil under roadside trees and agricultural fields in West Bengal, India. During rainy seasons, wild collections are gathered by the local people and sold on the Calcutta market as “Dudhi chata”, which means milky mushroom.

The natural occurrence of *C. indica* has been also reported from plains of Tamilnadu and Rajasthan in India (Doshi et al. 1989; Krishna Moorthy 1995). *C. indica* is a tropical mushroom that has not find a place in the global mushroom industry so far, but is presently commercially produced in India. It is cultivated in the tropical and subtropical parts of India during the summer months. This mushroom is particularly gaining popularity in South Indian states of Tamilnadu, Karnataka and Andhra Pradesh due to its similarity in colour and morphology to *Agaricus bisporus* and has readily been accepted by the people there as a “tropical variant” of the white button mushroom.

A. Substrate and Substrate Preparation

C. indica can be cultivated on non-composted lignocellulosic residues like *Pleurotus* spp. It can be grown on substrates containing lignin, cellulose and hemicelluloses. The substrate should be always fresh and dry; substrates exposed to rain or harvested premature (green plant material) are prone to various weed moulds, which may result in poor spawn growth and crop failure.

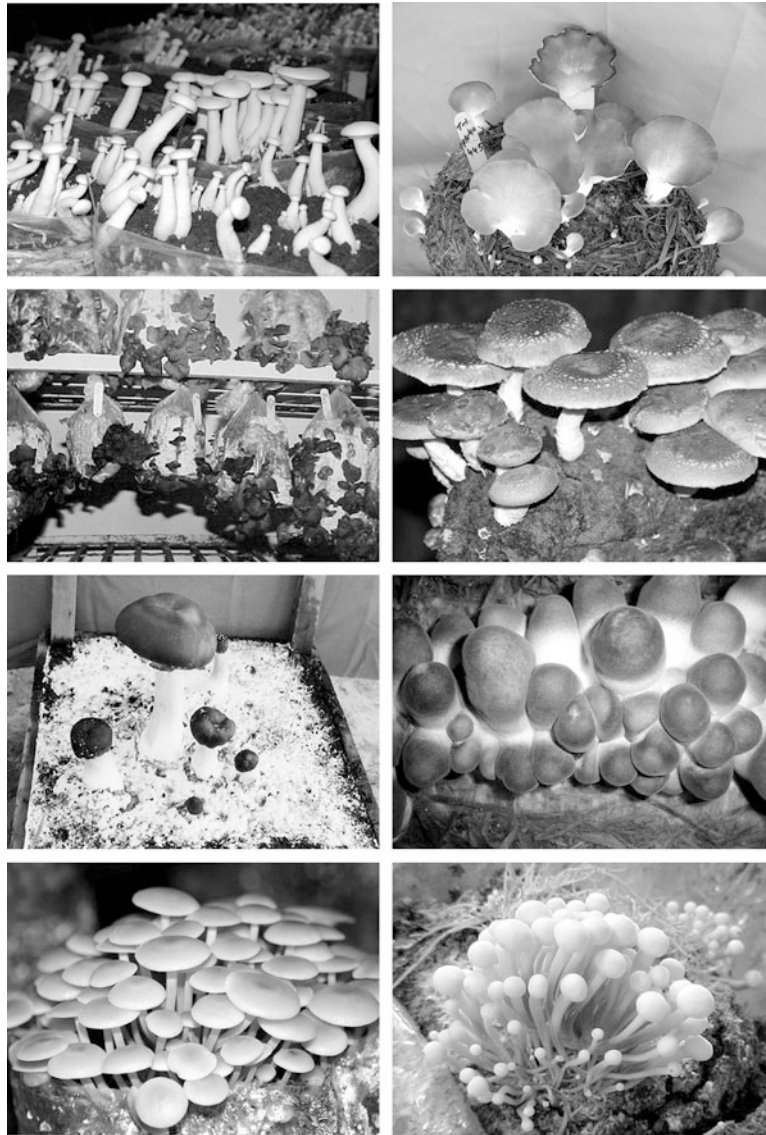
Suitable substrates for growing are straw of paddy, wheat, ragi, maize/bajra, sorghum, palm rosa grass, groundnut haulms, soybean hay, black gram hay, cotton stalks and leaves, sugarcane bagasse, cotton/jute waste, dehulled maize cobs, tea/coffee waste etc. However, cereal straw (paddy/wheat) easily available in large quantities is the most widely used substrate. Singh et al. (2009) reported higher mushroom yields from paddy straw followed by wheat straw, sugar cane bagasse and gram straw. Straw is chopped into small pieces (2–4 cm) and soaked in fresh water for 10–12 h. This period can be reduced when pasteurization is done by steam. Main purpose of soaking is to saturate substrate with sufficient moisture. It is easier to soak when the straw is filled into gunny bags and dipped into water.

The purpose of pasteurization is to kill harmful microbes. This can be achieved in three ways.

Hot Water Treatment: Water is boiled in a wide-mouth container and chopped wet straw filled into gunny bags is submerged into the hot water for 40 min at 80–90°C to achieve pasteurization. This is a very popular method particularly used by small growers.

Steam pasteurization: Wet straw is filled inside a insulated room or pasteurization tunnel either on perforated shelves or in wooden trays. Steam is released under pressure from a boiler and temperature inside chamber and substrate is raised to 60–65°C and maintained for 5–6 h. Air inside the room should be circulated to have uniform temperature in the substrate.

Fig. 4.1. Fruiting bodies of commercially used mushrooms. Left column, top down: *Calocybe indica*, *Auricularia polytricha*, *Stropharia rugoso-annulata*, *Flammulina velutipes* (wild type). Right column: *Pleurotus ostreatus*, *Lentinus edodes*, *Volvariella volvacea*, *F. velutipes* (cultivated type)



Sterilization: Substrate is filled in polypropylene bags (35×45 cm, holding 2–3 kg wet substrate) and sterilized. Once pasteurization/sterilization is over, straw is shifted to the spawning room for cooling, bag-filling and spawning.

similar to other mushrooms, however, incubation temperature for *C. indica* is higher ($30\text{--}35^\circ\text{C}$). The mycelial growth is slower than that of *Pleurotus* or *Volvariella* spp. and it takes 15–22 days for complete mycelial colonization of wheat grains.

B. Spawn Preparation

Cultures of *C. indica* are susceptible to low temperatures, and prolonged storage at low temperature ($+4^\circ\text{C}$) inhibits the culture vigour. The optimum temperature for culture storage is between 16 and 18°C . Pandey et al. (2000) found that wheat, ragi, sorghum and bajra grains can be used for spawn preparation. The spawn preparation method is

C. Spawning

Spawning methods are similar to oyster mushrooms, however, the spawn rate is higher. An amount of 5% spawn (wet weight of substrate) in cultivation bags should be used and spawn should not be older than 45 days. After autoclaving and

spawning, bags are shifted to the spawn running room and kept in the dark at 25–35 °C and relative humidity above 80%. Substrate supplemented with maize meal or wheat bran (5%) gives higher production. It takes about 20 days until the substrate is fully colonized and bags are ready for casing. Bags are shifted to special cropping rooms for casing and cropping. Yield reduction has been reported at temperatures below 25 °C during incubation, while supplementation of soybean meal (4%) at the time of spawning gives earlier and a higher number of basidiocarps (= sporophores, fruiting bodies) and yield (Singh et al. 2007).

D. Casing

As in the case of *A. bisporus*, casing is also required for fructification in *C. indica*. Casing means covering the top surface of bags with a pasteurized casing material after the mycelium has fully colonized the substrate.

The casing soil is applied after opening the bags on the top surface to a thickness of about 10–15 mm. The casing provides physical support and moisture and allows gases to escape from the substrate. The composition and quality of casing mixtures (pH, water holding capacity, C:N ratio etc.) directly affects the initiation of pinhead formation and further fruiting body development. Casing material (soil 75% and sand 25%) having a pH adjusted to 7.8–7.9 with chalk powder is pasteurized in compost tunnels at 60–70 °C for 4–6 h or chemically treated with formaldehyde (4%) for about a week in advance of casing. Formaldehyde solution should be added in amounts enough to saturate the soil. Casing soil is covered with a polythene sheet to avoid volatilization of chemicals and, at 2-day intervals, the casing soil is turned so that at the time of casing the soil is free from formalin fumes. A mixture of old spent compost, farmyard manure, sand and garden soil in a 1:1:1:1 ratio has been found to be the best material, giving the highest mushroom yields (Singh et al. 2007). The bag's top surface is made uniform by ruffling and sprayed with the fungicide carbendazim (0.1%, active against moulds) and formaldehyde solutions (0.5%). Casing material is spread in uniform layers of 1–1.5 cm thickness and the cropping room temperature is maintained at 30–35 °C and relative humidity of 80–90% after casing.

E. Cropping

It takes about 10 days for the mycelium to reach the top of the casing layer. Complete darkness during

incubation favours fruiting body formation. Light should be provided for 6–8 h. Krishnamoorthy et al. (2000) reported daily light of 1600–3200 lux for 6 h during daytime for higher mushroom production. The changes thus made in the fungus' environment result in the initiation of fruiting body formation. Pinheads start to appear within 3–5 days and usually mature in about a week. Mushroom heads (7–8 cm in diameter) are harvested by twisting, cleaned and packed in perforated polypropylene bags for marketing. Mushrooms can also be wrapped in clingfilm for longer storage.

F. Precautions during Cropping

- The substrate is the major source of weed moulds and disease-causing organisms. Hence substrates should be chopped and soaked at a distance from bag filling/spawn running and cropping areas. The worker chopping the straw should not be involved in bag filling and spawning without taking a bath and change of clothes.
- Bag filling and spawning rooms should be sprayed with formaldehyde (1%) twice a week. There should not be much air movement in the room. For large-scale production it is advisable to have air circulation through filters.
- At the time of casing, the open top surface should be sprayed with carbendazim (0.1%) plus formaldehyde (0.5%) before casing and repeated on casing soil and inside the room and again after a week; solutions should not be sprayed on the mushrooms. The insecticide malathion (0.1%) can be sprayed in the evening or next day to protect the material from flies.
- If any patch of mould (it may be green/blue/brownish) is noticed, spot treatment with formaldehyde (4%) should be performed with soaked cotton.
- During rainy seasons, controlled watering once a day may be enough; during summer months twice watering is necessary as the loss of water is higher and it becomes difficult to maintain the required humidity and moisture of the substrate. During such periods, one should spread sand on the floor and use mist sprayer and frequently check the moisture of the casing material by touching. Watering should also be done to maintain a relative humidity of 80–85% inside the cropping rooms.

G. Harvesting

The picking of fruit bodies is usually done at the stage of beginning cap opening. The first flush appears 2–3 weeks after casing and 4 weeks after spawning. Mature and fully grown mushrooms with expanded caps are picked up gently without disturbing the young pinheads. After a first harvest the trays or bags give their next flush within 7–12 days. The second flush yield is less than the first one. With a high-yield strain, 0.7–1.0 kg fresh mushrooms can be obtained from 1.0 kg dry substrate. The mature fruiting bodies contain about 15–17% protein on a dry weight basis. Chemical analysis revealed 12 amino acids and one amide. Among the detected amino acids, glycine appears to be predominant (Purkayastha and Nayak 1981). The fruit bodies of *C. indica* can be easily air/sun dried. No much work on the medicinal properties of this mushroom has been carried out. However, antibacterial properties against human pathogens, namely *Bacillus* spp., *Escherichia coli*, *Vibrio cholerae* and *Salmonella typhi*, have been reported from dried fruiting bodies. Significant levels of pyridine 3-carboxylic acid (nicotinic acid) were also observed in *C. indica* fruiting bodies (Mallavadhani et al. 2005). Selvi et al. (2007) found non-enzymatic antioxidant properties in both fresh and dried fruit bodies of the mushroom.

There are several advantages of growing *C. indica* namely:

- It is suitable for warmer climates and can be easily cultivated in tropical and subtropical areas of Asia, Africa and South America.
- The crop duration is short (4–8 weeks).
- The fresh mushrooms have a shelf life of 2–4 days and the fruiting bodies never turns brownish or black.
- No serious incidence of any mould or insect has been reported so far.
- The production costs are relatively low and therefore the fungus is suitable for “poor” farmers in developing countries.

V. Cultivation of *Pleurotus* spp. (Oyster Mushrooms)

Oyster mushrooms (Fig. 4.1) are the most suitable fungal organisms for producing protein-rich food from various agro-wastes without composting.

These mushrooms are cultivated in about 25 countries of Asia, Europe and America. It is the third most important cultivated mushroom in the world and annual world production was almost 900 000 t in 1997 (Chang 1999). China alone contributes to about 90% of the total world production. The other major producing countries are South Korea, Japan, Italy, Taiwan, Thailand and Philippines. *Pleurotus* mushrooms irrespective of the particular species are generally referred to as “oyster mushrooms”. Oyster mushrooms are lignocellulolytic fungi causing a white rot of wood and grow naturally in temperate, subtropical and tropical forests on dead wooden logs of deciduous and sometimes coniferous trees. They can also grow on decaying organic matter. Their fruiting bodies are distinctly shell-, fan- or spatula-shaped with different shades of white, cream, grey, yellow, pink or light brownish depending upon the species. However, the colour of the basidiocarps is extremely variable and influenced by temperature, light intensity and the nutrients of the substrate.

A. Advantages of Growing Oyster Mushrooms

Variety of substrates: *Pleurotus* mushrooms are white-rot fungi, degrading and growing on any kind of agricultural or forest waste material which consists of cellulose, hemicelluloses and lignin, and due to the secretion of a set of extracellular enzymes they can be used without fermentation or composting stage beforehand.

Choice of species: Among all cultivated mushroom genera, *Pleurotus* comprises the largest number of species and varieties. Most of them grow best at less than 20°C and some others prefer temperatures between 24 and 30°C. So cultivation of oyster mushrooms can be done round the year, and variation in shape, colour, texture and aroma can be achieved in dependence of the particular species/variant.

Simple cultivation technologies: *Pleurotus* mycelium can also grow on fresh straw and it does not require a specific substrate for growth. Substrate preparation for oyster mushrooms is quite simple and cultivation does not require controlled environmental conditions as in case of *A. bisporus*, because most *Pleurotus* species have a wide temperature, relative humidity and CO₂ tolerance.

Storage and shelf life: Unlike the white button mushroom, oyster mushrooms' fruit bodies can be easily dried and stored. Dried oyster mushrooms can be instantly used after soaking in hot water for 5–10 min or used in powdered form for several preparations. Fresh mushrooms have a shelf life of 24–48 h at room temperature.

Productivity: *Pleurotus* productivity is high as compared to all other cultivated mushrooms. One can harvest a minimum of about 500–700 kg of fresh oyster mushroom from one ton of dry wheat or paddy straw within 45–60 days (note: the same quantity of straw gives only 400–500 kg of the white button mushrooms within 100–120 days). Fruiting body yield can further be increased by supplementing the substrate with a suitable nitrogen source such as soybean and/or cotton seed meal or by using highly productive strains.

B. History of Oyster Mushroom Cultivation

The history of oyster mushroom cultivation is of recent origin in comparison to *Auricularia* spp. (600 A.D.), *Leptinula edodes* (1100 A.D.) and *Agaricus bisporus* (1650). The present day cultivation technology is a result of various successive steps evolved throughout the world during the twentieth century. A simple form of growing *Pleurotus* spp. was adopted by lumberman in Europe in the nineteenth century. They used to collect wooden logs and stumps showing fructification in nature and kept them in cool, moist places. It allowed them to harvest periodically oyster mushrooms from these logs under convenient conditions. The first successful modern cultivation of *Pleurotus ostreatus* was achieved in Germany by Falck in 1917. He inoculated tree stumps and wooden logs with mycelium of *P. ostreatus* (at that time *Agaricus ostreatus*) and could harvest fresh fruiting bodies, and later Etter (1929) produced fruiting bodies on different wood materials. On sawdust medium, formation of sexual spores of *P. corticatus* Fr. were reported by Kaufert (1935); and Block et al. (1958) cultivated *P. ostreatus* for the first time under laboratory conditions on saw dust. They used a mixture of oatmeal and saw dust for cultivation and found best results on *Eucalyptus* wood followed by pine saw dust. They observed some growth abnormalities in fruit bodies due to insufficient light conditions and no mushroom formation when the temperature was <10°C or >32°C. In India, cultivation of *P. flabellatus* on paddy straw was first reported by Bano and Srivastava in 1962. Corn cobs were used under sterile conditions for growing *P. ostreatus* (Toth 1970). This method was modified by Gyurko (1969) for non-sterile conditions. A Hungarian method for growing oyster mushrooms based on sterile production was patented in 1969 (HTTV patent). Stanek

and Rysava (1971) developed a method of application of thermophilic microorganisms in the fermentation of substrates for the subsequent cultivation of *P. ostreatus*. Zadrazil (1974) developed a method for continuous preparation of substrate and industrial production of *Pleurotus* mushrooms. Jandaik and Kapoor (1976) grew *P. sajor-caju* on various substrates including wheat and banana pseudo stems. Finally, Leong (1982) successfully developed a method for cultivation of *P. sajor-caju* using cotton waste from the textile industry.

C. Biology of the Oyster Mushroom

Visually the basidiocarps (fruiting bodies) of an oyster mushroom has three distinct parts – a fleshy shell or spatula shaped cap (pileus), a short or long lateral or central stalk called stipe and long ridges and furrows underneath the pileus called gills or lamellae. The gills stretch from the edge of the cap down to the stalk and bear the spores. If a fruiting body is kept on a paper directly (gills facing the paper), a dirty deposition of powdery spores will be seen. This spore print colour may be whitish, pinkish, lilac or grey. The spores are smooth, cylindrical or allantoid and germinate easily on any kind of mycological medium, and within 48–96 h, whitish thread-like colonies develop (primary mycelium). Fusion between two compatible primary mycelia (homothallic) develops into a secondary mycelium (heterothallic = dikaryotic), which has clamp connections and is again fertile; the primary mycelium is clampless and non-fertile. The mycelium of *Pleurotus* is pure white in colour except those of *P. cystidiosus*, *P. smithii* and *P. columbinus*, which form coremia-like stalked structures (asexual spores). *P. tuber-regium* forms a tuber-like structure in the substratum, which is also edible and has positive medicinal properties.

D. Varieties of Oyster Mushrooms

All the varieties and species of oyster mushrooms are edible except *P. olearius* and *P. nidiformis*, which were reported to be poisonous. There are 38 species of the genus recorded throughout the world (Singer 1986). In recent years, 25 species have been commercially cultivated in different parts of the world, among which the most important are as follows: *P. ostreatus* (Fig. 4.1), *P. flabellatus*, *P. florida*, *P. sajor-caju*, *P. sapidus*, *P. cystidiosus*, *P. eryngii*, *P. fossulatus*, *P. opuntiae*, *P. cornucopiae*, *P. yuccae*, *P. platypus*, *P. djamore*, *P. tuber-regium*, *P. australis*, *P. purpureo-olivaceus*, *P. populinus*, *P. levis*, *P. columbinus* and *P. membra-naceus*.

E. Cultivation

The procedure for oyster mushroom cultivation can be divided into the following four steps:

1. Preparation or procurement of spawn
2. Substrate preparation
3. Spawning of substrate
4. Crop management.

1. Preparation or Procurement of Spawn

The spawn preparation technique for oyster mushrooms is similar to that of the white button mushroom (*A. bisporus*). Pure cultures of *Pleurotus* spp. are transferred to sterilized wheat grains, which are then incubated for 10–15 days. It has been reported that jowar and bajra grains are superior over wheat grains. The mycelium of oyster mushroom grows fast on wheat grains and 25- to 30-day-old spawn already starts forming fruit bodies. Sometimes the mushroom farmers use active mycelium already growing on the substrate for spawning fresh oyster mushroom bags. This method can be used only on a small scale.

2. Substrate Preparation and Nutrition Quality

A large number of agricultural, forest and agro-industrial by-products are useful for growing oyster mushrooms. These by-products or wastes are rich in cellulose, lignin and hemicelluloses. However, yield of oyster mushrooms largely depends on the nutrition and nature of the substrate. The substrate should be fresh, dry, free from mould infestation and properly stored. Substrates exposed to rain and harvested immature with green chlorophyll patches inhibit the growth of *Pleurotus* mycelium. Oyster mushrooms can utilize a number of agrowastes including straw of wheat, paddy and ragi, stalks and leaves of maize, jowar, bajra and cotton, sugarcane bagasse, jute and cotton waste, dehulled corn cobs, pea nut shells, dried grasses, sunflower stalks, used tea leaf waste, discarded waste paper and synthetic compost of button mushrooms. It can also be cultivated using industrial wastes like paper mill sludge, coffee by-products, tobacco waste, apple pomace and dried leaves of deciduous trees. The cellulose and lignin contents are important components influencing the yield of *Pleurotus* fruiting

bodies; cellulose-rich substrates like cotton wastes are to prefer.

Methods of substrate preparation: The mycelium of *Pleurotus* is saprophytic in nature and it does not require specific substrates for its growth. Thus mycelial growth can take place on simply water-treated straw but there are always cellulolytic moulds present in straw, which may affect *Pleurotus* growth. These competitor moulds sometimes restrict the growth of *Pleurotus* mycelium due to secretion of toxic metabolites. There are various methods to get rid of undesirable microorganisms in the straw and to favour the growth of *Pleurotus* mycelium. The most popular methods of substrate preparation are as follows.

Steam pasteurization: Pre-wetted straw is packed in wooden trays or boxes and then kept in a pasteurization room at 60–65 °C for 4 h. Temperature of the pasteurization room is regulated with the help of steam through a boiler. The substrate, after cooling at room temperature, is seeded with spawn. The entire process takes 3–5 days. There are various minor variations of this methods adopted in Europe.

Hot water treatment: The substrate, after chopping (5–10 cm), is soaked in hot water (65–70 °C) for 1 h (Bano *et al.* 1987), or 60–120 min at 80 °C, or in the case of paddy straw at 85 °C for 30–45 min. After draining excess water, the spawn is added. The leached water contains a lot of soluble sugars and phenolic compounds. Hot water treatment makes compact substrates like maize cobs or stems softer so that mycelial growth can proceed more easily. However, this method is not suitable for large-scale cultivations.

Sterile technique: The chopped substrate, after soaking in cold water, is put in heat-resistant polypropylene bags and sterilized in an autoclave for 1–2 h, followed by spawning under aseptic conditions. This method is rather suitable for research labs than for large-scale production due to energy costs.

Fermentation or composting: This method is a modification of composting techniques used for the white button mushroom. It is most suitable for substrates like cotton stalks, maize stalks and leguminous stubbles. Both aerobic and anaerobic fermentation of the substrate is suitable for *Pleurotus* cultivation. Composting should be done on a covered area or shed. The chopped substrate (5–6 cm) is supplemented with ammonium sulfate or urea (0.5–1.0%) and lime (1%) on a dry weight basis of the ingredients. Horse or chicken manure (10% on a dry weight basis) can also be used instead of nitrogenous fertilizers. The addition of lime improves the physical structure of the compost that is sprinkled with water and put into triangular heaps (75–90 cm in height). After 2 days of incubation, the pile is turned over, and 1% superphosphate and 0.5% lime is added. The compost is ready after 4 days and can be spawned as such or used after pasteurization.

Chemical sterilization techniques: It has been observed that mould infestation due to various species of *Trichoderma*, *Gliocladium*, *Penicillium*, *Sclerotium*, *Aspergillus* and *Stysanus* is a common problem during oyster mushroom cultivation. Sometimes these moulds prevent

the growth of mushroom mycelium resulting in complete crop failure. When wheat straw is treated by steeping in a chemical solution of carbendazim 50% (37.5 ppm) and formaldehyde (500 ppm) for a period of 16–18 h, most of the competitor moulds are either killed or their growth is suppressed for 25–40 days. The technique was standardized in India by Vijay and Sohi in 1987. The authors of this chapter also obtained good results with lower concentrations of carbendazim.

Substrate supplementation: The nitrogen contents in most lignocellulosic substrates range between 0.5 and 0.8% and hence the addition of organic nitrogen to straw helps getting higher yields. Some of the common supplements (3–10%) are wheat bran, rice bran, cotton seed-meal, soybean cake, groundnut cake and ammonium nitrate. Supplements are thoroughly mixed with straw during spawning. It should be taken into account that supplements increase the substrate temperature, which can be risky during the summer season.

3. Spawning of Substrates

Freshly prepared, 20- to 30-day-old grain spawn is best for spawning. Old spawn (3–6 months) stored at room temperature (20–30 °C) forms very thick mats due to mycelium aggregation and, sometimes, young pinheads and fruit bodies start developing in the spawn bottle itself. The spawning should be done in a pre-fumigated room (48 h with 2% formaldehyde). The spawn should be mixed at 2–3% to the wet substrate, i.e. 300 g spawn is sufficient for 10–12 kg substrate. Spawn can be mixed thoroughly or in layers. Spawed substrates are filled into polyethylene bags (60 × 45 cm) of 125–150 gauge thicknesses. Ten to 15 small holes (0.5–1.0 cm in diameter) are made on all sides, especially two to four holes in the bottom to leach out excess water. Perforated bags give a higher and earlier crop (4–6 days) than non-perforated bags because of accumulation of CO₂, which inhibits fruiting. The bags can also be tightly pressed and tied with a nylon rope. Such blocks are incubated intact and, after mycelial growth, the polythene sheet is removed.

4. Crop Management and Incubation

The spawed bags or blocks are kept in incubation rooms for mycelial growth. They can be kept on a raised platform or shelf or hanged in cropping rooms for mycelial colonization. Although mycelium can grow at 10–30 °C, the optimum temperature lies between 22 and 26 °C. Higher temperatures (>30 °C) in the cropping room

inhibit growth and kill the mycelium. The daily maximum and minimum temperature of cropping rooms and beds should be recorded. The bed temperature is generally 2–4 °C higher than the room temperature. During mycelial growth, the bags are not to be opened and no ventilation is needed. Moreover, there is no need for high humidity or water spraying.

Fruiting body induction: Once the mycelium has fully colonized the substrate and formed a thick mycelial mat, it is ready for fruiting. Contaminated bags with moulds may be discarded while bags with patchy mycelial growth may be left for a few more days to complete mycelial growth. Bags should not be opened before days 16–18, except in the case of *P. membranaceus* and *P. djamor* var. *roseus* which form fruiting bodies within 10 days, even in closed bags with small holes. There is no need for casing the substrate. All the bundles, cubes or blocks are arranged on wooden platforms or shelves with a minimum distance of 15–20 cm between each bag. Some cultural conditions required for fruiting are as follows.

Temperature: Mycelial growth of all *Pleurotus* spp. can take place between 20 and 30 °C. However, for fruiting, different species have different temperature requirements. Depending upon the temperature requirement of a species, they can be categorized into two groups: winter or low-temperature species (10–20 °C) and summer or moderate-temperature species (16–30 °C). Summer varieties can fructify at low temperatures as well, but the winter varieties cannot do so at higher temperature; they need a low temperature shock to induce fructification. Commercial varieties which can be cultivated during summer are *P. flabellatus*, *P. sapidus*, *P. citrinopileatus* and *P. sajor-caju*. Low-temperature species are *P. ostreatus*, *P. florida*, *P. eryngii*, *P. fossulatus* and *P. cornucopiae*. The growing temperature affects not only the yield but also the quality of the product. The pileus (cap/cup) colour of *P. florida* is light brown when cultivated at low temperature (10–15 °C) but changes to white pale and yellowish at 20–25 °C. Similarly, the fruit body colour of *P. sajor-caju* when cultivated at 10–19 °C is white to dull white with a high dry matter content, while at 25–30 °C it is brownish to dark brown with a low dry matter content. The temperature requirements of different *Pleurotus* spp. are given in Table 4.2.

Relative humidity: All *Pleurotus* species require high relative humidity (70–80%) during fruiting. To maintain relative humidity, water spraying is to be done in the cropping rooms by hand or using humidifiers.

Oxygen and carbon dioxide requirements: The oyster mushroom can tolerate high carbon dioxide concentrations during a spawn run (up to 20 000 ppm, or 20–22%) while it should be less than 600 ppm or 0.6% during cropping. Therefore, sufficient ventilation should be provided during fructification. If the CO₂ concentration is high, the mushrooms will have long stipes and small convoluted pilei (like a trumpet).

Light: Light is required to initiate fruiting body formation. For primordia formation, the light requirement is 200 lux intensity for 8–12 h. Inadequate light conditions can be judged by long stalks, small caps and poor yield.

Table 4.2 Temperature requirement of different *Pleurotus* spp. and their yield performance (Upadhyay, unpublished data)

Species	Optimum temperature of mycelial growth on substrate (°C)	Optimum temperature for fruiting (°C)	Temperature range for growth	Yield performance (% B.E.)
<i>P. flabellatus</i>	25–28	22 ± 2	16–28	60–90
<i>P. sajor-caju</i>	25–28	24 ± 2	17–30	50–70
<i>P. sapidus</i>	25–28	24 ± 2	17–30	40–70
<i>P. membranaceus</i>	30	27 ± 2	20–30	60–80
<i>P. citrinopileatus</i>	30	26 ± 2	20–30	50–80
<i>P. djamor</i>	30	27 ± 2	16–32	70–90
Winter-cultivated species				
<i>P. ostreatus</i>	25–28	20 ± 2	18–22	30–50
<i>P. florida</i>	25–28	20 ± 2	12–22	60–90
<i>P. eryngii</i>	18–25	20 ± 2	12–22	50–70
<i>P. cornucopiae</i>	18–25	20 ± 2	12–25	40–70

The colour of the pileus is also influenced by light intensity and duration of exposure. Fruit bodies raised in bright light are dark brown, grey or blackish. If the light intensity is less than 100 lux, the mushrooms will be pale yellowish.

Hydrogen ion concentration (pH): The optimum pH during mycelial colonization is between 6 and 7, and the water for spraying should not contain too much salt. Rusted iron drums used for substrate treatment or storing water for spraying delay fructification due to the presence of excess iron in the water.

F. Harvesting and Post-Harvest Practice

Oyster mushrooms are harvested before spraying water. The right stage for picking can be judged by the shape and size of fruiting bodies. In young mushrooms, the edge of the cap is thick and the cap margin is enrolled, while the cap of mature mushrooms becomes flat and inward curling starts. It is advisable to harvest all the mushrooms at one time from a bag so that the next crop of mushrooms starts early. After harvesting, lower parts of the stalks/stipes with adhering debris should be cut using a knife. Stipes should be kept as short as possible because they are tough and not liked by the customers. Fresh mushrooms are packed in perforated polythene bags for marketing. They can also be dried in sunlight or drying rooms. Dried products with 2–4% moisture can be stored for several months.

G. Medicinal and Nutritional Value of Oyster Mushrooms

Oyster mushrooms are 100% vegetarian and their nutritive value is as good as that of other

edible fungi like the white button mushroom (*A. bisporus*), shiitake (*Lentinula edodes*) or the paddy straw mushroom (*Volvariella* spp.). They are rich in vitamin C and B complex. Protein content varies between 1.6 and 2.5% on a fresh weight basis (15–18% in dried mushrooms). It has most of the minerals required by the human body, such as potassium, sodium, phosphorus, iron and calcium. The niacin content is about ten times higher than that of common vegetables. A polycyclic aromatic compound with antibiotic properties, pleurotin, has been isolated from *P. griseus*.

H. Precautions While Growing Oyster Mushrooms

Oyster mushrooms produce millions of spores, which can be easily seen as spore clouds in the cropping rooms in early morning. Several growers working in cropping rooms have complained of headache, high fever, joint pains, nausea and coughing due to *Pleurotus* spores. Mushroom pickers are therefore advised to open the doors and ventilators or switch on exhaust fans 2–3 h before entering the cropping rooms. Furthermore, they should use respiratory masks in growing rooms and change clothes after coming out from growing rooms.

VI. Cultivation of *Auricularia* spp. (Black Ear Mushroom)

The genus *Auricularia* belongs to the “jelly fungi” and they are commonly known as wood ear or

black ear mushrooms (Fig. 4.1). The fungi cause a moderate white rot of different kinds of wood. The basidiocarps are gelatinous, mostly growing on logs, branches and twigs of deciduous or less frequently coniferous trees. The black ear mushroom is one of the oldest cultivated fungi and was already cultured 600 A.D. in China. It is widely distributed in tropical, sub-tropical and temperate forests where it grows both on fresh and dead wood. It is a traditional food in China and one of the main constituents of Chinese dishes. It is consumed fresh as well as after drying. *Auricularia* spp. are cultivated mainly in China, Taiwan, Thailand, Indonesia and Japan. Its annual world production is around 500 000 t/year and constitutes about 8% of the world-wide mushroom production (Chang 1999). During the monsoon season people in the North-eastern states of India collect wild *Auricularia* spp. and sell them on local markets.

A. Biology of Black Ear Mushrooms

The basidiocarps (fruiting bodies) of *Auricularia* spp. are 2–12 cm (in diameter), 1–3 mm thick and they are attached laterally to the substrate wood without a stipe. They are gelatinous, slimy, small cup-like and creamish brown to purple or reddish-brown in colour. The colour becomes darker after drying. There are 15 *Auricularia* spp. world-wide (Kirk et al. 2001), which differ in the shape, size and colour of the cup, length and thickness of basidiocarp hairs and its internal anatomy. The outer basidiocarp surface is mostly hairy while the inner part is bright and shining bearing the basidia and basidiospores. The basidiospores are hyaline, oblong, curved, cylindrical, smooth, heterothallic and readily germinate within 2–3 days. The basidiocarps rapidly appear during rainy season growing singly or in large bunch-like clusters on deciduous or coniferous wooden logs, branches and twigs or on the outer bark of living trees. Quimio (1984) observed a considerable decrease in viability of older spores from spore prints compared with fresh spores. Spores germinate best at 28–30°C, while at 10°C and 40°C, spore germination is totally inhibited.

Cultures of *Auricularia* spp. can be obtained by transferring basidiocarp pieces or basidiospores on natural, synthetic or semi-synthetic media. Best mycelial growth is obtained on glucose yeast extract, malt extract or yeast/potato/dextrose media. The colonies on agar plates are dirty white and flattened, changing to brownish during ageing. Glucose, fructose and galactose at 1% are well assimilable carbon sources and calcium nitrate, urea, asparagine or alanine are the best nitrogen sources. The spawn of *Auricularia* can be prepared on wheat grains or saw dust.

B. Cultivation Techniques

Several *Auricularia* species have been reported as cultivars: *A. auricula-judae*, *A. polytricha*, *A. delicata*, *A. mesentrica*, *A. cornea*, *A. rosea*, *A. peltata* and *A. fuscusuccinea*. Sohi and Upadhyay (1990) identified several light *Auricularia* spp. from the North-western Himalayas (India) suitable for commercial cultivation. There are mainly two cultivation methods: (1) conventional wood log cultivation and (2) compost bag or composted sawdust cultivation.

1. Wood Log Cultivation

Wood of most broad leaf trees can be used for black ear cultivation but *Quercus* spp. (particularly *Q. variabilis*, *Q. acutissima*) are preferred trees in Asia. Generally, the tree should be 6–10 years old and 10–15 cm in diameter and are cut during autumn into logs 1–2 m in length. The cut surface is smeared with Bordeaux mixture (copper sulfate, hydrated lime) to prevent attack by other wood-decay fungi. Then holes are made in the logs in two or three rows at a distance of 10–12 cm (holes are 1.0–1.6 cm in diameter, 1.0–1.5 cm in depth). The holes are inoculated with sawdust spawn and sealed with wax. The inoculated logs are arranged in shades and covered with grass to maintain humidity (35–40%), and during dry periods, watering once per week is necessary. The temperature required for fruiting is 15–27°C. The logs produce primordia after getting sufficient water by rain or through spraying water. After the first flush of fruiting bodies, the logs must be rested for a short time to get the next flush. The logs produce fruiting bodies again in the second year, and mushroom production just slowly reduces in the third and fourth year.

2. Composted Sawdust Method (“Synthetic Log” Cultivation)

Wood log cultivation is no longer feasible at larger scale due to non-availability of suitable logs and inconsistent yields. Moreover, it takes longer time to get fructification on logs so that this method has been replaced by the composted sawdust method, also named “synthetic log” cultivation. There are various formulations using treated sawdust, cereal straw or other lignocelluloses as basic material for synthetic log preparation, as well as sugars, salts and minerals as supplements.

3. Composting of Saw Dust, Spawning and Culture Conditions

Saw dust has to be composted prior to use. All ingredients are thoroughly mixed with sawdust and water is added to

a final moisture content of 65–70%. The wet material is piled in pyramidal shape and allowed to ferment for 6–10 days, with turning on alternate days. Composted sawdust is filled in polypropylene bags and sterilized for 2 h and, after cooling, is inoculated with the spawn. Cereal straw and other ingredients do not require composting but it may help by softening the straw for easy penetration of the mycelium.

Spawn of *Auricularia* spp. can be prepared on sawdust or cereal grains (e.g. wheat). Saw dust spawn is more suitable for wood logs and grain spawn for composted saw dust. The spawn should not be older than 30 days. Spawn bottles are thoroughly shaken so that the grains become loose. For 2 kg of composted saw dust or straw, about 25–30 g of spawn will be sufficient. The bags after spawning are plugged again. Spawned bags are incubated in dark growing rooms where the temperature is 20–25°C. The mycelial growth is inhibited and stopped if the temperature falls below 12°C or rises above 35°C. During the incubation stage, no fresh air is required and mycelial growth is better at higher CO₂ concentration. The spawn bags are full of whitish mycelium within 20–25 days and are then ready for fruiting. The bags are given four or five vertical slits with a knife and are hung in the growing room. Light is required for initiation of fruiting, and daily 2–4 h diffuse light is sufficient. Relative humidity at 75–80% in the cropping room is provided by the spraying of water. Fresh air circulation is maintained by a air-handling unit or simply by opening the windows. The pin heads develop scantily and they do not expand fully under deficient light conditions. It has been observed that, if there is excess carbon dioxide in the cropping room, the basidiocarps do not expand but form long and thin coral-like structures.

4. Harvesting

The mushrooms usually appear 15 days after slitting the bags. It takes 3–7 days from a pinhead to the mature cup suitable for harvesting. Mushrooms are harvested manually and the adhering straw or saw dust is removed from the base. Biological efficiencies of 137% and 174% have been reported to be reached within 4 and 8 weeks, respectively (Upadhyay 1999a, b). Interestingly, freshly collected wild-type strains gave higher yields than strains obtained from GenBank (Quimio and Guzman 1984). The yield of *A. auricula-judae* is generally somewhat lower than that of *A. polytricha*; and in the Philippines *A. fuscusuccinea* is the preferred and most productive species.

C. Nutritional Value of *Auricularia* spp

Black ear mushrooms are equally nutritional as other mushrooms despite their cartilage consistency. 100 g of dried *Auricularia* contains 14% protein, 1.4% fat, 72% carbohydrates, 4% fibres, and 5.4% ash; furthermore 0.2 mg thiamine, 0.6 mg riboflavin, 4.7 mg niacin, 240 mg

calcium, 256 mg phosphorus, 65 mg sodium and 72 mg iron and 984 mg potassium. There are some advantages of growing *Auricularia* spp. Harvesting can be prolonged up to 4 days, because these fruiting bodies do not rapidly lose quality as in the case of *Pleurotus*, *Volvariella*, and *Agaricus* spp. Bhandal and Mehta (1989) reported that *Auricularia* fruiting bodies remained in good condition even 1 week after harvest. The dried fruit bodies can be easily re-hydrated, which decreases transport cost and eliminate spoilage problems. *Auricularia* spp., besides being edible, have interesting medicinal effects. In traditional Chinese medicine, the black ear mushroom is considered mild and sweet in nature and is thought to activate blood and stop pain. It is often used to treat haemorrhoids, to stimulate bowel movement and to act as an anticoagulant (Ying et al. 1987). Polysaccharides of *A. auricula* have been claimed to have various positive effects on human health, e.g. they act against ulcer, diabetes and tumours, they are immunomodulatory and antibiotic, and they lower cholesterol and triglycerides (Hobbs, 1995).

VII. Cultivation of *Lentinula edodes* (Shiitake)

The wood mushroom *Lentinula edodes* (Fig. 4.1), commonly called shiitake, is the most popular mushroom in Japan and other East Asian countries. It is the second most important mushroom cultivated in the world after *A. bisporus*. In 1997, its annual production was about 1.6×10^6 t, of which almost 90% were produced in China (Chang 1999). The shiitake mushroom is liked by the consumers because of its unique taste and flavour and the presence of metabolites, which are thought to reduce the plasma cholesterol level.

A. Cultivation Technique on Wood Logs

Lentinula edodes is a white-rot fungus that grows in nature on dead logs of a number of hardwood trees, mainly *Quercus* spp. (Oak), *Castenopsis* spp. (*C. chinensis*, *C. tissa*, *C. fordil*, *C. lamontii* etc.), *Elaeocarpus* spp. (*E. chinenses*, *E. japonicus*, *E. lancaefolius*), *Lithocarpus* spp. (*L. calophylla*, *L. glaber*, *L. spicatus*), *Betula* spp. and *Carpinus* spp. It is commercially grown on oak wood logs or “synthetic logs” (see also *Auricularia*).

1. Log Preparation

The *Lentinula edodes* mycelium is saprophytic and cause a strong white rot. It mainly grows on dried wooden logs

absorbing nutrients from the cambium. The outer bark layer protects the growing mycelium from the mould competitors. Although it grows on any size and age of timber, logs 9–18 cm in diameter and 15–20 years in age are most suitable. The time of cutting the trees is also important. The most suitable period in East Asia is from autumn to early spring when the logs contain a maximum amount of carbohydrates and other organic substrates. The logs should have a moisture content of 44–55% at the time of felling. If the moisture content of the logs is <20%, there is no growth, likewise at moisture contents >60% (impending mould infections). The felled logs are left as such for 25–45 days, which results in the lowering of the moisture content to 40–45%.

2. Spawn Preparation and Spawning the Logs

There are two types of spawn: saw dust and wood plug spawn. The former is prepared by inoculating mixtures of water, saw dust, cereal bran, sugar and minerals with pure cultures of the fungus. The material is filled into either empty spawn bottles or in polypropylene bags and incubated for 30 days at $24 \pm 2^\circ\text{C}$. Wood plug spawn is prepared by inoculating mycelium on small wedge shaped or either small cylindrical wood pieces. When the fungal mycelium impregnates the wood pieces, they are ready for inoculation. The shiitake mycelium grows between 5 and 30°C with optimum temperature of $20\text{--}26^\circ\text{C}$. Low temperatures ($14\text{--}20^\circ\text{C}$) are favourable during spawning the logs. For spawn inoculation, small holes of 1×1 cm and 1.5–2.0 cm in depth are made on the logs with the help of a drilling machine. The holes are made at a distance of 20–30 cm (long axis) and 6 cm between each row. The holes between two rows alternate in their position. Saw dust spawn is filled into the holes or wood plug spawn. The holes are sealed with paraffin wax.

3. Crop Management

Inoculated logs are kept outdoors in flat piles at a place where the conditions are most favourable for mycelial growth. The pile is covered with either straw, or gunny bags to prevent excessive water loss of the logs. The vegetative growth in the logs will be completed within 8–12 months depending on the fungal strain and the type of wood. For fruiting body induction, temperature shock, high humidity and/or light exposure are required. The logs are either sprayed with cold water or immersed in a tank of cold water (1–3 days at $10\text{--}18^\circ\text{C}$). The cropping area is kept moist to maintain a high relative humidity (80–90%). Optimal temperature for fruiting is between 15 and 20°C at a humidity around 80–90%. Mushrooms can be harvested several times from one log; after a rest for 30–40 days, the logs are again watered to get a second flush. This can be repeated up to four times per year and logs may produce crops up to six years.

B. “Synthetic Log” Cultivation

This method is practiced in East Asia, Thailand, Sri Lanka, New Zealand, the United States and Europe, and it is becoming more and more important for commercial shiitake production. Cultivation is carried out in plastic bags or substrate blocks, also called “synthetic logs”.

1. Substrate Preparation

Commercial cultivation is mostly carried out on saw dust of oak (*Quercus* sp.), maple (*Acer* sp.), birch (*Betula* sp.) or other hardwood trees. Various formulations have been recommended for growing shiitake. These complex substrate mixtures consist of saw dust, cereal bran, grains, sugars, minerals (sulfates) and sometimes urea in different concentrations. The most suitable formulation can be selected after conducting productivity tests. The material is filled in plastic bags (1.5–4.0 kg) immediately after mixing and wetting the substrate. The bags are first loosely filled and later by putting some pressure giving them a cylindrical shape. Some growers make holes (15 mm in diameter, 20 mm in depth) before, others after heat treatment. The holes are covered with adhesive medical tape. The time between substrate preparation and sterilization should not exceed 6 h to avoid unwanted fermentation. Heat treatment can be carried out in an autoclave at 121°C for 1 h or on a brick- and cement-lined tower at $90\text{--}95^\circ\text{C}$ for 5–7 h.

2. Spawning and Spawn Run

If no holes had been made before sterilization, the bags are cleaned with 70% ethanol and forceps are used to make small holes. The amount of saw dust spawn per inoculation hole is about 1 cm^3 , i.e. a typical spawn bottle (750 g) can inoculate 25–30 bags. Grain spawn is introduced at concentrations of 2–5%. Spawn run may take 18–100 days. During this period, the bags are incubated in a 4 h/20 h light/dark cycles at $23\text{--}25^\circ\text{C}$. There are several stages of fruiting body formation:

1. **Mycelium formation.** A thick mycelial sheet/mat develops on the surface of the substrate. This usually occurs 2–4 weeks after inoculation.
2. **Formation of mycelial bumps.** Bumps are clumps of mycelium, commonly formed on the surface of mycelial mats. These bumps can turn into primordia at a later stage but most of them abort. Bump formation is promoted by fluctuation of temperature and high concentrations of CO_2 .

3. **Pigmentation.** The colour of the “synthetic logs” turns brownish 5–6 weeks after inoculation. At this stage, aeration should be provided to promote fruiting.
4. **Coat Hardening.** In the last stage, the outer part of the substrate including the mycelial mats becomes gradually hard whereas the inner part of the “synthetic log” stays soft and moist (80%); browning continues and encompasses the whole bag. At this stage, the plastic sheets are removed and fruiting body formation starts.

3. Fruiting and Harvesting

Factors affecting the induction of fruiting are: temperature fluctuation, humidity, CO₂ concentration, light and physical shocks. These parameters may vary in each stage (1–4) and from strain to strain. Some general data are summarized in Table 4.3. The “synthetic logs” do not require watering during incubation and fruiting. Humidity should be kept low (60–70%) to prevent microbial contamination when the protecting plastic sheets are removed. Deformed fruiting bodies obtained in the first flush are a sign of too high a CO₂ concentration during incubation and/or too short a spawn run. For harvesting, the stalks are held tightly and the whole mushroom is broken from the substrate. Generally, the mushrooms should be harvested at an early stage (young fruiting bodies with closed caps). Good yields range between 15% and 30% of the wet weight of the substrate.

4. Special Features of the Plastic Bag (“Synthetic Log”) Method

- The materials used to prepare synthetic logs are mainly saw dust and other agricultural wastes and by-products such as bagasse, sugarbeet

residues, cotton-seed hulls, peanut hulls and corn cobs.

- This method shortens the production period and gives high yields. Using natural logs, the time from spawning till first harvest is about 8–12 months and harvesting will be completed within 3–4 years. About 100 kg of natural logs can produce about 10–15 kg of fresh shiitake mushrooms. Using synthetic logs, mushrooms can already be harvested 80 days after spawning. Harvesting is possible over a period of 8 months and biological efficiencies of 80–145% can be reached within this period.
- Bag cultivation is easy to manage and does not require special logistics as in case of natural log cultivation.
- Negative effect: the quality of mushrooms produced on synthetic logs is poorer than those obtained from natural logs (in particular concerning the size and performance of fruiting bodies).

VIII. Cultivation of *Stropharia rugoso-annulata* (Wine-Cap *Stropharia*)

Stropharia rugoso-annulata (Farlow apud Murrill) was first described in the United States in 1922, and in the 1930s it was collected in Germany, Czechoslovakia and Japan (Szudyga, 1978). From the eco-physiological point of view, this agaric fungus has an intermediate position between the white-rot (i.e. wood-decaying) and litter-decomposing basidiomycetes; thus it grows well on bark and straw-like materials but not on logs (Steffen et al. 2000). Cultivation of *S. rugoso-annulata* was first attempted in East Germany (Puschal, 1969), then in Poland, Czechoslovakia and Hungary, and it has been cultured in India since the 1989 (Upadhyay and Sohi 1989). The mushroom is particularly popular among hobby farmers.

The basidiocarp of *Stropharia rugoso-annulata* is relatively large (up to 30 cm), agaricoid, stipitate and annulate (Fig. 4.1). The pileus (cap) is fleshy, 4–19 cm in diameter, differently coloured (withish, yellow, brown to wine-red), initially conic, then applanate or with slight depression. The stipe lies central, is cylindrical, 10–25 cm long and 1.0–2.5 cm in diameter, white to creamish in colour with a slightly swollen base. The form of basidiospores is broadly ovoid to sub-ellipsoid; they are smooth with a broad germ pore and violaceous to dark purple. Spore prints are purplish black.

Table 4.3. A schedule of various cultural requirements of *Lentinus edodes* for different stages of fruiting body formation (according to Upadhyay, unpublished data)

Stage/ activity	Days	Temperature (°C)	Light intensity (Lux)	Humidity (%)
Incubation	30–120	20–30	None	65–70
Induction	2–4	10–20	500–1000	85–95
Fruiting	7–14	12–18	500–1000	60–80
Rest	7–21	20–30	None	65–70
Induction	2–4	10–20	500–1000	85–95

Clamp connections are always present in the heterothallic mycelium. Spores easily germinate at 30–35°C within 2–4 days. *S. rugoso-annulata* has a bifactorial heterothallic mating system (Upadhyay, unpublished data). The mycelium can be grown on malt extract or potato dextrose agar and forms whitish cottony colonies. Maximum growth occurs between 20 and 30°C, with some variation between particular strains.

Spawn can be prepared on any kind of cereal grains, e.g. wheat, rye, sorghum or bajra, as described above for *C. indica*; sterilized corn cobs, wheat straw and pine bark are also suitable for spawn preparation (see also Chapter 22 of this book). Various agriculture residues, namely wheat straw, paddy straw, sugarcane bagasse or dehulled maize cobs, are suitable for fruiting body production.

The lignocellulosic materials are either sterilized (autoclaved) or composted prior to use and the fungus grows without supplements of organic nitrogen (Upadhyay and Sohi 1989). However, synthetic compost as used for *A. bisporus* does not support mycelial growth of *S. rugoso-annulata*. Composted wheat and paddy straw was found to give a biological efficiency of almost 130% BE (Upadhyay and Sohi 1989). There are reports recommending to cover the top surface of the straw substrates with soil to promote fructification; however, efficient fruiting body formation occurs also without casing (Upadhyay and Sohi 1989). However, casing may help to induce synchronized fruiting and the development of uniform mushrooms required for market. Mushrooms should be harvested in the button stage (i.e. before the caps starts to open), because mature (“over-ripe”) basidiocarps have a strong taste and may develop a pungent stench. Only a few pests and deformations have been recorded for *Stropharia rugoso-annulata*. Incidence with the fungal parasite *Mycogone rosea* causes damage to the primordia, gills and/or stipe (Sohi and Upadhyay 1989).

The wine-cap *Stropharia* has a promising potential as market mushroom, since it is easy to cultivate on cheap straw-like materials without nitrogen supplementation. To this end, it will be necessary to establish a collection of fast-growing wild-type strains isolated from suitable natural habitats in order to select new production strains. Furthermore strain selection/improvement should be done towards a more pleasant aroma.

IX. Cultivation of *Volvariella* spp. (Paddy Straw Mushrooms)

Paddy straw mushrooms (Fig. 4.1) are suitable market fungi for tropical and subtropical regions (e.g. south India, south-east Asia) and about

180000t were produced in 1997, mostly in China (Chang 1999). In India, *Volvariella* spp. were first cultivated by Thomas et al. in 1943 near Chennai. As *S. rugoso-annulata*, it is an intermediate species between white-rotters and litter-decomposers. The fungus requires higher temperatures than most other mushrooms (30–35°C), both for growth and fructification. It is a fast-growing mushroom, and it takes only 10 days to complete the cycle from fungal inoculum via mycelial growth on/in a substrate to full fruiting body formation. Although *V. volvaceae* is called the paddy straw mushroom, it was shown to grow also on other straw-like materials such as sorghum and wheat straw, maize debris, sugarcane bagasse as well as leaves of banana and water hyacinth (Chang and Mok 1971). The mycelium of *V. volvaceae* does seemingly not produce ligninolytic enzymes (neither lignin nor manganese peroxidases; see also Chapter 15 of this book) and growth is therefore inhibited by lignin-related phenolic compounds (Cae et al. 1993). For this reason, the fungus prefers lignocelluloses with low lignin and high cellulose content (e.g. straw, cotton wastes) and can also be grown on waste paper. Spawn is prepared analogously to *S. rugoso-annulata* or *C. indica*.

There are two methods for commercial growing of *Volvariella*: (1) out-door and (2) in-door cultivation. Out-door cultivation has several disadvantages like insect and pest damages, unstable yields and non-regulated environmental conditions. Nevertheless, this method is widely used in subtropical and tropical regions of Asia. The in-door method produces mushrooms in straw-beds in growing rooms. For one bed, 22 bundles of paddy straw are soaked overnight in water. These bundles are arranged on a raised platform in four layers of five bundles at a right angle, with two bundles on the top. On each layer, gram dal powder (1.5% of dry weight of straw) along with the spawn is added. The inoculated straw-bed is covered with a polyethylene sheet. Already, 10 days after spawning, fruiting bodies start to appear from all sides of the bed. Usually between 500 and 1000 g mushrooms can be harvested at one time from a bed. A new technique using cotton wastes was developed in the 1980s (Chang 1982). There are various substrate formulations combining cotton wastes as basic material with rice bran and limestone. The substrate is soaked in water and then stacked to form piles 1–2 m in height. After 2–4 days of composting, the material is transferred to a “mushroom house” made of plastic sheets and filled to a 10–20 cm layer. Then live steam is introduced to raise the temperature in the tent to 60–62°C and to sterilize the surface of the substrate. After cooling down to 35°C, the substrate is inoculated with fresh spawn. The biological efficiency of this method is 40% (Chang 1982).

X. Cultivation of *Flammulina velutipes* (Enokitake)

Flammulina velutipes (Curt. ex Fr.) Singer is also known as winter mushroom or golden mushroom and as enokitake in Japan (Fig. 4.1). It grows all over the world as a saprophyte (white-rot fungus) on trunks and stumps of deciduous trees, preferably on poplar trees (*Populus* spp.), elms (*Ulmus* spp.), willows (*Salix* spp.), plum trees (*Prunus* spp.), maple (*Acer* spp.) and birch (*Betula* spp.). Fruiting bodies typically develop from the end of autumn till early spring when the temperature ranges between -2°C and $+14^{\circ}\text{C}$ (Zadrazil 1999). *F. velutipes* was already cultivated as early as 800 AD in China and the mushroom has spread all over the world over the past 30 years. It is well suitable for growing in temperate regions as well as in hilly and mountain areas, where the temperatures are most of the time between $6-14^{\circ}\text{C}$. *F. velutipes* is known for its pleasant taste and aroma as well as its medicinal properties. The total world production of *F. velutipes* has increased from 143 000 t in 1990 to 285 000 t in 1997 and Japan is the biggest producer of this mushroom.

F. velutipes belongs to the Tricholomataceae of the order Agaricales. The fruit bodies are caespitose, arising in a large group. The cap or pileus is 1–3 cm in diameter and convex to hemispheric in the initial stages which later on flattens. The pileus is viscid with yellowish brown to dark brown in the centre, while the margin is lighter. The stipe is very long (4–10 cm), thin (3–7 mm) and velvety; its colour is light brownish at the top and dark brown at the base. Lamellae are adnexed, initially white to light cream and later light brown. The spores are smooth, hyaline, thin-walled and ellipsoid ($5-7 \times 3-4 \mu\text{m}$). The spore print is white and the spores germinate easily on common mycological media. The fungus has a bifactorial heterothallic mating system and clamp connections are always present in the dikaryotic hyphae.

The vegetative mycelium of *F. velutipes* is white and strandy. The optimum temperature for growth is around 25°C (though the fungus tolerates even temperatures below zero!). Spawn can be prepared on saw dust or cereal grains. The spawn can be prepared within 12–15 days, depending upon the vigour of the strains used. In earlier times, the cultivation of *F. velutipes* was carried out using wooden logs, but the production was just moderate and the quality of mushrooms not assured. Therefore, artificially composted substrates based on saw dust and rice bran were

developed in the 1920s (Morimoto 1928). Interestingly the saw dust of coniferous trees, *Chamaecyparis obtuse* (hinoki cypress) and *Pinus* spp. (pine trees), gave best yields. However, saw dust from other trees can be also used; cotton-seed hulls are nowadays the preferred substrate in China.

Synthetic substrate consisting of sawdust and rice bran (4:1 w/w) is mechanically mixed and, after soaking with water to a moisture content of 57–62%, it is filled into polypropylene bottles with a capacity of 800 to 1200 ml. The substrate can be also filled into polypropylene bags, vinyl bags, jars or filter bags. The sterilized containers are spawned with a mechanical mixers via a central hole made at the time of filling. The spawn rate should be 2–3% of dry substrate. The spawned bags are incubated in dark rooms with a temperature range of $20-25^{\circ}\text{C}$. The mycelium can grow at up to 34°C ; however, a low temperature during incubation helps to overcome the incidence of contaminants. The initiation of pinheads takes place in the dark; however, light is required for maturation of pinheads and pileus expansion. It takes 25–30 days for mycelium to spread on the substrate. Then the temperature of incubation rooms is lowered to $10-14^{\circ}\text{C}$, which is the ideal temperature for fructification. The stipe starts elongation, so a plastic collar is placed around the neck of bottle or bags to hold the mushrooms in place and straight. Aeration is required during cultivation because, when the CO_2 concentration is more than 5%, pileus expansion occurs. For better-quality mushrooms, the temperature is reduced to $3-8^{\circ}\text{C}$ until harvest. The relative humidity in the cropping room is maintained at 80–85% by spraying water. When the stipe length is 13–14 cm, the collar or rolled paper around neck is removed and fruiting bodies are harvested. Three or four flushes of mushrooms are obtained and the yield is about 80% in relation to the dry substrate. The yield of mushrooms in the first flush is 100–140 g from 250 g of dry substrate, while the second flush still gives 60–80 g, depending upon the strain quality. The entire crop cycle is around 90–100 days. The mushrooms have a long shelf life of 8–10 days and they are usually vacuum-packed for sale.

Gramss (1981) successfully used mixtures of beech saw dust, wheat straw, flour and bran as well as pea haulm to cultivate *F. velutipes*. Green mould caused by *Trichoderma harzianum* and related species is the most important pest associated with *F. velutipes*, causing poor mycelial growth. The addition of wood vinegar (a by-product from charcoal production using *Quercus acutissima*) to the saw dust helps to control the incidence of *Trichoderma* spp. and at the same time stimulates mycelial growth of *F. velutipes* (Chang et al. 1995). *F. velutipes* is nutritionally rich and contains over 30% protein, 6% fat and 3% fibres. Fresh fruiting bodies of *F. velutipes* are also rich in enzymes (peroxidase, superoxidase

dismutase) and bioactive compounds and have been claimed to prevent cancer and coronary heart diseases.

A. Future Prospects

- Fruiting bodies of *F. velutipes* are long-stiped with small pilei and very light; strains forming heavier mushrooms with larger pilei and smaller stipes would be helpful to mushroom growers.
- The fructification temperature is 12–16°C, which is a limiting factor for its cultivation in sub-tropical regions. Therefore new strains of *F. velutipes* (and closely related species) will have to be isolated aiming at fungi with fructification temperatures of 20–25°C.
- The available strains grow well on saw dust but their growth is limited regarding agricultural waste materials (Gramss 1981); improved/new strains colonizing different kinds of straw would be useful for *F. velutipes* production.

XI. Conclusions

Mushroom cultivation is one of the most suitable and safest commodities suitable for tropical, sub-tropical and temperate climatic conditions. It helps in recycling agricultural wastes and their conversion into protein-rich food. Mushroom farming is a labour-intensive activity, so it can help in employment generation, in particular in developing countries. The production of mushrooms during the past three decades has achieved a phenomenal growth not only regarding production as such but also in productivity. Thus, not only the total production but also the number of commercially cultivated fungal varieties have increased and several new edible species are under the process of “domestication” and commercialization throughout the world.

Against the background of a raised interest of consumers in fungal products, mushroom production will further increase in the course of this decade. Maybe molecular tools will help in the future to improve fruiting body formation (though the genetics of basidiomycetes is more complicated than that of bacteria, yeasts and ascomycetous moulds; Kothe 2001). It is equally important, however, to further study and protect fungal

biodiversity. This should ideally be done in world-wide programmes, because the mushroom flora is rapidly depleting everywhere due to global climate change, deforestation, soil erosion, forest fires, grazing by domestic animals and human interference. Still, it is possible to find new species and strains of edible mushrooms. Some of these fungi (and already available mushrooms as well) will be exploited, beyond fruiting body production, in the recycling of agricultural wastes, in bioremediation or for the development of new enzymes and bioactive molecules. Not least, cultures of edible mushrooms should be freely exchanged for their beneficial utilization in all countries.

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Metabolites and Enzymes

5 The β -Lactam Antibiotics: Current Situation and Future Prospects in Manufacture and Therapy

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I. Introduction

During the past ten years, the landscape of the β -lactam scenery has changed profoundly. Most of the marketed penicillins and cephalosporins are meanwhile generic and only very few novel structures like the very recently launched ceftobiprole (Zevtera), have reached the market. Most of the big pharmaceutical companies in the western hemisphere have abandoned or are divesting their β -lactam production and commodity businesses, and the gravity centres for research and production are moving eastwards. Accordingly, substantial parts of the respective patents and publications are written in Russian, Persian and particularly Chinese.

From all the formerly important β -lactam manufacturers in the western hemisphere, like GlaxoSmithKline, Merck, Eli-Lilly, Bristol-Myers-Squibb or Hoechst, only Sandoz, the generic brand of Novartis, to a lesser extent Antibioticos SpA and the Dutch DSM, formerly Gist-Brocades, are left as suppliers of appreciable quantities of β -lactam bulkware and intermediates, whilst increasing amounts are meanwhile provided by eastern companies.

Note that even the Japanese Fujisawa, which became a global β -lactam player through its acquisition of Asahi Pharmaceuticals, has meanwhile divested its β -lactam production and that also DSM is now trying to find an investor for its β -lactam production facilities.

Despite this development, β -lactams could however increase the share of the world market for systemic antibiotics from 50% in 1998 to now 65%, while their total sales volume increased from US \$11 to US \$18 billion. Sales leader are the

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cephalosporins which garner US \$10 billion, representing 30% of the total antibiotic market. The penicillins sales volume thus ranges at about US \$8 billion.

Irrespective of their age, β -lactams are still a substantial and valuable component in antibiotics and one of the top therapeutic categories in terms of pharmaceutical sales.

Accordingly, there are continued efforts to economize the production technologies and to keep their therapeutic position. Regarding these efforts, current states and future trends of manufacturing technologies and the prospects of β -lactams in antibiotics are reviewed and discussed in this chapter.

II. Manufacturing Aspects

In the past, the major cost reduction and leaps in productivity were achieved on the fermentation level and through improvements of the producer strains.

A. Strain Improvement

The productivity of the two fungal species mainly employed in industrial β -lactam production, the penicillin-producing *Penicillium chrysogenum* and the cephalosporin C producer *Acremonium chrysogenum* could have been augmented impressively during the past decades. Most of the progress was reached at first through conventional mutation and selection programs but since these developments came to a halt in the middle of the 1980s, the tools of genetic engineering got more and more included into the improvement programs and required a detailed characterization of the biosynthetic pathways.

To gain a profound basis for directed genomic alterations, studies were conducted at various corporations and universities to elucidate the mechanisms and genes involved in β -lactam biosynthesis to characterize the respective biosynthetic pathways. The results and achievements of these studies have already been presented and extensively discussed elsewhere (Brakhage and Caruso 2004; Brakhage et al. 2004; Schmitt et al. 2004; van den Berg et al. 2008; Teijeira et al. 2009), therefore current knowledge is just briefly summarized here.

1. Characterization of Biosynthetic Pathways

a) Cephalosporin C Synthesis in *Acremonium chrysogenum*

Biosynthesis (Fig. 5.1) starts with the polymerization of L- α -amino adipic acid, L-cysteine and L-valine to the linear tripeptide L- α -amino adipyl-L-cysteinyl-D-valine (ACV-peptide). This reaction is catalysed by ACV-synthase (MW about 420 kDa) through the following steps: (1) the ATP-dependent activation of these amino acids to bind them as thioesters, (2) the epimerization of L-valine and finally (3) condensation by a thio-template mechanism.

Cyclization of the ACV-peptide to the bicyclic isopenicillin N (IPN) occurs under oxygen-, Fe^{2+} -, ascorbate- and α -ketoglutarate-dependent action of the IPN-synthase (IPNS), which has a MW of about 38 kDa. Inhibitory to IPNS activity are cobalt ions and glutathione.

Further pathway reactions are:

1. IPN-epimerization to penicillin N (isopenicillinyl N-CoA synthase, isopenicillinyl N-CoA epimerase).
2. Penicillin N conversion to deacetoxycephalosporin C (DAOC) by expansion of the five-membered thiazolidine ring to a 6-C dihydrothiazine ring (DAOC-expandase).
3. The formation of deacetylcephalosporin C (DAC) by dehydroxylation and oxidation of the methylgroup in C3-position (DAC-hydroxylase).
4. As the final step, the acylation of DAC to cephalosporin C (DAC acetyltransferase).

DAOC-expandase and DAC-hydroxylase activities in *A. chrysogenum* are exerted by the same enzyme (MW 41 kDa), which like the IPNS belongs to the group of α -ketoglutarate-dependent dioxygenases.

The corresponding genes are organized in two separate clusters.

The first two enzymes, ACV- and IPN-synthase are encoded by the *pcbAB* and the *pcbC* gene, respectively. Both genes are linked to each other on chromosome VII by a 1.2-kb intergenic region carrying the putative promoter sequences from which they are divergently transcribed. The *pcbC*-promotor appears to be about five times stronger than that of the *pcbAB* gene. The enzymes performing the IPN epimerization,

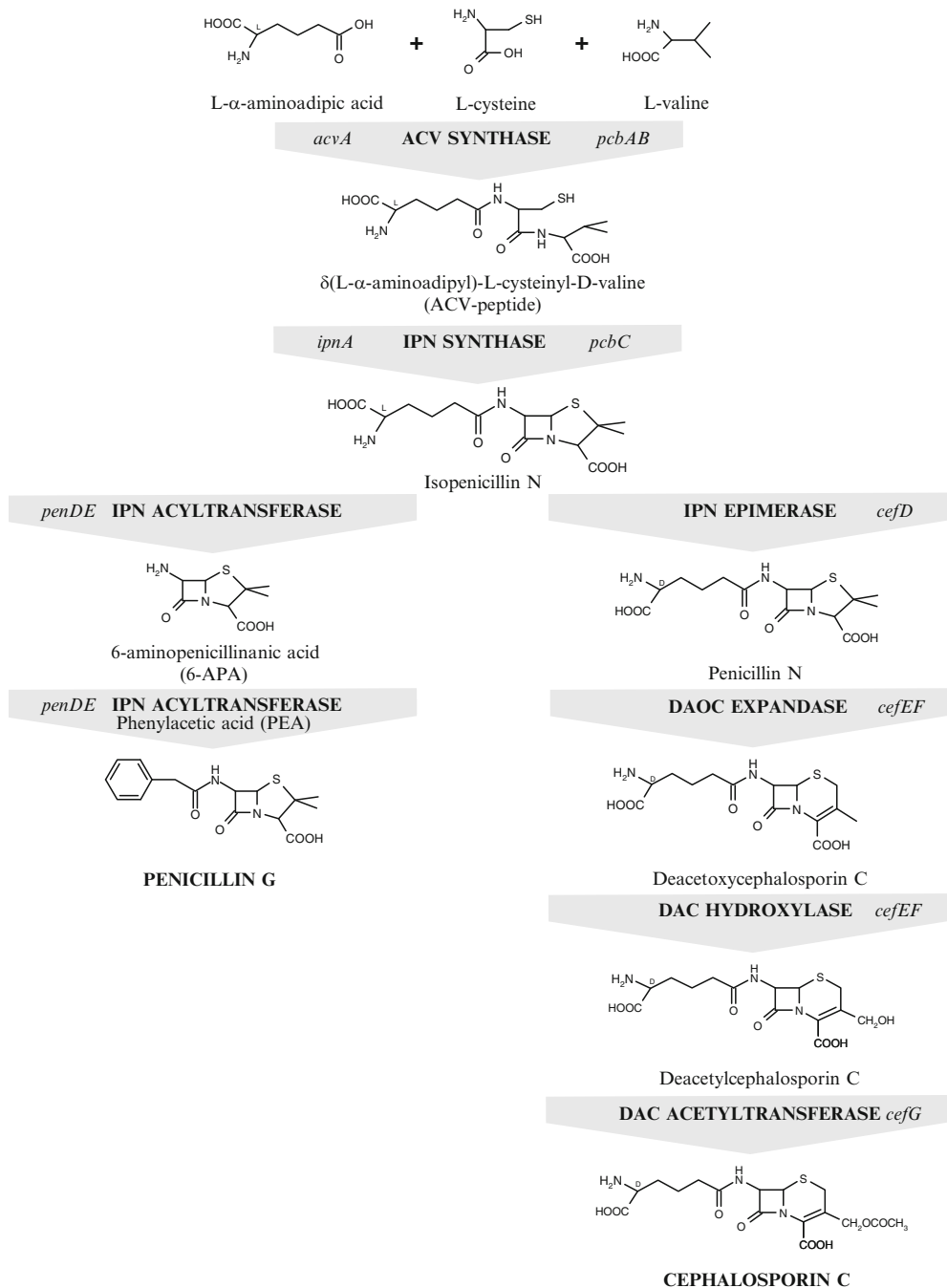


Fig. 5.1. Penicillin and cephalosporin biosynthetic pathways in *Penicillium chrysogenum* and *Acremonium chrysogenum*. Involved enzymes and genes have underlying grey arrows. The first reactions are common to both path-

ways. Cleavage of isopenicillin N to 6-APA and the subsequent acylation to penicillin G in *P. chrysogenum* and the expandase/hydroxylase activity in *A. chrysogenum* are exerted by bifunctional enzymes. For details see text

isopenicillinyl N-CoA synthase and isopenicillinyl N-CoA epimerase, are encoded by the *cefD1* and *cefD2* genes belonging to the same cluster.

Downstream of the *cefD1* gene is an open reading frame termed *cefM*, which encodes for a protein that is assumed to be involved in a possible translocation of penicillin N from microbodies, on which the epimerization takes

place, to the cytoplasm for further conversion to cephalosporin C (Teijeira et al. 2009).

The *cefEF* gene and the *cefG* gene encoding for the bifunctional expandase/hydroxylase and the DAC-acetyltransferase, respectively, are located adjacent to each other on chromosome I. Again the genes are separated by an intergenic region of 938 bp which is supposed to harbour the promoters from which they are transcribed in opposite directions.

The expression of cephalosporin biosynthetic genes is under control of a gene homologous to the gene *velvet* in *Aspergillus*, in which it exerts developmental functions (Dreyer et al. 2009). In contrast to the cephalosporin genes, the penicillin biosynthesis genes in *P. chrysogenum* are clustered in one single region.

b) Penicillin Formation by *Penicillium chrysogenum*

The first reactions of the penicillin biosynthetic pathway are identical to the ones in *A. chrysogenum* (Fig. 5.1). IPN however is not epimerized to penicillin N; instead it is converted to 6-aminopenicillanic acid (6-APA) by removal of the L- α -amino adipic acid side chain, which is substituted by a hydrophobic acyl group. Both steps are catalysed by the same enzyme, the acyl coenzyme A:IPN acyltransferase (IAT).

The enzymatic activity of IAT is believed to be the result of the processing of a 40 kDa monomeric precursor into a dimeric form consisting of two subunits with MWs of 11 and 29 kDa. Due to the broad substrate specificity of IAT, various penicillin derivatives are naturally synthesized by attachment of different acyl-CoA derivatives to the 6-APA core. For industrial purposes, synthesis is usually directed to the less hydrophilic penicillin V or penicillin G to facilitate subsequent extraction by organic solvents. This is achieved by addition of phenoxyacetic acid or phenylacetic acid as precursors to the culture broth.

The corresponding genes are: (i) the IAT encoding *penDE*-gene (also named *aata* in *Penicillium*) and *pcbAB* (also named *acvA*) and *pcbC* (*ipnA*) encoding the ACV-synthase and IPN-synthase, respectively, all located on chromosome I.

The enzymes involved in penicillin biosynthesis are distributed at different sites of the cell: ACV-activity was found to be bound to vacuole membranes, IPN-synthase occurs dissolved in the cytoplasm and IAT-activity is microbody associated.

2. Genetic Engineering

a) *Acremonium chrysogenum*

Early work at Eli-Lilly and Bristol-Myers-Squibb was destined for the amplification of the *cefEF* gene which was considered to be a bottleneck in the biosynthetic pathway in view of the accumulating side products penicillin N and DAOC. This approach resulted in partially significant increases of productivity in low- and medium-titre strains: improvements of the final yields by 50% have been reported (e.g. Skatrud 1992; Skatrud et al. 1997).

With progressing characterization of the regulatory mechanisms governing the biosynthetic pathway and of the methodologies in genetic engineering, it is to assume – though not adequately documented in the literature – that activities of all these genes have been increased over the last years, e.g. by amplification, enhancement of promoter strengths or altered regulation, and that engineered strains are meanwhile an established component in industrial cephalosporin production processes.

The created strains can be regarded as truly self-cloned, as the genes to be amplified have been isolated from production strains and the bacterial marker genes of the employed plasmids were also replaced by benomyl resistance conferring β -tubulin genes of *A. chrysogenum* prior to their interpropagation in *E. coli* and reinsertion into the fungal protoplasts of the very same *Acremonium* production strains. Thus, their treatment and fermentation should by definition not require any of the costly safety measures that result from the laws regulating the use of gene-recombinant organisms.

From the concentration of biosynthetic intermediates like penicillin N, DAOC (normally up to 5%) or DAC (normally up to 20%) in the culture broth of production strains which were not engineered, it may be deduced, that the removal of flux-limiting biosynthetic blockades leads to an increase of cephalosporin productivity at expense of these intermediates and now ranges between 30 g/l and 40 g/l (further aspects of metabolic engineering are discussed in Section D: Options for future production processes).

b) *Penicillium chrysogenum*

To get hints for more rational strain improvement approaches: (1) highly mutated production strains were genetically and physiologically compared with their less productive ancestors, (2) concentrations of pathway intermediates were determined

to identify potential pathway bottlenecks and (3) regulatory mechanisms were investigated.

In the course of these studies, high-performance strains of *P. chrysogenum* turned out to possess amplified copies of single genes like the *pcbC* gene or even copies of the whole cluster as well as increased steady-state transcript levels of pathway genes. In some strains, the amplifications were shown to be organized in tandem repeats, which presumably were generated by a hot spot TTTACA hexanucleotide (Fierro et al. 1995). Comparison of promoter strengths of these genes from high and low productive strains did not reveal any differences. This indicates the involvement of additional unknown *trans*-acting factors, as the amounts of increased mRNA did not correlate with the degree of gene amplification. Also a high specific activity of IPN synthase was reported in a more evolved *Penicillium* strain which was independent from transcript amounts and probably due to the higher enzyme stability. It was further found that high production strains contain enhanced microbody volume fractions (van den Berg et al. 2008).

Initial genetic engineering approaches at the former Gist-Brocades (now DSM) and at Panlabs were destined for pathway de-bottlenecking by the amplification of selected genes. Meanwhile, the complete genome and transcriptome of *Penicillium* have been sequenced and characterized (van den Berg et al. 2008) and regulator genes like *velvet* were investigated, which – as in *Acremonium chrysogenum* or *Aspergilli* – are involved in the regulation of key steps in secondary metabolism and therewith penicillin production. Chinese groups cloned the *Vitreoscilla* haemoglobin-like (VHb) gene leading to an improved ability of oxygen utilization (Xu et al. 2006) and an increased biomass and penicillin production (Li et al. 2006). The whole biosynthetic gene cluster has been cloned in high-titre strains leading to increases of productivity of more than 160% (Campos et al. 2008). Thus, it is to assume that engineered strains have become an established component in industrial penicillin production processes now exceeding titres of 50 g/l.

Despite these achievements, many physiological processes involved in the biosynthesis of β -lactams and intermediates remain to be elucidated at the molecular level and bear potential for further strain improvement.

3. Approaches and Goals for Further Strain Improvement

To fully realize the potential for further enhancement of productivity, current and future studies

will focus on: (1) the exploration of regulatory mechanisms and circuits on the transcriptional, translational and also on the posttranslational level, (2) the determination of the specific intermediate turnover rates resulting from specific enzyme activities, enzyme titres and enzyme stabilities, (3) the detailed investigation of linked and preceding pathways and (4) the investigation of intermediate transport and product secretion mechanisms (see also Evers et al. 2004).

From the experimental viewpoint, metabolic engineering faces mainly the following difficulties:

1. As both the direction and site of integration of the imported DNA cannot be controlled sufficiently and transformation efficiency is still quite low, the probability of finding new, higher-producing mutants, even in large-scale screenings, will be quite low.
2. The genetic instability and drift rises with the degree of genomic alterations, particularly during long-term vegetative propagation with numerous generation cycles in industrial large-scale fermentations.
3. As the complex interdependence with other metabolic areas is not yet fully understood, the removal of an obvious β -lactam pathway bottleneck, unknown reaction steps of the linked and preceding pathways may become flux limiting.

To investigate and overcome such flux limitations, RNA interference (RNAi) will play an important role in the future. RNAi offers approaches for the specific shut down of any gene of interest (further details and literature, see Schmidt 2005a) and may supply an explanation for observations made in the course of strain improvement programs, according to which antibiotic productivity can decrease drastically upon amplification of genes involved in antibiotic biosynthesis.

Strains emerging from the development programs are also selected for further beneficial alterations of genetic, physiological and morphological properties, which will contribute to an enhanced process economy.

Among these desired alterations are:

1. Decrease in the amount of side products which: (a) consume metabolic energy at the expense of the desired main product, (b) hamper the final product purification or (c) can even be inhibitory to the production organism. For instance, a significant reduction of DCPC concentration has been achieved by

knocking out esterases that hydrolyse cephalosporin C to DCPC during fermentation. Esterase inactivation was done by conventional mutagenesis as well as through gene disruption and by introduction of anti-sense genes.

2. Increase of the tolerance towards toxic side products and fed precursors like phenyl- or phenoxyacetic acid (see above).
3. Further reduction of feed-back inhibition by end products and metabolites (see below).
4. Further reduction of catabolite repression (see below).
5. Enhancement of genetic stability.
6. Enhancement of viability, stress resistance and life span.
7. Accelerated product formation and enhancement of time-specific productivity to shorten fermentation time.
8. Enhancement of the strain specific productivity to reduce the amount of biomass to be processed and disposed.
9. Increase of product secretion rate.
10. Improvement of the fermentation behaviour in terms of: (a) higher efficiency of substrate/precursor consumption or (b) diminished oxygen demand and (c) diminished shear sensitivity.
11. Improvement of filterability.

In summary, it can be stated that further success in strain improvement requires a deeper comprehension of the genetic and physiological parameters. But the future evaluation of the expected results will have to take account that every fungal strain behaves individually and that regulation mechanisms also depend on growth phases, on environmental influences and thus also on the performance of the fermentation process.

B. Fermentation

Industrial β -lactam fermentation is a fed-batch process carried out aseptically in stainless steel tank reactors with capacities between 2 m³ – mostly the scale of seed fermenters – and 100 m³ for the production stage. The fermentation usually involves two to three initial seed growth phases followed by a production phase having a time cycle ranging from 120 to 200 h. To ensure optimum conditions for product formation, the fermentations are highly computerized and automated.

All process relevant parameters like temperature, pH, dissolved oxygen, substrate concentration, biomass and antibiotic productivity, etc. are closely monitored and controlled, and liquified starch, soybean oil and ammonium sulfate are fed throughout the cycle in definite ranges. The precursor substances phenylacetic acid (for penicillin G) or phenoxyacetic acid (for penicillin V) are supplied con-

tinuously and sulfuric acid and ammonium are fed to adjust the pH of the fermentation liquid between 6.4 and 6.8 during the active production phase.

Though being optimized for decades, industrial β -lactam fermentation is still subjected to stepwise and continuous further improvement: (1) by varying and adapting process parameters to the physiological properties and needs of new strains emerging from the development programs, (2) by identifying productivity-enhancing substrates and compositions of the cultivation media and particularly (3) by optimizing process control to enhance process consistency.

Selected aspects of these topics, namely relevant physiological and economical implications of media composition and process control are presented in the following section.

1. Cultivation Media

a) Complex Ingredients

To be suitable for industrial purposes, fermentation media have to fulfil several, in parts somewhat contradictory criteria. They have to be: (1) inexpensive, (2) not too viscous to provide a sufficient oxygen transfer to the cell, (3) easily sterilisable and (4) have, according to various guidelines, to be free of animal-derived ingredients that bear the risk of transmitting encephalopathies. For the first-mentioned reason, commercial media are predominantly formulated with complex carbon and nitrogen sources like liquified starch, cornsteep, soybean and cottonseed meals which also contain all constituents needed for a rapid biomass production like vitamins and trace elements. About 65% of the carbon is metabolized for cellular maintenance, 20–25% for growth and 10–12% for product formation.

b) Synthetic Components

The natural ingredients usually employed, however, are disadvantageous in some ways. In most cases, they are not sufficiently characterized and not of constant quality. This inherent lot-to-lot variation can lead to process and yield deviations; the insoluble meal compounds and the generally enhanced viscosity hamper sterilization and oxygen transfer during fermentation, and the application of on-line analysis is difficult to realize (see below). To enhance process consistency and

to facilitate sterilization, the use of chemically defined (synthetic) media or at least some defined constituents in commercial fermentations has been suggested (Zhang and Greasham 1999). The costs of these media indeed can be justified in relation to the overall production expenses, if yield and product quality are improved and more consistent. For instance, in the case of penicillin V fermentation, much lower penicillin V degradation has been reported in a synthetic medium than in a complex one containing corn steep liquor (Christensen et al. 1994).

c) Amino Acids

It is obvious that the biosynthesis of β -lactams, being synthesized from amino acids, is also influenced and regulated by the extracellular amino acid pool. In the course of the rounds of mutation and selection, however, industrial strains have lost this dependence to a significant extent. For example, feedback inhibition of β -lactam production by lysine (peculiar also to the wild-type strains) is – if still present – much less pronounced in the actually employed high performance strains. Lysine and β -lactams are both end products of the aminoadipate pathway, the branching point of which is the ACV-peptide precursor L- α aminoadipic acid. Also the sensitivity to feedback-inhibition of the valine pathway has been drastically reduced. Varying lysine and valine concentrations in complex nitrogen sources is hence no longer a critical process parameter.

A milestone in economizing cephalosporin fermentation was the employment of methionine-independent strains, as the cultivation media no longer needed to be supplemented with methionine. The dependence of the original *Acremonium chrysogenum* production strains on methionine was removed through knocking out the regulatory feedback mechanism for methionine synthesis (Matsumara et al. 1982). Methionine, together with inorganic sulfate is the compound from which L-cysteine, the cephalosporin sulfur atom donor, is directly formed via the reverse trans-sulfuration pathway. Methionine-independent mutants exhibit an increased level of intracellular methionine concentration as well as a superior sulfate assimilation rate. Nevertheless, methionine was proven to exert still a favourable effect on cephalosporin biosynthesis (for a review, see Demain and Zhang 1998). It enhances the transcription of the biosynthetic pathway genes encoding for ACV synthase, IPN synthase and DAOC expandase. Due to their role as precursor and regulatory elements, these effects turned out to be rather important and increased the specific cystathionine- γ -lyase activity. The latter is an enzyme of the reverse trans-sulfuration pathway: norleucine, a non-sulfur analogue of

methionine that of course is not involved in this pathway, mimics its effect.

Other potential effects of methionine such as the repression of sulfate uptake, de-repression of sulfatase or inhibition of valine uptake are not related to its stimulation of productivity. In addition, methionine can also induce favourable morphological alterations. So it stimulates mycelial fragmentation into unicellular, yeast-like arthrospores, a process which is under control of the above-mentioned global developmental homologue of the *velvet* gene also steering the biosynthetic cluster (Dreyer et al. 2009), and is favourable for oxygen uptake (see below). This morphological change is correlated with the start of the main production phase when cephalosporin synthesis in the mycelial stage is negligible. The physiological mechanisms underlying this alteration remain to be explored.

d) Productivity-Enhancing Substrates

In the search for productivity-enhancing additives, Ariyo et al. (1997) identified alginate oligosaccharides as suitable agents to increase penicillin G production up to 150% by low- and high-titre strains. An exact explanation for this effect is still lacking. Oligosaccharides were shown to act as signaling molecules and to exert regulatory functions in plants, animals and bacteria (references in Ariyo et al. 1997). Ho et al. (1990) reported a penicillin-enhancing effect of about 50% through the addition of 2% *n*-hexadecane to the medium. This effect was attributed to an increase of oxygen solubility in the cultivation liquid.

e) Concentration of Carbon, Nitrogen and Phosphor Sources

The biosynthesis of penicillin and cephalosporin as products of the secondary metabolism is evidently under control of catabolite repression by rapidly metabolisable carbon, nitrogen and phosphate sources (for general articles, see Kuenzi 1980; Heim et al. 1984; Shen et al. 1984; Liras et al. 1990; Weil et al. 1995; Martin et al. 1999). Formation and activity of pathway enzymes like ACV synthase, IPN synthase, DAOC expandase and DAC hydroxylase were reported to be inhibited by glucose, ammonium and phosphate in both species (Lübbe et al. 1984; Revilla et al. 1984, 1986; Zhang et al. 1988, 1989; Hönlinger and Kubicek 1989; Feng et al. 1994). Catabolite

repression in industrial β -lactam production, however, only plays a minor role since it can be circumvented by the use of alternative substrates.

Penicillium strains are mostly selected for their lactose consumption ability. Extracellular lactose is not hydrolysed to galactose and glucose before being taken up by the mycelium. The intracellularly liberated glucose is immediately metabolized and thus not effective with regard to catabolite repression.

In *A. chrysogenum*, soybean oil was not only found to prevent catabolite repression, but also to have an additionally stimulating effect on cephalosporin production. Karaffa et al. (1999) found that this effect correlated with an increase of the respiration that proceeded via the alternative cyanide resistant non-phosphorylating pathway. This pathway, using succinate as the major substrate, was shown to occur exclusively in arthrospores (Karaffa et al. 1996) and is hence correlated with the production phase (see above).

However, in the course of the strain improvement cycles, catabolite repressing regulations were almost completely eliminated for both species. The deregulation has reached such a degree that: (1) a clear-cut discrimination between growth phase and production phase (tropo- and idio-phase) is no longer possible and (2) the concentration of glucose, sucrose or hydrolysed starch (which are mainly used for cost reasons), ammonia, ammonium and phosphate compounds can relatively easily be kept below the critical thresholds by appropriate feeding strategies during fermentation. The development of such feeding strategies requires the knowledge of substrate concentrations and the surveillance of appropriate substrate dosage rates, which is one of the key elements of fermentation process control. Among further important process parameters influencing product yield and quality, are pH and oxygen supply.

2. Physical Process Parameters

a) Influence of pH

The pH has multiple influences on the gene activity and product stability.

In *P. chrysogenum*, pH appears to have a drastic influence on the transcription of production genes. A pH-dependent expression of the *pcbC*-gene is mediated by the PACC transcription factor. PACC has been identified in *Aspergillus niger* as a three-zinc-finger protein consisting of 667 amino acids with a MW of nearly 71 kDa that cognates 5'-GCCARG-3' sequences and is activated by an alkaline ambient pH (McCabe et al. 1996). Also in *P. chrysogenum*, a

PACC homologue has been characterized, as well as seven of these consensus PACC binding-sequences, which are located in the *pcbAB-pcbC* intergenic region. Penicillin production and steady-state *pcbC* transcript levels are markedly increased at an alkaline pH (Suarez and Penalva 1996).

Studies conducted with *Aspergillus nidulans* revealed that PACC needs to be activated through specific proteolysis yielding a protein that consisted of about 40% of the N-terminal part. Activation is under control of six *pal*-genes activated under alkaline conditions. In its full length form, being prevalent under acidic conditions, PAAC prevents the transcription of genes, which are expressed at low pH values.

Besides its physiological influence on productivity, the pH also has an influence on product stability. Penicillin and cephalosporin are both known to undergo a pH-dependent chemical hydrolysis (for cephalosporin, see Usher et al. 1988). Cephalosporin additionally is attacked by esterases (see above) which also work in strong pH dependence.

It thus appears to be reasonable to develop distinct pH profiles that take into account the particular requirements of growth and production phases as well as the product stability during less-productive phases.

b) Oxygen Supply

As both organisms are strictly aerobic and some of the pathway enzymes (IPN synthase, DAOC expandase, DAC hydroxylase) are oxygen-dependent (see above), it is conceivable that a sufficient oxygen concentration has to be ensured during fermentation. As empirically found, the partial oxygen pressure should not decline below 40%. The cellular oxygen supply depends on various parameters like: (1) fermenter design, (2) aeration and stirring rate, (3) rheological properties of the cultivation media and finally (4) cellular morphology. For *Penicillium* fermentations, a pelletized growth turns out to be beneficial for oxygen uptake.

A pellet morphology can be induced by choosing: (1) an appropriate spore titre, usually lower than $10^9/\text{m}^3$ (Calam 1976; van Suijdam et al. 1980), (2) an appropriate agitation rate and (3) polymers like carbopol (Elmayergi and Moo-Young 1973; van Suijdam et al. 1980). The growth characteristics, of course, have to be already criteria for strain selection. In enhancing the oxygen supply, the pellet morphology permits a lower input of agitation energy, thus a more economical performance of the process as well as the maintenance of a higher amount of productive biomass can be realized. To maintain a sufficient oxygen transfer into the pellet nucleus, the pellet size should not exceed a diameter of 5–8 mm. Due to shearing effects and mycelial abrasion in most cases, the morphology in the fermenter, however, develops towards a mixed form containing both pellets and filaments.

A. chrysogenum is less critical with respect to oxygen limitation and shearing stress. The rheological behaviour of the culture broth after arthrospore formation approaches that of Newtonian fluids (Yong Kim and Young 1992). In contrast to the highly productive *Acremonium* arthrospores, the vacuolated yeast-like growth observed in *P. chrysogenum* is correlated with aging under carbon starvation and leads to a sharp decline in productivity (Pusztahelyi et al. 1997). In general, oxygen supply is the most critical issue of industrial large-scale fermentations and scale-up.

3. Scale-Up

The scale-up of fermentation processes as a central problem in biotechnology was first recognized and described during industrial penicillin production in the beginning of the 1940s. The changed geometry and physical conditions in larger scales lead to a less favourable mixing behaviour and to impaired physiological conditions, which in turn may lead to a decreased process constancy and reproducibility, to reduced specific yields and to an increase of unwanted side reactions and thus ultimately to a diminished batch-to-batch consistency and product quality (key issues in industrial production processes).

The problem of reduced mixing quality in larger scales is aggravated with increasing vessel sizes: the opposite substrate and oxygen gradients along the vessel height, which are formed as a result of the conventional fermenter design (according to which substrate feed usually occurs from the top and aeration from the bottom), are more pronounced in larger reactors due to: (1) longer distances to be covered leading to larger substrate and oxygen depletion zones, (2) larger volumes of culture broth to be stirred and therewith longer mixing times and (3) stronger hydraulic pressure gradients influencing the oxygen transfer rate.

Cells at the fermenter top are exposed to excess glucose concentrations and simultaneously suffer from oxygen limitations whereas those at the bottom are exposed to glucose starvation. Excess glucose concentrations result in acetate overproduction (overflow metabolism), a simultaneous oxygen limitation further induces the formation of ethanol, hydrogen, formate, lactate and succinate. The produced acids can become re-assimilated in oxygen-rich zones, but in any case, first lead to a temporary acidifica-

tion of the micro-environment (and later eventually also of larger regions).

A combination of a decreased transportation and elimination of carbon dioxide, detrimental metabolites and surplus heat generated by agitation and metabolic processes resulting in zonal overheating and the lower mixing rates on a large scale can lead to the formation of zones with enhanced stress conditions. A prioritized goal of process optimization and scale-up therefore deals with an appropriate process design that improves the physiological conditions and the metabolic accuracy by minimizing microbial stress exposure.

To reduce the stress exposure by a more uniform glucose distribution in large-scale tanks, a glucose feed was suggested at the dynamic zones of the fermenter bottom together with the injection of air. A step ahead in this regard would be a further enhancement of the mixing quality by a reactor and process design permitting a multilevel injection of both air and substrates into high turbulence zones (for literature, see Schmidt 2005b).

Issues related to the development of fermentation optimization and scale-up strategies are discussed in detail by Schmidt (2005b). As mentioned, the problem of oxygen supply in β -lactam fermentation can fairly well be coped with by the use of pelletized biomass and strains producing large-sized arthrospores, which are generally preferred because they facilitate filtration of the culture broth and lead to an improved downstream processing behaviour.

C. Downstream Processing

1. Recovery and Purification

Whereas slight progress can be noticed in strain development and fermentation, purification procedures remained basically unchanged throughout the past years. As most of the product is secreted and thus concentrated in the culture broth, the recovery process starts with filtration, usually with a rotary vacuum filter, followed by a cascade of solvent extraction steps.

Common penicillin extraction solvents are amyl and butyl acetate or methyl-isobutyl ketone. As penicillin is extracted as a free acid at pH 2.0–2.5 and the molecule is unstable at this pH, extraction occurs in a counter-flow

using Podbielniak and Luwesta centrifugal extractors to shorten the contact time with the solvent and to prevent product decomposition.

Multiple re-extractions in buffer and solvent at varying pH using counter-current contactors has led to considerable penicillin concentration in the early recovery stages of the purification process. Pigments and other broth impurities are removed by the use of activated charcoal. Nevertheless, the additional application of flocculants and ultrafiltration has periodically also been under investigation. The final purification is carried out by repeated crystallization of the antibiotics from an aqueous solution after alkalization. In contrast to penicillin, the hydrophilic cephalosporin is more suitable for solid-phase extraction.

For high yield and purity extraction, a combination of different chromatographic purification steps can be used. Hydrophobic interaction chromatography on neutral polyaromatic resins like Amberlite XAD 4, 16, 1180 or Diaion HP20 is widely used in combination with weak basic anion exchangers like Diaion WA-30, Amberlite IRA 68 or strong acidic cation exchangers like Amberlite XAD 2000 and Diaion SK-1B (for further details, see Gosh et al. 1996, 1997). The mentioned resins are recognized for their high sorption capacity and their long shelf-life.

Modern techniques, such as reactive extraction using liquid membranes, two-phase system partitioning, hollow-fibre and hybrid liquid-membrane separation, potentially offer advantages as the possibility of a direct extraction from the culture broth but will need further investigations to become an economic large-scale application. Following purification, CPC can either be isolated as a sodium or potassium salt, washed and dried, or the CPC in solution can be directly passed onto enzymatic conversion to 7-aminocephalosporanic acid (7-ACA).

2. Side Chain Cleavage

7-ACA and 6-aminopenicillanic acid (6-APA) are the key intermediates for the production of all semi-synthetic penicillins and cephalosporins and are obtained by removal of the acyl side chains. The originally used chemical deacylation has been meanwhile replaced worldwide by enzymatic processes for economical as well as for ecological reasons.

a) 7-Aminocephalosporanic Acid

Enzymatic 7-ACA splitting procedures were first developed and commercialized by the companies Asahi Chemical, Hoechst and Novartis in the 1980–1990s. The replacement of chemical deacylation processes as the imino ether or the nitrosyl chloride methods used by then resulted in a cost reduction of 80% and a decrease of the waste volume by a factor of 100 from 31 t/t to 0.3 t/t 7-ACA. Chlorinated hydrocarbons like dimethyl aniline and methylene chloride as well as heavy metal ions can be completely avoided. Instead via zinc salt formation, multiple silylation, formation of the imino chloride, imino ether and finally by an imino ether hydrolysis, the side chain is removed in two enzymatic steps (Fig. 5.2).

Cephalosporin C is first oxidized and deaminated by a D-amino acid oxidase (DAO; for structural studies, see Arroyo et al. 2007), which can be obtained from various fungal species, like the yeasts *Trigonopsis variabilis* and *Rhodotorula gracilis* or the ascomycete *Fusarium solani*. The resulting α -keto-adipyl-7-ACA, upon decarboxylation, is converted into glutaryl-7-ACA (G-7-ACA). DAO is a flavoenzyme containing flavin adenine dinucleotide as the prosthetic group and catalyses the oxidation of D-amino acids to their corresponding keto acids. In a second step, the glutaryl side chain of G-7-ACA is deacylated by a glutarylamidase from *Pseudomonas diminuta* (Binder et al. 1993; Sio et al. 2005). The molecular data of other potentially suitable enzymes and genes from various sources are given by Isogai (1997). It is noteworthy that the enzymatic splitting process could have only been rendered economical and therefore commercially applicable through a significant increase of glutarylamidase yield on the fermentation level by using a gene-recombinant *E. coli* strain. Further crucial steps and measures in developing this process were the elimination and inactivation of catalases and esterases, which were co-purified with the DAO (Bianchi et al. 1998).

If catalase is present as a contaminant, hydrogen peroxide generated in the course of the first DAO reaction step will be decomposed into water and oxygen and thus, cannot be used for the oxidation of α -keto-adipyl-7-ACA in the second DAO reaction step (Fig. 5.2). In consequence, this intermediate, which cannot be cleaved to 7-ACA, accumulates and the overall yield drastically decreases. To ensure the DAO oxidation step, the reaction mixture can additionally be supplemented with extra hydrogen peroxide.

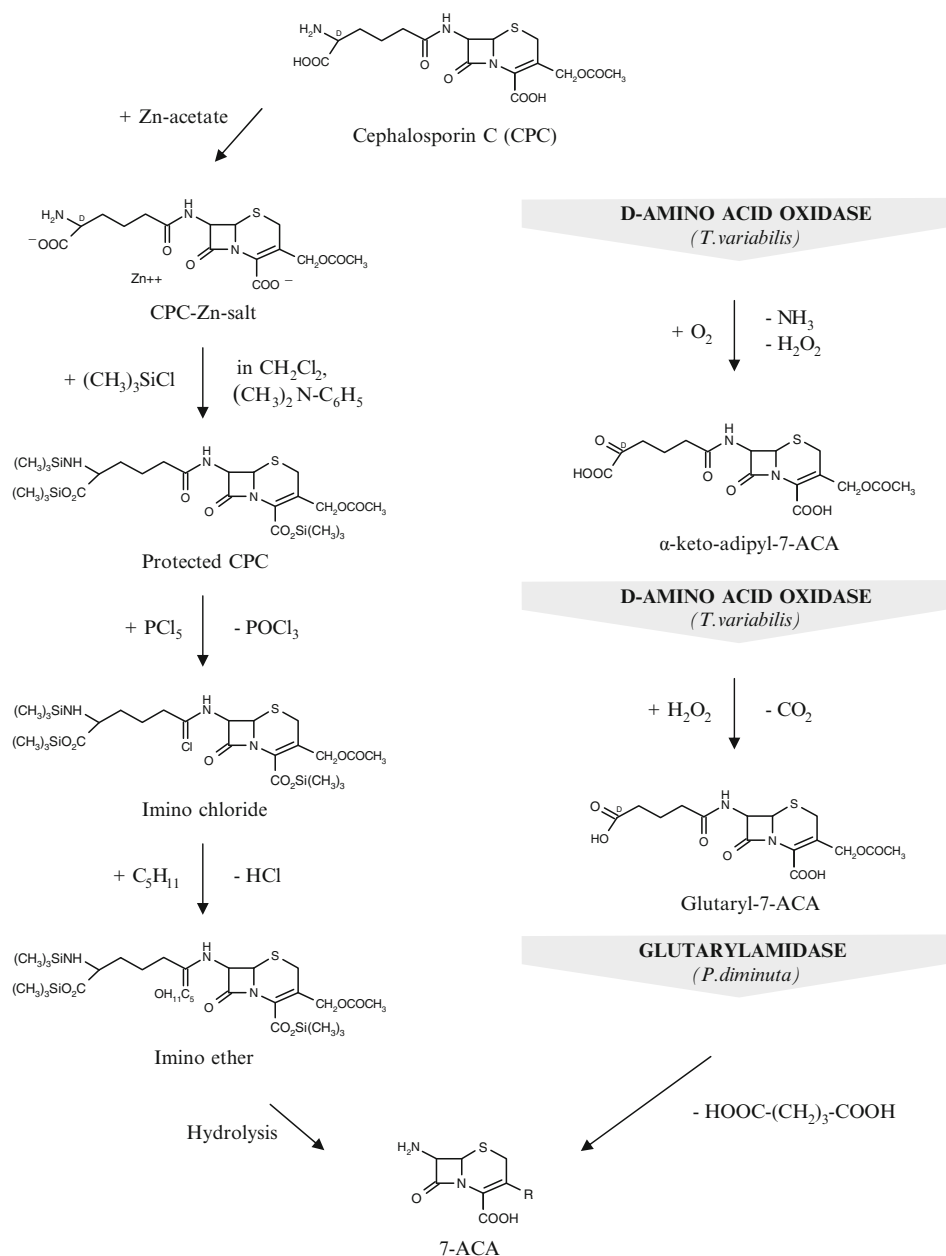


Fig. 5.2. Comparing reaction scheme of the imino ether method (*left*) and an enzymatic splitting procedure (*right*) for 7-ACA production (according to Bianchi et al. 1998;

extended). Enzymatic procedures are increasingly applied for evident economical and ecological reasons. For further explanations see text

Also the presence and activity of cephalosporin C esterases (see above) has to be avoided, as DAC, like α -keto-adipyl-7-ACA, is not further converted and processed. Meanwhile, selected strains of *T. variabilis* with diminished catalase and esterase activities have been introduced to DAO production. To enhance the efficiency of the oxidative deamination rate, the reaction vessel can be aerated with pure oxygen instead of air (Bianchi et al. 1998).

A detailed overview on industrial aspects of the enzymatic cleavage of cephalosporins is given in the review of Barber et al. (2004).

b) 6-Aminopenicillanic Acid

The enzymatic cleavage of penicillin V or penicillin G to form 6-APA is a process established now for decades. Though enzymes from various

microbial sources with penicillin acylase activities have been described, the most commonly applied procedure for 6-APA production is the deacylation of penicillin G with amidases from recombinant *E. coli* and *Bacillus* strains, which meet the high requirements for enzyme performance. With an annual turnover of 30 t, it is one of the most widely used biocatalysts in industry at all. Recent advancements made in penicillin cleavage technology have been summarized by Chandel et al. (2008).

3. Economization of the Cleavage Process

A significant milestone in the economization of the enzymatic cleavage processes has been reached by enzyme immobilization permitting the preservation and multiple use of the cleaving enzymes.

The presently mostly employed resins in industry for this purpose are epoxyacrylic acids (Eupergit) and silica gel derivatives (Deloxan). The general economic criteria for the preparation of biocatalysts are the production costs in relation to yield, turn-over rate, storage stability and operational/mechanical stability. Further criteria are outlined in the "Guidelines for the characterization of immobilized biocatalysts" by the European Federation of Biotechnology. Application criteria are filterability, sedimentation velocity and particle firmness.

Further economization has been achieved through a detailed characterization of their molecular structure and subsequent optimization of stability and reaction properties by site directed mutagenesis to enhance fission yields (Sio and Quax 2004; Arroyo et al. 2005; de la Mata et al. 2005). Directed mutagenesis and evolution are also employed to create novel enzymes with new specificities in the hydrolysis or synthesis of β -lactam antibiotics and may contribute to even more economical production procedures in the future.

D. Options for Future Production Processes

Besides attempts to develop processes enabling the performance of the two reaction steps in one reactor by co-immobilization of the two involved enzymes (Bianchi et al. 1998; Lopez-Gallego et al. 2005), the main focus currently aims at a direct one-step conversion of cephalosporin C and a direct expression of 7-ACA and derivatives by

accordingly engineered enzymes and production strains.

1. One-Enzyme Cleavage of Cephalosporin C

In addition to their glutarylamidase activity, acylases from various *P. diminuta* strains (e.g. V22, N 176) were shown to exhibit a cephalosporin C acylase (CC acylase) activity. These enzymes, with very low yields (5% compared to 100% glutarylamidase activity) convert cephalosporin C as well as deacetylcephalorin (DAC) directly into 7-ACA and 7-aminodeacetylcephalosporanic acid (7-ADACA), respectively (Isogai 1997).

DAC as the prevalent naturally occurring fermentation side product amounts up to 20% of the total cephalosporins produced in the culture broth. Since it is not cleaved and thus lost in the established cleaving procedures, the development of a DAC-splitting procedure evidently would bear an enormous potential for process economization. Such a procedure would require: (a) *Acremonium* strains that exclusively produce DAC, as performed by Basch et al. (2004) by esterase cloning or (b) the in vitro deacylation of CPC to DAC after fermentation, e.g. by immobilized esterases. Penicillin acylases are another group of enzymes investigated regarding the direct cephalosporin deacylation. Thus, Oh et al (2004) engineered a mutant penicillin G acylase with CC acylase activity.

2. Production of Adipyl-Cephalosporins in *Penicillium chrysogenum*

In trying to take advantage of the much higher productivity of *Penicillium* strains, Merck and Eli Lilly published the production of cephalosporin derivatives in recombinant *P. chrysogenum* strains expressing either: (a) the cephalosporin C expandase from *Streptomyces clavuligerus* (Ingolla et al. 1988; Cantwell et al. 1990; Conder et al. 1992; Crawford et al. 1995), or (b) the bifunctional *Acremonium* expandase/hydroxylase without or (c) together with its acetyltransferase (Conder et al. 1994; Crawford et al. 1995). Feeding of adipic acid to these strains resulted in the formation of adipyl-6-APA and subsequently, in dependence of the cloned activities, in formation of adipyl-7-aminodeacetylcephalosporanic acid (adipyl-7-ADOCA), aminodeacetylcephalosporanic acid (adipyl-7-ADACA) or adipyl-7-ACA (Fig. 5.3). The removal of the adipyl side chain, which turned out to be more susceptible to glutarylamidase cleavage than the aminoadipyl side chain of natural cephalosporins (Conder et al. 1994), yielded the corresponding intermediates 7-ACA, 7-ADACA and 7-ADOCA (Fig. 5.3).

3. Production of 7-ADOCA

7-ADOCA accounts for about 20% of the precursor substances for the production of all commercialized cephalosporins and is usually produced by a chemical ring expansion of penicillin G to phenylacetyl-7-ADCA (cephalosporin G). In addition to the approach of expressing

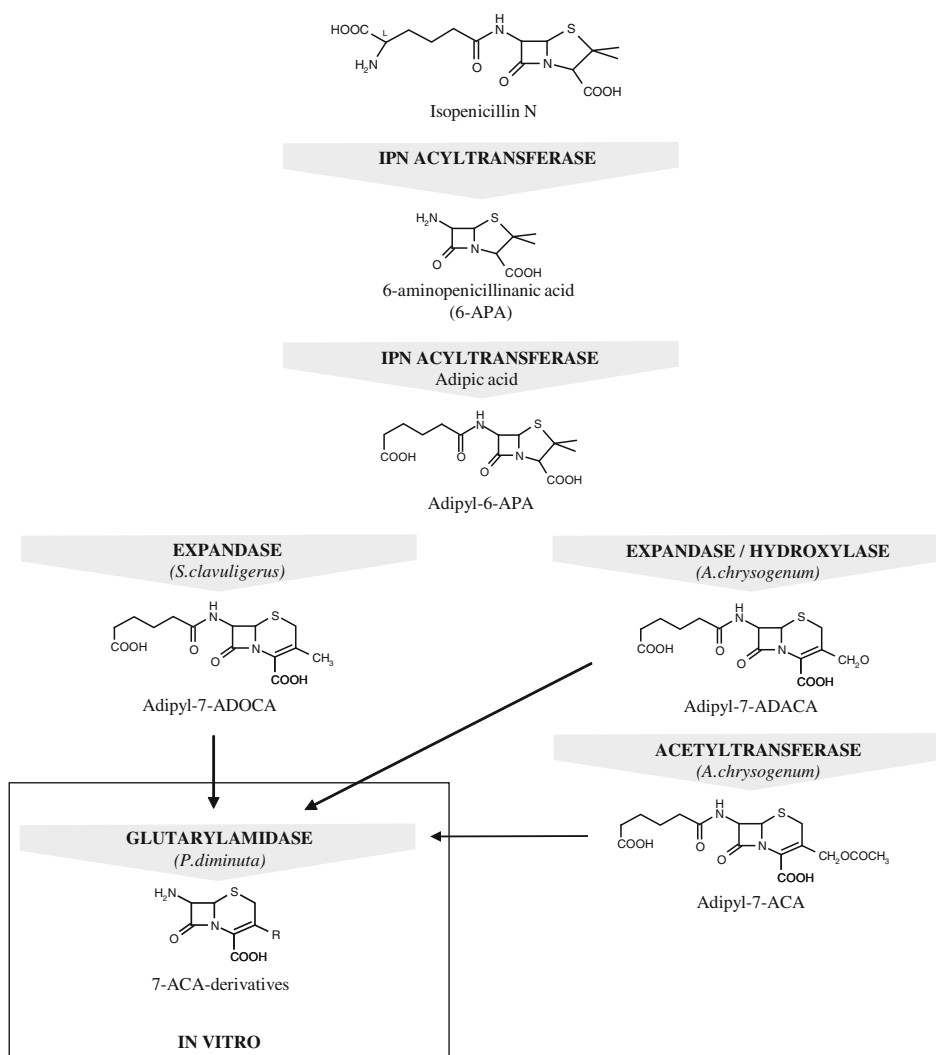


Fig. 5.3. Production of adipyl-cephalosporins by recombinant *P. chrysogenum* strains with cloned activities from *S. clavuligerus* or *A. chrysogenum* (according to Crawford et al. 1995; modified). In dependence of the cloned activities the intermediates adipyl-7-ACA, adipyl-7-ADACA or

adipyl-7-ADOCA are produced upon feeding with adipic acid. Cleavage to 7-ACA, 7-ADACA or 7-ADOCA is catalysed by immobilized glutarylaminidase from *P. diminuta*. For details see text

cephalosporins in *Penicillium* (see above), which is also pursued by DSM (Bovenberg et al. 2002), there are further options for replacing this expensive and polluting chemical multistep reaction by biotechnological measures.

Antibiotics published a method based on the silencing of the *cefEF* gene of *Acremonium chrysogenum*, encoding the bifunctional expandase/hydroxylase activity, by the expandase encoding *cefE* gene from *Streptomyces clavuligerus*. The DAOC production level was almost equivalent to total β -lactams biosynthesized by the parental strain (Velasco et al. 2000a, b; Rodriguez-Saiz et al. 2005). The subsequent DAOC deacylation was carried out by two final enzymatic bioconversions catalysed by D-amino acid oxidase and glutaryl acylase, yielding 7-ADCA. Goo et al.

(2008) report an optimization of the activity of engineered *Streptomyces* expandase on various penicillin substrates.

4. Direct Expression of 7-ACA in *A. chrysogenum*

The most elegant procedure for the production of 7-ACA production is the direct biosynthesis and secretion of 7-ACA by the production strains. To this end, Isogai and co-workers at Fujisawa Pharmaceuticals constructed a complete 7-ACA biosynthetic operon consisting of the genes from *F. solani* and *P. diminuta* encoding for DAO, glutarylaminidase and CC acylase activities (Fukagawa et al. 1991; Isogai and Fukagawa 1991; Isogai et al. 1991). The

promoter and regulatory elements originated from an alkaline *A. chrysogenum* protease. The transfer of these operons into *A. chrysogenum* resulted in an intracellular conversion of cephalosporin C to keto-adipyl-7-ACA, G-7-ACA and 7-ACA. When CC acylase from *P. diminuta* V22 was part of this operon, residues of not DAO-converted cephalosporin C and keto-adipyl-7-ACA intermediates were also cleaved directly to 7-ACA (Isogai 1997).

Even though there are many efforts underway to optimize enzymes, mostly glutarylamidases from *Pseudomonas* (Fritz-Wolf et al. 2002; Koller et al. 2002; Otten et al. 2002, 2007; Sio et al. 2002, 2003, 2005; Wei et al. 2003; Pollegioni et al. 2005) for a direct cleavage of cephalosporin C or to create fusion proteins of DAO and GA for one step cleavages (Luo et al. 2004), these procedures are still far from being commercialized due to their poor yields and low efficiency. The studies aiming at a direct 7-ACA expression in production strains demonstrate, however, the promising potential of pathway engineering approaches for future process economization and, in using β -lactam encoding genes from bacterial sources (for a review, see Martin 1998), also for the creation of novel β -lactam entities with an enhanced therapeutic value.

III. Therapeutic Aspects

By inhibiting a metabolic target unique in bacteria, namely the transpeptidase involved in peptidoglycan cell-wall biosynthesis, β -lactams only weakly interfere with the mammalian cell metabolism. They are thus nearly free of severe side effects and generally well tolerable. Due to their peptide structure β -lactams nevertheless can lead in a small minority of cases to an immunological sensitization and to allergic reactions. The most serious clinical problem, however, which is generally encountered now in antibiotic therapy, is the emergence and spread of multidrug resistant germs, which force the continuous development of novel antibacterial drugs to overcome and evade the resistance mechanisms (Schmidt 2004; Jagusz-Krynicka and Wyszynska 2008; O'Neill 2008).

A. Multidrug Resistance

The cellular mechanisms of antibiotic resistance are manifold (for details, see Skalweit Helfand

2008; Savjani et al. 2009). Beta-lactam resistance is mainly mediated in the following three ways:

1. Alteration of the penicillin binding protein (containing the target enzyme activity) with a lower β -lactam affinity, which is particularly common in Gram-positive germs.
2. A failure of compound up-take into the cell due to modified porous forming membrane proteins (porines; Pages et al. 2008; Delcour 2009) and an energy driven efflux (Poole 2007; Davin-Regli et al. 2008; Lomovskaya et al. 2008; Nishino 2008) play a major role in Gram-negative bacteria like *Pseudomonas* (mucoid strains are additionally shielded from host immune defences, antibiotics and even antiseptics and disinfectants by an alginate layer).
3. The most widely spread mechanism, however, particularly among Gram-negative bacteria, is the production of a variety of β -lactamases inactivating the antibiotics by hydrolysing the amide bond of the β -lactam ring (for a review, see Gupta 2007).

The 190 clinical relevant β -lactamases described so far can be grouped according to their function and β -lactamase inhibitor susceptibility (for nomenclature, see Jacoby 2006). Of particular clinical importance are β -lactamases with an extended substrate spectrum and class B metalloproteases, against which no inhibitor is available at present.

Successful approaches in overcoming the resistance problem were the introduction of β -lactamase inhibitors (see below) into therapy at the beginning of the 1980s as well as semisynthetic penicillins and cephalosporins with higher target affinities and enhanced β -lactamase resistance.

B. β -Lactamase Resistance

The first semisynthetic β -lactams were the penicillin derivatives methicillin and ampicillin introduced in the early 1960s by the Beecham Research Laboratories.

Methicillin was stable against staphylococcal β -lactamase and effective against penicillin resistant *Staphylococcus aureus* strains. Ampicillin was the first penicillin widely active against Gram-negative germs. The first antibiotic clinically useful against non-mucoid strains of *Pseudomonas aeruginosa* was carbenicillin introduced in 1967. At this time the choice of agents available for the treatment of *P. aeruginosa* infections was limited.

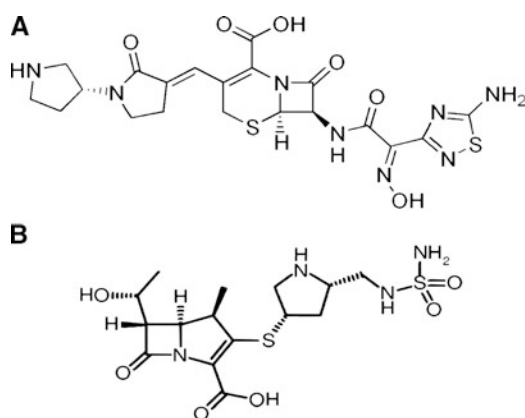


Fig. 5.4. New β -lactam compounds recently introduced into therapy: ceftobiprole (A), the first fifth-generation cephalosporin, administered as a water soluble prodrug, and doripenem (B), an ultra-broad-spectrum carbapenem

Active cephalosporins did not appear until the late seventies. Since then, each generation developed exhibited an extended spectrum of activity, particularly against Gram-negative germs, an improved pharmacology and a greater resistance to β -lactamases than the preceding generation (for details and compilations of the characteristics of the respective cephalosporin generations, see <http://en.wikipedia.org/wiki/Cephalosporin>). The most advanced compound in this regard is the recently launched first fifth-generation cephalosporin ceftobiprole (Zevtera; Fig. 5.4A), which was developed by Roche's subsidiary Basilea and co-commercialized with Johnson & Johnson's Janssen-Cilag and Ortho-McNeil. It is the first broad-spectrum cephalosporin with equal activities against Gram-positive and Gram-negative germs, in particular against methicillin-, penicillin- and multi-resistant strains of *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *Enterococci* (for reviews, see Anderson and Gums 2008; Del Pozo and Patel 2008; Derenski 2008; Kontou et al. 2008; Lodise et al. 2008).

Ceftobiprole inhibits most of the penicillin-binding proteins with high affinity and appears to be less susceptible to the development of further resistances. The water-soluble prodrug (ceftobiprole-medocartil) is suitable for oral and i.v. administration.

Carbapenems are another class of β -lactam antibiotics getting more and more into the focus

of interest when treating infections caused by penicillin- and cephalosporin-resistant bacteria. The most recent carbapenem introduced into therapy is the ultra-broad-spectrum injectable doripenem (Schafer et al. 2009, Keam 2008; Fig. 5.4B). It was launched by the Japanese Shionogi under the brand name Finibax in 2005 and has been marketed outside Japan by Janssen-Cilag under the tradename Doribax since 2007.

Carbapenem antibiotics were originally developed from thienamycin, a naturally derived product of *Streptomyces cattleya* and possess a carbon atom instead of the sulfur of penicillin or cephalosporin. Carbapenems exhibit a broad spectrum of antibacterial activity and are highly resistant to β -lactamases.

Their inherent high resistance against β -lactamases make them also suitable for the development of β -lactamase inhibitors (Mugnier et al. 1998; Nukaga et al. 2008; Prezelj et al. 2008).

β -Lactamase inhibitors are β -lactam derivatives without antimicrobial activity which work as "suicide inhibitors" forming an irreversible covalent complex with β -lactamases and are preferably combined with penicillins, which are inherently less resistant to β -lactamases than cephalosporins. The currently most successfully applied inhibitors are clavulanic acid from *Streptomyces clavuligerus*, e.g. combined with amoxicillin in GlaxoSmithKline's top-selling Augmentin and the penicillanic acid sulfones sulbactam in Pfizer's Unacid (with ampicillin) and tazobactam in Wyeth's Tazobac (with piperacillin; Fig. 5.5).

C. Future Developments

1. β -Lactams

As in the past, the main strategies for future β -lactam developments are directed to protect the β -lactam moiety against hydrolysing enzymes, to facilitate tissue penetration and uptake by Gram-negative germs and to improve the general pharmacological behaviour. Overviews on novel β -lactams and non- β -lactam antibacterials recently launched and currently under development including β -lactamase inhibitors have been summarized by Cornaglia and Rossolini (2009), Aksoy and Unal (2008), French (2008), Gales and Sader (2008), O'Neill (2008), Perez et al. (2008), Skalweit Helfand (2008) and Vergidis and Faladas (2008).

A promising candidate for an approval in the foreseeable future is ceftaroline (Wang and Mealy 2008), a broad-

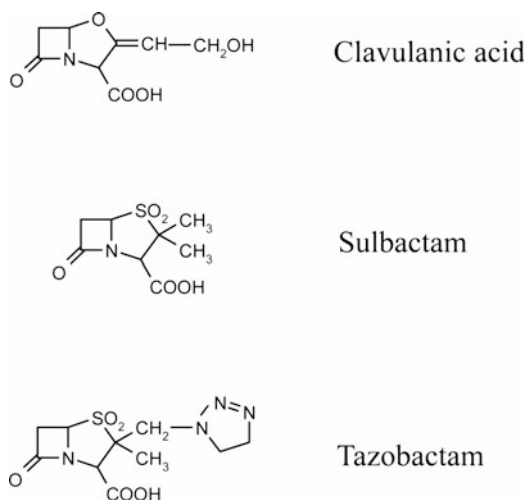


Fig. 5.5. Structures of the currently clinically most important β -lactamase inhibitors clavulanic acid, sulbactam and tazobactam. Clavulanic acid and sulbactam are orally and parenterally applicable whereas tazobactam is suitable only for parenteral administration

spectrum cephalosporin in-licensed from Takeda by Cerexa, now taken over by Forest Laboratories. Phase III clinical trials were recently successfully terminated and the United States Food and Drug Administration granted fast-track designation for the treatment of complicated skin infections caused by methicillin-resistant *S. aureus*.

2. Novel Therapeutic Approaches

Despite the development of such new compounds it cannot be ignored that the time intervals between their introduction into clinical practice and the emergence of resistant and less susceptible strains are getting shorter with every new generation of antibiotics launched. And since most of the antibiotics derive from microbial sources and are thus produced by the very same kind of organisms against which they are considered to act and which co-habit in nature, there always will be resistance before application, development and even discovery of a new compound. To evade this antibiosis cycle and the continuation of the arms race between microbes and humans, the focus of antibiotic research is currently shifting to the identification and inactivation of the disease-causing functions instead on the killing effect (e.g. Cegelski et al. 2008). Since these factors have no vital functions for the germs, they are supposed to be less prone to become resistant to inactivation and

thus to be ideal targets for novel therapies. Such a therapeutic concept would reduce the general global selective pressure and may be therefore of utmost importance to prevent the accelerated dissipation of resistances against antimicrobials in the future. In consequence, new targets will comprise not only metabolic functions that are essential for bacterial vitality, but will also include factors that are responsible for the colonization of tissues and the circumvention of the host's immune response (specific virulence and pathogenicity factors, cell communication).

A central role in this concept may play the inhibition of the cell to cell signalling (quorum sensing) bacteria use for controlling gene expression in response to population density (Ni et al. 2009). By production and exchange of extracellular signalling molecules (termed autoinducer) bacterial populations are able to coordinate their gene activities. Apart from activities like symbiosis, motility, competence, conjugation, sporulation and antibiotic production, they are also responsible for the formation of the medically relevant virulence factors.

Potential components of such novel therapeutic approaches targeting non-vital functions comprise monoclonal antibodies (Meinke et al. 2004; Bebbington and Yarranton 2008; Nagy et al. 2008; Rodrigues de Oliveira et al. 2008) and aptamers (Morrissey and Haerberli 2006; Bruno and Miner 2008; Bruno et al. 2008; Svarovski and Joshi 2008). Together with novel killing concepts like the use of peptide compounds (Schmidt 2004; Hou et al. 2009; Wang and Zheng 2009) and bacteriophages (Inal 2003; Cairns et al. 2009; Lu and Collins 2009), they might revolutionize antimicrobial therapy in the foreseeable future.

IV. Conclusions

Despite the development of such alternative and promising approaches in antimicrobial therapy presently appearing on the horizon, and despite the mentioned shifts in the production scenery and the markets, β -lactams will continue to be an important pillar in antibiosis, whereby the mentioned penicillin- β -lactamase-inhibitor combinations Tazobac and Augmentin and Abbott's Omnicef (cefdinir) will be the leading brands and growth drivers for the next couple of years (Business Insights Report 2007, 2008) and eastern companies and bulkware manufacturers will gain

further importance. While with decelerating frequency, novel structures and new generations of cephalosporins will be developed and available in the future, on the penicillin sector, research currently focuses more on the development of combinations with novel broad-spectrum β -lactamase inhibitors with more effective structures. Also, unlike cephalosporins, no grave improvements in production processes are expected to come in penicillin production; instead slight ameliorations in strain development and fermentation will contribute to a continuous penicillin process economization. In the long term, the economization of cephalosporin production will substantially profit from research programs targeted at one-enzyme 7-ACA-splitting processes and at a direct expression of 7-ACA in cephalosporin production strains. Such programs require close cooperation between academic institutions and industry and might, as far as the metabolic engineering is concerned, also comprise the development of novel β -lactam structures and therapies.

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6 Non- β -Lactam Antibiotics

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I. Introduction

Natural products still constitute an important source of chemical diversity for the development of new drugs (Lam 2007; Harvey 2008). Almost half of the drugs approved for clinical use in 1981–2007 are based on natural products covering almost all therapeutic areas (Newman et al. 2003; Newman and Cragg 2007) and over 200 natural product-derived compounds are currently undergoing clinical trials or are in preclinical development (Butler 2008). Some of the most important and clinically useful drugs are derived from fungal secondary metabolites such as the β -lactams (penicillins and cephalosporins), the potent immunosuppressants

cyclosporin and mycophenolic acid as well as the serum-cholesterol lowering drugs lovastatin and compactin. In addition, the development of the strobilurins as the most important class of agricultural fungicides was inspired by the discovery of natural antifungal compounds produced by various basidiomycetes (Anke and Steglich 1999). The great diversity of substrates and habitats which fungi can successfully colonize reflects an enormous richness of genetic and metabolic resources of these organisms, which still hold an enormous potential for the discovery of new biologically active compounds. During the past ten years, several new drugs based on fungal natural products have entered clinical practice or trials. These include the topical antibiotic retapamulin, a semi-synthetic derivative of pleuromutilin produced by the basidiomycete *Clitopilus scyphoides* (Daum et al. 2007) as well as caspofungin, anidulafungin, micafungin and aminocandin, a new class of potent semi-synthetic antifungal drugs originating from the echinocandins produced by various ascomycetes (Renslo 2007). Another example is irofulvene, a semi-synthetic derivative of the sesquiterpene illudin S from the basidiomycete *Omphalotus illudens*, which is currently in phase II clinical trials against prostate, ovarian and other cancers (Cai et al. 2009). Not least, natural fungal products provide valuable tools for the dissection of complex cellular pathways and functions (Dixon et al. 2007).

The following chapter focuses on new findings related to currently used and recently developed antifungal and antibacterial agents of fungal origin. Other important pharmacological activities reported for compounds originally detected and isolated because of their antibiotic activity are also included in this survey. Some fungal metabolites, which currently are used in the therapy of bacterial and fungal infections, were already described within the chapter “Non β -Lactam Antibiotics” in the first edition of *Mycota* vol. X (2002).

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II. Antifungal Drugs

During the past 30 years, the prevalence of life-threatening invasive fungal infections has dramatically increased owing to the increased number of immune-compromised patients due to AIDS related malignancies, hematological disorders, more aggressive chemotherapy, the receipt of transplants and advances in critical care (Enoch et al. 2006). About 300 fungi can cause diseases in humans (Woolhouse and Gaunt 2007), with *Candida*, *Aspergillus* and *Cryptococcus* species remaining the most common causes of invasive fungal infections in immune-compromised hosts, but infections with other opportunistic fungi, such as zygomycetes, *Fusarium*, *Scedosporium*, *Trichosporon*, *Blastoschizomyces*, *Malassezia* and the black molds, are being seen more frequently, particularly in severely immunosuppressed patients (Patterson 2005). Fungal infections remain a major direct cause of the death of patients who are treated for a malignant disease, and emerging resistance to currently available antimycotic agents is also an important problem (Anderson 2005; Cowen 2008; Monk and Goffeau 2008).

For many years, amphotericin B and fluconazole have been the standard therapy for the treatment of severe fungal infections (Mohr et al. 2008). However, serious side effects such as nephrotoxicity are associated with amphotericin B, while fluconazole suffers from such drawbacks as frequent interactions with coadministered drugs, a limited spectrum of activity (for example, it is not effective against *Aspergillus* spp.), and the increasing emergence of fluconazole-resistant *Candida* spp. (e.g., *C. krusei* and some strains of *C. glabrata*; Ostrosky-Zeichner et al. 2003; Panackal et al. 2006; Mandras et al. 2009; Segal 2009). Therefore, the development of new drugs and further improvement of currently used medications are essential.

A. Griseofulvin

The systemic antifungal antibiotic griseofulvin (Fig. 6.1) is produced by various species of *Penicillium*, including *P. griseofulvum* Dierckx, (Oxford et al. 1939), *P. janczewskii* (Brian et al. 1947; Grove and McGowan 1947), *P. urticae*, *P. raistrickii*, *P. raciborskii*, *P. kapuscinskii*, *P. albidium*, *P. melinii*,

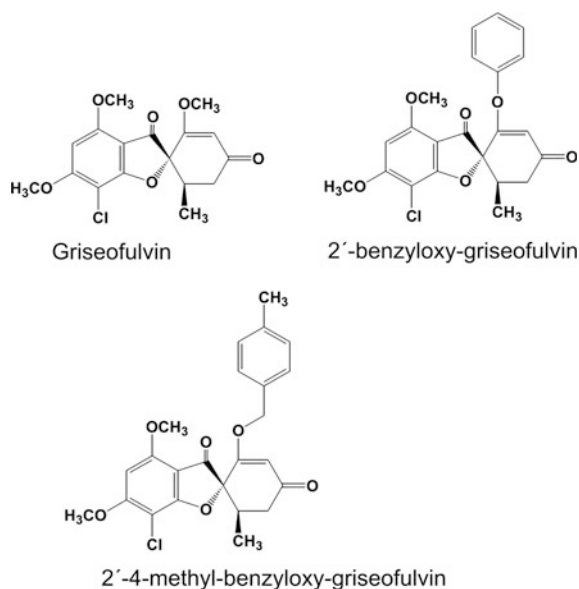


Fig. 6.1. Structures of griseofulvin and some derivatives

P. brefeldianum and some mutant strains of *P. patulum* (Brian 1951; Grove 1964; Broadbent 1966). In addition, *Aspergillus versicolor* (Kingston et al, 1976) and more recently *Nematospora coryli* (Taha and Chu 1991) have been shown to produce the antibiotic. The compound was launched in 1958 as the first systemic fungicide for the treatment of human mycosis caused by dermatophytic fungal genera like *Epidermophyton*, *Trichophyton* and *Microsporum*. Because of its capability to concentrate in the keratinous layer of the epidermis and its relatively low toxicity in man, griseofulvin is used by oral administration in the therapy of cutaneous mycoses like tinea capitis and tinea corpori in children (De Carli and Larizza 1988).

In sensitive fungi, griseofulvin causes characteristic nuclear and mitotic abnormalities followed by distortions in hyphal morphology. Although in fungi the exact mechanism of its action is still unknown, an interference with microtubule assembly is a widely accepted assumption. Interaction of griseofulvin and some of its derivatives with microtubules, and an inhibition of microtubule assembly and interference with mitotic spindle microtubule (MT) dynamics and function were proven by various studies in a variety of mammalian tissues and cells (Oda 2006).

Griseofulvin has also been shown to block cell cycle progression at G2/M and to induce apoptosis in human tumor cell lines and it has been suggested that the compound

suppresses spindle MT dynamics in a manner qualitatively similar to that of the vinca alkaloids and the taxanes albeit at much higher concentrations (Panda et al. 2005). More recently, griseofulvin was identified as an inhibitor of centrosome coalescence in human cancer cell lines with supernumerary centrosomes leading to multipolar spindles, mitotic arrest and subsequent apoptosis of tumor cells without affecting diploid cell lines with normal centrosome content (Rebacz et al. 2007). The structure activity relationship of 34 griseofulvin analogs with regard to anti-cancer activity revealed that the most active analogs were the 2'-benzyloxy and 2'-(4)-methylbenzyloxy analogs (Fig. 6.1) as well as the oxime of the former with a 25-fold increase of inhibitory activity on centrosomal clustering as compared to griseofulvin. Comparison of the results obtained in this work with prior reported growth inhibition data for dermatophytic fungi showed that the antifungal activity of griseofulvin analogs does not correlate directly to their activity in mammalian cells (Rønne et al. 2009).

B. Inhibitors of Fungal Cell Wall Assembly, (1,3)- β -D-Glucan Synthase Inhibitors

The fungal cell wall and the enzymes needed for its biosynthesis and maintenance offer a variety of targets for selective antibiotics. Since the fungal cell wall has no counterpart in mammalian cells, drugs targeting the main enzymatic activities essential for fungal cell wall assembly, such as chitin synthases, (1,6)- β -D-glucan synthases and (1,3)- β -D-glucan synthases, are ideal targets for the discovery of new antifungal agents. Chitin synthesis is the main target for nikkomycins and polyoxins. Inhibitors of glucan synthesis are the aculeacins, echinocandins, papulacandins and acidic terpenoids (arundifungin, enfumafungin, ascosteroside or ergokonin A), which have previously been reviewed (Sundriyal et al. 2006; Lórnrd and Kocsis 2007; Mathew and Nath 2009). (1,3)- β -D-Glucan synthases are targeted by several new antibiotics which are now in clinical use or at advanced stages of development. This field of research has been reviewed recently (Debono and Gordee 1994, Sable et al. 2008).

1. Echinocandins and Pneumocandins

The echinocandins and pneumocandins are antifungal lipopeptides composed of a cyclic hexapeptide core *N*-acetylated with different aliphatic carboxylic acids (Fig. 6.2). The echinocandin family is a group of related cyclic peptides originally derived from fermentations of *Aspergillus nidulans*

and *A. rugulosus* (echinocandins C and D; Traber et al. 1979) each possessing an *N*-linoleoyl side chain but having different hydroxylation patterns. The second group consists of the aculeacins from *Aspergillus aculeatus* (Mizoguchi et al. 1977; Mizuno et al. 1977; Sato et al. 1977) and the closely related lipopeptides from *Coleophoma empetri* (e.g. FR901379, FR901381, FR901382), which have the same peptide nucleus as echinocandin B but an *N*-palmitoyl side chain (Iwamoto et al. 1994a, b). The latter also differ from the echinocandins by having a sulfate moiety in the molecule thus rendering them more soluble than the echinocandins. The third group is that of pneumocandins, e.g. pneumocandin B from *Zalerion arboricola*, having a miristoyl side chain attached to the ring (Schwartz et al. 1989).

The echinocandins are non-competitive inhibitors of (1,3)- β -glucan biosynthesis by binding to the Fks1 subunit of the (1,3)- β -D-glucan synthase that is regulated by Rho1, a member of the Rac/Rho subfamily of Ras-like GTPases and elements of the calcineurin pathway. Blocking of (1,3)- β -D-glucan biosynthesis causes destabilization of the fungal cell wall, cell lysis and concomitant cell death. The echinocandins are active against a broad range of *Candida* spp. and *Aspergillus* spp. including those resistant to azoles and polyenes (Denning 2003). They are also active against the cyst form of *Pneumocystis carinii*, which expresses the catalytic subunit of the (1,3)- β -D-glucan synthase during cell wall formation. Other fungi that are inhibited by the echinocandins include *Scedosporium* spp., *Alternaria* spp., *Bipolaris* spp., *Cladophialophora bantiana*, *Phialophora* spp., *Exophiala* spp., *Fonsecaea pedrosoi*, *Paecilomyces variotii*, *Acremonium strictum* and *Blastomyces dermatitidis*. However, the echinocandins are not active against *Cryptococcus neoformans*, *Trichosporon* spp., *Fusarium* spp. or any Zygomycetes.

The echinocandin preparations that have been used to date are for intravenous use only. All echinocandins have linear pharmacokinetics after intravenous administration and are degraded mainly in the liver (also in the adrenals and spleen) by hydrolysis and *N*-acetylation (Balani et al. 2000). Unlike the azoles, the echinocandins are poor substrates for the cytochrome P450 enzymes and are not substrates for intestinal or tissue P-glycoprotein. Therefore, fewer drug interactions are described for these molecules than for the azoles and their tolerability is generally good (Dodds Ashley et al. 2006).

Due to undesired side effects encountered with the natural echinocandins, such as hemolytic activity, their structures were chemically altered

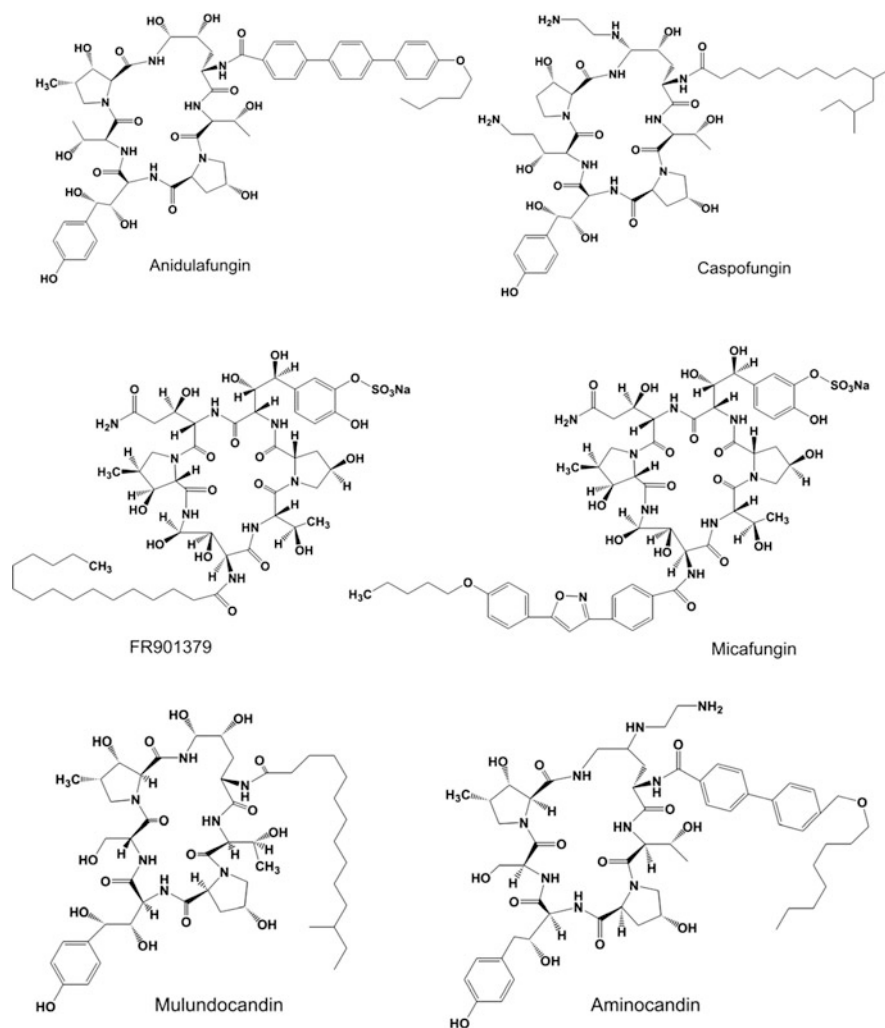


Fig. 6.2. The echinocandin class of antifungal drugs

to improve their absorption, distribution, metabolism and excretion characteristics. Lipophilic side chain replacement has been employed primarily to improve potency and reduce hemolytic potential, alternation of the cyclic peptide core influences chemical stability, aqueous solubility and pharmacokinetic profile, while also impacting potency and in vivo efficacy. While the first generation of clinical echinocandins resulted from either side chain (anidulafungin, micafungin) or peptide core (caspofungin) modification, a newer generation of clinical echinocandins (e.g. aminocandin) bearing modifications of both core and side chain is now progressing into clinical trials (reviewed by Renslo 2007). Semisynthesis of derivatives with an altered fatty acid side chain resulted in Cilofungin. This compound was subjected to

Phase II clinical trials, but was abandoned due to toxicity of the vehicle that was used to solubilize the compound, as well as low oral efficacy and a narrow spectrum of activity (Georgopapadakou and Tkacz 1995).

Caspofungin, a water-soluble semi-synthetic aminoderivative of pneumocandin B₀, was approved for the treatment of esophageal candidiasis, candidemia and other candida infections such as intra-abdominal abscess, peritonitis, pleural space infections and as salvage therapy for invasive aspergillosis (reviewed by Deresinski and Stevens 2003). The second approved antifungal agent of the echinocandin class is micafungin or FK463, a water-soluble semi-synthetic derivative of the natural occurring acylated cyclic hexapeptide FR901379 produced by the fungus *Coleophoma empetri* (Iwamoto et al. 1994a, b). It was prepared by enzymatic deacetylation of the natural sulfated echinocandin B derivative and reacylation of the

hexapeptide with an isoxazole containing benzoyl-like side chain (Fujie 2007; Hashimoto 2009). Micafungin is used for the treatment of *Candida* esophagitis in HIV-infected patients and for the prevention of *Candida* infections of patients undergoing hematopoietic stem cell transplantation (Chandrasekar and Sobel 2006). The third echinocandin derivative approved for the treatment of esophageal candidiasis and other *Candida* as well as *Aspergillus* infections is anidulafungin (V-echinocandin, VER002). It is a semi-synthetic echinocandin derivative in which the acyl sidechain was replaced by a terphenyl head and a C5 alkyl tail. Anidulafungin has potent *in vitro* fungicidal activity against a broad range of *Candida* species and is also effective against species of *Candida* that are intrinsically resistant to azoles (*C. krusei*), amphotericin B (*C. lusitanae*) or other echinocandins (*C. parapsilosis*). Anidulafungin has also shown excellent *in vitro* activity against several species of *Aspergillus*. Anidulafungin also demonstrates additive effects *in vitro* in combination with amphotericin B against *Aspergillus* species and *Fusarium* isolates and synergistic activity when combined with itraconazole or voriconazole against *Aspergillus* species. The compound has an extended half-life (18 h) compared with other members of the echinocandin class (reviewed by Vazquez and Sobel 2006; Cappelletty and Jung 2009).

Aminocandin (HMR3270) is a semi-synthetic fermentation product from *Aspergillus sydowi* structurally similar to mulunducandin but with the advantage of a half-life that is three to four times longer than that of other echinocandins. In *in vitro* studies aminocandin showed a potent activity against *Candida* spp. (including azoles- and amphotericin B-resistant species) and *Aspergillus* spp. (including itraconazole-resistant strains) but no activity against *Scedosporium* spp., *Fusarium* spp. and the Mucorales. In an immunocompromized mouse model of disseminated candidiasis, aminocandin has demonstrated activity comparable to amphotericin B with more than 70% survivors (Pasqualotto and Denning 2008).

Other echinocandin derivatives are the aerothricins (Aoki et al. 2000), the cyclic peptides reported from Fujisawa in a recent patent (Tojo et al. 2005) and the mulundocandins (Fig. 6.2) isolated from *Aspergillus sydowi*

(Mukhopadhyay et al. 1987a, b, 1992; Roy et al. 1987). Recently the sulfated echinocandin-like lipopeptides FR209602, FR209603 and FR209604 (Fig. 6.3) have been isolated from fermentations of *Coleophoma crateriformis* (Kanasaki et al. 2006a). The compounds showed *in vitro* antifungal activities against *C. albicans* and *A. fumigatus* with minimal effective concentrations (MEC) ranging from 0.02–0.04 $\mu\text{g/ml}$. The IC_{50} values for the inhibition of *C. albicans* 1,3- β -glucan synthase were 0.49, 0.64 and 0.72 $\mu\text{g/ml}$ respectively. FR209602 and FR209603 showed good efficacy by subcutaneous injection against *C. albicans* in a mouse model of systemic infection, with ED_{50} values of 2.0 and 1.9 mg/kg (Kanasaki et al. 2006b). The same group also reported the isolation of the echinocandin analogs FR220897, FR220899 from *Coleophoma empetri*, with altered amino acids in the cyclic peptide ring and the sulfate residue at a different position of the aryl ring, and the novel lipopeptides FR227673 and FR190293 isolated from *Chalara* sp. and *Tolypocladium parasiticum*, which have the same peptide nucleus as FR901379 but differ in the acyl side chains. These compounds showed antifungal activity against *A. fumigatus* and *C. albicans* and inhibited 1,3- β -glucan synthase. FR220897 was also active in a *C. albicans* mouse infection model (Kanasaki et al. 2006c, d).

Resistance to echinocandins has been described for some clinical isolates of *C. albicans* obtained from patients who failed or responded poorly to caspofungin and some spontaneous *C. albicans* mutants resistant to N2-PnB0, an analog of caspofungin. Biochemical analyses revealed that the decreased susceptibility to caspofungin is due to amino acid mutations in two defined regions of the highly conserved FKS1 gene, which renders the mutant enzyme 1000-fold less sensitive to the drug (reviewed by Perlin 2007). Recently it was shown that a single serine to proline substitution (S678P) in the Fks1p was sufficient to confer echinocandin resistance in *Aspergillus fumigatus* (Rocha et al. 2007).

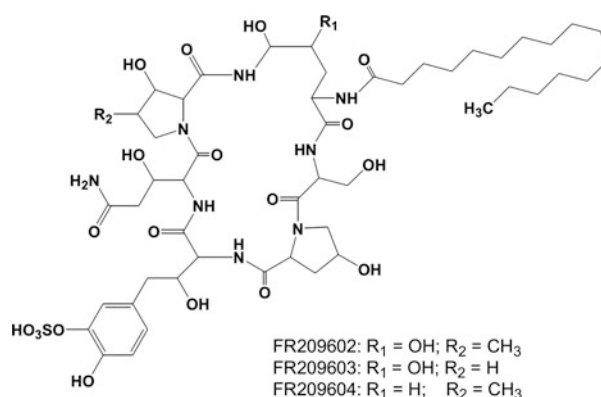


Fig. 6.3. Structures of FR209602, FR209603 and FR209604

2. Papulacandins

The second family of (1,3)- β -D-glucan synthase inhibitors are the papulacandins (Traxler et al. 1977a, b, 1980). These glycolipids consist of a modified spirocyclic diglycoside linked to two fatty acids (e.g. papulacandin B, chaetiacandin; Komori and Itoh 1985; Komori et al. 1985; Fig. 6.4). Papulacandin B and chaetiacandin are active *in vitro* against a variety of *Candida* species with minimum inhibitory concentrations (MICs) of 0.2 μ g/ml or less. However, papulacandin is not active against other pathogens, such as *Candida guilliermondii*, *A. fumigatus* and *C. neoformans*. Despite medicinal chemistry efforts to improve the efficacies of these compounds, the papulacandins have not been developed further because they have limited potency in animal models (Traxler et al. 1987; Georgopapadakou and Tkacz 1995; Yeung et al. 1996; Fostel and Lartey 2000).

3. Acidic Triterpenes

Screening for new inhibitors of glucan biosynthesis with improved pharmacokinetic properties resulted in a new class of antifungal triterpenes with a polar (acidic) moiety. The glycosidic triterpene ascosteroside A (Fig. 6.5) was originally isolated as an antifungal metabolite from *Ascotricha amphitricha* (Gorman et al. 1996). Recently, the closely related ascosteroside B has been isolated from fermentations of an endophytic fungus, taxonomically unrelated to the original producer (Weber et al. 2007).

The related glycoside, enfumafungin (Fig. 6.5) was described for *Hormonema* spp. (Schwartz et al. 2000). Another member of this class of antifungal triterpenes is arundifungin containing a succinate moiety, which was originally isolated from *Arthrinium arundis* (Apiosporaceae, Xylariales) by Cabello et al. (2001). It was also found in a wide range of other fungi (including endophytes) from diverse habitats and geographic origins (Caballo et al. 2001; Weber et al. 2007). Ergokonin A, a sulfate-derivatized amino acid containing triterpene, was reported for various *Trichoderma* species (Augustinak et al. 1991; Vicente et al. 2001).

Studies on the mode of action showed, that these compounds inhibit glucan synthesis in whole cells and in (1,3)- β -D-glucan synthase assays. The antifungal spectra of the acidic terpenoids are comparable to those of the known glucan synthase inhibitors echinocandins and papulacandins. *Candida* and *Aspergillus* are highly sensitive, while *Cryptococcus* and bacterial strains are not inhibited (Onishi et al. 2000). In addition to the *in vitro* antifungal activity, two of the compounds, ascosteroside and enfumafungin, have detectable activity against *C. albicans* in animal models.

Recently a novel triterpene glycoside, FR227244 (Fig. 6.5), was isolated from fermentations of *Myrothecium cinctum* by Fujisawa Inc.. The compound exhibits *in vitro* antifungal activity against *Aspergillus* spp., *Trichophyton* spp., *Candida utilis* and *C. parapsilosis* but shows low activity against *C. albicans*, *C. krusei* and *C. tropicalis*. Specifically, FR227244 exhibits *in vitro* and *in vivo* antifungal activity against *A. fumigatus*.

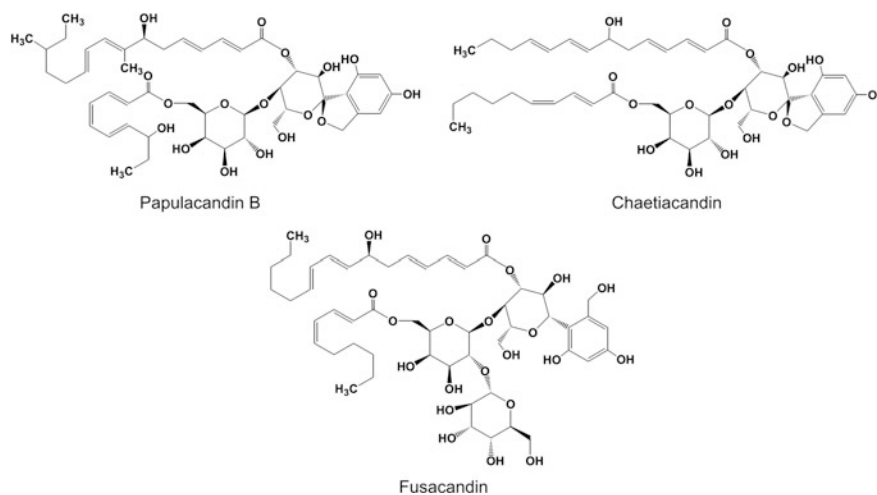


Fig. 6.4. Structures of some papulacandins

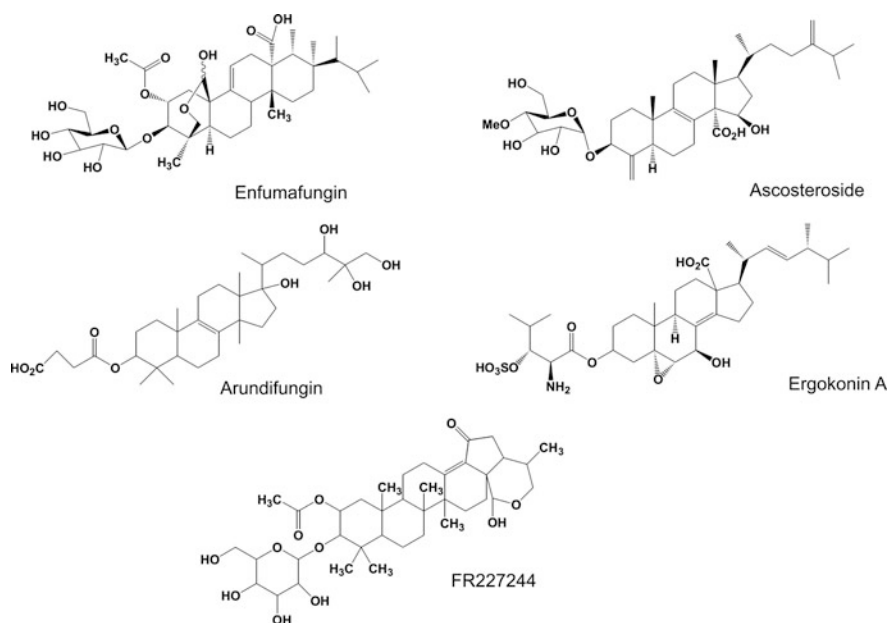


Fig. 6.5. Structures of some acidic triterpenes

The minimum effective concentration (MEC) of FR227244 against *A. fumigatus* was 0.031 $\mu\text{g}/\text{ml}$. FR227244 showed good efficacy after subcutaneous injection and oral administration against *A. fumigatus* in a mouse systemic infection model with ED_{50} values of 1.9 and 18 mg/kg, respectively. FR227244 inhibited glucan synthesis in a (1,3)- β -D-glucan synthase assay weakly and in whole cells strongly, but did not affect the synthesis of other macromolecules, including proteins, nucleic acids, mannan and chitin (Kobayashi et al. 2004).

Because of their solubility in water, the acidic triterpenes represent new promising lead structures for the development of oral active antifungal drugs and a variety of semi-synthetic derivatives of enfumafungin have recently been disclosed in a patent by Merck KGaA (Greenlee et al. 2007).

C. Sphingolipid Synthesis Inhibitors

Sphingolipids are a family of lipids that play pivotal roles as structural components of membranes in eukaryotic cells and also in the control of different cellular mechanisms, including cell proliferation, cell differentiation, apoptotic cell death, cell contraction, retraction and migration (reviewed by Hanun and Obeid 2008). Early on, it was recognized that sphingolipid signaling is important in human cancers. Later it was shown to play important roles in other human diseases,

such as diabetes and heart disease, microbial infections, neurological disorders including Alzheimer's disease and immune dysfunctions. In *Saccharomyces cerevisiae*, sphingolipid synthesis and signaling is essential for growth, stress response, cell wall integrity, endocytosis, drug resistance and translation (Dickson 2008). In pathogenic fungi such as *Cryptococcus neoformans*, *Candida albicans* and *Aspergillus nidulans*, sphingolipid signaling has been implicated in host-pathogen interactions and pathogenesis (Heung et al. 2006; Rhome and Del Poeta 2009). Inhibition of sphingolipid synthesis results in growth inhibition and cell death. The early steps in mammalian and fungal sphingolipid synthesis are conserved, but diverge thereafter to produce structurally and chemically different types of sphingoid bases, ceramides and complex sphingolipids (Dickson 2008). There are several enzymes unique for fungal sphingolipid synthesis and therefore, over the years, the sphingolipid biosynthetic pathway has been exploited as target for antifungal drugs against pathogenic yeasts (reviewed by Delgado et al. 2006; Pruett et al. 2008). Three key enzymes in the sphingolipid synthesis pathway are used to search for novel antifungals: serine palmitoyltransferase, ceramide synthase and inositol phosphoceramide (IPC) synthase.

1. Inhibitors of Serine Palmitoyltransferase

The early steps of the sphingolipid biosynthetic pathway take place in the endoplasmic reticulum and are conserved from fungi to mammals. The first step is the condensation of serine and palmitoyl CoA which is catalyzed by serine palmitoyltransferase (SPT)1. This enzyme belongs to a family of pyridoxal 5'-phosphate-dependent biocatalysts. The product, 3-ketodihydrosphingosine, is further metabolized to dihydrosphingosine followed by the formation of ceramide in mammals or phytoceramide in fungi. SPT-deficient *Saccharomyces cerevisiae* strains cannot grow in the absence of an exogenous supply of sphingolipids or sphingolipid precursors, indicating that sphingolipids are essential for cellular survival (Obeid et al. 2002; Cowart and Obeid 2007). Sphingoid base-like inhibitors of SPT are produced by a variety of fungi.

Myriocin (ISP1/thermozymocidin; Fig. 6.6) was originally isolated as an antifungal compound from fermentations of *Myriococcum albomyces* (Kluepfel et al. 1972) and an unidentified fungus (Craveri et al. 1972). Later ISP1 (which was identical with myriocin) was isolated from *Isaria sinclairii* (the imperfect stage of *Cordyceps sinclairii*) as a potent immunosuppressant (Fujita et al. 1993). Myriocin (ISP1) and the closely related 4-deoxy compounds, mycestericins isolated from *Mycelia sterilia* (Fujita et al. 1996), are strong inhibitors of both lymphocyte proliferation in mouse allogeneic mixed lymphocyte reaction (MLR) *in vitro* and generation of allo-reactive cytotoxic T lymphocytes in mice *in vivo*. They also induce apoptosis in cytotoxic T cells.

The sphingofungins A to F (illustrated in Fig. 6.6 by sphingofungin B) isolated from *Aspergillus*

fumigatus and *Paecilomyces variotii* are antifungal agents with a limited spectrum inhibiting the growth of various *Candida* species and *Cryptococcus neoformans* but were found to be inactive against filamentous fungi (Horn et al. 1992; VanMiddlesworth et al. 1992). Other compounds with similar structural features are fumifungin isolated from *Aspergillus fumigatus* (Mukhopadhyay et al. 1987a, b) malonofungin isolated from *Phaeramularia fusimaculans* (Berova et al. 1994) and sulfamisterin (Fig. 6.6) from a *Pycnidella* species, a sulfated 18-carbon myriocin-like analog (Yamashi-Hasegawa et al. 2005).

Myriocin, sphingofungin, mycesterin and sulfamisterin are potent and highly selective inhibitors of SPT, inhibiting mammalian SPT in cell-free preparations with IC_{50} values in the nanomolar range (Miyake et al. 1995). It has been suggested that inhibition occurs by reaction of these compounds with SPT to form adducts that mimic the natural intermediates of the catalytic reaction (Hanada 2003; Delgado et al. 2006). Interestingly micromolar concentrations of ISP1 (myriocin) were necessary for the inhibition of sphingolipid synthesis in *Saccharomyces cerevisiae* which were explained by the inactivation of ISP1 through the conversion to *N*-acetyl-ISP1 by the fungal *N*-acetyltransferase *SLII* (Momoi et al. 2004).

ISP1/myriocin has attracted much attention because of its immunosuppressive activity and several synthetic routes were elaborated for total synthesis and derivatization (reviewed by Liao et al. 2005). As treatment with myriocin was shown to cause severe gastrointestinal side effects, great effort has been put to the development of less toxic myriocin analogs. Among the numerous synthesized myriocin derivatives, FTY720 (Fig. 6.6) turned out to be most promising with a more potent immunosuppressive activity and less toxicity than myriocin and without inhibiting serine palmitoyltransferase (Fujita et al. 1996; Kiuchi et al. 2000). FTY720 is phosphorylated *in vivo* to its active form by the sphingosine kinase 2. FTY720-P exerts its effects as an antagonist of several sphingosine-1-phosphate

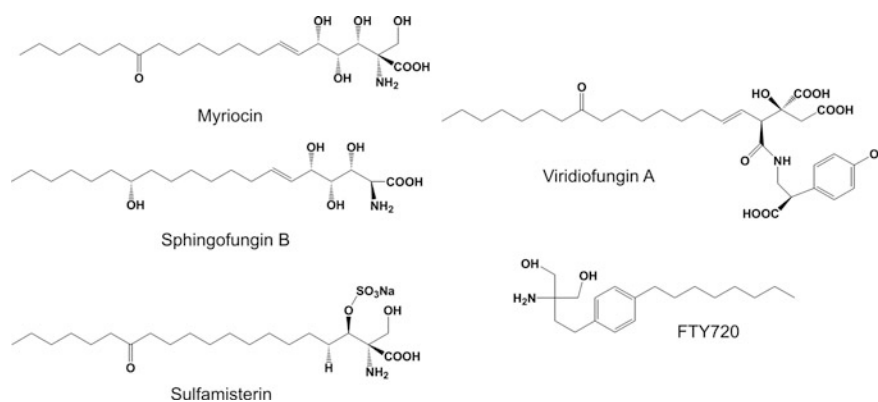


Fig. 6.6. Inhibitors of serine palmitoyltransferase

(S1P) receptors and induces internalization and degradation of the S1P₁ receptor which results in a prolonged receptor down-regulation and thereby depriving thymocytes and lymphocytes of an S1P signal necessary for their egress from secondary lymphoid tissue leading to a decrease in circulating lymphocytes. FTY720 is now being explored in Phase III studies for multiple sclerosis (reviewed by Zhang and Schluesener 2007; Kihara and Igarashi 2008; Takabe et al. 2008).

Viridifungins A (Fig. 6.6), B and C isolated from *Trichoderma viridae* are amino alkyl citrates with a broad-spectrum antifungal activity inhibiting the growth pathogenic fungi such as *Cryptococcus neoformans*, *Candida* spp. and *A. fumigatus*. The viridifungins exhibited potent inhibitory activity against serine palmitoyltransferase from *C. albicans* at nanomolar concentrations, whereas the *S. cerevisiae* SPT was resistant. In addition, these compounds also inhibit squalene synthase with IC₅₀ values in the micromolar range (Mandala et al. 1997a, b; Onishi et al. 1997).

2. Inhibitors of Ceramide Synthase

Ceramide synthase (CerS) catalyzes the acylation of the amino group of sphingosine, sphinganine and other sphingoid bases using acyl-CoA esters of varying chain length. CerS activity is found in microsomes and mitochondria (Hanada 2003; Delgado et al. 2006). Several sphingoid base-like inhibitors are produced by fungi (Du et al. 2008).

The fumonisins are family of mycotoxins produced by *Fusarium verticillioides* (formerly *F. moniliforme*) with fumonisin B1 (FB1; Fig. 6.7) being the most prevalent member of this class of compounds.

F. verticillioides is a common fungal contaminant of corn and some other grains and the consumption of fumonisins is associated with severe toxicological effects such as carcinogenicity, hepato- and renal toxicity, pulmonary edema, immunosuppression (and sometimes immunostimulation), neurotoxicity and more recently with birth defects (Desai et al. 2002; Gelineau-van Waes et al. 2005; Missmer et al. 2006). FB1 contains an amino-eicosapentenol backbone with two hydroxyl groups esterified with tricarballylic acid. A similar structure is present in the AAL-toxins (e.g. TA; Fig. 6.7) which are produced by *Alternaria alternata* sp. *lycopersici* and cause diseases in plants (Abbas et al. 1998). These compounds inhibit CerS by interaction of the aminopentol moiety with the binding site for the sphingoid base substrate as well as the polyanionic side chains with the binding site for the fatty acyl-CoA cosubstrate. The toxicity and carcinogenicity of fumonisins are attributed to the depletion of ceramide and complex sphingolipids and the concomitant accumulation of highly bioactive sphingoid bases. Removal of the tricarballylic acid(s) of FB1 diminishes the potency of the inhibition of CerS but converts the compound into a CerS substrate which is acetylated to the cytotoxic *N*-acylamino-pentols. These *N*-acetylated forms are potent inhibitors of CerS and this sequence of metabolic transformation may occur *in vivo* and play a role in the diseases caused by fumonisins (Desai et al. 2002; Delgado et al. 2006). Although fumonisin B1 inhibits fungal CerS *in vitro*, the fumonisins have very poor activity against whole-cell fungal sphingolipid synthesis or growth (Wu et al. 1995).

Australifungin (Fig. 6.7) isolated from the coprophilous ascomycete *Sporormiella australis* is a broad-spectrum antifungal compound, exhibiting activity against human pathogenic fungi with MICs against *Candida* spp., *Cryptococcus neoformans* and *Aspergillus* spp. between 0.015 and 1.0 μ g/ml.

Australifungin contains a unique combination of α -diketone and β -ketoaldehyde functional groups and

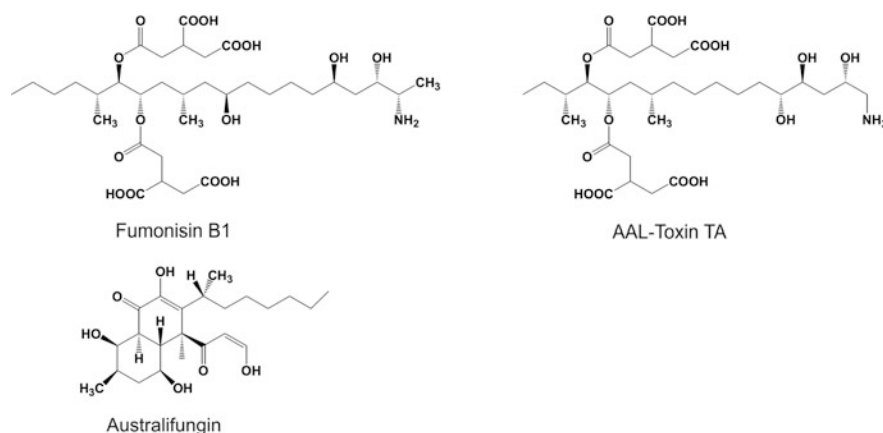


Fig. 6.7. Inhibitors of ceramide synthase

was the first non-sphingoid-based inhibitor described for the sphingolipid biosynthetic pathway. Australifungin was found to inhibit *C. albicans* ceramide synthase *in vivo* at nanomolar concentrations and caused an accumulation of early sphingolipid intermediates. Australofunginol, an analog isolated from the same fungus, showed a 50-fold less inhibitory activity against CerS (Mandala et al. 1995). Australifungin also inhibited ceramide synthesis in HepG2 cells, thus limiting its therapeutic use for the treatment of fungal infections (Mandala et al. 1997a, b).

3. Inhibitors of Inositol Phosphorylceramid Synthase

One enzyme that is unique to the fungal sphingolipid synthesis pathway is inositol phosphorylceramid (IPC) synthase. IPC synthase (or Ipc1) is responsible for the removal of an inositol-phosphate group from phosphatidylinositol and the transfer of that group to the terminal hydroxyl group of phytoceramide forming IPC and diacylglycerol. Ipc1 coded by the aureobasidin resistance gene 1 (*AUR1*) was shown to be essential in fungi and for the pathogenicity of *C. neoformans*. Downregulation of Ipc1 in *C. neoformans* diminishes melanin production and impairs intracellular growth within alveolar macrophages resulting in an attenuation of virulence in animal

models of cryptococcal meningitis. The production of diacylglycerol by IPC synthase is the major element in regulation of virulence since activation of protein kinase C by diacylglycerol is known to regulate melanin production, cell wall integrity and transcriptional activation of the antiphagocytic protein 1 (Shea and Del Poeta 2006; Rhome and Del Poeta 2009). As the first fungal-specific step in sphingolipid synthesis, IPC synthase has been pursued as a target for antifungal drug discovery (Sugimoto et al. 2004). Several natural compounds such as aureobasidins, khafrefungin, rustimycin and more recently pleofungins have been described as inhibitors of IPC synthase.

Aureobasidins A (Fig. 6.8) to R are antifungal cyclic depsipetides isolated from *Aureobasidium pullulans* and were found to strongly inhibit the growth of a variety of fungi including *Candida albicans*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae* but were considerably less effective against *Aspergillus fumigatus*. Their most potent representatives were described as aureobasidins A, B, C and E (Ikai et al. 1991; Takesako et al. 1991).

Later it was shown that aureobasidin A inhibits IPC synthase activities in various *Saccharomyces*, *Candida* and *Aspergillus* strains with IC₅₀ values ranging from

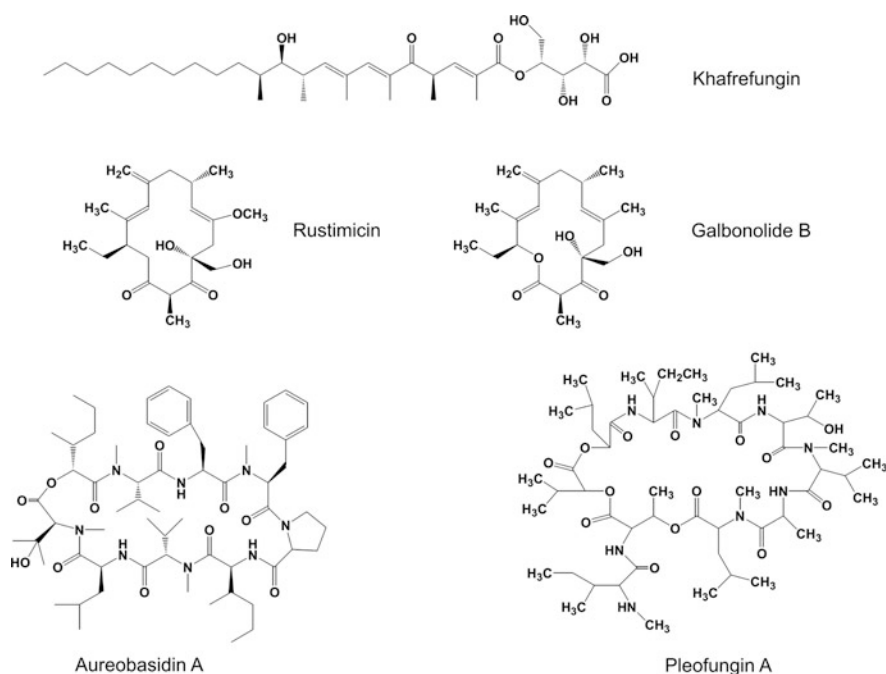


Fig. 6.8. Inhibitors of IPC synthase

0.2 to 4.9 nM (Nagiec et al. 1997; Zhong et al. 2000). Aureobasidin A showed a promising potential as therapeutic in a mouse model of systemic candidiasis with good efficacy and low toxicity (Takesako et al. 1993). However, modification of the depsipeptide of aureobasidin A, in order to increase the potency or the preparation of derivatives with structures that are less complex and retain the inhibitory properties of the native molecule, were largely unsuccessful (Rodriguez et al. 1996; Kurome et al. 1998). Recent investigations on the mode of action of aureobasidin A revealed that it is an irreversible, time-dependent inhibitor of IPC synthase in both *C. albicans* and *S. cerevisiae*. Moreover the entire intact structure of the compound appears to be important for both the affinity and the specific inhibitory mechanism of this compound (Aeed et al. 2009). The insensitivity of *A. fumigatus* and zygomycetous fungi to aureobasidin A was attributed to an increased efflux in the case of *A. fumigatus* and a different biosynthetic pathway present in zygomycetes (Zhong et al. 2000; Aoki et al. 2004).

The macrolides Rustimicin (galbonolide A), galbonide B and various analogs, isolated from fermentations of *Micromonospora* spp. (Takatsu et al. 1985; Harris et al. 1998) and the filamentous bacterium *Streptomyces galbus* (Achenbach et al. 1985), are also inhibitors of IPC synthase (Fig. 6.8).

Rustimicin showed a remarkable activity against *Cryptococcus neoformans* IPC synthase with an IC_{50} value of 70 pM and with IC_{50} values in the low nanomolar range against the enzymes from *Candida albicans* and *S. cerevisiae* (Mandala et al. 1998). However, in a mouse model of cryptococcosis, rustimicin A showed poor efficacy, which was probably caused by the rapid degradation into the inactive γ -lactone (Mandala et al. 1998).

The broad-spectrum antifungal compound khafrefungin (Fig. 6.8) was first isolated from the mycelium of a sterile endophytic fungus and consists of an aldonic acid moiety esterified at the C4 position with a C22 polyketide acid. Khafrefungin inhibits IPC synthases of *Candida albicans*, *S. cerevisiae* and *Cryptococcus neoformans* with IC_{50} values of 0.6, 7.0 and 31.0 nM, respectively, without affecting mammalian sphingolipid synthesis (Mandala et al. 1997a, b).

The absolute stereochemistry has been determined by total synthesis (Kobayashi et al. 2001; Wakabayashi et al. 2001) and studies of structure-activity relationships have revealed that the stereochemistry and the functional groups along the polyketide chain are crucial for the antifungal activity, as well as the ester group linking the above chain with the aldonic acid unit. Except for the lactone derivative, almost all derivatives lost antifungal activity indicating that, as in the case of aureobasidin A, the entire structure may have to be recognized by IPC synthase for an efficient inhibition (Nakamura et al. 2003).

Although aureobasidin A, rustimicin A and khafrefungin exhibit a highly potent and specific antifungal activity, their negligible activity against *A. fumigatus*, a major human fungal pathogen, is the major drawback for their application as antifungal drugs.

Recently, the novel depsipeptide IPC synthase inhibitors Pleofungins A, B, C and D have been described from fermentations of *Phoma* sp. SANK13899. Pleofungin A (Fig. 6.8), the most active compound, inhibited the IPC synthase of *S. cerevisiae* and *A. fumigatus* at IC_{50} values of 16 and 1.0 ng/ml, respectively. The inhibitor also suppressed the growth of *Candida albicans*, *Cryptococcus neoformans*, and *A. fumigatus* at MIC values of 2.0, 0.3, and 0.5 μ g/ml respectively. These biological properties indicate that pleofungins belong to a novel class of IPC synthase inhibitors also efficacious against *A. fumigatus* (Aoyagi et al. 2007; Yano et al. 2007).

A functional homolog of the fungal *Ipc1* gene was also identified in the pathogenic protozoan *Leishmania major* and it was shown that the corresponding IPC synthase was sensitive to the fungal IPC synthase inhibitor aureobasidin A (Denny et al. 2006). IPC synthase activities were also identified in *Trypanosoma cruzii* and *Trypanosoma brucei* (Denny et al. 2004; Figueiredo et al. 2005; Heung et al. 2006). Moreover, since unperturbed sphingolipid synthesis appears to be essential for infectivity of trypanosomes as well as *Toxoplasma gondii*, IPC synthase may represent a tractable target for the development of drugs for the treatment protozoan infections without detrimental effects to the mammalian host cells (McQuiston et al. 2006; Sonda et al. 2006; Suzuki et al. 2008).

D. Inhibitors of Protein Biosynthesis

Several antibiotics, such as aminoglycosides, tetracyclines, chloramphenicol and macrolides, that selectively interfere with prokaryotic protein biosynthesis are already in clinical use (Poehlsgaard and Douthwaite 2005). Quite contrary to the prokaryotes, fungal protein biosynthesis has – with the notable exception of cycloheximide – hardly been exploited for the development of antibiotics against fungal pathogens of plants, animals and men. A reason for this is the rather great similarity of the protein synthesizing apparatus of lower and higher eucaryotes including mammals. Most components are highly conserved and can be interchanged in cell-free systems with full retention of

functionality. A remarkable exception is the translation elongation factor 3 (eEF3) which is unique to fungi and essential for peptide chain elongation *in vitro* and *in vivo*. eEF3 is an ATP-binding protein that promotes the release of deacylated tRNA from the E-site, thereby increasing the affinity of aa-tRNA for the A-site (Triana-Alonso et al. 1995). The protein also physically and genetically interacts with eEF1A (Anand et al. 2003, 2006; Andersen et al. 2006). This promising target, however, has not yet been used for the development of antifungal drugs.

The most important family of antifungal compounds selectively interfering with fungal protein biosynthesis by interacting with elongation factor eEF2 and stabilizing the eEF2-ribosome complex are the sordarins and related compounds. Sordarin (Fig. 6.9) was originally isolated from the terrestrial ascomycete *Sordaria arenosa* (Hauser and Sigg 1971). All naturally

occurring sordarins contain the diterpene tetracyclic aglycon sordaricin connected to a sugar residue consisting usually of sordarose (Hauser and Sigg 1971), rhamnose – as in the recently isolated sordarin B (Weber et al. 2005) or alternatively a tricyclic uronic acid (Schneider et al. 1995; Okada et al. 1998). Also a further side chain can be attached.

Since the discovery of sordarin, several structurally related compounds sharing the common aglycone of sordarin have been isolated from diverse species of ascomycetous fungi like *Zopfiella marina* (Sordariaceae), an obligate marine species (zofimarin; Fig. 6.9; Ogita et al. 1987), a facultative marine strain of *Hypoxyton croceum* (hypoxysordarin; Daferner et al. 1999), *Graphium putredinis* (GR135402; Kennedy et al. 1998; Kinsman et al. 1998), *Xylaria longipes* (xylarin = SCH57404; Fig. 6.9; Schneider et al. 1995), *Penicillium minioluteum* (BE31405; Okada et al. 1998), an unidentified fungus (SCH57404 = xylarin; Coval et al. 1995), *Sordaria araneosa* (neosordarin; Davoli et al. 2002), *Podospora pleiospora* (sordarin B;

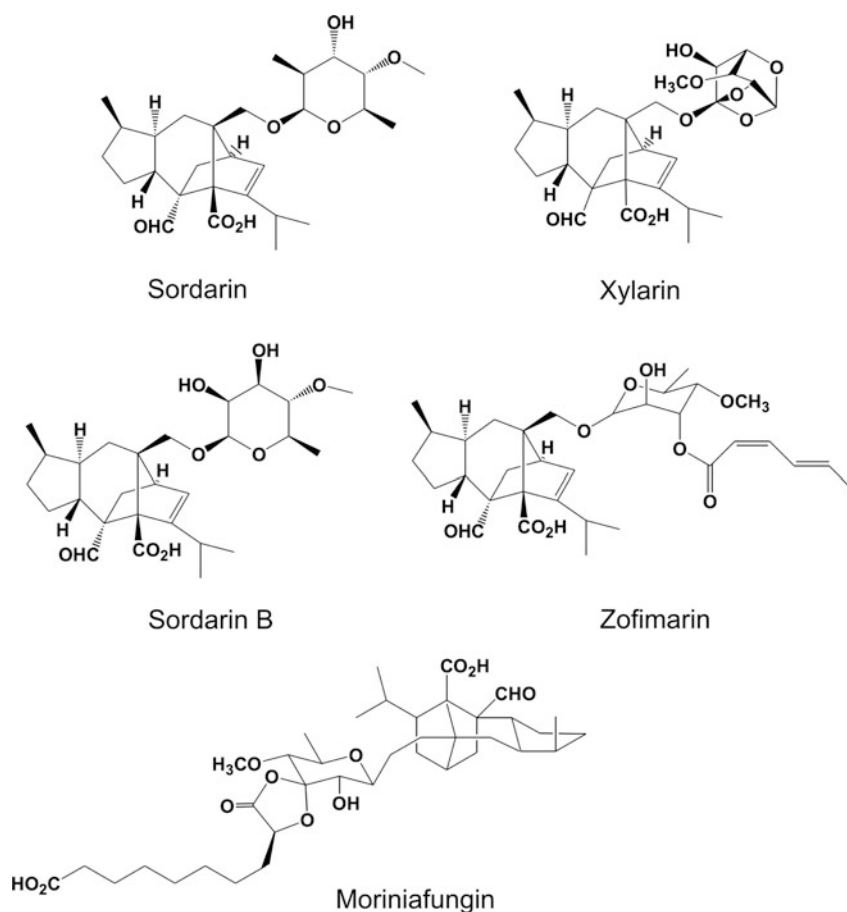


Fig. 6.9. Structures of some naturally occurring sordarins

Fig. 6.9; Weber et al. 2005) and more recently *Morinia pestalozzioides* (moriniafungin; Fig. 6.9; Basilio et al. 2006). A recent survey of the distribution of sordarins in various fungal taxa showed that all producing strains belong to the Xylariales being distributed across three families in this order, the Xylariaceae, the Amphisphaeriaceae and the Diatrypaceae (Vicente et al. 2009).

The sordarins show remarkable high and selective activities against a wide variety of fungi including *Candida albicans* (Herreros et al. 1998). All compounds of this class inhibited *in vitro* translation to various degree in *C. albicans*, *C. tropicalis*, *C. kefyr* and *Cryptococcus neoformans* but were inactive against *Candida glabrata* and *C. parapsilopsis*, with the notable exception of moriniafungin. These observations suggest a highly specific binding site of the sordarins, which may also be the basis for the selective inhibition of the fungal but not the mammalian protein synthesis.

The biochemical mechanism of action underlying the antifungal activity of sordarin was elucidated (Capa et al. 1998; Justice et al. 1998; reviewed by Domínguez and Martín 2004). Sordarins are selective inhibitors of fungal protein synthesis and impair the function of eEF2 thus stabilizing the fungal eEF2-ribosome complex. Genetic studies in *Saccharomyces cerevisiae* demonstrated that resistance to sordarin is conferred by mutations in eEF2 or the large ribosomal stalk protein rpP0, although eEF2 is the principal determinant of sordarin specificity (Gomez-Lorenzo et al. 1998; Justice et al. 1999). Since the binding of sordarin to eEF2 is greatly enhanced in the presence of ribosomes, the eEF2-ribosome complex involving 26S RNA, rpP0 and the G' subdomain of eEF2 must be considered as the functional target, where mutations in the stalk rpP0 protein induce resistance through allosteric effects (Domínguez and Martín 2004). These data suggest that the sordarin binding site seems to be quite susceptible to long-distance conformational changes.

Several strategies for the *de novo* chemical synthesis of sordaricin derivatives were reported and simplified agylcone analogs that resemble the pharmacophore of sordaricin were pursued; but so far, none of the simpler and more synthetically amenable structures have retained an interesting biological profile (reviewed by Liang 2008). However, new sordarin derivatives were obtained by derivatization of natural product intermediates as starting material. Sordaricin, sordarin and 4'-O-demethyl sordarin were derivatized at the sordarose moiety (e.g. acetylation, esterification, substitutions of hydroxyls by nitrogen or carbons etc.), including replacement by non-sugar arrangements.

Out of these, the semi-synthetic derivatives with different sugar portions such as GM 193663 (Fig. 6.10) and GM 237354 (Fig. 6.10) showed potent activity *in vitro* against *Candida* species with decreased fluconazole susceptibility and were also active in animal models of candidiasis, coccidiodomycosis, histoplasmosis and pneumocystosis but showed limited activity against *A. fumigatus* strains (Graybill et al. 1999; Clemons and Stevens 2000; Aviles et al. 2001; Martinez et al. 2001).

The azasordarins are a group of sordarins where the 4' sugar moiety was replaced by a 6-methylmorpholin-2-yl group with different *N*-4' substituents at position 8a of the sordaricin indacene ring system (illustrated in Fig. 6.10 by GW 479821). The azasordarins showed high activity against *Pneumocystis carinii* (MIC: 0.001–4.0 $\mu\text{g/ml}$) and most clinically important *Candida* species (MIC: 0.001–4.0 $\mu\text{g/ml}$), except *C. krusei* (Herreros et al. 2001). In addition they were also active against some emerging fungal pathogens, such as *Rhizopus arrhizus* (MIC: 1.0–4.0 $\mu\text{g/ml}$), *Blastoschizomyces capitatus* (MIC: 0.12–0.5 $\mu\text{g/ml}$) and *Geotrichum clavatum* (MIC: 0.12–0.5 $\mu\text{g/ml}$). The azasordarins were inactive against *Aspergillus* species and *Cryptococcus neoformans* (MIC: >16 $\mu\text{g/ml}$). Based on the lead structure GM 193663, a series of 3',4'-fused alkyl-tetrahydrofuran sordarin derivatives were prepared by replacement of an oxygen atom at the position C3' or C4' by a carbon atom (illustrated in Fig. 6.10 by compounds 6A, 9B). Several of these derivatives showed remarkable activity against *C. albicans*, *C. glabrata* and *C. tropicalis* but were inactive against *A. flavus* (Bueno et al. 2002). Chemical modification of the natural sordarin zofimarin led to R-135853 that possesses a *N*-methylpropenyl substituted 1,4-oxazepane ring moiety (Fig. 6.10). This compound exhibited potent activities against *C. albicans* including fluconazole resistant strains, *C. glabrata*, *C. guilliermondii* and *Cryptococcus neoformans*. The compound was highly absorbed by oral administration in mice and exhibited good *in vivo* efficacy in mouse models of hematogenous and esophageal candidiasis at oral doses of 20–50 mg/kg (Kamai et al. 2005). Recently a novel natural sordarin, moriniafungin (Figure 9), was isolated from *Morinia pestalozzioides* (Basilio et al. 2006). Although the compound has a broad antifungal spectrum including *Candida albicans*, *C. glabrata* and other clinically relevant *Candida* strains it was inactive in a mouse model of disseminated candidiasis with enhanced susceptibility to *C. albicans*. The chemical modification of the natural agylcone sordaricin resulted in the novel sordarin derivative FR290581 (Fig. 6.10) bearing a unique tri-substituted tetrahydrofuran ring. FR290581 exhibited good activity against *Candida* species, including azole-resistant *C. albicans* as well as azole-susceptible strains (MIC: 0.5–8.0 $\mu\text{g/ml}$) but showed no activity against *Aspergillus* species. FR290581 displayed potent *in vivo* activity, which, in mouse serum, was 100-fold superior to sordarin against *C. albicans*. FR290581 also showed a better pharmacokinetic profile and significantly longer half-life than sordarin and exhibited good efficacy in a mouse systemic candidiasis model of *C. albicans* (Hanadate et al. 2009).

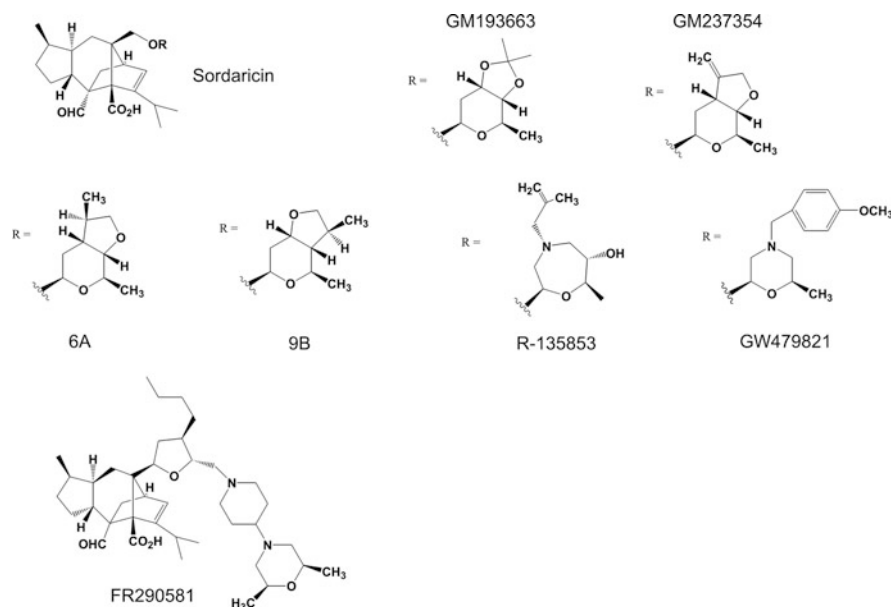


Fig. 6.10. Semisynthetic sordarin derivatives

Due to the broad-spectrum *in vivo* activity and the oral efficacy of some sordarins in animal models, they represent a promising group of new antifungal agents, although further studies will be needed to clarify their clinical usefulness.

E. Emerging Antifungal Therapies

Calcineurin is a heterodimeric Ca^{2+} -calmodulin-activated serine- and threonine-specific phosphatase consisting of a catalytic subunit (calcineurin A) and a regulatory subunit (calcineurin B), which is involved in various biological processes such as T cell activation and differentiation as well as different pathophysiological incidents.

In T cells, activation of the T cell receptor results in Ca^{2+} influxes into the cytosol from intracellular stores and calcineurin is bound by Ca^{2+} -calmodulin causing a conformational change that relieves repression of the catalytic site by an autoinhibitory domain. Activated calcineurin then dephosphorylates the cytoplasmic component of the nuclear transcription factor of activated T cells (NF-AT), which translocates to the nucleus and induces interleukin (IL)-2 transcription and T cell activation (Hogan et al. 2003; Macian 2005). The immunosuppressants FK506 and cyclosporine (CsA) block calcineurin's enzymatic function by forming intracellular complexes with FKBP12 and cyclophilin, which then bind to calcineurin and preventing NF-AT activation (McConnell and Wadzinski 2009). In pathogenic fungi, calcineurin plays a crucial role

in antibiotic resistance and virulence (Cowen 2008; Cowen and Steinbach 2008; reviewed by Stie and Fox 2008) and it has been shown that FK506-FKBP12 and CsA-cyclophilinA also inhibit calcineurin in pathogenic fungi including *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Indeed, cyclosporin A was first isolated because of its antifungal activity. The growth of *C. neoformans* at 37 °C was found to be sensitive to cyclosporine and FK506, and calcineurin mutants of *C. neoformans* and *Candida albicans* were attenuated in mouse systemic infection models. In *A. fumigatus*, calcineurin is required for hyphal growth, tissue invasion, and pathogenicity. Strains lacking calcineurin form short, blunted filaments resulting in a significant attenuation of virulence through a third distinct mechanism of action (reviewed by Steinbach et al. 2007; Kozubowski et al. 2009).

All these observations suggest that calcineurin could be an attractive antifungal target. Moreover various reports showed that *in vitro* FK506 and CsA chemosensitize *Candida albicans*, *A. fumigatus*, *Cryptococcus neoformans* and the dermatophyte *Trichophyton mentagrophytes* to azoles as well as other clinically available antifungals (e.g. caspofungin) and convert them into fungicidal compounds (reviewed by Steinbach et al. 2007; Bastidas et al. 2008 and references herein; Cowen and Steinbach 2008).

The clinical use of calcineurin inhibitors is limited by their immunosuppressive activity, however, the non-immunosuppressive FK 506 analog L-685,818 (Fig. 6.11) and the CsA analogs 211-810 and 209-825 (Fig. 6.11) were shown

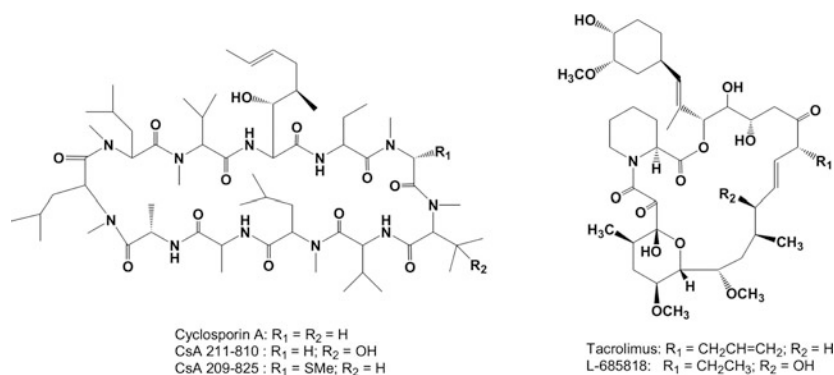


Fig. 6.11. Inhibitors of calcineurin signalling

to exhibit potent antifungal activity by inhibiting fungal calcineurin with concomitant diminished or absent immunosuppressive activities (Odom et al. 1997; Cruz et al. 2000; Onyewu 2003). In addition, other components which participate in calcineurin signaling such as the chaperone Hsp90 are currently investigated as potential antifungal drug targets. Genetic and protein interaction data suggest that calcineurin is a direct client of Hsp90 and a strong synergistic activity was observed between Hsp90 inhibitors (geldanamycin, radicicol) and azoles in *Candida albicans*, and with candins in *A. fumigatus* (Bastidas et al. 2008; Cowen et al. 2009).

These results establish fungal calcineurin and Hsp90 as attractive targets for the development of potent and specific inhibitors, even if the major challenge lies in discriminating between the signaling pathways and chaperone machineries of pathogen and host.

F. The Strobilurins and Oudemansins, Inhibitors of Fungal Respiration

The strobilurins are an important class of agricultural fungicides, representing about one-fifth of the world's fungicide market. At present nine different strobilurin derivatives have been successfully introduced into the fungicide market (Figs. 6.12, 6.13; Sauter 2007). They originate from the discovery of the strobilurins (Fig. 6.12) and oudemansins (Fig. 6.12), two natural fungicidal derivatives of β-methoxyacrylic acid. The strobilurins A and B were first detected in a screening of basidiomycete cultures for the production of antibioticly active compounds. Both compounds isolated from the basidiomycete *Strobilurus tenacellus* were shown to possess high and selective activity against several fungi, including phytopathogens (Anke et al. 1977).

The production of related compounds possessing the β-methoxyacrylate moiety, which is essential for the biological activity, was later described for various species of basidiomycetes such as *Agaricus* sp. 89139 (Zapf et al. 1993, Hellwig et al. 1999) as well as for the genera *Collybia* (Engler et al. 1998), *Crepidotus* (Weber et al. 1990a), *Cyphellopsis* (Weber et al. 1990b), *Favolaschia* (Zapf et al. 1995; Wood et al. 1996; Nicholas et al. 1997), *Filoboletus* (Simon et al. 1994), *Hydropus* (Bäuerle and Anke 1980), *Mycena* (Schramm et al. 1978; Bäuerle and Anke 1980; Daferner et al. 1998; Buchanan et al. 1999), *Oudemansiella* (Anke et al. 1979, 1990; Vondracek et al. 1983), *Pterula* (Engler et al. 1995), *Strobilurus* (Anke et al. 1977), *Xerula* (Anke et al. 1983) and an ascomycete, *Bolinea lutea* (Fredehagen et al. 1990a, b).

It is interesting that strobilurin producing species can be found all over the world in temperate and tropical climates. They can be detected in fruiting bodies and mycelial cultures on the natural substrates even in the natural habitat (Engler et al. 1998).

The strobilurins and oudemansins are potent and highly selective inhibitors of fungal mitochondrial respiration by binding to the ubihydroquinone oxidation (Q_p) center of the bc₁ complex and consequently, blocking the electron transfer between cytochrome b and cytochrome c₁ which, in turn, interrupts the energy cycle within the fungus by halting the production of ATP (Becker et al. 1981; Von Jagow et al. 1986).

The strobilurin-producing fungi are resistant against their own product. In *Strobilurus tenacellus* this is due to the exchange of three amino acid residues in positions 127, 254 and 261 within the ubihydroquinone (and strobilurin) binding site of cytochrome b (Kraiczky et al. 1996). Target side modification involving mutations in the cytochrome b gene CYTB, such as the substitution of glycine by alanine at position 143 (G143A), were observed in several phytopathogenic fungi resistant to strobilurins.

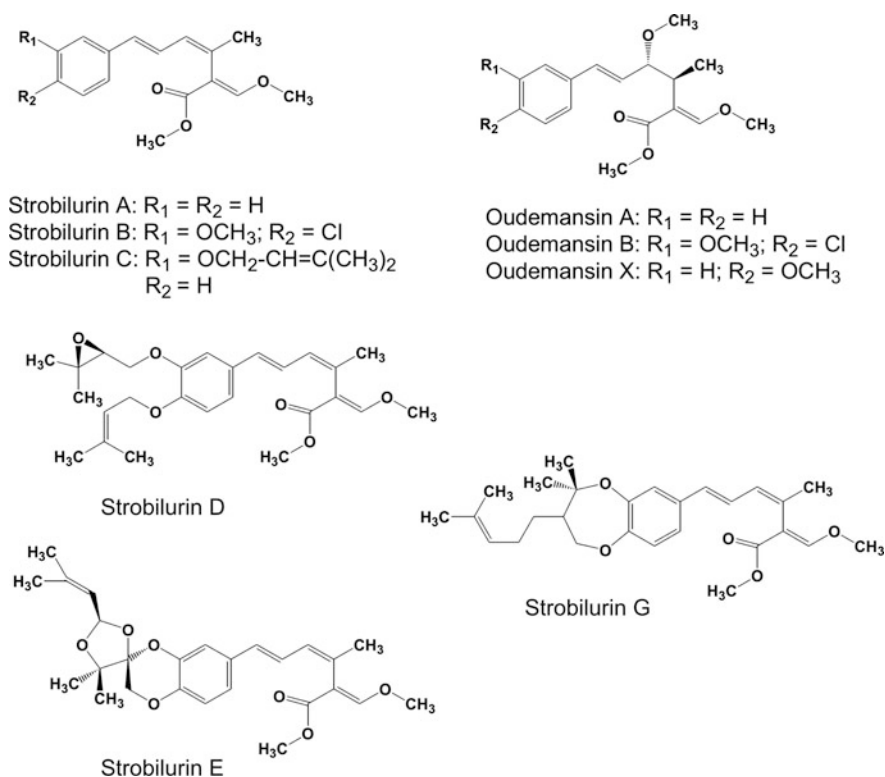
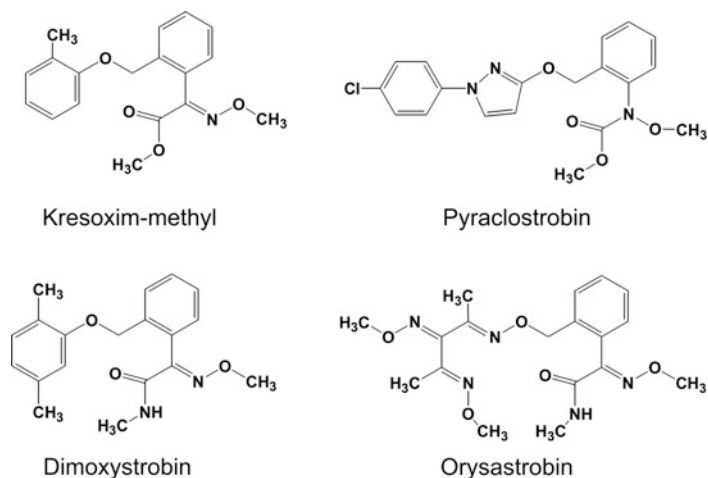


Fig. 6.12. Structures of some strobilurins and oudemansins

Fig. 6.13. Structures of some strobilurin derived agricultural fungicides



In addition other mechanisms including alternative respiration and efflux transporters may also be involved (Fernandez-Ortuño et al. 2008). Although the components of the respiratory chain and oxidative phosphorylation are highly conserved in eukaryotes, strobilurins A, B, C and X, as well as oudemansins A and B are relatively non-toxic for mice. According to different data, the peroral lethal dose, LD_{50} , for strobilurin A is 500 or 825 mg/kg. The intraperitoneal LD_{50} is 250 mg/kg and that for oudemansin A

exceeds 300 mg/kg (Zakharychev and Kovalenko 1998 and references herein).

Numerous synthetic studies to gain insight into the structural elements essential for the anti-fungal activity of the strobilurins, improvement of photostability, uptake into plants and molecular distribution have been reported and the reader is

referred to the excellent reviews by Sauter et al. (1999), Bartlett et al. (2002) and Sauter (2007), which give a comprehensive overview of the discovery, development and the chemical, biological and ecotoxicological properties of the strobilurins on the market.

III. Antibacterial Antibiotics

A. Fusidic Acid

The triterpenoid antibiotic fusidic acid (Fig. 6.14) was originally isolated from fermentations of the imperfect fungus *Fusidium coccineum* (recently referred to as *Paecilomyces fusidioides* and *Acremonium fusidioides*), and later other fungi, such as *Cephalosporium* spp., various dematophytes and *Isaria kogane*, were reported to produce the antibiotic (Godfredsen et al. 1962; Godfredsen et al. 1965; Haller and Loeffler 1969; Hikino et al. 1972; Perry et al. 1983). Fusidic acid is a

narrow-spectrum antibiotic used for the treatment of multiple-resistant staphylococcal infections or in combination with other antibiotics (Verbist 1990; Collignon and Turnidge 1999). Systemic application includes the treatment of septicemias, endocarditis, staphylococcal pneumonia, osteomyelitis and wound infections (Whitby 1999; Rigopoulos and Larios 2008).

Fusidic acid inhibits bacterial protein biosynthesis by interference with the elongation factor G (EF-G). In sensitive bacteria, fusidic acid prevents the dissociation of EF-G from the ribosome after hydrolysis of GTP and forms a stable complex of EF-G/fusidic acid/GDP/70S ribosome. The translocation step and the GTP cleavage are not inhibited. The inhibition of protein synthesis by fusidic acid is due to the drug-induced formation of a stable EF-G/fusidic acid/GDP/70S complex which cannot bind aminoacyl-tRNA. Therefore fusidic acid is not a direct inhibitor of translocation, but inhibits protein biosynthesis by preventing the functional binding of aminoacyl-tRNA to

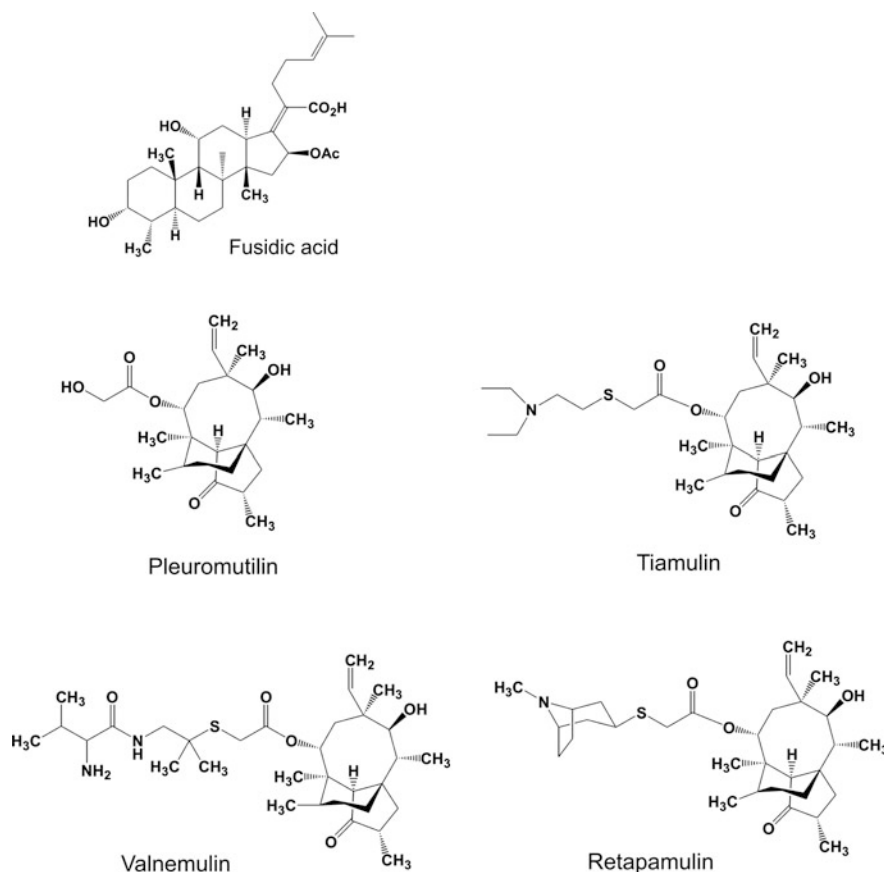


Fig. 6.14. Antibacterial antibiotics derived from fungi

the ribosome (SanMillian et al. 1975; Nierhaus and Wittman 1980; Spahn and Prescott 1996). Fusidic acid is effective in prokaryotes because they contain only one elongation factor. Eukaryotes, however, contain several other factors that are not inhibited by fusidic acid (Collignon and Turnidge 1999). Resistance to fusidic acid in clinical strains of *Staphylococcus aureus* occurs mainly by spontaneous mutations in the gene encoding for EF-G (*fusA*) or by horizontal acquisition of the *fusB* determinant, which encodes an EF-G-binding protein that protects the staphylococcal translation apparatus from inhibition by fusidic acid (O'Neill et al. 2004, 2007; O'Neill and Chopra 2006).

B. Pleuromutilins

A class of fungal antibiotics which have largely remained undeveloped for human clinical use is that of the pleuromutilins. The lead compound, pleuromutilin (Fig. 6.14), was first isolated from the basidiomycetes *Pleurotus mutilus* (syn. *Clitopilus scyphoides*), *P. passeckerianus* (Kavanagh et al. 1951) and later from *Clitopilus passeckerianus* (Brandl et al. 1968; Knauseder and Brandl 1976). Pleuromutilin is active against Gram-positive bacteria but the most interesting biological activity is its high effectiveness against various forms of mycoplasmas. Several semi-synthetic derivatives with improved antibacterial activity such as tiamulin, valnemulin and retapamulin (Fig. 6.14) were synthesized retaining a common tricyclic mutilin core, a C21 keto group, essential for antimicrobial activity (Egger and Reinshagen 1976a, b) and adding various substituents at C14, most of which are extensions of diverse chemical nature, such as thioacetates or thiocarbamates (Brooks et al. 2001).

The pleuromutilins (Fig. 6.14) have a special mode of action, which involves inhibition of bacterial protein synthesis by binding to the domain V of 23S rRNA at the 50S large prokaryotic ribosomal subunit and blocking peptide formation directly by interfering with substrate binding (Schlünzen et al. 2004; Long et al. 2006; Champney and Rogers 2007; Davidovich et al. 2007, 2008). Their binding site is unique compared to other ribosomally targeted inhibitors. Thus, pleuromutilins have no target-specific cross-resistance to other antibacterials (Yonath 2005). However, mutations in the genes encoding for the 23S rRNA and the ribosomal L3 protein proximal to

the peptidyl transferase center were shown to lead to a reduced susceptibility to tiamulin (Pringle et al. 2004; Long et al. 2006).

The semisynthetic pleuromutilin analogs tiamulin and valnemulin are currently used in veterinary practice for the control and treatment of serious infections in swine. Tiamulin is highly active against Gram-positive bacteria, *Mycoplasma* spp. and intestinal spirochetes ($MIC \leq 1 \mu\text{g/ml}$) and also shows potent activity against some staphylococci of human origin ($MIC_{50} \leq 0.5 \mu\text{g/ml}$). The C14-sulfanyl-acetate pleuromutilin derivative retapamulin (ointment 1%) has recently been approved in the United States and Europe for topical applications in the treatment of impetigo caused by *Staphylococcus aureus* and *Streptococcus pyogenes* and, also in Europe, for secondarily infected wounds (Daum et al. 2007). Retapamulin is highly active against staphylococci and β -hemolytic streptococci with MIC_{90} values of $0.12 \mu\text{g/ml}$ for *Staphylococcus aureus* and $< 0.03 \mu\text{g/ml}$ for *Streptococcus pyogenes*. No cross-resistance was observed for an organism subset resistant to oxacillin, erythromycin or mupirocin (Jones et al. 2006). Unfortunately, retapamulin has very little activity against Gram-negative bacteria and most *Enterococcus* species (Traczewski and Brown 2008).

Recently a series of novel pleuromutilin derivatives for human use bearing a purine ring linked by a piperazine ring spacer were synthesized. These compounds show a potent antibacterial activity in pulmonary infection models, including methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae* and *S. pyogenes*. In addition these compounds also show a better solubility in water, good pharmacokinetics and ADME properties and a better metabolic stability than the previous analogs (reviewed by Hu and Zou 2009). The generation of novel pleuromutilin conjugates with nucleosides or acyclic nucleoside derivatives in the side chain by a click chemistry approach were recently described by Lolk et al. (2008), which allows a convenient approach toward a library of active pleuromutilin derivatives in the search for future therapeutic antibiotics.

IV. Conclusions

There are relatively few antimicrobial structural scaffolds that are purely synthetic in origin, and humans rely heavily upon many natural products

and their derivatives as agrochemicals, antimicrobials, immunosuppressants and antineoplastic agents (Baker et al. 2007). Natural products derived from fungi still have an enormous impact on the development of new drugs for almost all therapeutic areas. The increase in multidrug-resistant pathogens to the widely used anti-infectiva is a great unmet medical problem, and new classes of antibiotics are in great need. Given the fact that only 7–8% of all fungal species are known (Hawksworth 2001), only a small portion of the chemical diversity of fungal compounds has been exploited so far and therefore less thoroughly investigated fungal taxa among the basidiomycetes, endophytic fungi, insect-associated fungi or marine fungi offer a great opportunity to discover new biologically active compounds (Lorenzen and Anke 1998; Zjawiony 2004; Gunatilaka 2006; Zang et al. 2006; Saleem et al. 2007; Blunt et al. 2009). In addition recent progress in fermentation technologies, characterization and genetic manipulation of biosynthetic pathways of fungal products, new screening methods (e.g. high-throughput techniques), the identification of new targets (e.g. by transcriptomic and proteomic methods, chemical genetics) as well as the advances in natural product chemistry have significantly furthered the discovery of novel chemical lead structures for the development of new medications and agrochemicals (Butler 2004; Spring 2005; Hanessian 2006; Harvey 2008; Schneider et al. 2008).

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7 Insecticidal and Nematicidal Metabolites from Fungi

HEIDRUN ANKE¹

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I. Introduction

In our last review (Anke and Sterner 2002), published in the first edition of this volume, we focused on the chemical diversity of nematicidal and insecticidal fungal metabolites and the producing organisms. Since then, only a few novel compounds have been described. However, many publications have appeared which describe the biological activities of some of the described compounds (other than insecticidal/nematicidal) or their mode of action. In addition, the ecological significance of some of the compounds has been partly elucidated, e.g. their role in insect pathogens during the colonization of the host. Therefore this chapter also includes some of these aspects.

II. Novel Compounds and Their Producers

A. Peptides, Cyclic Peptides, and Cyclic Depsipeptides

Cyclic peptides and depsipeptides produced by fungi, among them insect pathogenic fungi (e.g.

members of the genera *Aschersonia*, *Beauveria*, *Isaria*, *Metarhizium*, *Paecilomyces*, *Verticillium*), and their occurrence have been summarized in four comprehensive review articles (Anke and Sterner 2002; Zimmermann 2007a, b; Anke and Antelo 2009).

Since 2000, new producers of bioactive depsipeptides have been reported, for example, *Beauveria fellina* strains of marine origin (Lira et al. 2006), *Verticillium* sp. FKI-1033 (Monma et al. 2006), *Aspergillus carneus* (Capon et al. 2003), *Torrubiella luteoestrata* and its anamorph *Paecilomyces cinnamomeus* (both isolated from a scale insect; Isaka et al. 2007), *Verticillium hemipterigenum* (Nilanonta et al. 2003; Supothina et al. 2004), an *Aureobasidium* species from the tropical rain forest (Boros et al. 2006), an unidentified endophytic fungus (Huang et al. 2007), and a soil-borne *Phoma* species (Aoyagi et al. 2007). For a compilation of beauvericins and enniatins produced by *Cordyceps* species and their anamorphs as well as other insect pathogens, see Isaka et al. (2005a, b). Pseudodestruxins were found in *Nigrosabulum globosum* (Che et al. 2001) and reviews on destruxins and the producing organisms were published by Pedras et al. (2002) and Zimmermann (2007b). Some of the relevant compounds are listed in Table 7.1.

Chemical screening by HPLC-MS techniques led to the identification of five novel beauverolides from *Beauveria bassiana* and *Paecilomyces* spp. (Kuzma et al. 2001; Jegorov et al. 2004). Specific protocols for the detection and quantification of insecticidal cyclodepsipeptides in fungal cultures are now available (Jegorov et al. 2003).

Besides cyclopeptides and depsipeptides, fungi can also produce linear peptides with insecticidal activity; recently reported examples are efrageptin G and methylated peptides from a marine fungus associated with a sponge, neoefrapeptins from *Geotrichum candidum* (Nagaraj et al. 2001; Fredenhagen et al. 2006) and the Aib-containing

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Table 7.1. Examples of insecticidal and nematicidal compounds reported lately. The structures are given in Figs. 7.1–7.5

Producing fungus	Compound	Structure	References
<i>Geotrichum candidium</i>	Neoefrapeptin A	Fig. 7.1	Fredenhagen et al. (2006)
Marine isolate	Efrapeptin G	Fig. 7.1	Nagaraj et al. (2001)
<i>Cordyceps heteropoda</i>	Cicadapeptin I	Fig. 7.1	Krasnoff et al. (2005)
<i>Paecilomyces lilacinus</i>	Leucinostatin B	Fig. 7.1	Park et al. (2004)
<i>Omphalotus olearius</i>	Omphalontins E–I	Fig. 7.2	Liermann et al. (2009)
<i>Nigrosabulum globosum</i>	Pseudodestruxin A	Fig. 7.2	Che et al. (2000)
<i>Beauveria bassiana</i>	Beauverolide N	Fig. 7.2	Kuzma et al. (2003)
<i>Verticillium</i> sp. FKI-1033	Verticillide	Fig. 7.2	Momna et al. (2006)
<i>Gliocladium</i> sp. FTD-0668	Argifin	Fig. 7.3	Arai et al. (2000)
<i>Laptosphaeria maculans</i>	Sirodesmin PL	Fig. 7.3	Boudart (1989)
<i>Metarhizium anisopliae</i>	Serinocyclin	Fig. 7.3	Krasnoff et al. (2007)
<i>Epichloe typhina</i>	Epichlicin	Fig. 7.3	Seto et al. (2007)
<i>Fusarium</i> sp.	Apicidin	Fig. 7.3	Singh et al. (2002)
<i>Penicillium cluniae</i>	Paraherquamides H, I	Fig. 7.4	López-Gresa et al. (2006)
<i>Galiella rufa</i>	Pregaliellalactone	Fig. 7.4	Köpcke et al. (2002a, b)
<i>Penicillium</i> sp. FKI-2140	Quinololone B, yaequinolones D, F	Fig. 7.4	Uchida et al. (2006a, b)
<i>Penicillium bilaiae</i>	Penipratynolene, 2, 6-pyridinedicarboxylic acid	Fig. 7.4	Nakahara et al. (2004)
<i>Penicillium citrinum</i>	Quinolactacide	Fig. 7.4	Abe et al. (2005)
<i>Penicillium simplicissimum</i>	Peniprequinolone	Fig. 7.4	Kusano et al. (2000)
<i>Aspergillus niger</i>	Nafuredin- γ	Fig. 7.4	Omura et al. (2001)
<i>Nodulisporium</i> sp.	Nodulisporic acids B ₂ , C	Fig. 7.5	Onydeyka et al. (2003)
<i>Coronophora gregaria</i>	MK7924	Fig. 7.5	Kumazawa et al. (2003)
<i>Penicillium expansum</i>	Communesins C, D, E	Fig. 7.5	Hayashi et al. (2004)

cicadapeptins I and II from *Cordyceps heteropoda* (Krasnoff et al. 2005). Structures of some linear peptides are shown in Fig. 7.1, cyclic peptides and depsipeptides in Figs. 7.2 and 7.3. The neoefrapeptins comprise a large group of ten 16-residue and two 13-residue peptides containing also non-proteinogenic amino acids (Fredenhagen et al. 2006).

From protoplasts of a monokaryotic strain of the basidiomycete *Omphalotus olearius*, five novel hydroxylated omphalotin derivatives (omphalotin E–I) were isolated, complementing the already known omphalotins.

Interestingly the monokaryotic strain grew faster and produced higher amounts of these compounds than the dikaryotic parental strain from which it was obtained (Liermann et al. 2009). All *O. olearius* strains, irrespective of their geographical origin, seem to produce omphalotin derivatives (Anke et al., unpublished data). In fruiting bodies, omphalotins could not be detected. Omphalotins E–I exhibited similar nematicidal activities against *Meloidogyne incognita* (Kofoid & White) as omphalotin A. Antibacterial or antifungal activities were not detected and none of the compounds showed cytotoxic effects towards mouse leukemia cells (L1012 cells) or human colon adenocarcinoma cells (Colo 320 cells) at concentrations up to 50 $\mu\text{g/ml}$. The novel compounds were seemingly produced at the expense of the known omphalotins,

including omphalotin A, the amount of which decreased drastically towards the end of the fermentation (Liermann et al. 2009).

B. Novel Metabolites and New Derivatives of Insecticidal or Nematicidal Metabolites

A screening of some 500 endophytic fungi for the production of nematicidal metabolites led to the selection of 17 strains with selective activity towards *Meloidogyne incognita*, while *Caenorhabditis elegans* was less affected (Schwarz et al. 2004). In five strains identified as *Phomopsis phaseoli* and *Melanconium betulinum* (four strains), the nematicidal principle was found to be the simple metabolite 3-hydroxypropionic acid (Schwarz et al. 2004).

In the Sarcosomataceae (teleomorphs of the genera *Galiella*, *Urnula*, *Strumella*), the production of the galiellalactone precursors pregaliellalactone (Fig. 7.4) and structurally related hexaketides accounted for the nematicidal activity. Interestingly an endophytic strain of *Cistus salviifolius*, which according to its 18S rDNA sequences also belongs to the Sarcosomataceae, produced the same compounds (Köpcke et al.

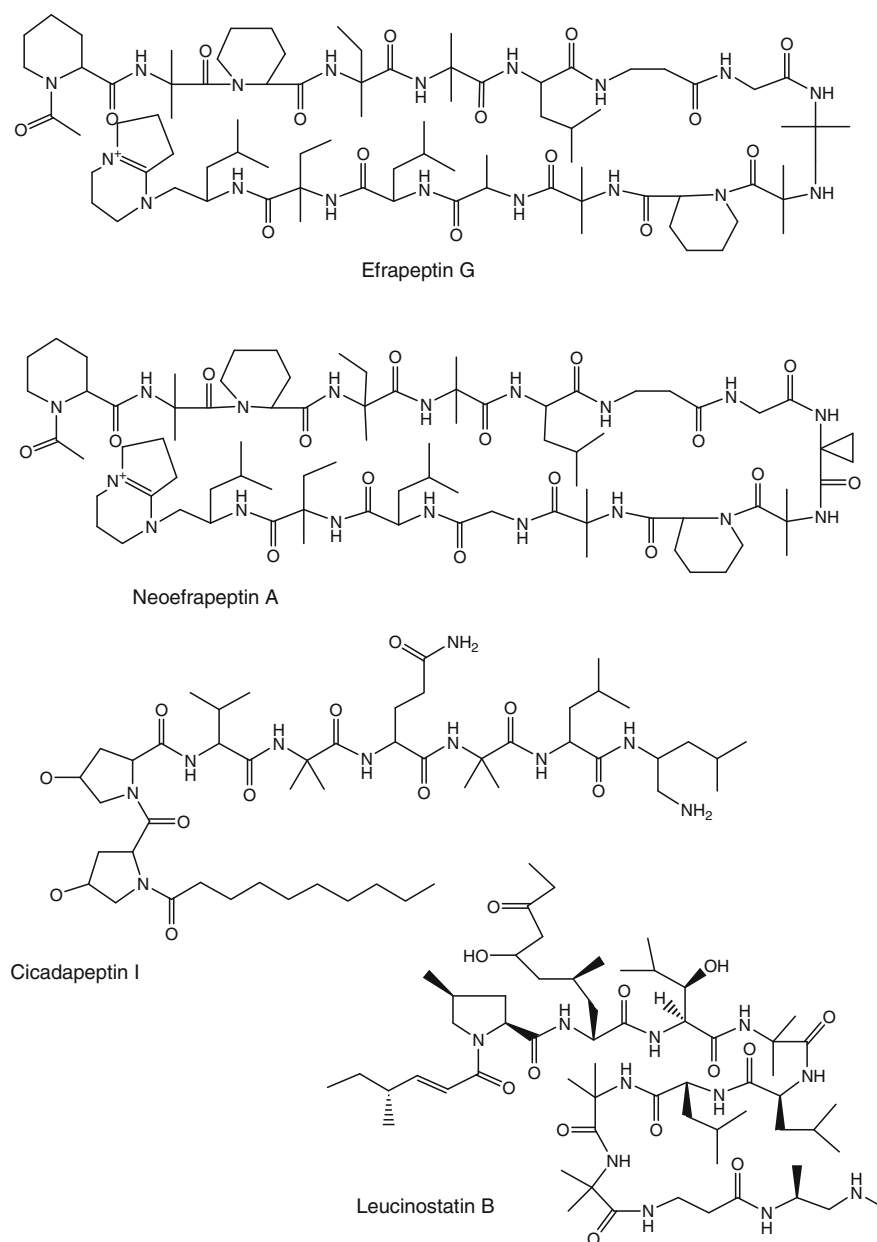


Fig. 7.1. Neofrapeptin A (produced by *Geotrichum candidum*). Efrapeptin G (from a marine isolate). Cicadapeptin I (from *Cordyceps heteropoda*). leucinostatin B (from *Paecilomyces lilacinus*)

2002a, b). A further nematicidal metabolite, MK7924 (Fig. 7.5), with weak activity against *Aspergillus niger* and *Caenorhabditis elegans* was isolated from *Coronophora gregaria* (Kumazawa et al. 2003).

Quinolactacide (Fig. 7.4), an insecticidal quinolone, was isolated from solid-state cultures of *Penicillium citrinum* (Abe et al. 2005). Using *Artemia salina* as test organism, seven novel yaequinolones

were isolated from *Penicillium* sp. FKI-2140, together with nine known and structurally related compounds, among them quinolinones A and B, penigequinolones A and B, and peniprequinolone (Uchida et al. 2006a, b).

Quinolone derivatives were also obtained from other *Penicillium* species, e.g. *P. janczewskii* (He et al. 2005) and *P. simplicissimum* (Kusano et al. 2000). Peniprequinolone, in addition to its strong insecticidal properties,

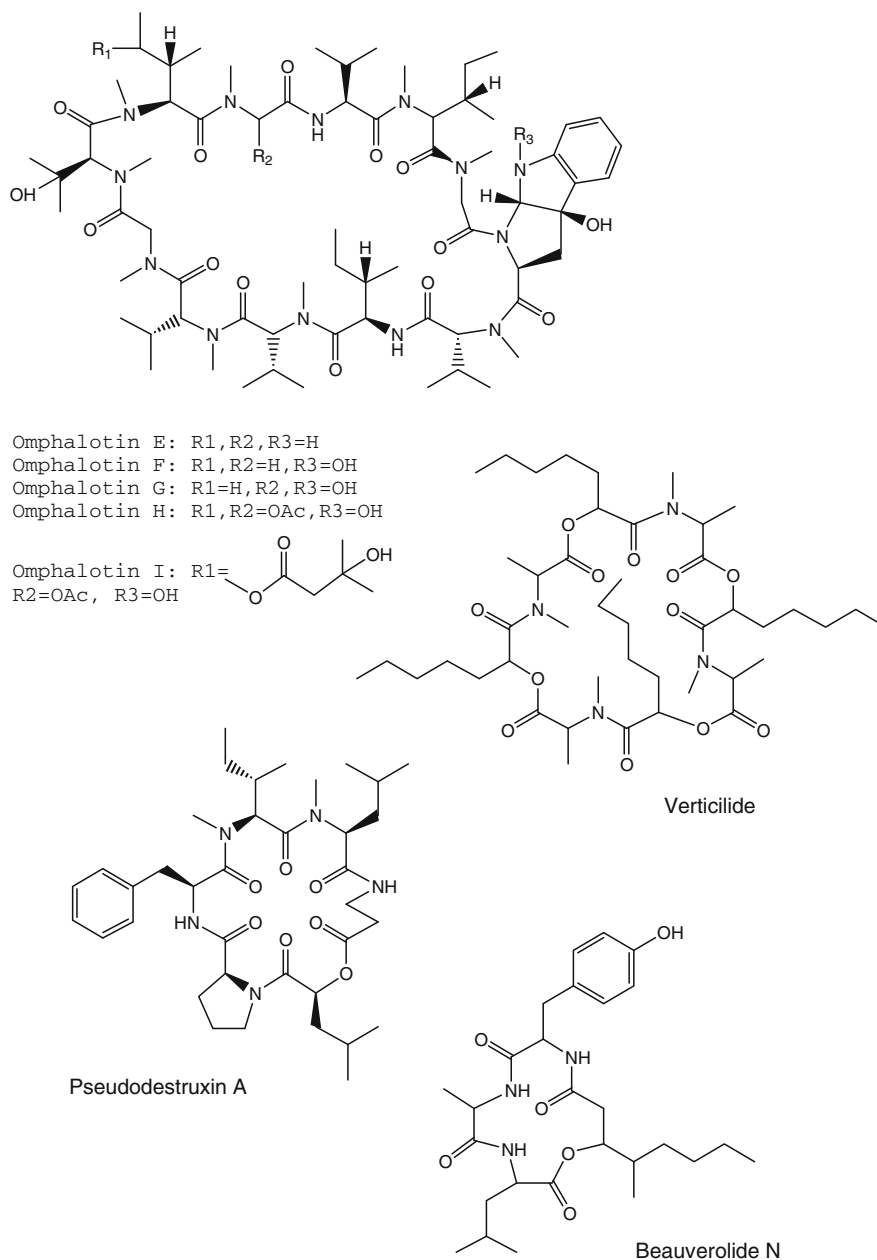


Fig. 7.2. Omphalontins E-I (from *Omphalotus olearius*). Pseudodestruxin A (from *Nigrosabulum globosum*). Beauverolide N (from *Beauveria bassiana*). Verticillide (from *Verticillium* sp. FKI-1033)

inhibits the root-lesion nematode *Pratylenchus penetrans* (Kusano et al. 2000). Some of the quinolones and their producers are listed in Table 7.1, and their structures are given in Fig. 7.4. Another *Penicillium* metabolite active against the nematode *Pratylenchus penetrans* is penipratinolene (Nakahara et al. 2004). The compound was isolated together with 6-methoxy-carboxypicolinic acid and 2,6-pyridinedicarboxylic acid (Fig. 7.4) from *Penicillium bilaiae*.

New nodulisporic acids were isolated from a *Nodulisporium* sp., namely nodulisporic acids B, B₁, B₂, and C, C₁, C₂, and Δ^{23} nodulisporic acid C₄ (Ondeyka et al. 2002, 2003; Singh et al. 2004). The compounds are active against fleas on dogs (Shoop et al. 2001) and comprise complex chemical structures (see Fig. 7.5). From *Penicillium expansum* Link MK-5, communesins C, D, and E (Fig. 7.5) were

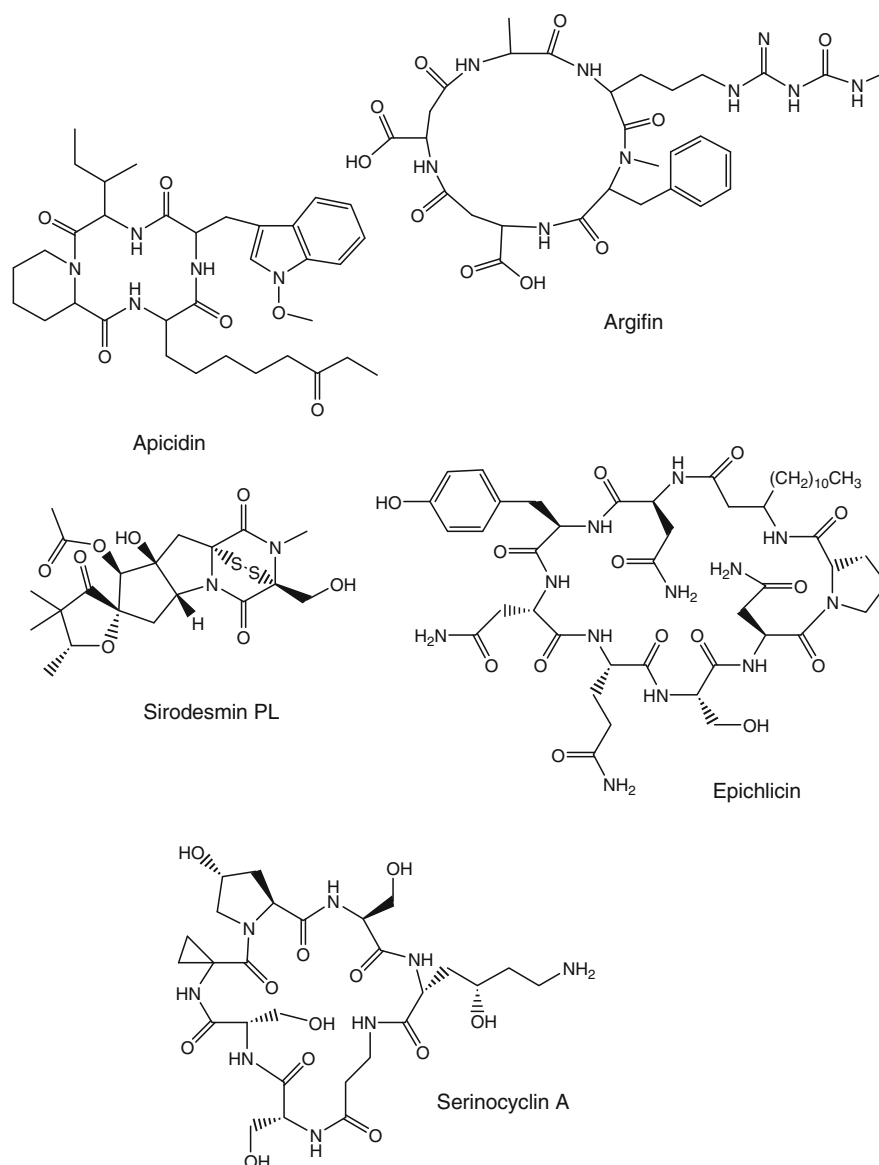


Fig. 7.3. Argifin (from *Gliocladium* sp. FTD-0668). Sirodesmin PL (from *Laeptosphaeria maculans*). Serinocyclin (from *Metarhizium anisopliae*). Epichlicin (from *Epichloe typhina*). Apicidin (from *Fusarium* sp.)

obtained, along with two known communesins (A, B). All novel communesins showed selective insecticidal activity against silkworms (Hayashi et al. 2004).

The large family of paraherquamide metabolites has gained new members from *P. cluniae* Quintanilla: paraherquamide H and I (Fig. 7.4) together with five known derivatives. Structure-activity relationships revealed paraherquamide E to be the most potent member, with a LD_{50} of 0.089 $\mu\text{g}/\text{nymph}$ of the milkweed bug *Oncopeltus fasciatus* Dallas (López-Gresa et al. 2006).

Nafuredin is one of the few insecticidal metabolites that does not contain nitrogen, i.e. it is not an alkaloid. The substance was isolated from a marine-derived *Aspergillus niger* strain using mitochondria of *Ascaris suum* as test system with the aim to find inhibitors of NADH-fumarate reductase from helminths (Omura et al. 2001). By weak alkaline treatment the compound was converted to its γ -lactone (Fig. 7.4) which also inhibits NADH-fumarate reductase (Shiomi et al. 2005).

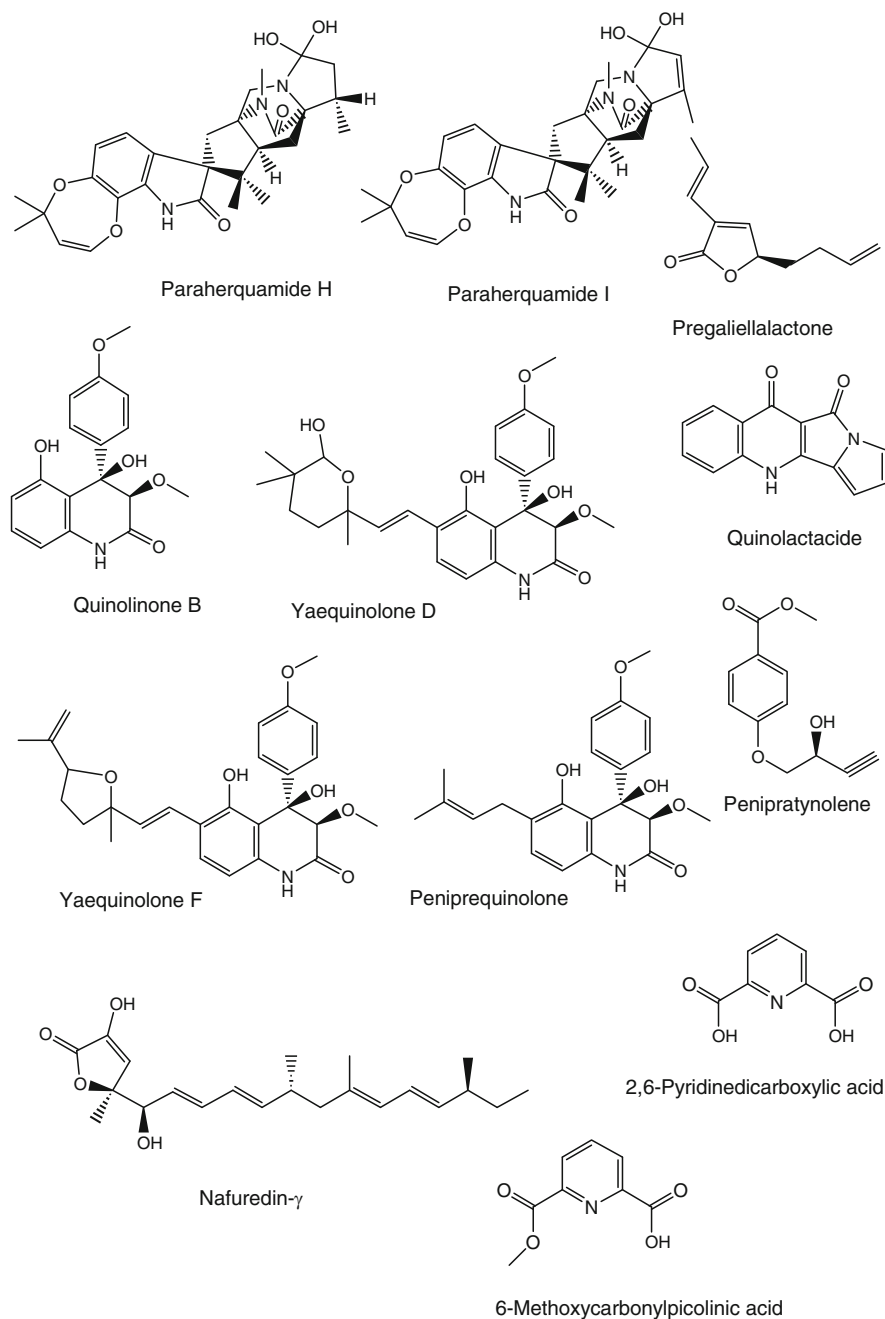


Fig. 7.4. Paraherquamides H, I (from *Penicillium cluniae*). Pregaliellalactone (from *Galiella rufa*). Quinolinone B, yaequinolones D, F (from *Penicillium* sp. FKI-2140). Penipratynolene and 2,6-pyridinedicarboxylic acid (from

Penicillium bilaiae). Quinolactacide (from *Penicillium citrinum*). Peniprequinolone (from *Penicillium simplicissium*). Nafuredin- γ (from *Aspergillus niger*)

Several reports on insecticidal activities of extracts obtained from fungal culture filtrates have been published without identification of the active components (Meyer et al. 2004; Mohanty and Prakash 2008). Thus, Chen et al.

(2003) reported on an extract obtained from an endophytic fungus with high activities against *Heliothis armigera*; the metabolites isolated, however, were not insecticidal (Yasui et al. 2006).

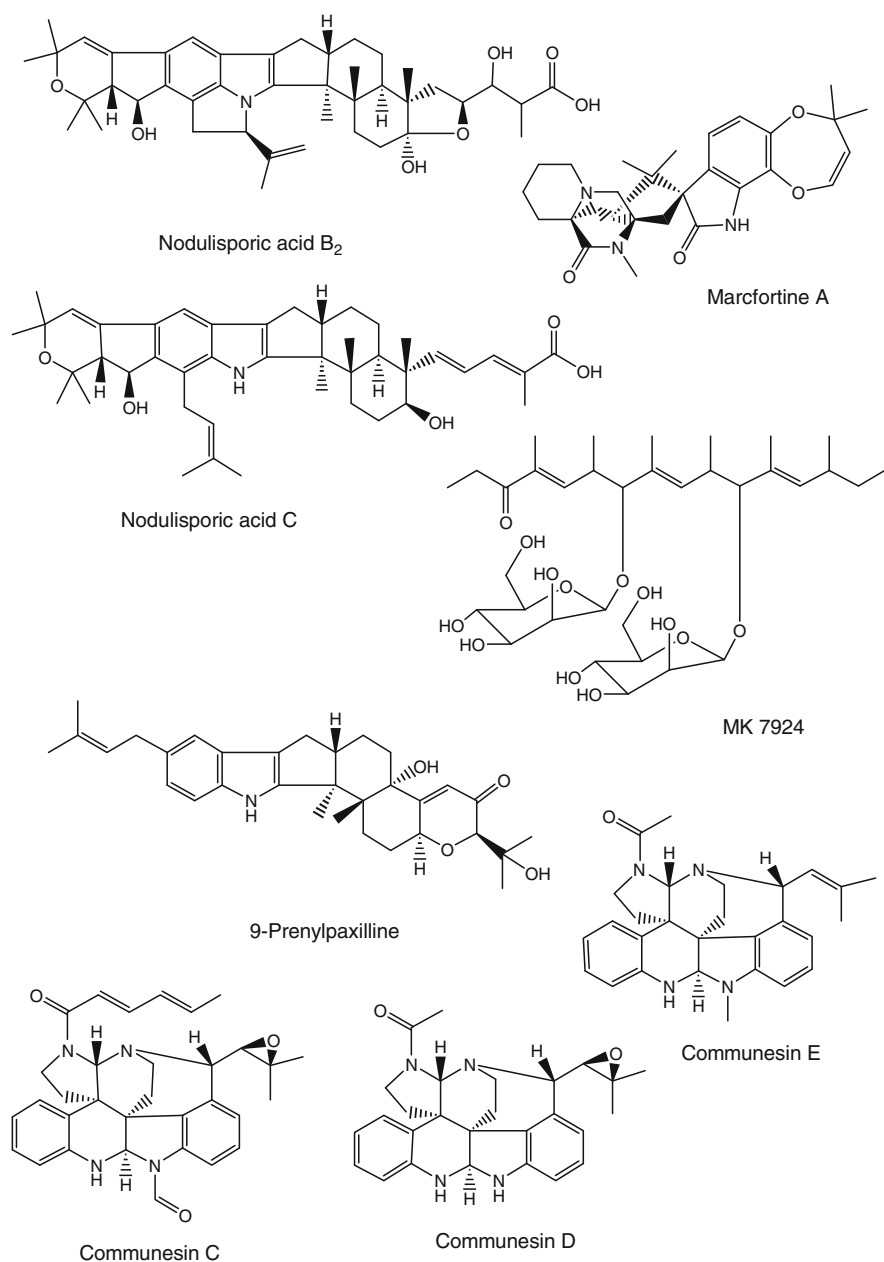


Fig. 7.5. Nodulisporic acids B₂, C (from *Nodulisporium* sp.). MK7924 (from *Coronophora gregaria*). Communesins C, D, E (from *Penicillium expansum*)

Many insecticidal or nematicidal metabolites, despite their complex structures, have been chemically synthesized during the last few years, for example, 9-prenylpaxilline (Smith and Cui 2003), marcfortine A, and structurally related metabolites of the paraherquamide group, many of which have potent insecticidal and antihelmintic properties (Williams 2002; Williams et al. 2003;

Trost et al. 2007); quinolactacide (Abe et al. 2006), nafuredin- γ (Nagamitsu et al. 2003), and verticillide (Momna et al. 2006).

The successful *in vitro* synthesis of enniatins with the multienzyme enniatin synthetase may open the way to novel derivatives, since the substrate binding pocket for the α -hydroxy carboxylic acid accepts chemically different acids (Glinski et al. 2001; Feifel et al. 2007).

III. Biological Activities and Mode of Action

Cyclic depsipeptides and their biological activities were thoroughly reviewed by Sarabia et al. (2004), insecticidal and other biological activities of destruxins, isariins, enniatins, and beauverolides by Anke and Sterner (2002), by Anke and Antelo (2009), and by Zimmermann (2007a, b).

Sirodesmin PL produced by *Leptosphaeria maculans* was found to have phytotoxic, antibacterial, and insecticidal properties (Rouxel et al. 1988; Boudart 1989). Serinocyclin A isolated from *Metarhizium anisopliae* conidia produced a sublethal locomotory defect in mosquito larvae (Krasnoff et al. 2007). Argifin and argadin, two cyclopentapeptides from a *Gliocladium* sp. and a *Clonostachys* sp. turned out to be potent inhibitors of chitinase B from the gram-negative bacterium *Serratia marcescens* (Arai et al. 2000). When injected into cockroach larvae, their moult was arrested (Houston et al. 2002).

Like many peptaibols, the efraeptins act as channel-forming ionophores and have insecticidal, antimalarial, and antiprotozoal activities (Nagaraj et al. 2001). In addition, they inhibit exocytosis but not endocytosis in eukaryotic cells (Muroi et al. 1996) and exhibit antibacterial and antifungal activities (Bandani et al. 2000).

Efraeptins bind strongly to V-ATPases in the brush border membrane of the mid-gut of the wax moth *Galleria mellonella* (Bandani et al. 2001). Leucinostatins A and B originally isolated from *Penicillium lilacinum* [now *Paecilomyces lilacinus* (Thom) Samson] were found to have nematocidal activities (Arai et al. 1973; Park et al. 2004). Like other peptaibols they act on bacteria, are uncouplers in the inner mitochondria membrane and inhibit mitochondrial ATP synthesis. Recently interesting and potent in vivo and in vitro antitrypanosomal activities of these compounds have been reported. Despite its toxicity, the strong in vivo antitrypanosomal activity of leucinostatin B makes the compound a promising lead structure for the development of drugs with a new type of action mode (Ishiyama et al. 2009).

Among nine beauverolides tested for acyl-CoA:cholesterol acyltransferase (ACAT) inhibitory activity in CHO-cells expressing ACAT1 or ACAT2, beauverolides I and III inhibited ACAT1 rather selectively, and no antimicrobial or cytotoxic activities were detected.

Both compounds produced by the entomopathogenic fungus *Beauveria bassiana* exert anti-atherogenic activity in

low-density lipoprotein receptor- and apolipoprotein E-knockout mice without any side effects and thus may serve as lead structures for new anti-atherosclerotic agents (Namatame et al. 2004). Contrary to the beauverolides, related beauvericin was clearly cytotoxic (Matsuda et al. 2004; Ohshiro et al. 2007). Cell lines derived from insects, e.g. *Spodoptera frugiperda* SF-9 cells, were also inhibited by beauvericin (Calo et al. 2003; Fornelli et al. 2004). The compounds induced rapid cell death in *Xenopus* oocytes via influx of Ca²⁺ (Tang et al. 2005).

Furthermore, significant effects were detected for destruxin E on insect haemocytes (Vey et al. 2002) and for different destruxins, e.g. destruxins A, B, and E, on human and insect cell lines (Skrobek and Butt 2005). Verticillide from moulds of the genus *Verticillium* inhibits the binding of ryanodine to its receptor (RyR) and, hence, has insecticidal activity (Monma et al. 2006).

The target of PF1022A, a fungal cyclo-octadepsipeptide, is a latrophilin-like receptor from the parasitic nematode *Haemonchus contortus* (Saeger et al. 2001). Emodepsin, a semi-synthetic depsipeptide derived from PF1022A, has already been successfully used against helminths in veterinary medicine (Conder et al. 1995; Dyker et al. 2004; Samson-Himmelstjerna et al. 2005). Due its limited availability and therefore a rather high price, its use is restricted to small pets. PF1022A is a metabolite of an endophytic fungus from the ornamental plant *Camellia japonica* (Sasaki et al. 1992; Scherkenbeck et al. 2002). Based on its 18S rRNA gene sequence, the endophyte was tentatively identified as a member of the ascomycetous family Xylariaceae close to *Xylaria polymorpha* and *Rosellinia necatrix* (Miyado et al. 2000).

Selective nematocidal properties were only reported for the omphalotins with high inhibitory activity towards *Meloidogyne incognita* and lower activities against *Caenorhabditis elegans* (Mayer et al. 1999). The nematocidal properties of hydroxylated omphalotins, some of which can be produced by monokaryotic strains generated from dikaryotic parent mycelia, were found to be higher than those of the unsubstituted compound, but unfortunately they were not stable (Büchel et al. 1998; Liermann et al. 2009). Their mode of action has not yet been elucidated.

Paraherquamides, potent anthelmintic agents isolated from various *Penicillium* species, were reported to possess promising activities against drug-resistant intestinal parasites (Williams et al. 2003).

Quinolactamide was also strongly active against *Myzus persicae*, and 250 ppm were lethal to 88% of the aphides tested; and, at a concentration of 500 ppm, 42% mortality towards the diamondback moth (*Plutella xylostella*) was recorded (Abe et al. 2006).

Nafuredin is a selective inhibitor of the helminth complex I (NADH-fumarate reductase). It showed only very weak inhibition of the mammalian complex I (NADH ubiquinone reductase from bovine liver) but was selectively active in vivo against the stomach worm *Haemonchus contortus* in sheep (Omura et al. 2001). The γ -lactone was almost equally active in the helminth complex I assay but less active in the in vivo tests (Shiomi et al. 2005). The differences between the human and helminth complex I make this an interesting target for the development of novel selective drugs against parasitic nematodes.

IV. Ecological Significance

Many secondary metabolites play a crucial role for fungi in their natural habitats. For example, endophytic fungi of grasses belonging to the genera *Neotyphodium*/*Epichloe* confer protection from mammalian and insect herbivores, or enhanced resistance to nematodes and phytopathogenic fungi (Schardl et al. 2004; Panaccione et al. 2006). Some of these beneficial effects are due to secondary metabolites.

Loline and peramine have been identified among the fungal metabolites with insecticidal activities in the plant host. Another metabolite from *Epichloe typhina*, epichlicin, efficiently prevents the germination (IC₅₀ value of 22 nM) of *Cladosporium phlei* spores, a host plant pathogen (Seto et al. 2007). Likewise, sirodesmin PL or zearalenone and other mycotoxins produced by *Leptoshaeria maculans* and *Fusarium* species, respectively, were detected in the plant hosts (Laser et al. 2003; Elliott et al. 2007). Some endophytic fungi produce 3-hydroxypropionic acid as a nematicidal principal (Schwarz et al. 2004). This might be the natural nematicide with the simplest chemical structure; however it remains to be elucidated whether the compound is also produced in planta.

The function of shearamide A, an insecticidal cyclopeptide isolated from the ascostromata of *Eupenicillium shearii* (Belofsky et al. 1998), and likewise sclerotiamide from *Aspergillus sclerotiorum* sklerotia (Whyte et al. 1996), may be to protect the reproductive or survival structures of

the fungi against insects or nematodes, similar to ergopeptides in the sclerotia of *Claviceps* species (Leistner and Steiner 2009).

Investigations on the role of destruxins in the pathogenicity of *Metarhizium anisopliae* against three species of insects revealed a direct relationship between the titre of destruxins produced by the strains in vitro and their destructive action (Kershaw et al. 1999).

However, in *M. anisopliae* mutants, incapable of destruxin production, virulence towards *Galleria mellonella* was unaltered (Amiri-Besheli et al. 2000). In the plant pathogenic mould *Alternaria brassicae*, destruxin B is a host-specific toxin. In three *Brassica* species the degree of their sensitivity to destruxin B positively correlated with their degree of susceptibility (Pedras et al. 2002).

From cultures of a number of fungi producing cyclic depsipeptides (e.g. *Beauveria bassiana*), also dipeptides composed of the same amino acids as the depsipeptides were isolated.

Other insect pathogens like *Verticillium* species and *Metarhizium anisopliae* as well as plant pathogenic fungi, e.g. *Colletotrichum gloeosporioides*, *Exserohilum holmi*, *Gliocladium deliquescens*, *Alternaria* and *Trichoderma* spp. were also found to produce dipeptides. An unidentified endophyte from mangrove leaves (*Rhizophora* spp.) produced two cyclic depsipeptides and three diketopiperazines (Huang et al. 2007).

It might be interesting to elucidate the insecticidal and nematicidal activities exhibited by cocktails of all these secondary metabolites of a pathogen. Such synergistic effects of different fungal metabolites have been largely neglected so far. In general, the role of dipeptides and depsipeptides in insect and plant pathogenicity is not fully understood. Further, it is intriguing that depsipeptides are widespread in phytopathogens (e.g. *Cochliobolus* with anamorphs *Helminthosporium* and *Bipolaris*, *Calonectria* with its anamorph *Cylindrocladium*, as well as *Fusarium* and *Alternaria*), insect pathogens (*Aschersonia*, *Beauveria*, *Cordyceps*, *Diheterospora*, *Fusarium*, *Hirsutella*, *Isaria*, *Metharizium*, *Paecilomyces*, *Tolypocladium*, *Verticillium*), and others (Zimmermann 2007a, b; Buckingham 2008). As molecular tools become more and more available, this question may be correctly addressed and respectively answered in the near future.

The efrapeptins produced by insect pathogenic *Tolypocladium* species are also produced

in vivo; the amounts, however, were found to be too small to cause the death of insects. Therefore, it was suggested that the compounds act in concert with additional, not yet known, pathogenicity factors (Bandani et al. 2000).

For the function of enzymes in entomopathogenic fungi and their role in disease development, see Khachatourians and Qazi (2008) in this context. The effects of secondary metabolites on the enzymes involved in pathogenesis in plants and insects alike is another innovative field, in which interesting results still wait to be elucidated.

V. Conclusions

Natural products derived from plants, animals, and microorganisms constitute only 7.6% of the global insecticides market (Elbert et al. 2007). However, more than 50 000 microbial metabolites are known. Half of them exhibit bioactivities, first and foremost antibiotic activity. Fungal metabolites represent about 40% of these natural products (Bérdy 2005; Buckingham 2008). So far, no secondary metabolite from a fungus has been developed into a marketable insecticidal or nematocidal product, despite the fact that most insect pathogens are fungi and many have successfully been screened for the production of “soft pesticides”. However, as it appears, many of the compounds isolated from these fungi are rather toxic, like the destruxins or efrapeptins. While they fulfil their ecological role very well, i.e. killing the host animal, they do not meet the requirements of modern agricultural pesticides regarding selectiveness, low costs, and environmental safety. Nevertheless, the example of PF1022A shows that fungal metabolites can be developed into drugs useful in agriculture and veterinary medicine. The many synthetic efforts using natural products as lead structures also point in this direction. Taking into account the overall number of fungal species, which is estimated to be around 1.5 million (Hawksworth 2001), and comparing this number with the number of about 50 000 species screened so far, it seems to be only a matter of time until the first insecticide from a fungus is introduced into the market.

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8 Immunomodulators

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I. Introduction

Immunomodulators are actually natural products of the immune system (Pirofski and Casadevall 2006). The immune system of healthy organism produces a diverse range of metabolites to keep the body in homeostasis condition. An immunomodulator may be defined as a substance, of biological or synthetic origin, which can stimulate, suppress or modulate any of the components of the immune system including both innate and adaptive arms of the immune response (Agawal and Singh 1999). In clinical practice, immunomodulators are classified into three main categories:

1. **Immunosuppressants** are agents that somehow inhibit the immune system. They can be used for the control of pathological immune response after organ transplantation and for the treatment of autoimmune diseases, hypersensitivity immune reactions as well as immune pathology associated with infections.
2. **Immunostimulators** are agents that stimulate the immune system by inducing the activation or increasing activity of any of its components. They enhance the body's resistance against allergy, infection, cancer and autoimmunity.
3. **Immunoadjuvants** are agents used to enhance the vaccine efficacy. This can be also considered as a specific immune stimulator effect.

The industrial importance of immunomodulators is based on their large market value. The market size of immunomodulators was evaluated at US \$43 billion in 2006, and is expected to grow at a compound annual growth rate (CAGR) of 13% to reach US \$80 billion by 2011 (Research and Market 2007). Although some potential immunomodulating substances can be chemically synthesized and have been successfully tested for modulation of the immune system [e.g. synthetic muramyl dipeptide (MDP) analogues], research activities

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in this field have been focused on immunomodulatory active compounds from natural resources. Among them, fungal immunomodulators represent the most interesting group of metabolites. This review outlines the current state of knowledge on fungal immunomodulators used already for different medical applications and discusses the future potential of new compounds of fungal origin.

II. Immunosuppressants

The importance of immunosuppressants arose in the mid of the twentieth century in the context of new developments in organ transplantation. The principal goal of immunosuppressant application in organ transplantation is to minimize the risk of allo-graft rejection or graft-dysfunction by achieving adequate immunosuppression, yet also to ensure that the level of immunosuppression does not contribute to long term morbidity (Patel and Kobashigawa 2008). The first organ transplantation was performed in 1933 when a kidney was transplanted from a cadaver. Total lymphoid irradiation was used for the immune suppression but the tissue was rejected and the patient eventually died. This was followed by the use of corticosteroids as immunosuppressive agents, but unfortunately, these steroids as such did also not give the positive results expected. In the early 1960s, cytotoxic agents such as modified corticosteroids were introduced to suppress the immune system after organ transplantation (Khan 2008). The first clinically used fungal immunosuppressive agent was introduced into the market in the mid of 1980s when cyclosporine became available for clinical applications after getting its approval from the United States Food and Drug Administration (US FDA). This was one of the most important milestones in the history of organ transplantation. Beside their important roles in organ transplantation, immunosuppressive agents are used for other applications, for example, in the prevention of the newborn Rh hemolytic disease (Contreas and DeSilva 1994) or for the treatment of some autoimmune diseases. The chemical structures of the three main fungal immunosuppressive agents (cyclosporine, mycophenolic acid, mizoribine), which all are used in organ transplantation and for other medical applications, are shown in Fig. 8.1.

A. Cyclosporins

Cyclosporins (Cys) are a family of neutral, high lipophilic, cyclic undecapeptides containing some unusual amino acids and having a remarkable spectrum of biological activities. The first member of this class of compounds was named cyclosporine A. To date, more than 30 members of this family of compounds have been isolated from natural resources and were classified as cyclosporins A to Z (CyA–Z; Traber et al. 1982, 1987). CyA was originally described as an antifungal peptide with a narrow spectrum of efficacy. However, the interest in this compound only increased significantly after the demonstration of its specific immunosuppressive activity. In 1983, Sandoz first introduced a cyclosporine-A-based drug, Sandimmun, into the market. The modified form of this drug with increased bioavailability, Neoral, became available on the market in 1994 in form of soft gelatins and oral applicable solutions. Since then, Sandimmun and Neoral have been Novartis' leading pharmaceutical products and these drugs generated a revenue of US \$1.216 billion in 1997 (Svarstad et al. 2000). Nowadays, based on market research data, only five members of the cyclosporin family, namely CyA (CAS 59865-13-3), CyB (CAS 63775-95-1), CyC (CAS 59787-61-0), CyD (CAS 63775-96-2) and CyH (CAS 83602-39-5) are commercially available for pharmaceutical applications as immunosuppressive agents.

According to their immunosuppressive mode of action, cyclosporins belong to the specific calcineurin inhibitors. Immunosuppressant activity is mediated through blocking the activation and proliferation of CD4⁺ and CD8⁺-T lymphocytes by inhibiting IL-2 production (Siekierka et al. 1989; Shibasaki et al. 2002). Under normal conditions, the binding of major histocompatibility peptides to the T-cell receptors results in the formation of an activated form of calcium/calmodulin-dependent serine/threonine phosphatase calcineurin. This leads to dephosphorylation and nuclear translocation of the nuclear factor of activated T-cells (NF-AT). Subsequently, NF-AT binds genes encoding pro-inflammatory cytokine IL-2, resulting in an up-regulated gene transcription (Schreiber and Crabtree 1992; Butch 2008). CyA freely crosses lymphocyte membranes and forms complexes with the specific cytoplasmic binding protein immunophilin cyclophilin A. The CyA-cyclophilin A complex inhibits calcineurin

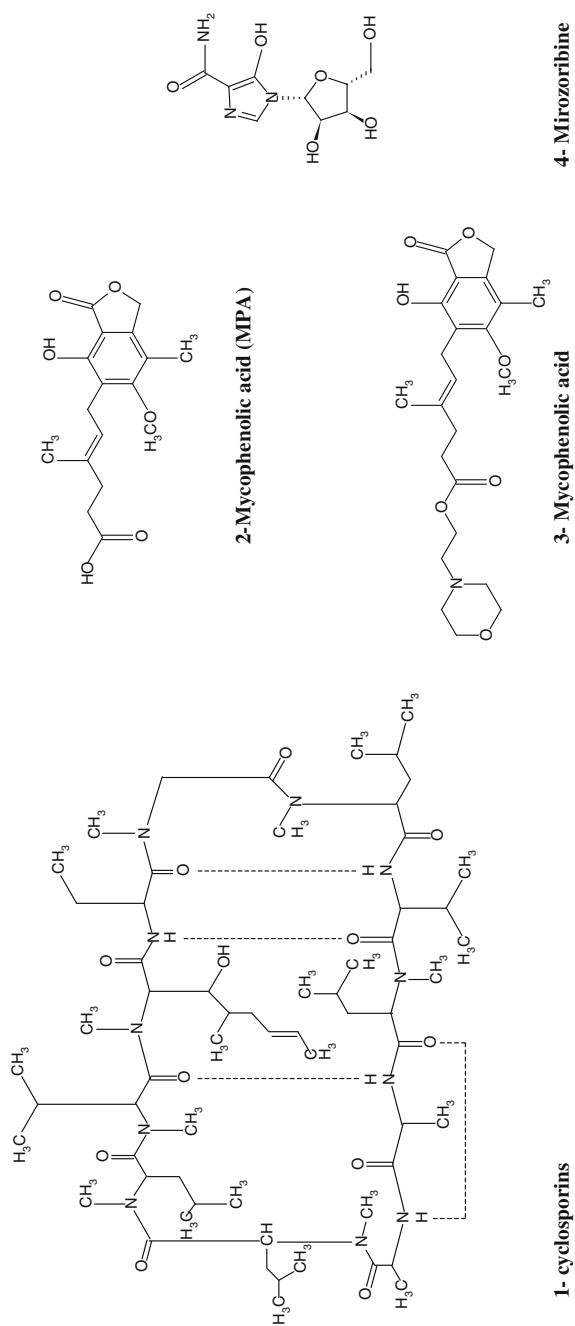


Fig. 8.1. Chemical structure of the main clinically important fungal immunosuppressives compounds

activity and the nuclear translocation of NF-AT. This leads to the down-regulation of the pro-inflammatory molecules gene transcription and subsequently halts the production of IL-2 and TNF- α (Jorgensen et al. 2003).

Currently, CyA is approved and used worldwide as an immunosuppressive drug to prolong organ and patient survival after kidney, liver, heart and bone-marrow transplants. CyA is available on the market under the trade name Sadimmune for both oral and intravenous applications. A nano-sized pre-concentrate formulation of CyA (CyA-MEPC or Neoral) exhibiting a better absorption characteristic is used orally in form of solutions or soft-gelatin capsules (Vonderscher and Meinzer 1994; Uchida et al. 2004). Besides the products of the market leader Novartis, several generic formulations are nowadays available and are often referred to as modified CyAs (Alloway 1999).

1. Chemistry

CyA, the most important member of the cyclosporin family, is a cyclic undecapeptide with a 33-membered ring composed of 11 lipophilic aliphatic amino acids, of which four are leucine and three are the non-proteogenic amino acids D-alanine, (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine (Bmt) and L- α -aminobutyric acid. The full chemical name of CyA is 32-ethyl-2-[(E,1R,2R)-1-hydroxy-2-methylhex-4-enyl]-3,6,9,12,14,17,21,27,30-nonamethyl-8,11,20,26-tetrakis-(2-methylpropyl)-5,23-di-(propan-2-yl)-3,6,9,12,15,18,21,24,27,30,33-undecaza-cyclotriactane-1,4,7,10,13,16,19,22,25,28,31-undecone. The molecular formula and molecular weight of CyA are C₆₂H₁₁₁N₁₁O₁₂ and 1202.61 g mol⁻¹, respectively. The non-proteogenic amino acids are found at the positions 1 (Bmt), 2 (L- α -amino butyric acid) and 8 (D-alanine). Remarkably, seven of the 11 peptide bonds are N-methylated, which has several important implications. First, the N-methylated peptide bonds and the cyclic structure of the molecule renders cyclosporins stable toward mammalian digestive and systemic proteases. Cyclosporin metabolism in animals and humans is exclusively carried out by cytochrome P450 enzymes catalyzing its oxidative transformation. Therefore, cyclosporins are not only well absorbed when given orally but also characterized by high and long-lasting

plasma levels. A second consequence of the N-methylation pattern is rigid conformation in the non-polar environment characterized by intramolecular hydrogen bonds being oriented towards the hydrophobic environment (Kallen et al. 1997).

Plain cyclosporin is difficult to crystallize on its own and therefore, was initially analyzed as crystalline iodo-cyclosporin. The structural analysis of such CyA crystals by X-ray diffraction revealed a rigid conformation (Loosli et al. 1985). The rigidity can be attributed to a number of unique structural properties. Predominantly, the four intra-molecular hydrogen bonds maintain it by stabilizing the backbone structure. Not least, this is evident from the increase in the number of backbone conformations observed in polar solvents due to the formation of inter-molecular hydrogen bonds with the solvent molecules (Kratochvil et al. 1999). In addition to the four intra-molecular hydrogen bonds, CyA exhibits a *cis*-amide bond between the N-methylleucine residues at positions 9 and 10. Moreover, the N-methyl moiety of MeVal in the loop makes backbone contacts, which further contribute to the rigidity of the structure (Velkov and Lawen 2003).

2. Biosynthesis

The biosynthesis of bioactive peptides like cyclosporins proceeds non-ribosomally and is catalyzed by complex multi-functional enzymes termed non-ribosomal peptide synthetases (NRPS). Cyclosporin synthetase (CySyn) is one of the best studied enzyme complexes of this type and capable of catalyzing a total of at least 39 different reaction steps in the synthesis of cyclo-undecapaptides via an assembly belt-like mechanism: 11 amino acyladenylation reactions, ten transpeptidations, seven N-methylations, ten chain elongation reactions and a final cyclization reaction (Dittmann et al. 1994; Velkov and Lawen 2003).

The enzyme consists of 11 protein modules, each being responsible for the recognition, activation and modification of one substrate (Lawen and Zocher 1990; Weber et al. 1994) and a small 12th module putatively responsible for cyclization. Based on the gene sequence and the established models for non-ribosomal peptide synthetases (Marahiel et al. 1997), each module of CySyn essentially consists of a central adenylation domain (A-domain;

recognition, activation), a thiolation domain (T-domain, covalent binding of adenylated amino acid on phosphopantethein) and a condensation domain (C-domain; elongation step). During elongation, the activated amino acids are linked by peptide bonds leading to enzyme-bound nascent peptide chains.

CySyn substrates include L-valine, L-leucine, L-alanine, L-glycine, α -amino butyric acid (Abu), 4-methylthreonine, and D-alanine. With the adenulation domain, cyclosporine synthetase generates the acyl-adenylated amino acids and then covalently binds the amino acid to phosphopantetheine through a thioester linkage. Seven of the substrate amino acids become N-methylated by S-adenosylmethionine via respective methyltransferase activities of CySyn. The final cyclization step releases CyA from the enzyme complex (Hoppert et al. 2001).

Several members of Cy family (like CyA) contain non-proteinogenic amino acids (D-alanine, Abu and unusual Bmt or a similar C9-amino acid; Fig. 8.2), which have to be synthesized by a pathway independent of the primary metabolism. Therefore, besides CySyn, the presence of some other enzymes is crucial for CyA biosynthesis as

well. The biosynthesis of Bmt is catalyzed by a polyketide synthase (PKS) that forms the polyketide backbone by the head-to-tail condensation of four acetate units, resulting in a 3(R)-hydroxy-4-(R)-methyl-6-(E)-octenoic acid thioester; the C-methyl in the carbon chain is derived from AdoMet (Offenzeller et al. 1993). The polyketide 3(R)-hydroxy-4-(R)-methyl-6-(E)-octenoyl-CoA is then transformed into the β -amino acid form which is utilized by CySyn as a substrate for cyclosporine biosynthesis. D-Alanine is provided by a distinct pyridoxal phosphate dependent alanine racemase (Hoffmann et al. 1994). The remaining amino acid constituents of the CyA molecule are synthesized by classic biosynthetic pathways, as confirmed by Senn et al. (1991) using ^{13}C -labeling experiments.

The massive CySyn polypeptide represents the upper limit of molecular size of the NRPS enzymes. A molecular mass of 1.69 MDa (15 281 amino acids), was delineated from the sequence of the CySyn gene, *simA*, which constitutes an intronless genomic open-reading frame (ORF) of 45.8 kb (Weber et al. 1994; Velkov and Lawen 2003). The role of this gene in CyA biosynthesis was proved by Weber and Leitner (1994). They demonstrated that the knock-out of the *simA* gene in *Tolypocladium inflatum* resulted in its inability to produce cyclosporins.

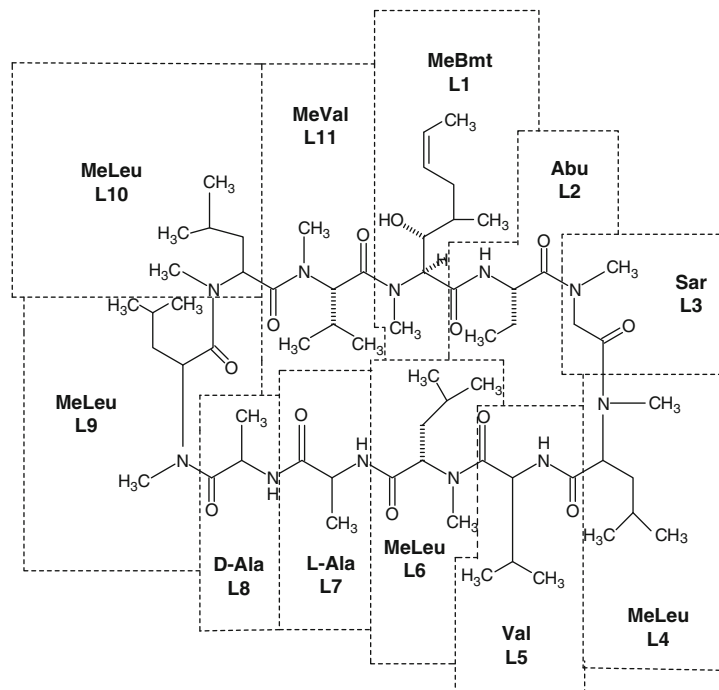


Fig. 8.2. The chemical structure of cyclosporin A including the numbering system. It is composed of 11 amino acid unit, with seven of the amide nitrogen methylated. The three non-proteogenic amino acids are: D-alanine, Abu (L-2 amino-butyric acid) and Bmt (4R)-4-[(E)-2-butyl]-4-methyl-L-threonine (modified from Velkov et al. 2006)

Transmission electron micrographs of negatively stained CySyn macromolecules showed large globular complexes of 25-30 nm in diameter, built up by smaller interconnected units associated with smaller particles of 7 nm length. Complexes of CySyn and D-alanine racemase are linked and localized at the fungal vacuolar membrane, where Cy synthesis is carried out (Hoppert et al. 2001). CySyn and D-alanine racemase seem to be located in close vicinity to each other, since D-alanine is the leading amino acid of the polypeptide chain synthesized by CySyn. Cyclosporin is subsequently accumulated inside the vacuoles and released slowly through vacuolar and cytoplasmatic membranes or rapidly upon cell lysis.

CySyn was prepared in purified form at pilot scale and used as a model to produce large amounts of CyA in vitro. The process included ammonium sulfate precipitation, gel filtration, hydrophobic interaction chromatography and anion exchange chromatography, and it yielded an electrophoretically homogenous cyclosporin synthetase preparation (Velkov et al. 2006). The obtained enzyme exhibited an optimal temperature range between 24 and 29 °C and a pH optimum around 7.6.

3. Production

The production of cyclosporins at the laboratory scale can be carried out using different aerobic filamentous fungi such as *Tolyposcladium inflatum*, *Fusarium solani* (Sawai et al. 1981), *Neocosmospora vasinfecta* (Nakajima et al. 1989), *Acremonium luzulae* (Moussaïf et al. 1997) and *T. cylindrosporium* (Sekar et al. 1997). The industrial production of cyclosporins is mainly performed using highly productive strains of *T. inflatum*.

This organism was originally mis-classified as *Trichoderma polysporum* Gams, however, later it turned out that it belonged to a new genus of ascomycetous molds, *Tolyposcladium* and coined the name *T. inflatum* (Gams 1971). In 1983, another research group found that *T. inflatum* was identical to *Pachybasium niveum*, and since the latter older name has priority under the rules of the International Code of Botanical Nomenclature, the strain was renamed as *T. niveum* (Bissett 1983). This fungus was again re-classified as *Beauveria nivea* (Von Arx 1986). Based on the research of Kathie Hodge, this strain was found to be the asexual state of *Cordyceps subsessilis* (Hodge et al. 1996). Due to the economic importance of this fungus, the classification as *T. inflatum* was nevertheless conserved for cyclosporin producers to avoid any confusion with other strains (Dreyfuss and Gams 1994).

In spite of some efforts to produce CyA with immobilized cells or by solid-state fermentation (SSF), the industrial production of this immunosuppressive agent is mostly carried out using free cells in submerged cultures in stirred-tank

bioreactors. The particular role of the type of strain on the production of CyA along with some characteristic morphological features was reported by several authors.

In case of *T. inflatum*, large intra-population variations in colony color and shape were observed on solid media. Thus, colony color can range from white to brownish (including yellow, orange and red colonies) (Aarnio and Agathos 1990). The production of a pink pigment was found to be associated with cyclosporin production in certain *T. inflatum* strains (Chun and Agathos 1989). Besides the selection of highly productive colonies of wild-type strains, attempts were undertaken to increase the strain productivity by mutation using chemical mutagens such as methyl sulphate, epichlorohydrin or nitrosoguanidine (Agathos et al. 1986). A recent study of Mi-Jin and coworkers (2009) has demonstrated the possibility of the improvement of *T. niveum* productivity by using random mutagenesis combined with protoplast transformation. The mutant strain, generated using a random UV method, produced more than ninefold higher amounts of CyA than the wild-type strain. Additionally, a bacterial gene of a *Vitreoscilla* spp. (hemoglobin gene, VHb) was transferred to the UV-irradiated mutant to increase oxygen uptake in liquid culture and led to an additional increase in CyA production of more than 30%. Besides the type-strain used, the production levels of CyA are dependent on several regulating factors such as inoculum type and size, medium composition and additives as well as process parameters such as temperature, pH and partial oxygen pressure. A high density of the spore-inoculum was found to be necessary for the development of small pellets, which is the preferred morphology for cyclosporin production (Dayfuss et al. 1976; Isaac et al. 1990). However, inoculum size is only one of more than 20 other factors controlling the fungal pellet formation (El Enshasy 2007).

The influence of the type and concentration of carbon and nitrogen sources on CyA production has been examined in wild-type and mutant strains of *T. inflatum*. Among different carbon sources tested, 3% sorbose gave the highest CyA titre (Agathos et al. 1986). A feeding strategy using the sequential addition of two carbon sources (sorbose and maltose) was also reported to be successful in attaining a higher volumetric production (Agathos et al. 1986). Another study showed that an optimal medium for CyA production can be developed by factorial experimental design and consisted of the three carbon sources glucose, sucrose and starch in different ratios (Abdel Fattah et al. 2007).

Biosynthesis of CyA was found to be heavily influenced by the external addition of amino acid

constituents of the molecule. Addition of L-valine increased the specific production of CyA by 60% in semi-synthetic media and even by 400% in synthetic media. Experiments using repeated addition of L-valine indicated that the amino acid has to be present in the exponential growth phase of the fungus for optimal CyA production (Lee and Agathos 1989). Based on this finding, a mathematical model for the production of CyA in the presence of supplemented L-valine was developed, which also considered kinetic information and mechanistic data on CyA biosynthesis (Agathos and Lee 1993). Concomitant addition of L-leucine and L-valine to a synthetic medium was found to stimulate CyA production as well (Balakrishnan and Pandey 1996). When fungal cells enter the stationary phase and CyA accumulates in the medium, they partially undergo lysis and CyA degradation sets in, especially under carbon source limitation. The intensity of cell lysis and CyA degradation in the bioreactor was higher than in agitated flasks, especially under an uncontrolled pH regime (El Enshasy et al. 2008).

Several attempts have been made to use immobilized cells for CyA production. One example is the successful production of CyA in high amounts using carrageenan-entrapped cells of *T. inflatum* in an airlift bioreactor (Foster et al. 1983). CyA was also produced in relevant amounts by a *Tolypocladium* sp. immobilized in calcium alginate beads in a packed-bed reactor (Sekar and Balaraman 1998a). Continuous production of CyA was realized using immobilized spores of *T. inflatum* on celite beads (Chun and Agathos 1989). The CyA productivity by cells immobilized on celite beads (100–500 μm) was reported to be 4–6 $\text{mg l}^{-1} \text{h}^{-1}$. This value is about six- to tenfold higher than those of batch fermentations in suspension cultures (Lee et al. 1997). Furthermore, attempts were made to produce CyA by solid-state fermentation (SSF) to reduce the production costs. So in a study, wheat-bran was used as a solid support and yielded up to 1400 mg CyA kg^{-1} substrate (Sekar et al. 1997). After optimizing different cultivation parameters, such as the type and design of tray, thickness of the solid substrate bed, type and size of inoculum as well as relative humidity, the CyA production increased to a value of 1920 mg kg^{-1} (Sekar and Balaraman 1998b). However, SSF up-scaling raises severe engineering problems due to difficulties of adjusting temperature, pH, oxygen and moisture content as well as of managing gradient formation inside the cultivation system. Recently, a novel process for CyA production by *F. solani* using a large-scale SSF bioreactor of an area of 226 m^2 has been developed (Khedkar et al. 2007). Besides the continuous optimization of the up-stream part of the production process, improvement of CyA extraction methods has also contributed to increase the overall yield of the process. CyA is a hydrophobic molecule with high solubility in

low-molecular-weight alcohols, and extraction could be optimized using different alcohols as solvent system and varying temperatures during the extraction process (Ly and Margaritis 2007; Ly et al. 2007).

4. New Generations

A recent study has demonstrated that CyA has a number of side-effects causing among others hypertension, dyslipidemia, hirsutism and chronic renal insufficiency that leads in 10% of cardiac transplant recipients to an end-stage renal disease (Patel and Kobashigawa 2008). Numerous analogues and derivatives of CyA have been tested in order to improve the drug's therapeutic properties. For example, CyG, a cyclosporin A analogue with a L-novoline substituent at position 2, displays equal immunosuppressive effects as CyA but with less nephrotoxicity (Hiestand et al. 1985). Another derivative SZZ IMM-125, which is a hydroxyethyl derivative of D-serine-8-cyclosporine, was found to be slightly more potent but far less nephrotoxic than CyA in both in vitro and in vivo models (Hiestand et al. 1992; Ferrareso and Kahan 1993). ISA_{TX}247 is a potent derivative with higher activity and lower nephrotoxicity compared to CyA (Gregory et al. 2004). Several other cyclosporin analogues with high immunosuppressive activity were obtained through the chemical modification of the side chains at the first amino acid and optionally at third amino acid (Molino and Yang 2006).

B. Mycophenolic Acid

Mycophenolic acid (MPA), [6-(4-hydroxy-6-methoxy-7-methyl-3-oxophthalanyl)-4-methyl-4-hexenoic acid; CAS 24280-93-1; Figs. 8.1–8.2], is one of the oldest known secondary metabolites. The compound was first detected in 1896 by Gozio in the fermentation broth of *Penicillium glaucum* and recognized as a lipid-soluble weak organic acid. This compound was also isolated from the culture filtrate of *Penicillium stoloniferum* Thom by Alsberg and Black (1913), who named it MPA (Alsberg and Black 1913; Jekkel et al. 2001). Since then, numerous reports have been published dealing with the production of MPA by different microorganisms. The complete chemical structure of MPA was first reported by Birkinshaw et al. (1952).

At the early stage of its biomedical applications, MPA was used as a broad-spectrum antibiotic due to its antibacterial, antifungal, antiviral and antiprotozoal activities (Abraham 1945; Ando et al. 1968; Cline et al. 1969; Noto et al. 1969). Moreover, it was found to exhibit also some antitumor and antipsoriasis as well as anti-inflammatory activities (Carter et al. 1969; Spatz et al. 1978; Epinette et al. 1987). MPA was produced during that time under the trademark Bialin (Vinkurova et al. 2005). However, it has not widely used in practice as antibiotic because most microbes were found to readily become resistant to this compound. In spite of its low acute toxicity to mammals, MPA was also described as a mycotoxin by some authors (Sanchis et al. 1988; Puel et al. 2005). New interest in MPA and its derivatives has remarkably grown after the discovery of their immunosuppressive properties.

The MPA 2-morpholinoethyl ester (also named mycophenolate mofetil, MMF) of the chemical structure 2-morpholinoethyl-(*E*)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexeneoate (CAS 128794-94-5; Figs. 8.1–8.3), is one of the most important MFA derivatives and approved by the FDA in 1995 as immunosuppressive agent for the prevention of acute renal allo-graft rejection and in 1998 for heart transplantation. A further improved generation of MPA drugs, based on sodium mycophenolate in controlled release formulations with good gastrointestinal absorption and bioavailability, was approved in 2005 (Xy and Yang 2007). In addition to the well established market for organs transplantations, MPA and its derivatives have been recognized by many physicians as an effective option for the treatment of immune-mediated diseases (Bentley 2000; Mydlarski 2005). Another pro-drug of MPA was developed in Japan during the early 1980s, initially as antitumor agent against various experimental cancers (Mitsui et al. 1981; Matsuzawa and Nakase 1984). In this derivative, the hydroxyl group of MPA was derivatized and the carboxylic functionality was replaced by an ethyl ester to produce ethyl-[*N*-(*p*-carboxyphenyl)-carbamoyl]-mycophenolate, abbreviated as CAM. The chemical structure of CAM was fully characterized and its crystal structure solved by Nawata and coworkers (Nawata et al. 1988; 1989). An early study using CAM as immunosuppressive drug demonstrated that it can suppress acute allergic cephalomelitis in Lewis rats (Mizobuchi et al. 1997). Based on this study, it was suggested that CAM might be also a useful adjunct for the long-term immunosuppressive therapy of inflammatory diseases of the central nervous system. At the same time, a study of Sawada and his group demonstrated the usefulness of CAM in bowel transplantation (Sawada et al. 1996). Using a rat model, the immunosuppressive activity of CAM was explained through the inhibition of the interphotoreceptor retinoid-binding protein (IRBP) mediated autoimmune

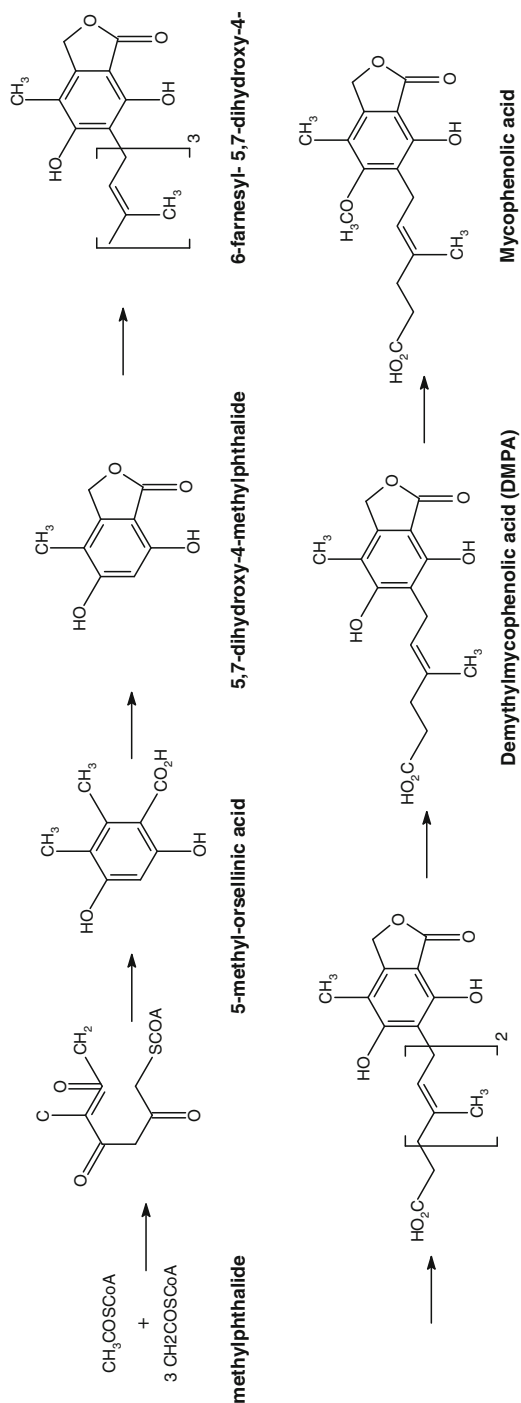


Fig. 8.3. Different steps of MPA biosynthesis

uveoretinitis by a decrease in cytokine production (Sakai et al. 1999). Compared to other immunosuppressive drugs, CAM has only minor adverse side-effects due to its relatively specific action on lymphocytes. Furthermore, this new derivative was more effective than MMF in prolongation of heart-graft survival in rats at each dose applied (Takazawa et al. 1995).

Nowadays, MPA derivatives used as immunosuppressant drug in organ transplantation are marketed under different trade names, such as CellCept (mycophenolate mofetil, Roche) and Myfortic (mycophenolate sodium; Novartis). In case of oral applications of MMF, the pro-drug mycophenolate mofetil is rapidly hydrolyzed to MPA after administration and suppresses the immune system via a non-competitive reversible inhibition of inosine-5'-monophosphate dehydrogenase (IMPDH; EC 1.1.1.205). This enzyme catalyzes the NAD-dependent oxidation of inosine-5'-monophosphate (IMP) to xanthosine-5'-monophosphate (XMP), which is the committed step in the de novo biosynthesis of guanosine monophosphate (GMP). This reaction is particularly important to generate the guanosine nucleotide levels needed to initiate a proliferation response of B- and T-lymphocytes to mitogens and antigens (Sintchak et al. 1996). Thus, MPA acts as a potent anti-proliferative agent (Hood and Zarembski 1997) affecting cytokine-dependent signals and causing in vivo the inhibition of lymphocyte reactions (Allison and Eugui 2000). In consequence, MPA and its derivatives are being widely used in the transplantation of different organs in humans at different ages (Budde et al. 2006; Tönshoff 2006; Aw et al. 2008).

Besides the wide application of MPA in organ transplantation, it is also used in the treatment of immune related diseases such as rheumatoid arthritis, lupus inflammatory bowel disease and other kidney or skin disorders (Liu and Mackool 2003; Appel et al. 2005; Hartmann and Enk 2005; Iaccarino et al. 2007). Moreover, MPA has recently been used in the treatment of rare diseases like interstitial nephritis (Preddie et al. 2006) and focal segmental glomerulosclerosis (Cattran et al. 2004).

In general, MMF has several advantages over cyclosporins as maintenance therapy of organ-graft recipients (Eugui and Allison 1993). Above all, MMF is well tolerated by the human body and has a lower toxicity and hence fewer side-effects than CyA.

1. Chemistry

Already before the discovery of its immunosuppressive activity, MPA was used as drug due to its wide biological activity against bacteria, parasites and viruses. Thus for many years modifications of the MPA structure were the subject of intensive research in order to increase its biological activity, bioavailability and the range of applications (Lee et al. 1990; Nelson et al. 1990; Rohloff et al. 1995).

Most studies focused on increasing the anti-tumor activity of MPA through the production of monocyclic analogues and carboxamide derivatives without any change in the aromatic ring and the surrounding side chains, since the free phenolic structure is an absolute prerequisite for MPA activity (McCorkindale and Baxter 1981; Anderson et al. 1996; Menza-Aviñ et al. 2005). The methoxy and methyl groups of the aromatic ring represent two other key structural elements influencing the activity of MPA (El-Araby et al. 2004). The study of Nelson et al. (1996) demonstrated that the aromatic methyl group of MPA is essential for its biological activity and the replacement of the methoxyl group by other ethers resulted in compounds with two- to four-fold higher potency in vitro and in vivo.

Further improvement of MPA activity was achieved by the development of new MPA analogues, which have overcome the drawback of glucuronidation of the phenolic hydroxyl group at C7 (Chen et al. 2007). In these derivatives, a truncated MPA is connected to an adenosine moiety via a linker (e.g. methylene *bis*-phosphonate) leading to mycophenolic adenine dinucleotide derivatives. The new molecules show a better biological activity and chemical stability. Moreover, a new series of IMPDH inhibitors based on the replacement of the benzofuranone moiety in MPA by a methoxy-(5-oxazolyl)-phenyl (MOP) moiety has recently been developed (Chen et al. 2008). Besides the different methods of chemical modification, several attempts were made to transform MPA using different microorganisms (Jekkel et al. 2001). In the course of these bioconversions, mycophenolic acid was found to undergo one or more of the following transformations: hydroxylation at the side chain or the lactone ring, amide or alcohol formation at the carboxylic acid group, oxidative cyclizations of the side chain or glycosylation (Jekkel et al. 2002).

2. Biosynthesis

The molecule of MPA consists of an acetate-derived aromatic nucleus, a terpenoid side chain, and two methyl groups (the 5-methoxyl and the 4-methyl group). Different schemes of MPA biosynthesis have been proposed by different authors (Bedford et al. 1973; Muth and Nash III 1975; Nulton and Campbell 1978). In 2000, Bentley summarized and updated the synthesis pathway in his excellent review (Fig. 8.3). Accordingly, MPA biosynthesis involves two major pathways of secondary metabolite formation: the polyketide and the isoprenoid pathway as well as methylation reactions on oxygen and carbon atoms.

In this process, a typical acetate-polymalonate condensation with a methylation prior to condensation leads to the aromatic structure, 5-methyl-orsellinic acid. It proceeds thereafter through lactone formation followed by addition of the C₁₅-farnesyl diphosphate unit and formation of the intermediate 6-farnesyl-5,7-dihydroxy-4-methylphthalide (Fig. 8.3). This compound undergoes several oxidative degradation steps to remove eight carbon atoms from the side chain by two possible mechanisms: one involves two oxidative cleavages at the two side chain double bonds removing levulinic acid and acetone from the aromatic ring. The other possible mechanism is a direct oxidation at the central double bond of the farnesyl side chain. The two pathways are regarded as being of equal importance (Bentley 2000). Early studies had already shown that the basic carbocyclic skeleton of the molecule was acetate-derived and that methionine provided the O- and C-linked methyl groups attached to the aromatic ring (Birch et al. 1958; Jaureguiberry et al. 1964). The last step of MPA biosynthesis was found to be the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to demethylmycophenolic acid (DMPA). This step is catalyzed by a specific SAM:DMP O-methyltransferase (Muth and Nash 1975).

3. Production

MPA was originally isolated from culture filtrates of *Penicillium glaucum* and *P. stoloniferum* as a weak acid with antifungal activity (Alsberg and Black 1913); later MPA production was reported for 12 strains of the species *P. brevicompactum* (Clutterbuck et al. 1932).

Since that time, many reports have been published dealing with the production of MPA using different species of the genus *Penicillium* (Vinokurova et al. 2005), such as *P. brevicompactum* (Doerfler et al. 1979; Ozaki et al. 1987a; Alani et al. 2009), *P. brunneostoloniferum* (Nakajima

et al. 1979); *P. roqueforti* (Lafont et al. 1979, Engel et al. 1982; Schneewis et al. 2000) and other molds like *Neocosmospora* spp. and *Byssoschlamys nivea* (Puel et al. 2005). Unfortunately, most of the studies on MPA production were carried out on a small scale and data on the detailed effects of media components and cultivation conditions are scarcely found in the literature.

Among different MPA producers, the highest productivity was obtained for strains of *P. brevicompactum* and *P. stoloniferum* which both are suitable for industrial fermentation (Queener and Nash 1978; Kida et al. 1984; Sircar et al. 2005). Several attempts have been made to improve MPA production by using antibiotic-resistant mutants with a high internal ergosterol level (Queener et al. 1982). Also, rational breeding procedures based on the biosynthetic pathway were used to select strains with improved MPA productivity. Among the different antibiotic-resistant mutants developed, a clofibrate and dodecyltrimethylammonium chloride double resistant mutant produced about 4.7 g l⁻¹ MPA (about three times more than the parent strain, *P. brevicompactum* ATCC 16024). A glutamate auxotroph of this antibiotic-resistant mutant was even able to produce up to 5.8 g l⁻¹ MPA. This strain was found to grow on L-aspartate instead of L-glutamate and exhibited only one-third of the pyruvate carboxylase activity of the parent strain (Ozaki et al. 1987a).

Cultivation in submerged culture showed that the production of MPA starts concomitantly with the hyphal aggregation phase just before pellet formation (Doerfler et al. 1978). Unlike most secondary metabolites, MPA is produced growth-associated in the exponential phase (in both batch and continuous cultures) and independent of the medium composition (Nulton and Campbell 1977; Doerfler et al. 1979). The production process is carried out either in submerged cultures or by solid-state fermentation (SSF).

For many years, the optimal medium for MPA production has been a semi-synthetic mixture composed of glucose (C-source), ammonium salts or casein (N-source), potassium dihydrogen phosphate (P-source), magnesium sulphate and trace elements. Some authors supplement other components like the amino acid glycine (Xu and Yang 2007) to further increase MPA production. More recently, it has been reported that, among different nitrogen sources, urea in concentrations up to 5 g l⁻¹ was the N-source of choice to support MPA production (Roh 2008). However all in all, only little efforts have been done to optimize media composition and cultivation conditions compared to the production of other important fungal metabolites.

Like other production processes involving fungal cells, growth morphology is a critical factor determining the growth rate and production yield. Fungi can grow either in form of pellets or mycelia and thus, controlling the growth morphology to a desired shape is important to improve the cell productivity. Altogether, more than 30 factors have been reported in the literature to influence the growth morphology; these include strain-dependent factors (type of strain, inoculum size, physiology, etc.), cultivation conditions (pH, temperature, osmotic stress, etc.) and medium composition (C-source, N-source, C/N ratio, surfactants, presence of insoluble particles) and many other factors (El Enshasy 2007).

Using spores as inoculum for MPA production in submerged culture, it was shown that the increase of spore density from 10^4 to 10^7 spores ml^{-1} resulted in significant reduction in pellet size with a concomitant increase in MPA production from 0.2 up to 4.8 g l^{-1} . Further, it was found that MPA can be continuously produced, independent of spore concentration, in the presence of 1% celite in the culture medium. In the case of celite addition, growth occurred in form of pellets (500 μm in diameter) regardless of the inoculated spore concentration (Ozaki et al. 1987b). New cultivation approaches try to overcome the problem of changed cell morphology by applying rotating fibrous-bed bioreactors (RFB). Accordingly immobilized cells in RFBs produced MPA up to a concentration of 5.7 g l^{-1} within 14 days using the standard wild-type strain *P. brevicompactum* ATCC 16024. Other advantages of RFB fermentation include the ease of product separation and purification from the fermentation broth as well as the possible repeated use of cells for long-term operation (Xu and Yang 2007).

Several attempts were made to use solid-state fermentation (SSF) as an alternative cultivation method. In general, filamentous fungi are well suited for SSF and a number of valuable metabolites can be produced under these conditions, since they perfectly reflect the natural habitats of the fungi (Krishna 2005).

An early study by Bartman et al. (1981) demonstrated that, when cells grew as surface culture, MPA production was associated with the aerial mycelium and its production ceased completely when the formation of aerial hyphae was blocked. However, the yield of MPA in this study was relatively low (only 0.3 mg g^{-1} wet weight). SSF production of MPA was optimized using a response surface methodology and the maximal yield achieved was 3300 mg kg^{-1} wheat bran (Sadhukhan et al. 1999). Further improvement was achieved by using a fed-batch strategy (Tiwari et al. 2003). Furthermore, a recent study of Alani et al.

(2009) has demonstrated that MPA production by SSF in a packed-bed bioreactor can lead to yields up to 6900 mg MPA kg^{-1} pearl barley within just 168 h.

Due to its low molecular weight and the relatively simple chemical structure, MPA and its derivatives can nowadays also be prepared chemically. Different procedures for MPA total synthesis have been published using different starting materials.

Patterson (1993) synthesized MPA using silyloxy-1,3-cyclohexadiene and allylic alcohol via an *ortho*-ester Claisen rearrangement. Another interesting method for a convergent synthesis of MPA via a palladium-tin coupling reaction between the alkyl side chain and the phthalide ring was described by Plé et al. (1997). A further method involves 2-geranyl-1,3-acetonedicarboxylate and 4-pivaloxy-2-butyral and a specific cyclization as key step (Covarrubias-Zúñiga and Gonzalez-Lucas 1998). The production of MPA using this new synthetic strategy is based on a ring annulation sequence involving a Michael addition reaction and an intra-molecular Dieckmann condensation in situ (Covarrubias-Zúñiga et al. 2003).

C. Mizoribine

The immunosuppressive antibiotic mizoribine or bredinin (5-hydroxy-1- β -D-ribofuranosyl-1H-imidazole-4-carboxamide; CAS 50924-49-7; Fig. 8.4) was first isolated from the culture medium of *Eupenicillium brefeldianum* isolated from soil samples on Hachijo island (Japan; Mizuno et al. 1974). Mizoribine (MZA) is an imidazole nucleoside and the metabolite MZ-5-P exerts its activity through selective inhibition of inosine monophosphate synthetase and guanosine monophosphate synthetase, resulting in the complete inhibition of guanine nucleotide biosynthesis (Shumpei 2002). Based on this immunosuppressive mechanism, mizoribine is superior to many

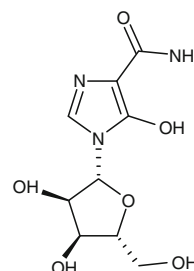


Fig. 8.4. Mizoribine

other clinically used drugs, since it may not cause damage to normal cells and nucleic acids.

In contrast to other immunosuppressive agents widely used at the time of its discovery (e.g. azathioprine), mizoribine was shown in animal experiments to lack oncogenicity and exhibited a clinically low incidence of side-effects such as hepatotoxicity and myelosuppression. These facts together supported its use in clinical application in long-term immunosuppression therapies. In 1984, MZR was first approved for the treatment of graft rejection after kidney transplantation (Takei 2002). Later, it was also approved for the treatment of other diseases including lupus nephritis, rheumatoid arthritis and primary nephritic syndrome. It is currently marketed in China, Korea and Japan under the trade name Bredinin (Tanaka et al. 2006). The drug is mainly produced by the fungi *E. brefeldianum* and *E. javanicum* in submerged culture under aerobic conditions, however, little information is available on the biosynthesis pathway and the production process (Mizuno et al. 1975; Benedetti et al. 2002).

D. Other Immunosuppressants

In addition to the three clinically approved fungal immunosuppressants, Cyclosporins, MPA and mizoribine, many other fungal metabolites possess also potent immunosuppressive activities (Fig. 8.5). Some of them were found to be not suitable for clinical applications due to their side-effects, whereas others are still subject of intensive studies and currently undergoing different levels of clinical trials till final approval by FDA.

1. Ovalicins and Fumagillins

The fungal metabolites ovalicin, fumagillin and their related derivatives belong to the most potent anti-angiogenic compounds. They bind covalently to the active site of the enzyme methionine-aminopeptidase type 2 (MetAP2) and irreversibly block its proteolytic activity (Liu et al. 1998; Turk et al. 1998).

Ovalicin or Graphinone (CAS 19683-98-8) is a sesquiterpene that was first isolated from culture filtrates of *Pseudurotium ovalis* in 1962 and found to have antimicrobial and cytotoxic activities. It was chemically characterized by Sigg and Weber (1968). The chemical structure of this

compound is related to the antibiotic fumagillin. The immunosuppressive properties of ovalicin were evaluated using the mouse hemagglutinin test, which reflects the degree of antibody production. It was found to be not toxic to the cells of bone marrow, which distinguished it from existing immunosuppressants at that time. Unfortunately, when tested in humans it later turned out to have other toxic side-effects.

A *Metarhizium* sp. isolated from soil in Japan was found to produce a 12-hydroxyovalicin (Kuboki et al. 1999), which was named Mer-f3. This compound was examined for its influence on mixed lymphocyte cultures, and showed a similar inhibitory activity as ovalicin. Mer-f3 had an immunosuppressant activity in the murine mixed lymphocyte test with $IC_{50} = 1$ nM which is even better than that of CyA (110 nM). Moreover, Mer-f3 had no inhibitory activity on leukemia L-1210 which indicates a low mammalian toxicity. Another *Metarhizium* sp. isolated from soil was reported to produce a novel immunosuppressive substance, which was named metacytofilin (Iijima et al. 1992); this compound had not any antimicrobial activity, while showing strong immunosuppressive effects.

Chlovalicin, a chlorinated compound derived from the epoxide ring attached to ovalicin, was discovered in the fermentation broth of the soil fungus *Sporothrix* sp. It inhibited the IL-6 dependent growth of MH60 cells and appeared to be a new IL-6 inhibitor (Hayashi et al. 1996). Two ovalicin related compounds, FR 65814 (CAS 103470-60-6) and Fumagillol (CAS 108102-51-8), were isolated from culture filtrates of the soil fungus *Penicillium jense-nii*. They both showed significant immunosuppressive activity at low concentrations (Hatanaka et al. 1988). A chiral and stereoselective total synthesis of FR 65814 using glucose as starting material was described by Amano et al. (1998, 1999), and fumagillol can be also synthesized using other starting materials (Kim et al. 1997, 2005a; Boiteau et al. 2001). TNP-470, a semisynthetic derivative of fumagillin, reduced the proliferation of endothelial cells with an IC_{50} value of 2.5×10^{-11} M. Therefore, it entered clinical trials as immunosuppressive and anti-tumor agent. The main drawbacks of its therapeutic properties were the short physiological half-life span and the severe side-effects such as ataxia, vertigo and agitation (Figg et al. 1997). The relation between the chemical structure and bioactivity of fumagillin and its derivatives was studied by several authors. It was shown that the spiro-epoxide is essential for the activity of fumagillin and its conversion into a methylene group results in a considerable reduction of efficacy (Logothetis et al. 2001). On the other hand, the epoxide of the side chain has no major effect on the biological activity. New synthetic analogues of ovalicin and fumagillin lacking reactive epoxy functionalities, which are thought to be responsible for the severe toxic side-effects, were synthesized by Mazitschek et al. (2005).

2. Gliotoxin

Gliotoxin (CAS 67-99-2) is a sulfur-containing antibiotic that belongs to the epipolythiodioxopiperazine group of secondary metabolites and

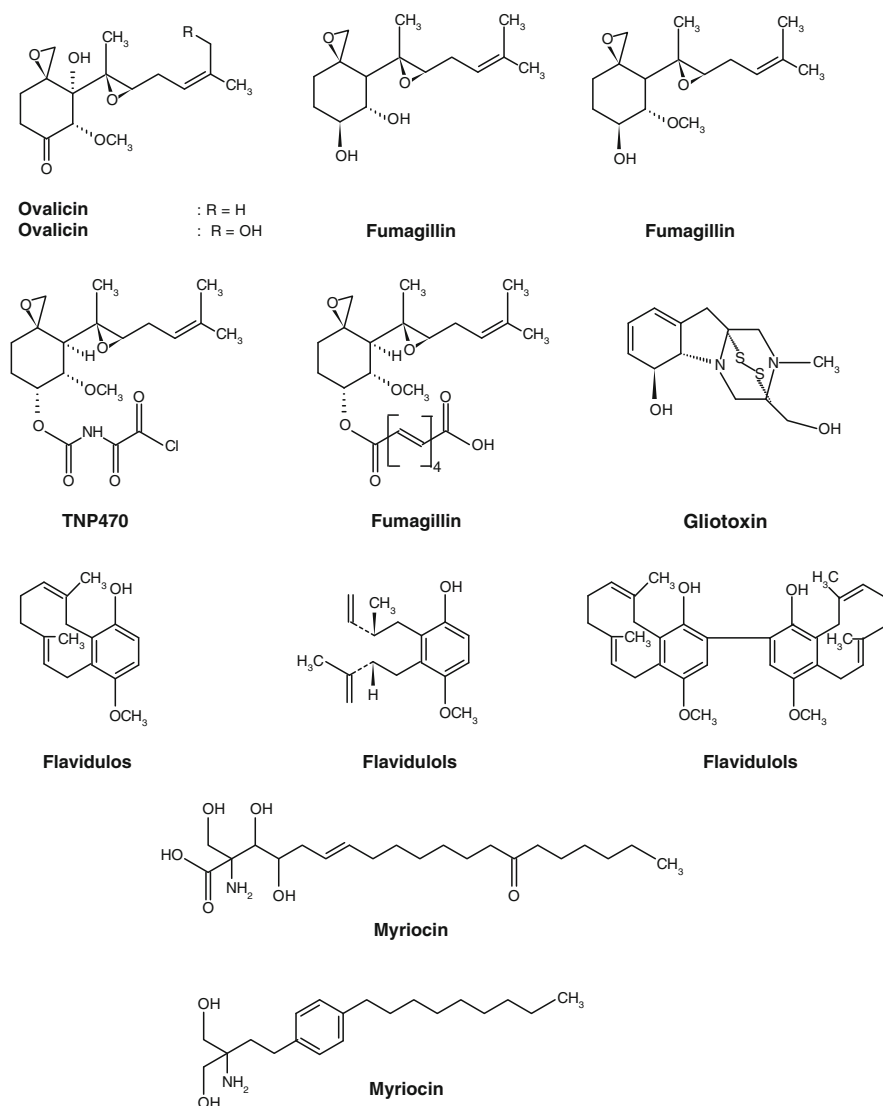


Fig. 8.5. Molecular structure of different types of fungal immunosuppressive agents

exhibits antifungal, antiviral as well as strong immunosuppressive activities. Gliotoxin was originally isolated from *Gliocladium fimbriatum* and named accordingly. It was reported thereafter that this compound is commonly produced by several genera of molds such as *Aspergillus*, *Trichoderma* and *Penicillium*. Gliotoxin was also claimed to be produced in yeasts of the genus *Candida*, however, a recent study being based on a screening of 100 clinical isolates of *Candida* doubted the occurrence of this compound in yeasts (Kupfahl et al. 2007).

The immunosuppressive effects of gliotoxin have been explained by different mechanisms. It suppresses cell activity and induces apoptosis in a variety of cell types including neutrophils, eosinophils and granulocytes (Ward et al. 1999). It inhibits the chymotrypsin-like activity of the 20S proteasome in a non-competitive manner (Kroll et al. 1999) and likewise the activation of NF- κ B in T- and B-cells when applied at nanomolar concentrations (Pahl et al. 1996). The immunosuppressive activity of gliotoxin was also attributed to the inhibition of perforin-dependent and Fas-ligand-dependent cytotoxic T-lymphocyte-(CTL)-mediated cytotoxicity (Yamada et al. 2000). Another study has furthermore demonstrated that gliotoxin suppresses the mast cells, which play a key

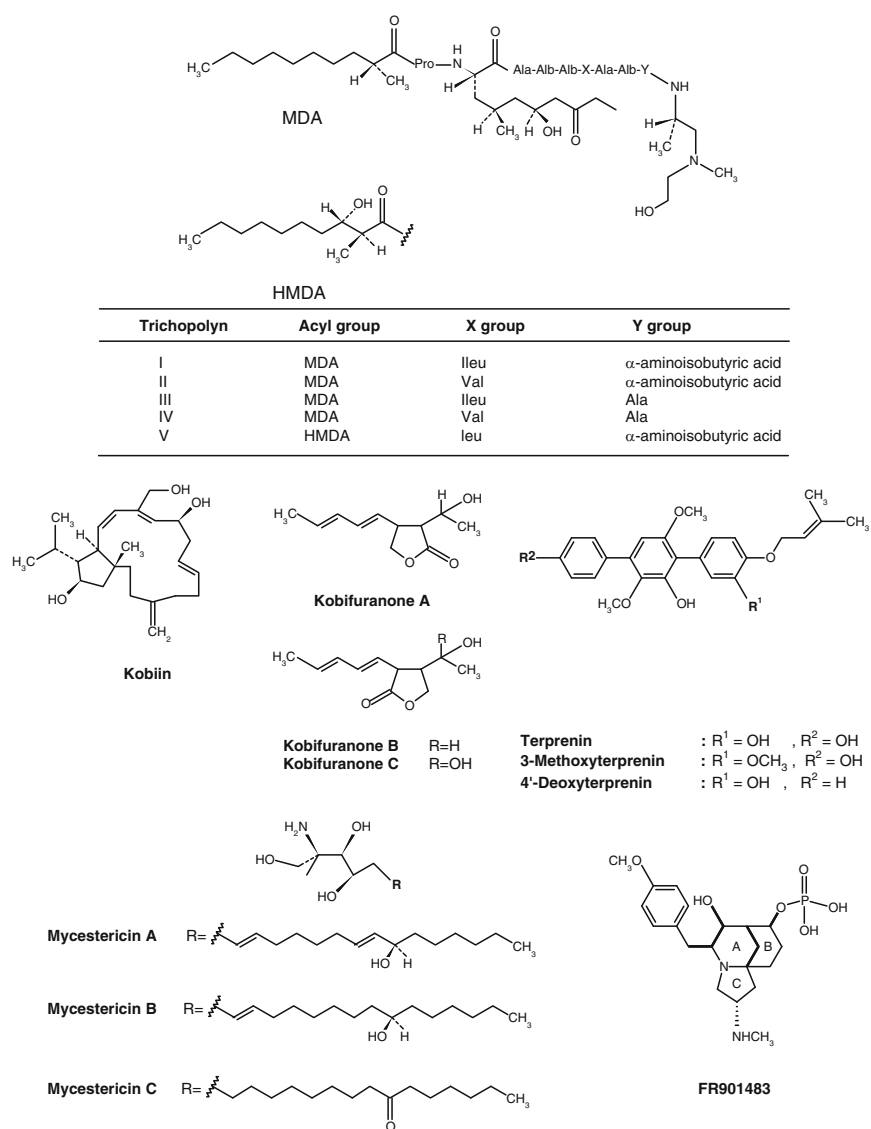


Fig. 8.5. (continued)

role in host defense and are important in both innate and adaptive immunity (Niido et al. 2006). As this compound is produced by many potential human pathogens in vivo during the course of infection, gliotoxin may also contribute to the etiology of fungal diseases (Waring and Beaver 1996).

3. Trichopolyns

Trichopolyns (TPs) are peptabiotic compounds produced by *Trichoderma polysporum*. TPs I and II were first isolated as new antifungal and antibacterial antibiotics in 1978 by Fuji and

coworkers. The chemical structures of TP I (CAS 66554-87-8) and TP II (CAS 6655-88-9) were identified as peptide antibiotics three years after their discovery (Fujita et al. 1981). Nowadays, the structures of five different trichopolyns (I–V) are known. Trichopolyns I and II are ten-residue peptides characterized by the presence of a 2-methyldecanoyl group at the N-terminus, and the C-terminal residue is protected by trichodiaminol. The other three analogues, TPs III–V differ from TPs I and II in that way that Aib (α -aminoisobutyric acid) is replaced by L-alanine. In contrast, TP V has the same amino acid sequence as

TP I, but the N-terminal acyl group is substituted by 3-hydroxy-2-methyldecanoic acid (instead of 2-methyldecanoic acid). These peptabiotics have been shown to suppress the proliferation of lymphocytes in mouse allogeneic mixed lymphocyte reactions (Lida et al. 1999). The TP I activity was even stronger than that of CyA.

4. Myriocin

Myriocin (antibiotic ISP-1 or thermozyomicidin; CAS 35891-70-4) was first isolated from the thermophilic fungus *Myriococcum albomyces* by Kluepfel and his group in 1972 and patented in the United States in 1975 (Kluepfel et al. 1975). The compound was recognized as an active antibiotic against yeasts and dermatophytes when applied in vitro. However, the compound appeared to be too toxic for therapeutic purposes in humans.

More than 20 years later, the same compound and its derivatives (mycestericins) were isolated from *Isaria sinclairii*, which is the imperfect stage of *Cordyceps sinclairii*, and showed strong immunosuppressive activities. Myriocin was 10- to 100-fold more effective than cyclosporin A both in vivo and in vitro tests (Fujita et al. 1994; Sasaki et al. 1994). *Isaria* sp. belong to the entomopathogenic fungi colloquially called “vegetable wasps and plant worms”, which have been used in oriental medicine for more than 1000 years (Im 2003). Myriocin was found to suppress both the production of antibodies against red blood cells of sheep and the induction of cytotoxic T-lymphocytes more strongly than cyclosporine A. It is also a potent inhibitor of serine palmitoyltransferase (SPT), the enzyme that catalyzes the first step in sphingosine biosynthesis (Miyake et al. 1995). Thus, it is used in biochemical research as a tool for depleting cells of sphingolipids. Myriocin can also be produced by chemical methods (Banfi et al. 1982; Oishi et al. 2002; Jones and Marsden 2008).

Fingolimod or FTY720 (CAS 162359-55-9) is a novel immunosuppressant obtained by chemical modification of myriocin (Adachi et al. 1995). A number of alternative ways for its preparation using shorter pathways for synthesis with higher overall yields have been published over recent years (Seidel et al. 2004; Adachi and Chiba 2007). Fingolimod was actually designed to eliminate the GI toxicity of the original compound myriocin. The exact mechanism of its immunosuppressive activity, however, remains still unclear. Some researchers have hypothesized that FTY720 may induce the apoptosis of lymphocytes (Suzuki 1996; Fujino et al. 2002). Others have proposed that the number

of lymphocytes decreases as a result of their movement towards secondary lymphoid organs such as lymph nodes and Peyer's patches (Sugito et al. 2005). It is certain that this novel immunosuppressant prolongs the survival of allo-graft transplants and is effective in the treatment of some immunological diseases. At the moment, FTY720 is being further developed by Novartis in phase II clinical trials. Not least, this compound may have a great clinical potential because of efficacy as oral drug for the treatment of multiple sclerosis (Gullo et al. 2006; Klatt et al. 2007).

5. Flavidulols

The immunosuppressive geranylphenols, flavidulols A (CAS 117568-32-8), B (CAS 117568-33-9) and C (CAS 117568-34-0), were originally isolated from fruiting body extracts of the mushroom *Lactarius flavidulus* in the course of a screening for new inhibitors of the proliferation of mouse lymphocytes (Takahashi et al. 1988). The chemical structure of these compounds was determined by NMR analysis (Takahashi et al. 1993); this paper also reported on the isolation of a new flavidulol D (CAS 156980-40-4). The suppressive effects of flavidulols A, B and C on the proliferation of mouse lymphocytes were stimulated in the presence of mitogens such as concavalin A (CoA) and lipopolysaccharides (LPS). Their IC₅₀ values for the inhibition of mitogen-induced concavalin A proliferation of mouse lymphocytes were between 9 and 36 $\mu\text{g ml}^{-1}$ and against lipopolysaccharide-induced proliferation between 7 and 28 $\mu\text{g ml}^{-1}$ (Fujimoto et al. 1993).

6. Kobiins

The sesterterpenetriol immunosuppressant kobiin and another three related 2-furanones named kobifuranones A, B and C were first isolated from the ascomycetes *Gelasinospora kobei* by Fujimoto et al. (1998). Kobiin possesses a bicyclic skeleton of five- and fifteen-membered rings. Kobifuranones A, B and C were supposed to be metabolites formed from a common intermediate biosynthesized through the acetate-malonate pathway. AcOEt extracts of fungal mycelia containing kobiin and the three kobifuranones were found to suppress proliferation of mouse spleen lymphocytes stimulated with the mitogens CoA

and LPS. After solvent fractionation followed by repeated chromatography, the purified kobilin preparation obtained showed the highest immunosuppressive activity.

7. Mycestericins

Mycestericins are a group of unique immunosuppressive compounds and chemically, hydroxylated α -hydroxymethyl α -aminoalkanoic acids. All known types of mycestericins were isolated from the cultures of *mycelia sterila* (i.e. filamentous fungi without any morphological structures, neither sexual organs and spores nor conidia and other asexual spores). The chemical structures of mycestericin A (CAS 128440-98-2), B (CAS 128341-87-7), C (CAS 37817-99-5), D (CAS 157183-67-0) and E were determined on the basis of comprehensive spectroscopic studies and chemical tests (Sasaki et al. 1994). Mycestericins suppress the proliferation of lymphocytes in the mouse allogeneic mixed lymphocyte reaction with a potency similar to that of myriocin. Further studies led to the isolation of two more active compounds, mycestericins F and G, from the same fungus (Fujita et al. 1996). The chemical structures of mycestericins F and G were identical to the respective dihydromycestericins D and E. Mycestericin A has also been chemically synthesized using simple tartrate as starting compound (Sato et al. 2008); total chemical synthesis of mycestericins E was accomplished by a cinchona alkaloid-catalyzed asymmetric Baylis-Hillman reaction (Iwabuchi et al. 2001). Mycestericins D–G can enzymatically be prepared using L-threonine aldolase from *Candida humicola* in the key step reaction (Nishide et al. 2000).

8. Terpenins

Terprenin (CAS 197899-11-9) was discovered by Kamigauchi et al. (1998) in the fermentation broth of *Aspergillus candidus* during a screening for natural immunosuppressants (Kamigauchi et al. 1998). It has a novel highly oxygenated *p*-terphenyl structure with a prenyloxy side chain. Two terprenin derivatives, 3-methoxy-terprenin and 4'-deoxyterprenin, were also isolated from the fermentation liquid and showed significant immunosuppressive effects when tested with respect to the proliferation of mouse spleen lymphocytes.

The most relevant activity of terprenin is its suppressive effect on the production of immunoglobulin E (IgE), which is a factor of 10^4 stronger than that of FK506, and interestingly, without any toxicological side-effect (Kawada et al. 1998). In mice experiments, terprenin suppressed IgE production in a typical dose dependent manner. Even after immunization with ovalbumin, when the IgE value had reached a high level, terprenin still exhibited a significant suppressive effect at 20–40 mg kg⁻¹ (Liu 2006). The total synthesis of terprenin is possible and was reported by different authors (Kawada et al. 1998; Yonezawa et al. 1998).

9. FR901483

A potent immunosuppressant, FR901483, was isolated in 1996 from the fermentation broth of *Cladobotryum* sp. by Fujisawa Pharmaceutical Co. in Japan (Sakamoto et al. 1996). It was found to exert a potent immunosuppressive activity in vitro and significantly prolonged graft survival in the rat-skin allograft model, apparently by the inhibition of purine nucleotide biosynthesis. This compound has an intriguing tricyclic structure possessing a phosphate ester in its molecule. Since its discovery, FR901483 has garnered significant attention from the organic chemists due to its biological activity and unique aza-tricyclic nature. Thus, different synthesis schemes were published for the total synthesis of this important immunosuppressant (Maeng and Funk 2001; Kropf et al. 2006; Carson and Kerr 2009).

10. Colutellin A

Colutellin A is a new immunosuppressive peptide recently isolated from *Colletrichum dematium*. It showed CD⁺ T-cell activation of interleukin 2 (IL-2) production with an IC₅₀ of 167 nM. Moreover, it exhibited no cytotoxicity to human peripheral blood mononuclear cells in respective in vitro tests. Thus, it could be medicinally used as a novel immunosuppressive compound in the near future (Ren et al. 2008).

III. Mushroom Immunomodulators

Nowadays, immunostimulators (biological response modifiers) are becoming increasingly more popular in the health and wellness industries

as people have started to realize the importance of a healthy immune system as a first barrier for the prevention of diseases. These pro-drugs or prophylactic medicines have a long history in traditional medicine, in particular in Asian and Mediterranean countries. Thus the medical use of mushrooms has a long tradition in Japan, China, Korea and Southeast Asia, whereas, in Europe and the United States, this field has just been developing since the early 1980s. The positive medicinal properties of mushrooms are based on various cellular compounds and secondary metabolites, which can be isolated from different parts of the fruiting body or from the mycelium during growth in solid-state or liquid cultures (Tang et al. 2007). The immunomodulating effects of mushroom metabolites are especially valuable in the prophylaxis as a mild and non-invasive form of a treatment, which can even prevent the proliferation of metastatic tumors, and is used as a co-treatment in combination with classic chemo- and radiotherapies (Wasser 2002). The most potent immunomodulators produced by mushrooms belong to the lectins, terpenoids and polysaccharides.

A. Lectins

The immunomodulatory activities of lectins (highly glycosylated proteins with specific binding capacities) from different organisms have been known for decades. Mushroom lectins are characterized by their particular antiproliferative and antitumor activities.

Boletus satanas lectin, bolesatine, was shown to have a potent mitogenic activity on human peripheral blood lymphocytes, and also to stimulate the release of IL-1 α , IL2 and TNF- α from mononuclear cell cultures (Licastro et al. 1993). A fruiting-body lectin of *Grifola frondosa* showed cytotoxic activity against HeLa cells, when applied at low concentration (Kawagishi et al. 1990). A heterodimeric melibiose-binding lectin from fruiting bodies of the oyster mushroom *Pleurotus ostreatus* was reported to be an in vivo inhibitor of sarcoma S-180 and hepatoma H-22 tumor cells (Wang et al. 2000). A specific lectin was identified in fruiting bodies and mycelia of the straw mushroom (*Volvariella volvacea*) and had a stronger immunomodulatory effect than concanavalin A (She et al. 1998). Two lectins, TML-1 and TML-2, with immunomodulatory and antitumor activities were isolated from *Tricholoma mongolicum*; however, when these

lectins were directly tested in vitro, no antitumor activity was observed. This suggests that the lectins are rather immunomodulatory substances than substances exerting acute cytotoxicity. Peritoneal macrophages in mice treated with TML-1 or TML-2 revealed – after LPS stimulation – an enhanced production of nitrite and TNF- α . Both compounds inhibited also the growth of P815 mastocytoma cells by stimulating peritoneal macrophages to produce more macrophage-activating factors including interferon- γ and some other cytokines (Wang et al. 1996, 1997).

B. Terpenoids

Terpenes are built up of isoprene sub-units consisting of five carbon atoms. Among the huge number of terpenes, are special triterpenoids which are exclusively found in certain macrofungi (mostly basidiomycetes) and are famous for their biological activities and medicinal properties. One example of such a triterpenoid compound is the highly oxidized lanostane which can be isolated from wood-decay fungi of the families Polyporaceae and Ganodermaceae (e.g. *Ganoderma lucidum*). This and related compounds show different biological activities including anti-infective, cytotoxic and immunomodulating efficacy (Moradali et al. 2007). Ganoderic, ganoderenic, ganodermic and applanoxidic acids, ganoderals, ganoderols, lucidone, ganodermanontriol as well as ganodermanondiol are the most common triterpenoids found in these mushrooms. Mixtures of these compounds can be prepared by the extraction of respective fruiting bodies with organic solvents. They were shown to have an antitumor activity that is comparable to that of certain β -D-glucans (see below). Fungal terpenoids can stimulate the NF- κ B pathway and modulate Ras/Erk, c-myc and CREB proteins as well as mitogen-activated protein kinases (Gao et al. 2003). In consequence, these activation mechanisms can lead to other immune stimulations which are finally effective against tumor cells.

C. Polysaccharides

Various polysaccharides of microbial and non-microbial origin have been widely used as potential biological response modifiers (BRMs) as reviewed by Leung et al. (2006). Mushroom derived polysaccharides are regarded as excellent

immunostimulators due to their suitable therapeutic properties, i.e. they are barely toxic and have just negligible side-effects compared to other immunostimulants. Respective polysaccharides occur in relevant amounts in the macroscopic fruiting bodies and cultured mycelia but also to some extent in the culture filtrates of fungi. Most macrofungal polysaccharides belong either to the homoglycans or heteroglycans, and can bind to structure proteins to form polysaccharide-protein complexes. In general, immunomodulator polysaccharides appear to be related to the fungal cell wall and comprise (1→3)- and (1→6)- β -glucans as well as (1→3)- α -glucans or polysaccharide complexes of the galactomannan- and glucuro-mannan-protein type.

Research on mushroom polysaccharides can be traced back to the 1960s when Ikegawa's group in Japan first investigated the host-mediated antitumor activity of hot-water extracts of several edible mushrooms against sarcoma 180 cells of mice (Ikekawa et al. 1969). Until the late 1980s, three antitumor-immunomodulators of the β -glucan type were isolated and characterized, namely lentinan, schizophyllan and a protein-bound β -glucan (PSK Kresin). They originate from the white-rot fungi *Lentinus edodes*, *Schizophyllum commune* and *Coriolus versicolor*, respectively, and have successfully been introduced into the probiotic and pharmaceutical market in Japan. A similar polysaccharopeptide as PSK, abbreviated as PSP is produced in China and widely used in the clinical treatment of tumors and in anticancer therapy (Ooi and Liu 2000). Although their mode of action against tumor cells is not yet fully understood, they have been demonstrated to act as biological response modifiers (BRMs), which are able to restore or enhance various immune responses in vitro and in vivo.

The mushroom polysaccharides or polysaccharide-protein complexes stimulate the non-specific immune system and thereby exert antitumor activities through the stimulation of the body's own defence mechanisms (Wasser and Weis 1999; Reshetnikov et al. 2001). They can activate effector cells like macrophages and T-lymphocytes or prompt NK cells to secrete cytokines like TNF- α , IFN- γ and IL-1 β . In turn, some of these cytokines are able to directly promote the cytotoxicity of macrophages. The production of cytokines by immune cells can be considered as a key event in the initiation and regulation of the body's immune response (Lull et al. 2005). In this context, mushroom polysaccharides can act as antiproliferative effectors and induce apoptosis in tumor cells.

Certain mushroom polysaccharides (e.g. BRMs) were shown to reduce the tumor size by more than 50% and considerably prolonged the survival of tumor-bearing mice (Wasser 2002). Though the exact mechanism of BRM action is not known, it has been proposed that they initiate a cascade of signal transduction that is responsible for the immune response. Since polysaccharides are not able to penetrate cells (due to their high molecular mass), the first step of the cascade may be the recognition of BRM and its specific binding to immunocell receptors. Some evidence exists that there are pattern recognition receptors (PRRs) for the molecular reception of the polysaccharide BRM (Lowe et al. 2001). The binding of the BRM-ligand to PRRs may initiate Rel/NF- κ B-mediated signaling events, which leads to the induction of gene expression and the stimulation of specific cellular functions of the innate immunity system (Leung et al. 2006). Whilst it is known that mushroom extracts have immunomodulatory activity, the standard approach has always been to isolate, characterize and administer pure active compounds. However, different types of polysaccharides in a mushroom extract may have synergistic activities (Borchers et al. 2004; Lull et al. 2005). The responses to different polysaccharides are mediated by different PRRs on the cell surface. An appropriate combination of strong responses involving different parts of the cell may provide greater therapeutic effects than a single polysaccharide. A brief list of immunomodulator polysaccharides and polysaccharide-protein complexes from mushroom is given in Table 8.1.

Mushroom polysaccharides greatly differ in their sugar composition, branching configuration, helical conformation and other physical properties. The structure relationship between immunomodulator and the anticancer activities of polysaccharides have been reviewed by several authors (Ooi and Liu 2000; Lull et al. 2005; Zhang et al. 2007; Ooi 2008). It has been stated that structural features such as (1→3)- β -linkages in the main chain of the glucan and additional (1→6)- β -branching points, represent important factors influencing their biological effectiveness. β -Glucans containing mainly (1→6)-linkages are less effective, maybe due to their inherent flexibility and the large number of possible conformations (Zhang et al. 2007). In general, β -glucans exhibit immunomodulatory and/or antitumor activities when their main chain ("backbone") forms a linear structure and do not have long branches. For example, pachyman, a branched (1→3)- β -D-glucan obtained from the brown-rot fungus *Poria cocos* is inactive, whereas pachyman obtained by the debranching of pachyman using selective periodate oxidation and mild hydrolysis, shows a pronounced activity (Chihara et al. 1970a).

Lentinan (2/5) and schizophyllan (1/3) are (1→3)- β -D-glucans with two or one branches for every 5-D-glucopyranosyl and 3-D-glucopyranosyl residue, respectively (Tabata et al. 1981; Chihara 1992). The polysaccharide moiety in PSK (1/5) is a (1→3)- β -(1→4)- β -D-glucan with one branch for every 5-D-glucopyranosyl residue (Tsukagoshi et al. 1984). Although the degree of their branches is different, their bioactivities are similar (Ooi and Liu 2000). Interestingly, debranched lentinan preparations were found to be more effective against cancer cells than the native lentinan when applied during in vivo studies

Table 8.1. Immunomodulator polysaccharides from mushrooms

Mushroom species	Polysaccharide source	Active component	References
<i>Coriolus versicolor</i>	Mycelium, Culture broth	Polysaccharopeptide	Cui and Chisti 2003
<i>Cordyceps sinensis</i>	Fruiting body, Mycelium, Culture broth	Glucan, heteroglycan, Cordyglucan	Yalin et al. 2005 Russell and Paterson 2008
<i>Flammulina velutipes</i>	Fruiting body, Mycelium	Glucan-protein complex, glycoprotein	Leung et al. 1997
<i>Ganoderma lucidum</i>	Fruit body, Culture broth	Heteroglycan, mannoglucan, glycopeptide	Miyazaki and Nishijima 1981 Gao and Zhou 2003
<i>Grifola frondosa</i>	Fruiting body, Culture broth	Proteoglycan, glucan, heteroglycan, galactomannan, grifolan	Cun et al. 1994 Yang et al. 2007
<i>Hericum erinaceus</i>	Fruiting body, Mycelium	Heteroglycan, Heteroglycan-peptide	Mizuno 1992
<i>Inonotus obliquus</i>	Fruiting body, Mycelium	Glucan	Kim et al. 2005b
<i>Lentinus edodes</i>	Fruiting body, Culture broth	Mannoglucan, glucan, Lentinan, polysaccharide protein complex	Chihara et al. 1970b Hobbs 2000
<i>Peziza veruculosa</i>	Fruiting body	Glucan, Proteoglycan	Mimura et al. 1985
<i>Pleurotus ostreatus</i>	Fruit body, Mycelium, Culture broth	Heteroglactan, Proteoglycan	Sarangi et al. 2006
<i>Polyporus umbellatus</i>	Mycelium	Glucan	Yang et al. 2004
<i>Polystrictus versicolor</i>	Fruiting body, Mycelium, Culture broth	Heteroglycan, glycopeptides, krestin (PSK)	Cui and Chisti 2003
<i>Sclerotinia sclerotiorum</i>	Sclerotium	Glucan, scleroglucan (SSG)	Palleschi et al. 2005 Survase et al. 2007
<i>Tremella aurantialba</i>	Fruiting body	Heteroglycan	Liu et al. 2003
<i>Tricholoma lobayense</i>	Culture broth	Polysaccharide- protein complex	Liu et al. 1996

(Sasaki et al. 1976). Overall, the relationship between the molecule's biological activity and the branching pattern/ratio of β -glucans seems to be rather complicate. The available data indicate that the (1 \rightarrow 3)- β -D-glucan backbone is essential and that the most active polymers have degrees of branching between 0.2 and 0.33 (Ooi 2008). The molecular weight of the polysaccharide plays also an important role for the bioactivity. For a (1 \rightarrow 3)- β -glucan extract of *G. frondosa* consisting of fractions of different size, the highest immunomodulatory activity was detected for molecular masses around 800 kDa (Adachi et al. 1990). When PSK was separated into four fractions (F1, <50 kDa; F2, 50–100 kDa; F3, 100–200 kDa, F4, >200 kDa) by successive ultrafiltration, the highest immunomodulatory activity was obtained with the high-molecular mass fraction F4 (Kim et al. 1990). Chemically modified (1 \rightarrow 3)- β -D-glucans, such as schizophyllan and lentinan having a linear “worm-like”, triple-helical structure and average molecular masses of <50 000 g mol⁻¹ or >110 000 g mol⁻¹ efficiently stimulated monocytes in vivo and caused the secretion of more TNF- α than the samples with molecular masses between 67 000 and 110 000 g mol⁻¹ did. Therefore, the actual

relation between the polysaccharide molecular mass and its immunomodulation activity remains to be clarified. Conformations of polysaccharides include single helices and triple-helices as well as random-coiled structures. A triple-helix conformation is usually more stable than a single-helix. Lentinan, schizophyllan and the glucan moiety of PSK have all triple helix structures. Also the cytokine-stimulating activity of (1 \rightarrow 3)- β -D-glucans was found to be associated with the triple-helix conformation (Falch et al. 2000). Therefore, the immunological activities of polysaccharides must be dependent on appropriate helical conformation.

To improve the biological activity of polysaccharides by chemical modification, carboxymethylated, hydroxylated, formylmethylated, aminethylated and sulfated products have been designed. For example, a hydroxylated schizophyllan was found to induce in vivo the production of higher concentrations of nitric oxide (NO) and TNF- α in macrophages than native

schizophyllan (Ohno et al. 1995). A sulfated (1→3)- α -D-glucan prepared by respective modification of native cell-wall glucan from *L. edodes* exhibited a strong antiproliferation activity against breast carcinoma cells, whereas its native water-insoluble precursor glucan had only moderate antitumor activity (Zhang and Cheung, 2002). Another study conducted by Zhang et al. (2004) showed that the high bioactivity of carboxymethylated β -glucan was mainly attributed to the increase of water-solubility and the latter was also the main factor that enhanced the efficacy of the hyperbranched β -glucan TM3b after sulfatation (Tao et al. 2006).

D. Fungal Immunomodulator Proteins

Recently, different mushrooms have been reported to produce a new family of fungal immunomodulatory proteins (FIPs) with possible applications in therapy (Chen and Wang 2007). These include Ling Zhi-8 (LZ-8) from *Ganoderma lucidum* (Kino et al. 1989), FIP-fve from *Flammulina velutipes* (Gr.) Sing (Ko et al. 1995), FIP-vvo and FIP-vvl from *Volvariella volvacea* (Bull.; Fr.) sing (Hsu et al. 1997), FIP-gts from *Ganoderma tsugae* Murr (Lin et al. 1997) and PCP from *Poria cocos* (Schw.) Wolf (Chang and Sheu 2007). All these compounds were grouped together in a distinct protein family based on similarities in their amino acids sequence and their effects on compounds of the immunological response system (Ko et al. 1995).

FIPs were found to be mitogenic in vitro for human peripheral blood lymphocytes (hPBLs) and mouse splenocytes. They induce a bell-shaped dose-response curve similar to that of lectin mitogens. In the course of in vivo studies, the FIP-like substance LZ-8 could act as an immunosuppressive agent through the prevention of systemic anaphylactic reactions and significantly decreased footpad edema during the Arthus reaction (Tanaka et al. 1989). Moreover, it suppressed autoimmune diabetic reactions in diabetic mice and increased graft survival in transplanted allogenic mouse skin and pancreatic rats without producing the severe toxic effects known for CyA (Van der Hem et al. 1994; 1996). FIP-fve isolated from fruiting bodies of *F. velutipes* stimulated mitogenesis of human peripheral lymphocytes, suppressed systemic anaphylaxis reactions and enhanced the transcription of interleukin-2 (IL-2) and interferon- γ (Ko et al. 1995). The induction mechanism of interferon- γ production was proposed to be mediated by a signaling pathway involving the p38 mitogen-activated protein kinase (Wang et al. 2004). FIP-gts was reported to significantly induce cytokine

secretion, cellular proliferation in human peripheral mononuclear cells (HPBMCs) and interferon- γ expression. The effect of FIP-gts may be caused by the activation of phosphatidylinositol 3-kinase (Hsiao et al. 2008). Finally, the immunostimulus initiated by the recently isolated FIP-PCP is mediated via an enhanced production of NO, IL-1 β , IL-6, IL-18 and TNF- α (Chang and Sheu 2007).

E. Industrial Production of Mushroom Immunomodulators

Immunomodulator metabolites can be isolated from fruiting bodies, cultured mycelia or culture filtrates. All medicinal mushrooms are lignocellulose degraders (white-rots, brown-rots, litter decomposers) and can utilize woody materials as growth substrates and for fruiting body production. One historical method of cultivation, that is still practiced mainly in Asia, is fungal cultivation on hardwood tree-logs. This process occurs over several years and yields two crops of mushrooms each year. It continues until the log physically “disappears” due to wood decay and lignocellulose decomposition. The use of polypropylene bags containing crushed lignocellulosics (including waste materials) and selected nutrients can be regarded as a modified version of the log method and actually represents a kind of solid-state fermentation (SSF). After autoclaving, the bags are inoculated with the mushroom mycelium of choice and can be incubated in the greenhouse under controlled conditions. This way, the production cycle for fruiting bodies can be shortened to 1–3 months (Smith et al. 2002). Mushroom production using SSF techniques was recently reviewed by Fan et al. (2008; see also Chapter 4 of this book). However, for the production of biomolecules, the production process should be carried out under more defined and controlled conditions to fulfil the strict requirements of the current good manufacturing practice (cGMP) for the production of active pharmaceutical ingredients (API). Quality control of mushroom cultivation poses several challenges, such as maintaining a constant substrate quality, temperature, moisture, a stable yield of the desired compounds and sterility.

To overcome these problems, more specific research was carried to cultivate mushrooms under submerged conditions. This method of cultivation has some advantages over SSF, for example, high yields in fungal mycelium under more defined conditions in a closed and well controlled

volume (higher space–time yields). Furthermore, sterility is easier to guarantee in a stirred-tank bioreactor than in logs or plastic bags (Lull et al. 2005). Nowadays, different bioactive metabolites from mushrooms can successfully be produced in submerged culture both in form of intracellular and extracellular products. However, in order to scale-up these methods to an industrial scale, various technical problems will have to be solved (Tang et al. 2007). Like in case of other fungal metabolites, medium composition governs the bioactive agents' production; for example, the production of mushroom polysaccharides was found to be regulated by the type and concentration of carbon and nitrogen sources (Wasser et al. 2003; Cui et al. 2006), the C/N ratio (Wang et al. 2005), by the ammonium ion concentration (Mao and Zhong 2006) and by different other components supplemented to the medium (Lim and Yun 2006). Other main key parameters influencing product yield are shear stress (Gong and Zhong 2005) and the mode of oxygen supply to the culture (Tang and Zhong 2003). The steady-state concentration of the latter in the culture medium was found to be very important for the over-production of mushroom metabolites in bioreactors (Mao and Zhong 2004). Among different cultivation strategies applied, fed-batch cultivation was proved to be the method of choice for most mushrooms tested so far (Kim et al. 2006; Zou 2006). The recent reviews of Zhong and Tang (2004) and Tang et al. (2007) summarize and discuss the latest developments in this field.

IV. Conclusions

Fungal immunomodulators act either as suppressors or stimulators, and they are equally important molecules for many medical applications. The number of known fungal immunomodulators is increasing every year through the discovery of new compounds from so-far ignored fungal species and the development of appropriate semi-synthetic derivatives. This has led to a rapid expansion of the respective market. Some of these agents have approved from FDA and are widely used as commercial preparations, some have reached the level of clinical trials and others are currently subject of detailed pre-clinical tests. Also in the future, the search for new safe immunomodulators (without severe side-effects) to substitute the currently used preparations will be an important goal for both the scientific and the industrial communities related to the health care sector.

Mushrooms are one of the most promising sources for the production of safe and effective immunomodulators. The number of mushroom species ("macrofungi") on earth has been estimated to be 140 000, suggesting that only 10%

are known so far. Assuming that the proportion of "useful" mushrooms among the undiscovered and unexamined species will be only 5%, this implies a number of 7000 promising species as a future source of immunomodulators (Lindequist et al. 2005). Moreover, it has to be taken into consideration that different strains of one species can produce different bioactive compounds. For example, strains of *G. lucidum* can produce more than 120 different triterpenes and in addition also bioactive polysaccharides and proteins.

Based on the increased knowledge of the biochemistry and molecular biology of bioactive metabolites as well as the advanced technology of high-throughput screenings using omic approaches (genomics, proteomics, metabolomics), a rapid development of this research field is expected and will lead to the discovery of many novel immunomodulators in the near future. Not least, the investigation of novel immunomodulators and their effects will widen our knowledge of the complex mechanisms regulating the immune system and body defense.

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9 Ergot Alkaloids

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I. Introduction

Ergot alkaloids are a class of indole-derived mycotoxins that have affected human health and agricultural productivity in both positive and negative

ways. The ergot alkaloids include a diverse group of secondary metabolites produced by several fungi. These alkaloids have a common biosynthetic origin and, in the cases studied, originate from a common set of biosynthetic reactions encoded by a cluster of shared genes (Tudzynski et al. 1999; Coyle and Panaccione 2005; Haarmann et al. 2005; Unsöld and Li 2005; Fleetwood et al. 2007; Lorenz et al. 2007). After these early, shared steps, pathways diverge in different fungi to yield the diverse structures observed in different ergot alkaloid producers. Additional diversity of structures results from inefficiency in the respective pathways such that intermediates and spur products accumulate to relatively high levels (Panaccione 2005). Thus the various ergot alkaloids observed in a fungus at a given time may include pathway end-products, pathway intermediates, and products of spur pathways off the main ergot alkaloid pathway of the particular fungus.

Ergot alkaloids have affinities for receptors of the monoamine neurotransmitters 5-hydroxytryptamine (5-HT, also called serotonin), dopamine, noradrenaline, and adrenaline² (Pertz 1996; Pertz and Eich 1999; Schade et al. 2007; Zanettini et al. 2007; Tfelt-Hansen and Koehler 2008). Because of their structural diversity, some ergot alkaloids act as agonists at these receptors, whereas others are antagonists. The interactions are further complicated by the fact that receptors for each of these neurotransmitters occur as different subtypes, and affinities and activities for different alkaloids at different subtypes vary. As a result, ergot alkaloids affect a variety of systems and often have undesirable side-effects.

Several fungi, representing two different orders and three different trophic strategies, produce ergot alkaloids. Historically, ergot

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²Noradrenaline and adrenaline are synonymous with norepinephrine and epinephrine, respectively.

alkaloids associated with the ergot fungus *Claviceps purpurea* have achieved notoriety because of inadvertent human poisoning in populations that relied heavily upon rye bread for a large portion of their calories. Clinically, ergot alkaloids have been used to treat migraines, Parkinson's disease, prolactinomas, and to stimulate uterine contractions and reduce bleeding during child birth (Maughan et al. 2006; Schade et al. 2007; Zanettini et al. 2007; Gallos et al. 2008; Tfelt-Hansen and Koehler 2008). However, their toxicity and often multiple side-effects have limited their utility as drugs. In agriculture, ergot alkaloids have directly and negatively affected plant quality through their antimammalian activities and indirectly and positively affected plant productivity as a result of their activities against invertebrate and vertebrate herbivores (Clay and Scharndl 2002; Panaccione 2005; Panaccione et al. 2006c; Scharndl et al. 2006; Potter et al. 2008). All these activities are discussed in detail below.

The main objectives of this review are to illustrate the diversity of alkaloids in the ergot group, propose ways by which these diverse alkaloids are formed, and discuss the biological significance of ergot alkaloids to the fungi that produce them.

II. Ergot Alkaloid-Producing Fungi

Ergot alkaloid-producing fungi have a long history of association with agricultural and medical problems. Three groups of ergot alkaloid-producing fungi have been studied extensively: the ergot fungi of the genus *Claviceps*, endophytic mutualists of the genus *Neotyphodium* (some with perfect states in the genus *Epichloë*), and the common saprophyte and opportunistic human pathogen *Aspergillus fumigatus* and a few related saprophytic fungi. These fungi represent two different lineages. *Claviceps* spp. and *Neotyphodium* spp. are grouped in the family Clavicipitaceae of the order Hypocreales, and *A. fumigatus* and related fungi belong to the family Trichocomaceae of the order Eurotiales. The ergot alkaloid producers display three trophic strategies that range from saprophyte (*A. fumigatus*), to parasite (*C. purpurea*), to mutualistic symbionts (*Neotyphodium* spp.).

A. Ergot Alkaloid Producers in the Clavicipitaceae

1. *Claviceps* Species

The most widely studied ergot fungus is *C. purpurea*, a pathogen of rye (*Secale cereale*) and other wild and cultivated grasses. Like other ergot pathogens in the genus *Claviceps*, *C. purpurea* infects flowers of grasses and can replace the developing seed with a hardened fungal overwintering structure known in mycological terms as a sclerotium (plural sclerotia) or more casually as an ergot. The fungus has a life cycle with an active infective stage restricted to flowers of grasses and an overwintering stage in its sclerotial form (reviewed in detail by Luttrell 1981; Tudzynski and Scheffer 2004). The initial infection results from ascospores that arise from perithecia embedded in stalked stroma, which develop in the spring on ergot sclerotia that had been produced during the previous year. Ascospores are actively discharged from perithecia and infect through open flowers. The fungus colonizes the floret and often produces a sugary, sticky fluid called honeydew during the early stage of infection, sometimes referred to as the sphaelial stage. Honeydew contains large numbers of conidia (the asexual, clonal spores of the fungus) that may serve as inoculum for other flowers when introduced to those flowers by insects, rain splash, or simply dripping down the infected grasses spikelet. Later in the season, as the uninfected flowers develop into seeds, the fungus in the infected florets forms the dense, typically darkly pigmented sclerotium, in which the fungus will rest idly through the fall and winter. The sclerotium is made up of densely packed hyphae of the fungus and is packed with oils and relatively high concentrations of ergot alkaloids.

The contamination of rye and other grain crops with ergot alkaloid-containing sclerotia of *C. purpurea* caused gangrenous and convulsive toxicoses known as ergotism or St. Anthony's fire. These toxicoses plagued people throughout recorded history until diets of affected cultures became diversified and screening of grain to remove ergot sclerotia became common practice (Matossian 1989). The ability of different ergot alkaloids to act as agonists or antagonists at different monoamine neurotransmitter receptors results in deleterious effects on nervous, circulatory,

reproductive, and immune systems (Pertz 1996; Gröger and Floss 1998; Pertz and Eich 1999).

Other species of *Claviceps* also accumulate ergot alkaloids in sclerotia that are produced in place of infected grain. These other *Claviceps* species have not been studied as intensively as *C. purpurea*, presumably because the grasses they infect are not as widely consumed by humans (e.g., sorghum for *C. africana*) or because the diseases have limited geographical distribution (e.g., ergot of maize caused by *C. gigantea*, which is restricted to regions of Mexico). *C. fusiformis* (which infects pearl millet, *Pennisetum glaucum*), *C. paspali* (which occurs on *Paspalum* species), *C. gigantea* (which causes ergot of maize), and one of the three *Claviceps* species (*C. africana*) that infects sorghum each has a life cycle similar to that described for *C. purpurea* and produces a characteristic profile of ergot alkaloids that differs from that produced by *C. purpurea*.

2. *Neotyphodium* Species

From ecological and agricultural perspectives, *Neotyphodium* spp. (imperfect states of fungi in the genus *Epichloë*) may be the most important of the ergot alkaloid-producing fungi. These fungi are typically found in mutualistic symbioses with grasses including important species of forage and turf grasses, where the fungi grow intercellularly and nondestructively, thus earning the name “endophyte”. The symbioses have been reviewed in detail by Scott (2001), Clay and Schardl (2002), and Schardl et al. (2004).

In the asexual phase, these fungi are not pathogenic, but rather live symbiotically in above-ground parts of the plant. Mycelium of the fungus generally proliferates between cells in the pseudostem of the grass and to a lesser extent in the leaf blades. Eventually, mycelium of the fungi colonizes developing seeds and is transmitted vertically to the next generation seedling through infected seed. In those species that also have a sexual state (classified in the genus *Epichloë*), sexual reproduction by the fungus results in eruption of the fungus through the host cuticle, damaging host tissue and preventing sexual reproduction by the host (Schardl et al. 2004). Thus when forming sexual structures, *Epichloë* species cross the line from mutualist to antagonist.

The *Neotyphodium* species that produce ergot alkaloids primarily accumulate those alkaloids in their hyphae rather than in any specialized structure. Hyphae growing in different tissue of their host plant all appear to accumulate ergot alkaloids but the profile of alkaloids produced is not always constant. The endophytes have significant effects on their host plant and on other organisms that interact with their host plant as

herbivores, parasites, or competitors. Grass–endophyte interactions that are of particular relevance to agriculture include the interactions between tall fescue (*Lolium arundinaceum*, synonym *Festuca arundinacea*) and *Neotyphodium coenophialum*, and between perennial ryegrass (*Lolium perenne*) and its endophytic fungi *Neotyphodium lolii* or, in some cases, the hybrid *Neotyphodium lolii* × *Epichloë typhina* (Schardl et al. 1994). Numerous other *Neotyphodium*/*Epichloë* species have been described from other wild and cultivated grasses (Schardl et al. 2004).

From an agronomic perspective, endophytic fungi are beneficial because they increase host fitness and stress tolerance (Bush et al. 1997; Schardl and Phillips 1997; Bacon and White 2000; Malinowski and Belesky 2000; Clay and Schardl 2002). However, endophytes are associated with a variety of animal toxicoses. Symptoms include the following: weight loss or poor weight gain, low fertility, reduced lactation, inability to control body temperature, low immunity, and gangrene of the limbs and tail (Schardl and Phillips 1997; Porter and Thompson 2002; Gadberry et al. 2003). Many of the symptoms of the endophyte-associated toxicoses are typical of the symptoms associated with human poisoning by ergot alkaloids. Several carefully performed experiments have shown that ergot alkaloids produced by the endophytes are major factors in the toxicoses associated with grazing endophyte-infected grasses (e.g., Hill et al. 1994; Filipov et al. 1998; Tor-Agbidye et al. 2001; Gadberry et al. 2003). However, it has not been clearly established which ergot alkaloid(s) among the complex ergot family are primarily responsible (Hill et al. 2001; Gadberry et al. 2003).

3. Other Clavicipitaceous Fungi

Some fungi in the genus *Balansia* also produce ergot alkaloids (Bacon et al. 1981, Powell et al. 1990). *Balansia* spp. grow intercellularly in infected warm season grasses and typically produce relatively large sclerotia which may be associated with vegetative or reproductive tissues of the host (Bacon et al. 1986). The use of ergot alkaloid-containing sclerotia of *Balansia cyperi* in obstetrics by an Amazonian culture (Lewis and Elvin-Lewis 1990) is analogous to the use of *C. purpurea* sclerotia for similar purposes in European cultures.

Another group of ergot alkaloid-producing fungi in this family has only recently been discovered. The association of ergot alkaloids with certain morning glories and related plants in the Convolvulaceae has been a puzzle for decades. The work of Leistner and colleagues has shown that

accumulation of ergot alkaloids is correlated with localized colonization of secretory glands by previously undiscovered fungi (Kucht et al. 2004; Steiner et al. 2006). The fungi have not been cultured or formally described yet but their DNA sequences indicate a phylogenetic placement in the Clavicipitaceae (Steiner et al. 2006). Elimination of fungi from the morning glory plants by fungicide treatment resulted in lack of ergot alkaloids in the fungus-free plants (Kucht et al. 2004). Moreover the fungi contain gene sequences for *dmaW*, the initial gene in the ergot alkaloid pathway and other pathway genes (Markert et al. 2008).

B. Ergot Alkaloid Producers in the Trichocomaceae

1. *Aspergillus fumigatus*

Aspergillus fumigatus is a fungus that exists in nature primarily as a free-living saprophyte on vegetation. It accumulates high concentrations of ergot alkaloids on its very small and highly buoyant spores (Panaccione 2005; Panaccione and Coyle 2005; Coyle et al. 2007). The fungus has been known and studied in its anamorphic *Aspergillus* state for decades, but its teleomorphic (sexual) state was just recently described as *Neosartorya fumigata* (O’Gorman et al. 2009). Despite its natural existence as a saprophyte of vegetation, from our perspective *A. fumigatus* is often considered a noxious mold of homes and buildings and as an opportunistic pathogen of the immunocompromised. In fact, *A. fumigatus* is the most common airborne fungal pathogen of humans (Latgé 1999).

The ability of *A. fumigatus* to cause disease in animals and humans may be considered an unfortunate accident, since infection of humans does not allow the fungus to complete its life cycle and thus is of no apparent ecological benefit to the fungus. Its pathogenic abilities may be due in part to its extremely small and buoyant spores which may be inhaled deeply into the lungs, its ability to grow at 37 °C, and its ability to sometimes avoid destruction by cells of the innate immune system. In individuals whose acquired immune system has been suppressed by drugs (such as the organ transplant drug cyclosporine) or virus infection (most notably HIV), the fungus can grow invasively in the lungs resulting in invasive aspergillosis. The infection may also spread from the lungs into other organs and tissues. In immunocompromised individuals, invasive aspergillosis is almost always fatal (Latgé 1999).

Aspergillus fumigatus also is an agriculturally important fungus. It can grow in silage and in wet hay, causing spoilage of these feeds (Cole et al. 1977; Dutkiewicz et al. 1989; Melo dos Santos et al.

2002; Sanchez-Monedero and Stentiford 2003). Its thermotolerance and good saprophytic abilities also allow the fungus to grow in compost. Proliferation of the fungus at large municipal composting facilities can be a health concern (Fischer and Dott 2003). The abundant spores that may be produced in composting facilities or agricultural conditions can cause clinical syndromes separate from invasive aspergillosis.

For example, aspergilloma (also called mycetoma or “fungus ball”) is a condition in which a colony of the fungus grows in a pre-existing cavity in the lung or sometimes the sinus. In most cases the colony grows without penetrating cells of the host tissue. Sometimes the condition may be asymptomatic and only diagnosed during examinations for other conditions. In this state the fungus is growing essentially as a saprophyte but within cavities in the human body. Other species of *Aspergillus* also may cause this condition, but the incidence of *A. fumigatus* is high (Latgé 1999) presumably because the spores of the fungus are common, abundant, and exceptionally small and buoyant (Panaccione and Coyle 2005). Spores of this fungus also may cause a severe allergic complication in patients who suffer from asthma or cystic fibrosis (Basica et al. 1981; Laufer et al. 1984). This condition has been termed allergic bronchopulmonary aspergillosis. Symptoms range from compounding of the symptoms of the initial disease to complete and fatal destruction of the lungs (Rosenberg et al. 1977; Patterson et al. 1982; Latgé 1999). The involvement of ergot alkaloids in any of these conditions has not been studied.

2. Other Trichocomaceous Fungi

Several other *Aspergillus* species and a few *Penicillium* species (also in the Trichocomaceae) have also been reported to produce ergot alkaloids (Flieger et al. 1997; Kozlovsky 1999; Boichenko et al. 2001). Among the *Penicillium* species, the production of ergot alkaloids by *P. commune* is noteworthy because this fungus is a saprophyte commonly associated with spoilage of dairy products and is the fungus most commonly associated with contamination of cheese (Lund et al. 1995, 2003).

III. Ergot Alkaloid Profiles and Pathways in Different Fungi

Individual ergot alkaloid producers often contain a diverse set of ergot alkaloids, and even greater diversity may be observed among producers from

different taxa. The output of the ergot pathway of any of these fungi is not a single toxin but rather a complex profile of alkaloids consisting of the pathway end-product, one or more intermediates that accumulate to relatively high levels, and, often, the products of pathway spurs from which some portion of an intermediate is modified to an alternate product (Panaccione 2005). In this section, the profile of ergot alkaloids observed in representative ergot alkaloid producers is described in the context of where those ergot alkaloids occur along the pathway. The ergot alkaloid pathway also is considered as a set of taxa-specific branches that arise from a common shared root.

Ergot alkaloids represent a continuum of structures of increasing complexity as they are produced along a long pathway. Traditionally they have been grouped into several structural classes: clavine alkaloids, simple amides of lysergic acid, and ergopeptines. Clavines are the simplest ergot alkaloids observed accumulating in fungi. In some fungi, such as *A. fumigatus* and *C. fusiformis*, clavines are the ultimate products of the ergot alkaloid pathway. In other fungi (e.g., *C. purpurea* and several *Neotyphodium* spp.), clavines accumulate to varying levels but also serve as intermediates on the pathway to lysergic acid, which is subsequently incorporated into lysergic acid amides and non-ribosomally synthesized peptides (ergopeptines).

A global scheme of the ergot alkaloid pathway and its branches in different groups of fungi is represented in Fig. 9.1. Comparisons of ergot alkaloid profiles of different fungi and the ergot alkaloid biosynthesis gene clusters in these fungi support the hypothesis of a shared early portion of the pathway followed by different terminal branches in different ergot alkaloid-producing fungi.

A. Early Pathway Steps Shared Among Producers

Much of the information on the early steps in the ergot alkaloid pathway comes from studies performed with synthesized precursors fed to cultures of *C. purpurea* or *C. fusiformis*, followed by characterization of the products that the cultures produced from those precursors (Floss 1976; Kozikowski et al. 1993; Gröger and Floss 1998). More recently molecular genetic analyses have provided additional information on some steps through functional analyses of genes and hypothetical

information on many more steps based strictly on DNA sequence analysis. A scheme for the early steps of the pathway based on these experiments is shown in Fig. 9.2 and summarized below. Studies on these early steps were reviewed in detail recently (Schardl et al. 2006).

The first committed step of the ergot alkaloid pathway is the prenylation of tryptophan by the enzyme 4- γ,γ -dimethylallyltryptophan synthase (DMATrp synthase) using dimethylallylpyrophosphate as co-substrate. The gene encoding DMATrp synthase (named *dmaW*) was first cloned in *C. fusiformis* and its catalytic capability demonstrated by expression in yeast (Tsai et al. 1995). Genetic evidence for the role of *dmaW* in ergot alkaloid production was provided by gene knockout and complementation analyses in *Neotyphodium lolii* \times *Epichloë typhina* isolate Lp1 (Wang et al. 2004). Additional genetic and biochemical analyses demonstrated that *dmaW* encodes the initial step in the ergot pathway of *A. fumigatus* (Coyle and Panaccione 2005; Unsöld and Li 2005). Phylogenetic analyses indicate *dmaW* genes in all ergot alkaloid producers have a common origin (Liu et al. 2009).

Since genes involved in a secondary metabolite pathway are frequently clustered in fungi (Keller and Hohn 1997; Walton 2000), the possibility that other pathway genes were clustered with *dmaW* has been investigated by sequence analysis in the genomes of ergot alkaloid-producing fungi. Clustering of *dmaW* with genes that encoded enzymes that had functions that could be ascribed to enzymes in the ergot alkaloid pathway was first shown in *C. purpurea* (Tudzynski et al. 1999). Functional analysis of one of the clustered genes, *lpsA* (encoding lysergyl peptide synthetase 1, a branch-specific enzyme required for ergopeptide biosynthesis), in *Neotyphodium lolii* \times *Epichloë typhina* isolate Lp1 further validated the hypothesis of an ergot alkaloid gene cluster (Panaccione et al. 2001).

Genes similar to *dmaW* have been cloned from several ergot alkaloid-producing fungi (Coyle and Panaccione 2005; Unsöld and Li 2005; Liu et al. 2009). In those fungi for which substantial genomic sequence is available (*A. fumigatus*, *C. purpurea*, *C. fusiformis*, *N. lolii*, *E. festucae*), the DMATrp synthase-encoding gene is clustered with genes for several other biosynthetic enzymes. Interestingly several of the genes in the ergot cluster are in common among all the producers for which significant genomic sequence is available. These genes include *dmaW*, *easA*, *easC*, *easD*, *easE*, *easF*, and *easG* (Fig. 9.3, Table 9.1).

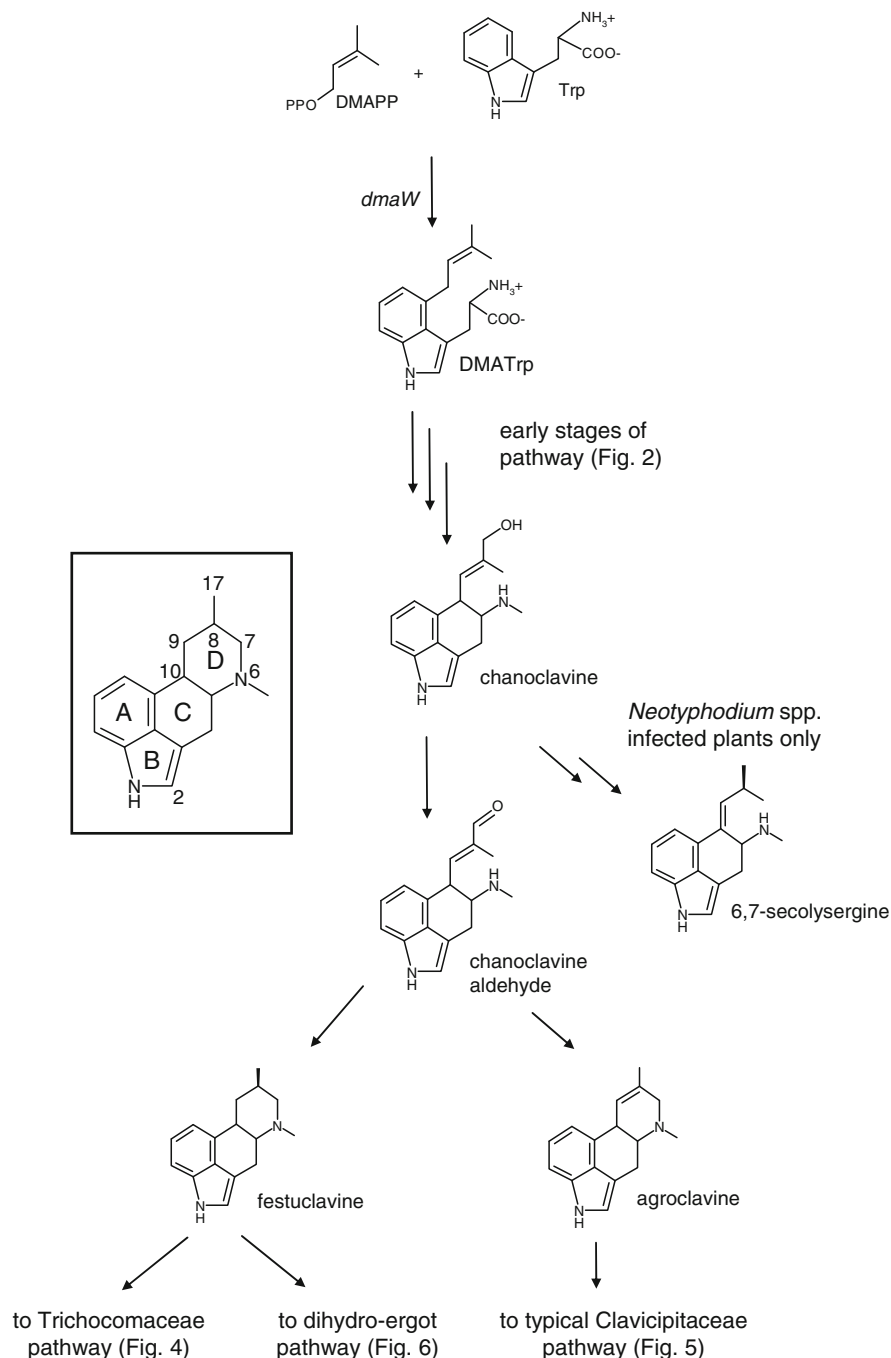


Fig. 9.1. Proposed scheme for divergence of ergot alkaloid pathways. Multiple *arrows* indicate steps omitted for brevity. *DMAPP* Dimethylallylpyrophosphate, *Trp* tryptophan,

DMATrp dimethylallyltryptophan. *Insert*: ring- and position-labeling; for simplicity, position numbering is shown only for atoms referred to in the text

The clustering of these genes with *dmaW* in all studied ergot alkaloid producers and sequence analysis of their predicted enzymes has provided evidence that they encode the early, shared steps of the pathway (Coyle and Panaccione 2005; Haarmann et al. 2005; Unsöld and Li 2005; Rigbers and Li 2008). The ability of the

easF product (FgaMT) to catalyze *N*-methylation of DMATrp was demonstrated by *in vitro* expression of the *A. fumigatus easF* homologue in *Escherichia coli* and biochemical characterization of the product (Rigbers and Li 2008). This finding is supported by knockout analysis of *easF* in *A. fumigatus* (Coyle et al. 2008).

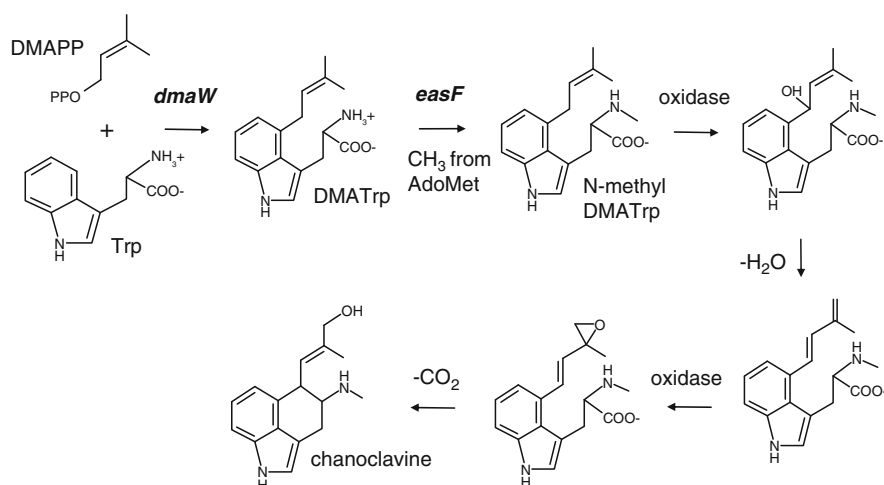
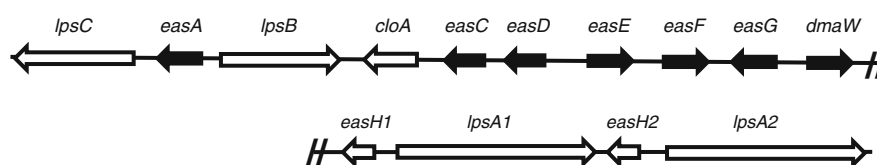


Fig. 9.2. Early shared steps of the ergot alkaloid pathway. *DMAPP* Dimethylallylpyrophosphate, *Trp* tryptophan, *DMATrp* dimethylallyltryptophan, *AdoMet* S-adenosylmethionine

Claviceps purpurea

(redrawn from Haarmann et al. 2005)



Claviceps fusiformis

(redrawn from Lorenz et al. 2007)



Aspergillus fumigatus

(redrawn from Coyle and Panaccione 2005; Unsöld and Li 2005)



Fig. 9.3. Graphic representation of ergot alkaloid associated gene clusters from three fungi. *Black arrows* denote genes common to all clusters. *White arrows* represent genes found in clavicipitaceous clusters that are not found in the *A. fumigatus* genome. Genes unique to the *A. fumigatus* cluster are marked with *gray arrows*. Refer to Table 9.1 for description of genes. The symbol // indicates the contiguous *C. purpurea* chromosome was broken for the purpose of drawing this figure. The symbol ψ indicates

pseudogenes. An additional partial cluster has been published for *N. lolii* containing *easH*, *easA*, *easG*, *easF*, and *lpsB* (Fleetwood et al. 2007); additional genes are present in the genome but their placement relative to the cluster has not yet been published. The clavicipitaceous epibiotic symbiont of the Convolvulaceae has an incompletely characterized ergot alkaloid cluster containing at least the following genes: *easE*, *easF*, *easG*, and *dmaW* (Markert et al. 2008)

Thus *easF* encodes the second step of the pathway. One of the two oxidation steps required between *N*-methyl-DMATrp and chanoclavine appears to be catalyzed by *easE*, since knockout of that oxidoreductase-encoding gene eliminates chanoclavine and alkaloids beyond chanoclavine in *A. fumigatus* (Coyle et al. 2008). Similarly, the catalase-encoding gene *easC* also is required prior to

chanoclavine (Coyle et al. 2008). The gene *easA* is required after chanoclavine but before the pathways diverge (Coyle et al. 2008). The functions of *easG* and *easD* have not been demonstrated at the time of writing but additional oxidation and reduction steps are still unaccounted for among the hypothesized early steps in the pathway.

Table 9.1. Genes found in ergot alkaloid clusters

Gene	Function or proposed function	Accession Number ^a
<i>cloA</i>	Elymooclavine oxidase	AJ884676
<i>dmaW</i>	Dimethylallyltryptophan synthase	AY259840, XM_751048
<i>easA</i>	Oxidoreductase	AJ703809, XM_751040
<i>easC</i>	Catalase	AJ703808, XM_751047
<i>easD</i>	Oxidoreductase	AJ011966, XM_751044
<i>easE</i>	Oxidoreductase	AJ011965, XM_751049
<i>easF</i>	N-Methyltransferase	AY836772, XM_751050
<i>easG</i>	Oxidoreductase	AY836771, XM_751041
<i>easH</i>	Dioxygenase	XM_751039
<i>easK</i>	FAD monooxygenase	XM_751042
<i>easL</i>	Reverse prenyl transferase	XM_751043
<i>easM</i>	P450 monooxygenase	XM_751045
<i>easN</i>	O-Acetyltransferase	XM_751046
<i>lpsA</i>	Lysergyl peptide synthetase 1	AJ011964
<i>lpsB</i>	Lysergyl peptide synthetase 2	AJ439610
<i>lpsC</i>	Lysergyl peptide synthetase 3	AJ884677

^aGenBank/EMBL accession numbers are provided for *C. purpurea* genes (accessions starting with A) and *A. fumigatus* gene (accessions starting with X). All *C. fusiformis* ergot cluster genes are contained under accession number EU006773

Expression of these early genes appears to be coordinated such that intermediates from the early, shared portions of these pathways do not accumulate to levels that would allow them to be detected by high-performance liquid chromatography (HPLC). The earliest intermediate detected in any of the ergot alkaloid producers is chanoclavine. Products of later, fungus-specific stages of the pathway often do accumulate to relatively high levels giving alkaloid profiles that are characteristic of the producing fungus (Panaccione 2005). These profiles and the inefficiency in the pathway which results in them are discussed under sub-headings for different ergot alkaloid-producing fungi below.

B. Ergot Alkaloid Profile Diversification Through Pathway Divergence

1. Terminal Branch in Trichocomaceae Ergot Alkaloid Producers

Festuclavine represents the key intermediate in the Trichocomaceae-specific branch of the ergot

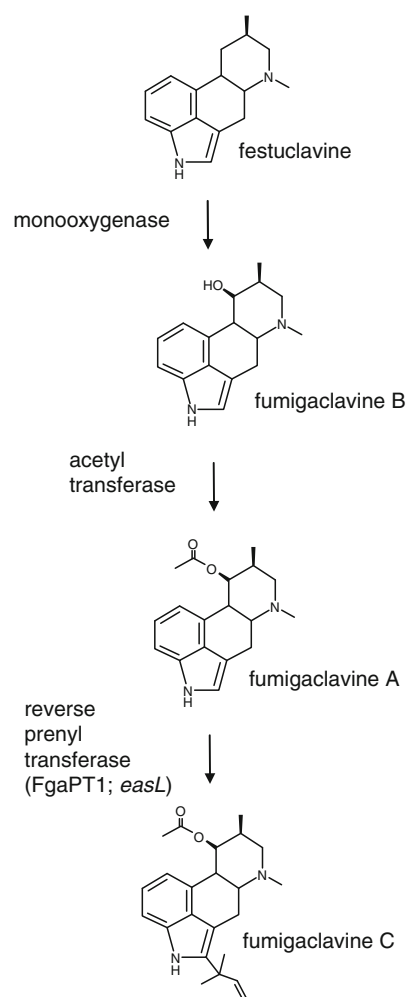


Fig. 9.4. Trichocomaceae-specific terminal branch of the ergot alkaloid pathway. *Aspergillus fumigatus* completes the illustrated branch, whereas *P. commune* stops at the fumigaclavine A level

alkaloid pathway (Fig. 9.4). Festuclavine differs from agroclavine, the key intermediate in the typical clavicipitaceous pathway, in having a fully saturated D ring (Fig. 9.1). In *A. fumigatus* and certain *Penicillium* spp., festuclavine is oxidized at position 9 (refer to Fig. 9.1, insert) to form fumigaclavine B, and this oxygen is then subsequently acetylated to form fumigaclavine A (Fig. 9.4). In *A. fumigatus* only, reverse prenylation of fumigaclavine A at position 2 results in the formation of fumigaclavine C, the ultimate product of the ergot alkaloid pathway of *A. fumigatus* (Fig. 9.4). Positions 9 and 2 are not the sites of activity for any known enzyme in the pathway branches for clavicipitaceous fungi.

In the clavicipitaceous fungi oxidations occur at position 17, and, in some cases, position 10.

Evidence for genes catalyzing these trichocomaceo-specific steps has come from examination of an *A. fumigatus* gene cluster (Coyle and Panaccione 2005; Unsöld and Li 2005). In particular, genes found in the *A. fumigatus* cluster but not in the clusters of *C. purpurea*, *C. fusiformis*, or *N. lolii* are likely candidates to catalyze the trichocomaceo lineage-specific steps. The ergot cluster in *A. fumigatus* contains two monooxygenase genes that do not have homologues in the clusters of other ergot alkaloid-producing fungi (Fig. 9.3, Table 9.1). Hypothetically one of these may function to oxidize festuclavine to fumigaclavine B, a function for the other is not readily apparent considering the lack of a need for an additional oxidase in the trichocomaceo-specific branch. The *A. fumigatus* cluster also contains an *O*-acetyl transferase encoding gene (*easN*) that has no homologues in any of the other ergot alkaloid gene clusters (Fig. 9.3, Table 9.1). The product of this gene presumably acetylates fumigaclavine B to form fumigaclavine A. The final step of the *A. fumigatus* branch is the reverse-prenylation of fumigaclavine A at position 2 to yield fumigaclavine C. A gene encoding a reverse prenyl transferase (FgaPT1; Unsöld and Li 2006) that catalyzes this reaction is located in the ergot alkaloid gene cluster (labeled *easL* in Fig. 9.4). The coding sequences of this gene have been expressed in *Escherichia coli* and the product catalyzed the prescribed reverse prenylation (Unsöld and Li 2006). The reverse prenyl transferase has sequence similarity to the gene encoding DMATrp synthase.

Penicillium commune and several other *Aspergillus* and *Penicillium* species that produce ergot alkaloids appear to have their pathways stopped at fumigaclavine A and thus presumably lack the reverse prenyl transferase FgaPT1.

2. Typical Terminal Branch in Clavicipitaceous Ergot Alkaloid Producers

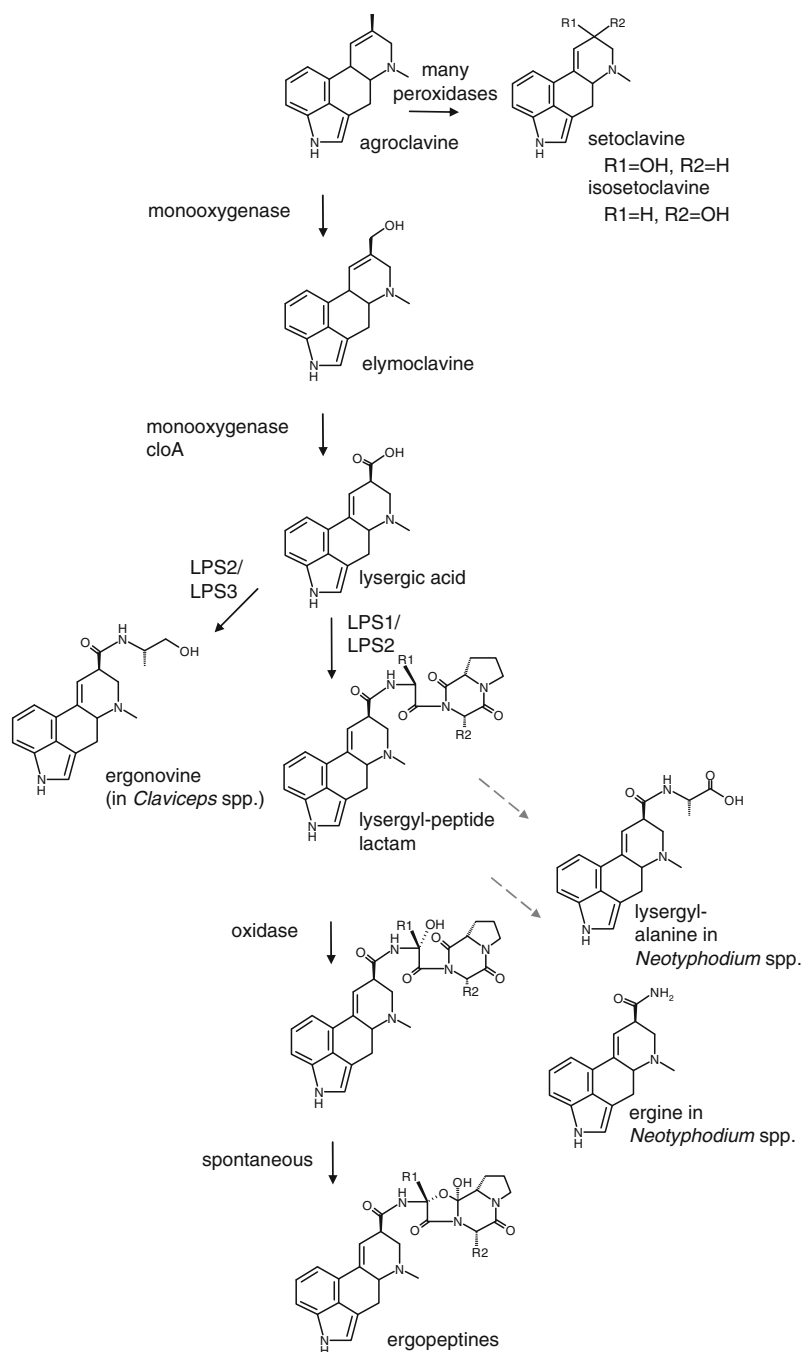
In the typical clavicipitaceous pathway (observed in *C. purpurea*, *C. fusiformis*, several *Neotyphodium* spp.), agroclavine is the key intermediate that is further oxidized and modified to yield different ergot alkaloid profiles in different producers (Fig. 9.5). Agroclavine is oxidized at position 17 to yield elymoclavine. An enzyme with this activity was partially purified and named agroclavine hydroxylase (Kim et al. 1981), but the enzyme has not been characterized further and the gene encoding the enzyme has not been identified. In *C. fusiformis*, elymoclavine serves as the end-product of the ergot alkaloid pathway. In *C. purpurea* and in ergot alkaloid-producing *Neotyphodium* spp., elymoclavine is further oxidized at

position 17 to yield paspalic acid, an isomer of lysergic acid that has a double bond in the $\Delta 8,9$ position. During or immediately after the oxidation of elymoclavine to paspalic acid, the double-bond arrangement isomerizes to the $\Delta 9,10$ position to yield lysergic acid (Floss 1976; Gröger and Floss 1998; Tudzynski et al. 2001). The gene *cloA* (clavine oxidase A) encoding the enzyme that catalyzes the oxidation of elymoclavine to lysergic acid has been cloned from *C. purpurea*, and its function has been demonstrated by gene knock-out analysis (Haarmann et al. 2006).

Clavicipitaceous fungi that produce lysergic acid then efficiently convert it into any of several different amides. Often an individual fungus will produce more than one lysergic acid amide. These amides can be classified into two groups, simple amides and ergopeptines, depending on their complexity. The ergopeptines are a family of non-ribosomally synthesized peptides containing lysergic acid and three amino acids. With one exception the second amino acid is L-proline, and variation at the first and third amino acids produces a family of different ergopeptines. The sequence of amino acids in some commonly encountered ergopeptines is indicated in the legend to Fig. 9.5. The ergopeptines are formed through the interaction of two peptide synthetases (Riederer et al. 1996; Walzel et al. 1997). The two-component peptide synthetase is unique among fungal peptide synthetases, which are usually comprised of a single multifunctional polypeptide (Walton et al. 2004). The three amino acids are recognized and incorporated into the ergopeptide by lysergyl peptide synthetase 1 (LPS1), a multifunctional non-ribosomal peptide synthetase, whereas lysergic acid is recognized and activated for incorporation into the ergopeptide by an enzyme named lysergyl peptide synthetase 2 (LPS2; Riederer et al. 1996; Walzel et al. 1997). Functional analysis of the role of LPS1 in ergopeptide formation in *N. lolii* × *E. typhina* isolate Lp1 was demonstrated by gene knockout (Panaccione et al. 2001). Genetic evidence for the role of LPS2 was provided in *C. purpurea* by Correia et al. (2003) and in *N. lolii* by Fleetwood et al. (2007).

Simple amides of lysergic acid include ergine (also called lysergic acid amide, LSA, or lysergamide), lysergyl alanine, ergonovine (also called ergometrine, ergobasine), and lysergic acid α -hydroxyethylamide. The existence of a separate enzyme for lysergic acid activation (LPS2) allows for an elegant combinatorial system in *C. purpurea* in which LPS2 combines with LPS1 to form ergopeptines (as described above) and with a second peptide synthetase (LPS3) capable of activating alanine (and encoded by *C. purpurea* ergot cluster gene *lpsC*; Haarmann et al. 2005; Ortel and Keller 2009) to form the simple amide ergonovine (Ortel and Keller 2009).

Fig. 9.5. Typical Clavicipitaceae-specific terminal branch of the ergot alkaloid pathway. Not all possible spur products or alternate end-products are shown. *Dashed arrows* represent hypothetical branches. Ergopeptines differ by amino acid side chains at positions R1 and R2. For example, in ergotamine R1 = alanine and R2 = phenylalanine, in ergovaline, R1 = alanine and R2 = valine, in ergosine, R1 = alanine and R2 = leucine, in ergocristine, R1 = valine and R2 = phenylalanine



The alanine-activating peptide synthetase LPS3 contains a reductase domain in its carboxy-terminal region to convert the lysergyl-alanine intermediate into ergonovine. In contrast the endophyte *N. lolii* \times *E. typhina* isolate Lp1 appears to lack a homologue of LPS3 (based on lack of a homologue in the genome of its ancestor *E. festucae*) and accumulates different simple amides, ergine and lysergyl-alanine (Panaccione et al. 2003). Panaccione et al. (2003)

hypothesized that these particular simple amides form as breakdown products during the synthesis of ergopeptines, because a strain of *N. lolii* \times *E. typhina* isolate Lp1 in which the peptide synthetase LPS1 was knocked out accumulated no ergine or lysergyl-alanine. The lysergyl-peptide lactam product of LPS1/LPS2 and its oxidized derivative (Fig. 9.5) are potential stages that might yield the simple amides in *Neotyphodium* spp.

The alkaloid profiles of individual clavicipitaceous fungi can be accounted for by each of them having a certain subset of functional genes in this branch. *Claviceps purpurea* with its functional *cloA* (to convert elymoclavine into lysergic acid) and full complement of peptide synthetase-encoding genes typically accumulates the simple amide ergonovine and several ergopeptines. *C. fusiformis* follows a branch similar to *C. purpurea* but stops at elymoclavine. Data indicate that *C. fusiformis* lacks functional copies of peptide synthetase genes required making lysergic acid derivatives (Panaccione et al. 2001; Lorenz et al. 2007).

Interestingly, the gene *cloA*, which encodes a p450 monooxygenase required to oxidize elymoclavine into lysergic acid (Haarmann et al. 2006), is present in the *C. fusiformis* ergot gene cluster and is transcribed in culture but does not encode a functional enzyme (Lorenz et al. 2007). *C. paspali* produces simple amides of lysergic acid (ergonovine, lysergic acid α -hydroxyethylamide, ergine) but not ergopeptines. Such a profile could be explained by a battery of ergot alkaloid biosynthetic genes similar to those found in *C. purpurea* but lacking the peptide synthetase LPS1 encoded by *lpsA*. This explanation is hypothetical because ergot pathway genes have not yet been analyzed in *C. paspali*.

Neotyphodium spp. endophytes apparently have a functional *cloA* because they typically produce ergopeptines and the simple amides ergine and lysergyl-alanine (Porter et al. 1981; Lyons et al. 1986; Panaccione et al. 2003). The lack of ergonovine presumably results from the lack of a homologue for *lpsC*. The profiles of some *Neotyphodium* spp. endophytes also contain relatively high concentrations of chanoclavine (Lyons et al. 1986; Panaccione et al. 2006a) and of similar clavine describe as 6,7-secolysergine (Panaccione et al. 2003). The clavine 6,7-secolysergine appears to be a pathway spur product derived from chanoclavine or from a precursor to chanoclavine.

3. Dihydroergot Alkaloid Branch in Clavicipitaceous Fungi

The sorghum ergot pathogen *C. africana* produces a family of dihydroergot alkaloids in which the D ring is completely saturated (Fig. 9.6). In *C. africana*, a series of oxidations analogous to those described in *C. purpurea* occurs at position 17, but with festuclavine as opposed to agroclavine as the substrate (Barrow et al. 1974). The resulting products are first dihydroelymoelavine and then

dihydrolysergic acid. In *C. africana*, dihydrolysergic acid is then incorporated into the dihydroergopeptine dihydroergosine. The maize ergot fungus *C. gigantea*, follows a similar path from festuclavine, but its pathway ends at dihydroelymoelavine (Agurell and Ramstad 1965).

The most parsimonious explanation of the origin of the dihydroergot alkaloids observed in *C. africana* and *C. gigantea* would be a pathway that incorporates enzymes of the typical clavicipitaceous branch but with festuclavine serving as the entry point into that pathway. This simplest of explanations would be based on the premise that the presence or absence of a double bond in the D ring is not

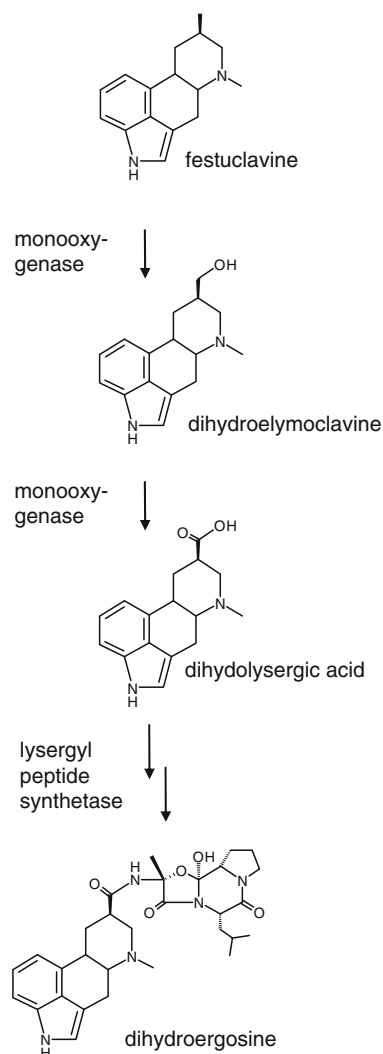


Fig. 9.6. Dihydroergot alkaloid terminal branch of the ergot alkaloid pathway. *Claviceps africana* follows the pathway to its completion, *Claviceps gigantea* does not proceed past dihydroelymoelavine

a significant factor for recognition by the enzymes that act on substrates downstream of agroclavine/festuclavine. One observation in support of this hypothesis is that LPS1 of *C. purpurea* has the same K_m for dihydrolysergic acid, which contains an unsaturated D ring, as it does for its natural 8,9-unsaturated substrate lysergic acid (Riederer et al. 1996). An alternative, less parsimonious explanation would be that *C. africana* and *C. gigantea* have evolved enzymes with specificity for saturated D rings for each of the steps that occur after festuclavine formation. Experiments to address these questions have not yet been performed, but genes and technology to do so should soon be available.

The relationship of the profiles of the maize ergot fungus *C. gigantea*, whose pathway ends at dihydroelymoclavine, to that of *C. africana*, which produces a dihydroergopeptine, is analogous to the relationship of the comparative profiles of *C. fusiformis* to *C. purpurea*. Molecular analyses of genes in *C. africana* or *C. gigantea* ergot pathways have not yet been reported.

C. Regulation of Clustered Ergot Pathway Genes

In *C. purpurea*, the production of ergot alkaloids can be repressed by high concentrations of phosphate (Robbers et al. 1972). The physiological significance of this phosphate effect is not clear, but it has provided a useful means to demonstrate the coordinated regulation of ergot cluster genes.

Haarmann et al. (2005) investigated the accumulation of mRNA from ergot cluster genes in cultures of *C. purpurea* grown in media containing different phosphorus concentrations. They found that mRNA from all six ergot cluster genes that they investigated were detectable by northern analysis in cultures grown at 0.5 g/L phosphate, but transcripts were not detectable or present at much lower concentrations when cultures were grown at 2.0 g/l phosphate.

Fungal endophytes of grasses produce ergot alkaloids reliably only when grown in planta, during which time they grow exclusively as vegetative hyphae. Fleetwood et al. (2007) used semiquantitative RT-PCR to show that transcripts from six ergot cluster genes were present in higher concentration (relative to mRNA of a β -tubulin gene) when *E. festucae* was cultivated in its host plant (perennial ryegrass) as compared to in culture medium in which the fungus did not produce ergot alkaloids.

In *A. fumigatus* ergot alkaloids are found in very high concentrations in conidia (Panaccione and Coyle 2005). Conidia are abundantly produced under all culture conditions and in all media tested,

making tests of whether ergot alkaloids were also produced in vegetative mycelium impractical.

To generate non-conidiating cultures of the fungus, Coyle et al. (2007) disrupted the regulatory gene *brlA*, which encodes a DNA-binding protein (BrlA) that promotes transcription of many genes associated with conidiation (Adams et al. 1998). Strains of the fungus, in which *brlA* was disrupted, produced abundant vegetative mycelium but no conidia (Coyle et al. 2007). The *brlA* mutant strains produced no detectable ergot alkaloids when analyzed by HPLC, whereas the wild-type strain accumulated high concentrations of ergot alkaloids. The data demonstrate that ergot alkaloids are exclusively associated with conidiation and are not produced in vegetative mycelium of the fungus. One implication of this finding is that ergot alkaloids are not important to the fungus during the invasive stage of human or animal infection (invasive aspergillosis), since during that phase of disease the fungus grows exclusively as a vegetative mycelium. Whether ergot alkaloids present in or on conidia are important to survival of inhaled conidia prior to establishment of an invasive mycelium in the host has not yet been addressed. Microarray analysis showed that transcripts of ergot alkaloid cluster genes were not detectable in the transcriptome of a *brlA* knockout strain but were abundant in the wild type (Twumasi-Boateng et al. 2009). Interestingly the ergot alkaloid gene cluster is the only secondary metabolite gene cluster that is regulated by BrlA in *A. fumigatus* (Twumasi-Boateng et al. 2009).

IV. Pathway Inefficiency as a Means to Diversify Alkaloid Profiles

A. Inefficiency in the Ergot Pathway Provides Diversified Alkaloid Profiles

The ergot alkaloid pathway appears unusually inefficient in that certain intermediates do not flow rapidly through the pathway to an ultimate end-product. Instead there are typically points along the pathway at which intermediates may accumulate to concentrations approaching or exceeding those of the pathway end-product (i.e., preset, step-specific reductions in flux; Table 9.2). Also, the pathway in certain producers contains shunts along which intermediates may be diverted to alternate products. The accumulation of intermediates and alternate products (rather than their rapid conversion to the ultimate pathway product) suggests that these alkaloids provide some benefit to the producing fungus that differs from those conferred by the pathway end-product.

The lineage-specific steps in the *A. fumigatus* pathway do not work together efficiently in a way

Table 9.2. Inefficiency in the ergot alkaloid pathway of several fungi

Fungus and source	Ergot alkaloid ^a	Percentage of total, molar basis
<i>Aspergillus fumigatus</i> whole cultures	Festucaclavine	56
	Fumigaclavine B	0.1
	Fumigaclavine A	0.6
in vitro (Coyle et al. 2007)	<i>Fumigaclavine C</i>	37
	Chanoclavine	47
	6,7-Secolysergine	20
<i>Neotyphodium lolii</i> × <i>Epichloë typhina</i> isolate Lp1 in perennial ryegrass leaves (Panaccione et al. 2006a)	Setoclavine/ isosetoclavine	0.4
	Ergine	0.7
	<i>Ergovaline</i>	23
	Festucaclavine	0.8
<i>Claviceps africana</i> sclerotia and sphacelia in sorghum (Blaney et al. 2003)	Dihydroelymoclavine	25
	<i>Dihydroergosine</i>	67
	Festucaclavine	0.7
<i>Claviceps gigantea</i> sclerotia on maize (Agurell and Ramstad 1965)	Chanoclavine	65
	Miscellaneous other clavines	13
	<i>Dihydroelymoclavine</i>	15
	Festucaclavine	0.7

^aIntermediates and spur products are shown in regular type face. Pathway end-products are italicized

that would lead to rapid conversion of intermediates to end-products. For example festucaclavine was found to accumulate to relatively high levels in isolated spores (Panaccione and Coyle 2005) and even higher levels when entire sporulating cultures were analyzed (Coyle et al. 2007; Table 9.2).

Fumigaclavine B and fumigaclavine A are readily detectable in *A. fumigatus* cultures but are present in lower concentrations than is festucaclavine. The pathway end-product fumigaclavine C is sometimes detected at concentrations lower than the concentration of festucaclavine (Coyle et al. 2007). These observations suggest that the unidentified oxidase that forms fumigaclavine B from festucaclavine (Fig. 9.4) is present in low concentration, has a low catalytic rate, or is spatially separated from its substrate by cell type or intracellular compartmentalization. The lower accumulation of the next two intermediates, fumigaclavines B and A, would indicate that the enzymes that catalyze the formation of these alkaloids are less restricted from catalyzing their reactions, though some inefficiency is still evident. As a result *A. fumigatus* has a characteristic profile of four relatively abundant ergot alkaloids: festucaclavine, fumigaclavine C, fumigaclavine A and fumigaclavine B, listed in typical descending order of concentration (Table 9.2).

In comparison to other ergot alkaloid-producing fungi, *C. purpurea* is relatively efficient

in channeling intermediates into end-products. The alkaloids typically detected in sclerotia of *C. purpurea* are ergopeptines and the simple amide ergonovine, representing alternate end-products of the *C. purpurea* alkaloid pathway (Fig. 9.5). If one of the benefits of inefficiency is to generate a collection of alkaloids some of which provide benefits not provided by the other, then *C. purpurea* may achieve the same benefit by channeling its intermediates to alternate end-product.

The ergot alkaloid pathways of grass endophytes that have been studied in detail are very inefficient in converting certain intermediates to their next step and in converting intermediates into end-product (reviewed by Panaccione 2005). For example, leaf blade tissue of perennial ryegrass infected with *N. lolii* × *E. typhina* isolate Lp1 contains significantly more chanoclavine (early pathway intermediate) and 6,7-secolysergine (spur product) than it does ergovaline (end-product of pathway; Panaccione et al. 2006a; Table 9.2). Recent results indicate that the clavine intermediates and lysergic acid-derived pathway end-products provide different benefits to endophytes and their grass hosts (Panaccione et al. 2006a, b; Potter et al. 2008). These studies are summarized below (Section V).

The dihydroergot alkaloid branch observed in *C. africana* and *C. gigantea* also shows inefficiency in converting intermediates to end-product. Based on data published by Blaney et al. (2003) and converted here to moles of alkaloid, about two-thirds of the ergot alkaloid measured in samples of sorghum ergot (*C. africana*) was in the pathway end-product dihydroergosine (Table 9.2). The pathway intermediates festucaclavine and dihydroelymoclavine made up one-third of the sample on a molar basis. The alkaloids extracted from *C. gigantea* sclerotia showed an even more pronounced bias toward pathway intermediates, with approximately two-thirds of the ergot alkaloids represented as festucaclavine (Agurell and Ramstad 1965; Table 9.2).

B. Other Pathways that Appear to have Preset, Step-Specific Reductions in Flux

Another pathway that demonstrates inefficiency in the conversion of intermediates to end-product and for which intermediates and end-product

have different activities is the catecholamine pathway, which leads to sequential production of the neurotransmitters dopamine, noradrenaline, and adrenaline (Schulz et al. 2004). Detailed analyses of this pathway may provide some clues about the mechanism by which pathway inefficiency may be generated.

If one were to assay the central nervous system of a mammal as a whole, it would appear that the catecholamine pathway simply has a reduced flux because dopamine, noradrenaline, and adrenaline would all be detectable. However, on a smaller scale, it would become apparent that the pathway terminates at different steps in different cell types depending upon the availability or expression of the relevant enzymes. For example, phenylethanolamine *N*-methyl transferase (PNMT), which methylates noradrenaline to adrenaline, is expressed only in chromaffin cells of the adrenal medulla and adrenergic neurons. Whereas the enzyme is cytosolic, the substrate, noradrenaline, is contained in storage vesicles and only noradrenaline that leaks from the vesicle can be acted upon by PNMT. Similarly dopaminergic neurons do not express dopamine- β -hydroxylase and thus accumulate dopamine instead of hydroxylating it into noradrenaline (Schulz et al. 2004). Interestingly the ergot alkaloids act as partial agonists or, in other cases, antagonists of certain dopamine and adrenergic receptors.

The methyl-jasmonate pathway of plants can be considered somewhat analogous, as accumulating intermediates in the pathway have activities that differ from the activities of the end-product (Liechti and Farmer 2002).

For example, methyl-jasmonate (the pathway end-product) is required for male floral organ development and thus male fertility (Stintzi and Browse 2000). In contrast, the cyclopentenone intermediate 12-oxophytodienoic acid (OPDA) and its reduced product, jasmonic acid, do not affect male flower production but can regulate expression of plant defense genes (Stintzi et al. 2001). The jasmonate pathway molecules differ from those in the ergot pathway in being hormonal in nature and acting as regulators of transcription, but they do provide a precedent for pathway intermediates providing different benefits from pathway end-products.

V. Activities of Ergot Alkaloids

The biological activities of ergot alkaloids have been investigated from different perspectives. One perspective is to consider how the alkaloids affect humans as toxins and as pharmaceuticals. A second, more ecological perspective is to consider the benefits that the alkaloids provide

the producing fungus and organisms with which the fungi associate in nature or agriculture.

A. Effects on Humans

As described in the introduction, ergot alkaloids exert a variety of effect on humans through their interaction with receptors for monoamine neurotransmitters (i.e., 5-HT, dopamine, adrenaline, noradrenaline). In certain cultures and during certain periods of time when people were dependent upon rye bread for a large proportion of their calories, poisoning by ingestion of ergot sclerotia that often contaminated rye grain was reported (Matossian 1989; Schardl et al. 2006).

Among the first symptoms of ergot poisoning noted were tingling or burning sensations in the extremities due to the vasoconstrictive activities that resulted from ergot alkaloids acting as agonists at certain 5-HT receptors. The vasoconstriction could become so severe that hands and feet felt as if there were burning (which in part gave rise to the names holy or St. Anthony's fire for this toxicosis), turned black from the loss of blood, and self-amputated from the body. Other symptoms included uncontrolled muscle contraction, miscarriages, hallucinations, and other problems with the central nervous system. Implementation of grain screening (ergot sclerotia are typically larger than the viable grains) and diversification of diets due to the availability of potatoes and other crops that could be substituted or grown in addition to rye has made poisoning by ergot a very rare occurrence during recent history (Matossian 1989).

The affinity of individual ergot alkaloids for monoamine neurotransmitter receptors makes ergot alkaloids attractive as pharmaceuticals. They can be biotechnologically produced for the direct use as drugs or as precursors for other pharmaceuticals using wild-type strains (e.g., *C. purpurea*) and both surface or submerged liquid cultures and solid-state fermentation systems (Keller and Tudzynski 2002; Krishna 2005; Haarmann et al. 2009). However, the propensity for ergot alkaloids to interact with receptors for multiple neurotransmitters often results in undesirable side-effects and limits their utility.

For example, the semisynthetic ergot alkaloid pergolide was withdrawn from the market in the United States by the Food and Drug Administration due to dangerous side-effects that resulted from its interaction with multiple neurotransmitter receptors (United States Food and Drug Administration 2007). Pergolide had been prescribed for the treatment of Parkinson's disease because of its activity as a dopamine agonist (strong affinity for dopamine

receptor subtypes D₁, D₂). However, the drug also interacts with 5-HT receptors (of subtype 5-HT_{2B}) causing thickening and stiffening of heart valves due to excessive growth of cardiac fibroblasts, thus leading to valvular heart disease (Schade et al. 2007; Zanettini et al. 2007).

Ergot alkaloids, in particular ergotamine and dihydroergotamine, also have been used extensively for the treatment of migraine headaches (Tfelt-Hansen and Koehler 2008). Their strong affinity for 5-HT receptors results in the vasoconstriction of blood vessels in the brain, reducing the symptoms and potential cause of migraines. In the past decade, ergot alkaloids have been supplanted by the triptan family of 5-HT agonists (which, like ergot alkaloids, are indole derivatives) as the preferred treatment for migraines (Tfelt-Hansen and Koehler 2008). Ergot sclerotia have historically been administered during childbirth to stimulate contraction and control hemorrhaging. In many cases, the ergot alkaloid methylergonovine is used for these purposes (Gallos et al. 2008), although oxytocin has become the drug of choice in the United States (Maughan et al. 2006).

No description of the effects of ergot alkaloids on humans would be complete without a brief mention of lysergic acid diethylamide (LSD). The complicated interaction of this semisynthetic ergot alkaloid with multiple neurotransmitter receptors induces hallucinations and strong changes in sensory perception at very low concentrations (Hofmann 1980). Once considered as a candidate for treatment of a variety of psychoses, this powerful alkaloid has become infamous for its illegal use as a recreational drug (Kavalier 1965; Hofmann 1980).

B. Ecological Roles

In nature, ergot alkaloid-producing fungi occur as saprophytes (*A. fumigatus*, *P. commune*), opportunistic pathogens of humans and animals (*A. fumigatus*), pathogens of plants (*Claviceps* spp.), and mutualistic symbionts of plants (*Neotyphodium* spp.). These varied trophic strategies would seem to preclude some specialized function of ergot alkaloids. Among the broader possibilities by which ergot alkaloids might benefit the producing organisms would be self-preservation and resource preservation.

In addition to their effects on humans (documented above), ergot alkaloids affect other mammals, insects, and nematodes, all of which have a similar complement of mono-amine neurotransmitters (Brownlee and Fairweather 1999; Osborne 1996; Libersat and Pflueger 2004) and all of which might provide more immediate competition for resources. Some clavine ergot alkaloids (but not lysergic acid derivatives) possess antibiotic activity (Schwarz and Eich 1983; Eich et al. 1984; Eich and Pertz 1999; Panaccione 2005). Eich and Pertz (1999) suggested that the antibiotic activity may stem from inhibition of DNA synthesis.

It is relatively straight-forward to show that an alkaloid has an effect against an organism with which the ergot alkaloid producer may compete for resource in nature; however, it is much more difficult to demonstrate that the alkaloid has an ecologically important role in the interaction of those organisms. Gene knockouts resulting in truncation of the ergot pathway at specific points result in elimination of ergot alkaloids or alteration of the ergot alkaloid profile. When such modified fungi are then put back into relevant settings, the effects resulting from the alteration of the ergot alkaloid profile can be observed.

A series of experiments based on this strategy in the perennial ryegrass endophyte *N. lolii* × *E. typhina* isolate Lp1 has provided information on the ecological roles of ergot alkaloids in this fungus. In this case, the gene knockout approach allows separation of endophyte effects in general from the effects of particular toxins. Studies were conducted with perennial ryegrass infected with the wild-type fungus (producing the full complement of ergot alkaloids; Table 9.2), endophyte-free perennial ryegrass (which has no fungus and no ergot alkaloids), and perennial ryegrass containing two different ergot pathway knockouts. A strain of the fungus in which the first pathway gene, *dmaW*, was knocked-out produced no ergot alkaloids at all (Wang et al. 2004). A strain in which peptide synthetase (LPS1) gene *lpsA* was knocked-out produced no ergovaline or lysergic acid amides but did accumulate clavines, primarily chanoclavine and 6,7-seco-lysergine (Panaccione et al. 2001, 2003). Choice tests in which rabbits (as a model animal) were fed these grass-endophyte combinations demonstrated that clavines serve as feeding deterrents to rabbits (Panaccione et al. 2006a). In these studies, rabbits indicated a strong preference for endophyte-infected but ergot alkaloid-free grasses, indicating that in the absence of clavines, endophyte-infected plants would be grazed disproportionately compared to endophyte-free plants. In contrast, in studies with black cutworm (*Agrotis ipsilon*), clavines were ineffective feeding deterrents, and late pathway products ergovaline and/or lysergic acid amides were required for effective

anti-insect activity (Potter et al. 2008). The endophytes producing ergovaline and lysergic acid amides also had significantly greater insecticidal activity than did the *lpsA* knockout mutant accumulating only clavines. Together these studies show that pathway intermediates and spur products (clavines) provided benefits that differed from those of the pathway end-products, and thus supported the hypothesis that pathway inefficiency is beneficial to ergot alkaloid-producing fungi.

A set of perennial ryegrass-*Neotyphodium* endophyte combinations has been used to study the role of ergot alkaloids in the suppression of lesion nematodes population on roots of perennial ryegrass. The population of lesion nematode (*Pratylenchus scribneri*) supported on roots of perennial ryegrass in greenhouse experiments was greatly inhibited by endophyte presence (Panaccione et al. 2006b). However, elimination of ergovaline and lysergic acid amides (as in the *lpsA* knockout strain) or all alkaloids (as in the *dmaW* knockout strain) did not significantly reduce the endophyte-associated population suppression (Panaccione et al. 2006b). These data show that ergot alkaloid production and lesion nematode population suppression are separable traits.

VI. Conclusions

Results of biochemical, molecular genetic, and genomics studies have successfully been combined to answer important questions about the biosynthesis and function of ergot alkaloids. Ergot alkaloids are produced by fungi from at least two distinct lineages. Comparative genomic analyses along with biochemical or molecular genetic characterization of individual genes indicate that ergot alkaloids produced by fungi in these two lineages have a common biosynthetic and genetic origin (Wang et al. 2004; Coyle and Panaccione 2005; Panaccione 2005; Unsöld and Li 2005; Scharld et al. 2006; Fleetwood et al. 2007; Lorenz et al. 2007; Coyle et al. 2008; Rigbers and Li 2008). Identification of the potential ability of other fungi to produce ergot alkaloids will be facilitated by the availability of data from the ergot alkaloid gene clusters which provide a means to quickly screen additional fungal genomes, as data from those genomes becomes available.

After the early, shared steps, the pathways of different ergot alkaloid-producing fungi diverge to follow different terminal branches, resulting in unique profiles of ergot alkaloids. Recent data from gene knockout and expression analyses indicate that enzymes catalyzing these lineage

specific steps are also part of an individual fungus's ergot alkaloid gene cluster (Panaccione et al. 2001; Correia et al. 2003; Haarmann et al. 2006; Unsöld and Li 2006; Ortel and Keller 2009).

The composition and complexity of an individual fungus's ergot alkaloid profile is controlled not only by which terminal branch its pathway follows but also by inefficiency in converting pathway intermediates into end-product. Pathway inefficiency allows for certain intermediates to accumulate to high levels, sometimes exceeding the concentration of the pathway end-product (Panaccione 2005). Similarly, pathways followed in some fungi allow for combinations of enzymes to provide alternate end-products (as is the case with the peptide synthetases of *C. purpurea*; Ortel and Keller 2009). At least some alternate end-products or accumulating intermediates have activities that differ from those of the ultimate end-products (Panaccione 2005). Thus, pathway inefficiency provides a means for fungi to derive multiple benefits from a single biochemical pathway. A demonstration of this concept was provided by recent studies showing differences in mammalian feeding deterrence and anti-insect activities of intermediates and spur products versus pathway end-products in the perennial ryegrass endophyte *N. lolii* × *E. typhina* isolate Lp1 (Panaccione et al. 2006a; Potter et al. 2008).

The next decade promises to provide much more information on the biosynthesis and biological significance of ergot alkaloids. Progress will be made on several topics by application of existing resources, tools and technologies. For example, several of the ergot cluster genes have not yet been functionally characterized in the published literature. Genomic sequence data of many ergot alkaloid producers has not been collected or investigated. Moreover, biologically relevant characterization of fungi containing mutations in ergot alkaloid pathway genes remains to be done.

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10 Production of Organic Acids by Filamentous Fungi

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I. Introduction

Fungi, in particular members of the genus *Aspergillus*, are well known for their potential to over-produce various organic acids. It is generally assumed that this ability provides the fungi with an ecological advantage, since they grow rather well at pH levels below 4.0, and some species even tolerate pH values as low as 1.5. Yet significant organic acid accumulation only occurs under a number of defined conditions and, in several

cases, conditions have been found that result in an almost quantitative conversion of the carbon substrate into acids. This is today exploited in the industrial production of citric, gluconic and itaconic acids at a large scale. Table 10.1 lists the most important organic acids for which a production process employing fungi has been described.

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid; EU no. E330) is a true bulk product (estimated global production 1 500 000 t in 2008). It is mostly produced using the mitosporic fungus *Aspergillus niger*, although a minor fraction is made with the yeast *Yarrowia lipolytica*. Citric acid is the most widely used organic acid in foods, beverages, pharmaceuticals and technical applications. Applications are based on three properties: (1) acidity and buffer capacity, (2) taste and flavour and (3) chelation of metal ions. Because of the three carboxylic groups with pK values of 3.1, 4.7 and 6.4, citric acid is able to produce a very low pH in solution, but is also useful as a buffer over a broad pH range (2–7). Citric acid has a pleasant acid taste that leaves little aftertaste. It sometimes enhances flavour, but is also able to mask sweetness, such as the typical aspartame taste in diet beverages. Chelation of metal ions is a very important property that has led to applications as antioxidant and preservative. An advantage, which citric acid shares with most other organic acids, is that it is “natural” and fully biodegradable. Applications of citric acid are summarised in Table 10.2.

In this chapter, we discuss different aspects of organic acid production, including the biochemistry and genetics of its over-production, the production process and product applications. Since citric acid is by far the most important organic acid, in terms of production volume and knowledge available, emphasis is given to the production of citric acid by *Aspergillus niger*. The production of itaconic acid and gluconic acid is discussed in less detail. Finally, the reader is referred to a number of excellent reviews that have been written on organic acid production by fungi (Röhr et al. 1996a–c; Matthey 1992; Zidwick 1992; Kristiansen et al. 1999; Karaffa and Kubicek 2003; Kubicek and Karaffa 2006).

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Table 10.1. Organic acids for which fungal production has been reported

Acid	Producing organism(s)	Application(s)
Citric	<i>Aspergillus niger</i> <i>Yarrowia lipolytica</i>	Many (see Table 10.2)
Gluconic	<i>Aspergillus niger</i>	Food additive Cleaning metal surfaces and glassware Therapeutic metal salts
Itaconic	<i>Aspergillus terreus</i> <i>Aspergillus itaconicus</i>	Co-polymer Detergents
Kojic	<i>Aspergillus oryzae</i> <i>Aspergillus flavus</i>	Skin whitening Precursor for food additives
Malic	<i>Aspergillus</i> spp. <i>Saccharomyces cerevisiae</i>	Food additive Synthetic polymers
Fumaric	<i>Rhizopus oryzae</i>	Food additive Synthetic polymers
Lactic	<i>Rhizopus</i> spp.	Food additive Synthetic polymers
Gallic	<i>Aspergillus</i> spp.	Dyeing
Epoxy succinic	<i>Aspergillus fumigatus</i>	Precursor for tartaric acid

Table 10.2. Applications of citric acid

Application	Function/property
Food/beverage	pH adjustment, provides acidity, provides tart taste, enhances flavour, reduces sweetness, antioxidant (stabilises colour/taste/flavour), preservative (inactivates micro-organisms)
Pharmaceutical/cosmetic	pH adjustment, provides effervescence together with carbon dioxide, anticoagulant, antioxidant (e.g. in vitamin preparations), facilitates rapid dissolution of active ingredients
Technical	pH adjustment, removal of oxidation products from metal surfaces, complexing agent in textiles and oil well treatment, retards setting of concrete, hardener in adhesives

II. Citric Acid

A. Biosynthesis of Citric Acid

The metabolic pathway of citric acid formation in *A. niger* is well established. Tracer studies by Martin and Wilson (1951) and Cleland and Johnson (1954) showed that citric acid is mainly formed by the glycolytic catabolism of hexoses (usually glucose and fructose) with subsequent condensation of a C4-unit with a C2-moiety. The process can be summarized as follows: (1) uptake of the sugar substrate, (2) glycolytic catabolism of glucose to form 2 mol of pyruvate, (3) their subsequent conversion to oxaloacetate and acetyl-CoA, (4) condensation of these two precursors to citric acid and (5) finally excretion of citric acid (Fig. 10.1).

Little knowledge is yet available on how sugars are taken up during citric acid production. Torres et al. (1996a) studied glucose uptake by *A. niger* ATCC 11414. Kinetic analysis showed that this strain possesses at least two glucose transporters. High-affinity glucose transport (K_m , 0.3 mM) was detected after growth at a low glucose concentration (1% w/v), whereas an additional low-affinity permease was only found in mycelia cultured in the presence of high glucose concentrations (15% w/v). Based on specific glucose uptake rates at different glucose concentrations in gluco-stat experiments, Wayman and Matthey (2000) concluded that, at glucose levels higher than approximately 250 mM, entry of glucose into the cells by simple diffusion (i.e. not carrier-mediated) could explain glucose consumption during citric acid production. However, their data can be fitted adequately assuming a carrier with low apparent affinity. Perhaps simple diffusion does contribute to the glucose uptake at very high glucose concentrations, but carrier-mediated transport will always play a major role, in particular, at lower glucose levels. Since fructose is a component of sucrose (molasse) based processes, fructose transport is also relevant for citric acid production.

A major hexose transporter of *A. niger* (MstA) has been studied in detail: it is able to transport D-fructose (K_m 4.5±1.0 mM), D-xylose (K_m , 0.3±0.1 mM), D-mannose (K_m 60±20 M) and D-glucose (K_m 25±10 M), indicating a role as a high-affinity transporter for D-glucose, D-mannose and D-xylose. MstA is subject to CreA-mediated carbon catabolite repression and pH regulation mediated by PacC

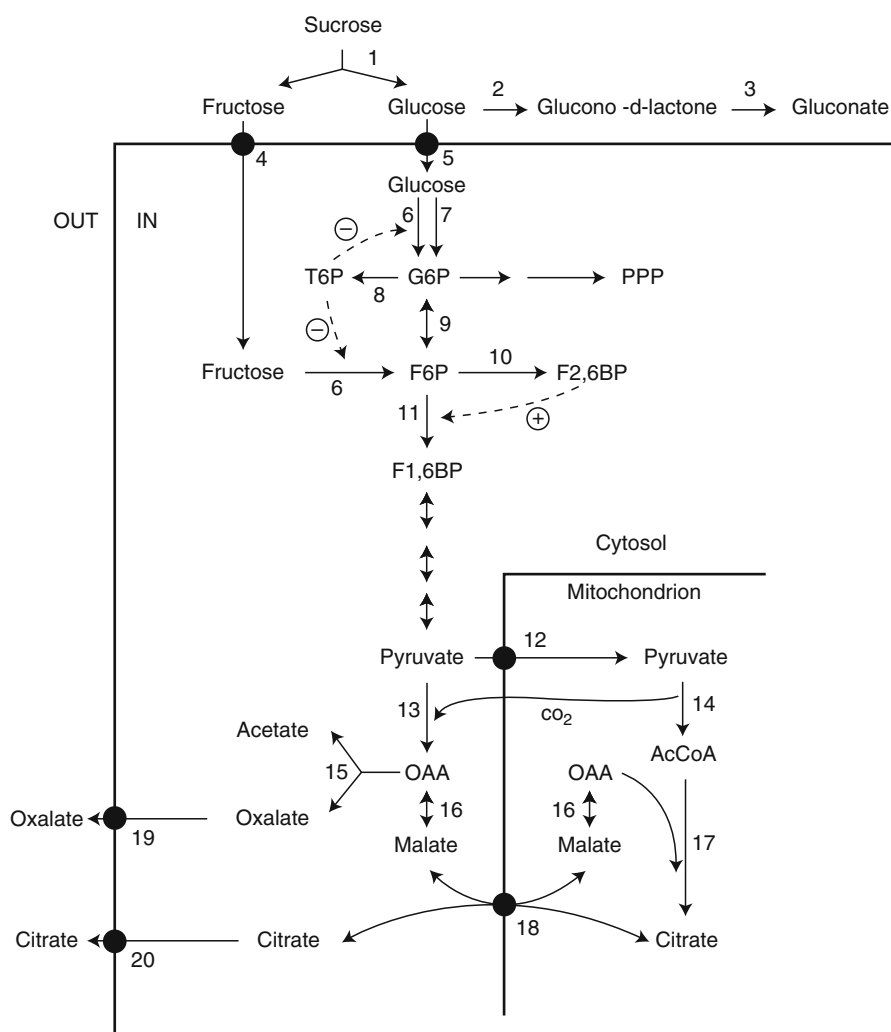


Fig. 10.1. Schematic representation of metabolic steps and some regulatory interactions in the biosynthesis of organic acids in *Aspergillus niger*. The following steps are depicted: 1 invertase, 2 glucose oxidase, 3 lactonase, 4 fructose transport, 5 glucose transport, 6 hexokinase, 7 glucokinase, 8 trehalose 6-phosphate synthase, 9 phosphoglucose isomerase, 10 6-phosphofructo-2-kinase, 11 6-phosphofructo-1-kinase (phosphofructokinase), 12 pyruvate trans-

port, 13 pyruvate carboxylase, 14 pyruvate dehydrogenase, 15 oxaloacetate hydrolase, 16 malate dehydrogenase, 17 citrate synthase, 18 tricarboxylate carrier, 19 oxalate transport, 20 citrate transport. PPP Pentose phosphate pathway. Dashed arrows Regulatory interactions. For convenience, glycolytic metabolism from fructose 1,6-P₂ (F1,6BP) to pyruvate is not fully depicted. Acetate released by oxaloacetate hydrolase is reconsumed (Ruijter et al. 1999)

(Van Kuyk et al. 2004). Knock-out of *mstA* resulted in a two- to fivefold reduction in affinity for glucose and led to the expression of another low-affinity glucose transporter gene, *mstC*, at high growth rates (Jørgensen et al. 2007). *MstA* and yet two other high-affinity glucose transporter genes, *mstF*, were expressed irrespectively of the growth rates in chemostat cultures, whereas only *mstC* was expressed in batch cultures. These data suggest a complex regulation of hexose transporters in *A. niger*. However, the genome sequence of *A. niger* (Pel et al. 2007) contains >70 putative hexose transporters, which renders an understanding of sugar transport in this fungus extremely difficult.

Phosphorylation of the sugar is the first metabolic step after its uptake. Two hexose phosphorylating enzymes exist in *A. niger*, a hexokinase and a glucokinase, and the genes for both enzymes have been cloned (Panneman et al. 1996; 1998). Both enzymes are present under citric acid-producing conditions, but their contribution to the phosphorylation of glucose and fructose is distinct. *A. niger* hexokinase is a “classic” hexokinase, able to phosphorylate both glucose and fructose, whereas

glucokinase has a very low affinity for fructose and has no physiological function in fructose phosphorylation. The affinity of glucokinase for glucose is very high (K_m 0.06 mM) and modelling studies indicate that glucokinase probably accounts for the phosphorylation of a large fraction of glucose (Panneman et al. 1998). Hexokinase is strongly inhibited by trehalose 6-phosphate, an intermediate in trehalose biosynthesis (Panneman et al. 1998); and Arisan-Atac et al. (1996) have successfully attempted to increase flux through glycolysis by removing this inhibition. A strain lacking trehalose-6-phosphate synthase activity, due to disruption of the gene encoding it (*tpsA*), showed a higher rate of citric acid production from sucrose in the early phase of acid accumulation. However, since glucokinase is not inhibited by trehalose-6-phosphate, this approach may not be entirely successful in a process using glucose as the substrate.

At the level of glucose 6-phosphate, an important metabolic branch point exists. Carbon metabolism can either proceed by glycolysis or by the pentose phosphate pathway. Early in the fermentation⁵, when active growth occurs, about 80% of the carbon is metabolised via the pentose phosphate pathway (Legisa and Matthey 1986) to provide the cell with building blocks and NADPH. Later in the fermentation, during citric acid accumulation, the pentose phosphate pathway accounts only for a minor fraction (<20%) of metabolised carbon (Legisa and Matthey 1986), while the major fraction of carbon is metabolised via glycolysis.

Phosphofructokinase-1 is one of the most intensively studied enzymes of the citric acid biosynthetic pathway. It is regulated by a large number of metabolites, both inhibitory (citrate, PEP, ATP) as well as stimulatory ones (NH_4^+ , AMP, fructose-2,6-bisphosphate = fructose 2,6- P_2).

Allosteric activation of phosphofructokinase-1 by fructose 2,6- P_2 is probably the most relevant factor in the regulation of the enzyme (Arts et al. 1987). Under physiological conditions, phosphofructokinase-1 is not active in the absence of fructose 2,6- P_2 (Ruijter et al. 1997). Some experimental data suggest that phosphofructokinase-1 might have flux control in the citric acid

biosynthetic pathway: in mutants that exhibited an increased citric acid production rate, phosphofructokinase-1 and hexokinase activity were twofold higher than in the parental strain (Schreferl-Kunar et al. 1989). However, amplification of phosphofructokinase-1, individually or in combination with pyruvate kinase, did not increase the rate of citric acid production in *A. niger* N400 (Ruijter et al. 1997). Interestingly, recombinant strains with increased phosphofructokinase-1 consistently had a 40% lower intracellular concentration of fructose 2,6- P_2 . In vitro measurements mimicking intracellular conditions, assuming an intracellular pH of 6.8, showed that such a reduction in the fructose 2,6- P_2 level could decrease the specific activity of phosphofructokinase-1 in the cell significantly. Thus, the fungus seems to adapt to over-expression of phosphofructokinase-1 by decreasing the specific activity of the enzyme through a reduction in the level of fructose 2,6- P_2 . The level of the enzyme producing fructose 2,6- P_2 , 6-phosphofructo-2-kinase, was comparable in the parental strain and the recombinant strains, suggesting a regulation of its activity. However, this enzyme is only poorly regulated in *A. niger* (Harmsen et al. 1992), rendering unclear the mechanism by which the level of fructose 2,6- P_2 is decreased.

Intracellular pH is clearly an important factor, as many enzymes are sensitive to changes in the pH range 6.0–7.5. Although one would assume that the intracellular pH during citric acid production is below 7.0, it is indeed even higher. Hesse et al. (2000) determined intracellular pH in *A. niger* using ³²P-NMR in a perfusion set-up and found that *A. niger* cells were able to maintain the intracellular pH at 7.7 over a pH range in the hyphal environment from 1.5 to 6.0 (Hesse et al., unpublished data).

Another mechanism for the regulation of phosphofructokinase-1 (PFK1), phosphorylation by a cAMP-dependent protein kinase, was originally proposed by Legisa and Bencina (1994). A drop in pH may raise the intracellular concentration of cyclic AMP to activate a cAMP-dependent protein kinase, which in turn phosphorylates PFK1. More recently, the authors extended their model to imply a serine protease, which would also be induced by this drop in the intracellular pH, to produce a proteolytical fragment of PFK1 which is insensitive towards inhibition by citrate (Mesojednik and Legisa 2005). However, this is in contrast to the data mentioned above. They further concluded that the shorter PFK1 fragment, arisen by proteolysis, seems to be the enzyme form most likely responsible for generating undisturbed metabolic flow through glycolysis during citric acid production. However, such changes in pH are not detectable in vivo (see above), and solid proof of this model by reversed genetics is still pending.

As already mentioned, catabolism of glucose via glycolysis yields two moles of pyruvate, which are subsequently converted to the precursors of citrate (oxaloacetate and acetyl-CoA). Cleland

⁵Fermentation is here used in a more general sense and means a “metabolic production process” as such and does not refer to an anaerobic sugar conversion process leading mainly to ethanol (see alcohol fermentation in Chapter 2).

and Johnson (1954) were the first who showed that *A. niger* re-uses the CO₂ released in the decarboxylation of pyruvate, to form oxaloacetate (Fig. 10.1). This reaction is of utmost importance to the high citric acid yields commonly obtained (80%), because oxaloacetate could otherwise only be formed by one turn of the tricarboxylic acid cycle. This would result in the loss of two moles of CO₂ with a concomitant maximal theoretical yield of 67%. The enzyme catalysing this reaction has later been shown to be pyruvate carboxylase (Woronick and Johnson 1960; Bloom and Johnson 1962; Feir and Suzuki 1969; Wongchai and Jefferson 1974). Unlike the enzyme from several other eukaryotes, pyruvate carboxylase of *A. niger* has been reported to be localised in the cytoplasm (Bercovitz et al. 1990; Jaklitsch et al. 1991a) and pyruvate should therefore be converted to oxaloacetate in the cytoplasm. However, the recently published genome sequence of *A. niger* (Pel et al. 2007) refines this model: *A. niger* in fact contains two pyruvate carboxylases (a cytoplasmic and a mitochondrial one) and a mitochondrial oxaloacetate transporter was also found.

McIntyre and McNeil (1997) investigated whether increased levels of dissolved CO₂ obtained by treating cultures with gas mixtures containing up to 15% CO₂ would give rise to improved citric acid production rates. The rationale was to supply pyruvate carboxylase with a higher concentration of CO₂, but the approach was not successful. Productivity was unaffected at low CO₂ levels and decreased when CO₂ was above 3% (v/v).

Peksel et al. (2002) demonstrated that pyruvate carboxylation is dependent on the sugar concentration in the medium: during cultivation at high glucose levels (14% w/v), most of the label from ¹³C-glucose was found in the citric acid carbons C2 and C4, which is consistent with the operation of pyruvate carboxylase. However, a significant portion of ¹³C was also found in the positions C1 and C5, which may be explained by the partial re-consumption of glycerol and erythritol. Moreover, the early fermentation phase is characterized by a high rate of anaplerosis from oxaloacetate to pyruvate, which decreases in the further course. At low glucose concentrations (2% w/v), the extent of citrate labelling at C2 and C4 was found to decrease and that at C1 and C5 increased. Growth on 2% glucose, is also characterized by an appreciable scrambling of mannitol and considerable back-flux from mannitol to trehalose (indicating strong glycolytic control at the fructose-6-phosphate level) and an

increased anaplerotic formation of pyruvate from oxaloacetate. These data indicate that cultivation on high sugar concentrations shifts the control of glycolysis from the fructose-6-phosphate step to the glyceraldehyde-3-phosphate step.

Cytosolic oxaloacetate is converted to malate and may serve as the co-substrate of the mitochondrial tricarboxylic acid carrier, which probably catalyses the transport of citrate from the mitochondrion to the cytoplasm. Possibly, an increased cytosolic malate concentration stimulates the export of citrate from the mitochondrion (Kubicek 1988).

The effect of inserting genes involved in the reductive branch of the tricarboxylic acid (TCA) cycle on citrate production by *A. niger* was evaluated by de Jongh and Nielsen (2008). Several different genes were inserted individually and in combination, i.e. malate dehydrogenase (*mdh2*) from *Saccharomyces cerevisiae*, two truncated, cytosolic targeted fumarases (*Fum1s* and *FumRs*) from *S. cerevisiae* and *Rhizopus oryzae*, respectively, and the cytosolic soluble fumarate reductase (*FrdS1*) from *S. cerevisiae*. It turned out that all the transformant strains showed enhanced yields and productivities for citrate compared with the wild-type strain, and an over-expression of *FumRs* and *FrdS1* resulted in the best citrate-producing strain, giving a maximum yield of 0.9 g citrate g⁻¹ glucose and a maximum specific productivity of 0.025 g citrate g⁻¹ dry weight h⁻¹. Over-expression of *mdh2* alone resulted in an increased citrate production only in the initial phase of fermentation compared with the other transformants and the wild-type strain.

Biosynthesis of citrate requires at least one mitochondrial step that is catalysed by citrate synthase located exclusively in the mitochondria (Jaklitsch et al. 1991a). Recently, the *A. niger* gene encoding the mitochondrial citrate synthase was cloned and over-expressed (Ruijter et al. 2000). An up to 11-fold overproduction of citrate synthase did not increase the rate of citric acid production in *A. niger* N400; however, this may be due to the fact that *A. niger* has one cytosolic and three putative mitochondrial citrate synthases. A similar redundancy was found for aconitase, for which two putative cytoplasmic and two mitochondrial forms were described (Pel et al. 2007). Finally it contains two ATP-citrate lyases and one eukaryotic β -chain mitochondrial citrate lyase. This indicates a high degree of conservation of the “metabolic pathway” of citrate formation. Because one of the ATP-citrate lyases is mitochondrial, a futile cycle of citrate formation and degradation needs to be prevented. The identification of

genes involved in citric acid metabolism and transport provides excellent opportunities to study and understand the efficient production of citric acid by *A. niger* in much more detail.

Export of citric acid from the cells may be a very important step in flux control. Cornish-Bowden et al. (1995) proposed a strategy to improve metabolite production based on a "supply and demand" principle. In their view, the cell produces a metabolite for a purpose and regulatory mechanisms will ensure that production of a metabolite is decreased when the intracellular level is sufficient. Thus, in order to keep the production rate of the metabolite high, the best approach would be to remove it from the cell. Apparently, *A. niger* possesses an efficient mechanism to export citric acid, since it is able to accumulate this metabolite at a high concentration in the medium. Matthey and co-workers (Matthey 1992; Kontopidis et al. 1995) proposed that citrate efflux from the cells may occur by (facilitated) diffusion of the citrate²⁻ anion, driven by the large pH gradient between the cytosol and the extracellular medium. If this assumption is correct, less citrate would be secreted at higher external pH. Netik et al. (1997), however, showed that citrate export requires ATP and that its V_{\max} is not strongly affected by the external pH, which makes the diffusion hypothesis rather unlikely.

An intriguing question for many researchers has been why does *A. niger* accumulate citric acid in the medium to such high concentrations? Many have sought the solution in a block in the metabolism downstream of citrate, but in most cases this has been shown to be incorrect. For example, low activity of aconitase due to iron deficiency was thought to cause citric acid accumulation (Szczodrak and Ilczuk 1985). However, the activity of this enzyme during citric acid accumulation has clearly been demonstrated by others (La Nauze 1966; Kubicek and Röhr 1985). Similarly, inhibition of the NADP-dependent isocitrate dehydrogenase by glycerol (Legisa and Matthey 1986) was proposed to decrease the flux through the tricarboxylic acid cycle and, because of the K_{eq} of aconitase, this resulted in an accumulation of citrate. However, this hypothesis had to be rejected after studying the effect of increased intracellular glycerol concentrations on the oxidation of [1,5-¹⁴C]-citrate by intact cells and isolated mitochondria of *A. niger* (Arisan-Atac and Kubicek 1996). The appearance of ¹⁴CO₂ – which, because of the labelling position applied, can only be released during the metabolic conversion of citrate to α -ketoglutarate – was unaffected by high glycerol concentrations. Furthermore, unlike the enzyme in crude cell-free extracts (Legisa and Matthey 1986), purified NADP-dependent

isocitrate dehydrogenase was not inhibited by citrate (Arisan-Atac and Kubicek 1996). These results speak out against a prominent role of glycerol in initiation of citric acid production. Clearly, the rapid and almost exclusive flow of carbon from hexoses to citric acid is the result of the combined properties of the enzymes involved in citric acid biosynthesis, but a metabolic block, i.e. the absence of a certain enzyme activity, is not necessary to explain citric acid accumulation. An alternative explanation might be that the unique presence of a certain enzyme, such as a citric acid exporter, results in increased acid production. If the enzyme kinetics of this exporter are favourable, most of the carbon will be channelled to extracellular citric acid.

A. niger forms an alternative respiratory pathway under conditions that promote citric acid production (Zehentgruber et al. 1980; Kirimura et al. 1987). In the course of a citric acid fermentation, the activities of the normal respiratory enzymes decrease, whereas the activity of alternative oxidases increases (Kirimura et al. 1987). Sun et al. (2007), comparing the genomes of different *A. niger* strains, identified additional copies of the *aox1* gene encoding the alternative oxidase; but Hattori et al. (2009), analysing a different citric acid producer strain of *A. niger* (WU 2223L), found only a single copy. The alternative oxidases do not pump protons concomitantly with electron transport through the mitochondrial membrane and their physiological function is thought to be removal of excess reducing equivalents (NADH, FADH₂). Such a function is in agreement with the presence of alternative oxidases during citric acid production. Conversion of hexoses to citric acid results in net production of ATP and NADH. Since there is not much growth at the stage of citric acid production, the cells probably do not require much ATP, and a switch from normal respiration to alternative oxidases would enable the fungus to re-oxidise its NADH without concomitant ATP production.

Citric acid production by *Yarrowia lipolytica* can be done with either *n*-alkanes or glucose as the substrates. The pathways for citric acid production from glucose in *Y. lipolytica* are similar, if not identical, to those in *A. niger* (Fig. 10.2). The importance of the anaplerotic reaction catalysed by pyruvate carboxylase was shown by Aiba and Matsuoka (1979) and, like in *A. niger*, *Y. lipolytica* pyruvate carboxylase is located in the cytoplasm (Sokolov et al. 1995).

The metabolism of *n*-alkanes by *Y. lipolytica* is schematically shown in Fig. 10.2. Following uptake of the hydrocarbon, the first metabolic step is probably its hydroxylation by a cytochrome P450 monooxygenase. The resulting 1-alkanol is oxidised to the aldehyde and subsequently to

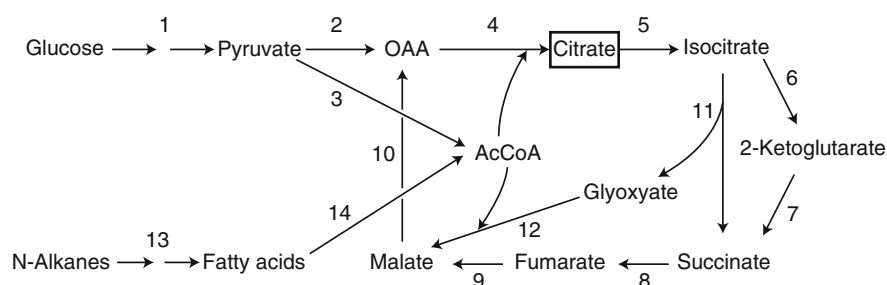


Fig. 10.2. Simplified scheme of the central metabolism involved in citric acid biosynthesis from glucose or *n*-alkanes by *Yarrowia lipolytica*. The following steps are depicted: 1 glycolysis, 2 pyruvate carboxylase, 3 pyruvate dehydrogenase, 4 citrate synthase, 5 aconitase, 6 isocitrate

dehydrogenase, 7 2-ketoglutarate dehydrogenase, 8 succinate dehydrogenase, 9 fumarase, 10 malate dehydrogenase, 11 isocitrate lyase, 12 malate synthase, 13 initial *n*-alkane catabolism, 14 β -oxidation

the corresponding fatty acid by NAD^+ -dependent alcohol and aldehyde dehydrogenases (Osumi et al. 1974). Fatty acids are converted to acyl-CoA esters and are degraded by the peroxisomal β -oxidation pathway to give acetyl-CoA, which is used to produce malate in the glyoxylate cycle. Isocitrate lyase and malate synthase, the enzymes making up the glyoxylate cycle, are induced by growth on *n*-alkanes (Nabeshima et al. 1977).

Initially, the major problem with production of citric acid from *n*-alkanes was the simultaneous production of considerable amounts of isocitric acid. Wild-type *Y. lipolytica* strains were found to produce approximately equimolar amounts of citric acid and isocitric acid from *n*-alkanes, whereas less isocitric acid is produced from sugar substrates (Finogenova et al. 1986). Akiyama et al. (1973a) reasoned that a low activity of aconitase, the enzyme catalysing the conversion of citric acid into isocitric acid, was essential to reduce production of isocitric acid. They selected a mutant that was more sensitive to fluoroacetate than the wild-type strain. This mutant had merely 1% of the wild-type aconitase activity and produced virtually no isocitric acid (Akiyama et al. 1973a, b).

B. Fermentation Conditions

A “good” citric acid fermentation, i.e. with high rate and yield, is only obtained when a number of culture conditions are met. These constraints are summarised in Table 10.3 and discussed in more detail in the next paragraphs. A first systematic study on the influence of culture conditions on citric acid production was done by Shu and Johnson (1948a, b) and most of their results have been used since then and are still valid. It must be noted that most of the data publicly available come from academic studies and, although the conditions employed in industrial processes are generally similar to those described here, industrial strains may be less sensitive to certain media requirements.

Table 10.3. Conditions for citric acid production by *Aspergillus niger*

Parameter	Component	Range
Carbon	Glucose, sucrose	140–220 g/l
Nitrogen	Ammonium salts	Equivalent of 0.4–0.6 g/l N
Phosphorus	Potassium phosphate	Equivalent of 0.4–1.0 g/l phosphate
Metal ions ^a	Mg^{2+} Fe^{2+} Zn^{2+} Mn^{2+}	20–100 mg/l 1.3–1.5 mg/l 0.3–0.5 mg/l <1 $\mu\text{g/l}$
Oxygen	Air	>100 mbar partial O_2 pressure
pH		<2
Temperature		30–35 °C

^aMagnesium, iron and zinc are added as sulfate salts to provide sulfur.

Sugar substrates which can be used in submerged citric acid production are sucrose, glucose and fructose; these carbon sources are rapidly consumed by the fungus resulting in fast citric acid production (Xu et al. 1989). Any feedstock containing a sufficiently high level of these sugars is suitable (Kubicek and Röhr 1986), but the most commonly applied complex substrates are molasses, (semi)refined sucrose or glucose syrups prepared by hydrolysis of starch. Sucrose is probably not taken up directly by *A. niger* but hydrolysed to glucose and fructose by an extracellular invertase (Boddy et al. 1993). In general, a high substrate concentration (14–22% w/v) is required to obtain a satisfactory rate and yield (Shu and Johnson 1948a).

A high yield facilitates an easy recovery of the product. Yet an interesting biochemical effect of a high sugar concentration was reported by Kubicek-Pranz et al. (1990), who found that the level of fructose 2,6-P₂ was elevated using high sugar concentrations. As described above, fructose 2, 6-P₂ stimulates phosphofructokinase-1 (see Section II.A), which might explain a higher production rate. Polysaccharides give low productivities in

submerged fermentation processes (e.g. Begum et al. 1990), supposedly because their rate of hydrolysis is too slow to produce the high levels of sugar required for a fast sugar conversion into citric acid. An explanation may be that during the submerged cultivation processes, the pH is usually very low (<2), which can result in a low activity of hydrolases. In addition, the monosaccharides would never accumulate in high amounts in the medium. Just the semi-solid “Koji” process uses agricultural raw materials containing polysaccharides, such as starch and cellulose (see Section II. D).

The concentration of phosphate and nitrogen is usually kept low in media designed for citric acid fermentation (Shu and Johnson 1948a). The reason is that growth has to be restricted in order to get accumulation of citric acid and not (too much) biomass. In various literature reports, both nitrogen- and phosphate-deficiency are claimed to be effective in inducing citric acid accumulation (Shu and Johnson 1948a; Kubicek and Röhr 1977; Kristiansen and Sinclair 1979). Growth limitation can also be achieved by deficiency of trace metals and the most effective method probably depends on the particular *A. niger* strain and the conditions in general. For example, since molasses contain relatively large amounts of assimilable nitrogen, it is probably easier to achieve growth restriction by phosphate limitation. Nitrogen is usually added in the form of ammonium nitrate or ammonium sulfate. Consumption of ammonium early in the fermentation decreases the pH of the growth medium, which is another constraint for citric acid fermentation. In a fed-batch process where nitrogen was added continuously at a relatively low rate, the productivity was increased twofold (Dawson et al. 1988).

Low pH is important for citric acid production by *A. niger*, because it prevents the production of other acids. In addition to citric acid, *A. niger* can readily accumulate gluconic and oxalic acid. Gluconic acid is formed by the extracellular glucose oxidases (see Section III). Oxalic acid is produced by oxaloacetate hydrolase (Ruijter et al. 1999), which is a cytoplasmic enzyme (Kubicek et al. 1988). Both glucose oxidase and oxaloacetate hydrolase expression is optimal at pH 5–6, whereas only low activities can be detected at pH 2–3 (Kubicek et al. 1988; Witteveen et al. 1993; Ruijter et al. 1999; Pedersen et al. 2000). In pure sugar fermentations, the production of gluconic and oxalic acids can thus be kept to a minimum by starting the fermentation at a relatively low pH. In fermentations using molasses as a substrate, an

initial pH of 5–6 is commonly employed, because the amino acids present in the molasses make the adjustment of a lower pH expensive. Therefore, in processes using molasses, production strains lacking glucose oxidase and oxaloacetate hydrolase are advantageous.

An *A. niger* *goxC* mutant did not possess a functional glucose oxidase and strains carrying *goxC* did not produce gluconic acid from glucose (Witteveen et al. 1990). Another *A. niger* mutant, *prtF*, lacking oxaloacetate hydrolase activity was described by Ruijter et al. (1999) and a *goxC-prtF* double mutant, lacking both glucose oxidase and oxaloacetate hydrolase, accumulated citric acid from sugar substrates in a regular synthetic medium at pH 5. Remarkably, under these conditions, production is completely insensitive to Mn^{2+} (see below). Pedersen et al. (2000) cloned and disrupted the *oahA* gene encoding oxaloacetate hydrolase, which resulted in a strain unable to produce any oxalic acid. This is an important fact, as it illustrates that this reaction sequence is the major one of several possible pathways for oxaloacetate formation under citric acid producing conditions (cf. Pel et al. 2007).

A critical requirement for citric acid fermentations is a high dissolved oxygen tension (DOT), which is necessary to provide the stoichiometric amounts of oxygen needed for the conversion of the high amounts of sugar into citric acid. Productivity increases with DOT at least up to a partial pressure of 150 mbar (15 kPa), which is equivalent to approximately 75% saturation of the growth medium with air (Kubicek et al. 1980). Interruptions in the air supply cause an immediate decrease in productivity without any harmful effect on mycelial growth (Kubicek et al. 1980; Dawson et al. 1986). Depending on the time in fermentation and the duration of the interruption in aeration, production may be irreversibly impaired. The biochemical explanation for this observation may be that the presence of the alternative respiratory pathway (see Section II. A) is dependent on a high DOT (Kubicek et al. 1980; Zehentgruber et al. 1980; Kirimura et al. 1987, 1996).

The importance of correct levels of the trace elements iron, zinc, copper and manganese in the medium has been known for more than 60 years (Shu and Johnson 1948b). Their effect is not absolute, but depends on their relative proportion to other nutrients, particularly phosphate (Shu and Johnson 1948b). The effect of manganese ions (Mn^{2+}) is particularly striking, as concentrations as low as 2 ppb reduce citrate accumulation by about 20% (Clark et al. 1966). Such concentrations

are easily introduced into the medium by other nutrients, such as the carbon source, and therefore manganese must be removed from the feedstock. In pure sugar fermentations, manganese is usually removed by cation exchangers, whereas in molasses, manganese is precipitated with ferrocyanide (Clark et al. 1965). Another method for decreasing the detrimental effect of manganese is the addition of copper ions, which is explained by the inhibition of active manganese uptake by copper (Hockertz et al. 1987b). Also the addition of lower alcohols was proven to be successful (Moyer 1953).

Manganese deficiency has multiple effects on the fungal physiology and can, for example result in an increased protein turnover (Ma et al. 1985), impaired DNA biosynthesis (Hockertz et al. 1987a) and altered composition of plasma membranes (Meixner et al. 1985) and cell walls (Kisser et al. 1980), which indicates that the manganese effect is not clearly related to a particular cellular function. Manganese deficiency has a strong effect on *A. niger* morphology, although it must be noted that morphology is also influenced by other factors such as pH and agitation. Mycelia grown under manganese-deficient conditions are strongly vacuolated, highly branched, contain strongly thickened cell walls and exhibit a bulbous appearance (Kisser et al. 1980), and these phenomena may be related to a loss of orientation of apical growth. Dai et al. (2004) used suppression subtractive hybridization to identify 22 genes whose expression responded to Mn^{2+} . These differentially expressed genes, which have a tentatively identified function, could be assigned to two general categories: those involved in amino acid or protein metabolism (i.e. in cell growth) and those involved in cell regulation. They concluded that the rapid hyphal growth associated with the switch to filamentous morphology observed upon induction by sufficient Mn^{2+} levels probably requires an increased protein production as well as the degradation and utilization of proteins required for the maintenance of the pellet growth state. A functional analysis of one of the unknown genes – *brsa-25* – indicated that it was indeed involved in the regulation of fungal morphology.

Netik et al. (1997) reported that the citrate export is strongly increased in mycelia grown under manganese deficiency. The reason for the requirement of manganese deficiency for citrate export is not clearly understood, but may be related to an absolute requirement of the citrate uptake for manganese ions (Netik et al. 1997). Uptake of citrate by the cells in the production phase was shown for *A. niger* (Kontopidis et al. 1995) and, if manganese deficiency prevented this, a higher productivity could be obtained. An *A. niger* mutant lacking both glucose oxidase and oxaloacetate hydrolase accumulated citric acid at pH 5 and, under these conditions, production was completely insensitive to Mn^{2+} (Ruijter et al. 1999). However, in a traditional citric acid fermentation medium at low pH, this mutant behaved like a wild-type strain and did not produce any citric acid when Mn^{2+} was added. This finding

implies that the requirement for Mn^{2+} deficiency is related to the specific conditions during traditional citric acid fermentation.

The main differences between the fermentation with *A. niger* and *Y. lipolytica* are the following: (1) *Y. lipolytica* requires a pH of about 5 for optimal production, (2) it is not sensitive to Mn^{2+} and (3) citric acid production by *Y. lipolytica* is usually initiated by nitrogen limitation. If the pH is not kept above 5, erythritol and mannitol accumulate at the expense of citrate (Tabuchi and Hara 1970). The fact that Mn^{2+} is not relevant has some parallels with the *A. niger* mutant described by Ruijter et al. (1999), which is also insensitive to Mn^{2+} during production at pH 5 (see above). A peculiarity of the *n*-alkane process is the requirement for thiamine; if it is omitted, α -keto acids (mainly α -ketoglutarate) will accumulate. The biochemical basis of this effect is not fully understood, but may be related to an increased requirement for the cofactor thiamine pyrophosphate by the enzymes, which are involved in the oxidative decarboxylation of these α -keto acids.

C. Strain Breeding

Strain improvement can be achieved in different ways. Nowadays, much of this work involves a classical genetic approach. Mutants can arise spontaneously or be produced by mutagenic treatment, such as exposure to chemicals, UV light, γ - or X-ray radiation. To minimise the chances of introduction of unwanted mutations, the mutagenic treatment should be performed in such a way that a high percentage of survival is ensured. Afterwards, mutants that perform better than the parent strain have to be identified by appropriate screening methods employing plate tests, small liquid cultures or, in case of “high-throughput” screenings, microtiter plate methods, which have an even higher capacity than plate tests, as they can be automated to a large extent. Beneficial characteristics of different strains may be combined by cross-breeding or protoplast fusion. The objectives in strain-breeding programmes are usually directed towards the two major aspects of the production process: productivity and yield.

Many studies have been aimed at an improved citric acid production on polysaccharides without the need of prior hydrolysis (e.g. starch) in the course of liquid

fermentation process (Rugsaseel et al. 1993; Suzuki et al. 1996). Some respective mutants were found to give improved yields on starch, but these yields have still been too low compared with those obtained on glucose or sucrose. Although *A. niger* almost quantitatively converts the sugar substrate into citric acid, some by-products may be formed and, thus, a decreased formation of by-products may improve the productivity, yield and recovery of citric acid from the broth. Possible by-products of citric acid fermentation have already been mentioned above (i.e. gluconic acid, oxalic acid), but in addition, also polyols (glycerol, erythritol, mannitol; Röhr et al. 1987), enzymes (e.g. pectinases; Bohdziewicz and Bodzek 1994) and polysaccharides (Kirimura et al. 1999b) can be formed. A mutant that is unable to produce both gluconic acid and oxalic acid was described by Ruijter et al. (1999). Polyol compounds are formed from sugars, but will be re-consumed once the sugar substrate is exhausted. Therefore, polyol compounds are probably not a major problem for the final yield in citric acid as long as the sugar substrate is completely converted. The subsequent production and re-consumption of polyols may, however, reduce productivity. Other strategies in strain improvement have been directed at the decrease or the removal of Mn^{2+} sensitivity (Gupta and Sharma 1995), a high productivity at relatively low dissolved oxygen tension, or the improved ability of the fungus to perform under suboptimal conditions (e.g. low pH, high osmotic pressure).

In addition to mutagenesis and screening, which is largely a trial and error process, genetic engineering would be a promising approach. To this end, quantitative metabolic knowledge is required in order to decide which enzymatic steps should be altered in order to increase productivity. Torres (1994a, b) and Torres et al. (1996b) analysed the flux control of citric acid biosynthesis in *A. niger*. Initially, modelling of the first part of the pathway (i.e. up to pyruvate; see Fig. 10.1) suggested that sugar transport and phosphorylation, which had been lumped into one single step in the model, represent the most important step in controlling the flux through the pathway. Experimental data suggested that hexokinase indeed has a flux control. In a study with a strain lacking trehalose 6-phosphate inhibition of hexokinase, due to disruption of the gene encoding trehalose 6-phosphate synthase (*tpsA*), a higher rate of citric acid production from sucrose in the early phase of acid accumulation was observed (Arisan-Atac et al. 1996). To date, this is the only successful attempt published to increase flux through glycolysis by genetic engineering. In a subsequent study (Torres et al. 1996b), it was concluded from flux optimisation calculations that the simultaneous

over-production of seven enzymes would be required for a significant increase in flux. For practical reasons, this cannot be accomplished at the moment. Simultaneous over-expression of seven enzymes in a controlled way is experimentally difficult to achieve. Notably, this model has not incorporated the metabolic steps from pyruvate to extracellular citric acid. Hexokinase seemingly have a certain flux control in the conversion of glucose to pyruvate, but the control of the complete pathway (from hexose to citric acid) may lie in later steps, i.e. between pyruvate and citric acid.

D. Production Processes for Citric Acid

Originally, citric acid was solely produced from lemons and the global market at that time was dominated by an Italian cartel for most of the nineteenth and the beginning of the twentieth century. For this reason, other means of citric acid production were sought. Chemical synthesis of citric acid was possible, but not suitable due to expensive raw materials and a complicated synthesis process with low yield. The first description of citric acid production by *A. niger* is found in a United States patent (Zahorski 1913). Subsequent work by Currie (1917) led to a rapid development of the fermentation process, which about a decade later accounted for a large part of the global production of citric acid (about 10 000 t year⁻¹ at that time).

There are three different types of fermentation processes, which are in use for production of citric acid: the surface process, the submerged process and the solid-state process. Originally, surface cultures were most commonly used. This process is very labour-intensive, but – although the major fraction of citric acid is now produced by submerged cultivation – is still in use, because it is less sensitive against Mn^{2+} , the major weak point of the submerged process. Surface fermentation is usually carried out in flat trays filled with the nutrient medium to a depth of 10–20 cm. Following conidiospore dispersion over the liquid, the mycelium develops as a mat on the surface. Sterile air is passed over the trays, serving both as an oxygen supply and as a cooling aid.

Nowadays, submerged fermentation prevails because it is more efficient in terms of space-time yield, labour and productivity. Two types of

bioreactors are in use: stirred-tank and air-lift fermenters, both constructed of high-grade stainless steel to prevent contamination with metal ions. Aeration occurs from the bottom and is very important considering the specific requirements of citric acid production by *A. niger*. Because profound aeration results in excessive foaming of the culture, measures have to be taken to counteract foam formation, e.g. by adding antifoam agents. In the laboratory, the bioreactors can be inoculated directly with spores but, in industry, a seed culture (pre-germinated spores in a separate tank) is used. The latter ensures optimal use of the production tanks. Fermentations are run at 30–35 °C. Once the rate of citric acid production has reached a point where it becomes uneconomic to proceed (usually after 5–10 days) the fermentation is stopped.

In East Asian countries, solid-state fermentation (the “koji” process) is used for citric acid production. The advantages of solid-state fermentation are the lower energy requirement and the cheap growth substrates, i.e. all kinds of agricultural materials such as cereal brans (Shankaranand and Lonsane 1994), tubers (Lu et al. 1997) and fruit wastes (Tran and Mitchell 1995) can be used as substrates. While the use of polysaccharides is uneconomic in submerged fermentations, high productivities can be obtained with them in solid state. The polymeric substrates, mostly starch as well as cellulose and hemicelluloses, are degraded by the hydrolytic enzymes produced by the fungus. Productivity can be improved by application of mutants with decreased protease activity, resulting in reduced degradation of polysaccharide degrading enzymes such as gluco-amylase (Sarangbin and Watana-pokasin 1999). In most cases, a packed bed configuration, either shallow or deep, is used with a water content of about 70–80% and an initial pH of 4–5. In deep beds, aeration is important for supplying the fungus with oxygen and for cooling. Little is known about the physiology of the fungus during solid-state fermentation, but it is clearly different from submerged fermentation. For example, similarly to the surface fermentation, *A. niger* is less sensitive to metal ions including Mn^{2+} during solid-state fermentation (Shankaranand and Lonsane 1994). After 5–8 days, the koji is harvested and placed in percolators to extract the citric acid with water. Further purification occurs by the same procedures as for the surface or submerged fermentations (see below).

Citric acid production by yeasts is exclusively performed in submerged culture. The yeast process came into use because of the possibility to use cheap (in those days), crude-oil-based hydrocarbons as growth substrate, but after the sudden increase in feedstock prices in 1973 and because

yeasts turned out to be flexible with respect to their substrates, a switch was made to sugars as the major carbon source.

During an industrial fermentation, the process is constantly monitored and, if necessary, adjusted to maintain optimum production. Relevant process parameters are nutrient and product concentrations (e.g. carbon substrate, citric acid, NH_4^+ , O_2 , CO_2), pH, temperature and fungal morphology. Also monitoring the redox potential, changes of which are a consequence of several processes and biochemical reactions, has been suggested to be useful (Berovic 1999). One of the most prominent features of submerged fermentation is fungal morphology: germinating spores form stubby, forked and bulbous hyphae aggregating to small (0.2–0.5 mm) pellets, which have a smooth surface. This particular morphology is a well known criterion for a “good” acid production and dependent on an appropriate nutrient composition, in particular concerning manganese (Kisser et al. 1980). It is therefore a simple and convenient indicator for assessing the conditions of the fermentation (e.g. by quantitative photographic imaging).

Recovery of citric acid from the surface process starts with filtration to separate the mycelium from the culture liquid and washing of the mycelial cake, which may still contain a significant fraction of the citric acid produced. Filtration of the mycelium from the submerged process may require the use of filter aids due to the formation of slimy polysaccharides as by-products. Recovery of citric acid from the culture broth (Fig. 10.3) is then accomplished by either one of two procedures: precipitation or solvent extraction (Gluszc and Ledakowicz 1999).

Precipitation involves addition of lime to the citric acid solution and filtration to obtain calcium citrate. Treatment of the calcium citrate with concentrated sulfuric acid and removal of the resulting $CaSO_4$ precipitate yields a citric acid solution, from which residual impurities are removed, e.g. by active carbon filtration or ion-exchange steps. Finally, citric acid is crystallised from the purified solution using vacuum crystallisers. This method requires large amounts of lime and sulphuric acid and results in a huge quantity of gypsum, therefore alternative technologies have been sought. A very successful one is solvent extraction, making use of a solvent containing an amine (e.g. King 1992). The solvent, which does not mix with water, takes up the citric acid from the mother liquor and is then washed with water to recover the citric acid. Crystallisation is again the last step.

Although alternative technologies for citric acid production have been explored, batch-wise production is still the most economic method.

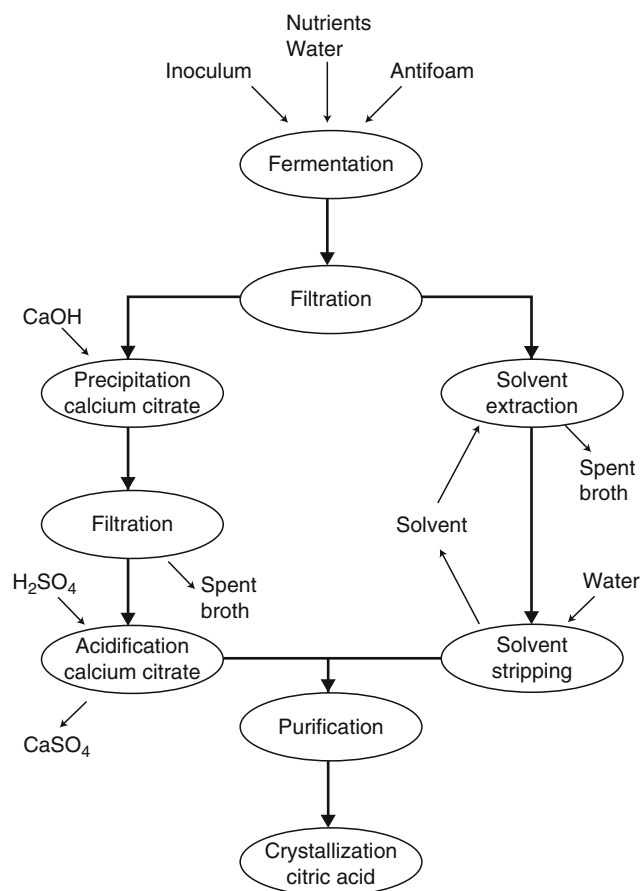


Fig. 10.3. Flow diagram of citric acid fermentation and the recovery of citric acid. Two alternative procedures are shown for the downstream processing: the traditional precipitation method (*left*) and the solvent extraction process (*right*)

Continuous production is also possible (Kristiansen and Sinclair 1979) but not suitable because of a high residual sugar level and low citric acid concentrations in the outflow. Another approach is the immobilisation of fungal material in or on supports of various kinds, enabling the re-use of the biomass (e.g. Eikmeier and Rehm 1984). These systems were found to have a low productivity and are difficult to scale up to industrial production, and are therefore not competitive with the classic batch processes (Federici and Petruccioli 1997).

III. Gluconic Acid

Gluconic acid is a non-volatile acid with a very low toxicity and low corrosivity and is able to form water-soluble complexes with a variety of di- and trivalent metal ions in alkaline solution.

It is considered as a generally permitted food additive in the European Union and has GRAS status according to the FDA in the United States. It is used as an additive in food, for the cleaning and finishing of metal surfaces and as an additive in cement. An important pharmaceutical application of gluconic acid is its use as a counterion in calcium and iron salts for the therapy of deficiencies of these metals. The annual world-wide production has been estimated to be 50 000–100 000 t, of which approximately 45% is used in the construction industry for metal cleaning. The total market value in 2004 was estimated to be about US 330×10^6 (Business Communication Co. 2004). The economic and societal need for sustainable processes has recently revived interest in the fermentation processes of organic acids, including gluconic acid, as illustrated by some recent review articles (Ramachandran et al. 2006, Singh and Kumar 2007).

A. Biochemistry of Gluconic Acid Formation

Gluconic acid is derived directly from glucose by oxidation. In bacteria, gluconic acid is formed by a membrane-bound D-glucose dehydrogenase, which uses pyrroloquinoline quinone (PQQ) as a coenzyme and converts extracellular glucose into extracellular gluconic acid. Fungal gluconic acid formation is a two-step process involving the oxidation of β -D-glucose to D-glucono- δ -lactone by glucose oxidase and the subsequent hydrolysis of the lactone to gluconic acid, which can either occur spontaneously or be catalysed by a lactonase (Röhr et al. 1996b). In *A. niger*, both enzymes are located outside the hyphae (extracellular biocatalysts), mainly in the cell wall (Witteveen et al. 1992).

Glucose oxidase is both N- and O- glycosylated, a homodimeric flavoprotein with two tightly (but non-covalently) linked FAD moieties which uses molecular oxygen in its reaction and produces hydrogen peroxide that is subsequently decomposed by catalases. The enzyme properties and its mechanism (particularly that of GOX from *A. niger*) were investigated in detail in the 1960s (e.g. see Gibson et al. 1964; Kleppe 1966; Weibel and Bright 1971). The 3-D structures of both the *A. niger* and the *Penicillium amagasakiense* GOX proteins were solved and published (Hecht et al. 1993; Wohlfahrt et al. 1999). Further details on the catalytic mechanism of GOX were given by Gerd et al. (2004) and Leskovacs et al. (2005). There are numerous applications of glucose oxidase other than for gluconate production. For a recent review, we can refer to Wong et al. (2008). Regulation of glucose oxidase expression, facilitated by the cloning of the *A. niger* glucose oxidase gene *goxA* (Kriechbaum et al. 1989), and its influence on gluconic acid production were studied in detail (Witteveen et al. 1993). The enzyme is strongly induced by high levels of glucose and a pH around 5.5. The availability of a series of mutants that either lack or overproduce glucose oxidase or show altered regulation of its expression (Swart et al. 1990) has greatly facilitated the development of a model for glucose oxidase induction. Glucose oxidase, lactonase and two catalases are induced by H₂O₂ in a co-ordinated way, probably mediated by a regulatory gene, *goxB* (Witteveen et al. 1993). This may explain the conditions for optimal induction: both glucose and O₂ are required for H₂O₂ formation. The requirement for a relatively high pH may be attributed to the fact that glucose oxidase is inactivated at pH below 3 and, hence, cannot be induced since no H₂O₂ is formed. The genome sequence of *A. niger* (Pel et al. 2007) indicates that our understanding of the *A. niger* metabolism is far from complete. Genes are present that encode one intracellular and three secreted (putative) glucose oxidases. Furthermore, 11 ORFs encoding catalases have been annotated, two of which contain a signal sequence. This illustrates the necessity that *A. niger* has to ensure protection against high concentrations of hydrogen peroxide. Finally, at least one of the four putative lactonases is supposed to be an extracellular enzyme.

B. Production of Gluconic Acid

Gluconic acid can be produced by various fungi such as *A. niger* and *Penicillium* spp. as well as by bacteria including *Pseudomonas* spp. and *Gluconobacter* spp. (see e.g. Ramachandran et al. 2006) but most industrial production processes involve *A. niger* or *Gluconobacter*. Recently, the yeast *Aureobasidium pullulans* was advocated as an alternative production organism and was positively evaluated both in continuous and discontinuous fermentation configurations (Anastassiadis et al. 2003, 2005; Anastassiadis and Rehm 2006). Gluconic acid production with *A. niger* was already developed in the 1930s. It was found that, obviously, the conditions resulting in high glucose oxidase expression are also optimal for industrial gluconic acid production. Thus, high glucose (12–15% w/v for calcium gluconate, 20–25% w/v for sodium gluconate, respectively) and oxygen levels should be used and the pH maintained around 5, which was traditionally achieved by the addition of calcium carbonate (chalk) to neutralise the acid formed. Other nutrients (e.g. phosphate and nitrogen) are added in limiting amounts in order to restrict growth of the fungus. In the 1950s, a process was developed for the production of sodium gluconate, which has a much higher solubility than calcium gluconate, using NaOH for neutralisation (Blom et al. 1952). Productivity is proportional to the dissolved oxygen tension (Reuss et al. 1986), which is logical considering the stoichiometry of the reaction and, therefore, increased pressure has been used to increase the partial pressure of O₂.

So far, little molecular work and genetic engineering for strain improvement has been reported, which is not surprising in view of the almost quantitative yields obtained in process times of less than 24 h. However, provided that increased oxygen levels are achieved by high pressure, glucose oxidase-overproducing strains may further improve fermentation characteristics. Such strains with increased glucose oxidase activity were constructed in two ways: by mutagenesis (Fiedurek et al. 1986; Markwell et al. 1989; Swart et al. 1990) and by transformation of *A. niger* with *goxA*, the gene encoding glucose oxidase (Whittington et al. 1990; Witteveen et al. 1993). Increasing *goxA* expression levels by transformation using genetic “background” strains was described by Swart et al. (1990) and may offer further perspectives to reduce the fermentation time. Another fungal system more recently explored at a laboratory scale is that of *Penicillium variable* P16, a strain which was found to efficiently secrete glucose oxidase. Both strain improvement by mutagenesis

to increase GOX production (Petruccioli et al. 1995) and fermentation optimization (Crognale et al. 2008) for gluconate production were investigated.

For product recovery in the industrial process with *A. niger*, the mycelium is removed by filtration and sodium gluconate is precipitated after concentration to a 45% w/v solution by raising the pH to 7.5. Today sodium gluconate is the main manufactured form of gluconic acid. Free gluconic acid is prepared from the sodium salt by ion exchange.

IV. Itaconic Acid

Itaconic acid (methylene succinic acid) is commercially produced by *Aspergillus terreus*. Also basidiomycetous yeasts (blastomycetes) have been reported to produce the metabolite (e.g. Levinson et al. 2006). Because of its slight toxicity, itaconic acid is only used in industrial applications and not for food applications. The methylene group is able to participate in polymerisation reactions, which makes itaconic acid a profitable product applied in various types of synthetic polymers. Due to its acid groups, the resulting heteropolymers have hydrophilic properties. Based on its application potential, itaconic acid is one of the 12 top value-added chemicals identified in a study by the United States Department of Energy (Werpy and Petersen 2004). Annual global production is estimated at 70 000–80 000 t.

A. Biochemical Pathways of Itaconic Acid Synthesis

Itaconic acid formation follows a pathway which is similar to that involved in the accumulation of citric acid by *A. niger*, i.e. sugar catabolism via glycolysis, anaplerotic formation of oxaloacetate by pyruvate carboxylase and condensation of oxaloacetate with acetyl-CoA. In addition, and in contrast to *A. niger*, *A. terreus* contains an additional enzyme activity, *cis*-aconitate decarboxylase, which forms itaconate from *cis*-aconitate (Bentley and Thiessen 1957a-c; Bonnarme et al. 1995, Dwiarti et al. 2002). Although biochemical studies had already identified the enzyme more than 50 years ago, the encoding gene was only recently characterized by reverse genetics (Kanasama

et al. 2009) and transcriptomics-based approaches (van der Werf et al. 2009). The encoding gene was shown to be a member of the MmgE/PrpD family of proteins, previously known to include mainly methylcitrate dehydratases. Since the *cis*-aconitate decarboxylase reaction is considered to be localised in the cytosol and because citrate synthase and aconitase are traditionally considered to be exclusively localised in the mitochondria, it has been suggested that *A. terreus* transports *cis*-aconitate, rather than citrate, in exchange with malate out of the mitochondria (Jaklitsch et al. 1991b). Analogously to the presumed citrate exporter in *A. niger* (Netik et al. 1997), *A. terreus* probably has a transport protein that is capable of excreting itaconic acid (Gyamerah 1995).

With the recent availability of the annotated *A. terreus* genome at NCBI (<http://www.ncbi.nlm.nih.gov/genome/guide/aspergillus/>), a more detailed analysis of the itaconic acid production pathway can be carried out using genome-based approaches. For *A. niger*, this type of analysis has already shown that many pathways in primary carbon metabolism, including that of organic acid production, may be much more complex than previously anticipated (Flipphi et al. 2009; see also Chapters 19, 20).

B. Production of Itaconic Acid

Itaconic acid is commercially produced mainly by submerged fermentation processes employing strains of *A. terreus* or *A. itaconicus*. The fermentation conditions for itaconic acid production by *A. terreus* are similar to those for citric acid production by *A. niger*, i.e. it requires an excess of an easily metabolisable carbon source (glucose syrup, molasses), a high dissolved oxygen tension (DOT), a limitation in metal ions and ammonium as the nitrogen source (Miall 1978; Riscaldati et al. 2000; Wilke and Vorlop; 2001).

Fermentations are usually run at 35–42 °C. The optimal pH regime for the fermentation is still controversial. In many studies, the pH in the production phase was below 2 (e.g. Bentley and Thiessen 1957a), but it was also reported that the pH, after falling to values below 2 during the initial stage of fermentation, has to be raised to and maintained at around 3 in the actual production phase to prevent formation of the by-product itatartaric acid (Miall 1978). Recent studies confirmed the importance of pH for an optimum productivity (van der Werf et al. 2009).

Since itaconic acid is relatively easy to crystallise, recovery of the acid from filtered broth is usually performed by direct crystallisation. With the cloning of the *cis*-aconitate decarboxylase gene and the identification of other pathway-related genes, optimization of the production process can now be envisaged by using specific gene-based strain improvement approaches (Li, Punt and van der Werf 2009, unpublished data).

V. Other Acids

Kojic acid (5-hydroxy-2-hydroxymethyl- γ -pyrone) is produced by many *Aspergillus* species, but commercial production from glucose is performed with *A. oryzae* and *A. flavus*. It is produced on a much smaller scale than the other acids and is mainly used for skin whitening and as a precursor for synthesis of flavour enhancers, such as maltol. The biochemistry of kojic acid formation has not been completely established yet.

Tracer experiments showed that the labelled positions in glucose were mostly retained in kojic acid, indicating a direct route from glucose to kojic acid (Arnstein and Bentley 1953). Bajpai and co-workers (1981) studied the activities of enzymes potentially involved in this conversion and proposed a metabolic scheme for kojic acid production (Fig. 10.2). The conditions for kojic acid production in submerged culture are similar to those employed for citric and itaconic acid production: a high initial glucose level, profound aeration to maintain a high dissolved oxygen tension, ammonium as the nitrogen source and a low pH. Yields are generally not very high (<50%), but very high levels of kojic acid in the culture broth, up to the saturation point, can be obtained in a fed-batch process (Kwak and Rhee 1992).

Fumaric acid is derived exclusively from petroleum-based materials, since microbiological production is not competitive with the chemosynthetic route (Gangl et al. 1990). Production from glucose by a fermentation employing *Rhizopus oryzae* has been reported and might still be of interest with increasing application of fumaric acid as a food additive. An interesting aspect is that the metabolic pathway of fumaric acid biosynthesis is located completely in the cytosol, since *Rhizopus oryzae* has a cytoplasmic fumarase isoenzyme (Peleg et al. 1989).

Malic acid can be produced from sugar feedstocks by *Aspergillus* species (e.g. Battat et al. 1991) but this process is also not economic. Instead,

malic acid is produced either chemically from maleic acid or fumaric acid or by biotransformation of fumaric acid employing the fumarase reaction. The latter has the advantage that it is stereospecific, i.e. yields L-malic acid, whereas chemical synthesis gives the racemate. Many different organisms are reported to be capable of efficiently biotransforming fumarate to malate, mostly bacterial species (e.g. *Brevibacterium flavum*; Takata et al. 1980). Yields are near-quantitative with process times of hours. Interestingly, amplification of fumarase in *S. cerevisiae* has been successfully used to establish a highly efficient bio-conversion process with a process times of less than 1 h (Peleg et al. 1990; Neufeld et al. 1991).

Lactic acid can be produced by *Rhizopus* species (Soccol et al. 1994), but industrial production is routinely done with lactic acid bacteria or by chemical synthesis (Miall 1978). There are some other acids for which fungal production is known (Table 10.1), such as gallic acid or epoxy-succinic acid, but very few data are available on these products.

VI. Conclusions

Although many organic acids are known to be produced by fungi, in particular by members of the Aspergilli, only a few are produced on an industrial scale. Citric acid makes up the major fraction, perhaps as much as 80–90%, and is mainly applied in foods and beverages. It is unlikely that new, large-scale applications will be developed for citric acid and the increase in the production volume will therefore probably follow economic growth. However, the availability of the full sequence of several *Aspergillus* spp. may offer the introduction of genes that modify citric acid or convert it into another acid that may then be produced at similar high yields. Also, the production of other tricarboxylic acids, e.g. L-malate and fumarate, may grow faster as they gain more and more interest as food additives. There is a growing demand for succinic acid, which is so far only produced by yeasts and whose production e.g. by Aspergilli may become attractive.

The biochemical pathways of organic acid formation are in most cases well established and, particularly, the properties of some of the enzymes involved have been adequately studied.

However, new pieces of information obtained from recent genome sequencing projects have indicated that the involved mechanisms are much more complex than originally thought, thus explaining why the biochemical basis of over-production of organic acids is still not fully understood.

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11 Production of Vitamin B₂ and a Polyunsaturated Fatty Acid by Fungi

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I. Introduction

Vitamins are organic nutrients essential for the vital functions of humans or domestic animals because their own anabolism does not synthesize them adequately. Since they fulfil catalytic or hormone-like functions, an intake of small amounts is sufficient but because storage is limited they should be on the menu every day. Although at least in the so-called developed countries a more than sufficient and seasonally independent food supply allows adequate intake of all vitamins and vitamin-like compounds, up to 50% of the people tested in large scale studies were found to be short in vitamin B₂, folate, vitamin A, vitamin D, vitamin E, and vitamin C (Aranceta et al. 2001). Between 1930 and 1950 all 13 vitamin groups were determined in their structure after extraction, purification, and chemical synthesis.

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Today the technical production of thousands of tonnes per year is performed by chemistry, extraction, fermentation or bioconversion, or combined processes (Table 11.1).

Arachidonic acid, vitamin B₂, and vitamin B₁₂ are produced by fermentation exclusively. Since biotechnical vitamin B₂ production has almost completely replaced chemical synthesis, other processes might follow. The replacement of chemical by microbiological and/or enzymatic processes, nowadays often traded under the name “white biotechnology”, is encouraged by the increasing costs for disposing of wastes and a shift towards renewable educts to fulfil the requirements of sustainability. This chapter focuses on vitamin B₂ and long-chain polyunsaturated fatty acids because these processes are perfect examples for the application of fungi on an industrial scale. Only the most important physiological features and biotechnological strategies for their improvement are discussed.

II. Vitamin B₂

Riboflavin is the most frequently used name of 7,8-dimethyl-10-(D-1'-ribityl)isoalloxazine because it indicates its yellow (Latin *flavus*) color as a pure solid. Synonyms are lactoflavin, lactochrome and ovoflavin, referring to the sources it derives from. Riboflavin or its derivatives have key functions in energy metabolism, maintenance of a healthy skin and muscles, support of the immune and nervous systems, and promotion of cell growth and division. It is the precursor for the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), important electron carriers in redox reactions. Additionally, both flavo-coenzymes participate in bioluminescence, light sensing, phototropism, DNA protection against UV light damage, and reset of the circadian clock

Table 11.1. Scale and methods for industrial production of vitamins and vitamin-like compounds with an impact of microorganisms

Compound	Production method				World production (t year ⁻¹)
	Chemical synthesis	Extraction from plants	Microbial	Combined	
Vitamin C				+	> 100 000
Vitamin B ₂			+		< 10 000
Carotenoids	+	+	+	+	> 1000
PUFAs		+	+		> 1000
Vitamin B ₁₂			+		> 10

(Cashmore et al. 1999). Since higher eukaryotes cannot produce riboflavin, it has the status of a vitamin. Because of light sensitivity and poor resorption, riboflavin deficiency is often a result of surveys on the nutritional status. Therefore supplementation of food (Fletcher et al. 2004) and feed is performed. Overdosing due to dietary supplementation is impeded by the direct excretion of riboflavin with the urine. In industrialized countries processed food is often fortified by the use of riboflavin as a colorant (E 101). About 70% of industrially produced riboflavin is applied in animal feed, since productive livestock, especially poultry and pigs, show growth retardation and diarrhea in the case of riboflavin deficiency. According to a SRI Consulting Report from 2005, the need for industrially produced riboflavin was estimated to be 7000 t year⁻¹. Deficiency symptoms like dermatitis can be avoided by a nutritional intake of 0.3–1.8 mg day⁻¹ for humans and 1–4 mg kg⁻¹ for animals (Eggersdorfer and Adam 1996). One cup of milk per day is sufficient to fulfil this requirement.

For about half of a century riboflavin production was in the domain of the chemical industry and followed a multi-step process starting with 3,4-xylydine and D-ribose. The latter was provided by chemical conversion of glucose or more recently by fermentation from glucose using transketolase-deficient *Bacillus* spp. strains. The first commercial single-step riboflavin fermentations came up in the 1940s, employing the anaerobic Gram-positive bacterium *Clostridium acetobutylicum* or the more efficient hemiascomycetes *Eremothecium ashbyi* or *Ashbya gossypii* (Wickerham et al. 1946; Perlman 1979). All companies manufacturing riboflavin by fermentation shut down their production plants in the 1960s, being competitively disadvantaged by the chemical processes (Lago and Kaplan 1981).

Today, riboflavin production by modern microbiology has become a paradigm of environmentally superior white biotechnology replacing

classic chemistry. After Merck (USA) resumed fermentation by *A. gossypii* in 1974, the success story started with an *A. gossypii* production plant of BASF (Germany) in 1990. The market share of riboflavin produced by the microbial processes increased from 5% in 1990 to 75% in 2002. Initially the two naturally vitamin B₂-overproducing fungi *A. gossypii* and *Candida famata*, and later the metabolically engineered Gram-positive bacterium *Bacillus subtilis*, were used. Today *C. famata* is no longer used and chemical riboflavin production came to an end, leaving only *A. gossypii*- and *B. subtilis*-based production methods. Both processes are the outcome of classical strain improvement, genetic engineering, and process optimization. The *B. subtilis* process was recently compared with the *A. gossypii* process by Hohmann and Stahmann (2010). Because *A. gossypii* seems to be much better in productivity, the major part of this chapter deals with it and a minor part discusses relevant data of *C. famata*.

A. *Ashbya gossypii*

1. The Riboflavin Biosynthesis Pathway

Riboflavin biosynthesis was found in plants and most microorganisms but not in higher eukaryotes. Two precursors, guanosine triphosphate, also needed for RNA, and ribulose-5-phosphate, provided by pentose phosphate pathway, are the roots of this anabolism (Fig. 11.1; Bacher 1991). Despite the dephosphorylation of the ribityl residue, which is catalyzed by an unspecific phosphatase, all enzymes are known and all genes are cloned for *A. gossypii* (Revuelta et al. 1995). While reduction of the ribosyl residue comes prior to deamination of the diamino pyrimidinone in fungi, the sequential order of these

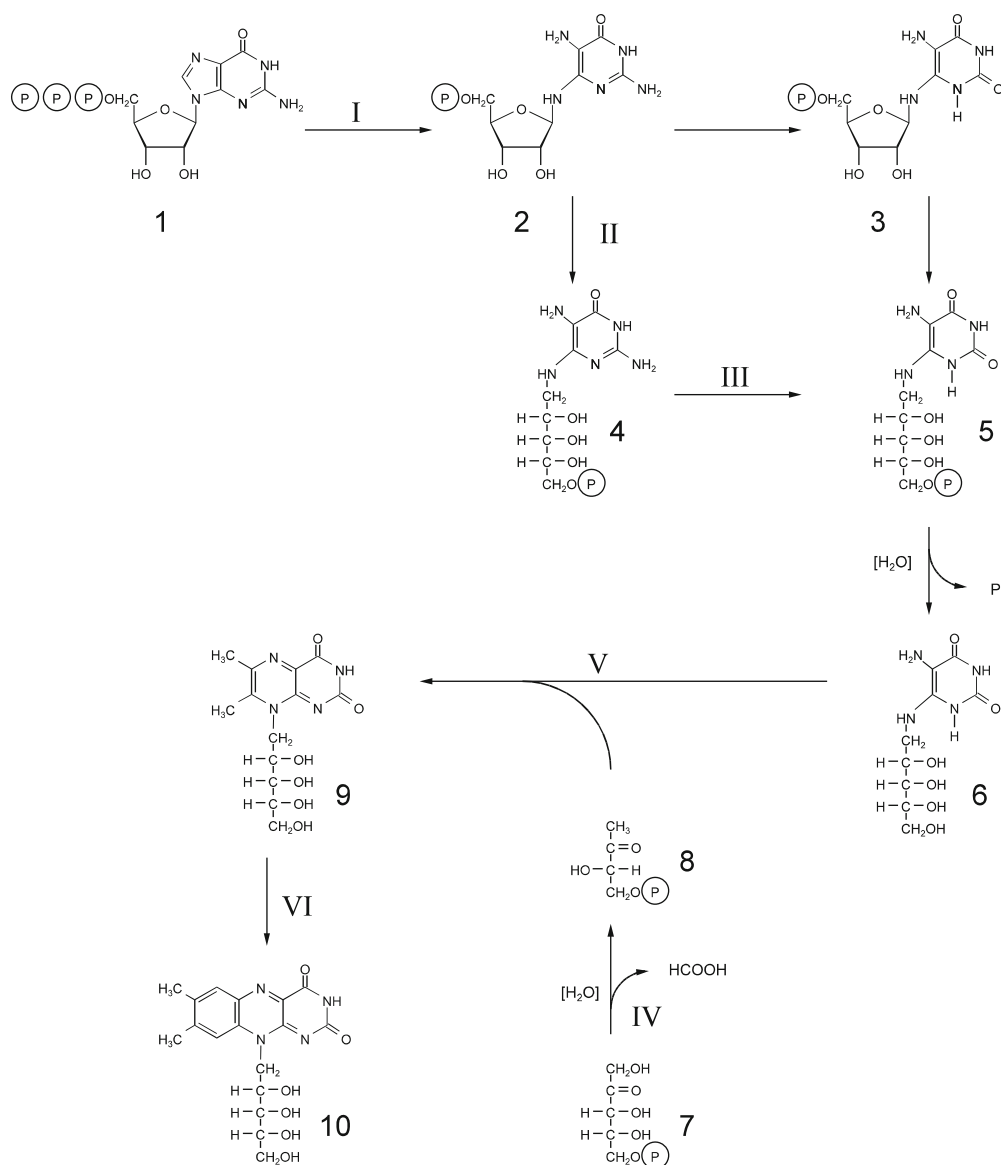


Fig. 11.1. Riboflavin biosynthesis pathway in *Ashbya gossypii* or bacteria where desamination takes place before reduction. An enzyme catalysing a dephosphorylation of ArPP is not characterized. Structures are adapted from Bacher (1991). 1: guanosine triphosphate; 2: 2,5-diamino-6-ribosylamino-4 (3H)-pyrimidinone-5'-phosphate; 3: 5-amino-6-ribosylamino-2,4 (1H,3H)-pyrimidinedione-5'-phosphate; 4: 2,5-diamino-6-ribitylamino-4 (3H)-pyri-

midinone-5'-phosphate; 5: 5-amino-6-ribitylamino-2,4 (1H,3H)-pyrimidinedione-5'-phosphate; 6: 5-amino-6-ribitylamino-2,4 (1H,3H)-pyrimidinedione; 7: ribulose-5'-phosphate; 8: 3,4-dihydroxy-2-butanone 4-phosphate; 9: 6,7-dimethyl-8-ribityllumazine; 10: riboflavin. I: Rib1p, GTP cyclohydrolase II; II: Rib7p, DARPP reductase; III: Rib2p, DArPP deaminase; IV: Rib3p, DHBP synthase; V: Rib4p, DRL synthase; VI: Rib5p, riboflavin synthase;

reactions is inverted in bacteria (Burrows and Brown 1978). Because the last step, formation of riboflavin, is a dismutation of two molecules of 6,7-dimethyl-8-ribityllumazine, in total two molecules of ribulose-5-phosphate and one molecule of guanosine triphosphate are needed. Therefore the

activity of 3,4-dihydroxy-2-butanone-4-phosphate synthase must be double in comparison to the other enzymes. Detailed insight into riboflavin biosynthesis and the catalytic mechanisms of the involved enzymes is described in comprehensive reviews (Fischer and Bacher 2005).

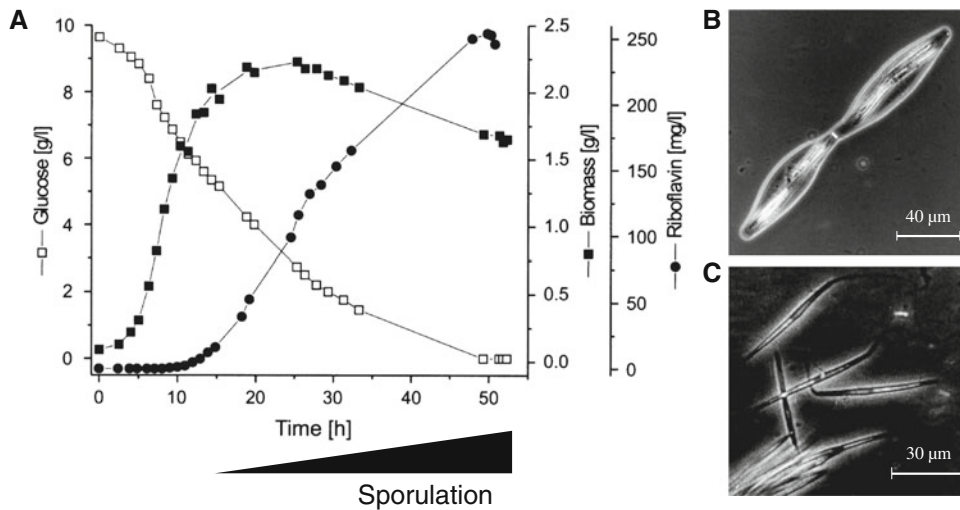


Fig. 11.2. Time course of growth and sporulation in a culture of the *A. gossypii* wild-type strain. Riboflavin production starts when the growth rate declines (A). A medium was

used where other nutrients but not glucose limited growth. Some cells differentiate into asci (B). They liberate spindle-shaped ascospores (C)

2. Isolation of *RIB* Genes

The yeast *Saccharomyces cerevisiae* played an important role in the genetic analysis of riboflavin biosynthesis. Mating between riboflavin-auxotrophic mutants obtained by random mutagenesis and the screening of haploid strains revealed six complementation groups (Oltmanns and Bacher 1972). The *S. cerevisiae* genes *RIB1* to *RIB5* and *RIB7* were then cloned by functional complementation of the respective auxotrophic mutants (Revuelta et al. 1994). The *RIB* genes of *A. gossypii* could not be identified based on the *S. cerevisiae* *RIB* sequence information because neither the genome of *S. cerevisiae* nor that of *A. gossypii* was available at that time. Functional expression of *A. gossypii* cDNA or genomic DNA libraries in the *S. cerevisiae* riboflavin auxotrophs allowed cloning of the *A. gossypii* *RIB* genes (Revuelta et al. 1995).

3. Regulation of Riboflavin Overproduction

Overproduction of riboflavin by *A. gossypii* cultivated on glucose as carbon and energy source was found to be linked to the formation of ascospores (Fig. 11.2). Supplementation of the medium with cAMP or non-metabolizable derivatives of cAMP arrested the fungus in the state of a vegetative mycelium without riboflavin overproduction. This phenomenon is an argument for a cAMP-

dependent signaling cascade controlling riboflavin overproduction (Schlösser et al. 2001). An independent approach to study regulation was performed by chemostatic cultivation. *A. gossypii* did not overproduce riboflavin at constant dilution rates. Dilution rate down-shifts causing nutritional stress resulted in sporulation of the culture and riboflavin overproduction. Increased *RIB3*, *RIB4*, and *RIB5*, but not *RIB2* and *RIB7*, mRNA levels were detected after the nutritional downshift (Schlösser et al. 2007). In batch culture, the DHBP synthase activity increased 50-fold when growth rate declined due to an increased transcription initiation, as revealed by promoter-reporter fusion experiments (Schlösser et al. 2001). Whereas the molecular mechanism of *rib* gene regulation in *A. gossypii* has still to be elucidated, glucose repression could be excluded (Schlösser et al. 2001).

Since the initiation of *RIB* gene expression in *A. gossypii* correlates with sporulation a linked regulatory mechanism for asci development and the induction of riboflavin overproduction can be suggested. *A. gossypii*, originally described as severe but today a negligible plant pathogen, was isolated from plant tissues like cotton balls (*Gossypium hirsutum*). They were reported to become yellowish (Batra 1973) probably due to pigmentation with riboflavin when infected by the fungus. Riboflavin can protect the hyaline *A. gossypii* spores against UV-induced damage (Stahmann et al. 2001). *A. gossypii* is unable to penetrate the epidermis of plant tissue and

therefore depends on insects with piercing-sucking mouthparts like *Dysdercus nigrofasciatus*. They take up and distribute the spores while they feed on the plants. A riboflavin pigmentation of *A. gossypii*-infected tissue, probably also taking place in citrus fruits (Dammer and Ravello 1990), might preferentially attract the insects to infected plants.

4. Classical and Molecular Tools for Strain Improvement

The wild type of *A. gossypii* isolated by Ashby and Novel (1926) is a natural overproducer of riboflavin, accumulating 100 mg g⁻¹ biomass under growth-limiting cultivation conditions (Schlosser et al. 2001). The selection of improved production strains is possible by visual inspection of colonies obtained by the cultivation of a randomly mutagenized *A. gossypii* population on agar plates. Submerged production of haploid monokaryotic spores, which can be easily harvested by extraction with lipophilic solvents, allows the generation of classical mutants at a high throughput.

Nowadays, a broad spectrum of molecular tools is available including plasmid transformation, replacement mutagenesis with dominant marker genes, restriction enzyme-mediated chromosomal integration, and transposon mutagenesis (Wright and Philippsen 1991). Replacement mutagenesis with a high site-specificity is possible with less than 50 bp homology regions flanking the DNA fragment, which has to be integrated into the genome (Wendland et al. 2000). Pop-out of dominant markers, e.g. the *kan* gene which provides geneticin resistance, is possible via short repetitive DNA sequences. This option allows a repeated use of the same marker for consecutive chromosomal DNA manipulations.

Today the *A. gossypii* genome sequence is publicly available (Gattiker et al. 2007). Previously, functional complementation of *Saccharomyces cerevisiae* mutants with *A. gossypii* genomic libraries was the method of choice to isolate genes. This was possible because introns are rare in the *A. gossypii* genome.

5. Engineering of Pathways

a) Riboflavin Pathway

Additional copies of *RIB3*, *RIB4*, and *RIB5* were integrated into the genome of *A. gossypii* (Althöfer et al. 1999) by use of a geneticin resistance cassette controlled by the *A. gossypii* *TEF* promoter (Steiner and Philippsen 1994). The original *RIB*

promoters caused more than a doubling in productivity when the itaconate-resistant mutant ItaGS-01 (Schmidt et al. 1996b) was used as the parental strain (Althöfer et al. 1999). Integration of additional copies of *RIB1*, *RIB2*, *RIB 4*, and *RIB7* into the genome of *A. gossypii* LU21 also resulted in a significant increase in riboflavin production (Althöfer et al. 2003). Flanking of the geneticin resistance gene by two *TEF* promoter elements facilitated a pop-out of the resistance marker. The marker-free strains fulfill the legal requirements for self-cloning strains according to EC Council Directive 98/81. The above-mentioned two examples are applications of restriction enzyme-mediated integration. Although successful, the method has the disadvantage of risking a disruption of the loci involved in product formation (Casas-Flores et al. 2004). Site-specific insertion of linear DNA by double cross-over into the *A. gossypii* genome is possible in *A. gossypii* (Steiner et al. 1995). The low frequency of homologous recombination needs larger screening efforts but provides strains with a defined molecular structure.

b) Riboflavin Precursor Supply

Purine and Glycine Metabolism

An important step in the purine biosynthesis pathway, phospho-ribosylamine synthesis catalyzed by phosphoribosyl-pyrophosphate amidotransferase, is negatively regulated at the transcriptional level by adenine in *A. gossypii*. For a deregulated expression of this amidotransferase in *A. gossypii*, the encoding *AgADE4* gene was functionally linked to the strong constitutive promoter of the glyceraldehyde 3-phosphate dehydrogenase gene. Furthermore a feed-back resistant amidotransferase mutein designed by three amino acid exchanges was constructed by site-directed mutagenesis. Overexpression of the mutein gene in the wild type of *A. gossypii* led to an increased riboflavin production in shaking flask cultivation (Jimenez et al. 2005).

Glycine supplementation is known to stimulate riboflavin overproduction by *A. gossypii* (Demain 1972), presumably by overcoming a metabolic limitation in de novo biosynthesis of the riboflavin precursor GTP (Fig. 11.3).

This idea is supported by the effect of the glycine analog aminomethyl phosphonic acid which did not increase, but competitively reduced riboflavin production (Monschau

et al. 1999), suggesting that glycine did not induce riboflavin production via a regulatory function, but alleviated as a substrate a possible glycine shortage. Overexpression of *GLY1* encoding a threonine aldolase increased riboflavin production only if the medium was supplemented with L-threonine. Since *A. gossypii* prefers uptake of L-threonine and excretion of glycine, the boosting effect exceeds simple supplementation of the medium with the precursor of purine biosynthesis (Fig. 11.4; Monschau et al. 1998).

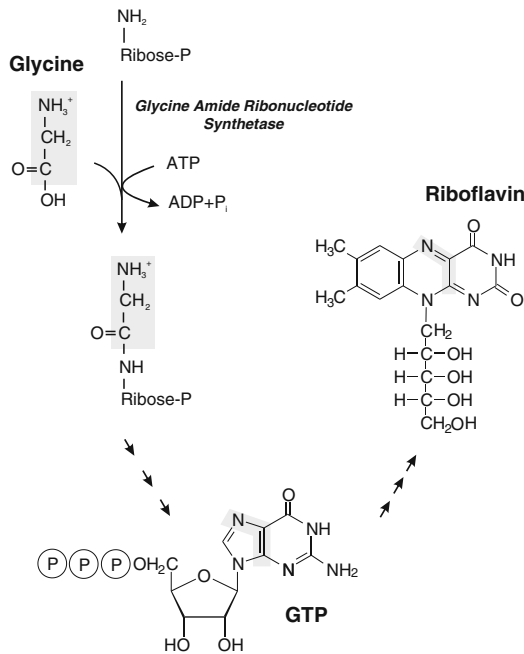


Fig. 11.3. Incorporation of glycine into the riboflavin biosynthesis pathway was shown by Plaut (1954)

Elevated glycine supply also explains the enhanced riboflavin overproduction of *SHM2*-disrupted *A. gossypii* mutants. *SHM2* encodes the cytosolic serine hydroxymethyltransferase catalyzing the conversion of glycine into serine by consumption of methylene tetrahydrofolate (Schlüpen et al. 2003). In vivo ^{13}C -labeling experiments confirmed that this cytosolic serine hydroxymethyltransferase was a C-1 compound producer during growth but a glycine-consuming enzyme during the riboflavin production phase (Fig. 11.4).

Isocitrate Lyase and Isocitrate Dehydrogenase

Since technical production uses plant triglycerids as carbon and energy source, the glyoxylate pathway came under consideration as a flux-limiting bottleneck. Indeed, *A. gossypii* mutants resistant to inhibitors of isocitrate lyase, e.g. itaconate, showed increased riboflavin production (Schmidt et al. 1996a, b). Isocitrate lyase and malate synthase constitute the anaplerotic glyoxylate shunt providing malate from acetyl-CoA, which is derived e.g. from β -oxidation of fatty acids.

The genes of both enzymes are induced upon glucose depletion (Maeting et al. 1999) and assimilation of the intracellular reserve lipids (Fig. 11.5; Stahmann et al. 1994) or upon growth on plant lipids. The mutation leading to itaconate resistance increased isocitrate lyase-specific activity (Schmidt et al. 1996b), probably favoring anaplerosis but also 3,4-dihydroxy-2-butanone 4-phos-

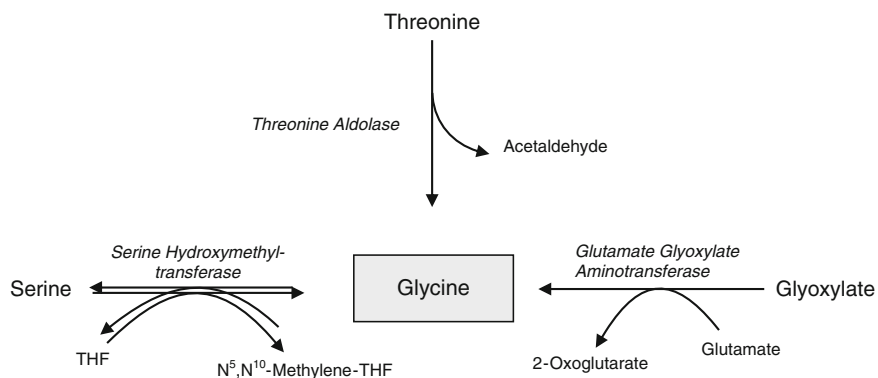


Fig. 11.4. Glycine pathways in *Ashbya gossypii*. Glycine, a flux limiting precursor of riboflavin, can be produced by at least three pathways. For the threonine aldolase pathway, enzyme activity and the corresponding gene (*GLY1*) were investigated. Evidence for the existence of a serine hydroxymethyltransferase and a glyoxylate aminotransferase

using glutamate as amino donor was given by enzyme activity assays with crude extracts. Increased riboflavin production of a mutant disrupted in *SHM2*, encoding the cytosolic serine hydroxymethyltransferase, was explained by a reduced flux from glycine to L-serine

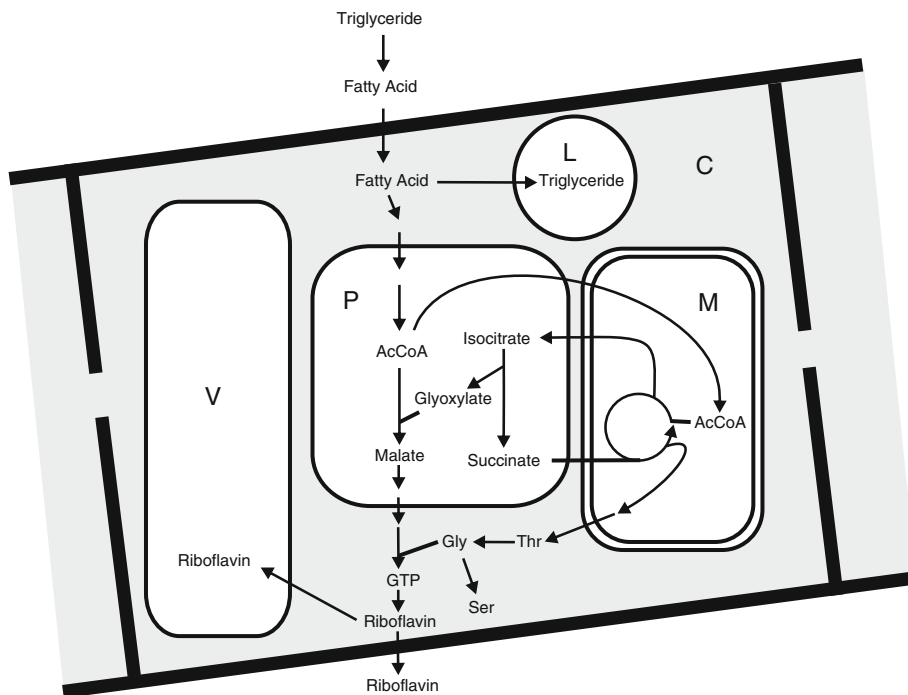


Fig. 11.5. Metabolic model of pathways relevant for riboflavin formation in a cell of the mycelial fungus *Ashbya gossypii*. Subcellular compartments: C cytosol, L lipid

droplet, V vacuole, P peroxisome, M mitochondrion. Thr Threonine, Gly glycine, GTP guanosine triphosphate, AcCoA acetyl coenzyme A

phate synthase specific activity, was significantly increased (Schlösser et al. 2001). The itaconate resistance might be the result of a pleiotropic mutation, affecting up-regulation of both the glyoxylate shunt and the riboflavin pathway. A first transcription factor identified to have an impact on riboflavin production was AgBAS1. A truncation of amino acids 632–743 led to an enhanced productivity but avoided an auxotrophy for adenine, which was seen when the gene was replaced with a dominant marker (Mateos et al. 2006).

Isocitrate lyase and malate synthase are transported into the peroxisomes of *A. gossypii* (Maeting et al. 1999) an organelle in which β -oxidation takes place (Maeting et al. 2000). Interestingly, a knock-out of the gene encoding a peroxisomal isocitrate dehydrogenase led to a reduced production of riboflavin. Overexpression of the gene using the constitutive *TEF* promoter led to an increase in riboflavin production. A higher activity of the peroxisomal NADP-dependent isocitrate dehydrogenase may lift the intracellular levels of NADPH, which in turn might be limiting in *A. gossypii* cells assimilating unsaturated fatty acids liberated from plant oils (Althöfer et al. 2001).

6. Riboflavin Excretion and Vacuolar Accumulation

Kinetic studies of riboflavin influx and efflux with riboflavin auxotrophic or overproducing *A. gossypii* mutants indicated the presence of active riboflavin transport systems (Förster et al. 2001). Inhibition of riboflavin uptake by extracellular FMN or FAD led to an increase in the apparent riboflavin efflux during the early production phase, indicating the presence of a separate excretion carrier.

A. gossypii transports the yellow pigment not only into the cultivation medium. A significant amount is transported into the vacuole and is accumulated there so that crystals can be formed. Disruption of *VMA1* encoding a subunit of the vacuolar ATPase did not interfere with the viability of *A. gossypii*, but no vacuolar riboflavin was detectable and instead all was excreted into the culture medium (Förster et al. 1999).

7. Production and Downstream Processing

The industrial riboflavin production process utilizes soybean oil and soybean meal as carbon and

energy source. Since these are present in the fermentation broth as osmotically inactive emulsified lipid droplets or solid particles a high nutrient load is possible. *A. gossypii* secretes a 35-kDa lipase (Stahmann et al. 1997) hydrolyzing the triglycerides in the culture medium into free fatty acids, which are then ingested by the fungus. In stirred or shaken systems the lipase is inactivated within minutes due to aggregation at the gas/water and lipid/water interfaces. Furthermore, elevated concentrations of free fatty acids interfere with lipase production (Stahmann et al. 1997).

Riboflavin-overproducing cells accumulate significant amounts of the product as intracellular crystals. A heating step after completion of the fermentation run induces self-lysis and liberates riboflavin from the biomass (Kurth 1992). Heating of the fermentation broth and slow cooling over several hours promote the formation of riboflavin crystals in the broth, facilitating separation of the crystals from the supernatant by decantation (Faust et al. 1991). A partial purification is carried out by resuspension of the riboflavin-containing precipitate in diluted acid, heating, and decantation (Grimmer et al. 1992). Since *A. gossypii* production strains with increased copy numbers of homologous genes are classified as self-clones, remains of the production organism in the feed application product are tolerated.

B. *Candida famata*

Ten years ago *Candida famata*, considered synonymous to *Candida flareri* by the ATCC, was one of two fungi applied for riboflavin production (Stahmann et al. 2000).

Production strains, improved by COORS (USA) and afterwards used by ADM (USA), were described to produce more than 20 g l⁻¹ riboflavin (Heefner 1988). *C. famata* was one of the organisms used for early studies of riboflavin biosynthesis (Audley and Goodwin 1962). A special feature is that it can grow on *n*-alkanes, allowing the composition of a defined medium with biotin for riboflavin production (Olczyk 1978).

Like *A. gossypii* this fungus is also a natural riboflavin overproducer. It does not grow with hyphae causing high viscosity but grows as a yeast, and therefore it has advantages in stirred vessel cultivation concerning gas exchange. *A. gossypii* and *C. famata* have in common that

their overproduction of riboflavin, as found in wild-type strains, can be enhanced by supplementation of the medium with glycine (Heefner 1988).

Probably the regulation of the *RIB* genes is negatively affected by iron ions in *C. famata*. Improvement of production was observed with mutants resistant to increased concentrations of iron (Heefner et al. 1988). But keeping the iron concentration in the culture medium below 15 μM to prevent its negative effect needs an extra effort. Unfortunately, the iron effect has not been studied on a physiological or molecular level yet.

Mutants of *C. famata* resistant to 2-deoxyglucose (Heefner et al. 1993) are probably mutated in a transcription factor that has an impact on riboflavin biosynthesis genes. The very first step in metabolism, i.e. uptake of the carbon source, as well as glucose repression mechanisms are the targets of this antimetabolite.

Antimetabolites were reported to be useful in the selection of mutants with improved riboflavin production. Tubercidin, an inhibitor of purine biosynthesis, is an example found in patent literature (Heefner et al. 1992). A transformation system was established for *C. famata* using a *leu2* mutant as recipient and the *LEU2* gene of *Saccharomyces cerevisiae* as marker (Voronovsky et al. 2002). The *C. famata* *RIB* genes were identified by functional complementation of *C. famata* *RIB* mutants. The genes were also found to be functional in *Pichia guilliermondii* auxotrophs (Dmytruk et al. 2004). An interesting product for the future might be FMN, which was found to be overproduced when the flavin kinase gene was overexpressed (Yatsyshyn et al. 2009).

III. Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs) are vitamin-like compounds with structural function in cell membranes and lipid tissue. They serve as precursors for the biosynthesis of hormones like leucotrienes, prostaglandins, and thromboxanes. PUFAs are aliphatic monocarboxylic acids with two to six double bonds in a biologically active *cis*-configuration within their C₁₈- to C₂₄-backbone. They are categorized as ω-3 or ω-6, depending on the position of the first double bond, counting from the terminal carbon atom of the fatty acid. Their biosynthesis starts from oleic acid (18:1) and is continued by a sequence of desaturase and elongase reactions (Fig. 11.6; Marszalek and Lodish 2005). Since humans cannot introduce double bonds at the ω-3 or ω-6 position, linoleic

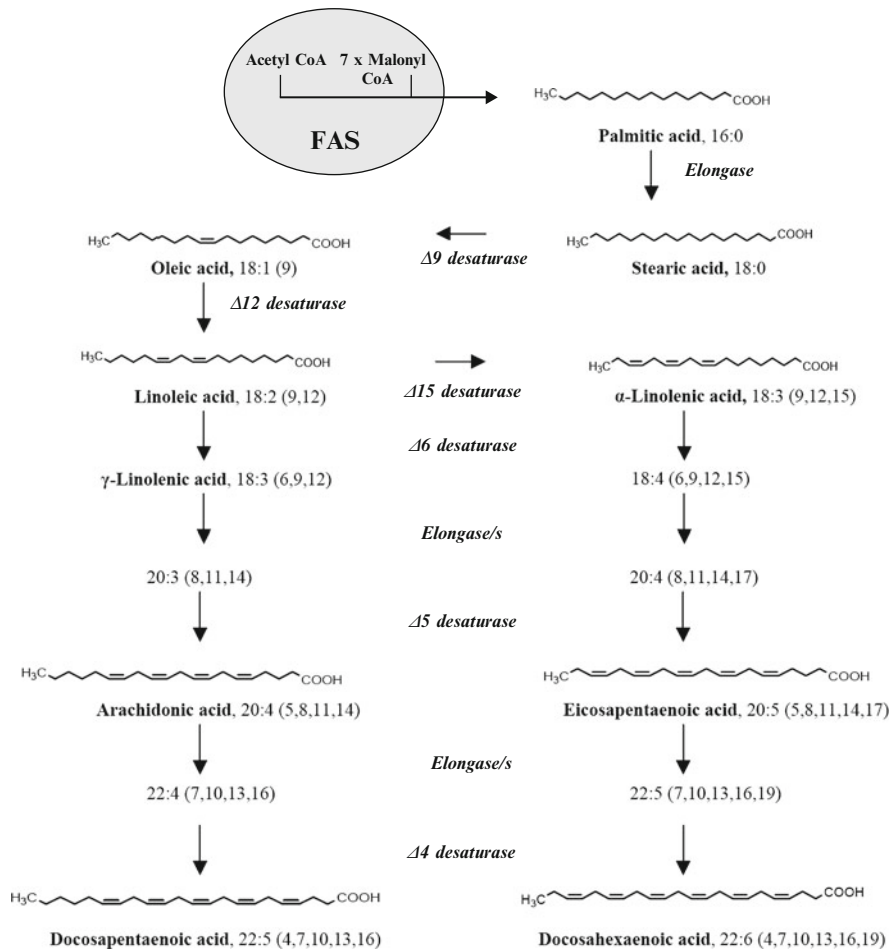


Fig. 11.6. Eukaryotic biosynthesis of fatty acids starts with fatty acid synthetase (FAS) in the cytosol (Ratledge 2004). Parallel elongation and desaturation steps take place in the membranes of the endoplasmic reticulum

acid (18:2, ω -6) and α -linolenic acid (18:3, ω -3) are essential. They belong to the vitamin F group.

Arachidonic acid (20:4, ω -6; ARA) and docosahexaenoic acid (22:6, ω -3; DHA) are not essential but of commercial interest. They are needed for the developing brain, neuronal tissue, and retina of the human fetus and are therefore contained in human breast milk. Studies on supplemented infant formula suggested a correlation between PUFA content in the cerebral cortex of breast-fed infants and better cognitive and visual functions of infants fed with infant formula supplemented with long-chain PUFAs (Heird and Lapillonne 2005).

For DHA and ARA, commercial-scale processes based on natural isolates or classically derived microbial strains are available. Attempts to employ molecular engineering techniques in PUFA production were successful. When

Saccharomyces cerevisiae, not a natural PUFA producer, was provided with the appropriate desaturase and elongase genes one or several biosynthetic steps towards eicosapentaenoic acid (20:5, ω -3; EPA) or DHA starting from the corresponding precursor fatty acids were possible (Parker-Barnes et al. 2000). A series of patent applications from DuPont published in 2004 and 2005 disclosed EPA production in the genetically modified oleogenic yeast *Yarrowia lipolytica*.

DHA is found in phospholipids of different cell types, e.g. in the photosensitive part of the retina DHA, accounts for more than 60% of the total fatty acid content. Functioning as signal molecule precursor, beneficial effects of DHA on the prevention of cardiovascular diseases, probably by down-regulation of the intracellular mecha-

nism leading to the expression of pro-atherogenic genes, are well documented. Fish is the most important source of DHA in human nutrition. It cannot synthesize DHA by itself but marine microorganisms at the lower end of the food chain are the genuine DHA producers. In view of the over-exploitation of marine fish resulting in ever-reducing catch size, fish oil is not considered a sustainable source for DHA so that microbial alternatives are needed.

Cryptocodinium cohnii, a marine microalga, consists of triglycerides with 30% DHA. In flask cultivations with ethanol as the carbon source 83 g l⁻¹ biomass accumulated with a lipid triglyceride content between 30% and 50% (Swaaf et al. 2003). The genera *Schizochytrium*, *Thraustochytrium*, and *Ulkenia*, marine heterotroph protists that are classified as Labyrinthulida, are preferred sources of DHA. In laboratory-scale fermentations with a *Schizochytrium* sp. strain high cell densities of 63 g l⁻¹ dry biomass were obtained with a fatty acid content of 70%, of which 37% were DHA (Ganuza et al. 2008). DHA-containing products acquired from protist fermentations are offered by Martek Biosciences and Advanced BioNutrition (both USA) focusing on the human nutrition and aquaculture market, respectively.

ARA, also a natural component of breast milk, supports neonatal eye and brain development (Alessandri et al. 2004). Breast-fed infants have higher ARA blood levels than formula-fed infants. Infant formulas fortified with ARA and DHA have been sold in Europe for more than 10 years. After permission by the FDA, they were introduced in the United States in 2002.

In contrast to DHA, which can be obtained from fish oil or microbial processes, industrial ARA production solely relies on fermentation. First publications about the mycelial fungus *Mortierella alpina* as ARA producer appeared in the late 1980s.

A. *Mortierella alpina*

Mortierella alpina, a filamentous zygomycete, produces lipids rich in ARA when grown at 18 °C (Lindberg and Molin 1993). Its presense in the spores and preferred degradation during their germination suggests a role as a store for carbon and energy (Lounds et al. 2007). The pattern of the fatty acids produced could be influenced by the addition of a specific delta 5-fatty acid desaturase inhibitor, i.e. dioxabicyclo [3.3.0] octane derivatives

like sesamin, or a selection of mutants with absent or reduced delta 5-fatty acid desaturase activity (Kawashima et al. 1992).

A number of *M. alpina* genes encoding enzymes, which are involved in the synthesis of polyunsaturated fatty acids, were cloned, e.g. delta 5-fatty acid (Knutzon et al. 1998) delta 6-fatty acid (Sakuradani et al. 1999a), delta 9-fatty acid (Sakuradani et al. 1999b), and delta 12-fatty acid (Huang et al. 1999) desaturases. Convincingly, verification of the genes functionality by expression, e.g. in *Aspergillus oryzae*, resulted in a shift to more than 70% of linoleic acid (18:2, ω-6) in the cell's fatty acid composition (Sakuradani et al. 1999c). Two transformation systems were established, one working with uracil auxotrophs (Takeno et al. 2004) and a second using zeocin as dominant marker (Takeno et al. 2005). By use of the latter, ARA production was increased after transformation with a gene encoding a rate-limiting elongase (Takeno et al. 2005). More details concerning breeding of *M. alpina* is presented by a recent mini-review (Sakuradani et al. 2009).

The accumulation of lipid starts in *M. alpina* cells when growth becomes limited, e.g. after consumption of the nitrogen source, and depends on the high activity of malic enzyme needed for NADPH supply in fatty acid biosynthesis (Zhang and Ratledge 2008). The highest titers with glucose as carbon source reported so far are 20 g l⁻¹ of PUFAs, with up to 70% ARA produced by *M. alpina* mutants (Sakuradani et al. 2009).

Since the production of biodiesel today makes glycerol available at low costs, strains were screened to use this carbon source for ARA production (Hou 2008). The morphology of the fungal mycelium in the reactor is of specific concern as only small pellets with a diameter of 1–2 mm allow sufficient mass transfer and keep the broth viscosity at an acceptable level. After fermentation the mycelium is harvested by filtration, dried, and solvent-extracted. Alternatively, the mycelium is homogenized as an aqueous suspension and extracted with a water-immiscible organic solvent. Safety of *M. alpina* concerning its products in food was investigated more than a decade ago (Streekstra 1997) and recently ARA-containing biomass in rats (Nisha et al. 2009).

Fungal ARA is produced and marketed as ARASCO by Martek Biosciences (USA). In 2004 Cargill (USA) entered into a joint venture with Wuhan Alking Bioengineering Co. Ltd (China), an ARA producer serving the Chinese infant formula business. Suntory, a leading Japanese beverage manufacturer and a pioneer in microbial PUFA production, provides a fungal ARA oil under the trade name SUNTGA.

IV. Conclusions

Mineral oil-based chemical riboflavin production, successfully used for half a century, has been completely replaced by two microbial processes for almost a decade. These save half of the costs, reduce waste and energy requirements, and use renewable resources like sugars or plant oils. A major point is that more than 100 biochemical conversions take place in a single cell and these work in a single vessel. A complex multi-step plant was replaced by a single-step process. The key to this remarkable success were decades of strain development and improvement. A combination of selection after random mutagenesis and genetic engineering after rational design resulted in strains not only improved by “removal of a bottle-neck” but by generation of a “network of anabolic highways” and a deletion of unwished reaction. A sensible use of “high-tech” like NMR or genome sequencing has allowed phenomena to be explained, but simple techniques are sometimes more powerful, like a comparison of the yellow color of different strains on agar plates allowing the prediction of which one will be better in the 100 m³ vessel.

The coexistence of a fast bacterial process using *Bacillus subtilis* and a much slower fungal process applying *A. gossypii* can mean that both are much more efficient than chemistry and not under pressure now because the market is growing and the selling price is high. This is probably true for both riboflavin and ARA, because they are needed in small dosages. The design of both *A. gossypii* and *M. alpina* strains is not at an end. Each step has improving potential and some steps are not known at all.

An open point is the transport of riboflavin and its precursors across membranes. Neither the transporter accumulating the pigment in the vacuole nor the carrier excreting it against a concentration gradient are known. Also little is known about the facilitation of shuttle processes in the cell, e.g. between peroxisomes and mitochondria.

A problem in studying fatty acid biosynthesis is the lack of in vitro assays for desaturases and elongases which are probably working on membranes. Overexpression and in vivo data do help indirectly but determination of specific activities would shift the research on the most relevant level for their direct comparison.

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12 Fungal Flavours

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I. Introduction

The formation of volatile flavours belongs to the most remarkable and exciting properties of fungi. Human interest and fantasy have been stimulated by aroma compounds from fruiting bodies since ancient times, and unique aroma impression are often reflected in Latin genus or species names. Attributes like *butyrace-* (butter-like), *odor-/osm-* (fragrant), *delicat-* (delicious), *olid-* (ambrosial), *suav-* (sweet), or *nidoros-* (pungent) point to specific aroma characteristics. Additionally, trivial names often give hints to aromatic, spicy, or floral flavour impressions in many different languages. Distinctive odours have long been and are still used as taxonomic markers for mushroom species (Larsen 1998). The pleasant aroma, along with further nutritional benefits, has stimulated the world-wide industrial production of various edible fungi. Prominent species include e.g. *Agaricus bisporus*, *A. arvensis*, *Agrocybe aegerita*, *Auricularia auricula-judae*, *Coprinus cornatus*, *Flammulina velutipes*, *Grifola frondosa*, *Hericium erinaceus*, *Lentinula edodes*, *Lepista nuda*, *Pholiota nameko*, *Pleurotus eryngii*, *P. ostreatus*, *P. salmoneostramineus*, *P. citrinopileatus*, *Hypsizygus ulmarius*, *H. tessulatus*, and *Stropharia rugosannulata*. In one sense, this represents an enormous agro-biotechnological production of fungal flavours (see also Chapter 4).

Only sophisticated analyses and comprehensive knowledge on the respective flavour profile allow for an efficient optimisation of the substrate composition and the growth conditions. The volatile compounds emitted by the fruiting bodies of basidiomycetes are usually analysed by gas

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chromatography coupled to mass spectrometry (GC/MS) or by GC-olfactometry. GC-olfactometry requires dynamic headspace (DHS) concentration through cryotrapping, solvent extraction, simultaneous distillation, and solvent extraction (SDE), or solid phase microextraction (SPME).

For a number of important savoury fungi, adequate industrial production systems are still not available. For example, the highly appreciated mycorrhizal fungi *Tuber aestivum* (summer truffle) or *T. melanosporum* (black truffle), various wild *Boletus* species, and *Cantharellus cibarius* (chanterelle) are collected from natural habitats. Their aroma directly determines the market prices, and thus their economic value.

Based on their respective flavour profiles, edible mushrooms may be divided into three groups. Carbon-eight (C₈) derivatives, like oct-1-en-3-ol, oct-2-en-1-ol, octan-3-ol, octan-1-ol, oct-1-en-3-one, and octan-3-one impart the “characteristic mushroom flavour” and dominate the volatiles’ profile of the first group (Cronin and Ward 1971; Pyysalo and Suihko 1976; Fischer and Grosch 1987; Venkateshwarlu et al. 1999). Fungi rich in terpenoid volatiles account for the second group, and the last one is rich in sulfur-containing odour compounds.

This chapter reviews current knowledge on flavours emitted from fruiting bodies as well as on volatiles formed in liquid culture systems. As a number of excellent reviews are available on the biotransformation of external administered substrates (e.g. Cheetham et al. 1993; Berger 1995; Berger et al. 2002), this chapter focuses on the de novo biosynthesis of volatile flavour compounds. To avoid redundancies, a strong focus is made on publications since 1990. For extensive summaries of earlier work on fungal flavours, see Maga (1981), K nderlerer (1989), Berger et al. (1992), Janssens et al. (1992), and Mau et al. (1994). Further, this chapter reviews the deliberate generation of volatile flavour compounds by asco- and basidiomycetous fungi; yeasts have not been considered.

II. Volatile Flavours Emitted from Fruiting Bodies

A. Volatiles from Sporocarps Emitted by Basidiomycetous Fungi

1. *Agaricus bisporus*

a) General Remarks

The well known edible basidiomycete *Agaricus bisporus* (white button mushroom) belongs to

the most often cultivated and consumed mushroom species in the world. *A. bisporus* natively grows as a litter-decomposer on grasslands and pastures in Europe and North America, and is cultivated in more than 70 countries on special compost materials. The volatile constituents from *A. bisporus* have been widely studied (Le Loch-Bonazzi and Wolff 1991). As for most other mushroom species, the characteristic flavour is attributed mainly to C₈ volatiles. The concentration of C₈ compounds relative to the total volatiles varies widely (44–98%; Mau et al. 1994). This might be due to differences between mushroom types, production or post-harvest conditions, or caused by different analytical approaches. Among the C₈ compounds, oct-1-en-3-ol represents the most important volatile in *A. bisporus* (Cronin and Ward 1971). In recent years, headspace samples (SDE) and liquid-liquid extracts of *A. bisporus* were investigated by means of GC/FID, GC/FTIR/MS, and GC/O (Buchbauer et al. 1993). Because of different vapour pressures, C₈ concentrations obtained via headspace (HS) techniques typically differ from those resulting from SDE. In total, over 150 different volatiles have been identified in the button mushroom (Le Loch-Bonazzi and Wolff 1991).

The flavour profiles were compared to those of other common edible mushrooms, i.e. *A. campestris* (meadow mushroom), *Lepiota procera* (parasol mushroom), *Armillaria mellea* (honey mushroom), *Boletus edulis* (king bolete), and *Cantharellus cibarius* (chanterelle). Over 70 flavour compounds were identified in these species, and oct-1-en-3-ol was found to be crucial for the overall flavour impression of the investigated mushrooms (Buchbauer et al. 1993). Further C₈ derivatives contributed significantly to the odour of most of the samples as well. These findings were confirmed by Venkateshwarlu et al. (1999): 22 different volatiles were tentatively identified by means of SDE and GC/FID/O. Again, the most abundant compound was oct-1-en-3-ol. Other predominant components were octan-3-ol, octan-3-one, and oct-2-en-1-ol (Venkateshwarlu et al. 1999). Pelusio et al. (1995) noted a pronounced difference between the concentrations of oct-1-en-3-ol and octan-3-one in the cap and the stalk of *A. bisporus*. While octan-3-one was the predominant C₈ volatile in the caps, oct-1-en-3-ol dominated in the stalks. In contrast, more oct-1-en-3-ol was produced in the cap and gills than in the stipe when the mushrooms samples were incubated at room temperature for 5 min after homogenisation with an Ultra-Turrax (Wurzenberger and Grosch 1983; Mau et al. 1992).

Next to C₈ volatiles, aromatic compounds like benzyl alcohol, benzaldehyde, *p*-anisaldehyde (4-methoxybenzaldehyde), benzyl acetate, and phenyl ethanol play an important role in the

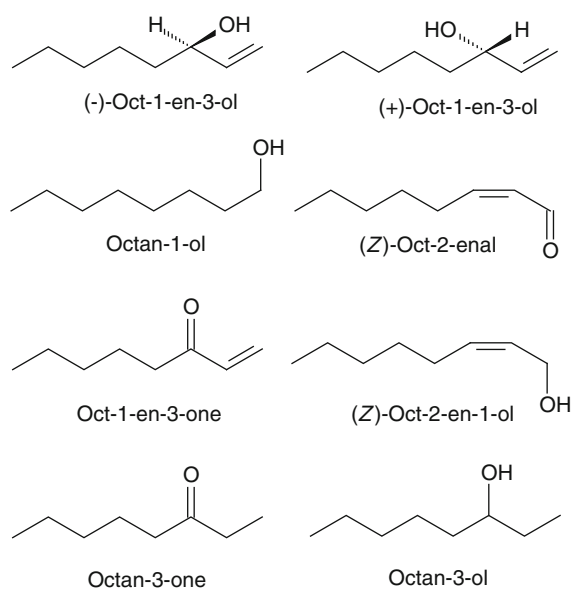


Fig. 12.1. Common C₈ compounds contributing to the characteristic “mushroom flavour”

flavour of *A. bisporus* (Buchbauer et al. 1993; Venkateshwarlu et al. 1999).

b) Enzymatic Pathways of C₈ Volatiles in Fungi
 As discussed above, volatile C₈ compounds like octan-1-ol, octan-3-ol, octan-3-one, oct-1-en-3-ol, oct-2-en-1-ol, and oct-1-en-3-one (Maga 1981; Mau et al. 1994; Chiron and Michelot 2005; Fig. 12.1) are of utmost importance for fungal flavour profiles. These C₈ flavour compounds are found ubiquitous in the fungal kingdom and are formed by oxidation and subsequent cleavage of poly-unsaturated fatty acids. Oct-1-en-3-ol, together with oct-1-en-3-one, is supposed to be the most important fungal flavour (Dijkstra and Wikén 1976; Pyysalo and Suihko 1976; Maga 1981; Fischer and Grosch 1987). Oct-1-en-3-ol was first isolated as the predominant volatile of *Tricholoma matsutake*, and therefore was called matsutake alcohol (Murahashi 1936). Later, it was structurally identified as oct-1-en-3-ol (Murahashi 1938).

Since then, oct-1-en-3-ol has been found in several non-fungal sources, e.g. black currants (Andersson and von Sydow 1964), cranberries (Anjou and von Sydow 1967), and potatoes (Nursten and Sheen 1974). In dairy products, oct-1-en-3-ol was identified as a mushroom off-flavour resulting from lipid oxidation (Stark and Forss 1962). The chiral oct-1-en-3-ol occurs in two optically active forms. The naturally predominant (*R*)-(-)-oct-1-en-3-ol possesses a fruity mushroom-like and more intense odour than its

(*S*)-(+)-isomer, which exhibits a mouldy, grassy note (Dijkstra and Wikén 1976; Mosandl et al. 1986). Despite significant differences in the oct-1-en-3-ol concentrations, the optical purity of (*R*)-(-)-oct-1-en-3-ol of several analysed basidiomycetous fungi was very high. In *A. bisporus* 99% (*R*)-(-)-oct-1-en-3-ol were found; *Xerocomus badius* (also known as *Boletus badius*) showed with 82% the lowest optical purity (Zawirska-Wojtasiak 2004).

The odour impressions of racemic alcohols with the general formula R-CH(OH)-CH=CH₂, with R varying from methyl to pentyl were evaluated. Beside oct-1-en-3-ol, only hept-1-en-3-ol exhibited a faint smell of mushrooms. Additionally, a weak mushroom flavour was noted for octan-3-ol (Ney and Freytag 1978). The odour of oct-1-en-3-one was described as “boiled mushroom” with a metallic impression at higher concentrations. During heating, oct-1-en-3-ol was converted into its corresponding ketone and is likely responsible for the change of the flavour impression of *A. bisporus* (Picardi and Issenberg 1973).

Fatty acids as the assumed direct precursors of C₈ flavours are key components in a variety of lipids (Combet et al. 2006). The respective lipid composition varies with the species, the age of the culture, and the culture conditions (Sumner 1973). A comparison of the lipid composition of fruiting bodies and mycelia of *A. bisporus* revealed quantitative, but no significant qualitative differences between the two culture systems (Byrne and Brennan 1975). The oxidation of fatty acids provides mammals, plants, and fungi with a wide pool of biologically active compounds (Hamberg and Gardner 1992; Hamberg 1993; Blée 2002).

In fungi, these so-called oxylipins include fatty acid hydroperoxides as well as their breakdown products, the C₈ volatiles. Oxylipins are involved in a broad range of biological processes, such as reproduction, growth, and pathogen interactions (Combet et al. 2006). For example, mushrooms emit C₈ volatiles to attract flies and mosquitoes to help distributing their spores (Chiron and Michelot 2005). Oct-1-en-3-ol is discussed as an attractant to tsetse flies (*Glossina palidipes*, *G. morsitans*), and acts as an aggregation hormone for certain beetles (Hall et al. 1984; Pierce et al. 1989; Fäldt et al. 1999). The same reason may be responsible for the increase of 1-octen-3-ol and/or octan-3-one levels of two polypores (*Fomitopsis pinicola*, *Fomes fomentarius*) during their sporulation phase. Production of C₈ volatiles was also observed with ascomycetous *Aspergillus* and *Penicillium* species (Börjesson et al. 1993), and oct-1-en-3-ol was described as an inhibitor of *Penicillium paneium* and *Penicillium expansum* (Okull et al. 2003; Chitarra et al. 2004). Furthermore, it was suggested to act as a fungal hormone (Chitarra et al. 2005).

The enzymatic cleavage of fatty acids via hydroperoxides and the subsequent formation of short-chain volatiles were observed in a wide range of organisms. Early evidence of such a hydroperoxide cleavage system was found e.g. in cucumbers (Wardale et al. 1978), tomatoes (Galliard and Matthew 1977), banana (Tressl and Drawert 1973), and tea (Hatanaka et al. 1977). In the late 1970s, it was suggested that oct-1-en-3-ol was formed in *A. bisporus* from linoleic acid (18:2, $\Delta^{9,12}$) by oxidation, followed by an allylic rearrangement (Varoquaux et al. 1977). Linoleic acid accounts for 63–74% of the fatty acids in *A. bisporus* (Holtz and Schisler 1971; Proštenik et al. 1978) and thus is a readily available substrate for enzymatic transformations. It is not yet known whether linoleic acid is required to be present in the cell as a free fatty acid, or if the oxidising enzyme targets esterified fatty acids. If linoleic acid had to be released prior to the oxidation reaction, lipases would play an important role in the substrate's bioavailability (Combet et al. 2006).

In green beans, linoleic acid was identified as the precursor of oct-1-en-3-ol. Next to oct-1-en-3-ol, small amounts of oct-1-en-3-one were found. The latter was easily reduced to oct-1-en-3-ol by homogenates of whole green beans and mushrooms but it was not possible to reverse this reaction. The mechanism for the cleavage of lino-

leic acid and the formation of oct-1-en-3-ol was not established (de Lumen et al. 1978).

Tressl et al. (1981) proposed a three-step enzymatic pathway for oct-1-en-3-ol formation in *A. bisporus* (Fig. 12.2). For the initial step, the authors assumed the involvement of a lipoxygenase. Lipoxygenases catalyse the incorporation of two atoms of oxygen into polyunsaturated fatty acids providing (*Z,Z*)-penta-1,4-dien structures. In plants, the heterolytic cleavage of linoleic acid generally proceeds via the corresponding 9- and 13-hydroperoxides (Combet et al. 2006). In analogy, Tressl et al. (1981) suggested a lipoxygenase catalysed formation of (9*Z*,11*E*)-13-hydroperoxyoctadeca-9,11-dienoic acid (13-HPOD) and (10*E*,12*Z*)-9-hydroperoxyoctadeca-10,12-dienoic acid (9-HPOD) in the presence of oxygen. In a subsequent step, a hydroperoxide lyase would catalyse the formation of oct-1-en-3-one and 10-oxodecenoic acid (10-ODA) from 13-HPOD. Analogously, 9-HPOD could be transformed to oct-2-enal and 10-oxodecanoic acid. The last step involved the reduction of oct-1-en-3-one to oct-1-en-3-ol and the reduction of oct-2-enal to (*Z*)-oct-2-en-1-ol by an alcohol oxidoreductase. Several further C_8 and C_{10} compounds were found as cleavage products (Tressl et al. 1982). The incubation of ^{14}C -labelled linoleic acid and 13-HPOD with homogenates of *A. bisporus* proved that linoleic acid was cleaved into oct-1-en-3-ol and (*E*)-10-oxodec-8-enoic acid (Wurzenberger and Grosch 1982), as previously observed by Tressl et al. (1982). In contrast, the suggested intermediate 13-HPOD was not transformed to oct-1-en-3-one nor to oct-1-en-3-ol. Only to the corresponding hydroxy fatty acid was found when incubated with mushroom homogenate. As a consequence thereof, Wurzenberger and Grosch (1982) concluded that the cleavage of linoleic acid in

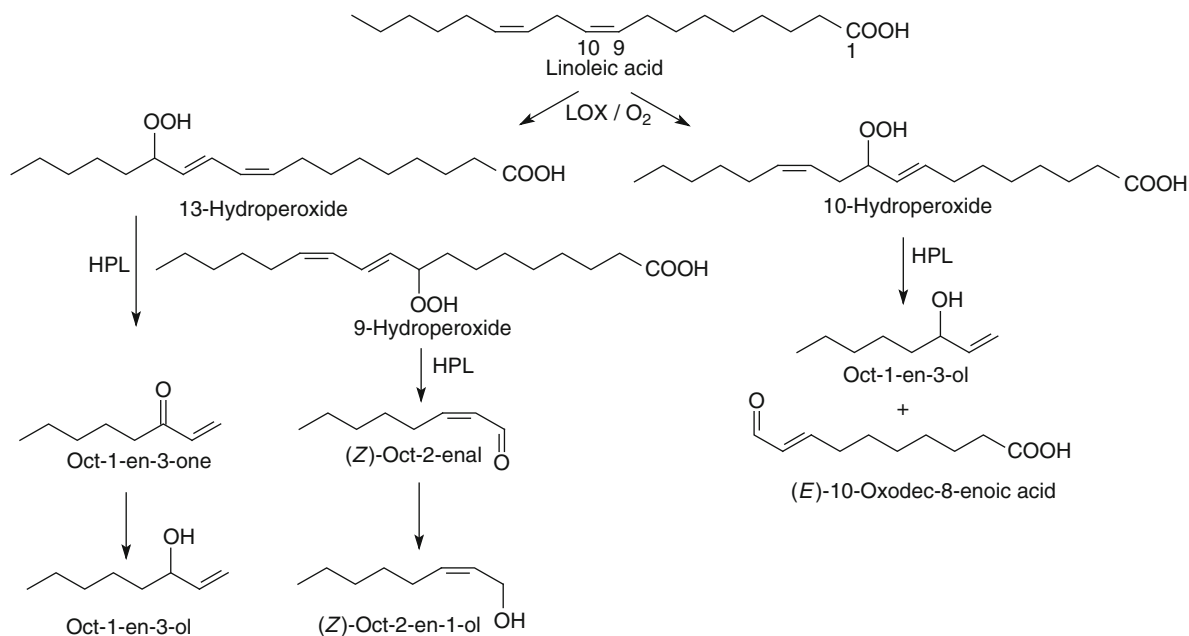


Fig. 12.2. Hypothetical pathways towards C₈ volatiles in mushrooms

mushrooms probably proceeds via a 10-HPOD intermediate (Fig. 12.2). In a subsequent work, the 9-, 10-, 12- and 13-HPOD isomers of linoleic acid were prepared by a photosensitised oxidation, and the hydroperoxyoctadecadienoic acids were incubated with an enzyme preparation of *A. bisporus*. As expected, only the 10-HPOD was cleaved to oct-1-en-3-ol and 10-ODA (Wurzenberger and Grosch 1984c). Interestingly, only the 10-(S)-HPOD isomer was accepted by the hydroperoxide lyase of *A. bisporus* as a substrate (Wurzenberger and Grosch 1984b), and the incorporated oxygen in oct-1-en-3-ol originated from the vapour phase (Wurzenberger and Grosch 1984a). The optimum linoleic acid and protein concentrations for the formation of oct-1-en-3-ol with homogenates of *A. bisporus* were determined to be 1.5 mM and 1.5 mg ml⁻¹, respectively (Husson et al. 2001).

After incubation of mycelial homogenate of submerged grown *Pleurotus pulmonarius* with linoleic acid, oct-1-en-3-ol, (*E*)-10-oxodec-8-enoic acid, and 13-HPOD were identified as the major metabolites. The product concentrations depended on the initial linoleic acid concentration. As in *A. bisporus*, 13-HPOD was excluded to be the precursor of oct-1-en-3-ol (Assaf et al. 1995, 1997). Most probably, oct-1-en-3-ol and 13-HPOD were generated via two distinct enzymatic pathways during the cleavage of linoleic acid. Chen and Wu (1984) studied the enzymatic reduction of oct-1-en-3-one by means of *A. bisporus* extracts. In contrast to the results of Tressl et al. (1981), beside oct-1-en-3-ol octan-3-one was formed in comparable quantities. Although numerous studies have been done to elucidate the enzymatic pathways leading to C₈ volatiles in fungi, the corresponding enzymes still have not been fully identified nor characterised.

2. *Pleurotus florida*

Besides *A. bisporus*, several *Pleurotus* species are highly appreciated for their culinary value. *P. florida* is a common species in tropical West Africa and Southern part of Asia. The edible fruiting bodies of this white-rot fungus develop in large numbers as a group on fallen trees, logs of wood and wooden poles (Adenipekun and Gbolagade 2006). The group of Venkateshwarlu et al. (1999) analysed the volatile compounds of *P. florida* in parallel to those of *A. bisporus* and found oct-1-en-3-ol to be the predominant flavour in both species.

3. *Pleurotus ostreatus*

A near relative of *P. florida*, the basidiomycete *P. ostreatus* (Jacq.: Fr.) Kummer (oyster mushroom)

grows widespread in temperate and subtropical forests throughout the world. It develops in compact clusters on stumps and trunks of leaved trees, where it causes a white rot and forms fruiting bodies during the rainy season (Nyegue et al. 2003). *P. ostreatus* was investigated for volatile constituents by GC/MS using organic solvent extraction, and 28 volatile components were identified. As for *A. bisporus* and *P. florida*, the major odorous compounds were C₈ components like oct-1-en-3-ol, octan-3-ol, octan-3-one, octanal, oct-1-en-3-one, (*E*)-oct-2-enal, and octan-1-ol. Additionally, benzaldehyde (almond odour), benzyl alcohol (sweet-spicy odour) and 2-phenylethanol (rose odour) as well as monoterpenes, i.e. linalool and linalool oxide, also contributed to its pleasant flavour (Nyegue et al. 2003; Tsai et al. 2009).

In contrast to the data reported by Nyegue et al. (2003), the major volatile reported by Tsai et al. (2009) was oct-1-en-3-one; oct-2-en-1-ol was identified additionally, but none of the two C₈ aldehydes were detected. Comparative analyses of the volatile profiles of fruiting bodies of *P. ostreatus* and its mycelium grown in liquid cultures, on agar surface, and on solid support were performed by Kabbaj et al. (2002). The authors employed dynamic headspace technique (DHS) combined with GC/MS and GC/O. The major flavour compounds produced by the fruiting bodies of *P. ostreatus* were octan-3-one (80% of the total volatiles) and octan-3-ol (14% of the total volatiles). The authors proposed that octan-3-one with its sweet, fruity, or at high concentrations mildewy odour, was responsible for the fruity flavour of *P. ostreatus*. In contrast, only small amounts of oct-1-en-3-ol were determined in the fruiting bodies, and other C₈ compounds were absent. The concentrations of oct-1-en-3-ol and benzylic acid increased slightly during the maturation of the fruiting bodies, whereas those of 2-methylbutan-1-ol and 2-methylbutanal decreased.

The obvious discrepancies in the described main volatiles of fruiting bodies between these different studies might be due to specific strains, culture conditions, or the analytical techniques used.

4. *Pleurotus eryngii*

Pleurotus eryngii (king oyster mushroom, almond oyster mushroom, trumpet mushroom, umbel oyster mushroom, scallop mushroom, or boletus of the steppes), is a typical fungus of the flora of the subtropics and steppes. It grows in Mediterranean regions of Europe, the Middle East, and

North Africa, but also in certain parts of Asia. The white-rot fungus is easily recognised by its peculiar habitat; in Europe, it attacks the roots of *Eryngium campestre* L. (field eryngo).

To investigate the flavour profile, *P. eryngii* was grown in plastic jars using moist sawdust supplemented with rice bran as a substrate. The volatile flavour compounds were extracted using SDE followed by GC/MS analysis (Mau et al. 1998). The predominant compounds were octan-3-one, oct-1-en-3-one, octan-3-ol, oct-1-en-3-ol, octan-1-ol, oct-2-en-1-ol, and benzaldehyde. Instead of a C₈ compound, the major volatile compound in the investigated fruiting bodies was benzaldehyde. Additionally, the volatile compositions of large and small fruiting bodies growing on the same base were compared, but no significant differences were observed (Mau et al. 1998).

Whereas Venkateshwarlu et al. (1999), Nyegue et al. (2003), Tsai et al. (2009), and Mau et al. (1998) investigated the flavour profiles of fresh fruiting bodies of *Pleurotus* sp., Lizrraga-Guerra et al. (1997) used aroma extract dilution analysis (AEDA; Schieberle and Grosch 1987) of organic solvent extracts and static headspace (SHS) extraction to identify the most potent odorants in cooked *Pleurotus* samples. Nineteen odorants were identified by AEDA, and eleven of them were also present in the headspace samples. The most potent odorant was 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon) with a flavour dilution (FD) factor of 1000. The authors concluded that only sotolon and a not further identified second compound played an important role in the flavour of cooked oyster mushrooms. The hydroxy furanone derivative sotolon is one of the most important high-value flavours, with the typical smell of fenugreek or curry at high concentrations, and maple syrup, caramel, or burnt sugar aroma at lower concentrations. It is the character impact compound of *Trigonella foenum-graecum* seeds, a key flavour compound in French flor sherry wine, and imparts the burnt flavour to old saké. It has also been identified in soy sauce, sugar molasses, and barley malt used in the manufacturing of beer. Sotolon is widely used in food, e.g. in artificial maple syrup and curry, as well as in the tobacco industry (Rapior et al. 2000a).

5. *Fistulina hepatica*

Fistulina hepatica (Schaeffer: Fr.) Fr. is an annual edible brown-rot fungus with reddish to brown, fleshy and juicy fruiting bodies, with a slightly sour tannic taste. It is distributed in temperate and subtropical hardwood forest ecosystems. It grows on numerous hardwood species, such as oak trees. Because of its appearance, it is aptly

and commonly named as beefsteak polypore, ox-tongue fungus, or poor man's beefsteak.

Volatiles from the fruiting bodies of wild *F. hepatica* were isolated by continuous liquid liquid (CLLE) extraction, and investigated by GC/MS, GC-atomic emission detector (GC/AED), and GC/O. Forty-eight volatile compounds were identified and semi-quantified. Among them, 11 compounds significantly contributed to the overall flavour of *F. hepatica*: oct-1-en-3-one, oct-1-en-3-ol, linalool, 2-phenylacetaldehyde, butanoic acid, an unidentified volatile compound with a mouldy odour, (*E*)-2-methylbut-2-enoic acid, methyl (*E*)-cinnamate bisabolol oxide B and 2-phenylacetic acid (Wu et al. 2005a).

De Pinho et al. (2008) used headspace solid phase micro-extraction (HS-SPME) as well as dichloromethane extracts to identify 35 volatile and semivolatile compounds in *F. hepatica*. Surprisingly, oct-1-en-3-one was not detected in this study. Beside *F. hepatica*, de Pinho and co-workers analysed ten other wild edible mushrooms (*Amanita rubescens*, *Boletus edulis*, *Cantharellus cibarius*, *Hygrophorus agathosmus*, *Russula cyanoxantha*, *Suillus bellini*, *S. granulatus*, *S. luteus*, *Tricholoma equestre*, *Tricholomopsis rutilans*) for the correlation between sensory descriptors and released volatiles. Approximately 50 volatiles were identified and 13 others were tentatively identified.

6. *Boletus edulis*

Boletus edulis is widely distributed in the northern hemisphere and is known as porcini in Italy, cep in France, Steinpilz in Germany, and king bolete in English-speaking countries. It is a highly valued edible ectomycorrhizal fungus and considered a delicacy. Cooked as well as dried, *B. edulis* possesses an attractive and strong aroma and taste. Due to its mycorrhizal growing nature, *B. edulis* has so far resisted attempts to cultivate it commercially (Chang and Miles 2004). In 1973, Thomas detected approximately seventy volatiles in an extract of dried *B. edulis* by means of GC/MS. Among them, nine pyrazines and seven 2-formylpyrroles were identified. Recently, the volatiles of dried, cooked, and canned *B. edulis* samples were compared by GC/O and GC/MS. In this study, approximately 50 compounds were identified (Misharina et al. 2009a, b).

The well known C₈ volatiles were the predominant flavours and responsible for the mushroom notes. In canned *B. edulis*, the amounts of oct-1-en-3-ol (64 mg kg⁻¹), octan-1-ol (16 mg kg⁻¹), and octan-3-ol (12 mg kg⁻¹) were elevated, while boiled samples revealed higher concentrations of

octan-3-one (2 mg kg^{-1}). In the dried fungi, oct-1-en-3-one was the predominant constituent (87 mg kg^{-1}), followed by γ -octalacton (14 mg kg^{-1}), octa-2,4-dien-1-ol (13 mg kg^{-1}), and octanal (11 mg kg^{-1}). Additionally, methional, substituted furans, pyrazines, and pyrroles contributed to the overall flavour of *B. edulis*. The effect of preliminary processing and storage on the sensory quality of frozen *B. edulis* was investigated by Jaworska and Bernaś (2009) over a period of 12 months. Blanching in water proved to be a suitable pre-processing method to assure a high sensory quality of the frozen products.

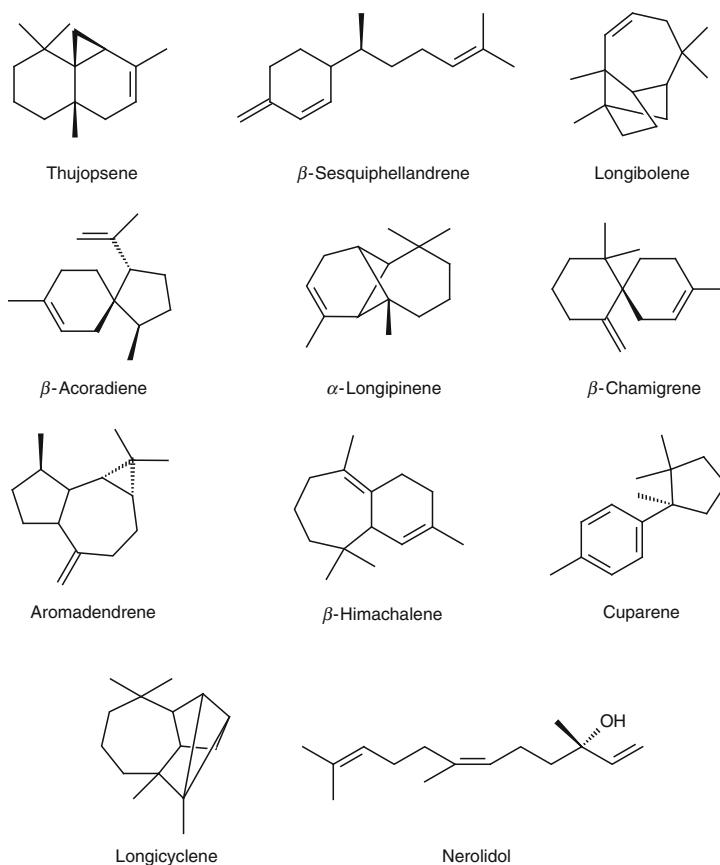
7. *Calocybe indica*

Calocybe indica, the so-called milky mushroom, represents a further edible fungus with predominant C_8 volatiles. Its flavour composition was analysed by means of SDE and GC/FID/O by Venkateshwarlu et al. (1999).

8. *Hygrophorus* spp

All known *Hygrophorus* species are edible litter-decomposing (i.e. saprobic) mushrooms. *H. eburneus* and *H. chrysodon* are consumed especially in Greece as high-quality mushrooms

Fig. 12.3. Uncommon sesquiterpenoid compounds identified in fruiting bodies of *Hygrophorus russocoriaceus*



with a unique aroma. Like *H. russocoriaceus*, which is known for its distinctive cedar odour, they have a slimy, viscous cap and gills with a distinct waxy or silky feel. Their flavour profiles were analysed by HS-SPME combined with GC/MS. Forty-five volatiles were detected, including alcohols, aldehydes, ketones, hydrocarbons, esters, and terpenes.

The most abundant compounds in the three species were 3-methylbutanal, hexanal, *p*-cymene, octan-3-one, oct-1-en-3-one, octan-3-ol, oct-1-en-3-ol and methyl benzoate. The fraction of C_8 compounds varied between 24% and 61% of the total volatiles among these three species (Ouzouni et al. 2009). The presence of seldom-found sesquiterpenes (thujopsene, β -sesquiphellandrene, longifolene (V4), β -acoradiene, α -longipinene, β -chamigrene, aromadendrene, β -himachalene, cuparene, longicyclene, nerolidol; Fig. 12.3) only in *H. russocoriaceus* confirmed the characteristic distinctive cedar odour of this species.

9. *Termitomyces shimperi*

Nyegue et al. (2003) investigated the flavour of *Termitomyces shimperi*, an edible fungus found in termite nests,

that is popular in Africa. The volatiles of the whole fungus were compared with those of the cap and stem. Twenty-four compounds were identified by means of organic solvent extraction and GC/MS. Besides oct-1-en-3-ol, 2-phenylethanol and hexanal were the dominating constituents of fresh fruiting bodies of *T. shimperi*. No significant differences between the caps and stems were observed.

10. *Lentinula edodes*

Shiitake (*Lentinula edodes*²) is the second most produced mushroom world-wide. This white-rot fungus has been cultivated in China and Japan for about 2000 years (Silva et al. 2007). While fresh fruiting bodies exhibit only a slight odour, a characteristic sulfurous aroma develops during processing (Yasumoto et al. 1976). Lenthionine (1,2,3,5,6-pentathiepane, C₂H₄S₅), a sulfur-containing cyclic compound was identified as the primary odour (Morita and Kobayashi 1966; Wada et al. 1967). Its odour threshold was determined to be 0.27–0.53 mg l⁻¹ water (Wada et al. 1967). Dimethyl disulfide, dimethyl trisulfide, 1,2,4-trithiolane (C₂H₄S₃), 1,2,4,6-tetrathiepane (C₃H₆S₄), and 1,2,3,4,5,6-hexathiepane (CH₂S₆) were identified as further sulfurous metabolites in dried shiitake (Morita and Kobayashi 1967; Charpentier et al. 1986). Later, 18 different non-cyclic and cyclic sulfur compounds were extracted from homogenates of fresh shiitake, including lenthionine, 1,2,4,5-tetrathiane (C₂H₄S₄), 1,2,3,5-tetrathiane (C₂H₄S₄), and 1,2,4-trithiolane (C₂H₄S₃; Chen and Ho 1986). Yasumoto et al. (1976) postulated an enzymatic pathway from the precursor lentinic acid to lenthionine (Fig. 12.4).

When the volatiles of young, immature, mature, and old shiitake mushrooms were extracted by SDE using organic solvents and analysed by GC/MS, approximately 130 compounds were identified. The most abundant flavours were oct-1-en-3-ol, octan-3-ol, octan-3-one, and oct-4-en-3-one. The characteristic sulfur-containing flavours, like dimethyl disulfide, dimethyl trisulfide and 1,2,4-thiolane, were found in samples of all development stages. The concentration of oct-1-en-3-ol decreased with increasing age of the fruiting bodies, while the octan-3-one levels increased. The C₈ compounds summed up to 71, 64, 64, and 60% of the total volatiles in the four growth stages, respectively. The maximum concentration of sulfur-containing compounds was observed in mature shiitake samples (Cho et al. 2003). It was reported that γ -irradiation of

fresh or dried shiitake mushrooms impairs their flavour significantly (Yang et al. 1998; Lai et al. 1994). The encapsulation of shiitake flavours by spray drying was investigated by Shiga et al. (2004), and the nutrient composition, covering the tentative identification of some volatiles of *L. edodes*, was published by Çağlarmak (2007).

11. *Volvariella volvacea*

Straw mushrooms [*Volvariella volvacea* (Bull. ex Fr.) Sing.], are widely cultivated in China and other regions of South-east Asia and cause a white rot in straw-like materials. They are commonly harvested in early growth phases, i.e. as egg-shaped and bell-shaped stages. To identify differences in the respective flavour profiles, straw mushrooms were harvested, sorted into five maturity categories, and the volatile constituents were analysed by organic solvent extraction followed by GC analysis. The flavours identified were limonene, oct-1,5-dien-3-ol, octan-3-ol, oct-1-en-3-ol, octan-1-ol, and oct-2-en-1-ol, with oct-1-en-3-ol being the most abundant volatile compound (72–83% of the total volatiles).

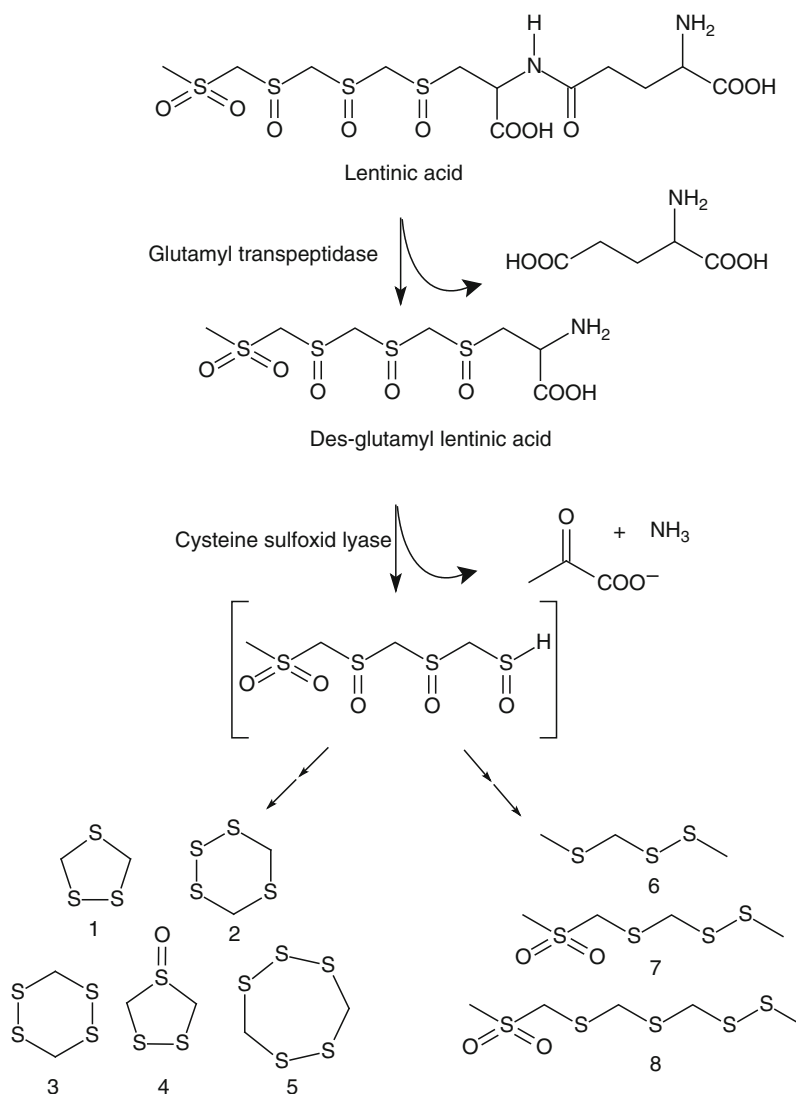
The total concentration of the investigated volatiles ranged from 8 to 19 mg kg⁻¹ fresh weight. The more mature stages of *V. volvacea* possessed higher aroma concentrations, but the different stages of life did not show noteworthy differences in their flavour profiles beside changes in the corresponding oct-1-en-3-ol concentrations (Mau et al. 1997).

12. *Polyporus sulfureus*

In contrast to the only slight changes in the aroma of straw mushrooms during maturing, studies with the brown-rot fungus *Polyporus (Laetiporus) sulfureus* showed that the volatile composition of fruiting bodies from the same species may greatly vary with age. Wu et al. (2005b) compared the odorous compounds of fresh fruiting bodies of wild *P. sulfureus* (Bull.:Fr.) Fr. with the volatile composition of aged fruiting bodies. Forty major volatiles were identified and semi-quantified in young samples of the edible basidiomycete, and oct-1-en-3-one, oct-1-en-3-ol, 3-methylbutanoic acid, 2-phenylethanol, and 2-phenylacetic acid proved to be responsible for the characteristic flavour of young *P. sulfureus*. Contrary, 2-methylpropanoic acid, butanoic acid, 3-methylbutanoic acid, and 2-phenylacetic acid were

²Formerly known as *Lentinus edodes* (IFIS 2009)

Fig. 12.4. Proposed pathway for the formation of sulfurous flavour compounds from lenthinic acid in *Lentinus edodes*. 1 1,2,4-Trithiolane, 2 1,2,3,5-tetrathiane, 3 1,2,4,5-tetrathiane, 4 1,2,4-trithiolane-4-oxide, 5 lenthionine, 6 2,3,5-trithiahexane, 7 2,3,5,7-tetrathiaoctane-7,7-dioxide, 8 2,3,5,7,9-pentathiadecane-9,9-dioxide



determined as the characteristic odorants of aged species. Oct-1-en-3-one was not detected in the latter at all, and oct-1-en-3-ol did not contribute significantly to the overall aroma of aged *P. sulfureus*.

13. *Tricholoma matsutake*

Tricholoma matsutake Sing. (pine-mushroom) is a highly valuable, ectomycorrhizal mushroom species, exhibiting a characteristic and delicate flavour. It is used in a wide range of local dishes, especially in South Korea, including stews, soups, and steamed dishes. Even though they are commonly consumed cooked, they may also be eaten raw, thus better preserving their unique flavour.

Quality parameters of pine-mushrooms, such as aroma, taste, texture, and colour, vary with their specific grades. Cho et al. (2007) compared the aroma characteristics of raw fruiting bodies of *T. matsutake* of four different grades by using GC/O and sensory analysis. Piny, meaty, and floral attributes were strongly correlated with each other, and were the most important descriptors for defining the pine-mushrooms of the highest grade. Among 23 identified flavour compounds, (*E*)-dec-2-enal, α -terpineol, phenylethyl alcohol, and ethyl 2-methylbutanoate contributed most to these attributes. In contrast, the major aroma characteristics of the pine-mushrooms of the lowest grade were like wet soil, alcohol, metallic, mouldy, and fermented. These characteristics

were strongly associated with the presence of oct-1-en-3-one, oct-1-en-3-ol, octan-3-ol, octan-3-one, (*E*)-oct-2-en-1-ol, and methional (Cho et al. 2007).

In another study, the characteristic flavour compounds in raw and cooked pine-mushrooms were compared by GC/O and AEDA. Oct-1-en-3-one was the predominant constituent in raw fruiting bodies of *T. matsutake* and had the highest flavour dilution (FD) factor, followed by ethyl 2-methylbutyrate, linalool, methional, octan-3-ol, oct-1-en-3-ol, (*E*)-oct-2-en-1-ol, and octan-3-one. In contrast, methional, 2-(methoxymethyl)-1,3-thiazole, 3-hydroxybutan-2-one, and 2-phenylacetaldehyde together with C₈ compounds were identified as the major aroma-active compounds in cooked pine-mushrooms (Cho et al. 2006).

14. *Phallus impudicus*

Phallus impudicus (common stinkhorn) is a widespread saprobic fungus with phallic shape when mature and a very strong putrid odour, described as resembling carrion. Despite its unpleasant smell, *P. impudicus* is not poisonous, and the young mushroom is consumed in certain parts of Europe. Borg-Karlson et al. (1994) compared the putrid odour of *P. impudicus* with the similar odour released from the flower of the voodoo lily *Sauromatum guttatum* Araceae by means of headspace GC/MS analysis. In both species dimethyl disulfide and dimethyl trisulfide were found as major constituents. Further important volatiles of *P. impudicus* were linalool, (*E*)-ocimene, and phenyl acetaldehyde.

15. *Marasmius alliaceus*

Marasmius alliaceus (garlic mushroom) is characterised by its strong garlic-like odour which predestines the fungus as a condiment. The flavour profile of fresh carpophores of this saprobic fungus was analysed by organic solvent extraction and DHS concentration in conjunction with GC/MS and GC/O to identify the compounds responsible for its garlic odour (Rapior et al. 1997a). Sixteen and 27 volatile components were identified by solvent extraction and headspace methods, respectively. Seven volatile compounds were identified by both methods: 1,3-dithietane, benzaldehyde, 2,3,5-trithiahexane, 2,3,4,6-tetrathiaheptane, dimethyl disulfide, dimethyl trisulfide, and dimethyl tetrasulfide. The major linear sulfur-containing compounds identified in *Marasmius* species were 2,4,5,7-tetrathiaoctane and 2,3,5-

trithiahexane (solvent extraction), and 2,4-dithiapentane, 3,4-dithiahexane, and *S*-propyl methanethioate (headspace analysis).

16. *Lactarius helvus*

Another mushroom occasionally used as a spice is the ectomycorrhizal mushroom *Lactarius helvus* ("Maggi-pilz"). *L. helvus* possesses a strong smell reminiscent of chicory and fenugreek odour. It is mildly toxic if consumed raw, but can be used in small quantities after drying. Dried fruiting bodies of *L. helvus* were analysed by means of GC/MS, and 38 volatile components were identified (Rapior et al. 2000a). The major constituents were capric acid, sotolon, and 2-methylbutanoic acid, with sotolon being the key compound responsible for the typical odour.

17. Flavour Profiles of Miscellaneous Fungi

The volatiles of 18 different fresh wild mushrooms (*Boletus aereus*, *B. calopus*, *Chroogomphus rutilus*, *Gomphidius glutinosus*, *Leccinum aurantiacum*, *L. lepidum*, *L. pulchrum*, *L. quercinum*, *L. versipelle*, *Paxillus atrotomentosus*, *P. involutus*, *Suillus bovinus*, *S. collinitus*, *S. granulatus*, *S. grevillei*, *S. luteus*, *S. variegatus*, *Xerocomus subtomentosus*) were identified after organic solvent extraction with dichloromethane followed by GC/MS analysis. Four fungi (*L. versipelle*, *L. lepidum*, *L. pulchrum*, *P. atrotomentosus*) possessed no determinable volatiles. The predominant components of the remaining fungi were oct-1-en-3-ol, oct-1-en-3-one, oct-2-en-1-ol, octan-1-ol, octan-3-ol, octan-3-one, benzaldehyde, limonene, N(2-phenylethyl)acetamide, geranyl acetone, farnesyl acetone, and (*E,E*)-farnesol. Eucalyptol, camphene, α -humulene, and germacrene D were identified for the first time in fresh mushrooms (Rapior et al. 1996b, 1997b). The sesquiterpene α -humulene, accompanied by α -copaene, and β -caryophyllene were identified as important constituents of the aroma of *Cantharellus cibarius* (Bareau et al. 1998).

18. Fungi Emitting Anis-Like or Bitter Almond-Like Odours

Several studies were performed to identify the volatiles responsible for anis-like or bitter almond-like odour impressions of basidiomycetous fungi. GC/MS analysis after hydrodistillation

and solvent extraction techniques revealed the flavour compounds of fresh wild fruiting bodies of *Clitocybe odora*, *Lentinellus cochleatus*, and *Agaricus essettei* (Rapior et al. 2002). The main volatile compounds identified were *p*-anisaldehyde, methyl *p*-anisate, benzaldehyde, and benzyl alcohol.

Thus, *p*-anisaldehyde was identified as the key odorous component of fresh *C. odora* (Rapior et al. 2002) which was previously detected in significant amounts in frozen fruiting bodies of *C. odora*, too (Rapior et al. 1996a). The intense anise aroma of *p*-anisaldehyde masked other detected flavouring compounds such as benzaldehyde and oct-1-en-3-ol. *p*-Anisaldehyde, methyl *p*-anisate, methyl (*Z*)-*p*-methoxycinnamate, and methyl (*E*)-*p*-methoxycinnamate were responsible for the aniseed smell of *L. cochleatus*. A mixture of benzaldehyde and benzyl alcohol shaped the complex odour of fresh and wild fruiting bodies of *A. essettei* (Rapior et al. 2002), *A. augustus* (Wood et al. 1990), and *Gyrophragmium dunalii* (Rapior et al. 2000b). Rösecke and König (2000) analysed lignin-derived volatiles in hot-water extracts of fruiting bodies of *Gloeophyllum odoratum*. The authors identified 16 aromatic compounds and claimed that some of them were responsible for the pleasant anise- and fennel-like odour of *G. odoratum*.

19. Key Flavour Compounds of Miscellaneous Fungi

Numerous further studies have been carried out to identify key compounds specific for the typical odour impressions of certain mushroom species or single flavours of special interest. Geosmin was found to be the character impact compound for the musty-earthly aroma of fresh fruiting bodies of *Cortinarius herculeus*, *Cystoderma amianthinum*, and *C. carcharias* (Breheret et al. 1999). In fruiting bodies of *Clitopilus prunulus*, *Catathelasma ventricosa*, and *Tricholoma virgatum*, Wood et al. (1994) identified (*E*)-non-2-enal to be responsible for their cucumber odour. The key compound of the candy-like aroma of the fruiting bodies of *Nolanea fructufragrans* was found to be 1,3-dimethoxybenzene (Largent et al. 1990). Another aromatic flavour compound, 2-aminobenzaldehyde, was identified in organic solvent extracts of *Hebeloma sacchariolens* and described as the source for its “sweet odour” (Wood et al. 1992). Skatole was the major contributor to the disagreeable odour of *Coprinus picaceus* (Laatsch and Matthies 1992). Indole, which was found in very

small amounts in *C. picaceus* too, together with 3-chloroindole and oct-1-en-3-ol were identified as the source of the unpleasant and faeces-like odour of *Hygrophorus paupertinus* (Wood et al. 2003).

B. Volatiles Emitted from Ascomycetes

1. *Tuber* spp

Tuber aestivum (summer truffle), *T. melanosporum* (black truffle, “truffle du Périgord”), *T. magnatum* Pico (white truffle), and other truffles of the genus *Tuber* F.H. Wigg are subterranean ectomycorrhizal fungi. They are highly appreciated because of their unique and characteristic aroma (Díaz et al. 2002). Truffles can associate to numerous plants, mainly of the genera *Quercus* L., *Corylus* L., *Pinus* L., *Tilia* L., *Ostrya* Scop., and *Cistus* L. (Díaz et al. 2003). In the past two decades, the aroma qualities of different truffle species and their changes as a function of preservation methods have been studied. For analysis mainly HS-SPME GC/MS (single or triple quadrupole and ion trap MS) as well as purge and trap GC/MS methods have been employed. Additionally, proton transfer reaction mass spectrometry (PTR-MS) was used in some studies. Analysis of the truffle aroma has been suggested as a tool for authenticating different truffle species and varieties.

For example, Gioacchini et al. (2005) described a rapid method to identify six different truffles (*T. magnatum* Pico, *T. borchii*, *T. dryophilum*, *T. aestivum*, *T. mesentericum*, *T. brumale*) with similar morphological characteristics but different organoleptic qualities and economic value. The differentiation was achieved by means of SHS-SPME GC/MS using a DVB/CAR/PDMS fibre. The corresponding mass spectra of the volatiles were presented as “fingerprints”, and a stepwise factorial discriminant analysis afforded a limited number of characteristic fragment ions that allowed the classification.

2. *Tuber aestivum* and *T. melanosporum*

Díaz et al. (2002) optimised the extraction of volatile compounds from *T. aestivum* by means of HS-SPME, and performed a qualitative and quantitative comparison of the volatile flavours of different truffles species [*T. aestivum* (Soria), *T. aestivum* (Valladolid), *T. melanosporum*; Díaz et al. (2003)]. In total, 89 different compounds were identified in the three truffle samples. The most characteristic

volatiles were sulfur compounds, especially dimethyl sulfide and dimethyl trisulfide.

T. melanosporum contained higher concentrations of esters and benzene derivatives than *T. aestivum*. Among the latter, *T. aestivum* collected in Soria, Spain, showed a significant different aroma profile and higher concentrations of total volatiles than those from Valladolid, Spain. These findings correlated well with the intensity of the respective odour impressions of the two *T. aestivum* species. Among the identified compounds in *T. aestivum* of the different geographical origins, some volatiles were considered to be strongly correlated to the origin (Valladolid: propanal, 3-methylpent-3-en-2-one, octan-2-one, 1,3,4-trimethyl-2-pyrazoline, 3-ethyl-4,5-dihydro-1H-pyrazol, heptan-1-ol, dimethyl sulfoxide, 2-methylhexanoic acid; Soria: ethyl-3-methylbutanoate, dimethyl disulfide, octanal, octane-2,3-dione, hept-2-enal, octan-3-ol, furan-2-carbaldehyde, decanal, prop-2-enoic acid, dodecanal, phenol). These volatiles might thus be used to discriminate among *T. aestivum* species from different geographic origins (Díaz et al. 2003). Two aroma components detected in *T. aestivum* from Soria (furan-2-carbaldehyde and decanal) as well as one volatile in *T. aestivum* from Valladolid (3-methylpent-3-en-2-one) had not been identified in other truffle species before.

Pacioni et al. (1990) analysed the aroma composition of black truffles (*T. melanosporum* Vitt.) from Italian provenance. By SHS analysis, ten oxygen-containing substances (short chain alcohols, aldehydes, ketones, esters) were identified, but the most abundant compound again was dimethyl sulfide. Pelusio et al. (1995) used a HS-SPME technique to profile the aroma of Italian *T. melanosporum* Vitt. and compared the results to those of traditional headspace Tenax adsorption technique. The authors concluded that HS-SPME GC/MS was a suitable technique for the detection of volatile organic sulfur compounds. However, besides two sulfurous volatiles (dimethyl sulfide and 2,4-dithiapentane) only two further components (butan-2-one, butan-2-ol) were detected by the SPME method. Interestingly, no traces of butan-2-ol were detected by Pacioni et al. (1990). With the Tenax adsorption technique 17 flavour compounds were identified in black truffle samples, including four additional sulfur compounds: 1-(methylthio)propane, 1-(methylthio)prop-1-ene, dimethyl disulfide, and dimethyl trisulfide. Ethanol, acetone, propan-2-ol, butan-2-one, butan-2-ol, and C₈ compounds were the most abundant non-sulfur compounds.

The strong characteristic sulfurous truffle aroma disappeared rapidly when the samples were left open to the air, while the pattern of non-sulfur components was much

more stable (Pelusio et al. 1995). This resulted in a distinct mushroom odour caused by oct-1-en-3-ol, octan-3-one, octan-3-ol, and oct-1-en-3-one. The sulfurous volatiles may primarily serve to round off the flavour of Perigord truffles (Bellesia et al. 1998). A number of studies identified 2- and 3-methylbutan-1-ol together with 2- and 3-methylbutanal as important components of the truffle aroma (Pacioni et al. 1990; Bellesia et al. 1998; Díaz et al. 2003). Out of 72 identified compounds, 2- and 3-methylbutanal accounted for more than 50% of the total volatiles analyzed in black truffle from Spain (Díaz et al. 2003). In contrast, these aldehydes were only found in traces by the group of Pelusio et al. (1995), and the corresponding alcohols were not detected at all. The obvious differences in the obtained aroma profiles might be traced back to regional and environmental differences (Bertault et al. 1998), the analytical technique applied, or to the freshness of the investigated samples. For example, Pelusio et al. (1995) stored the truffles up to 1 month at 5°C, while Bellesia et al. (1998) observed significant changes in the corresponding aroma profile already within 7 days of storage.

3. *Tuber magnatum* Pico

The flavour of white truffle (*T. magnatum* Pico) was first analysed in 1967 by Fiecchi et al.. They characterised 2,4-dithiapentane as the most important component. Since then, several further sulfur-containing volatiles of white truffle have been identified, e.g. dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, 1,2,4-trithiolane, 2,3,5-trithiahexane, and tris(methylsulfanyl)methane, dimethyl sulfoxide, methylsulfonylmethane, (methylsulfanyl)(methylthio)methane, dimethyl sulfone, 1,3-benzothiazole, and (methylthio)dimethyl sulfoxide (Pelusio et al. 1995; Bellesia et al. 1996; Piloni et al. 2005; Aprea et al. 2007). Dimethyl sulfide and 2,4-dithiapentane were confirmed as predominating flavour compounds. Significant variations of the flavour composition of different truffles collected on the same day at the same place were noticed (Bellesia et al. 1996). Varying ratios of compounds between gleba and peridium were observed as well. Different ripening stages or microbiological transformations during the short storage time were considered as potential reasons.

Additionally, the changes of the volatile profiles of *T. magnatum* Pico during storage were analysed. Non-sulfur compounds affected the aroma of fresh samples to a lesser extent, but their significance increased over time as a function of storage conditions. At 0 °C C₅ alcohols (especially 2-methylbutan-1-ol) were released, while the conversion of 2,4-dithiapentane into dimethyl disulfide became the most relevant alteration of the flavour at room temperature (Bellesia et al. 1996). Eighteen white

truffles from six different Italian regions were collected and the corresponding aroma profiles were analysed by headspace analysis and proton transfer reaction mass spectrometry (Aprea et al. 2007). It was possible to differentiate *T. magnatum* Pico samples from different origins by use of PTR-MS spectra as anonymous fingerprints. PTR-MS was thus shown to be a new tool for the rapid, quantitative, and non-invasive characterisation of white truffle by direct headspace injection without any pre-concentration. Aprea et al. (2007) suggested to restrict the time-consuming GC analysis to an exploratory phase, and to use the faster and easier PTR-MS as an instrument for extensive sampling campaigns or routinely for quality control of truffles.

4. *Tuber borchii*

The truffle *T. borchii* Vitt. is morphologically similar to the more appreciated *T. magnatum* Pico, but it lacks the latter's typical sulfurous fragrance. Nevertheless, it is often used as a substitute of *T. magnatum*, sometimes as an adulteration.

The volatile profile of fresh samples of the gleba and peridium of *T. borchii* were determined by DHS extraction and GC/MS analysis by Bellesia et al. (2001). While the total concentration of flavour compounds was about 12–13 g kg⁻¹, oct-1-en-3-ol, further alcohols, and aldehydes were the most abundant flavours. 2- and 3-methylthiophene were detected as sulfur compounds, but no open-chain sulfide or polysulfide were identified. The absence of 2,4-dithiapentane, dimethyl disulfide, and dimethyl trisulfide clearly distinguished *T. borchii* from the preferred *T. magnatum*.

III. Flavour Profiles of Fungi Grown in Submerged Cultures

Due to fast and reproducible growth rates, the use of cheap media, and the independence from climatic conditions, submerged fungal cultures have been intensely studied in recent years concerning flavour production. Based on the enormous biochemical potential of basidio- and ascomycetes, submerged cultures represent an interesting production system for these compounds.

A. Basidiomycetous Fungi

1. Flavour Profiles of Miscellaneous Fungi

A broad screening for flavour compounds formed by basidiomycetes grown in shake flasks was performed by Abraham and Berger (1994). The volatiles in the culture media (modified standard

nutrition solution; Sprecher 1959) of 20 submerged grown lignolytic fungi were concentrated by adsorption to Levatit and analysed by GC/FID, GC/MS, and GC/O. In total, 123 compounds were identified, including 40 different alcohols, 11 aldehydes, 12 ketones, 14 lactones, and 19 esters. Most of the basidiomycetes produced the higher alcohols butan-1-ol, 2-methylpropan-1-ol, and 3-methylpropan-1-ol when grown submerged. In general, only small amounts of C₈ alcohols were formed. Wood-rot fungi growing in synthetic nutrition media often prefer aromatic compounds in anabolic and catabolic routes. Thus, a number of aromatic volatiles were found, e.g. 2-phenylethanol and anise alcohol. Benzaldehyde was identified as the predominant aldehyde. Most of the ketones detected were aliphatic. 3-Hydroxybutan-2-one, octan-3-one, 4-methylpent-3-en-2-one, and 1-phenylethanone were preferentially generated, but in small absolute amounts only (generally less than 1 µg l⁻¹).

The only noteworthy lactone producers were *Nigroporus durus*, *Polyporus umbellatus*, and *Tyromyces sambuceus*. The former was able to generate 4-hexanolide, 4-octanolide, and oct-2-en-4-olide in concentrations above 1 mg l⁻¹. The most interesting strains with regard to ester generation were *Polyporus* sp. and *Sarcodontia setosa*. The latter generated methyl 3-methylpentanoate, methyl phenylacetate, and methyl 2,4-dihydroxy-3,6-dimethylbenzoate. *Polyporus* sp. produced phenols such as 3-methoxy-2,5-dimethylphenol, 5-methoxy-2,3-dimethylphenol, 3-methoxy-5-methylphenol, and 3-hydroxy-5-methylphenol. While mono- and sesquiterpenoids were generally identified in only low concentrations, *Kuehneromyces mutabilis* generated four furanoid and pyranoid linalool oxides, and linalool was detected in cultures of *Lentinula edodes*. In a further investigation, 117 mushrooms grown submerged in liquid culture media (PGY) were screened for pleasant flavour impressions (Kawabe and Morita 1993). The white-rot fungus *Polyporus tuberaster* K2606 was most highly rated by sensory analysis. Its odour was described as fruity and floral, and 47 compounds from the culture medium were identified after Porapak Q adsorption, concentration, and GC/MS analysis. The most abundant flavour compound and important characteristic odour of the submerged culture was benzaldehyde (61% of the total volatiles). Further aromatic compounds comprised benzonitrile, acetophenone, benzyl alcohol, 2-phenylethanol, 2-phenylpropanol, and 3-phenylpropanol. In contrast to *Ischnoderma benzoinum*, which accumulated *p*-anisaldehyde (Berger et al. 1987) in addition to benzaldehyde, anisaldehyde was not identified in the culture supernatant of *P. tuberaster*. It is noteworthy that the C₈ volatiles oct-1-en-3-ol, oct-1-en-3-one, and octan-3-one were not found. Kawabe and Morita (1993) traced this back to weak lipoxygenase and hydroperoxide lyase activities of the submerged culture, but no experimental proof

of this hypothesis was provided. 2-Formylpyrrole, a constituent of cocoa and bread flavour, was the only nitrogen-containing volatile identified.

2. *Pleurotus florida*

The volatiles of *Pleurotus florida*, grown submerged in a modified standard nutrition solution, were analysed after SDE by means of GC/FID. Nineteen compounds were identified by comparing the analytes' Kovats indices with those of authentic standards (Venkateshwarlu et al. 2000). The most abundant compounds were *p*-anisaldehyde, 3-methyl-butan-1-ol, 2-methyl-propan-1-ol, benzaldehyde, benzyl acetate, and oct-1-en-3-one. All of them were previously found in the culture media of the closely related *P. sapidus* and further white-rot fungi (Abraham and Berger 1994). The obtained flavour profile differed significantly from the profile of fresh fruiting bodies of the same species. Submerged grown mycelial pellets of *P. florida* omitted a sweet anise and almond-like odour attributed mainly to *p*-anisaldehyde and benzaldehyde. These flavour compounds were previously not detected in *P. florida* sporophores (Venkateshwarlu et al. 1999). Similar data accounted for 2-methyl-propan-1-ol and 3-methyl-butan-1-ol which were found in pronounced amounts in submerged cultures, but only in low concentrations in fruiting bodies.

On the other hand, the major flavour compound of naturally grown *P. florida*, oct-1-en-3-ol (65% of the total volatiles, 20 mg kg⁻¹ fresh mushroom), was only a minor constituent in the liquid media. The addition of linoleic and linolenic acid did not enhance the concentration of these flavour compounds. Thus, the enzymatic conversion of unsaturated fatty acids may be impaired in liquid medium (Venkateshwarlu et al. 2000). This was in good accordance with the studies done by Abraham and Berger (1994). Out of 20 strains, only *Sarcodontia setosa* produced oct-1-en-3-ol in concentrations above 100 µg l⁻¹.

3. *Pleurotus ostreatus*

With *Pleurotus ostreatus*, the pronounced shift from predominant C₈ volatiles to aromatic flavour compounds was not observed when the fungus was grown in liquid Raper medium (Raper et al. 1972). Mycelium of *P. ostreatus* grown in this medium produced most of the major flavour

compounds involved in the aroma of the fruiting bodies, but their ratios and concentrations changed significantly with the culture conditions (Kabbaj et al. 2002). The odour intensity perceived from submerged cultures was very low compared to that of the fruiting bodies. Nevertheless, even in the liquid medium the predominant flavours were oct-1-en-3-ol and octan-3-one. In good agreement with other fungi grown in submerged cultures, elevated amounts of 3-methylbutan-1-ol and 2-methylbutan-1-ol were observed. The authors attributed the changes in the volatile profiles to the differences in the mode of growth. In liquid culture, mycelia growth involved pellet formation and growth under stress, particularly owing to the low concentration of dissolved oxygen. Sheer forces may play an additional role.

4. *Nidula niveo-tomentosa*

Growing in submerged cultures, the basidiomycete *Nidula niveo-tomentosa* (a "bird's nest fungus") de novo synthesised the characteristic impact compound of raspberries, 4-(4-hydroxyphenyl)butan-2-one (raspberry ketone) together with the corresponding alcohol (betuligenol). If this highly sought flavour compound was extracted from raspberries, it would cost several million dollars per kilogram, based on the price of the fruit alone. To open up a biotechnological route towards natural raspberry ketone, a systematic attempt was made to improve raspberry ketone production by *N. niveo-tomentosa*. Optimising the composition of the nutrient medium increased the yields significantly (Böker et al. 2001). Surprisingly, exposition of the growing cultures to periodic irradiation with UV-A ($\lambda_{\text{max}} = 365$ nm) resulted in increased growth rates and high product concentrations of > 100 mg l⁻¹ (Fig. 12.5). Consequently, the proteome response of *N. niveo-tomentosa* to UV-A irradiation was investigated by comparison of cytosolic protein patterns of light- and dark-grown cultures. The analysis of differentially expressed proteins by 2-D electrophoresis and ESI-tandem mass spectrometry revealed a sophisticated stress-defence machinery of *N. niveo-tomentosa*. The spectrum of UV-A light-induced enzymes comprised several stress-related proteins like heat-shock proteins, catalases, glutathione S-transferases, and proteasomes (Taupp et al. 2008). GC/AED as well as

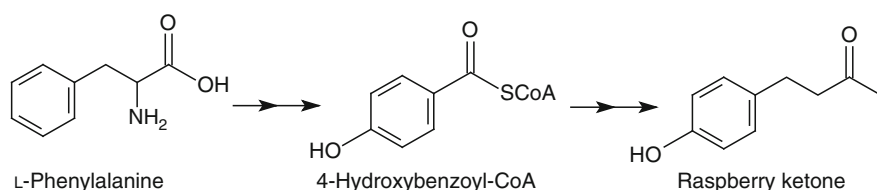
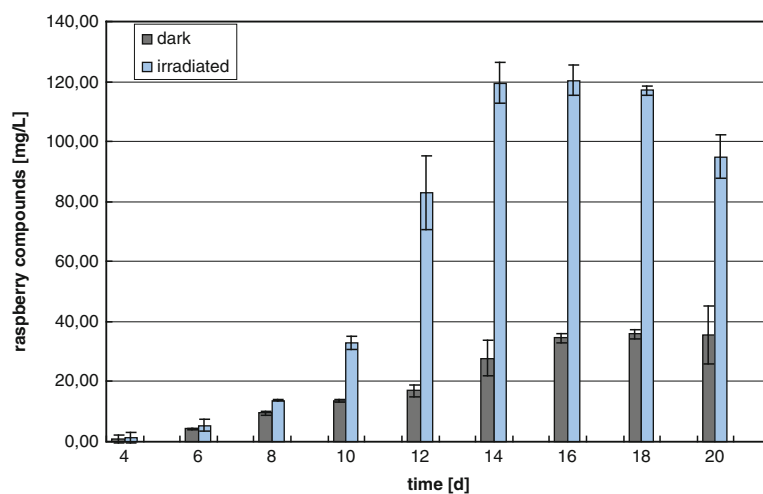


Fig. 12.5. De novo synthesis of raspberry compounds (raspberry ketone, raspberry alcohol) from L-phenylalanine in submerged cultures of the basidiomycete *Nidula niveo-tomentosa*; comparison of cultures grown in the dark and periodically illuminated cultures

GC/MS was used to elucidate the biochemical pathway to raspberry ketone from ^2H - or ^{13}C -labelled L-phenylalanine and from $[1-^{13}\text{C}]$ glucose. The $\text{C}_6\text{-C}_3$ precursor L-phenylalanine was degraded to the $\text{C}_6\text{-C}_1$ structure hydroxy benzoyl CoA, and the benzoate moiety was side-chain elongated subsequently according to the poly- β -keto. This fungal pathway differs from the route established for plant tissues (Zorn et al. 2003).

5. *Fistulina hepatica*

As a follow-up to the investigation of the odour of wild *Fistulina hepatica* fruiting bodies (Wu et al. 2005a; see above), the flavour profile of a *F. hepatica* surface culture grown on oak wood powder was compared to a submerged culture (Wu et al. 2007). The surface cultures produced approximately 50 volatile compounds, while about 40 volatiles were found in the culture grown in a modified standard nutrition solution. Twenty compounds, mainly long-chain fatty acids and methyl esters thereof, and short chain aliphatic alcohols, were common to both systems. The surface culture produced several aldehydes, methoxybenzenoids

and terpenes, which were absent in the submerged cultures. Again, oct-1-en-3-ol was detected in submerged cultures in significant lower amounts ($\leq 10 \mu\text{g kg}^{-1}$ wet mycelia) than in fresh fruiting bodies ($500\text{--}1000 \mu\text{g kg}^{-1}$) of wild *F. hepatica*.

Comparable concentrations have been reported for submerged cultures of the basidiomycete strains *Kuehneromyces mutabilis*, *Pleurotus sapidus*, *Nigroporus durus*, and *Polyporus umbellatus* (Abraham and Berger 1994; see above). Compared to submerged cultures, the most notable changes in the volatile profiles concerned the presence of methoxybenzenoid compounds and terpenoids in *F. hepatica* grown on oak wood powder (Wu et al. 2007). The authors suggested an attack on lignin as a reason for the former.

B. Ascomycetous Fungi

1. *Tuber borchii*

In addition to wild *Tuber borchii* Vitt. (see above), the odour of mycelium grown in modified Melin-Norkrans liquid medium was investigated by headspace purge and trap methods as well as GC/MS (Tirillini et al. 2000). Out of 29 identified volatiles, only two had been mentioned as flavour

compounds of the genus *Tuber* before. The most abundant components were 3-methylheptane, butan-2-one, ethynylbenzene, and octan-3-one. Dimethyl trisulfide was the only sulfur compound detected in submerged cultures of *T. borchii* Vitt. The authors assumed that it was derived from the conversion of 2,4-dithiapentane to dimethyl disulfide through a hydrolytic-oxidative pathway already observed in *T. magnatum* (Bellesia et al. 1996).

2. *Penicillium vulpinum*

Larsen and Frisvad (1995) investigated the volatiles produced by the mould *Penicillium vulpinum* when cultured in Czapek yeast autolysate (CYA). Different extraction techniques were compared: diffusive sampling from headspace, purging and trapping of headspace, and SDE. The qualitative and semiquantitative composition of mainly unsaturated compounds like mono- and sesquiterpenes collected by diffusive sampling were comparable to the volatiles obtained by purging and trapping. SDE resulted in a significantly different flavour profile. Lipid degradation products like oct-1-en-3-ol and octan-3-one which were absent in the headspace samples dominated the SDE extracts. The authors concluded that these compounds were either confined within the fungal biomass, or were generated during the SDE procedure, where the fungal biomass was partially destroyed by heating and stirring. Thus, the latter method was not recommended as a screening method for fungal volatiles.

3. *Ceratocystis moniliformis*

The major aroma compounds produced by submerged cultures of the ascomycete *Ceratocystis moniliformis* were analysed by means of GC/FID. Ethyl acetate, propyl acetate, isobutyl acetate, isoamyl acetate, citronellol, and geraniol were identified, and an integrated bioprocess (IBP) for production and recovery of these de novo synthesised flavours was published (Bluemke and Schrader 2001).

IV. Conclusions

As a promising flavour production system, growing fungi in submerged cultures have become an interesting alternative to fruiting bodies over recent years. Fast and reproducible growth, de-

finer media composition, and independence from climatic conditions are general advantages of mycelium and flavour production in bioreactors. However, problems arising from significant differences in the flavour profiles of submerged cultured and traditionally grown fungi will have to be avoided before this method comes to a broad application (Wu et al. 2007). The genetic and biochemical principles behind this phenomenon still remain to be elucidated and may help to regulate flavour synthesis in bioreactors. Considering the popularity and the economic importance of edible fungi (mushrooms), surprisingly little is known about the biochemical pathways of flavour formation in fungi but hopefully this situation will change in the near future.

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13 Industrial Applications of Fungal Enzymes

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I. Introduction

Micro-organisms and their enzymes have served mankind for a long time. The ancient Babylonians and Sumerians were brewing as early as 6000 B.C. and reliefs on tombs dating from 2400 B.C. document beer making in Egypt (Young 2009). Based on archeological evidence, the Egyptians were probably also the first to bake leavened bread. The fermentation processes in Western cultures to a large extent were based on various kinds of yeasts, like *Saccharomyces* spp., which for this purpose may be considered as a “package” of enzymes. Eastern cultures have in addition utilized a range of other micro-organisms, including

mycelial fungi. For example, the Asian food products Shoyu (soy sauce) and Miso (fermented soybean paste) are in part based on fermentations by *Aspergillus oryzae* (Tanaka 2000).

Unlike yeasts the filamentous fungi secrete some enzymes from the hyphae to act in the medium, which may therefore be used as a source of enzymes apart from the intact organism. The oldest example of isolation and use of enzymes in a process separate from the source organism is the processing of milk. Rennets were thus used already in ancient times for coagulation of proteins during cheese production. Rennet or rennin is a mixture of chymosin and pepsin extracted from the gastric mucosa of young mammals, e.g. calves and lambs. The content of the highly specific endo-proteinase, chymosin, depends on the age and species of the animal.

The use of enzymes, isolated and separate from their producing organism, has increased steadily since World War II. Indeed, enzymes have today found use in several large industrial processes. The dawn of biotechnology played a crucial role in this development and recombinant enzymes are now readily available in bulk quantities. The workhorses of the biotechnology industry are the micro-organisms. Often these represent both the source of the genetic material and the host for production of the encoded enzymes. The production organism typically belongs either to the genus *Bacillus* (gram-positive bacteria) or to the genus *Aspergillus* (filamentous fungi), but other microbial production hosts are also used, such as *Streptomyces*, *Trichoderma* and *Penicillium*.

II. Fungal Enzymes in Industry

Members of the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) have commercialized more than 260 enzymes. Approximately 60% of these commercial enzymes

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originate from fungi and they are also produced in a fungal host organism (Figs 13.1, 13.2). With representatives from more than 25 fungal genera, the origin of the donor organisms is obviously more diverse than the selection of fungal host organisms. More than 25% of all industrial enzymes stem from *Aspergillus*, which is thereby the unambiguous champion amongst the micro-organisms of industrial importance. The top five fungal donors also include *Trichoderma*, *Penicillium*, *Rhizopus* and *Humicola*, accounting for another 20% of the industrial enzymes. More than 20 genera are represented in the remaining 14% of commercial enzymes of fungal origin, but each genus is represented by merely one or two products.

Enzymes of fungal origin are particularly well suited for industrial application for several reasons. The fungi are heterotrophic organisms. Most species grow as multicellular filaments called hyphae, but some species (e.g. yeasts) also grow as single cells. Heterotrophy is the utilization of extracellular sources of organic energy and material for maintenance, growth and reproduction. The source of energy may be simple sugars, polypeptides or more complex carbohydrates. The fungi can only absorb small molecules through their cell wall and an enzymatic digestion outside the

mycelium is therefore often required. The fungi capable of utilizing a variety of energy sources will initially absorb the simplest compounds (e.g. soluble sugars). Starch, pectin, cellulose, lignin and waxes are then digested in succession. The fungi secrete a complex battery of enzymes required for the digestion. Secretion makes it relatively simple to produce and isolate the enzymes on a large scale. Extracellular enzymes have also evolved naturally to work under harsh conditions, making them ideal candidates for industrial catalysts. The biochemical reactions catalysed by these enzymes are described in greater detail by their industrial application. Enzymes of bacterial origin are mentioned where required for the understanding of the industrial process. Applications depending solely on enzymes of bacterial origin are not addressed in this chapter.

III. Safety of Fungal Production Organisms

The fungi *Aspergillus niger*, *A. oryzae* and *Trichoderma reesei* are important production organisms in industrial fermentations. For more than 2000 years, *A. oryzae* has been used in China and Japan

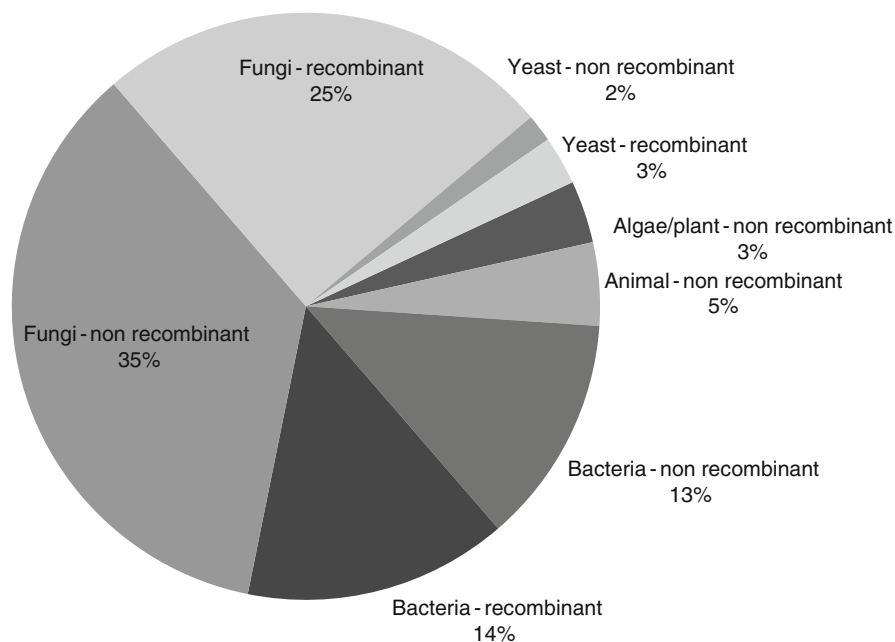


Fig. 13.1. Distribution of host organisms for production of recombinant/non-recombinant industrial enzymes based on data from AMFEP (2009)

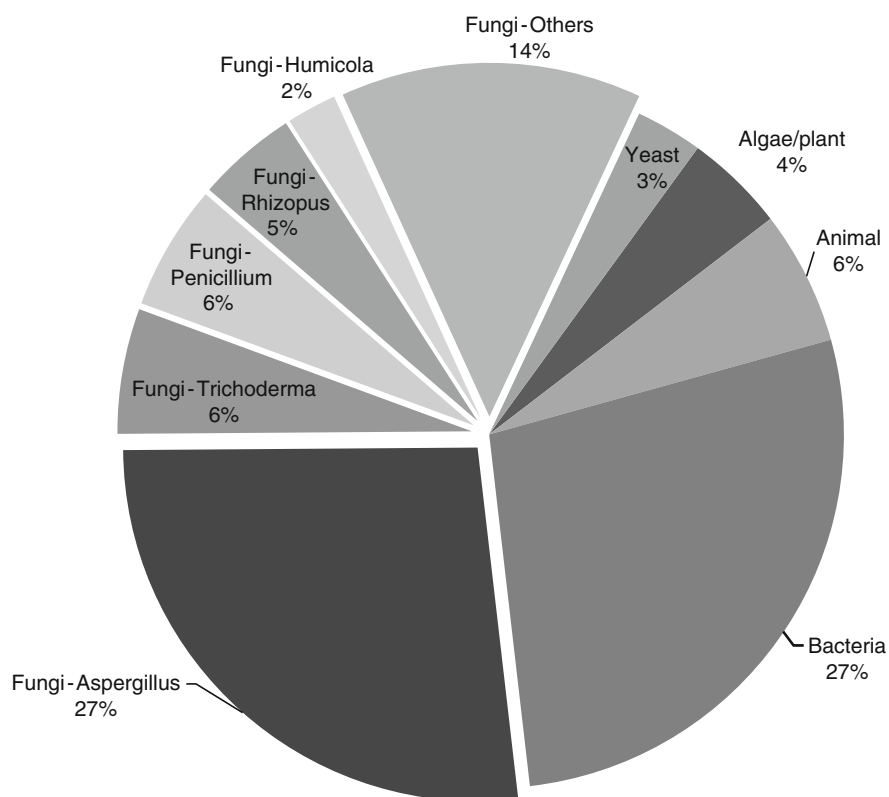


Fig. 13.2. Distribution of industrial enzymes by origin of donor organism based on data from AMFEP (2009) with specification of the top five fungal organisms

for the fermentation of rice and soy products (Barbesgaard 1992). For a century, it has also been used for the production of enzymes for baking and brewing. *A. niger* acquired industrial importance in 1919 for its ability to produce citric acid and it later became an important source of enzymes (Schuster 2002). The history and development of *Aspergillus* as an expression host was recently reviewed by Lubertozzi and Keasling (2009). *T. reesei* was first isolated in 1944 and it proved especially useful in the production of cellulases. The safety of these three fungi as production organisms has thus been demonstrated by a long history of use. Indeed, numerous enzyme preparations from these fungi are considered generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) in the United States.

Industrial production organisms are carefully selected, maintained and monitored to ensure lack of mycotoxins and other toxic secondary metabolites. *A. niger* and *A. oryzae* do produce some mycotoxins, e.g. ochratoxin A, but the production of toxin is both strain- and growth condition-

dependent (Blumenthal 2004). Non-toxic strains may therefore be selected as production organisms and production conditions may be controlled to exclude mycotoxins in the product. *Trichoderma* has been reported to produce trichothecene T-2 and its presence should therefore be tested in *Trichoderma*-derived enzyme preparations. Stringent toxicity tests are employed to confirm that enzyme products are safe for use in their intended application. The species have never been identified as the primary cause of disease in man. The risk of allergic hypersensitivity to inhaled spores is controlled by elimination of exposure to spores in the production processes. In conclusion, these three fungi are safe production organisms, provided good manufacturing practices are observed.

IV. Enzyme Classification System

Enzymes are named and classified by the reactions they catalyse (Enzyme Nomenclature 2009). There are six classes in the standard Enzyme

Table 13.1. List of industrial enzymes, and the reactions catalysed, listed according to the standard enzyme classification system

Class	Industrial enzyme	Reaction profile
EC 1: Oxidoreductases	Catalase, glucose oxidase, laccase, peroxidase	Redox reaction involving transfer of electrons from one molecule to another
EC 2: Transferases	Fructosyltransferase, Glucosyltransferase, Transglutaminase	Transfer of groups of atoms from one molecule to another
EC 3: Hydrolases	Amylase, amyloglucosidase, cellulase, β -glucanase, lactase, lipase, mannanase, pectin methyl esterase, pectinase, phospholipase, phytase, protease, pullulanase, xylanase	Hydrolysis, the cleavage of substrates by water
EC 4: Lyases	Pectate lyase, α -acetolactate decarboxylases	Addition of groups to double bonds or the formation of double bonds through the removal of groups
EC 5: Isomerases	Glucose isomerases	Transfer of groups from one position to another in the same molecule
EC 6: Ligases	Not used at present	Join molecules together with covalent bonds

Classification System maintained by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Table 13.1). The vast majority of the industrial enzymes are of the hydrolase class (Fig. 13.3), which includes enzymes responsible for the degradation of relative simple biopolymers. A large group of these hydrolases are of fungal origin and produced in a fungal host. The second-most populated class is the oxidoreductases, which includes the enzymes responsible for breaking non-hydrolysable carbon-carbon and ether bonds. The class is predominantly populated with fungal enzymes, both in terms of origin and production host. The third-largest class is the lyases, which has a fairly even population of bacterial and fungal enzymes. Enzymes of bacterial origin dominate the transferase class, while the isomerases are exclusively of bacterial origin. The sixth class, the ligases, is currently not represented among the industrial enzymes (but is used in molecular works at a laboratory scale). The majority of enzyme products in the portfolio of the AMFEP members are destined for the food industry (Fig. 13.4). However, enzymes are really used in a multitude of industrial application, as illustrated in Table 13.2.

It should be noted that many enzymes are destined for multiple industrial applications, including the food industry, while a number of enzymes are applied exclusively in the food sector. In terms of sales, products for the technical industries (detergents, starch, textile, fuel ethanol, leather, pulp and paper) constitute the bulk of sales, as illustrated by sales figures from Novozymes (Fig. 13.5).

V. Enzymes for Detergents and Personal Care

The use of enzymes in detergents contributes a range of benefits: e.g. enabling a lower washing temperature reduces the energy consumption. The water consumption is likewise reduced by a more effective dirt removal. Enzymes are a renewable resource and are readily biodegradable. Historically, the application of enzymes in detergents began in 1913 with a patent by the German scientist Otto Röhm for the use of pancreatic enzymes (Röhm 1913). However, enzymes and detergents were not particularly compatible until 50 years

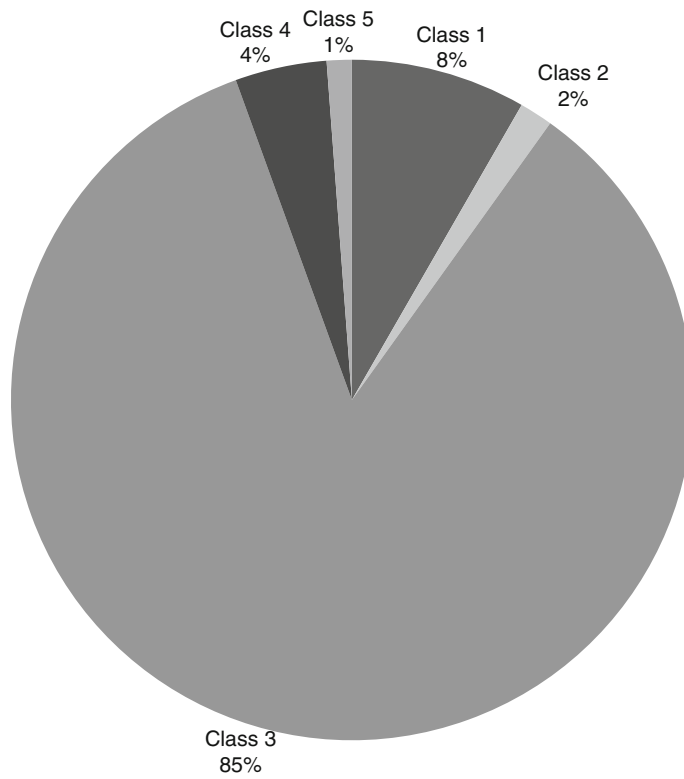


Fig. 13.3. Distribution of industrial enzymes, manufactured by AMFEP members, according to the standard enzymes classification system

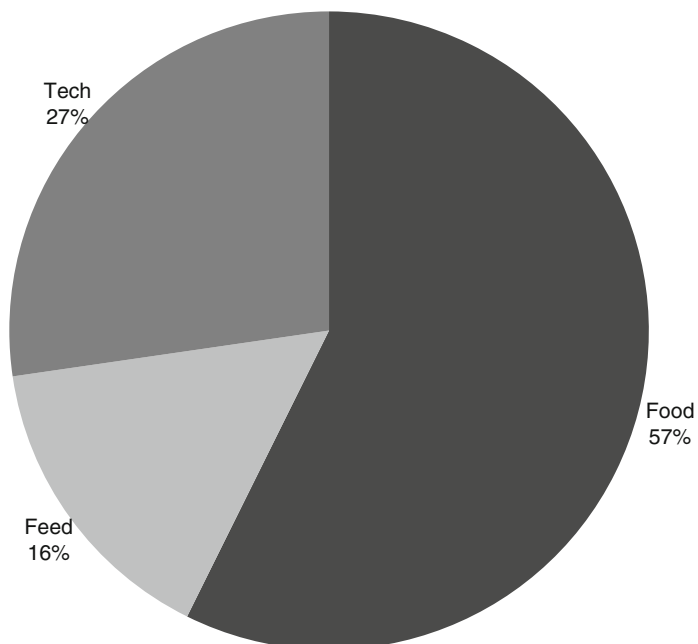


Fig. 13.4. Distribution of enzyme products, manufactured by AMFEP members, according to the industrial application

Table 13.2. List of fungal enzymes produced by AMFEP members and their industrial applications

Enzyme	Origin	Industry
Glucose oxidase:EC 1.1.3.4	<i>Aspergillus</i> spp.	Personal care, baking
Laccase:EC 1.10.3.2	<i>Myceliophthora</i> spp., <i>Trametes</i> spp., <i>Thielavia</i> spp.	Personal care, textile
Catalase:EC 1.11.1.6	<i>Aspergillus</i> spp., <i>Scytalidium</i> spp., <i>Thermoascus</i> spp.	Textile
Lipase:EC 3.1.1.3	<i>Aspergillus</i> spp., <i>Candida</i> spp., <i>Fusarium</i> spp., <i>Humicola</i> spp., <i>Rhizomucor</i> spp., <i>Thermomyces</i> spp.	Detergents, leather, forest products, biocatalysis, baking, dairy, lipid modification
Pectinase:EC 3.1.1.11 EC 3.2.1.15 EC 4.2.2.10	<i>Aspergillus</i> spp.	Fruit juice and wine
Phospholipase:EC 3.1.1.32 EC 3.1.4.1	<i>Aspergillus</i> spp., <i>Fusarium</i> spp., <i>Thermomyces</i> spp.	Lipid modification
Hemicellulase:EC 3.1.1.73	<i>Aspergillus</i> spp.	Fruit juice and wine
Phytase:EC 3.1.3.8 EC 3.1.3.26	<i>Aspergillus</i> spp., <i>Peniophora</i> spp.	Animal feed
α -Amylase:EC 3.2.1.1	<i>Aspergillus</i> spp., <i>Thermoactinomyces</i> spp.	Forest products, baking, brewing, fruit juice and wine
Amyloglucosidase/ glucoamylase:EC 3.2.1.3	<i>Aspergillus</i> spp., <i>Talaromyces</i> spp., <i>Trichoderma</i> spp.	Personal care, fuel ethanol, sweetener production, distilling, fruit juice and wine
Cellulase:EC 3.2.1.4 EC 3.2.1.91	<i>Humicola</i> spp., <i>Myceliophthora</i> spp., <i>Thielavia</i> spp.	Detergents, textile, forest products, fuel ethanol, distilling
β -Glucanase:EC 3.2.1.6	<i>Thermoascus</i> spp., <i>Trichoderma</i> spp.	Animal feed, fuel ethanol, brewing, distilling
Xylanase:EC 3.2.1.8	<i>Actinomadura</i> spp., <i>Aspergillus</i> spp., <i>Thermomyces</i> spp., <i>Trichoderma</i> spp.	Forest products, animal feed, fuel ethanol, baking, brewing, distilling
Lactase:EC 3.2.1.23	<i>Aspergillus</i> spp., <i>Kluyveromyces</i> spp.	Dairy
Pullulanase:EC 3.2.1.41	<i>Hormoconis</i> spp.	Fuel ethanol, distilling
Protease:EC 3.4.2x.xx	<i>Aspergillus</i> spp., <i>Cryphonectria</i> spp., <i>Fusarium</i> spp., <i>Rhizomucor</i> spp., <i>Trichoderma</i> spp.	Leather, dairy, protein hydrolysis

later when Novo introduced the protease Alcalase (Aunstrup 2001). The characteristics required for an enzyme to work well in today's laundry detergents are (Broze 1999):

- Alkaline pH optimum
- Efficient at low temperature (20–40 °C)
- Stability at high temperature (<60 °C)
- Stability in the presence of other ingredients
- Broad specificity

The most widely used enzymes in detergents today are of the hydrolase class and address soiling based on proteins, lipids and polysaccharides. The typical laundry dirt originates from either the body or from food and beverages. All members of the detergent enzyme family, i.e. proteases,

lipases, amylases, mannanases and cellulases, offer specific benefits for laundry, while mainly proteases and amylases are applied in automatic dishwashing. The basic detergent mechanisms are the same despite regional differences in type of dirt, detergent composition and washing process. Dirt, spots and stain are removed by mechanical agitation assisted by enzymes, surfactants and builders. Some dirt and stains are relative easy to remove in water, but enzymes add extra cleaning power to the otherwise solely physical action. The enzymes work by degrading dirt into smaller and more soluble components. Protein and starch stains are thus degraded by proteases and amylases, respectively. The removal of grease spots (lipids), difficult to remove at low temperature,

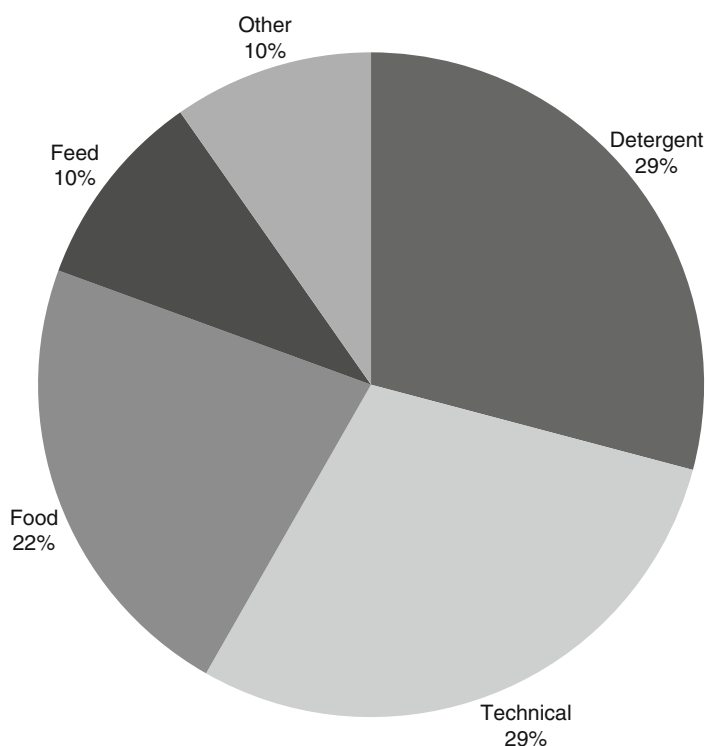


Fig. 13.5. Distribution of Novozymes' 2007 sales by industry

is facilitated by lipases. Mannanase helps to remove various food stains containing guar gum, which is a commonly used stabilizer and thickening agent in food products. Cellulases work indirectly by hydrolysing 1,4- β -D-glycosidic bonds of the textile. Particulate dirt attached to cellulose microfibrils are thus removed along with the fibrils on the surface of the cotton yarn. An added benefit of cellulase action is a greater softness and improved colour brightness of worn cotton surfaces.

Many of the commercial detergent enzymes are of bacterial origin though, as their characteristics make them more suited for this particular application. The detergent proteases (EC 3.4.21.61), amylases (EC 3.2.2.1) and mannanases (EC 3.2.1.78) are thus all from bacteria. The detergent enzymes of fungal origin includes lipases (EC 3.1.1.3) that catalyse the hydrolysis of triglycerides to more hydrophilic mono- and diglycerides, free fatty acids and glycerol (Fig. 13.6). The first commercial lipase product, Lipolase, was isolated from *Thermomyces lanuginosus* (Huge-Jensen 1989). The lipase enzyme was subsequently improved for detergent application by

protein engineering to generate e.g. Lipex with improved first-wash performance over a broad range of temperatures and pH values. The detergent cellulases, which cleave β -1,4-glycosidic bonds in cellulose (Fig. 13.7), are also from fungi. The enzyme class is divided into endo- (EC 3.2.1.4) and exo-cellulases (EC 3.2.1.91). Both types have been isolated from *T. lanuginosus* and are commercially available.

The personal care sector is another industry with large potential for enzyme applications. The area of dental hygiene has already today embraced enzyme technology; and various enzymes are incorporated in some brands of toothpaste. For example, Zendium claims to include lysozyme (EC 3.2.1.17), lactoperoxidase (EC 1.11.1.7), glucose oxidase (EC 1.1.3.4) and amyloglucosidase (EC 3.2.1.3) in their toothpaste. The main role of these enzymes is to eliminate *Streptococcus mutans* and *Lactobacillus* spp., the bacteria responsible for initiating caries.

The enzymes achieve this partly by removing the carbohydrates required for bacterial growth. The glucose oxidase concomitantly generates hydrogen peroxide. In addition to its function as a

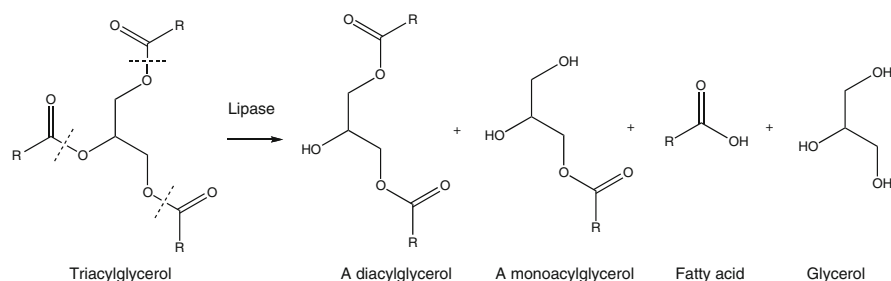


Fig. 13.6. Hydrolysis of lipids (triacylglycerols) catalysed by lipase

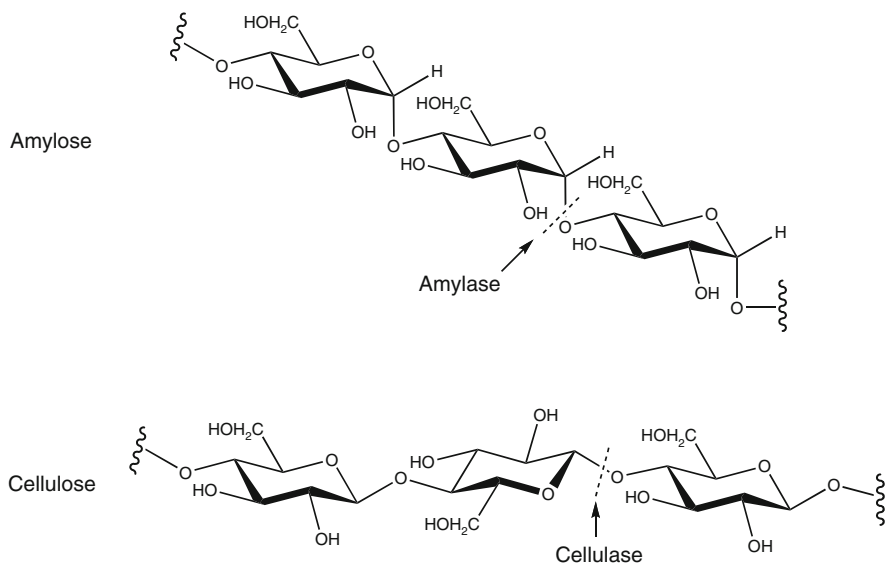


Fig. 13.7. Hydrolysis of starch and cellulose: α -amylases catalyse endohydrolysis of 1,4- α -D-glycosidic linkages in polysaccharides of three or more 1,4- α -linked glucose

units. Cellulases catalyse hydrolysis 1,4- β -D-glycosidic linkages in cellulose

mild bleaching agent, peroxide also plays a role in the bactericidal system. The thiocyanate anion (SCN^-) occurs in secretions such as saliva. The oxidation of the anion catalysed by lactoperoxidase generates short-lived oxidation products with antimicrobial activity (Kussendrager 2000). Lysozymes damage bacterial cell walls by hydrolysis and therefore have antimicrobial activity. Glucose oxidase and amyloglucosidase of fungal origin are available as commercial products (Fig. 13.8). Lactoperoxidase is a native protein to milk, while lysozyme is commercially available from chicken eggs. Other dental products, e.g. some chewing gums, include laccase (EC 1.10.3.2) from e.g. *Trametes* spp. for breath freshening. Laccases oxidize thiols, sulfides and amines. The enzymatic system thereby eliminates the molecules causing the malodor of halitosis.

VI. Enzymes for Other Non-Food Industries

A. Textiles

Enzymes have found widespread application in the textile industry (Araujo 2008). The steps in the finishing process for a textile product is illustrated in Fig. 13.9, where both fungal and bacterial derived enzyme products are applied. Cotton is the dominant textile fibre and its fibres are individual cells growing from the cotton seed. The interior of the fibre is nearly pure cellulose, while the outer primary cell wall contains waxes and hemicelluloses. The harvested fibres are mechanically cleaned to remove plant debris, then spun into yarn and made into fabrics. The warp yarn (longitudinal thread) of fabrics is often

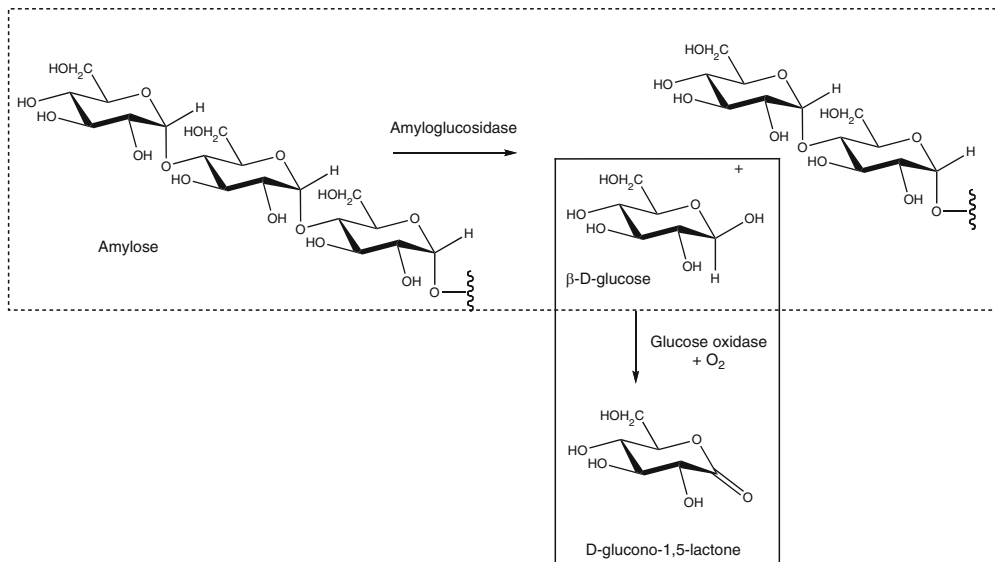


Fig. 13.8. Amyloglucosidases catalyse hydrolysis of terminal 1,4-linked α -D-glucose residues successively from non-reducing ends of the chains with release of β -D-glucose.

Glucose oxidases catalyse oxidation of the generated β -D-glucose to D-glucono-1,5-lactone

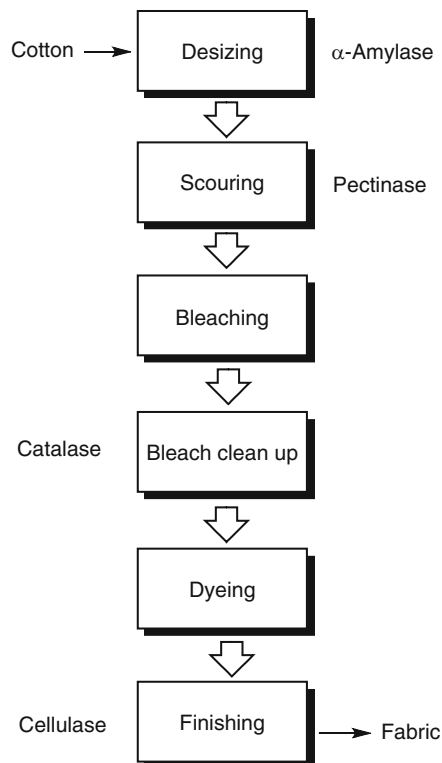


Fig. 13.9. Several steps in the industrial processing of raw cotton to finished textile involves use of enzymes

coated with starch to prevent it from breaking during weaving. Desizing, the use of α -amylases (EC 3.2.1.1) to remove starch size, is the first and one of the oldest enzyme applications in textile processing. However, the enzymes used are mainly of bacterial origin, especially from *Bacillus* spp.

Scouring is the process of removing non-cellulytic compounds from the fibre to make it hydrophilic before it is dyed. A pectate lyase (EC 4.2.2.2) from *Bacillus* has proved most effective for the degradation of pectin in cotton and is thus most effective for assisting the removal of waxes, oils and other impurities. The scoured cotton fabric has to be bleached before dyeing. The bleaching may be done using hydrogen peroxide, which needs to be removed before dyeing to prevent bleaching of the dye itself. Several fungal catalases (EC 1.11.1.6) from *Aspergillus*, *Scytalidium* and *Thermoascus* spp. are commercially available for this “bleach/clean-up” application.

Cotton finishing can be achieved with cellulases (EC 3.2.1.4), which prevent pilling and improve smoothness and colour brightness. Pills are entangled microfibrils protruding from the cotton fabric surface. Cellulases have also been applied to replace the pumice stone process to obtain abrasion of denim. A fungal cellulase from *Trichoderma reesei* has proved very effective for these applications. The cellulose-based denim abrasion

suffers a drawback: the re-deposition of released indigo dye onto the un-dyed white weft yarns. The enzymatic bleaching process based on laccase (EC 1.10.3.2) and a mediator eliminates this problem. The enzymatic system oxidizes the indigo in solution and part of the indigo attached to fabric to colourless and soluble products. Various fungal laccases are available as industrial enzymes, e.g. from *Myceliophthora* spp., *Trametes (Polyporus)* spp. and *Thielavia* spp.

B. Leather

Enzymes have always been an integrate part of leather-making (Fig. 13.10). The tanning process converts the protein of raw skin or hides into a stable, durable and versatile natural material that will not putrefy. The raw hide is prepared for tanning by a number of preparatory stages in the so-called beam-house. Enzymes play an important role in several of these processes (Thanikaivelan 2004). Soaking is the process to restore moisture in the raw hide, making the hide flexible, removing salts and globular proteins and improving the penetration of chemicals later used. Proteases improve the water absorption by degrading non-collagenous protein. A lipase is used to disperse the fat, thereby providing a synergistic effect.

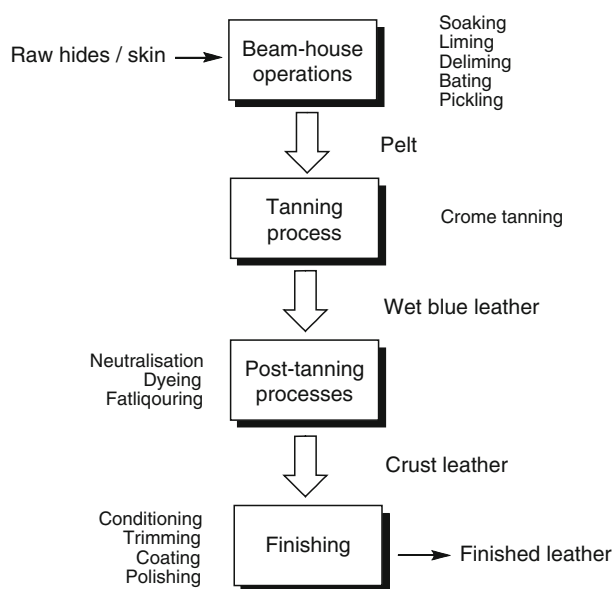


Fig. 13.10. Leather processing involves a series of operations as shown in the flow diagram (adapted from Thanikaivelan 2005)

Liming is a process to degrade the epidermal structure of the hide, including the hair. Alkaline proteases and lipases are used in this process as auxiliaries to speed-up the reactions. Bating is a treatment with proteolytic enzymes to make the leather pliable and takes place before tanning. The treatment dissolves and washes out residues of non-collagen protein. A range of microbial enzymes from fungal and bacterial sources is commercially available for the leather industry, e.g. Novobate, Greasex and Novocor.

C. Forest Products

The pulp and paper industry has incorporated some enzymatic processes and many more have been described in the literature (Kenealy 2003). Paper is mainly made from wood. Three polymers constitute the major components of wood: cellulose, hemicelluloses and lignin. The initial step in papermaking is the formation of a pulp containing free fibres. Pulping is either a mechanical or chemical process. A mechanical pulp retains all the wood components, while chemicals in a chemical pulp dissolve the lignin. The chemical pulp still contains lignin residues and these cause a darkening of the pulp. The pulp therefore needs to undergo a bleaching process before it can be used for manufacture of paper. The bleaching itself is typically based on the use of chemical bleaching agents, but prior treatment of the pulp with xylanases has a bleach-boosting effect. Xylanases open the hemicellulose structure and thus release bound lignin and lignin-carbohydrate complexes. It is thereby possible to wash out more lignin and the pulp becomes more susceptible to the bleaching chemicals. Several xylanases (EC 3.2.1.8) of fungal origin are available at an industrial scale, e.g. from *Trichoderma* spp., *Aspergillus* spp. and *Thermomyces* spp.

A mechanical pulp includes a resinous material called pitch, which can cause problematic sticky deposits on e.g. the rolls of the production line. The problem can be alleviated by addition of lipase (EC 3.1.1.3) to catalyse hydrolysis of the triglyceride. The resulting glycerol and free fatty acids can then be washed away from the pulp. A range of fungal lipases are commercially available, e.g. from *Candida* spp., *Fusarium* spp., *Hemicula* spp., *Thermomyces* spp. and *Aspergillus* spp.

Finally, paper may also be coated with starch to improve the gloss, smoothness and printing properties. A modified starch solution of low viscosity is used in this process. The reduced viscosity may be obtained using commercial α -amylases (EC 3.2.1.1). Amylases may also be applied in the process of paper recycling. They effectively degrade the starch coating and thereby release ink particles from the fibre surface. Cellulases (EC 3.2.1.4) also increase the efficiency of de-inking by defibrillation of the microfibrils attached to the ink. Amylases and cellulases of fungal origin are readily available as industrial enzymes.

D. Animal Feed

The addition of enzymes to feed can improve the digestibility, as many feed ingredients are only partially digested by livestock. Cereals constitute the major component in animal feed but the intestinal flora of non-ruminant animals, e.g. pigs and poultry, does not produce the endogenous enzymes required to degrade the contained fibres. Furthermore, the presence of non-starch polysaccharides interferes with digestion and their removal thus improves the feed utilization. Xylanases (EC 3.2.1.8; Fig. 13.11) are commonly used feed enzymes and degrade the xylan structure of plant cell walls to short-chain sugars. Xylanases from *Trichoderma* spp. and *Aspergillus* spp. have been commercialized for this application (Polizeli 2005). β -Glucanases (EC 3.2.1.6, Fig. 13.11) degrades the β -glucan structure found in barley to produce free glucose and oligosaccharides. β -Glucanases of fungal origin (again from *Trichoderma* spp. and *Aspergillus* spp.) are likewise commercially available for animal feed. An important storage of phosphorus in plants is phytate, but again monogastric animals do not have the enzymes to break it down. Addition of phytase to the feed circumvents this deficiency and makes the phosphorus available to the animals while reducing the environmental impact of phosphorus (Haefner 2005). Phosphorus is used for formation and maintenance of the skeleton and involved in many metabolic processes. Two types of phytase (Fig. 13.12), 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26), are commercially available from fungal sources (e.g. *Aspergillus* spp., *Peniophora* spp.).

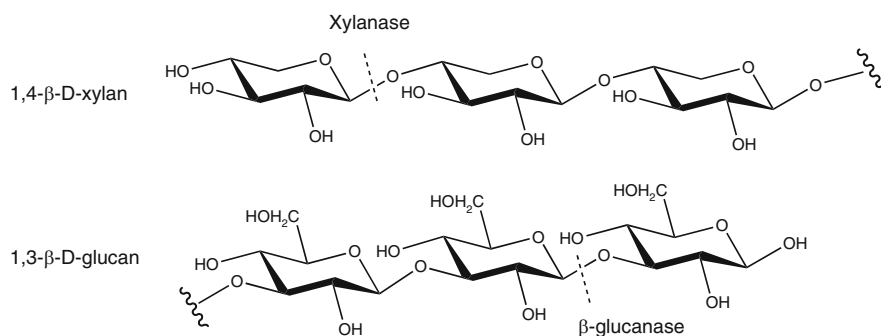


Fig. 13.11. Xylanases catalyse endohydrolysis of 1,4-β-D-xylosidic linkages in xylans. β-Glucanases catalyse endohydrolysis of 1,3- or 1,4-linkages in β-D-glucans when the

glucose residue, whose reducing group is involved in the linkage to be hydrolysed, is itself substituted at C-3

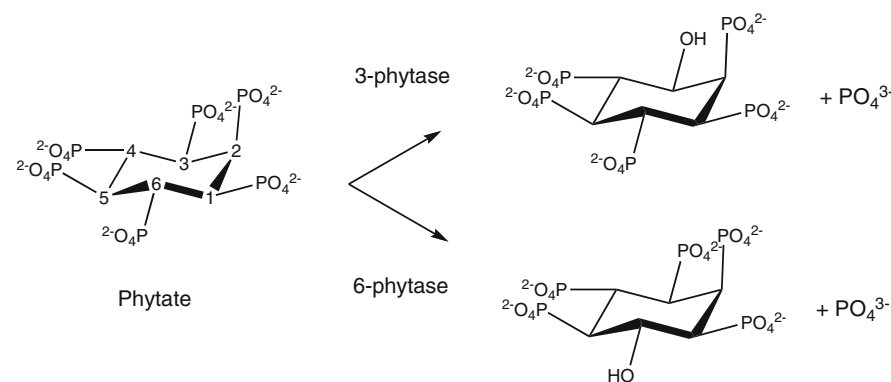


Fig. 13.12. Phytases catalyse hydrolysis of myo-inositol hexakisphosphate into myo-inositol 1,2,3,4,5-pentakisphosphate or myo-inositol 1,3,4,5,6-pentakisphosphate with concomitant release of inorganic phosphate

E. Fuel Ethanol

The majority of our food is provided by agricultural practices, yet our energy needs (i.e. coal, oil, gas) are mainly served by the hunter-gatherer principle. However, numerous technologies are under development that will enable us to harvest energy from renewable sources. An example is ethanol produced from biomass that may be used as a substitute or extender to traditional fossil fuels. The process of converting biomass to biofuel involves a series of different bacterial and fungal enzymes.

Sugar-based raw materials can be fermented directly, but starch-based materials must be hydrolysed to fermentable sugars. The starch-based raw material is typically whole grains ground down in a dry-milling process (Bothast 2005; Fig. 13.13). Starch has traditionally been hot-cooked in the process of liquefaction that gelatinizes the starch and makes it possible for

conventional enzymes to break down the starch into dextrins. An alternative “cold cook” or raw starch hydrolysis process, called the Broin Project X (BPX), was recently developed to eliminate the energy-demanding high-temperature cooking process (Lewis 2004). The process exploits the synergistic effect between glucoamylases (EC 3.2.1.3) and α-amylases, whereby the glucoamylase makes glucose from free non-reducing ends and the α-amylase makes more free non-reducing ends. Special amylases have been developed which start working efficiently even under the conditions found in dry mills. Phytases may be added to release calcium required for the function of α-amylases. Other enzymes, β-glucanase, xylanase and cellulase, address the problem of increased viscosity caused by non-starch polysaccharides, such as β-glucan, xylans and cellulose. The processed starch is subsequently subjected to saccharification, whereby the dextrins are broken down to fermentable sugars like glucose and

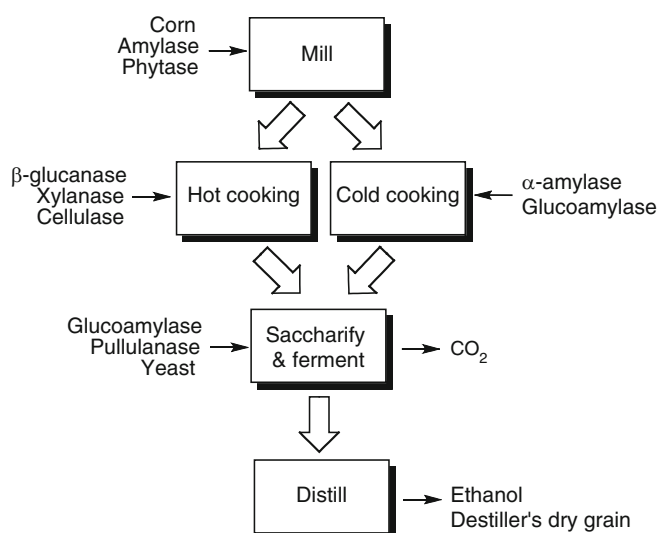


Fig. 13.13. Ethanol production process by dry grind (adapted from Bothast 2005)

maltose. The reaction is catalysed by glucoamylase and pullulanase (EC 3.2.1.41) of bacterial or fungal origin with a yield of 95–97%. During fermentation, the sugars are converted to alcohol by yeast and its enzymes. Saccharification and fermentation may also be combined in a simultaneous process (simultaneous saccharification and fermentation; SSF) to save tank capacity.

One of today's great challenges is the development of the second generation of biofuels produced from cellulosic biomass, which makes up the majority of the cheap and abundant non-food materials available from plants. There is great potential in the use of this plant biomass to produce liquid biofuels. Plant biomass is comprised mostly of plant cell walls, of which typically 75% is composed of polysaccharides. These polymeric carbohydrates are contained in a complex matrix, including not only crystalline cellulose, but also hemicelluloses and lignin. The hemicelluloses and lignin (together lignocelluloses) prevent access of cellulolytic enzymes to the cellulose thus decreasing the efficiency of the ethanol production. A large battery of new enzymes, including cellulase and hemicellulase, will therefore be required for production of second-generation biofuel. Indeed, much of the current research effort is focused on development of enzymatic and microbial solutions for degradation of lignocelluloses, increasing the level of fermentable sugars and increasing the final yield of ethanol. The ethanol may thereby be made from the non-food part of bio-renewables

and it will effectively reduce the emission of greenhouse gases (Demain 2009).

F. Biocatalysis – Enzymes in Organic Synthesis

The application of enzymes in organic synthesis has been the focus of much research, which has resulted in establishment of the technology within the chemical industry (Pollard 2007). Potentially, biocatalysis offers a number of advantages in organic synthesis:

- Highly efficient catalysts
- Regio- and stereoselectivity
- Protection/deprotection of functional groups not required
- Mild reaction conditions (pH 5–8, 20–40°C, aqueous media)
- Green technology
- No byproducts
- Easy purification

Enzymes are often capable of catalysing reactions quite different from their native biological function. It is exactly this property that is exploited in biocatalysis, where enzymes transform non-natural compounds. The most commonly used enzymes for organic synthesis are various hydrolases and especially lipases. Lipases are by nature designed to operate at an oil–water interface and they are therefore highly compatible with organic solvents. Lipases can also catalyse

reactions that are very different from hydrolysis of triglycerides. The B-component of the lipase from the yeast *Candida antarctica* has proved to be a particularly efficient enzyme for regio- and enantioselective synthesis (Kirk 2002). Other hydrolases of high importance include nitrilases, esterases, amidases and proteases. Several oxidoreductases (e.g. laccases, peroxidases) have also been applied in organic synthesis for the preparation of enantiomerically enriched compounds (Woodley 2008).

VII. Enzymes for Food Industries

A. Sweetener Production

The starch industry is heavily dependent on enzymatic reactions in its processes (Guzman-Maldonado 1995; Fig. 13.14). The enzymes involved are predominantly of bacterial origin with a single exception: glucoamylase. Starch is the most abundant storage polysaccharide of plants and the chief source of carbohydrates for humans and is therefore of considerable economic importance.

Polysaccharide is deposited in the plants in form of small insoluble particles called starch granules. Natural starch may be separated into two gross fractions, called amylose and amylopec-

tin. It consists of chains of glucose molecules, which are linked together by $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ glycosidic bonds. Amylose appears to be an essentially unbranched $\alpha(1\rightarrow4)$ glucan chain, while amylopectin is a branched molecule also containing some $\alpha(1\rightarrow6)$ linkages.

The majority of industrially processed starch is either used as starch or chemically modified starch in food or technical industries. The rest is modified by enzymatic hydrolysis to form special types of syrup. Syrups can be tailor-made by choosing the right enzyme and reaction conditions. Starch from corn is the most common raw material. The syrups are high fructose corn syrup, glucose syrup and various maltose syrups. The production is not possible by conventional chemical hydrolysis but rather comprises three enzymatic steps: liquefaction, saccharification and isomerization. The native starch is gelatinized and liquefied to make it susceptible to hydrolysis by bacterial α -amylases (EC 3.2.1.1). The enzyme hydrolyses α -1,4-glycosidic bonds in the gelatinized starch, producing maltodextrins. The dextrins may be sold as commercial products or processed further. The maltodextrins can be saccharified further using a fungal glucoamylase (EC 3.2.1.3) for stepwise removal of glucose units from the non-reducing end of the molecules. Bacterial pullulanases (EC 3.2.1.41) are employed to

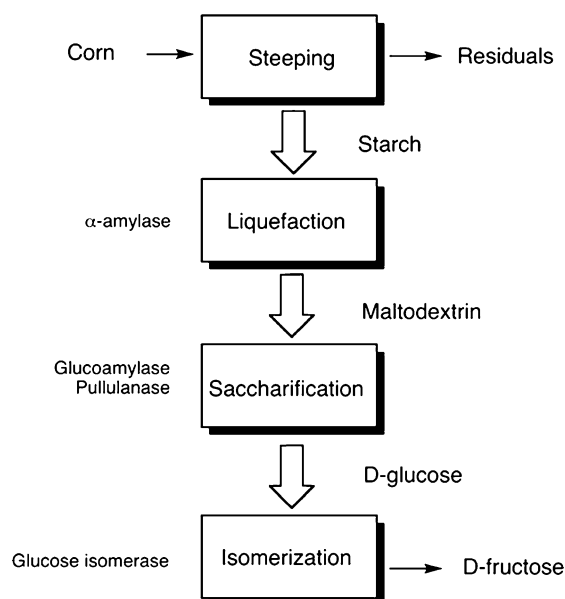


Fig. 13.14. Industrial processing of starch for sweetener production involves numerous enzymatic reactions

catalyse hydrolysis of the α (1 \rightarrow 6) linkages in amylopectin. Total hydrolysis yields 95–97% of glucose, which can be isomerized to fructose using a commercial bacterial glucose isomerase (EC 5.3.1.5). An industrial product containing 55% fructose can thereby be obtained with a sweetness similar to sucrose.

B. Baking

Fungal enzymes play nowadays an important role in bread making. The first enzymatic application in baking was historically the addition of malt to supplement the endogeneous α -amylases present in flour. The malt was subsequently replaced by fungal α -amylases with a better thermostability. The added α -amylase compensates for natural variations in the flour, but enzymes may also add value by producing ingredients in situ from compounds in the flour. Enzymes may thus replace a number of questionable chemical additives otherwise used.

Bread dough typically consists of flour, water, yeast, salt and possibly ingredients such as sugar and oil. Flour consists of gluten, starch, non-starch polysaccharides, lipids and traces of minerals. The yeast starts to work once the dough is made. Fermentable sugars are thereby converted to alcohol and carbon dioxide which makes the dough rise. Amylases degrade starch to produce small dextrins. The effect is a reduction in viscosity of the dough. The small dextrins also allow the yeast to work continuously during proofing and early baking thus improving the bread volume. Finally, the presence of more dextrins also enhances the Maillard reactions responsible for the browning of the crust and the flavour of baked bread (Martínez-Anaya 1996). Fungal amylases (EC 3.2.1.1), such as Taka-amylase from *Aspergillus oryzae*, are readily available as industrial enzymes for the baking industry. Amylases are also applied to prevent the staling of baked goods by modifying the starch granules during baking when the starch starts to gelatinize. The resulting modified starch granules remain more flexible during storage, but the most effective amylase for this application is from *Bacillus amyloliquefaciens*.

Enzymatic modification of other flour constituents is also important for the quality of bread. Non-starch polysaccharides constitute 2.5–3.5%

of flour and xylanases (EC 3.2.1.8) catalyse endo-hydrolysis of β -1,5-D-xylosidic linkages in the contained arabino-xylan. The result is an improved dough handling and stability and improved crumb structure and bread volume.

The mechanism has not been clearly demonstrated, but arabino-xylan may itself absorb water and produce viscosity. Wheat flour also contains 2–3% wheat lipids, which lipases (EC 3.1.1.3) hydrolyse into glycerol, free fatty acids, monoglycerides and diglycerides. The effect of lipase addition is given by the effect of the hydrolysis products. Some product of the hydrolysis may thus introduce or improve surface-active properties that can stabilize air bubbles in the gluten matrix. The result is a better crumb structure, improved dough stability and increased volume.

Oxidative reactions are also important in baking. Chemical oxidants, such as ascorbic acid and bromates, have been widely used to strengthen the gluten when making bread. Oxidases, e.g. glucose oxidase (EC 1.1.3.4) from *Aspergillus niger*, can partially replace these chemical oxidants (Wong 2008). Glucose oxidase catalyses the oxidation of glucose, with the consumption of molecular oxygen, to produce gluconolactone and hydrogen peroxide. The hydrogen peroxide reinforces the gluten network by increasing the number of inter- and intra-molecular disulfide linkages. The result is improved gas retention and less sticky dough. Current investigations indicate that also fungal laccase and tyrosinase can improve the dough properties.

Each enzyme described for baking has its own substrate, but there is often a synergistic effect by combining them (Leon 2002). Some beneficial effects are not even seen if only a single enzyme is used. The xylanase activity alone, for example, may result in a dough that is too sticky, which is circumvented by addition of glucose oxidase.

C. Dairy

The processing of milk with enzymes has a long history (Fox 1993). Coagulation during cheese production was earlier obtained using calf rennet, a mixture of chymosin and pepsin (EC 3.4.23.1). Coagulants of fungal origin are today readily available and produced from e.g. *Rhizomucor miehei* and *Cryphonectria parasitica* by fermentation in bioreactors. Bovine chymosin is also available

as a recombinant enzyme produced in *Aspergillus niger*. The microbial version of the enzyme tends to be more proteolytic than rennet, which results in a larger loss of casein fragments in the whey fraction.

The fresh curd obtained by milk clotting is separated from the whey by drainage. Minerals and lactose are not retained in the curd, which is rather composed of fat droplets entangled in a casein network. These compounds have a very mild taste in their natural state. The cheese flavour is developed during the ripening period by controlled hydrolysis of these compounds: Proteolysis of casein and lipolysis of triglycerides. Enzymes produced by micro-organisms in the curd play a major role in these biochemical reactions. The huge variety of cheeses available is created using different microflorae (Sandine 1970). The use of industrial enzymes during ripening is a problem, because, apart from being difficult to control, added enzymes usually end up in the whey fraction. An exception is the lipase used in Italian cheeses to develop their piquancy. The flavour was originally produced by the action of endogenous milk lipoprotein lipase (EC 3.1.1.34), which was incorporated in the curd due to its association with casein. The endogenous lipases are today inactivated by pasteurization of the raw milk and enzyme therefore needs to be added from an external source. Suitable lipases are present in the rennet pastes, but they are also available as industrial fungal enzymes from e.g. *Rhizomucor miehei*.

Cow's milk contains 5% lactose, a disaccharide consisting of a glucose moiety and a galactose moiety. The enzyme, lactase or β -galactosidase (EC 3.2.1.23; Fig. 13.15), is required to hydrolyse lactose. The level of the enzyme in humans is high at birth, but some people develop a deficiency later in life. Microbial lactases from e.g. *Aspergillus* and *Kluyveromyces* are used to hydrolyse lactose thus increasing digestability of dairy products

among people with lactose intolerance. The enzyme is also used to improved sweetness in milk-based beverages and to prevent lactose crystallization in ice cream. Cow's milk also contains proteins that can induce sensitization or an allergic reaction in infants. Proteases have therefore been used for many decades to produce infant milk formulas from cow's milk. Breaking internal peptide bonds in the proteins eliminates the epitopes and it also increases the nutritional value of the infant milk. Microbial endo-proteases with a preference for hydrolysis of peptide bonds within hydrophilic regions are used for this application.

D. Protein Hydrolysis

Proteins in their natural state do not contribute to the flavour in foods, but the products of their hydrolysis, peptides and amino acid, have flavour (Kirimura 1969). Classic examples are cheeses as described above and the hydrolysis of soy protein during microbial fermentation to produce soy sauce and related products. The products of the protein hydrolysis can also react with other food components to create other flavours. The Maillard reaction between reducing sugars and amino acids is an example of such a chemical reaction.

Hydrolysed proteins are ingredient in a wide range of common products, such as soups, stock cubes and savoury sauces, but glutamic acid (monosodium glutamate) is by far the most widely used flavour enhancer. Glutamate is also known as the fifth basic taste-sense or umami as suggested by its discoverer, the Japanese chemist Kikunae Ikeda, in 1908. It is used at a concentration of 0.2–0.8% in a variety of foods such as soups, broths, sauces, canned meats, and readymade dishes. Protein hydrolysates are produced from meat using a mixture of fungal exopeptidases and endopeptidases.

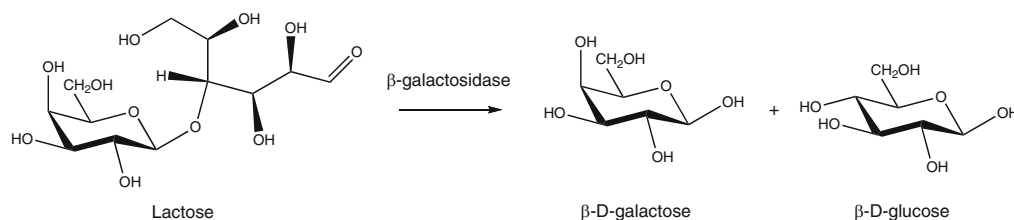


Fig. 13.15. Lactase or β -galactosidase catalyse hydrolysis of terminal non-reducing β -D-galactose residues in β -D-galactosides

E. Brewing

Brewing is an ancient and very traditional process whereby starch of a cereal is converted into alcohol (Fig. 13.16; Bamforth 2009). The process is divided into two stages: (1) the hydrolysis of starch into fermentable sugars and (2) the conversion of sugars into alcohol and carbon dioxide. Malted barley is a key ingredient in brewing and contains a series of enzymes: proteases, amylases, glucanases and cellulases.

During malting, the cell wall material is degraded by hemicellulases and β -glucanase, while protein-based materials are degraded by proteases. The amylases are now able to act on the starch granules. The crushed malted barley is mixed with hot water in a large vessel, the mash tun. Other cereals rich in starch (adjuncts) are also added to the mash. The addition of adjuncts will dilute the concentration of endogeneous enzymes in the mash tun (see Chapter 2). Too little enzyme activity during the mashing process is undesirable and auxiliary enzymes are therefore added as required.

Fungal α -amylase (EC 3.2.1.1) ensures simpler liquefaction and shorter process time. It rapidly hydrolyses the starch into smaller fragments that can be processed by the barley β -amylase (EC 3.2.1.2). Added fungal β -glucanase (EC 3.2.1.4) and xylanase (EC 3.2.1.8) breaks down β -glucan and xylan, thus reducing the wort viscosity and improving the filterability. The mash is filtered into a copper to make the sweet wort, which is then boiled with hops to make hopped wort. The cooled liquid is then transferred to the fermentation vessel and yeast is added. Enzymes may even be added during the fermentation process. Slow fermentation due to incomplete saccharification during mashing may be corrected by addition of fungal α -amylase directly in the fermenter.

F. Distilling

Production of beverage alcohol from starch-based raw materials has been practised for ages. The raw materials differ around the world: North

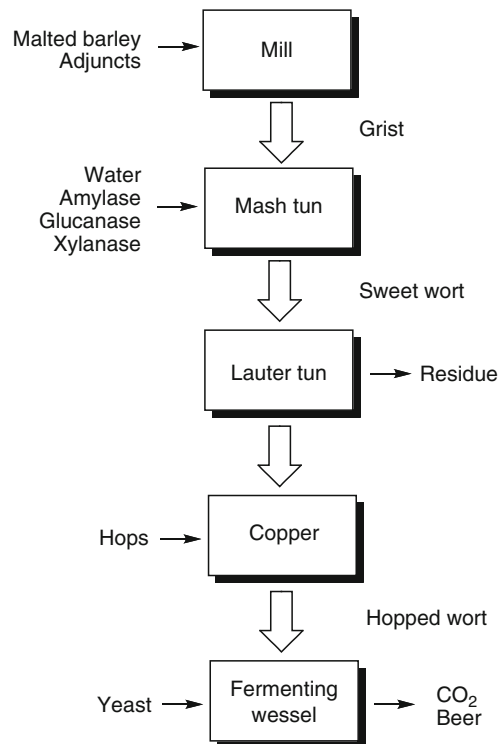


Fig. 13.16. Brewing involves a series of operations as shown in the flow diagram

American whisky is based on corn and rye, whereas Scotch malt whisky is based on barley. Rice is used to make sake in the Far East. Potatoes and grain are used for other types of spirits such as vodka and gin. The contained starch is typically hydrolysed in two enzymatic steps: liquefaction and saccharification. Yeast can then convert the fermentable sugar into ethanol. The enzymes used for beverage alcohol production are the same as those earlier described for production of first-generation fuel ethanol.

G. Fruit Juice and Wine

Enzymes are widely used in the juice industry to ease clarification of the juice and decrease the viscosity of the mash to shorten processing time. The fruit cell walls are composed of cellulose fibres with strands of hemicellulose attached. The fibres are embedded in a pectin matrix linked to structural proteins. The pectin binds with water in the fruit mash, thereby increasing the viscosity and making it difficult to separate liquid and solid in the press. Many micro-organisms produce enzymes that degrade fruit cell walls. Commercial pectinases for the fruit processing industry originate from strains of *Aspergillus* spp. and include a battery of different enzymes. The pectinases are classified according to their reactivity (Fig. 13.17). Pectin lyase (EC 4.2.2.10) is a pectin depolymerase, pectin methyl-esterase (EC 3.1.1.11) removes

methoxyl groups from pectin and polygalacturonase (EC 3.2.1.15) catalyses the hydrolysis of glycosidic linkage of galacturonans. Pectins are composed of galacturonic acid units linked by glycosidic bonds to form polygalacturonic acid. Fungal hemicellulase hydrolyses arabinogalactans, galactans, xyloglucans and xylans incorporated into the pectin molecule. Fungal acid α -amylase and amyloglucosidase are used to process fruit containing starch to prevent post-bottling haze formation. The amylase hydrolyses amylose and amylopectin to produce dextrans, which in turn are hydrolysed to glucose by the amyloglucosidase. Tailor-made enzyme preparations are available for specific fruits: apples, berries, citrus fruits, or wine grapes.

H. Lipid Modification

Lipids may be modified using lipases, which catalyse hydrolysis, esterification, transesterification and interesterification (Xu 2005). Fats with lower content of calories can be produced by elimination of one fatty acid from the triglyceride. Cooking oils enriched in 1,3-diglycerides are commercially available in Japan. The pancreatic lipase hydrolyses this diglyceride with no formation of 2-monoglyceride – a building block for serum lipid that could contribute to higher serum lipid levels. Specific fatty acids such as omega 3, believed to have health benefits, can also be

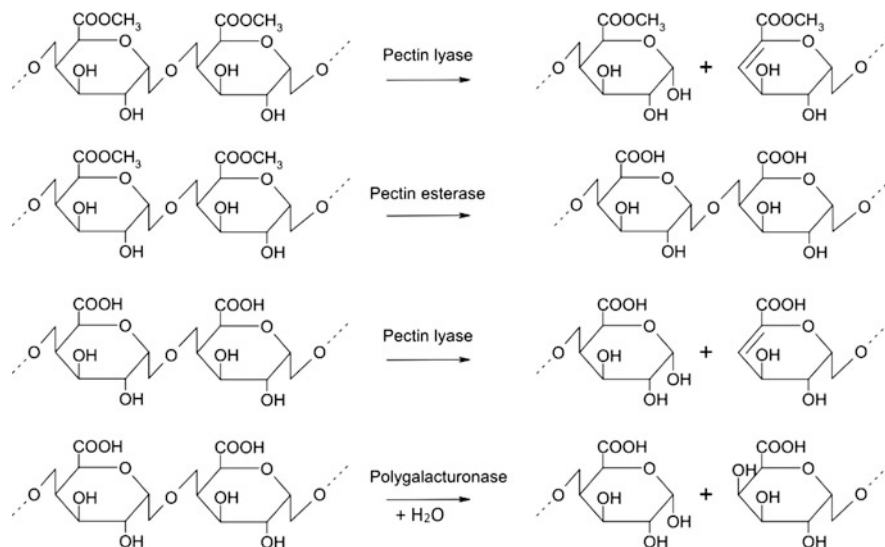


Fig. 13.17. Mode of action of the main pectolytic enzymes

substituted for other fatty acids in fats using a lipase. Also, lipase-catalysed interesterification is used for production of cocoa butter substitutes, whereby the fatty acids at positions 1 and 3 in high-oleate sunflower oil is exchanged with stearic acid. The reaction is catalysed by a lipase from the fungus *Rhizomucor miehei*. A range of other fungal lipases (e.g. from *Fusarium* spp., *Aspergillus* spp., *Humicola* spp., *Thermomyces* spp.) are also commercially available for these applications.

Crude vegetable oils contain phospholipids, triglycerides, diglycerides, free fatty acid and some other minor components. The phospholipids are usually removed to secure a reliable processing and thus a stable and good oil quality. The traditional degumming process includes treating the oil with small amounts of water followed by centrifugal separation. The separated gummy solid of phospholipids gave the process its name.

The phosphate-ester group of phospholipids is water-soluble, but the diacylglycerol backbone is oil-soluble. Phospholipids therefore function as emulsifiers and reduce the separation efficiency of the oil and water phases. Intact phospholipids are entwined with triglycerides representing a loss during degumming. Some non-hydrating phospholipids also remain in the oil and additional processes are required to lower the content. Enzymatic hydrolysis of the phospholipids by phospholipase A1 (EC 3.1.1.32) provides an attractive alternative to these multiple step processes (de Maria 2007). The phospholipase catalyses hydrolysis of fatty acids at one specific position and forms the more water-soluble lysophospholipids (Fig. 13.18). Phospholipase C (EC 3.1.4.1)

separates the oil component (diacylglycerol) from the water-soluble component and increases the oil yield even further by capture of 1,2-diacylglycerol in the oil phase. Several fungal phospholipases are commercially available, e.g. from *Fusarium* spp., *Aspergillus* spp. and *Thermomyces* spp.

VIII. Conclusions

Enzymes of fungal origin are used in a multitude of industrial applications. The benefits of using fungi in industry are manifold and fungi are a rich source of enzymes with valuable properties in industrial processes. A good example is their remarkable high stability, as they have naturally evolved to work in a relatively hostile extracellular environment. The fungi can utilize common “waste” as their source of carbon and energy and secrete the enzyme product from their cells into the medium. The cost of enzyme production in fungi is therefore another attractive parameter and an essential prerequisite for the exploitation of these catalysts in large-scale industrial settings. The market for industrial enzymes was estimated in 2008 by Novozymes to have a value around US \$3 billion. Two major players dominate the industry: Novozymes A/S and Danisco A/S, holding market shares of 47% and 21%, respectively (Fig. 13.19). The market for industrial enzymes has been growing steadily over the past decades, which may be illustrated by the increased sales of enzymes from Novozymes (Fig. 13.20). The net sales of Novozymes thus

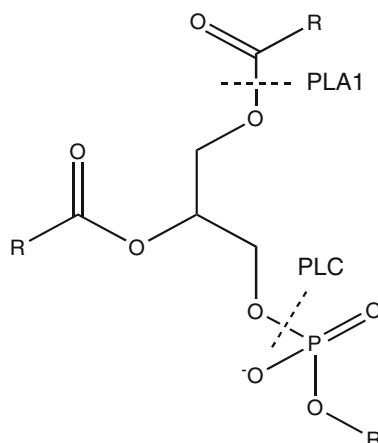


Fig. 13.18. Phospholipases catalyse hydrolysis of phospholipids at specific positions

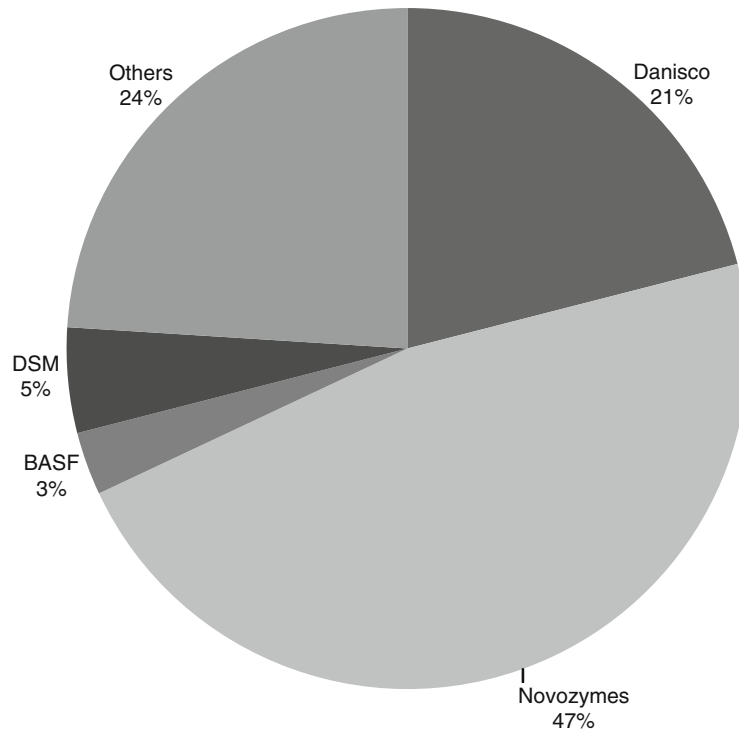


Fig. 13.19. Distribution of market shares among producers of industrial enzymes based on estimates by Novozymes A/S in 2007

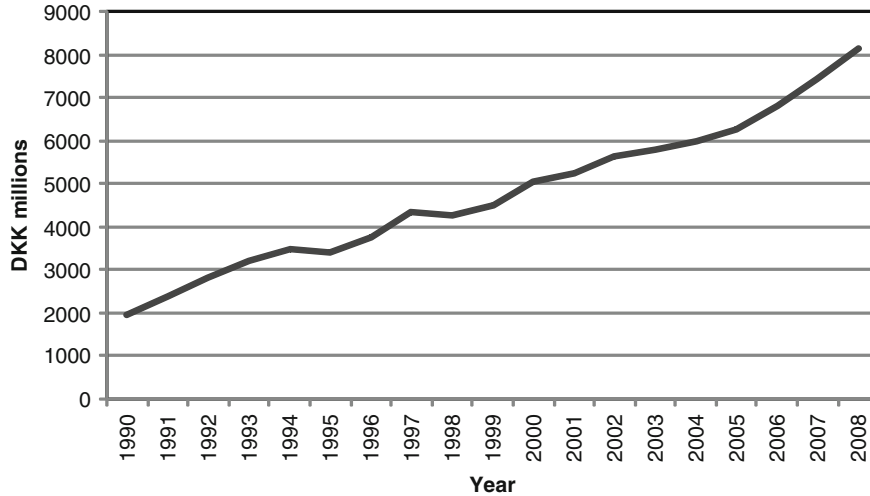


Fig. 13.20. Development in Novozymes' sales (given in million Danish Kroner; DKK) during the years 1990–2008

resulted in a compound annual growth rate of 8% for the period 1990–2008.

This positive development is likely to continue, as industrial enzymes offer very attractive sustainable technologies (Zika 2007). Recent years have seen an ever-increasing focus in the general

public and amongst politicians on environmental issues. Human activities have added significantly to the amount of greenhouse gases in the atmosphere. One of the main greenhouse gases added by humans is carbon dioxide, which has increased from 280 ppm in the year 1750 to 390 ppm today

(United Nations 2009). This accumulation threatens to change the climate of the Earth unpredictably. The application of enzymes allows processes to run at lower temperatures, thus reducing energy consumption and carbon dioxide emission (EuropaBio 2009). A very good and well known example is the use of enzymes in the detergent industry, where even cold-wash detergents have been introduced to the market in recent years. Another important driver for the enzyme industry is the limited reserve of fossil fuels and the resulting price increases (Soetaert 2006). Much of today's industry is based on raw materials and energy coming from fossil fuels. White biotechnology will undoubtedly play a crucial role in the development of alternative and more sustainable industrial production methods based on biorenewable resources (Wohlgemuth 2009).

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**Biotransformations, Lignocellulose Conversion and Recovery
of Metals from Solution**

14 Fungal Biotransformations in Pharmaceutical Sciences

WOLFGANG HÜTTEL¹, DIRK HOFFMEISTER²

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I. Introduction

Filamentous fungi have widely and successfully been used for biocatalysis. Examples include the depolymerization of lignocellulose, food production, and the bioremediation of contaminated soil through the breakdown of environmentally hazardous compounds. In this chapter, we focus on the biocatalytic capacities of fungi to derivatize bioactive small molecules, mainly from natural sources, highlighting examples from the pharmaceutical sciences. We also present selected examples at the interface of biotransformation and pathway engineering using recombinant organisms.

Whole-cell biotransformation represents a sustainable process, and the term “green chemistry” describes well its advantages: biotransformation occurs at ambient or moderately elevated

temperatures, so the use of energy is reduced. Also, the consumption of solvents may be reduced and, hence, hazardous waste which has problematic and expensive disposal. Tedious and time-consuming multi-step synthetic procedures can be circumvented, and expensive cofactors are regenerated by the cells’ intrinsic metabolism. Microbial whole-cell biotransformation is ideally suited for pharmaceutical purposes as it capitalizes on the regio- and stereoselectivity of many enzymatic reactions. Also, microbial diversity and, consequently, the potential realm of metabolic capabilities are vast and even more enhanced by genetic engineering and recombinant techniques. In the pharmaceutical sciences, biotransformations using fungi offer insights into the metabolic fate of a drug or drug candidates and how these are metabolized in eukaryotic systems, or how a microbial natural product is assembled, to learn more about its biosynthesis.

However, whole-cell biotransformation also comes with some drawbacks, such as side reactions which lead to undesired by-products. The work-up of the (desired) product may be complex and include multi-step procedures, such as biomass removal or extraction of the culture liquid. Also, biotransformation is an empirical process, which requires screening and method development for each new substrate to be converted biocatalytically, although experience, extensive libraries and databases are available and help accelerate development. For further and more specific information on biotransformation and the use of fungi and their enzymes for this purpose, we refer readers to a number of excellent broad or specialized review articles covering topics such as microbial biocatalysis for industrial purposes (Zaks 2001; Schoemaker et al. 2003), biocatalytic hydroxylation reactions (Holland and Weber 2000) and specifically hydroxylations of steroids (Holland 1999), fungal epoxide hydrolases (Archelas and Furstoss 1999), biotransfor-

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mations using species of the genus *Botrytis* (Aleu and Collado 2001) or *Candida* (Gamenara and Dominguez de Maria 2009), dehydrogenation with yeast dehydrogenases (Stewart 2006) and the comprehensive book chapter on biotransformation in pharmaceutical biotechnology by Chartrain and Sturr (2005), which also extensively covers practical and experimental considerations which, therefore, do not need to be repeated here.

II. Types and Properties of Biotransformations

The term biotransformation describes the chemical modification of a compound by an organism. In a narrower sense, biotransformation only refers to reactions inside the living cell. However, in case of unicellular or filamentous fungi, it can often not be distinguished whether a reaction takes place within a cell or is catalyzed outside by secreted enzymes or enzymes from lysed cells. Consequently, all chemical reactions carried out by the organism are considered biotransformations. In biotechnology, the term biotransformations often refers to any enzyme-catalyzed reaction, including those with cell extracts or isolated enzymes.

In general, two different types of biotransformations can be distinguished. On the one hand, there are xenobiotic transformations in which the substrate is completely unknown to the microorganism; on the other hand, there are biosynthetically patterned transformations in which the substrate is related to a natural intermediate of a biosynthetic pathway intrinsic to the organism (Hanson and Royal Society of Chemistry 2008). When setting up a biocatalytic process, the state in which the biocatalyst will be applied must be considered. Biotransformations can be carried out with vital cells in a growing or resting state, with lysed cells, cell-free extracts or isolated enzymes. If vital cells are used, enzymes and cofactors will be supplied continuously by the cells. However, the reaction conditions are limited by the demands of the organism and products often need to be isolated from a huge amount of culture medium. In contrast, the use of dead biomass allows any reaction condition, provided the enzymatic activity remains intact, and even organic solvent may be used. Except for some consecutive reactions, all conversions are catalyzed by enzymes.

The enzyme database BRENDA currently lists 483 200 registered amino acid sequences of enzymes allocated to 4932 different catalytic activities (BRENDA 2009). However, only 14 960 (3%) of these characterized enzymes are from fungi (2385 different catalytic activities). Given the large number of species, the different life forms and the remarkable taxonomic diversity within the fungal kingdom, a much higher number should be expected, indicating that the knowledge on enzyme catalysis by fungi still lags behind that of other groups of organisms, especially bacteria. According to the International Union of Biochemistry and Molecular Biology (IUBMB), enzymes are classified by the reaction they catalyze. All enzymatic activities are ordered in six main enzyme classes (EC), which are further categorized into subclasses (Table 14.1). Each enzymatic catalytic activity can be assigned a four-digit code, of which the first digit stands for the main enzyme class, followed by three different levels of subclassification (Webb 1992). Due to the specific environment in the active site, most enzymes catalyze reactions on a substrate with high selectivity towards a single product. The most important terms to describe the selectivity of chemical reactions are briefly summarized in Table 14.2. Detailed explanations are provided by textbooks of organic chemistry or the IUPAC recommendations (McNaught et al. 1997).

Reactions common to fungal biotransformations are summarized in Fig. 14.1. Depending on the substrate, most frequently observed are dealkylations, especially demethylation, ester or amide hydrolysis and oxidations, such as hydroxylations, nitrogen or sulfur oxidations, dehydrogenations and oxidations of alcohols to ketones and carboxylic acids, respectively. The following two examples may illustrate some important aspects of biotransformations with fungi.

1. Since a large number of enzymes is expressed in any cell, it is likely that more than one enzyme catalyzes reactions on a substrate. Consequently, more than one product is found in the extract. The reduced selectivity, however, can be very disadvantageous for biotechnological applications. β,δ -Dihydroxyhexanoates are valuable building blocks for the synthesis of various important pharmaceuticals, e.g., the statins (Müller 2005). A straightforward synthetic approach to these compounds is the regio- and stereoselective reduction of the δ -keto group of 3,5-diketohexanoates, such as **1** with alcohol dehydrogenases, followed by a stereospecific chemical reduction to obtain **3** (Fig. 14.2). In contrast to the (S)-enantiomer, there was no enzyme for the stereoselective reduction to the (R)-enantiomer available before 2001 (Wolberg

Table 14.1. Main enzyme classes according to the Enzyme Commission classification

Enzyme class	Number of enzymes (number of sequences) ^a	Prominent subclasses	Type of reactions	Utility ^b (% research)
1. Oxidoreductases	1323 (261 272)	Dehydrogenases, oxygenases	Electron transfer, i.e., oxidations or reductions in which oxygen and hydrogen are gained or lost	+++ (25%)
2. Transferases	1318 (253 343)	Kinases, acetyl-, methyl-, aminotransferases, polymerases	Transfer of functional groups	+ (~5%)
3. Hydrolases	1487 (131 729)	Lipases, esterases, nucleases, glucosidases, ATPases	Hydrolysis	+++ (60%)
4. Lyases	471 (92 795)	Decarboxylases, aldehydylases, hydrolyases, synthases	Removal or addition of atom groups without hydrolysis	++ (~7%)
5. Isomerases	182 (41 632)	Racemases, epimerases, isomerases	Rearrangement of atoms within a molecule	+ (~2%)
6. Ligases	151 (63 469)	Synthetases	Formation of C-C-, C-S-, C-O- or C-N- bonds through condensation and ATP breakdown	± (1%)

^aBRENDA (2009)

^bThe estimated “utility” of an enzyme class for the transformation of non-natural substrates ranges from +++ (very useful) to + (little use). The percentages indicate the amount of research performed with enzymes from a given class for the 1987–2003 period (Faber 2004)

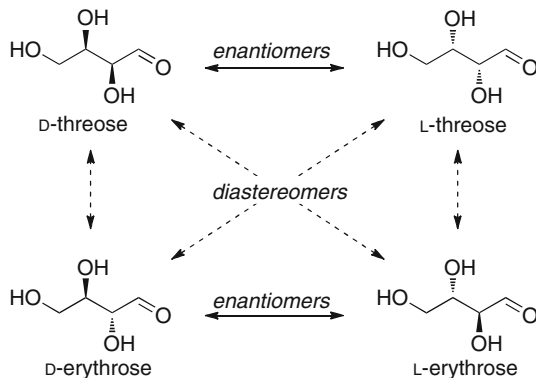
et al. 2001). However, whole-cell biotransformation with baker's yeast (*Saccharomyces cerevisiae*) gave the (*R*)-product in 52% yield, but the enantiomeric excess was rather poor (41% enantiomeric excess; *ee*). By applying two-phase systems the enantiomeric excess could be increased up to 96%. Yet, a recrystallization step was required to obtain the enantiopure compound. Later, a library of baker's yeast ketone reductases, assembled from the analysis of the yeast genome, was screened for catalytic activity on substrate **1** (Wolberg et al. 2004). Among the 12 most promising reductases three NADPH-dependent enzymes were active. They all converted **1** into the (*R*)-product **2** with *ee* values of 96–>99%.

2. Compound **6** is a phosphodiesterase inhibitor developed to treat asthma and should be synthesized stereoselectively in preparative scale for further evaluation (Chartrain et al. 2000). The stereoselective reduction of bisarylketone **4** to the chiral alcohol **5** followed by a chemical conversion into the product **6** was considered to be the most straightforward synthetic approach

(Fig. 14.3). However, the sterically demanding phenyl rings on either side of the keto-moiety made it a very difficult substrate for enzymes, and chiral chemical reagents were unlikely to react stereoselectively. Thus, a collection of microorganisms was screened for activity. A total of 310 microorganisms (53 bacteria, 113 yeasts, 144 filamentous fungi) were evaluated for activity. Eight strains (five filamentous fungi, three yeasts, no bacteria) were able to reduce **4**. Remarkably, both enantiomers were obtained by different fungi with high enantiomeric excess (*S*: 96% *ee*; *R*: > 99% *ee*). For preparative production, the yeast *Rhodotorula pilimanae* ATCC 32762 producing the (*S*)-enantiomer with 96% *ee* was selected. The bio-reduction process was scaled up to a 16-l fermentation. Approximately 10% of the substrate (16 g) were converted into the corresponding alcohol within 48 h. After extraction of the culture broth and purification, 0.92 g of pure (*S*)-alcohol were isolated (5.7% yield, *ee* > 96%). This exemplifies that fungal biotransformations have significant potential for novel activities, even with very demanding substrates.

Table 14.2. Definition of terms describing chemical selectivity

Term	Description
Stereoisomers	Isomeric molecules that have the same molecular formula and sequence of bonded atoms (constitution), but which differ only in the three-dimensional orientations of their atoms in space.
Enantiomers	Two stereoisomers which are non-superimposable mirror images of each other.
Diastereomers	Stereoisomers which are not enantiomers.



Chemoselectivity Preferential reaction of a chemical reagent with one of two or more different functional groups.
Regioselectivity Preference of one direction of chemical bond making or breaking in all other possible directions.
Stereoselectivity The preferential formation in a chemical reaction of one stereoisomer over another. If the stereoisomers are enantiomers, the phenomenon is called enantioselectivity, which is quantitatively expressed by the enantiomeric excess (*ee*). If they are diastereoisomers, it is called diastereoselectivity, which is quantitatively expressed by the diastereoisomeric excess (*de*; applicable for two diastereomers only).

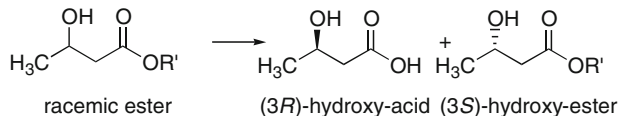
$$\text{Enantiomeric excess: } ee = \frac{|c_{E+} - c_{E-}|}{c_{E+} + c_{E-}} \cdot 100\% \quad \begin{array}{l} c_{E+}: \text{concentration of (+)-enantiomer} \\ c_{E-}: \text{concentration of (-)-enantiomer} \end{array}$$

$$\text{Diastereomeric excess: } de = \frac{|c_{D1} - c_{D2}|}{c_{D1} + c_{D2}} \cdot 100\% \quad \begin{array}{l} c_{D1}: \text{concentration of diastereomer 1} \\ c_{D2}: \text{concentration of diastereomer 2} \end{array}$$

Racemate or racemic mixture A mixture of two enantiomers in equal concentration; *ee* = 0.

Stereospecificity 1. The stereochemical course of a reaction is given by the reaction mechanism, e.g., S_N2 -reactions.
 2. In the context of enzyme catalysis, an enzyme accepts only one stereoisomer as substrate.

Kinetic resolution of a racemate Due to its stereospecificity an enzyme converts one stereoisomer much faster than the other, thereby creating an excess of the less reactive enantiomer.



III. Biotransformation of Pharmaceutically Relevant Small Molecules

A. Alkaloids

The term alkaloid describes a structurally and biosynthetically heterogeneous group of natural

products whose basic character stems from a nitrogen-containing heterocycle. These important microbial or plant secondary products exhibit pronounced bioactivities, and many of these compounds are relevant for medical and pharmaceutical purposes. Among the most prominent examples are: (1) the benzylisoquinolines, which include the narcotic morphine, as well as codeine

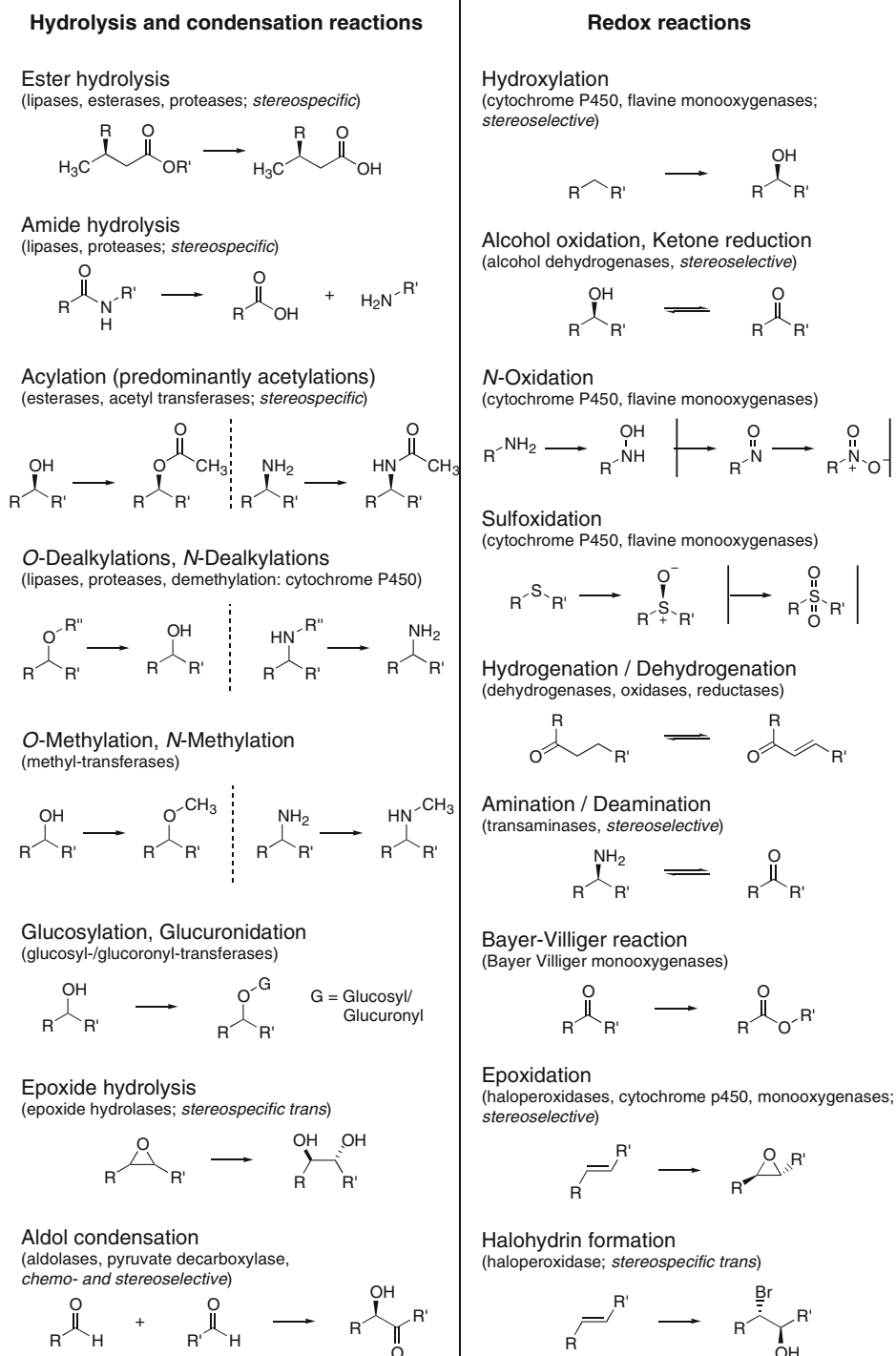


Fig. 14.1. Overview of enzymatic reaction types common to fungal biotransformation

(7) and thebaine (8), and (2) the L-tryptophan-derived indole alkaloids, e.g., the structurally complex ergot alkaloids.

Filamentous fungi have successfully been used in the bioconversion of alkaloids. With respect to the benzyloquinolines, Abel et al. (2003) tested

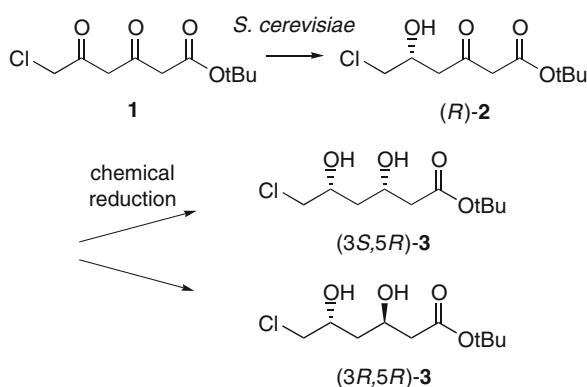


Fig. 14.2. Stereoselective chemoenzymatic reduction of 6-chloro-3,5-diketohexanoate to *syn* and *anti* diols

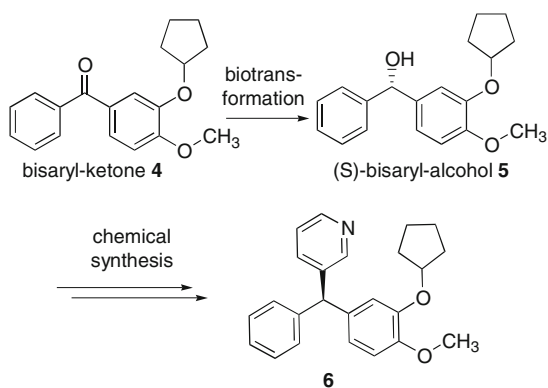


Fig. 14.3. Stereoselective reduction of a bisarylketon to an alcohol by biotransformation en route to the synthesis of phosphodiesterase inhibitor 6

various *Cunninghamella* strains for their ability to *N*- and *O*-demethylate thebaine derivative 9 (Fig. 14.4). This was done to biocatalytically generate precursors 10a–c, potentially useful for the synthesis of buprenorphine, a potent μ -opioid receptor agonist. *C. echinulata* NRRL 1384 turned out as the optimal strain.

N- and *O*-demethylation was carried out in batch cultures and as a function of temperature and pH. Optimal demethylation, i.e., a 25% conversion, occurred at pH 5 and 28 °C. The process was also run in the presence of cytochrome P₄₅₀ inhibitors (5 mM 1-aminobenzotriazole and 1 mM metyrapone, respectively), both of which completely suppressed demethylation, lending further support to the notion that these reactions are P₄₅₀-mediated.

Two decades prior, biocatalytic *N*-demethylation of codeine (7) into norcodeine (11) was reported (Gibson et al. 1984), using cell-free extracts of *C. bainieri*. The authors proposed that

the *N*-oxide is not a direct precursor to 11, as potassium cyanide, an inhibitor of *N*-oxide demethylases, did not affect the conversion, while P₄₅₀ inhibitors did. Another member of the mucoromycetes, *Mucor piriformis*, efficiently *N*-dealkylates thebaine (8) and a set of *N*-alkyl derivatives (e.g., 12) in yields of above 80% (Madyastha and Reddy 1994).

The psychotropic effect of the basidiomycetous *Psilocybe* species, also dubbed “magic mushrooms”, mainly stems from the indole alkaloid psilocybin (4-phosphoryloxy-*N,N*-dimethyltryptamine, 13a, Fig. 14.5), which, after uptake, is dephosphorylated. For biotransformation, the synthetic precursor mimic diethyl-*N,N*-tryptamine (14) was fed to undifferentiated or fruiting mycelia of *Psilocybe semilanceata* and *P. cubensis*, respectively (Gartz 1989). The mycelia biotransformed the indole system by hydroxylation at position 4 and subsequently formed the phosphoric acid ester, to yield 4-phosphoryloxy-*N,N*-diethyltryptamin (15). Hydroxylation and esterification also occur as terminal steps of the regular intrinsic pathway leading to psilocybin and upon feeding of the natural precursor *N*-methyltryptamine (16), which is then transformed to baecocystin (13b).

A transgenic approach was taken to biotransform tryptamine (17) and secologanin (18) into strictosidine (19; Fig. 14.6), a complex indole alkaloid produced by the apocynaceous plant *Catharanthus roseus*. 19 is a central intermediate to a wide variety of monoterpene indole alkaloids, including pharmaceutically relevant small molecules such as serpentine and the antiarrhythmic agent ajmaline. From the native producer, *C. roseus*, the cDNAs for strictosidine synthase and strictosidine β -glucosidase were cloned and heterologously expressed in the yeast *Saccharomyces cerevisiae* (Geerlings et al. 2001). This recombinant strain yielded titers of up to 2 g strictosidin per liter medium. Addition of the precursor secologanin was not necessary when the yeast was grown on snowberry extracts (*Symphoricarpos albus*) as secologanin source. Pentoxifylline [1-(5-oxohexyl)-3,7-dimethylxanthine, 20, Fig. 14.6] is a xanthine used in the treatment of vascular occlusive diseases. The above-mentioned *Cunninghamella* strain NRRL 1384 was also used for enantioselective reduction/oxidation processes pertaining to 20 and propentofylline (21; Pekala et al. 2009).

Fig. 14.4. Examples of biotransformations with benzyloisoquinoline alkaloids

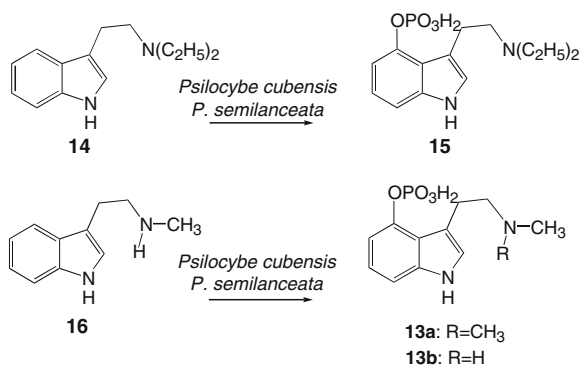
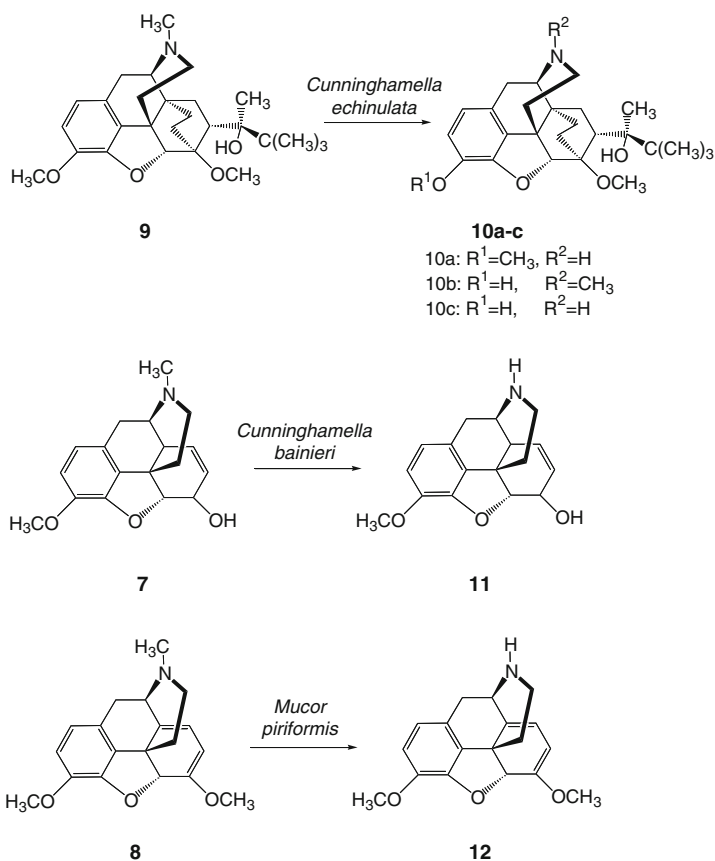


Fig. 14.5. Conversion of indol alkaloids by *Psilocybe* species

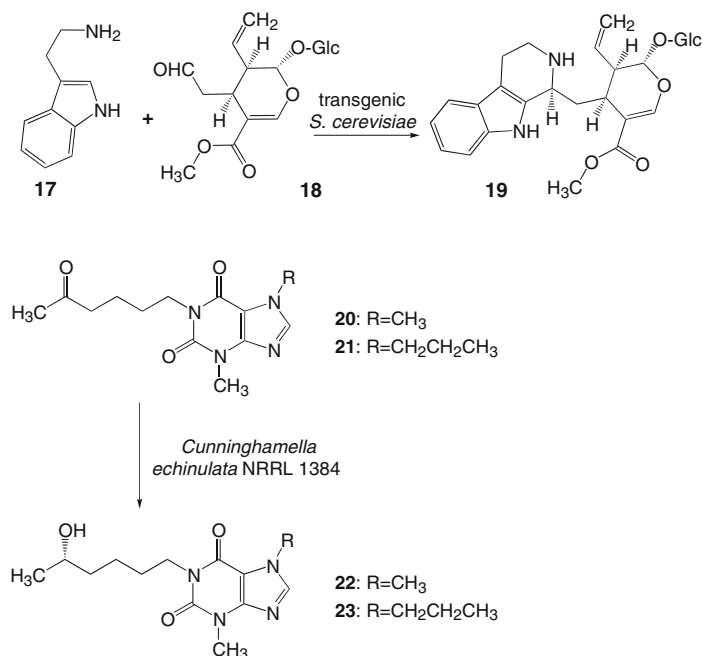
In agitated cultures, the fungus stereoselectively reduced the carbonyl of **20** and its analog **21** to the corresponding (*S*)-hydroxy analogs **22** and **23** (*ee* 98% and 65%, respectively). Conversely, from racemic mixtures of hydroxypentoxifylline and hydroxypropentofylline, an enantioselective (*S*)-oxidation to **20** and **21** was observed (*ee* 86% and 83%, respectively, calculated by quantifying the (*R*)-enantiomers of the substrate that remained unconverted in the medium).

B. Sterols, Taxanes and Other Terpenes

One of the major classes of bioactive and, thus, pharmaceutically relevant natural compounds are the terpenes. In this section, we focus on biotransformation regarding two particularly outstanding classes of terpene compounds: the triterpenoid sterols and the diterpenoid anticancer agent paclitaxel. Numerous other examples of biotransformation of mono-, sesqui- and diterpenes by fungal cells have been reported and reviewed (Ishida 2005; Arantes and Hanson 2007; Simeo and Sinisterra 2009).

The biotransformation of sterol substrates has been extensively investigated for about 60 years. The introduction of alcohol functionalities to different carbons is a very common biotransformation, e.g., catalyzed by *Mucor plumbeus*, as summarized by Lamm et al. (2007). Other types of transformations catalyzed by fungi include selective deglycosylation, dehydrogenation and reduction, isomerization, and C-C-bond cleavage. A biotechnological classic and undoubtedly one

Fig. 14.6. Strictosidine assembly by transgenic yeast (*upper reaction*) and stereoselective reduction of xanthine alkaloids (*lower reaction*)



of the most eminent examples of biotransformation in general is represented by the P₄₅₀-catalyzed hydroxylation of the non-activated carbon atom 11 of progesterone (24) to 11 α -hydroxyprogesterone (25; Fig. 14.7) by *Rhizopus nigricans* (and other fungal species, such as *Aspergillus ochraceus*). 25 is a key precursor in the production of the anti-phlogistic cortisones. This discovery, first published by Peterson et al. (1952), and subsequent development into and implementation of an industrial process allowed its production at reasonable costs.

The substrate for this biotransformation (either diosgenine from *Dioscorea* species, a monocotyledonous plant, or from stigmasterol from soybean) is available in a five-step synthesis and, given the availability of the fungal biocatalysis step, eventually translated into a 13-step synthesis to cortisone, as opposed to the total synthesis with 26 steps. The 11 β -hydroxylation by fungi is also well described, e.g., for *Aspergillus tamarii* (Brannon 1965).

Another hydroxylation capability harnessed on a production scale is the introduction of a secondary alcohol in the 15 α -position into 18-methyl-4-estrene-3,17-dione (26) by *Penicillium raistrickii* ATCC 10490 (Hofmeister et al. 1986; Fig. 14.7). The 15-hydroxy derivative 27 is then further converted by chemical means to gestodene (28), a hormonal contraceptive. 14 α -hydroxy-androstenedione (29) has been produced biocatalytically by

Curvularia lunata CBE, using an equimolar androstenedione (30)/methylcyclodextrin complex as educt, at an androstenedione load of 5 g/l. Maximum conversion (70%) was found after 30 h. However, this example also illustrates, once more, the formation of by-products, in this case the unwanted side reactions led to formation of 11 β -hydroxy-androstenedione (31) along with 11 β -hydroxy-testosterone (32; Yaderets et al. 2009).

Cinobufagin (33) is a cytotoxic 14 β ,15 β -epoxy-bufadienolide and major constituent in a traditional Chinese medicine preparation, obtained from the skin secretion of a toad. Ye et al. (2004) investigated a panel of filamentous fungi and used *Alternaria alternata* AS 3.4578 to simulate mammalian transformation of cinobufagin and to test bioactive derivatives for more favorable (i.e., less lipophilic) chemical and pharmacological properties.

Five new natural products were identified as the result of fungal bioconversion (Fig. 14.8): 12 β -hydroxy desacetylcinobufagin (34), 3-oxo-12 β -hydroxycinobufagin (35), 3-oxo-12 β -hydroxy-desacetylcinobufagin (36), 12-oxo-cinobufagin (37) and 3-oxo-12 α -hydroxycinobufagin (38). The hydroxylation occurred promptly and was complete within 8 h. The authors suggest that the 12 α -hydroxyl group may occur due to oxidation of the newly introduced 12 β -OH to a carbonyl, and subsequent stereospecific reduction to the 12 α -hydroxyl functionality.

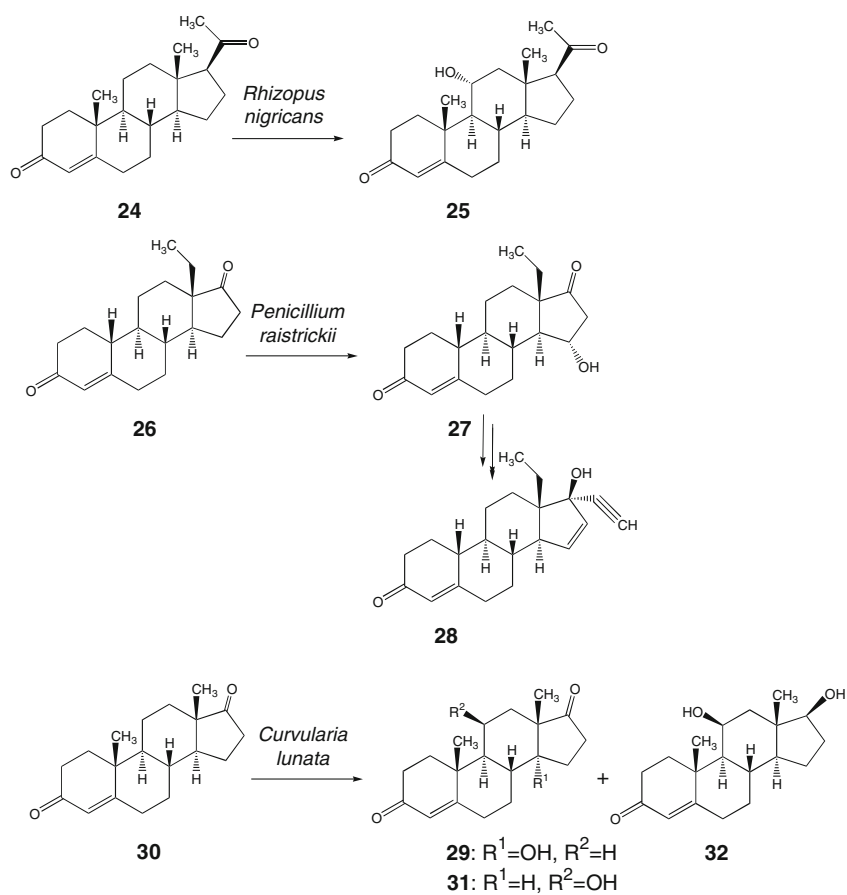


Fig. 14.7. Steran hydroxylations by fungi

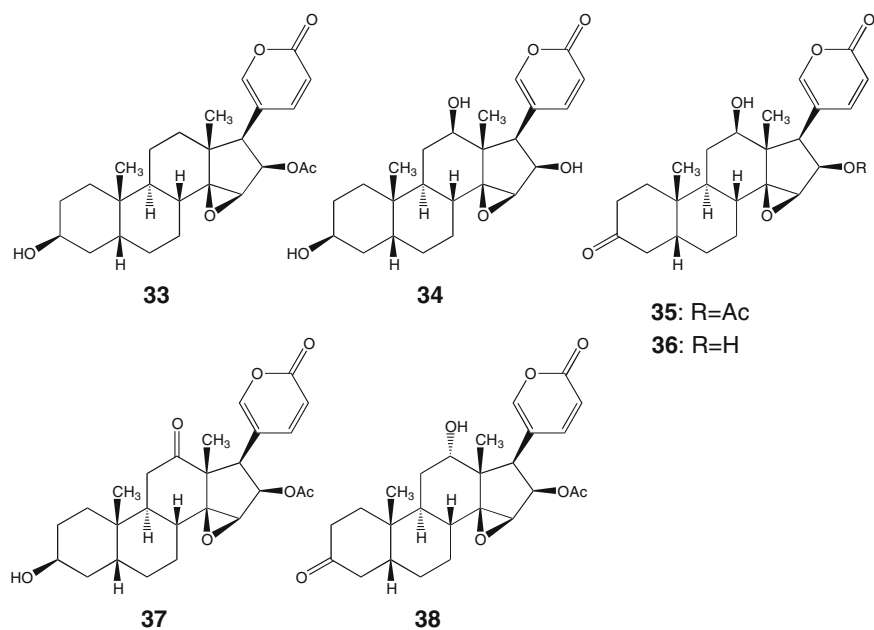


Fig. 14.8. Chemical structures of cinobufagin 33 and derivatives obtained through biotransformation

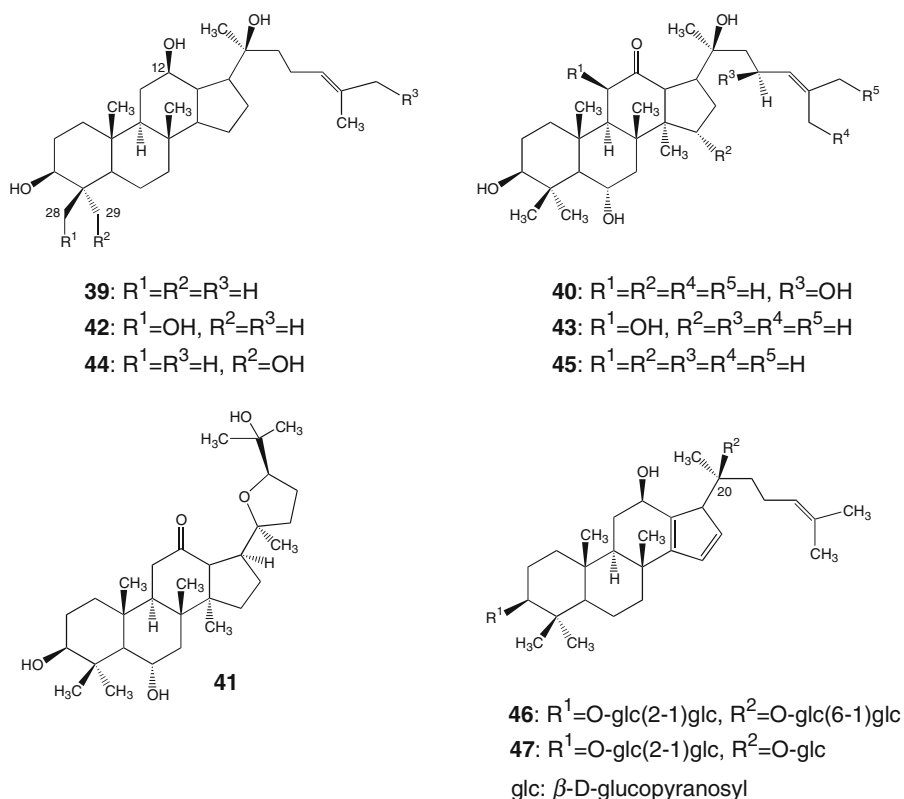


Fig. 14.9. Bioconversion of protopanaxatriol **39** into products **40–45**, and selective deglycosylation of ginsenoside Rb₁ **46** into ginsenoside Rd **47**

Another example of a compound of the traditional Chinese medicine whose bioactive principle possesses a sterane ring system and for which cytotoxic effects have been reported are the ginsenosides, produced by the plant *Panax ginseng*. One major direct breakdown product after oral uptake that indirectly mediates cytotoxicity on tumor cells is 20-(S)-protopanaxatriol (**39**). *Mucor spinosus* AS 3.3450 served as biocatalyst to convert this metabolite and to establish a preliminary structure–activity relationship by determining the bioactivity of the follow-up products (Zhang et al. 2007; Fig. 14.9).

The fungal bioconversion resulted in ten products, six thereof being new compounds: 12-oxo-23 β -hydroxy-20(S)-protopanaxatriol (**40**), 20(S),24(R)-epoxy-dammaran-3 β ,6 α -25-triol-12-one (**41**), 29-hydroxy-20(S)-protopanaxatriol (**42**), 12-oxo-11 β -hydroxy-20(S)-protopanaxatriol (**43**), 28-hydroxy-20(S)-protopanaxatriol (**44**), 12-oxo-20(S)-protopanaxatriol (**45**). Along with other derivatives, these new protopanaxatriol derivatives helped identify or confirm that the presence of hydroxyl groups at either

carbon 28 or 29 and a ketone at carbon 12 correlate with increased activity of ginsenosides.

Also pertaining to the ginsenosides is a report on a remarkably selective hydrolysis of the $\beta(1\rightarrow6)$ -glycosidic bond by *Cladosporium fulvum*, to convert ginsenoside Rb₁ (**46**) to ginsenoside Rd (**47**) in high yield (Zhao et al. 2009). Background for this study was that **46** is the quantitatively dominant natural product in the ginseng root, yet the potentially more interesting drug candidate is **47**, which differs in the saccharide decoration at carbon atom 20: **46** carries a D-glucose (6 \rightarrow 1)-D-glucose chain, while **47** is decorated only with a D-glucose monomer. Previous attempts to bioconvert **46** into **47** suffered from low selectivity, so other glycosidic bonds were hydrolyzed as well, resulting in a low yield of ginsenoside Rd. As with numerous other examples, this biotransformation process required optimization of fermentation conditions, maximum yields (86%) were observed at pH 5–6, with 250 mg/l, after 8 days.

Over the decades, various fungi, e.g., *Aspergillus tamarii* QM1223 and *Gliocladium catenulatum* ATCC10523, have been identified which stereospecifically degrade the 24 β -acetyl side chain to biotransform this compound into D-ring lactones

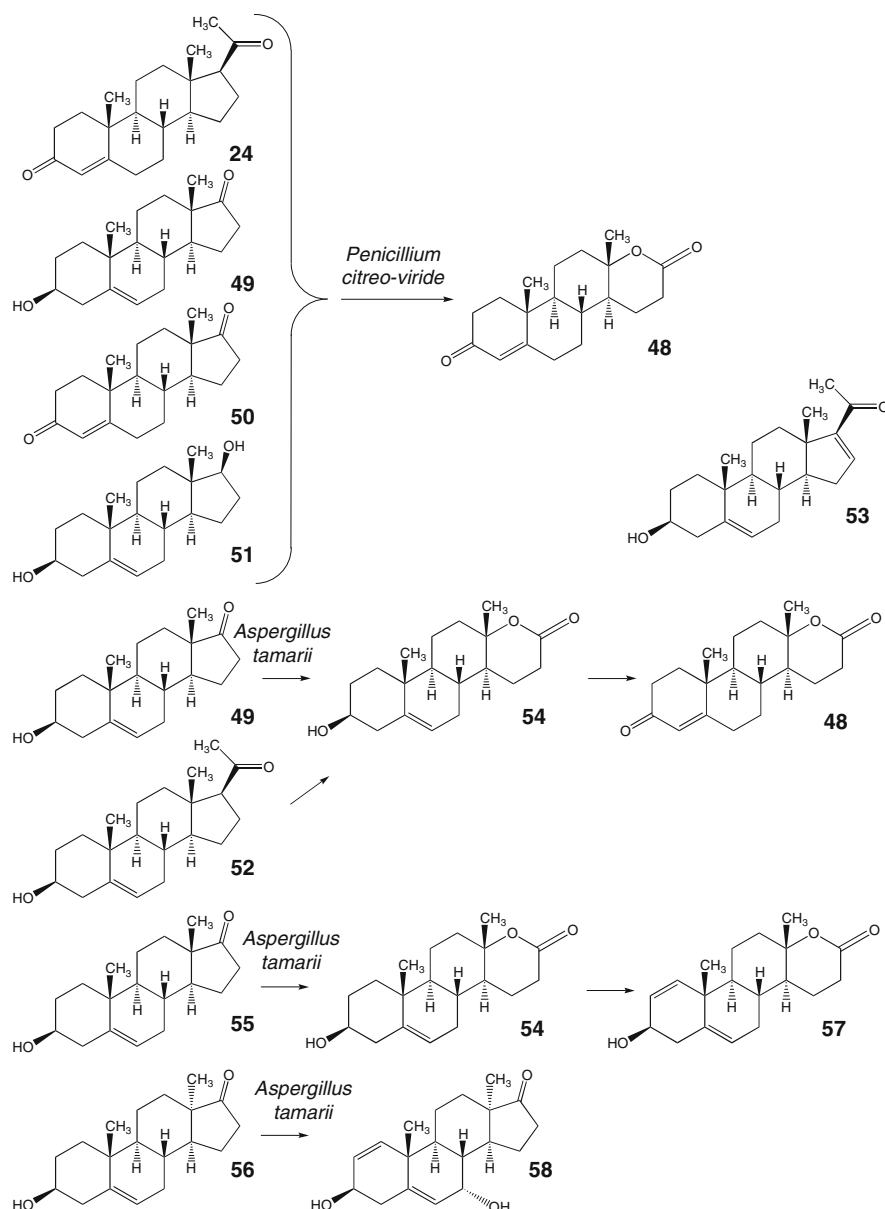


Fig. 14.10. Steran biotransformation by *Penicillium citreo-viride* and *Aspergillus tamarii*

(Peterson et al. 1952b; Brannon et al. 1965; Hunter et al. 2009). The pathway from 24 to testolactone (48; Fig. 14.10) represents a sequential four-step conversion.

The transformation of various steroids – 24, dehydroepiandrosterone (49), 4-androstene-3,17-dione 50, 5-androstene-3,17-diol 51 – fed to cultures of *Penicillium citreo-viride* ACCC 0402 converged in the production of 48 (Liu et al. 2006; Fig. 14.10), while pregnenolone (52) and 3 β -hydroxy-5,16-pregnadien-20-one (53) in contrast were not converted by this fungus. For *Aspergillus tamarii*,

Hunter et al. (2009) also described a structure-dependent turnover and provided evidence for 3 β -hydroxyl dehydrogenase/ Δ^5 - Δ^4 -isomerase activity: 5-ene steroids 49 and 52 fed as substrates were transformed into the corresponding 3-one 4-ene product 48 (via intermediate 54), following the D-ring lactone formation described above. 3 β ,17 β -Dihydroxyandrost-5-ene (55) and 3 β -hydroxy-13 α -androst-5-en-17-one (56) underwent C1–C2 double-bond formation, resulting in products 57 (via intermediate 54) and 58.

Hydroxylation of a set of steroids [24, 49, 52, testosterone (59), cortisone (60), prednisone (61),

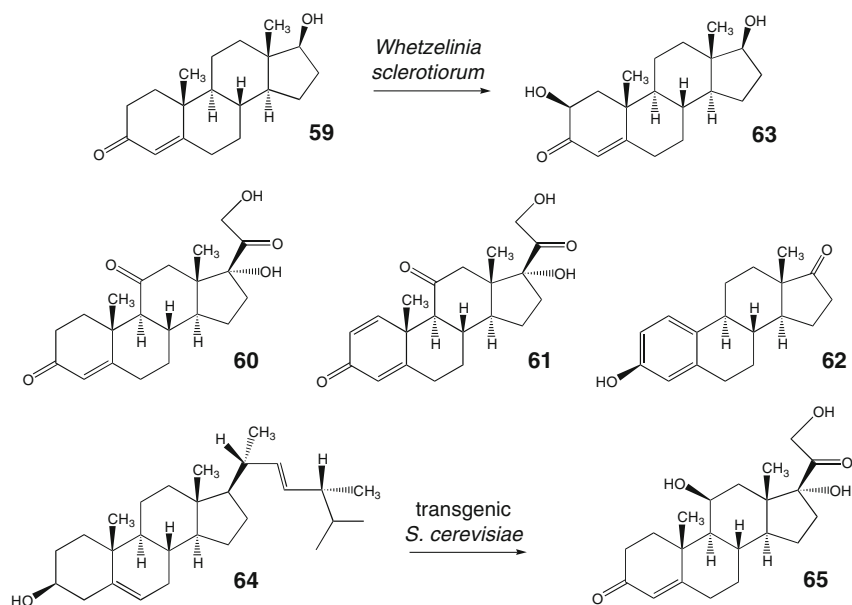


Fig. 14.11. Hydroxylation by *Whetzelinia sclerotiorum*, and hydrocortisone 65 production through genetic engineering of ergosterol 64 metabolism in *Saccharomyces cerevisiae*

estrone (62); Fig. 14.11] was accomplished by *Whetzelinia sclerotiorum* and yielded predominantly β -hydroxyls at carbons 2, 6, 7, 15, 16, as exemplified, e.g., by the conversion of 59 to 2 β ,17 β -dihydroxyandrost-4-en-3-one (63; Lamm et al. 2007). Dumas and coworkers reported a very elegant transgenic strategy to re-route ergosterol (64) metabolism in *Saccharomyces cerevisiae* to produce the mammalian steroid hydrocortisone (65; Szczebara et al. 2003). A set of eight mammalian proteins (four P₄₅₀ enzymes, a 3 β -hydroxyl dehydrogenase/isomerase and three electron transporters, encoded by human and bovine cDNAs) was overexpressed in an engineered host strain optimized to eliminate shunt product formation. Optimum production was accomplished with *S. cerevisiae* strain UCYI whose major steroid (69%, or 11.5 mg/l) produced due to this manipulation was 65.

The diterpene paclitaxel (Taxol, 66; Fig. 14.12) is a member of the taxane class of terpenoid natural compounds and was first discovered in the bark of pacific yew (*Taxus brevifolia*). Paclitaxel has been approved to treat patients with breast, lung, ovarian and other cancers. Due to the low concentration in the bark and as yew extracts usually represent a complex mixture of related metabolites, the production of large quantities proved difficult and raised environmental concerns.

Alternative approaches included isolation from yew suspension cell cultures, or fungi and, as the most viable route, partial synthesis. Microbial bioconversion, was also the object of scientific research on taxanes: the reactions catalyzed by fungi included regio- and stereoselective hydroxylation, backbone rearrangement, hydrolysis and epimerization.

Two *Cunninghamella* isolates, *C. elegans* AS 3.2033 and *C. echinulata* AS 3.1990, were chosen to convert C-14 oxygenated taxanes (Hu et al. 1996). After 48 h growth of *C. elegans*, 2 α ,5 α ,10 β ,14 β -tetraacetoxy-4(20),11-taxadiene (67) was added and subjected to a 9-day whole-cell transformation. After this, three novel 6-hydroxylated and deacetylated products were isolated: 5 α ,10 β -dihydroxy-2 α ,6 α ,14 β -triacetoxy-4(20),11-taxadiene (68), 6 α -hydroxy-2 α ,5 α ,10 β ,14 β -tetraacetoxy-4(20),11-taxadiene (69), 5 α ,6 α ,10 β -trihydroxy-2 α ,14 β -diacetoxy-4(20),11-taxadiene (70). After 11 days incubation of 70 with *C. echinulata* at pH 8 two new deacetylated taxanes were identified [5 α ,10 β ,14 β -trihydroxy-2 α -acetoxy-4(20),11-taxadiene (71), 5 α ,6 α ,10 β ,14 β -tetrahydroxy-2 α -acetoxy-4(20),11-taxadiene (72)], along with further previously described ones. In addition to selective 1 β - and 14 β -hydroxylation reactions, *Absidia coerulea* performed a ring rearrangement. 5 α ,7 α ,9 α ,10 β ,13 α -pentaacetoxy-4(20),11-taxadiene (73; which is relatively readily to obtain from yew trees) served as substrate. When fed to this fungus (50 mg/l, 5-day incubation), two derivatives hydroxylated at the 1 β - or 14 β -position (74, 75) resulted from this experiment, in yields of 39% and 26%, respectively (Hu et al. 1997). The third biotransformation product was the 11(15 \rightarrow 1)*abeo*-taxane derivative (76) of 73.

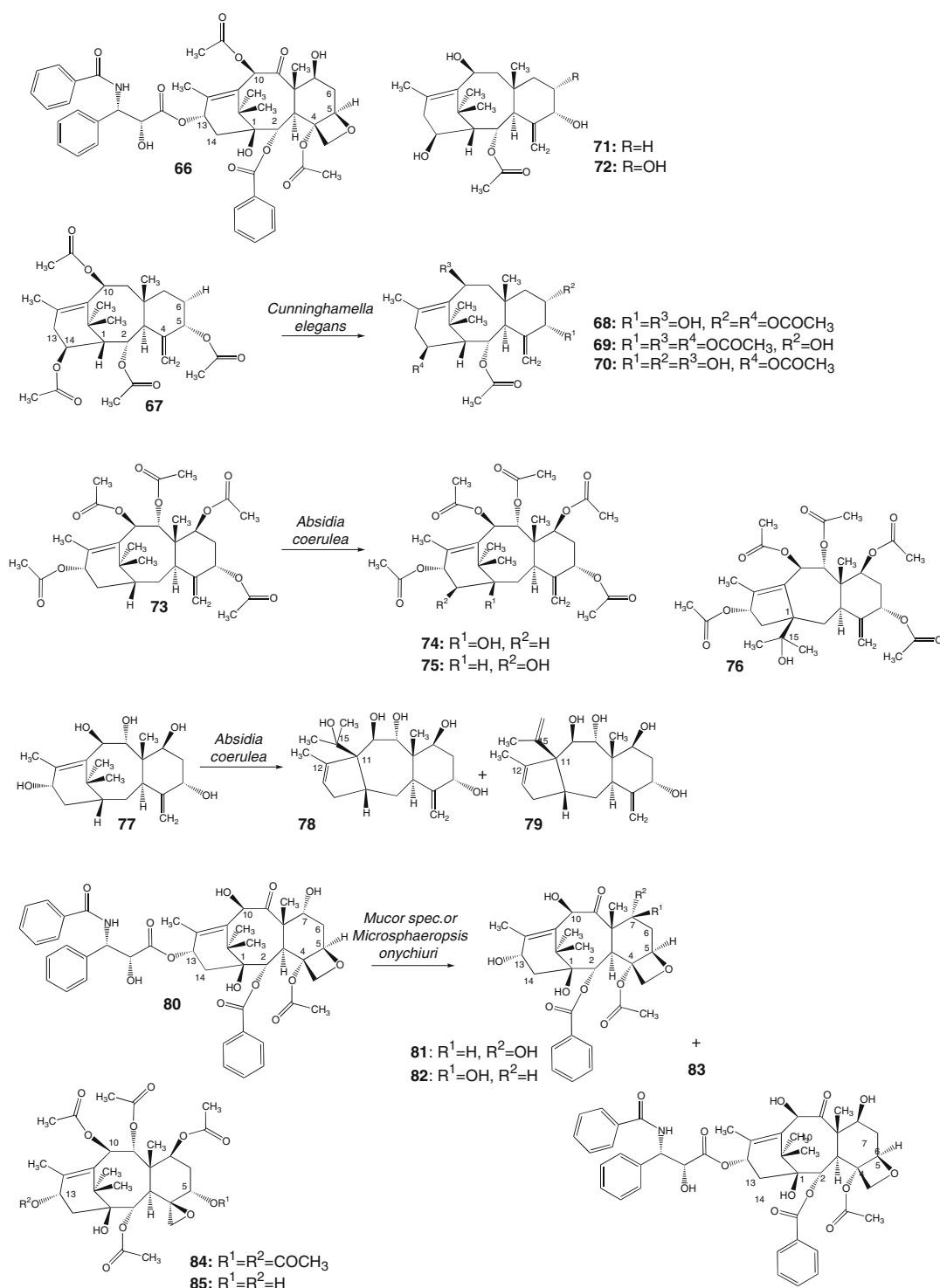


Fig. 14.12. Examples of taxane modification using fungal systems

Ring rearrangements using *Absidia coerulea* ATCC 10738a were also reported by Sun et al. (2001), who used semisynthetic $5\alpha,7\beta,9\alpha,10\beta,13\alpha$ -pentahydroxy-4(20),11

(12)-taxadiene (**77**) as a substrate, which was rearranged to 1 (15→11)*abeo*-analog **78** and **79**, in yields of 42% and 4%, respectively.

droxy-3-methylbutanoyl-*N*-methyl-*L*-phenylalanine dipeptidol building blocks. Beauvericin exhibits insecticidal and antimicrobial activities and received attention for its cytotoxic effects. In a biotransformation approach aimed at redirecting the intrinsic biosynthesis, Xu et al. (2007) generated modified derivatives *in vivo* by feeding substrate mimics of either monomeric building block (*D*-hydroxyisovalerate or *L*-phenylalanine) to submerged cultures. The substitution of one, two, or all three *D*-hydroxyisovalerate units by 2-hydroxybutyrate yielded beauvericins G₁–G₃ (87–89) whereas feeding of 3-fluorophenylalanine led to the corresponding fluorinated analogs, beauvericins H₁–H₃ (90–92). Along with the creation of modified beauvericins, which would have been very difficult to accomplish chemically, these bioconversions also shed more light on structural requirements of the pharmacophore, as beauvericin of the G-series showed decreased antihaptotactic (i.e., cell migration inhibitory) properties, while the H-series beauvericins were somewhat increased in cytotoxicity.

Following-up on these results, Xu et al. (2009) published a mutasynthesis strategy based on a *Beauveria bassiana* strain deficient in the ketoisovalerate reductase. In the wild type, this enzyme supplies the biosynthetic pathway with *D*-hydroxyisovalerate, which is needed for monomer assembly. “Combinatorial” feeding of *D*-2-hydroxybutyrate, 2-hydroxy-3-methylvalerate and fluorinated *L*-phenylalanine analogs directed the engineered biosynthetic machinery to a set of 14 novel beauvericins. Similar approaches to redirect fungal small peptide pathways by feeding precursor analogs also include antileukemic communesins and the lipid-lowering squalenestatsins: A *Penicillium* species known to produce communesins accepted 6-fluorotryptophan as building block which was incorporated and yielded the corresponding monofluoro analogs 93 and 94 (Fig. 14.13) of communesins A and B (95, 96; Wigley et al. 2006). A *Phoma* species fed with fluorinated benzoic acids incorporated those into squalenestatin (97) to yield the difluoro analog 98 (Cannell et al. 1993), while a *Fusarium* species (isolate F13945) converted 97 into its monomethyl ester 99 (Middleton et al. 1995).

Due to their beneficial toxicity profile and their effectiveness, the β -lactams represent antibiotics of utmost importance. A very informative review on industrial β -lactam production was written by Elander (2003). In *Aspergillus*, *Penicillium* and other fungi, β -lactam biosynthesis is initiated by the synthesis of a linear tripeptide, catalyzed by the key enzyme, δ -(*L*-*R*-aminoadipyl)-

L-cysteinyl-*D*-valine synthetase, the ACV-synthetase, which is encoded by the *acvA* gene in *Aspergillus nidulans*. Like the steroids, β -lactams have a long record within biotransformation and, in addition, attracted early attention to increase and optimize the production of drugs or their precursors by genetic engineering. An early example of directed penicillin biosynthesis is described by Ballio et al. (1960), who added a set of dicarboxylic acids to separate fermentations of *Penicillium chrysogenum* Wis 51.20 to alter the penicillin side chain.

Acremonium chrysogenum is used for the large-scale production of cephalosporin C (100), which then serves as starting material for semi-syntheses by chemical conversion into 7-aminocephalosporanic acid (101). As 100 is susceptible to non-enzymatic breakdown, deacetylation to deacetylcephalosporin C (102) may increase the yield of the cephalosporin nucleus in fermentations by 40% (Basch et al. 2004). *Rhodospiridium toruloides* produces an esterase which catalyzes exactly this reaction. For a whole-cell approach, this fungus is added – as live or heat-killed cells – to fermentations. Based on the findings in biotransformation, a heterologous approach included transfer of the esterase gene to *Acremonium chrysogenum*. Active enzyme was successfully expressed, yielding 102.

Another recent example includes a *Penicillium chrysogenum* strain blocked in the final step of penicillin G biosynthesis. This strain served as host to overexpress the cephalosporin-specific genes *cefEF* and *cefG*, i.e., the bifunctional expandase/hydroxylase and deacetylcephalosporin-acetyltransferase, respectively, along with *cefD1* and *cefD2* (isopenicillin N-CoA synthetase, isopenicillin N-CoA epimerase) from *Acremonium chrysogenum* (Fig. 14.14) to epimerize isopenicillin N (103) into penicillin N (104) and then extend the metabolic flow further to 102 (Ullán et al. 2007).

D. Polyketides

The biosynthetic potential of *Beauveria bassiana* ATCC 7159 was already highlighted above for the derivatization of beauvericin. The same fungus was also utilized for the biotransformation of anthraquinones, i.e., polyketide natural products which display a wide variety of bioactivities (e.g., antimicrobial, anticancer). Feeding of

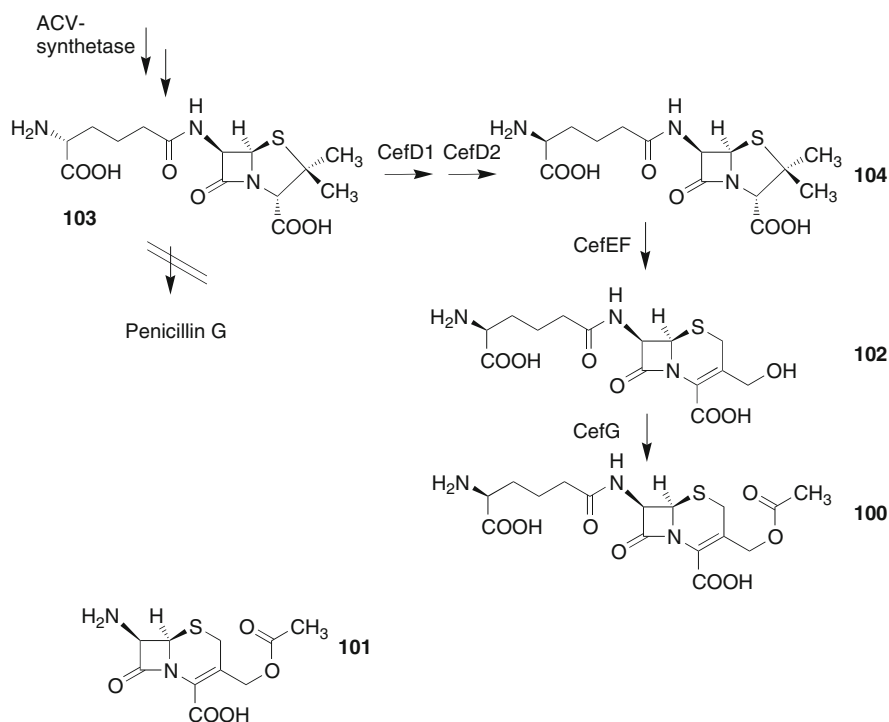


Fig. 14.14. Schematic representation of cephalosporin C **100** biosynthesis and chemical structure of 7-aminocephalosporanic acid **101**

1-amino-2-hydroxyanthraquinone (**105**), 1,2-diaminoanthraquinone (**106**), 1,8-dihydroxyanthraquinone (**107**) and 1,2-dihydroxyanthraquinone (**108**) to submerged cultures of *Beauveria bassiana* ATCC 7159 resulted in the selective glycosylation of the 2-amino group of 1,2-diaminoanthraquinone and remarkable yields (46–72%) of other *N*- and 4'-*O*-methyl-glucosylated anthraquinones **109–112** shown in Fig. 14.15 (Zhan and Gunatilaka 2006).

The polyketide natural product lovastatin (**113**) and natural, semisynthetic and synthetic derivatives thereof are medicinally used lipid-lowering agents. Natural statins, such as **113** and mevastatin (**114**), are produced by a variety of fungal species (Manzoni and Rollini 2002). Initially, biotransformations with cell-free extracts of one of the fungal producers, *Monascus ruber*, have been used to clarify biosynthetic events (Kimura et al. 1990; Nakamura et al. 1990; summarized by Auclair et al. 2001). Although the bacterial biotransformation protocol proved more effective and eventually suitable for the industrial scale, pravastatin (**115**) production fungi have also been used for this bioconversion: **115** differs from **114** in its hydroxy group at C-6.

Upon feeding **114** to *Mucor hiemalis*, selective and efficient 6 β -hydroxylation was found (Manzoni and Rollini 2002; Fig. 14.16). However, as statins also exhibit anti-fungal activity, the fungi tolerated only low substrate concentrations (0.05%). While elucidating the biosynthetic events for lovastatin assembly in the producer *Aspergillus terreus*, Hutchinson and coworkers heterologously over-expressed the genes for the key enzymes, the polyketide synthase LovB and LovC (an additional protein required for proper LovB activity) in *Aspergillus nidulans*. Due to this manipulation, its secondary metabolism was then successfully re-programmed to produce dihydromonacolin L (**116**), the first recognized intermediate of the lovastatin pathway (Kennedy et al. 1999). Follow-up work on this engineered strain identified a new derivative, monacolin N (**117**; Sorensen and Vederas 2003). Whole-cell catalysis using the lovastatin biosynthetic enzyme LovD, heterologously overexpressed in *Escherichia coli*, was performed to produce the clinically relevant semisynthetic simvastatin (**118**; Xie et al. 2006; Fig. 14.16), which differs from **113** in its α -dimethylbutyryl side chain (**113**; α -methylbutyryl). As a final step in lovastatin biosynthesis, the *A. terreus* acyltransferase LovD catalyzes the regioselective transfer of α -methylbutyrate from the second lovastatin polyketide synthase LovF to the C-8 hydroxy group of monacolin J (**119**). In vitro work showed that LovD does not have strict substrate requirements. Upon feeding both **119** and the *N*-acetylcysteamine (SNAC) thioester of α -dimethylbutyrate as LovD substrates, 35% of the former was biotransformed to the desired product **118**. Subsequent

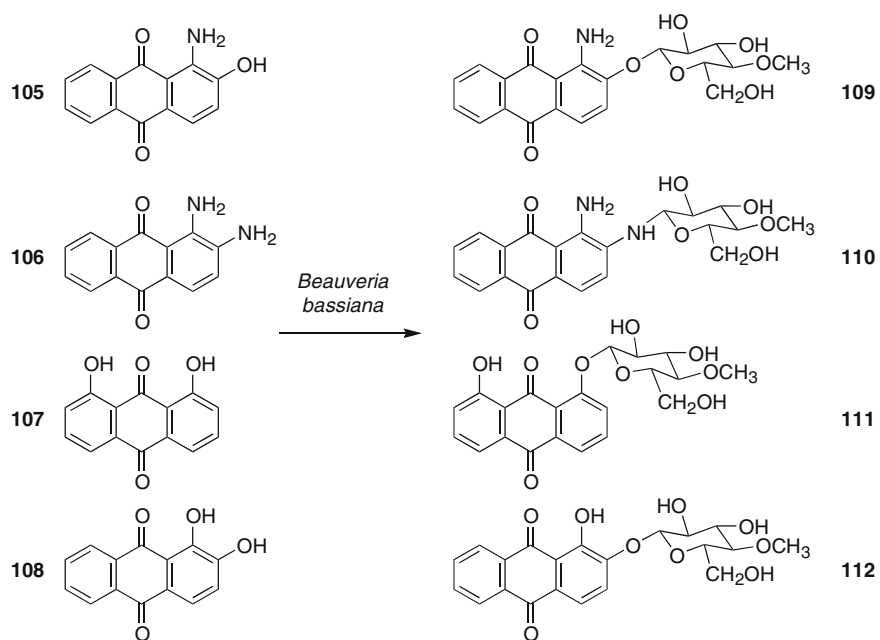


Fig. 14.15. Biotransformation of anthraquinones

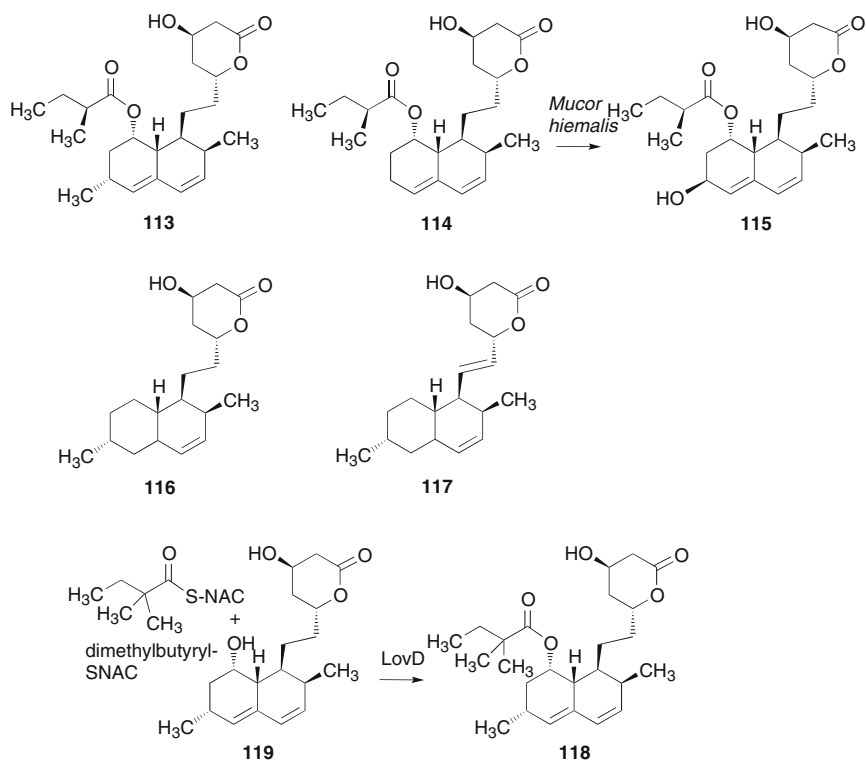


Fig. 14.16. Structures of lovastatin 113 and derivatives

optimization of this process involved replacement of the SNAC-ester as donor: Feeding of α -dimethylbutyryl-S-methyl-mercaptopropionate resulted in almost complete conversion of 119 to 118, very likely due to an increased membrane permeability (Xie and Tang 2007). Without involving any chromatographic steps, the authors achieved a recovery of 1.13 g (i.e. 90%) of 98% pure 118 from a 2-l fermentation.

IV. Biotransformation of Drugs and Various Synthetic Small Molecules

Fungal biotransformations can principally be divided into analytical and preparative applications. Analytical transformations greatly help modeling the metabolic fate of a drug in higher eukaryotes. As fungi subsist on absorption of nutrients, they possess a complex metabolism to degrade compounds, which is, to some extent, reminiscent to mammalian metabolism of xenobiotics. In contrast to mammalian systems, many fungal species are easy to handle, and fermentation methods established from biotechnology with bacteria can be adopted, so that research into the metabolic processes is significantly facilitated.

Preparative applications include the generation of derivatives of bioactive compounds as well as catalytic steps in their synthesis. First, in fungal bioconversions, pharmaceutically interesting compounds are modified in a way which is often not feasible by means of chemistry. Among these derivatives, compounds with novel or enhanced bioactivity can be identified and may expand the knowledge of structure–activity relationships. Second, although a large number of enzymes with various activities are expressed in vital cells, xenobiotic substrates are often transformed with high selectivity to a single product. Some of these selectivities are unique to fungi and can be applied for the production of pharmaceuticals or chiral building blocks. The scope of applications for fungal transformations in synthesis ranges from preparations of novel compounds in the milligram scale for research purpose up to large-scale industrial production of drugs or pharmaceutical intermediates. The following section provides some recent examples for fungal biotransformations of pharmaceuticals or pharmaceutically relevant compounds of synthetic origin.

A. Biotransformations for Preparative Purposes

Ketoprofen (120) is a therapeutically used non-steroidal anti-inflammatory drug (NSAID) and has analgesic and antipyretic effects due to inhibition of the body's production of prostaglandin. There is one stereocenter in the chemical structure of 120 and, although the enantiomers have different physiological effects, it is mostly used as racemate in therapy. The (*S*)-enantiomer, which is marketed under the name dexketoprofen (INN), is produced by enzymatic racemic resolution, e.g., of the (*R,S*)-ethyl ester 121. Besides conversions with isolated enzymes, whole-cell biotransformations have been applied and deliver the (*S*)-enantiomer preferentially as free acid. A conversion with the fungus *Citeromyces matriensis* CGMCC 0573, however, yielded the (*R*)-enantiomer as free acid at 42.6% conversion and 93% *ee* (Fig. 14.17; 120–121; Gong et al. 2002). A complementary racemic resolution with a mutant strain *Trichosporon laibachii* CBS 5791 gave the corresponding (*S*)-enantiomer with 47% conversion and 94% *ee* (Zhang et al. 2005). Notably, in both transformations the addition of Tween 80 significantly enhanced the enzymatic activity.

Like 120, flurbiprofen (122) is a cyclooxygenase-inhibiting NSAID. The main activity relies on the (*S*)-enantiomer, whereas antitumor activity has been found in animal models for the (*R*)-enantiomer. A racemic resolution of the (*R,S*)-flurbiprofen was possible via a (*R*)-specific esterification into ethyl ester 123 (Fig. 14.17; 122–123), catalyzed by *Aspergillus oryzae* (Spizzo et al. 2007). Dry mycelium and organic solvents were applied for this transformation. Best results were obtained with mycelium of *A. oryzae* strain MIM and *n*-heptane as solvent. Of a 10 mM (*R,S*)-flurbiprofen solution with 10 mM ethanol, 40% of the (*R*)-enantiomer were converted to the corresponding ethyl ester within 24 h. The application of organic solvents facilitated the recovery of the organic compounds significantly.

The stereoselective reduction of β,γ -diketoacids with baker's yeast was already discussed in Section II. The stereochemical outcome of such reductions is further complicated if the substrate already contains a chiral center. For example the reduction of a β -ketobutanoate with a methyl group at C-2 can afford four diastereomers: two *cis*- and two *trans*-enantiomers. This demanding stereoselective reduction has been investigated, both with isolated enzymes and with whole cells

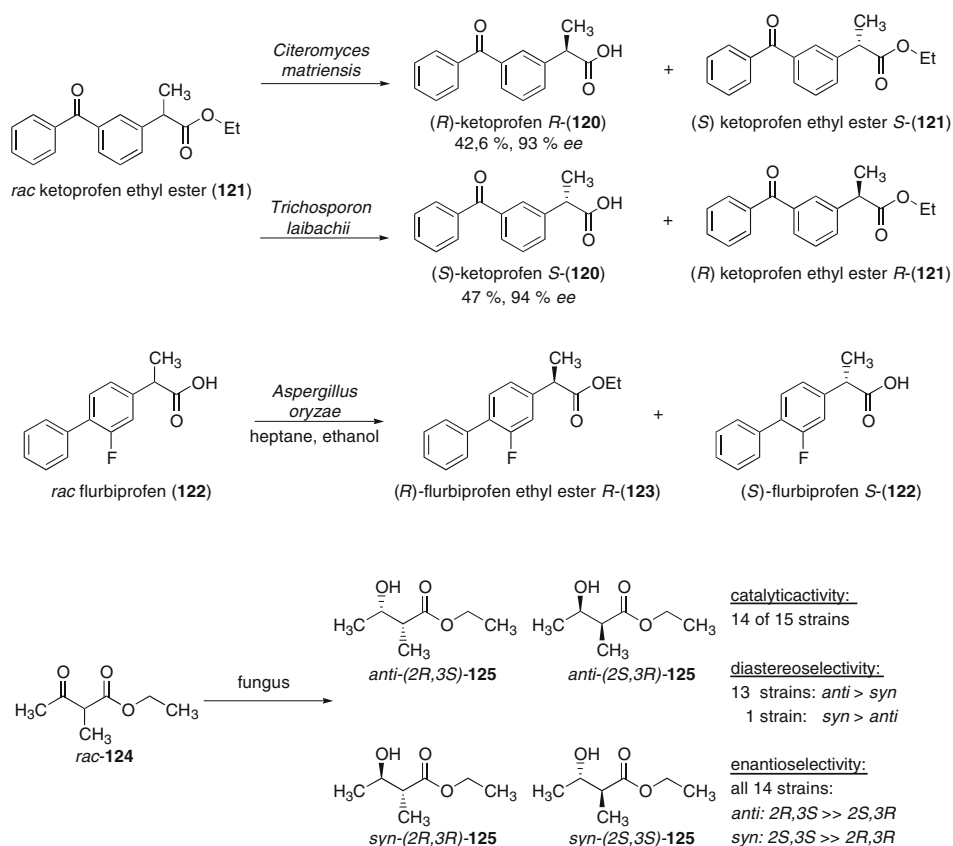


Fig. 14.17. Racemic resolution of non-steroidal anti-inflammatory drugs (NSAIDs) and 2-methyl-3-ketobutanoate by fungi

as catalysts. In a recent study, the reduction of α -methyl- β -oxobutanoate (124) with 15 filamentous fungi and yeast strains was reported (Ravia et al. 2009; Fig. 14.17; 124–125). All but one fungus showed good reduction activity (46–100% conversion). A moderate diastereoselectivity for the *anti*-product was observed (*syn:anti* from 27:73 to 3:97). Interestingly, one strain (CCM B4) of the white-rot fungus *Phanerochaete chysosporium* preferentially produced the *syn*-product (*syn:anti* = 70:30). The enantiomeric excess was moderate to high with a preference for the same enantiomers for all transformations [*syn*-(2*R*,3*S*): 60–99% ee; *anti*-(2*S*,3*S*): 84–99% ee].

B. Fungi as Models for Mammalian Xenobiotic Metabolism

The main intention of studies on the xenometabolism in fungi is to establish straightforward models

for human metabolism. Thus, in the first instance, the utility of a fungal species can be evaluated by the similarity of its xenometabolism to that of humans and mammals. Some species of filamentous fungi of the genus *Cunninghamella*, especially *C. elegans*, *C. blakesleeana* and *C. echinulata*, fulfill this requirement well. Since 1952, more than 90 biotransformations of drugs and organopollutants have been communicated, most of them in the past two decades. A comprehensive overview on bioconversions with *Cunninghamella* was published recently (Asha and Vidyavathi 2009).

The following section illustrates the multitude of studies on fungal xenometabolism, using the example of fluoroquinolone antibiotics which have been investigated frequently. In mammals, common xenobiotic transformations of fluoroquinolone antibiotics include additions of formyl-, acetyl-, sulfate-, methyl- and glucuronide groups as well as oxidation and breakdown of the piperazine ring (Parshikov et al. 2001a).

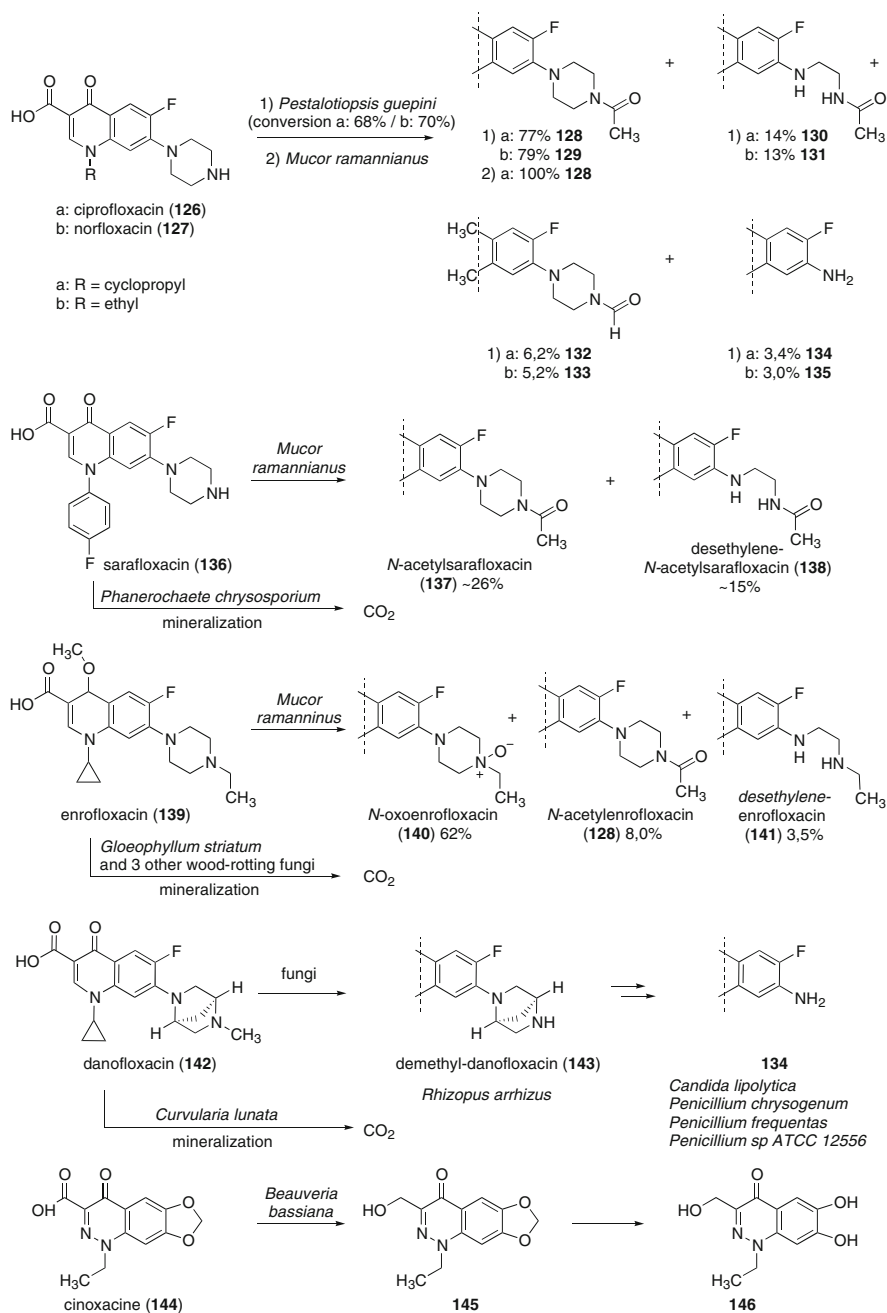


Fig. 14.18. Degradation of fluoroquinolone and related antibiotics by fungi

Three of these derivatizations were observed in biotransformations of the antibiotics ciprofloxacin (**126**) and norfloxacin (**127**) with *Pestalotiopsis guepini* (Parshikov et al. 2001b). For both compounds the conversion and metabolite patterns were very similar (Fig. 14.18). The predominantly observed transformation products were *N*-acetylated derivatives (**128**, **129**), followed by *N*-acetylated desethylened congeners (**130**, **131**), *N*-formyl-products (**132**, **133**) and 7-amino compounds (**134**, **135**). In a previous

biotransformation of **126** with *Mucor ramannianus*, the *N*-acetyl-derivative **128** was obtained as the only product (Parshikov et al. 1999). Notably, the same fungus was able to convert structurally closely related compounds to further metabolites: in a biotransformation with sarafloxacin (**136**), the desethylened-*N*-acetyl-derivative **137** was found in a considerable amount (15%) besides *N*-acetylsarafloxacin (**138**; Parshikov et al. 2001a). Enrofloxacin (**139**) was converted to three metabolites by *M. ramannianus*

(Parshikov et al. 2000). The main product was enrofloxacin-*N*-oxide (140; 62%), a metabolite whose analogs have not been detected in transformations with 126 or 136. Furthermore, an ethyl-acetyl exchange (128; 8.0%) and ethylene cleavage (141; 3.5%) were observed. These diverse results illustrate that the outcome of fungal biotransformations strongly depends on individual compounds, even though their structures are closely related. The degradation of danofloxacin (142) was investigated with a library of 72 soil microorganisms (Chen et al. 1997). Two metabolites in the cultures could be identified as demethyl-danofloxacin (143) and the 7-amino derivative 134 (Fig. 14.18). Whereas 143 was obtained from *Rhizopus arrhizus* and several bacteria species, the complete hydrolysis of the piperazine ring was exclusively catalyzed by fungi (*Penicillium* spp., *Candida lipolytica*).

The antibacterial agent cinoxacin (144), structurally related to both nalidixic acid and oxolinic acid, was transformed with *Beauveria bassiana* ATCC 7159 (Parshikov et al. 2002). Two metabolites were found. In the major product the carboxylic acid was reduced to the corresponding alcohol 145. Putatively in the second step, the methylene

bridge was cleaved so that an *ortho*-diphenol 146 was isolated as the minor metabolite.

Besides conversions to distinct congeners, some fungi are able to degrade pharmaceuticals entirely to carbon dioxide and other small molecules, a process referred to as mineralization. Fungi with this physiological capability are particularly interesting for the detoxification of pharmaceutical wastes or the degradation of environmental pollutants containing pharmaceuticals. In biotransformation experiments these mineralization processes can easily be detected by feeding ^{14}C -labeled substrates and trapping ^{14}C -enriched carbon dioxide ($^{14}\text{CO}_2$) and other volatiles.

Within the scope of a screening for microorganisms which are able to convert danofloxacin (142) one fungus, *Curvularia lunata*, was identified as being able to degrade the antibiotic to carbon dioxide (Chen et al. 1997). Approximately 31% of the radiolabel was recovered as volatiles. Microbial mineralization was also tested for sarafloxacin (136; Marengo et al. 2001). Four soil fungi were tested in biotransformations with the ^{14}C -labeled antibiotic (*Aspergillus*, *Penicillium*, *Chaetomium*, *Phanerochaete*).

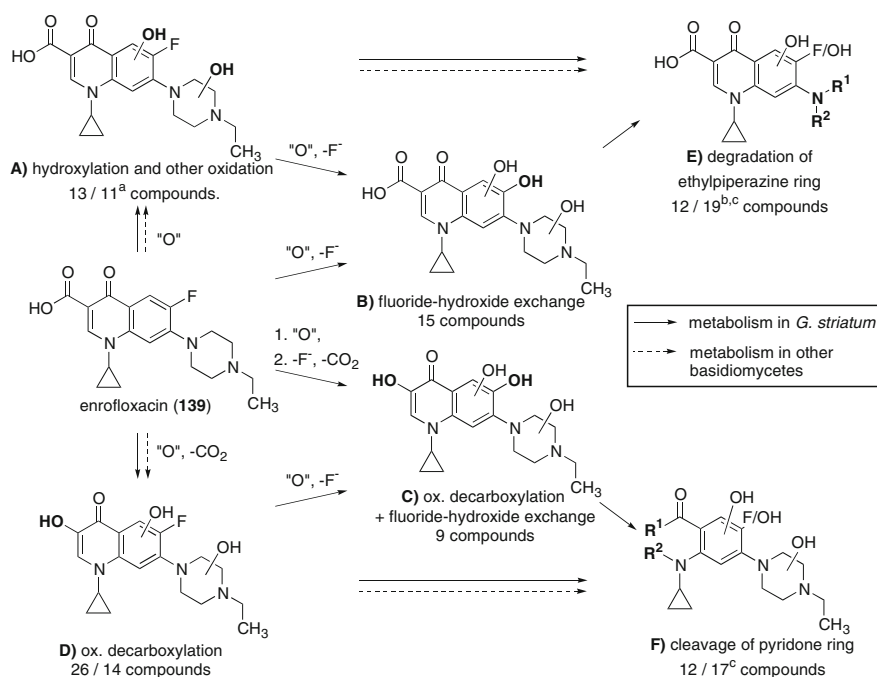


Fig. 14.19. Systematic analysis of enrofloxacin (139) degradation in *Gloeophyllum* and other basidiomycetes species. Each structure stands for a larger number of detected metabolites. The characteristic functional groups of each metabolite class are shown in bold. The number of detected compounds is given below each structure, the first number stands for metabolites detected in *Gloeophyllum* metabolism, the second for other basidiomycetes. Solid arrows describe the putative metabolism in *Gloeophyllum*, dashed arrows that of the other basidiomycetes. Note: Despite some bonds are not further specified, the structures do not include the whole diversity of the metabolite groups, but are based on typical compounds. (a) No aromatic hydroxylation. (b) Almost no aromatic hydroxylation. (c) No defluorination products

The best activity for biodegradation was observed with *Phanerochaete chrysosporium*. 136 was degraded into at least six products. The maximum cumulative ^{14}C production was 17.3% of the applied dose. Four wood-rotting fungi species were tested for their ability to mineralize enrofloxacin (139; Martens et al. 1996). Best results were obtained with three strains of the brown rot fungus *Gloeophyllum striatum* (^{14}C recovery = 53% in 8 weeks).

In a subsequent study the degradation of 139 by the three *Gloeophyllum* strains was analyzed in detail (Karl et al. 2006). By means of ^{14}C -labeled substrate and a highly sensitive HPLC-HRMS assay, it was possible to detect metabolites even in the lowest concentrations. As many as 87 congeners were identified in the crude extract of the fungi cultures. Although the structural transition between the diverse structures was rather smooth, they could be divided into six sections depending on the different key reactions undergone by the substrate (Fig. 14.19A–F). These were: (A) oxygenations at the aromatic or piperazine ring without further key transformations, (B) fluoride–hydroxide exchange, (C) oxidative decarboxylation and fluoride–hydroxide exchange, (D) oxidative decarboxylation, (E) degradation of the ethylpiperazine ring and (F) cleavage of the pyridone ring. A series of analogous transformation experiments was performed with seven other basidiomycetes indigenous to agricultural sites which were assigned to six families of three orders (Wetzstein et al. 2006). The patterns of major metabolites of the seven fungi were surprisingly similar; however, they differed significantly from those of *G. striatum* (Fig. 14.19). In total, 61 compounds were detected, 48 were new while 13 were known from *G. striatum* transformations. The main reason for the difference was the almost complete absence of both aromatic hydroxylations at the quinolinecarboxylic acid moiety and defluorination reactions. Instead, the diversity of modifications at the piperazine ring was enlarged.

Advanced investigations of fungal biotransformations of model compounds like fluoroquinolone antibiotics not only provide a detailed insight into the degradation of pharmaceuticals, they also allow comparative studies on the metabolism of diverse fungi (e.g., zygo-, asco-, basidiomycetes; soft-, brown-, white-rot fungi) and their activities, both non-enzymatic and enzymatic.

V. Conclusions

Biotransformation with fungal cells enables or facilitates access to complex bioactive compounds – both commercial drugs and candidates – due to their capacity to catalyze regio- and stereoselective reactions on small molecules. Also, fungi help expand our knowledge on how pharmaceuticals are metabolized. For drug discovery and production

purposes, whole-cell biotransformation historically relies on the intrinsic metabolic capabilities of a microbial strain. Numerous examples, some of them presented in this chapter, show that “traditional” biotransformation remains a viable and feasible option to derivatize small-molecule natural products in a stereo- and regioselective way, although finding a fungal species able to specifically perform the desired process remains a bottleneck. To circumvent this drawback, for example, lovastatin and the β -lactams alike demonstrate how genetically engineered organisms have expanded the biocatalytic repertoire and enabled custom-made biosyntheses as an elegant and straightforward form of performing challenging chemistry with living cells.

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15 Fungal Biodegradation of Lignocelluloses

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I. Introduction

Wood and lignified gramineous and other annual plants are generally called lignocellulose because they are composed of the three main natural polymers: cellulose, hemicelluloses and lignin. Lignocellulosic biomass is renewable, and huge amounts of lignocellulose are annually synthesized and degraded in nature. It has been estimated that annual worldwide production of terrestrial biomass is 200×10^{12} kg (Foust et al.

2008). In Earth's carbon cycle, especially in a forest ecosystem, saprotrophic wood-decaying and litter-decomposing fungi perform an essential role. Among them certain basidiomycetes, so-called white-rot fungi, have a special role since they are the only organisms that can efficiently degrade and even mineralize the most recalcitrant natural polymer, lignin.

Cellulose is considered to be one of the most abundant biopolymers on Earth. It is the main constituent of wood, and approximately 40% of the dry weight of most wood species is cellulose, which is located predominantly in the secondary cell wall (Sjöström 1993). Cellulose is a homopolysaccharide composed of β -D-glucopyranoside units which are linearly linked together by (1 \rightarrow 4)-glycosidic bonds. Cellulose can be crystalline, sub-crystalline and even amorphous, depending on the tissue source in native plant, or the way that cellulose is isolated (Ding and Himmel 2008). The structural integrity of cellulose is one of the main obstacles of enzymatic hydrolysis of cellulose. For the isolation of cellulose harsh extraction methods involving sequential acid and alkaline treatments are usually employed. Fiber aggregation caused by sample processing and found in isolated cellulose does not necessarily represent the cellulose structure, and the detailed molecular structure of plant cell wall cellulose remains unknown (Ding and Himmel 2008).

Hemicelluloses in wood consist of relatively short, mainly branched heteropolymers of glucose, xylose, galactose, mannose and arabinose as well as uronic acids of glucose, galactose and 4-O-methylglucose linked by (1 \rightarrow 3)-, (1 \rightarrow 6)- and (1 \rightarrow 4)-glycosidic bonds. Galactoglucomannans are the principal hemicelluloses in softwoods, which also contain arabinoglucuronoxylan. Xylose-based hemicelluloses are often termed xylans in both softwoods and hardwoods. Depending on hardwood species, the xylan content varies within the limits of 15-30% of the dry

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wood. Acetyl groups are present as substituents particularly in the glucomannans of gymnosperms and the xylans of angiosperms (Sjöström 1993). Hemicelluloses are reported to be linked to lignin through cinnamate acid ester linkages, to cellulose through interchain hydrogen bonding, and to other hemicelluloses via covalent and hydrogen bonds (Decker et al. 2008).

Lignin is a complex, amorphous, three-dimensional aromatic polymer. Lignins are synthesized from the oxidative coupling of *p*-hydroxycinnamyl alcohol monomers, dimethoxylated (syringyl, S), monomethoxylated (guaiacyl, G) and non-methoxylated (*p*-hydroxyphenyl, H) phenylpropanoid units. The molecular weight of lignin is difficult to determine because lignins are highly polydisperse materials (Argyropoulos and Menachem 1997). New bonding patterns have been described in softwood lignin, e.g. dibenzodioxocin structures (Karhunen et al. 1995). Recent studies show that lignin can incorporate many more monolignols than the traditional three basic units (Vanholme et al. 2008), e.g. acetylated lignin units have been identified in non-woody plants (Martínez et al. 2005). The isolation of native lignin is complicated if possible at all (Buswell and Odier 1987). Isolated lignin usually has a brown color but in sound non-degraded wood it is obviously colorless because the wood of many tree species is almost white, and after attack by white-rot fungi, by definition, the modified lignin in residual wood and cellulose is also white.

Uncertainties in the basic structures of especially lignin but also other components in lignocellulose make fungal biodegradation studies a challenging task. The following properties are important in terms of microbial or enzymatic attack: (1) lignin polymers have compact structures that are insoluble in water and difficult to penetrate by microbes or enzymes, (2) the intermonomeric linkages that account for the rigidity of lignin comprise many kinds of C–C and C–O bonds with the β -aryl ether linkage being the most significant and (3) intermonomeric linkages in lignin are not hydrolyzable. A conclusion from the above items is summarized as follows: (1) polymeric lignin degradation requires extracellular enzymes and/or small molecular weight mediators or factors such as radicals, (2) the lignin degrading system must be unspecific and (3) the enzymes must be oxidative, not hydrolytic.

II. Fungal Degradation of Lignocellulose

A. White-Rot Fungi

White-rot fungi are a heterogeneous group of fungi that usually belong to basidiomycetes, although there are ascomycetous fungi that cause pseudo-white rot (also designated as soft-rot type II), such as fungi belonging to the family Xylariaceae (Blanchette 1995; Liers et al. 2006). Basidiomycetous white-rot and some related litter-decomposing fungi are the only organisms which are capable of mineralizing lignin efficiently (Kirk and Cullen 1998; Hatakka 2001). More than 90% of all wood-rotting basidiomycetes are of the white-rot type (Gilbertson 1980). White-rot fungi are more commonly found on angiosperm than on gymnosperm wood species in nature. Usually syringyl (S) units of lignin are preferentially degraded, whereas guaiacyl (G) units are more resistant to degradation. Many white-rot fungi colonize cell lumina and cause cell wall erosion. Eroded zones coalesce as decay progresses and large voids filled with mycelium are formed. This type of rot is referred as non-selective or simultaneous rot. Calcium oxalate and MnO₂ accumulate when the decay proceeds (Blanchette 1995). *Trametes* (syn. *Coriolus*, *Polyporus*) *versicolor* is a typical simultaneous-rot fungus (Eriksson et al. 1990). Some white-rot fungi degrade lignin in woody plant cell walls relatively to a higher extent than cellulose, and they are called selective white-rot fungi. In nature they may cause white-pocket or white-mottled types of rot, e.g. *Phellinus nigrolimitatus* (Blanchette 1995). There are also fungi, e.g. the tree pathogen *Heterobasidion annosum*, that are able to produce both types of attack in the same wood (Eriksson et al. 1990).

In a screening to find suitable fungi for wood chip pretreatment for biopulping, 90 white-rot fungi were cultivated in spruce (*Picea abies*) wood blocks for 10 weeks, and about 20% of these fungi degraded more lignin than cellulose (Hakala et al. 2004). The selectivity depends on the wood species, cultivation time, temperature and many other things. This work and several other screening studies have shown that e.g. *Ceriporiopsis subvermispora*, *Dichomitus squalens*, *Phanerochaete chrysosporium*, *Phellinus pini*, *Phlebia radiata*, *Phlebia tremellosus* (syn. *Merulius tremellosa*), *Phlebia subserialis*, *Physisporinus rivulosus*, *Pleurotus eryngii*, *Pleurotus ostreatus* and *Pycnoporus cinnabarinus* are lignin-selective fungi at least under certain conditions (Eriksson et al. 1990; Akhtar et al. 1998; Hatakka 2001; Hakala et al. 2004).

B. Brown-Rot Fungi

Brown-rot fungi are basidiomycetes that degrade wood to yield brown, shrunken specimens that typically exhibit a pattern of cubical cracks and easily disintegrate upon handling. Only a small proportion – roughly 7% – of all wood decay basidiomycete species falls into this group, which occurs most frequently on gymnosperm wood. Nevertheless, brown-rot fungi are essential biomass recyclers in coniferous forests and are the most important cause of decay in man-made wooden structures (Gilbertson and Ryvarden 1986; Eriksson et al. 1990). Recent phylogenetic analyses based on ribosomal RNA sequences suggest that most brown-rot fungi evolved repeatedly from white-rot fungi, most likely by the selective loss of some biodegradative mechanisms. Six or more separate brown-rot groups, including the frequently studied genera *Gloeophyllum* and *Postia*, have probably arisen independently from the white-rot lineage via loss of some decay capabilities. One possible exception is the white-rot genus *Grifola*, which may have evolved from a brown-rot ancestor (Hibbett and Thorn 2001).

The hallmark of brown-rot is rapid strength loss in the wood before extensive weight loss has occurred. This observation was first explained by Cowling (Cowling 1961), who chemically delignified sweetgum wood that was undergoing fungal decay to obtain holocellulose fractions (i.e. the cellulose and hemicelluloses), and then determined their degree of polymerization by measuring their viscosities after solubilization. The results showed that the brown-rot fungus *Poria monticola* (= *Postia placenta*) decreased the average chain length of the wood polysaccharides about fourfold at only 10% weight loss. Since a principal function of the lignin in wood is to shield the structural polysaccharides from enzymatic attack, this result led to the conclusion that the biodegradative agents responsible for incipient brown-rot are low-molecular-weight species that can penetrate the lignin in sound wood despite its low porosity (Cowling 1961; Koenigs 1974).

During brown rot, the hemicelluloses in wood are degraded most rapidly, after which virtually all of the cellulose is removed, leaving behind a complex, aromatic ring-containing polymer derived from the original lignin. Experiments with *P. placenta* grown on cotton have shown that carbonyl and carboxylic acid groups are introduced into the residual cellulose during decay, which suggests that depolymerization of the

polysaccharides involves not only hydrolytic enzymes, but also an oxidative component (Kirk et al. 1991). Analyses of lignin from brown-rotted wood have likewise shown that it becomes oxidized, partially via demethylation of the aromatic rings, which increases the phenolic hydroxyl content, and partially via introduction of new carbonyl and carboxyl groups. Nevertheless, the residual lignin is still polymeric, and most of it appears to remain in situ (Kirk and Adler 1970; Kirk 1975). These findings have led to two conclusions: (1) brown-rot fungi have little capacity to degrade lignin and (2) the low-molecular-weight species they use to initiate decay are oxidants, with oxygen-centered free radicals such as the hydroxyl radical ($\cdot\text{OH}$) being the prime candidates (Koenigs 1974; Eriksson et al. 1990; Hammel et al. 2002).

The apparent persistence of the lignin after extensive brown-rot of wood is consistent with earlier proposals that low-molecular-weight agents that can penetrate the secondary cell wall must play a central role, but is also perplexing because brown-rot fungi clearly produce cellulases and hemicellulases (Herr et al. 1978, Ritschkoff et al. 1994; Mansfield et al. 1998; Machuca and Ferraz 2001; Cohen et al. 2005), which can presumably operate only if some of the lignin is degraded beforehand. Recent work based on nuclear magnetic resonance spectroscopy of dissolved wood samples have clarified the picture somewhat by revealing that many of the intermonomer linkages of lignin actually disappear during brown rot (Yelle et al. 2008). These results suggest that the lignin may be transiently depolymerized, which might facilitate the access of polysaccharide hydrolases.

C. Soft-Rot Fungi

Ascomycetes and mitosporic fungi usually cause soft-rot decay of wood (Nilsson et al. 1989; Blanchette 1995). The decayed wood is brown and soft, and the residue is cracked when dry. Soft-rot fungi form cavities within secondary walls or erosion but then the middle lamella is not attacked. Xylariaceous ascomycetes from genera such as *Daldinia*, *Hypoxylon* and *Xylaria* are grouped with the soft-rot fungi because they cause a typical erosive soft rot. Compared to basidiomycetous fungi, the knowledge about

lignocellulose degradation by ascomycetes is limited, and very little is known about how they degrade lignin. They mainly degrade hardwood, and weight losses up to 53% of birch wood were found within 2 months after decay by the most efficient fungus of this group, *Daldinia concentrica* (Nilsson et al. 1989).

III. Fungal Degradation of Wood Polysaccharides

The ability to degrade plant biomass and cellulose as its major component is widespread in fungi. *Trichoderma reesei* is a mesophilic soft-rot fungus that is extensively used as a source of cellulases and hemicellulases for various applications. It has also long been a model system for the degradation of plant cell wall polysaccharides. It was shown to be an anamorph of the pantropical ascomycete *Hypocrea jecorina*. The sequence data of the whole genome of this fungus has become available recently (Martinez et al. 2008). Thus the cellulolytic systems of *T. reesei* and those of the first studied white-rot basidiomycete, *Phanerochaete chrysosporium* (Martinez et al. 2004), and the first brown-rot basidiomycete, *Poria placenta* (Martinez et al. 2009), the genomes of which are available, give a new basis for comparisons.

The concept of how fungi degrade cellulose (and hemicelluloses) is almost totally based on the enzyme system of *T. reesei*, while only rather incomplete data are available on other ascomycetes. Cellulolytic enzymes of basidiomycetes were recently reviewed (Baldrian and Valášková 2008). The fungal degradation of cellulose is catalyzed by: (1) cellobiohydrolases (CBHs, EC 3.2.1.91) and (2) endoglucanases (EGs EC 3.2.1.4), of which CBHs cleave the polymeric cellulose from reducing or non-reducing ends, and EGs randomly endo-wise along the glucose chain. The cellulolytic system also contains (3) extra- or intracellular β -glucosidases (EC 3.2.1.21), which hydrolyze the resulting cellobiose or cello-oligosaccharides to glucose. The hydrolysis products can also be oxidized by cellobiose dehydrogenase (CDH). In addition, numerous other carbohydrate-active enzymes are produced by fungi. In the carbohydrate-active enzyme database CAZy (<http://www.cazy.org>) glycoside hydrolases are grouped in as many as 115 families (Cantarel et al. 2009).

Table 15.1 summarizes the characteristics of the sequenced genomes of lignocellulose-degrading basidiomycetes. Taxonomically and ecologically very different fungi have been used as models for lignin and cellulose/hemicellulose degradation, namely the white-rot basidiomycete *P. chrysosporium* and the mitosporic (ascomycete) fungus *T. reesei* (syn. *H. jecorina*), respectively. The latter fungus cannot degrade lignin while the former fungus degrades lignin but in addition also efficiently cellulose. Taking into account the ecological importance of wood-rotting basidiomycetes in carbon cycling and wood cellulose degradation, the databanks contain only surprisingly few genes encoding cellulases of basidiomycetous fungi.

P. chrysosporium has an unexpectedly high number of cellulase encoding genes compared to *T. reesei* (Martinez et al. 2008; Table 15.2). While *T. reesei* has 10 genes encoding cellulolytic enzymes, one CBH1 (Cel7A), and one CBH2 (Cel6), the *P. chrysosporium* genome has seven CBH1 and one CBH2 encoding genes. Only three of these CBHs have been purified and characterized (Uzcategui et al. 1991a, b). Many other ascomycetous fungi such as *Aspergillus nidulans*, *A. fumigatus* and *A. oryzae*, *Magnaporthe grisea*, *Neurospora crassa* and *Fusarium gramineum* have a higher number of cellulases, and even 1–5 of the most efficient CBHs (Table 15.2). *T. reesei* has rather many genes encoding hemicellulose-degrading enzymes, 16, and *P. chrysosporium* has 19 (Table 15.1). There are 34–44 hemicellulase encoding genes in the ascomycetes *Aspergillus nidulans*, *A. fumigatus* and *A. oryzae*, *Magnaporthe grisea* and *Fusarium gramineum* genomes, indicating that these saprotrophic and plant pathogenic fungi rely on hemicellulose degradation (see also Chapter 16).

It was originally reported that many brown-rot fungi grow poorly on pure cellulose and that crude cellulases obtained from cultures degrade amorphous cellulose but lack activity on crystalline cellulose, which is relatively recalcitrant to hydrolysis (Nilsson and Ginns 1979; Eriksson et al. 1990). These results have been interpreted to mean that brown-rot fungi lack cellobiohydrolases, the exo-acting enzymes that are required for the operation of a complete, synergistic cellulase system. In agreement with this original picture, almost all cellulases purified from brown-rot fungi are endoglucanases, i.e. enzymes that lack the processive activity characteristic of cellobiohydrolases. However, it is now clear that some brown-rot fungi can utilize crystalline cellulose as a sole carbon source (Cohen et al. 2005; Yoon et al. 2008),

Table 15.1. Characteristics of the sequenced genomes of lignocellulose-degrading basidiomycetes (modified from Lundell et al. 2010)

Fungus	Type of fungus	Genome size ^a , Mbp	Number of (putative) genes encoding:					Number of CAZyme modules ^b			Reference
			Proteins	Laccase	LiP	MnP	VP	Glycoside hydrolase	Carbohydrate-binding	Cellulose-binding	
<i>Coprinopsis cinerea</i> (<i>Coprinus cinereus</i>)	Litter-decaying	37.5	13 544	17	- ^c	- ^c	- ^c	211	26	1	<i>C. cinereus</i> genome homepage (http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/MultiHome.html)
<i>Phanerochaete chrysosporium</i>	White-rot	35.1	10 048	- ^c	10	5	- ^c	180	89	46	Vanden Wymelenberg et al. (2006) (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html)
<i>Pleurotus ostreatus</i>	White-rot	34.3	11 603	12	Several	Several	Several	? ^e	? ^e	? ^e	<i>P. ostreatus</i> genome homepage (http://genome.jgi-sf.org/PleosPC15_1/PleosPC15_1.home.html)
<i>Postia placenta</i>	Brown-rot	90.9 ^d	17 173	2	- ^c	- ^c	- ^c	144	6	- ^c	Martinez et al. (2009) (http://genome.jgi-psf.org/Posp11/Posp11.home.html)
<i>Schizophyllum commune</i>	White-rot	38.5	13 181	8	? ^e	? ^e	? ^e	? ^e	? ^e	? ^e	<i>S. commune</i> genome homepage (http://genome.jgi-psf.org/Schco1/Schco1.home.html)

^aHaploid, if not otherwise depicted^bAdapted from Martinez et al. (2009)^cNot present^dDikaryon^eNot yet determined

Table 15.2. The number of genes encoding cellulytic enzymes in fungi (data adopted from Martinez et al. 2008)

Fungus	Type	CBH1	CBH2	EG1	EG2	EG3	EG4	EG5	Sum
		Cel7A	Cel6	Cel7B	Cel5	Cel12	Cel61	Cel45	
<i>Aspergillus fumigatus</i>	A	2	1	2	3	3	7	1	19
<i>A. nidulans</i>	A	2	2	1	2	1	9	1	18
<i>A. oryzae</i>	A	2	1	1	2	2	8	0	16
<i>Fusarium gramineum</i>	A	1	0	1	2	2	13	1	23
<i>Hypocrea jecorina</i> (syn. <i>Trichoderma reesei</i>)	A	1	1	1	2	1	3	1	10
<i>Magnaporthe grisea</i>	A	3	2	2	2	3	17	1	30
<i>Neurospora crassa</i>	A	2	3	3	1	0	14	1	23
<i>Phanerochaete chrysosporium</i>	B/WR	7	1	2	2	1	14	0	27

Types: A ascomycetes, B basidiomycetes, WR white-rot fungus

Enzymes: CBH1 exocellobiohydrolase I, CBH2 exocellobiohydrolase II, EG1 endoglucanase I, EG2 endoglucanase II, EG3 endoglucanase III, EG4 glycoside hydrolase family, EG5 endoglucanase V

which raises the possibility that their non-processive cellulases act in concert with low-molecular-weight oxidants to enable complete substrate degradation.

Although there was one early report that *Coniophora puteana* produces cellobiohydrolases (Schmidhalter and Canevascini 1992), these enzymes should be re-examined because the assay used, cleavage of *p*-nitrophenyl lactoside, is not completely specific. In addition, it has been reported that one endoglucanase from *G. trabeum* has processive activity, as shown by the high ratio of soluble to insoluble reducing sugars it produced from crystalline cellulose (Cohen et al. 2005). Additional work is needed to check this conclusion, because measurements of insoluble reducing sugars are technically challenging and require careful replication (Irwin et al. 1993; Medve et al. 1998). Even if future work confirms some exceptions such as these, it appears that the cellulytic systems of brown-rot fungi are generally less complex than those of white-rot fungi. In this connection, it is noteworthy that the genome of *P. placenta* apparently encodes neither cellobiohydrolases nor endoglucanases with cellulose-binding modules (Martinez et al. 2009).

IV. Fungal Degradation of Lignin

A. White-Rot and Brown-Rot Fungi

Lignin is a large macromolecular and heterogeneous polymer the degradation of which is difficult due to its chemical structure, e.g. it does not contain by definition any hydrolysable linkages (Kirk and Cullen 1998). The enzymes must be therefore oxidative (i.e., oxidoreductases), and due to heterogeneity of the polymer and many types of linkages (C–C, C–O), also nonspecific.

Lignin is also stereo-irregular, thus differing from e.g. cellulose or hemicelluloses.

Due to difficulties in the isolation and analysis of lignin preparations, various lignin model compounds, e.g. dimeric β -O-4 model compounds and synthetic lignin (dehydrogenation polymerizate of coniferyl alcohol or other lignin precursors, DHP) are commonly used in microbiological and enzymatic studies. These model systems do not contain any linkages to wood polysaccharides. All lignin preparations have disadvantages in reproducibility of preparation, or exhibit altered structure or molecular weight compared to natural lignin (Buswell and Odier 1987). One of the most reliable methods to determine fungal lignin-degrading ability is based on ^{14}C -lignins (Haider and Trojanowski 1975; Kirk et al. 1975; Kirk et al. 1978). Usually the evolution of $^{14}\text{CO}_2$ from ^{14}C -DHP or other ^{14}C -(lignin)-lignocelluloses is monitored. These studies have given information on the suitable conditions for lignin degradation by white-rot fungi such as *Phanerochaete chrysosporium*, *Phlebia radiata* and many others (Kirk 1975; Hatakka and Uusi-Rauva 1983; Hatakka et al. 1983; Kirk and Farrell 1987; Hatakka 2001), as well as by litter-decomposing basidiomycetous fungi (*Stropharia* spp., *Agrocybe praecox*; Steffen et al. 2000). Dimeric lignin model compounds attached to e.g. polyethylene glycol (Kawai et al. 1995), thus mimicking polymeric lignin, have allowed the use of efficient analytical tools (e.g. NMR) for more defined characterization of degraded lignin structures.

Solubilization (formation of water-soluble lignin fragments) and mineralization (evolution of $^{14}\text{CO}_2$) of

¹⁴C-labelled natural and synthetic lignins have been demonstrated for various white-rot fungi (Hatakka and Uusi-Rauva 1983; Hatakka 2001). A high mineralization of ¹⁴C-(ring)-DHP was observed in the case of *Phlebia radiata* that released up to 71% ¹⁴CO₂ from ¹⁴C-DHP in 37 days when grown under optimal conditions for lignin degradation, i.e. under 100% oxygen atmosphere in a low nutrient nitrogen liquid medium (Hatakka et al. 1983). *Phlebia* sp. Nf b19 (former name *Nematoloma frowardii* b19, Hildén et al. 2008) caused even higher mineralization (75%) of ¹⁴C-DHP during growth on wheat straw while only a small percentage of the initial radioactivity (6%) was incorporated into the residual straw and the fungal biomass (Hofrichter et al. 1999b). Usually only the end-product of lignin degradation, ¹⁴CO₂, is determined. However, in the case of certain fungi or under special conditions, the rate-limiting steps may be the reactions after the initial attack on polymeric lignin, in which case soluble oligomers may be detectable. The variety of different ligninolytic enzymes and the isoenzymes and isoforms produced by the fungus may also influence the production of ¹⁴CO₂.

1. Ligninolytic Peroxidases of White-Rot Fungi

The extracellular enzymes involved in lignin degradation are peroxidases and laccases, with their accessory enzymes (Hatakka 1994; Kirk and Cullen 1998; Hammel and Cullen 2008). Peroxidases include: (1) lignin peroxidases (LiPs, “ligninases”, EC 1.11.1.14) and (2) manganese peroxidases (MnPs, “Mn-dependent peroxidases”, EC 1.11.1.13), which were discovered in the early 1980s, and (3) versatile peroxidases (VPs, EC 1.11.1.16), which were found in the 1990s (Martínez 2002) and apparently represent hybrids of LiPs and MnPs. Fungal oxidoreductases are listed in the FOLy database (<http://folly.esi.univ-mrs.fr>; Levasseur et al. 2008).

The plant and microbial peroxidase superfamily covers three classes of peroxidase families of which class I includes intracellular prokaryotic peroxidases, class II consists of secretory fungal peroxidases, i.e. lignin peroxidases (LiPs), manganese peroxidases (MnPs) and versatile peroxidases (VPs), while class III includes secretory plant peroxidases (Morgenstern et al. 2008). The analysis of the genes suggests that the class II sequences constitute a monophyletic gene family, and that they diversified extensively in the basidiomycetes. LiPs evidently arose only once in the Polyporales, which harbors many white-rot taxa, whereas MnPs and VPs are more widespread and may have multiple origins. The phlebioid clade of Polyporales includes such fungi as *Phanerochaete chrysosporium* and *Phlebia radiata*, which are well known producers of LiP. The *Phanerochaete chrysosporium* whole genome has ten *lip* genes and five *mnp* genes (Table 15.1), of which

two were previously unknown (Martínez et al. 2004), and the corresponding proteins were not characterized. The data suggest that *P. chrysosporium* also has a versatile peroxidase gene the sequence of which shares residues common to both *mmps* and *lips*. Phylogenetic comparisons between some well-known peroxidases show that white-rot and litter-decomposing fungi exhibit a continuum of variability in their peroxidases (Lundell et al. 2010). Even a single fungal species can produce several different peroxidases as exemplified by the white-rot basidiomycetes *P. radiata* (Hildén et al. 2005) and *Physisporinus rivulosus* (Hakala et al. 2006).

The fungal peroxidases LiPs, MnPs and VPs are all heme-containing glycoproteins which require hydrogen peroxide as an oxidant and have heme (protoporphyrin IX) as their prosthetic group coordinated by two highly conserved histidine residues (Martínez 2002). The heme cofactor is located in an internal cavity connected by two access channels. The main channel is used by hydrogen peroxide and the second is the site where MnP and VP oxidize Mn²⁺ to Mn³⁺ (Ruiz-Dueñas et al. 2009). Typically for LiP activity, the amino acid residue needed is a tryptophan, Trp171 in the isozyme LiPA (LiP H8) of *P. chrysosporium*. Tryptophan exposed on the LiP protein surface is conserved in LiP sequences and also in VPs (Martínez 2002; Pérez-Boada et al. 2005). It is assumed that it takes part in long-range electron transfer (LRET) from a protein radical at the surface of the enzyme, which would act as the substrate oxidizer, to the heme cofactor (Ruiz-Dueñas and Martínez 2009). This could allow the enzyme to oxidize bulky substrates such as polymeric lignin that cannot directly contact the oxidized heme in the active centre of LiP or VP.

Most white-rot fungi secrete several isoenzymes into their cultivation medium (Hatakka 1994). The molecular weight of the LiP, MnP and VP varies between 35–48 kDa, 38–62 kDa and 42–45 kDa, respectively. Lignin-modifying peroxidases have typically acidic pI values of 3.0–4.0 (Hatakka 2001), while also neutral MnPs have been detected from litter-decomposing fungi (Steffen et al. 2002).

LiP oxidizes non-phenolic lignin substructures by abstracting one electron and generating aryl cation radicals that then decompose chemically (Kirk and Farrell 1987). Reactions of LiP using a variety of lignin model compounds and synthetic lignin have thoroughly been studied, catalytic mechanisms elucidated and the enzyme’s capability for C_α-C_β bond cleavage, ring opening

and other reactions demonstrated (Kirk and Farrell 1987). MnP oxidizes Mn(II) to Mn(III) which then oxidizes phenolic rings to phenoxy radicals, leading to the decomposition of the structures (Gold et al. 2000). Studies with white-rot fungi have shown that the expression of MnP is more common than that of LiP (Orth et al. 1993; Hatakka 1994; Vares et al. 1995; Hofrichter 2002; Rainio, Maijala, Hatakka et al., unpublished data). VP has been reported so far only from *Pleurotus* spp. and *Bjerkandera* spp. (Ruiz-Dueñas and Martínez 2009). Crystal structures of substrate binding site mutants of MnP indicate that there is only one major Mn-binding site (Sundaramoorthy et al. 1997). This proposed site consists of a heme propionate, three acidic ligands and two water molecules.

The characteristics and potential applications of MnPs and VPs were extensively studied in the 1990s and frequently reviewed (Hatakka 2001; Hofrichter 2002; Martínez 2002; Ruiz-Dueñas and Martínez 2009), while LiPs were less studied. MnP has an important role in the depolymerization of lignin and chlorolignin as well as in the demethylation of lignin and bleaching of pulp (Hatakka 2001). Moreover, the enzyme mediates initial steps in the degradation of high-molecular-weight lignin (Perez and Jeffries 1992). MnP oxidizes Mn(II) to Mn(III) that is stabilized by organic acids such as oxalate, malate, lactate or malonate via chelation (Kishi et al. 1994; Kirk and Cullen 1998). Chelated Mn(III) in turn oxidizes various compounds, including lignin. In the presence of unsaturated fatty acids, it contributes to lipid peroxidation, and the formed peroxy radicals may act as selective oxidants. Significant mineralization of synthetic ^{14}C -labelled lignin (DHP), up to 16% of the applied ^{14}C as evolved $^{14}\text{CO}_2$, was measured in an in vitro system (without fungal mycelium) consisting of a mixture of MnP, linoleic acid, Mn and H_2O_2 (Hofrichter et al. 1999a; Kapich et al. 1999).

In addition, some accessory enzymes are involved in lignin degradation. These enzymes include H_2O_2 -generating enzymes such as aryl alcohol oxidase (AAO, EC 1.1.3.7), glyoxal oxidase (GLOX), and pyranose-2 oxidase (EC 1.1.3.10; Hatakka 2001; Kersten and Cullen 2007).

2. Peroxidases of Brown-Rot Fungi?

The components most often cited as missing in brown-rot fungi are the secreted enzymes generally thought to have a key role in delignification by white-rot fungi. As far as ligninolytic heme-containing peroxidases are concerned, the situation appears straightforward so far. The currently known lignin and versatile peroxidases (LiP, VP) of white-rot fungi, i.e. those enzymes that can cleave non-phenolic lignin structures directly, all

contain an exposed tryptophan required for catalysis (Martínez 2002) and no brown-rot fungus has yet been shown to produce a peroxidase with this essential residue. Similarly, manganese peroxidases (MnPs), which oxidize Mn(II) to the ligninolytic agent Mn(III), all contain an essential manganese-binding site comprised of acidic amino acid residues (Martínez 2002), and no brown-rot fungus peroxidase has been found with this property. Additional white-rot peroxidases that may have a ligninolytic function have been described recently (Martínez 2002; Miki et al. 2009) but none of them has a brown-rot counterpart so far. Perhaps most pertinent, no currently known type of ligninolytic peroxidase is encoded in the *P. placenta* genome (Martínez et al. 2009).

3. Laccases

Laccase (EC 1.10.3.2, *p*-diphenol:oxygen oxidoreductase) has been studied since the 1880s when it was first described in the lacquer tree (Thurston 1994; Baldrian 2006). Laccase is a copper-containing oxidase that utilizes molecular oxygen as oxidant and also oxidizes phenolic rings to phenoxy radicals (though the redox potential is somewhat lower than that of peroxidases; Thurston 1994; Baldrian 2006). Most white-rot fungi typically produce laccase (Käärik 1965; Bollag and Leonowicz 1984), and the enzyme is common also in higher plants and in other fungi, and even in bacteria and insects laccase-like multicopper oxidases have been described (Giardina et al. 2010).

Laccases belong to a superfamily of multicopper oxidases (MCOs; Hoegger et al. 2006), forming a phylogenetically divergent group of so called "blue oxidases" that all contain four copper atoms per mol of enzyme, arranged into three metal centres between three structural domains formed by a single polypeptide of about 500 amino acids in length (Ducros et al. 1998; Bertrand et al. 2002; Piontek et al. 2002; Lundell et al. 2010). Phylogenetically, multicopper oxidases may be classified as true fungal laccases and separated from other laccase-like enzymes (Lundell et al. 2010). In fungi laccases or laccase-like copper enzymes can be involved in lignin degradation, but in fungal physiology laccases may also have other functions, for example, in pigmentation, fruiting body formation, sporulation, pathogenicity and detoxification (Thurston 1994). The crystal

structures of ascomycete and basidiomycete laccases were solved for enzymes of *Melanocarpus albomyces* (Hakulinen et al. 2002) as well as *Coprinus cinereus* (Ducros et al. 1998), *Trametes versicolor* (Piontek et al. 2002) and *Lentinus tigrinus* (Ferraroni et al. 2007), respectively.

Laccases catalyze the oxidation of a wide range of organic substrates, such as phenols, aromatic amines and heterocyclic compounds, usually restricted by low redox potential characteristics (< 0.8 V). They catalyze the complete four-electron reduction of dioxygen (O_2) to water with concomitant electron withdrawal from the reducing organic substrate compounds (Thurston 1994; Call and Mücke 1997). Laccase catalyzes the cleavage of the C_α - C_β bond in phenolic β -1 and β -O-4 lignin model dimers by oxidizing C_α -carbon and by splitting the aryl-alkyl bond (Eriksson et al. 1990). Due to its rather low redox potential, it is not able to directly oxidize non-phenolic lignin units, which have a high redox potential (> 1.2 V). Laccases can, however, oxidize monomeric lignin-like phenols and anilines of redox potentials ranging from 0.5 V to even 1.9 V for one electron abstraction, and the efficiency of oxidation correlates to the redox potential but also to steric properties of the enzyme (Tadesse et al. 2008).

In native wood, less than 20% of all lignin units are phenolic (Higuchi 1990), and as a consequence, laccases are incapable of depolymerizing macromolecular lignins. In the presence of oxidation mediator compounds (redox mediators), such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate, (ABTS; Bourbonnais and Paice 1990) or organic N-OH compounds such as 1-hydroxybenzotriazole (HBT), which were discovered in the 1990s by Call and others (Call and Mücke 1997), laccase-aided oxidation of non-phenolic compounds and even delignification is promoted. There have been attempts to find possible natural mediators (Eggert et al. 1996), but except in one case (3-hydroxyanthranilic acid from *Pycnoporus cinnabarinus*), no such compound has been identified. Synthetic laccase mediators have widely been investigated for various applications in the pulp and paper/forest sector, the most common being HBT and violuric acid (Call and Mücke 1997; Widsten and Kandelbauer 2008). There is a vigorous search for safer and cheaper mediator molecules, e.g. among naturally occurring aromatic compounds. Syringaldehyde and other related compounds have shown promising results as charge-transfer mediators in laccase catalyzed reactions (Kawai et al. 2004; Camarero et al. 2005; Nousiainen et al. 2009). In nature, hardwood lignin could be a source of syringyl compounds, but even if they would act as redox mediators in nature, in softwood, these substructures are not present. Although white-rot fungi typically prefer hardwood species, many of them grow and degrade also softwood and its lignin.

More than 100 laccases have been purified from fungi and more or less characterized in detail (Baldrian 2006). Typically, laccases are inducible enzymes with pH optima between 3.0 and 5.7.

Laccases of some soil inhabiting basidiomycetes have higher pH optima (pH 7.0), e.g. those of *Rhizoctonia praticola* (Bollag and Leonowicz 1984) and *Coprinus cinereus* (Schneider et al. 1999). The optimal temperature may be as high as 75°C as found using the laccase of the litter-decomposing fungus *Marasmius quercophilus* (Dedeyan et al. 2000). Laccases from white-rot fungi usually have molecular masses of 60–80 kDa, acidic pIs (2.5–5.0) and are glycosylated (Thurston 1994).

Laccases have long been proposed to have a role in lignin biodegradation (Eriksson et al. 1990) but the exact role of laccase in wood and non-wood (grass) lignin degradation is still unclear. Although laccase is very common in wood-rotting white-rot fungi, it was confirmed that the whole genome of *P. chrysosporium* (a model white-rot fungus) does not contain laccase-encoding genes, although some other multicopper oxidase encoding genes have been identified in its genome. The role of laccase in lignin degradation becomes even more questionable, since the whole genome of the dung-dwelling, non-lignin-degrading basidiomycete *C. cinerea* contains several (17!) laccase-encoding genes (Table 15.1). Moreover, the recent whole-genome sequence analysis of *Poria* (*Postia*) *placenta* indicated the presence of true laccases also in a cellulolytic brown-rot fungus (Martinez et al. 2009).

Nevertheless, laccases are apparently rare in brown-rot fungi, as shown by the long usage of the Bavendamm reaction to differentiate between white-rot from brown-rot basidiomycetes (Bavendamm 1928). This test depends on the development of a color reaction after application of a phenol oxidase substrate such as gallic acid to the mycelium, and most brown-rot fungi give a negative result. However, recent work has revealed the presence in the growth medium of some brown-rot fungi of uncharacterized substances that oxidize laccase substrates, and the genomes of some of these fungi harbour laccase-like gene sequences (D'Souza et al. 1996; Lee et al. 2004). Furthermore, at least one brown-rot fungus, *P. placenta*, clearly expresses a true laccase on wood, as shown by peptide fingerprinting of extracted proteins and by heterologous expression of the responsible gene (Wei et al. 2010).

Even when laccases are present in brown-rot fungi, it does not necessarily follow that this decay type involves enzyme-catalyzed delignification of the substrate. Although there is a long association in the literature between fungal ligninolysis and the presence of laccases, there is little evidence

that these enzymes can truly depolymerize lignin directly. Instead, they rather cause additional polymerization when tested on model lignins *in vitro*, unless suitable redox mediators are present (Rochefort et al. 2004). It may be that natural laccase mediators do occur and simply remain to be discovered (perhaps even in the case of brown-rotters), or that laccases actually have no role in fungal ligninolysis, perhaps being involved instead in melanin formation or in the unspecific detoxification of phenols via polymerization (Bollag et al. 1988; Galhaup and Haltrich 2001). Alternatively, the laccases of brown-rot fungi may participate in the generation of biodegradative oxygen-centered radicals, as discussed below (Wei et al. 2010).

Thus the present genomic information strongly suggests that the role of laccase in fungal physiology, that is in the fruiting body and spore formation as well as pigmentation and detoxifying of phenolic compounds derived from lignin, and in other similar reactions, may be in nature more important than the participation of laccase in lignin biodegradation. Even if its role in lignin biodegradation is not clear, this enzyme has undoubtedly more potential than other fungal

oxidoreductases in various industrial applications (Widsten and Kandelbauer 2008), and because its heterologous production in industrial fungal hosts has been developed, its use on a relatively large scale is possible even now (Yaver et al. 1996; Berka et al. 1997; see also Chapters 14, 21).

4. Role of Small Oxidants in Incipient Decay

The chemical changes that occur in lignin and cellulose during brown-rot, in combination with the apparent lack of ligninolytic enzymes or complete cellulase systems in these fungi, suggest the involvement of small oxidants in decay. The best-known and perhaps most likely candidate is Fenton reagent, i.e. the oxidant that is produced when Fe^{2+} reacts with H_2O_2 (Fig. 15.1; Koenigs 1974). The Fenton oxidant is generally depicted as the hydroxyl radical ($\cdot\text{OH}$), although it is possible that the actual species is a similarly reactive iron-oxygen complex in which the iron has a formal charge of +4 or +5 (Halliwell and Gutteridge 1999). Essentially, all a fungus requires for the production of extracellular Fenton reagent is a mechanism to reduce extracellular Fe^{3+} to Fe^{2+} , because Fe^{2+} auto-oxidizes in most biological

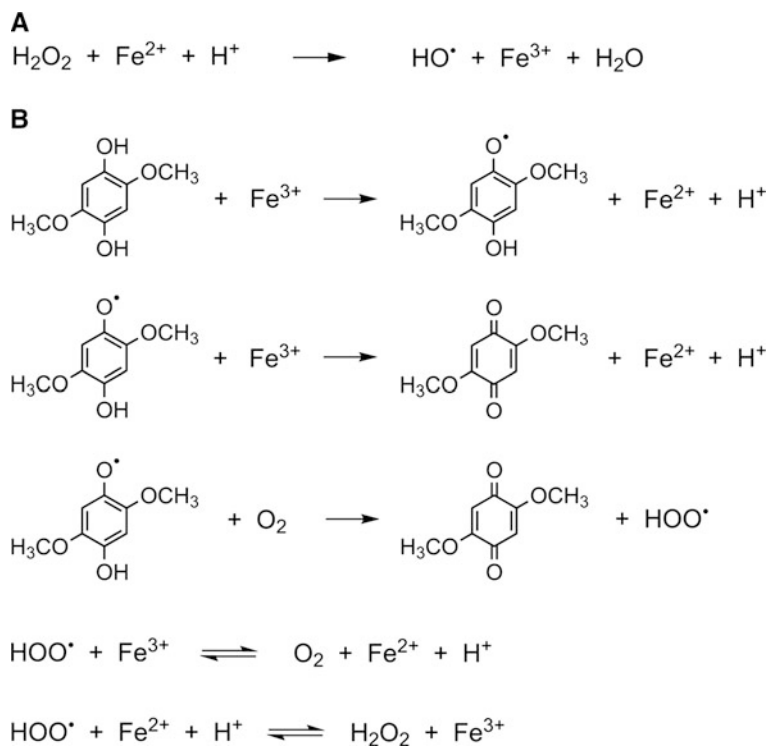


Fig. 15.1. Extracellular free radical reactions proposed to have a role in incipient brown-rot. **A** The Fenton reaction. **B** Hydroquinone-dependent processes that produce the Fe^{2+} and H_2O_2 required for Fenton chemistry

environments to produce the perhydroxyl radical/superoxide acid–base pair ($\bullet\text{OOH}/\text{O}_2\bullet^-$), which rapidly dismutates or oxidizes Fe^{2+} to produce H_2O_2 . Alternatively, Fenton chemistry will occur if the fungus has a system that generates extracellular $\bullet\text{OOH}/\text{O}_2\bullet^-$, because these radicals are sufficiently reducing to convert most forms of Fe^{3+} to Fe^{2+} (Fig. 15.1; Halliwell and Gutteridge 1999).

Fenton reagent is the strongest oxidant that can occur in water, and reacts non-selectively with virtually any organic compound it meets. It abstracts hydrogens from aliphatic structures such as polysaccharides, thus depolymerizing them and introducing oxygen functional groups, in agreement with the chemical changes that have been observed in brown-rotted cellulose. Fenton reagent also oxidizes aromatic rings, causing ring hydroxylation and scission of adjacent aliphatic structures, which may explain why brown-rot fungi are apparently capable of some ligninolysis (Hammel et al. 2002). In the process of these reactions, an array of new substrate-derived oxidants is produced, especially alkoxy ($\bullet\text{OR}$) and peroxy ($\bullet\text{OOR}$) radicals, which are less reactive than Fenton reagent but able to cause additional lignocellulose oxidation (Hammel et al. 2002). Several hypotheses have been advanced to explain how brown-rot fungi initiate Fenton chemistry.

5. Hydroquinones

Work over the past decade has shown that extracellular hydroquinones are produced by diverse, phylogenetically distinct brown-rot fungi, including *G. trabeum*, *P. placenta* and *Serpula lacrymans*. In *G. trabeum*, both 2,5-dimethoxyhydroquinone (2,5-DMHQ) and 4,5-dimethoxycatechol occur, whereas *P. placenta* and *S. lacrymans* appear to produce only 2,5-DMHQ (Kerem et al. 1999; Paszczynski et al. 1999; Shimokawa et al. 2004; Suzuki et al. 2006). The significance of this finding is that hydroquinones bearing electron-donating substituents (such as methoxyls) auto-oxidize rapidly in the presence of most Fe^{3+} salts, thus generating Fe^{2+} and semiquinone radicals. Some of the resulting semiquinones then reduce additional Fe^{3+} , or react further with O_2 to produce quinones and $\bullet\text{OOH}/\text{O}_2\bullet^-$. This last species then dismutates or oxidizes some of the Fe^{2+} to produce H_2O_2 (Fig. 15.1). The Fe^{2+} and H_2O_2 thus generated then undergo the Fenton reaction, as described above.

Some studies done with decaying wood support a role for hydroquinone-driven Fenton chemistry in incipient brown-rot. The hydroquinones are present in wood undergoing decay by *G. trabeum* and *P. placenta*, and are maintained in the reduced state despite their tendency to oxidize rapidly when removed from the wood (Suzuki et al. 2006; Wei et al. 2010). This result indicates that the hydroquinones must be present in a steady state, i.e. that they are continuously oxidized and regenerated in the wood. Computer modeling of these reactions has provided evidence that the quantity of Fenton reagent produced via this redox chemistry is large enough for the hydroquinones to have a role in polysaccharide scission during incipient decay by *G. trabeum*. However, the quantity appears insufficient to account for all of the cleavage that occurs, and thus other process must also contribute (Suzuki et al. 2006). An additional difficulty is that some brown-rot fungi, including *P. placenta*, produce large quantities of oxalic acid, a strong Fe^{3+} chelator that makes the reduction of Fe^{3+} by 2,5-DMHQ thermodynamically infeasible (Green et al. 1991; Park et al. 1997). In this case, it appears that *P. placenta* laccases rather than Fe^{3+} are the initial oxidants of 2,5-DMHQ. The resulting semiquinone radicals then react with O_2 to produce $\bullet\text{OOH}/\text{O}_2\bullet^-$, a species which, unlike 2,5-DMHQ, is sufficiently reducing to convert Fe^{3+} to Fe^{2+} in the presence of a high oxalate concentration, thus generating all components needed for a complete Fenton system (Wei et al. 2010).

6. Cellobiose Dehydrogenases

Cellobiose dehydrogenases (CDHs, EC1.1.99.18) are heme- and flavin-containing oxidoreductases that many fungi secrete. They catalyze the oxidation of cellobiose and some other sugars with concomitant reduction of an electron acceptor, molecular oxygen originally being considered the physiological substrate. Subsequent work with the enzyme from the white-rot fungus *P. chrysosporium* showed: (1) some Fe^{3+} salts are better electron acceptors, (2) the Fe^{2+} thus formed auto-oxidizes to produce the H_2O_2 precursor $\bullet\text{OOH}/\text{O}_2\bullet^-$ and (3) a complete Fenton system can thus be produced. Most of the work on CDH-catalyzed Fenton chemistry has focused on white-rot fungi and the possible role of reactive oxygen species in their decay processes (Henriksson et al. 2000). Genes encoding CDH have been described in the white-rot fungi *P. chrysosporium*, *Pycnoporus cinnabarinus*, *Trametes versicolor* and *Ceriporiopsis subvermispota*. In the last fungus it has been speculated that the poor degradation of cellulose is due to its lack of cellobiohydrolases or and CDHs. Recently, a CDH from *C. subvermispota* was purified and characterized (Harreither et al.

2009). The fungus produces it in later stages of cellulose degradation, and therefore it was concluded that CDH does not participate in lignin degradation but it could help the weak cellulolytic system of this fungus, lacking cellobiohydrolases, to degrade cellulose at a late stage of growth on cellulose. Thus, even now the exact role of this enzyme is not known.

This mechanism may also be relevant to the brown-rot fungus *Coniophora puteana*, which produces a CDH (Hyde and Wood 1997), but it must be absent in *P. placenta* because no CDH is encoded in this genome (Martinez et al. 2009). One problem with a role for CDH in Fenton chemistry is that it is no stronger a reductant than are hydroquinones such as 2,5-DMHQ (Hyde and Wood 1997), and thus like them it cannot reduce Fe^{3+} efficiently in the presence of high oxalate concentrations. In addition, there is a “chicken or egg” problem with CDH as a source of biodegradative oxidants – presumably, these low-molecular-weight species are needed during incipient decay to release sugars for the fungus to grow on, yet the sugars are required beforehand as electron donors for CDH to operate.

7. Redox-Active Glycopeptides

Nearly 20 years ago, wood-grown cultures of *G. trabeum* were reported to produce extracellular Fe^{2+} -containing glycopeptides that reduced O_2 to $\cdot\text{OOH}/\text{O}_2\cdot^-$ and also used H_2O_2 to produce a Fenton system. These substances also utilized Fe^{3+} , provided a reductant such as NADH or ascorbate was included in the reactions. The glycopeptides appeared somewhat heterogeneous, as shown by their chromatographic properties, but had a sufficiently low-molecular-weight mass range of 1–5 kDa to suggest they could have a role in incipient brown rot (Enoki et al. 1992). Subsequent work showed that similar substances were produced by a variety of brown- and white-rot fungi (Enoki et al. 1997; Tanaka et al. 2007). Recently, genes that apparently encode these peptides were also identified in the white-rotter *P. chrysosporium* (Tanaka et al. 2007), and it is interesting that similar genes are present and expressed by the brown-rotter *P. placenta* (Martinez et al. 2009).

However, so far some features of these substances are difficult to reconcile with a biodegradative role. First, the inferred molecular masses of the encoded peptides are around 14 kDa, much larger than reported for the substances that were first isolated from colonized wood.

Indeed, the possibility has not been ruled out that some of the originally reported glycopeptides might simply be heterogeneous mixtures of partially degraded extracellular fungal proteins. Second, as with other proposed fungal Fenton systems, a reductant is needed to return the bound iron to its active ferrous state. Since the physiological reductant is unlikely to be either NADH or ascorbate, a key part of the biodegradative system remains unidentified. Finally, it is not clear what advantage is conferred by having the iron bound to a glycopeptide, since Fenton chemistry occurs equally well with iron salts bound to much simpler chelators such as oxalate. One possibility worth investigating is that the Fenton systems involving glycopeptide-bound iron might oxidize polysaccharide or lignin structures more selectively than simple Fenton systems do. Another is that the ferric glycopeptides might be able to undergo reduction by donors that do not work with simpler Fe^{3+} chelates. In this connection, it is interesting that the *G. trabeum* glycopeptide was reported to undergo reduction by cellobiose (Wang and Gao 2003), but very few details of this reaction were provided. Additional work is clearly needed with these potentially relevant substances.

B. Soft-Rot Fungi

Ascomycetes are usually thought to degrade mainly carbohydrates in soil, forest litter and compost, but they may also degrade lignin in these environments (Rodriguez et al. 1996; Regalado et al. 1997; Tuomela et al. 2000; Kluczek-Turpeinen et al. 2003). Thus, some ascomycetes/deuteromycetes (“molds”) were found to be able to mineralize grass lignins (Haider and Trojanowski 1975) and e.g. *Penicillium chrysogenum*, *Fusarium oxysporum* and *F. solani* (Rodriguez et al. 1996) mineralized in 28 days up to 27% of a ^{14}C -labelled lignin prepared from milled wheat straw. Unlike model white-rot basidiomycetes such as *Phanerochaete chrysosporium* and *Phlebia* spp., which degrade lignin during secondary metabolism (Kirk and Farrell 1987; Hatakka 2001), the degradation by molds was maximal during primary metabolism (Regalado et al. 1997).

Pine wood was degraded very little, only showing 2.5% weight loss (Nilsson et al. 1989). The high concentration of guaiacyl units in the middle lamella of coniferous wood may cause its resistance to decay by soft-rot fungi because they may not have the oxidative potential to attack the more condensed recalcitrant guaiacyl lignin. In contrast, syringyl lignin apparently is readily oxidized and mineralized by soft-rot fungi (Nilsson et al. 1989). Usually these fungi, e.g. *P. chrysogenum* and *F. proliferatum*, mineralize less than 10% of the applied ^{14}C -labelled guaiacyl type

synthetic lignin (DHP; Rodriguez et al. 1994; Regalado et al. 1997). *Paecilomyces inflatus* mineralized 6.5% of a synthetic $^{14}\text{C}_\beta$ -labelled lignin in 12 weeks during solid-state cultivation of the fungus in autoclaved compost and 15.5% was converted into water-soluble fragments (Kluczek-Turpeinen et al. 2003). Two wood inhabiting fungi, *Xylaria hypoxylon* and *X. polymorpha*, when growing on beech wood meal mineralized 9% of the same DHP as above, and the major fraction (65.5%) was polymerized into water and dioxin insoluble material (Liers et al. 2006). These fungi formed large lignocellulose fragments unlike the basidiomycetous white-rot fungus *Bjerkandera adusta*, which released smaller lignocellulose fragments. This might have connections to the finding that *Xylaria* spp. produced high levels of hydrolytic enzymes, like esterase and xylanases.

There are no reliable reports on the presence of ligninolytic heme peroxidases in ascomycetes. The genome of the efficient cellulose degrading fungus *Hypocrea jecorina* (*Trichoderma reesei*) does not contain genes encoding laccase or ligninolytic peroxidases (Martinez et al. 2008). Laccase activities in lignin-degrading ascomycetes such as *Xylaria* spp. (Liers et al. 2006) and *P. inflatus* (Kluczek-Turpeinen et al. 2003) have usually been low. Laccase, aryl-alcohol oxidase and superoxide radicals were detected in liquid cultures of *F. proliferatum*, but neither MnP nor LiP were present (Regalado et al. 1999). Interestingly, in the ascomycete *Petriellidium fusioideum*, the specific inhibition of hydroxyl radical production was found to decrease the mineralization of ^{14}C -labelled synthetic lignin (DHP; Gonzales et al. 2002).

V. Biopulping as an Example of Potential Applications of White-Rot Fungi

Fungal or enzymatic treatment of wood or wood chips combined with mechanical or chemical pulping is a process known in a broad sense as biopulping, or more precisely as biomechanical, or biochemical pulping, depending on the pulping process which the biological process is combined with (Akhtar et al. 1998; Ferraz et al. 2008). In chemical pulping, such as soda or Kraft pulping, strong chemicals and high pressure are applied to remove lignin. Chemical pulping produces pulp with high strength but the process is polluting, hemicelluloses are also removed and the overall

yield is relatively low (40–50%). Mechanical pulping uses mechanical forces to separate wood fibers, and the yields are higher (up to 95%); it results in paper with good printing properties – newspaper is typically manufactured from mechanical pulp – but the process requires a lot of electrical energy for refining, the fibers have poor strength properties, and the high content of lignin causes a tendency to high color reversion (yellowing). Biological (fungal) treatment is usually combined with mechanical pulping to save refining energy (Akhtar et al. 1998), and its compatibility is not good with alkaline (Kraft) pulping, as the most useful white-rot fungi produce oxalic acid and other organic acids when growing in wood or straw (Galkin et al. 1998; Hofrichter et al. 1999b; Mäkelä et al. 2002; Hakala et al. 2005), and the neutralization of these acids causes a need for extra alkali.

The use of a lignin-degrading white-rot fungus to delignify wood is not a new idea as reviewed by Akhtar et al. (1998) and Gramss (1992). Already in the 1940s, Luthardt in East Germany started comprehensive studies to prepare softened myco-wood (“Mykoholz”) for the production of pencils using white-rot fungi (e.g. *Kuehneromyces mutabilis*, *Trametes versicolor*; summarized by Luthardt 1969, 2005). In 1957 a United States pulp and paper company screened wood-rotting fungi for delignification, and in 1972, it was found that treatment (cultivation) with a common white-rot fungus, *Rigidoporus ulmarius*, reduced the electrical energy needed for papermaking and produced stronger fibers from aspen wood chips. At the same time a pioneering work was published from the Swedish Forest Products Laboratory (STFI; Ander and Eriksson 1977). This screening identified some promising biopulping fungi from which also cellulaseless mutants were produced. These fungi were *Phlebia radiata*, *Pycnoporus cinnabarinus* and *Sporotrichum pulverulentum* (an anamorph of *P. chrysosporium*). For biopulping experiments and lignin biodegradation studies, the researchers from the United States used a strain of the same fungus, *P. chrysosporium* Burdsall. The next generation of biopulping research was started in the Forest Products Laboratory, USDA (Madison, Wis., USA) in 1987 by a large international biopulping consortium (Akhtar et al. 1998).

A key factor in successful biopulping is the use of a competitive and fast-growing but at the same time efficiently lignin-degrading fungus. Extensive screenings of white-rot fungi (Otjen and Blanchette 1987; Blanchette et al. 1988, 1992) resulted in the selection of the highly effective species *Ceriporiopsis subvermispora*, and also fungi of the genus *Phlebia* have been frequently

studied for biopulping purposes, e.g. strains of *P. tremellosa*, *P. subserialis* and *P. brevispora*. *C. subvermispora* was efficient during both hardwood and softwood treatment, and since then, it has been a target for lively research, and its lignin and cellulose degrading enzymes have been thoroughly studied (Lobos et al. 2001; Heidorne et al. 2006).

In fungal treatment of lignocelluloses, it would be useful to regulate the relationship of the degradation of polysaccharides versus lignin. To better understand the regulation of lignocellulose degradation, *Phlebia radiata* was grown under different conditions and the degradation and mineralization of ^{14}C -labelled tobacco cellulose, ^{14}C -labelled wheat-straw hemicelluloses and ^{14}C -(lignin)-labelled wheat straw was monitored. The results indicate that the degradation of lignin and hemicelluloses proceeds under similar conditions while the degradation of cellulose occurred under different conditions (Cho et al. 2009). For the acceleration of lignin degradation and the simultaneous repression of fungal attack on cellulose cultivation of the fungus in an oxygen atmosphere, addition of a small amount of glucose and supplementation of the medium with vanillic acid has been suggested.

In a study (Hakala et al. 2004) to find suitable biopulping fungi for the treatment of Norway spruce (*Picea abies*), about 300 white-rot fungi were initially screened on agar plate tests, and about 90 well-growing fungi were then cultivated for 10 weeks on spruce wood blocks. About 15 species of these pre-selected fungi degraded more lignin than cellulose (Hakala et al. 2004); some fungi such as *Phellinus viticola* even converted almost three times more lignin than cellulose. However, the weight losses were usually rather low, indicating slow growth. The most promising fungus in this screening was *Physisporinus rivulosus*, which was able to grow and degrade wood also at rather high temperature (37°C). *P. rivulosus* produces readily MnP and secretes also oxalate (Hakala et al. 2005, 2006). Both *C. subvermispora* and *P. rivulosus* caused losses of Klason lignin and an increase in acid-soluble lignin when growing in spruce wood. Two MnP isoenzymes were found in *P. rivulosus* and they are differentially regulated (Hakala et al. 2005, 2006).

Recently the so-far most intensive biopulping studies, both regarding technological and mechanistic aspects, have been started in Brazil (Ferraz et al. 2008) with the aim to treat *Eucalyptus grandis* and *Pinus taeda* with *C. subvermispora*.

Mill-scale pilot studies at the 50-t scale have shown which kind of practical difficulties biopulping must overcome. The same level of energy savings as at laboratory scale were possible to attain at mill-scale, i.e. 18–27%, depending on the mechanical pulping process and freeness. There is, however, still a need for more resistant and competitive fungal species, since the main drawback has been contamination of wood-chip piles with other fungi (“molds”). Earlier studies by the biopulping consortium (Akhtar et al. 1998) showed that the extent of lignin removal is not related to energy savings in biomechanical pulping, but Ferraz and others (Ferraz et al. 2008) have shown that there are good correlations between biopulping effects and intense lignin depolymerization observed during the initial stages of wood biotreatment by selective white-rot fungi.

The dominant oxidative enzyme that the potentially most applicable biopulping fungi produce on wood chips is (again) MnP (Hatakka et al. 2003; Maijala et al. 2008; Cunha et al. 2010). For industrial purposes, fungal biopulping is still considered too slow and technically demanding, and the direct application of enzymes on wood chips has become a more attractive alternative. When Scots pine (*Pinus sylvestris*) wood chips were treated with an MnP-lipid peroxidation system, 11% less energy was consumed while fiber strength and optical quality were maintained (Maijala et al. 2008). The treatment also increased the number of carboxyl groups on the surface of wood fibers while the total number of carboxyl groups was not affected. This indicates that the enzyme may act only on the surface of wood.

Most white-rot fungi readily produce oxalate during growth on lignocelluloses such as straw (Galkin et al. 1998; Hofrichter et al. 1999), and *Physisporinus rivulosus* started to secrete oxalate on the fifth day when growing on spruce wood (Hakala et al. 2004). Oxalate may directly contribute biopulping, namely it was found that esterification of oxalate produced by the fungus to the fibers may improve fiber saturation and correlates well with energy savings in biomechanical pulping (Hunt et al. 2004). Finally oxalate may have also many other roles in wood-rotting fungi (Mäkelä et al. 2002, 2009). In addition to MnP and oxalate, white-rot fungi secrete also an array of other oxidative and hydrolytic enzymes into the surrounding wood matrix, which all may be involved in the alteration of the microstructure of woody cell walls.

VI. Overview of Fungal Lignin Degradation and Outlook

Despite intensive research, especially on fungal oxidoreductases, it is still not clear in detail how lignin-degrading white-rot fungi actually attack lignin. The peroxidases involved in lignin degradation are rather well-known and their role in lignin degradation is generally accepted, while the role of laccase is not certain (Hatakka 2001; Hammel and Cullen 2008). Analogously to the degradation of polysaccharides (cellulose, hemicelluloses), it is assumed that lignin fragments produced by the peroxidase attack are taken up by the fungal hyphae and metabolized intracellularly. This process requires active uptake of lignin fragments through the fungal cell wall and membranes, which means that the fungus must possess transport mechanisms (Shary et al. 2008). This assumption is supported by the capability of the model fungus *P. chrysosporium* to metabolize aromatic compounds and de novo to synthesize veratryl alcohol (Lundquist and Kirk 1978). It was found at the end of the 1970s that mycelial pellets of *Sporotrichum pulverulentum* (the anamorph of *P. chrysosporium*) metabolize vanillic acid supplied to the cultivation medium, and several intermediates were analyzed from the extracellular medium (Ander et al. 1980). The fungus most probably utilizes intracellular or membrane bound cytochrome P450 enzymes, since it has as many as 154 cytochrome P450 encoding genes in its genome (Martinez et al. 2004). However, only a few transporters were upregulated under ligninolytic conditions (Shary et al. 2008). The uptake of lignin fragments may be an inefficient process. Apparently the lignin fragments cannot provide the fungus enough carbon and energy for growth since white-rot fungi cannot grow on polymeric lignin as a sole carbon source, i.e. lignin degradation by *P. chrysosporium* is a co-metabolic process associated with secondary metabolism and not linked to the growth of the fungus (Kirk and Farrell 1987).

All lignin-degrading white-rot fungi studied so far are apparently able to produce MnP (Hofrichter 2002). Laccase is also produced by almost all white-rot fungi, but not by the model fungus *P. chrysosporium*. This makes the role of laccase difficult to understand. A minor subset of white-rot fungi produce LiP or VP, the enzymes that can directly attack lignin (Ruiz-Dueñas and Martínez 2009). Thus the majority of lignin-degrading fungi seem to utilize a com-

ination of MnP and laccase (Hatakka 1994). This distribution was apparent also in a recent screening of about 50 lignin-degrading white-rot and litter-decomposing fungi, which were efficient degraders of wood lignin on wood blocks (Hakala et al. 2004) or in other test systems. MnP was the most common ligninolytic peroxidase, being even more commonly expressed by the fungi than laccase, but only about half of the species, such as those belonging to the genera *Cerrena*, *Phlebia*, *Pleurotus*, *Phanerochaete* and *Trametes* bleached the dyes Azure B and Reactive Black 5 indicative for LiP and/or VP production (Rainio, Majjala, Hatakka et al., unpublished data). As pointed out earlier it was concluded that, among more than 150 agaricomycetes, LiPs evidently arose only once in the Polyporales, whereas MnPs and VPs are more widespread and may have multiple origins (Morgenstern et al. 2008). The production of MnP is apparently distinctive to basidiomycetous fungi, since there are no serious reports on bacteria, yeasts and molds, nor mycorrhiza-forming basidiomycetes producing MnP (Hofrichter 2002). Studies claiming the contrary (i.e. production of “true” ligninolytic peroxidases by asco- and deuteromycetes, bacteria and other organisms) should be regarded with suspicion because they may either rely on incorrectly identified fungi or suffer from incorrect/insufficient enzyme assays (examples of such maybe misleading reports are Ferrer et al. 1992; Kanayama et al. 2002; Bermek et al. 2004). Last but not least, VP has only been reported from *Pleurotus* spp. and *Bjerkandera* spp.

The widespread production of MnP by white-rot fungi suggests its key role in lignin degradation. It was proposed that lignin can be mineralized outside the cell by an “enzymatic combustion” mechanism based on MnP acting as a radical pump (Hofrichter et al. 1999a; Hatakka 2001; Fig. 15.2). The most common way of attack on the lignin polymer would occur by oxidized and chelated manganese (Mn^{3+} -di-oxalate chelates; Fig. 15.2). The suggested mechanism involves indirect oxygenation of phenolic rings (ether peroxide formation), spontaneous ring opening to produce muconic acid derivatives and decarboxylation of the formed carboxyl groups to carbon dioxide (Hofrichter 2002). This mechanism does not necessarily produce small aromatic lignin fragments since the aromatic rings are gradually eroded outside the fungal cell.

According to the traditional model of lignin degradation, the action of extracellular heme peroxidase through one-electron and hydrogen abstractions from lignin units (the key step in the degradative process) produces unstable aryl cation radical intermediates, which undergo different reactions including breakdown of C_{α} - C_{β} and C4-ether linkages releasing the corresponding aromatic aldehydes (vanillin in the case of guaiacyl units) that can be intracellularly mineralized (Kirk and Farrell 1987; Ruiz-Dueñas and Martínez 2009). It should be kept in mind here that vanillin is toxic to the fungus already at low concentrations and probably oxidized and polymerized by peroxidases (Ander et al. 1980). In the case of *P. chrysosporium* LiP, lignin attack requires the presence of veratryl alcohol, probably as an enzyme-bound mediator, and hydrogen peroxide is mainly generated by glyoxal oxidase. In this traditional model concept there is no role for MnP (or laccase), and there is still the question how fungi that do not produce LiP (or VP) can also mineralize

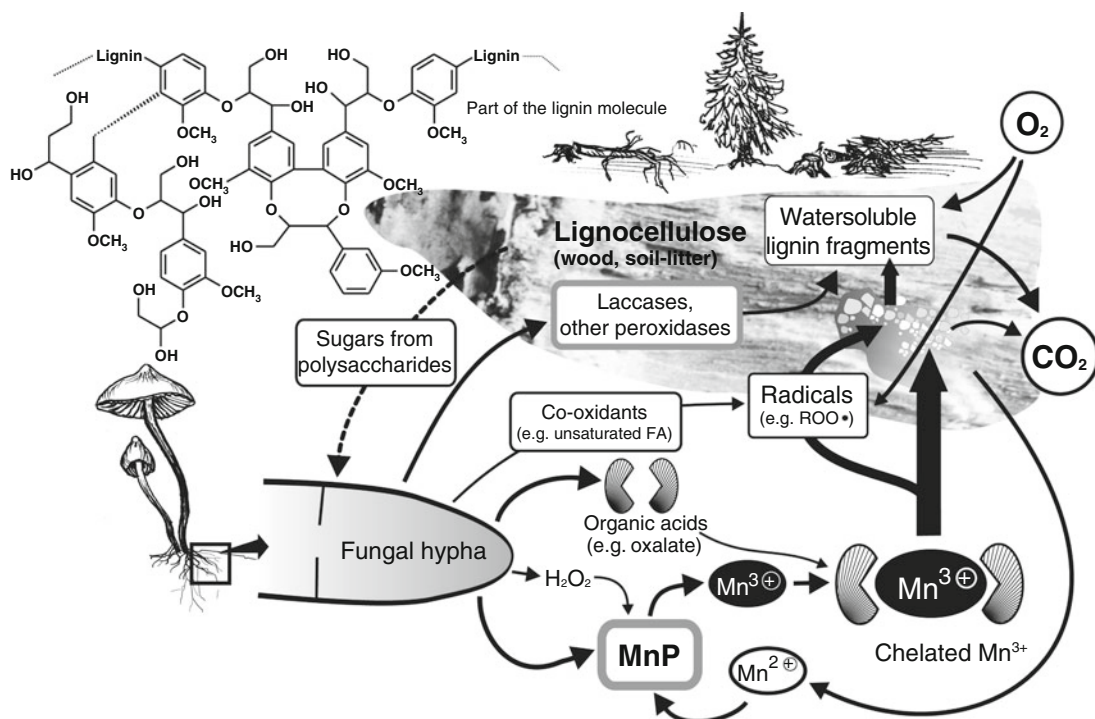


Fig. 15.2. Proposed mechanism of lignin degradation based on the radical-mediated reactions initiated by manganese peroxidase (MnP) acting as a radical pump (modified according to Fritsche and Hofrichter 2004)

lignin. If lignin fragments were (preferably) metabolized intracellularly, at least some energy and carbon should be gained from lignin for the fungus, and the fungus should be able to grow on lignin.

It is possible that white-rot fungi use both mechanisms to varying extents so that their role is important in fungi producing LiP and/or VP, and these fungi have an efficient metabolism of aromatic acids (e.g. vanillin) with necessary membrane transport mechanisms, while in other fungi the role of MnP is more significant. MnP-promoted lipid peroxidation causes the gradual extracellular breakdown of polymeric lignin (Bao et al. 1994), ring opening and formation of muconic acid residues, which can be decarboxylated by MnP, resulting in the production of carbon dioxide (Hofrichter et al. 1999a; Kapich et al. 1999, 2005; Hofrichter 2002).

The evidence is good that the earliest stage of brown-rot is oxidative and relatively non-selective, but there are also some differences between brown-rot species in the relative extents to which they modify lignin and cellulose (Irbe et al. 2001;

Filley et al. 2002; Niemenmaa et al. 2008), and the only other clear unifying feature in this decay type is that the fungi fail to mineralize much of the lignin. Given the diverse phylogenetic origins of brown-rot, one question is whether it has arisen repeatedly simply via the loss of ligninolytic enzymes that are essential for white-rot. If this is the case, there is presumably an underlying suite of oxidative components that are able by themselves to attack lignocellulose, albeit less efficiently where the lignin is concerned, and it should then theoretically be possible to convert any white-rot fungus to a brown-rot fungus by knocking out components of its ligninolytic apparatus. Alternatively, some brown-rot fungi may have evolved new decay mechanisms that were not already present in the white-rot lineages whence they arose. Presently, we have too little information to draw firm conclusions, but it is interesting that some proposed biodegradative components, e.g. cellobiose dehydrogenases and redox-active glycopeptides, have been reported in both decay types, whereas oxidant-generating hydroquinones have been found so far only in brown-rot fungi.

Despite all the progress of the past three decades, many questions regarding lignocellulose biodegradation by fungi are still open. Thus, more effort will be necessary to elucidate above all the regulation of ligninolysis both on molecular and enzymatic levels. Not least this will be the prerequisite for the further development of biopulping technologies and other applications of wood-rot fungi and their biocatalytic systems.

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16 (Hemi-)Cellulose Degrading Enzymes and Their Encoding Genes from *Aspergillus* and *Trichoderma*

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I. Introduction

A. Plant Cell Wall Composition

The plant cell wall is a complex structure composed of different polymeric compounds. Most of these compounds (up to 90%) are polysaccharides, such as cellulose, hemicellulose and pectin

(McNeill et al. 1984), but the cell wall also contains proteins and lignin, a polymeric compound consisting of aromatic residues.

Cellulose is a linear polysaccharide of $\beta(1\rightarrow4)$ -linked D-glucopyranose residues. Several (10–250) glucose chains are condensed to crystalline structures, called microfibrils, by the formation of hydrogen bonds (Kolpak and Blackwell 1976) and are linked together by hemicelluloses (Carpita and Gibeaut 1993). Cellulose also contains non-crystalline (amorphous) regions within the microfibrils. The relative amounts of crystalline and non-crystalline cellulose vary depending on the origin (Lin et al. 1987).

Three hemicelluloses have been identified in plant cell walls: arabinoxylan, xyloglucan and galactomannan. The most abundant and most heterogeneous hemicellulose is arabinoxylan (Biely 1985). This polysaccharide has a $\beta(1\rightarrow4)$ -linked D-xylopyranose backbone and can be substituted with different residues, depending on the origin of the xylan (Wilkie 1979). Single L-arabinofuranose residues and short arabino-oligosaccharides can be linked to O2 or O3 of the xylose residues. Glucuronic acid and its 4-O-methyl ether are attached to the xylose main chain by an $\alpha(1\rightarrow3)$ -linkage. This is the major substituent of hardwood xylans, whereas arabinose is the most abundant substituent in most other xylans. Depending on the origin of the xylan, acetyl-esters can be attached to O2 or O3 of the xylose residues (Wilkie 1979). Ester-linked feruloyl and *p*-coumaroyl residues can be present at O5 of terminal arabinose residues. Small amounts of D-galactopyranose have also been detected in certain xylans linked either to xylose or arabinose residues (Ebringerov et al. 1990, Wilkie and Woo 1977).

Xyloglucan has a backbone of $\beta(1\rightarrow4)$ -linked D-glucopyranose residues that are substituted with α -1,6 linked D-xylopyranose residues in a regular pattern (Hayashi 1989). Apart from single

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xylose substitutions, short oligosaccharides are also present as side chains of xyloglucan containing D-xylopyranose, D-galactopyranose, L-fucopyranose and L-arabinofuranose.

Galactomannan consists of a backbone of $\beta(1\rightarrow4)$ -linked D-mannose residues, which can be substituted with D-galactose residues via an $\alpha(1\rightarrow6)$ -linkage and can also contain small amounts of D-glucopyranose (Timell 1967). It is the main hemicellulolytic compound of softwood, but is only found in minor quantities in other plants. Pectin is comprised of structurally different regions, creating a highly complex structure (Albersheim et al. 1996). As pectin degradation is discussed in Chapter 20, we need not deal with its structure in detail here.

McCann et al. (McCann and Roberts 1991) have proposed a model for the primary plant cell wall of onion based on electron microscopy studies. In this model, the cellulose microfibrils are linked together by hemicellulose and pectin molecules. The resulting rigid structure gives the strength to the plant structure and as such functions as a skeleton.

B. *Aspergillus* and *Trichoderma*

Aspergilli are ascomycete fungi of the class Eurotiomycetes, which were first described by Micheli (1729). The genus consists of a large number of species and has received much attention over the past 75 years. The genus *Aspergillus* contains both pathogenic and non-pathogenic species. The best-studied pathogenic species are *A. fumigatus*, *A. flavus* and *A. parasiticus*. Amongst the non-pathogenic species *A. nidulans*, which is widely used as a model organism for mycelial fungi, and the group of black Aspergilli (e.g. *A. niger* and *A. tubingensis*) have received most attention. Products from several members of the latter group have a generally recognised as safe (GRAS) status, allowing these products to be used in food and feed industry. The black Aspergilli are particularly interesting for industrial applications, because they have good fermentation capabilities and are able to secrete large amounts of proteins and metabolites. They produce a wide range of plant cell wall degrading enzymes allowing bio-processing of crude biomass for food and non-food applications. The recent availability of *Aspergillus* genome sequences has revealed that they contain a much larger number of (hemi-)cellulases than

was previously described (Machida et al. 2005; Pel et al. 2007).

Trichoderma reesei (*Hypocrea jecorina*) is a member of the class Sordariomycetes of the Ascomycota and is with *Aspergillus* the most important industrial filamentous fungus. It has mainly been used for the production of cellulases and hemicellulases and it produces in particular high levels of cellulases, which has made *T. reesei* cellulose preparations the product of choice for many industrial applications that involve the degradation of plant polysaccharides. In contrast to the Aspergilli, genome sequencing of *T. reesei* only resulted in a small number of previously unidentified (hemi-)cellulases (Martinez et al. 2008; Ouyang et al. 2006).

C. CAZy Database

The CAZy database (www.cazy.org) is a sequence database devoted to enzymes that are active on carbohydrates (Cantarel et al. 2009). In this database, enzymes are divided into families based on amino acid sequence modules. The database contains five groups of families: the glycoside hydrolases (GH), glycosyl transferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE) and carbohydrate-binding modules (CBM). While some families contain only a single enzyme activity, other families contain several activities, which are often referred to as subfamilies. The CAZy database also contains information from public genome sequencing projects, but only if the resulting sequences have been submitted to GenBank.

In this chapter we only refer to those families that are relevant to our topic.

II. Galacto- (Gluco)mannan Degrading and Modifying Enzymes

A. Endomannanases and β -Mannosidases

Two classes of enzymes are responsible for the hydrolysis of the mannan backbone. Endomannanases (EC 3.2.1.78) cleave internal linkages resulting in the production of manno-oligosaccharides and belong either to family GH5 or GH26. The oligosaccharides generated by the endomannanases are further degraded by β -mannosidases (EC 3.2.1.25) that belong to GH1 and GH2. Some endomannanases contain carbohydrate binding modules. For

T. reesei Man5A it was shown that removal of this domain resulted in a significant reduction of the activity of the enzyme (Hagglund et al. 2003).

The terminal β -1,4-linked glucose residues are released from the oligosaccharides by β -glucosidases (EC 3.2.1.21) that belong to GH1 and GH3. All three classes of enzymes hydrolyse the substrate by retention of the anomeric configuration. Endomannanases are most active on galactomannans with a low substitution of the backbone (Civas et al. 1984) and release predominantly mannobiose and mannotriose from the polymer (Ademark et al. 1998; Civas et al. 1984; Eriksson and Winell 1968; Reese and Shibata 1965). The action of a β -mannosidase from *A. niger* was shown to be significantly reduced by the presence of galactose residues on the terminal mannose, but this inhibition is depended on the length of the oligosaccharide (Ademark et al. 1999).

B. Accessory Enzymes

The mannan backbone from galacto(gluco)mannan can contain acetyl and galactose side chains (see Section II.A). The acetyl residues are released by acetylglucosaminidases, which have been described for *A. niger* (Puls et al. 1992) and *A. oryzae* (Tenkanen 1998; Tenkanen et al. 1995). The *A. oryzae* enzyme is less specific than the one purified from *A. niger*, but both are active on polymers and oligomers and significantly enhance the activity of endomannanase. The galactose residues are removed by α -galactosidases (EC 3.2.1.22) that are assigned to GH27 and GH36.

Several α -galactosidases were described for *A. niger* with significant differences in substrate specificity (Ademark et al. 2001a, b; Manzanares et al. 1998) and several enzymes were also detected in *T. reesei* (Margolles-Clark et al. 1996). The presence of terminal β -linked galactose residues in some galactoglucomannans (Sims et al. 1997), suggested that β -galactosidases may also play a role. These enzymes are assigned to GH1, GH2 and GH35.

C. Presence of Mannan-Related Genes in Genomes of *Aspergillus*

CAZy annotation of genomes from several *Aspergillus* species provided insight in the total number of mannan related genes in these species. A recent study compared the polysaccharide degrading functions in *A. niger*, *A. nidulans* and *A. oryzae*

Table 16.1. Mannan-related function in the genomes of *Aspergillus niger*, *A. nidulans* and *A. oryzae*

Enzyme	<i>A. niger</i>	<i>A. nidulans</i>	<i>A. oryzae</i>
Endomannanase (GH5, GH26)	2	9	2
β -Mannosidase (GH2)	3	3	4
α -Galactosidase (GH27, GH36)	7	7	6
Total	12	19	12

and assigned functions to all putative genes identified in these species that could be assigned to CAZY families (Coutinho et al. 2009). Results of those analyses for mannan-related functions are summarized in Table 16.1. Based on this analysis, *A. nidulans* has the best developed mannanolytic system, while *A. niger* and *A. oryzae* are very similar.

III. Xylan Degrading and Modifying Enzymes

A. Endoxylanases and β -Xylosidases

Two groups of enzymes are responsible for the hydrolysis of the xylan backbone. Endoxylanases (EC 3.2.1.8) cleave internal linkages resulting in the production of xylo-oligosaccharides. The endoxylanases either belong to family GH10 or GH11. The 3-D structure of family 10 is characterised by a $(\beta/\alpha)_8$ fold whereas family 11 enzymes show a typical β -jelly roll structure. β -Xylosidases (EC 3.2.1.37) belong to GH3 and GH43. Xylanases hydrolyse the substrate by retention of the anomeric configuration.

All endo-splitting glycanases are composed of multiple subsites and the *A. niger* xylanase, which has been characterised in this respect, forms no exception to this rule having seven subsites (Biely et al. 1983). The individual endoxylanases have different action patterns in the degradation of xylan. Some enzymes cut randomly between unsubstituted residues, whereas the activity of others depends strongly on the substituent on the adjacent xylose residues. Endoxylanase I and III from *A. awamori* have different activities to linkages adjacent to singly substituted xylose residues (Kormelink et al. 1993a).

The oligosaccharides generated by the endoxylanases are further degraded by β -xylosidases. β -Xylosidase acts specifically on small xylo-oligosaccharides, resulting in the production of xylose.

Its action is inhibited in the presence of substituents on the xylose residues (Kormelink et al. 1993a). This enzyme therefore depends strongly on the presence of endoxylanases and accessory enzymes for the efficient production of xylose.

B. Accessory Enzymes

Due to the heterogeneity of the xylan structure, a wide range of accessory enzymes is needed to degrade this polysaccharide. *Aspergillus* and *Trichoderma* produce a complete spectrum of accessory enzymes, allowing complete hydrolysis of xylan by these fungi.

Arabinose residues are removed from the xylan main chain by α -L-arabinofuranosidase (EC 3.2.1.55) and arabinoxylan arabinofuranohydrolase. Arabinofuranosidases are also involved in the hydrolysis of pectin, but arabinoxylan arabinofuranohydrolase acts specifically on xylan-derived oligosaccharides and is not active on pectin. While arabinofuranosidases have been identified in many *Aspergilli*, arabinoxylan arabinofuranohydrolases have only been described for *A. awamori* (Kormelink et al. 1991) and *A. niger* (Gielkens et al. 1997).

However, recent data from genome annotations has indicated that this function is present in most filamentous ascomycetes, including *Aspergillus* and *Trichoderma*. The two groups of enzymes can easily be distinguished by using the synthetic substrate *p*-nitrophenyl- α -L-arabinofuranoside. Arabinoxylan arabinofuranohydrolases have no or very low activity against this substrate, whereas it can be readily hydrolysed by arabinofuranosidases.

α -Glucuronidases (EC 3.2.1.139) release glucuronic acid and its 4-O-methyl ether from xylan. They were first purified from *A. niger* (Uchida et al. 1992) and *A. tubingensis* (de Vries et al. 1998) but were later detected in all *Aspergilli* and *Trichoderma* and are assigned to GH67. The *A. niger* enzyme was reported to be an intracellular enzyme, but the purification procedure described does not exclude the possibility of the enzyme being in fact extracellular but bound to the cell wall.

Strong synergy exists between *A. tubingensis* α -glucuronidase and *A. tubingensis* endoxylanase A (de Vries et al. 2000). This α -glucuronidase has very low activity on polymeric substrates (de Vries et al. 1998) and prefers short xylo-oligosaccharides. It has been shown that *A. tubingensis* α -glucuronidase hydrolyses the substrate in a single step via the inverting mechanism (Biely et al. 2000). Re-

cently, the gene encoding an α -glucuronidase active on polymeric xylan was identified for the basidiomycete *Schizophyllum commune* (Chong and de Vries, unpublished data). This gene defined a new glycosyl hydrolase family (GH115) that also contains uncharacterised genes from *Aspergillus* and *Trichoderma*.

Acetyl xylan esterases (EC 3.1.1.72) remove acetyl residues from C2 and C3 of the xylose residues in the xylan main chain. They have only been purified from a limited number of species (Kormelink et al. 1993b; Koseki et al. 1997; Sundberg et al. 1990), but are present in most filamentous fungi. They have a high activity on intact xylan and are therefore believed to have a significant influence on the degradation of the xylan backbone by endoxylanases.

Feruloyl esterases release ferulic acid from C5 of terminal arabinose residues linked to the xylan main chain. Their activity depends strongly on the hydrolysis of the xylan backbone by endoxylanases (de Vries et al. 2000). Several feruloyl esterases with different characteristics, but all active on xylan, have been purified from *Aspergillus* sp. (de Vries et al. 1997, 2002a; McCrae et al. 1994; Tenkanen et al. 1991). Both FaeA and FaeB from *A. niger* have been shown to be active on xylan and pectin, but the former has higher activity on xylan, while the latter has higher activity on pectin (de Vries et al. 2000, 2002a).

A recent study including *A. niger*, *A. fumigatus*, *A. terreus* and *A. nidulans* demonstrated that orthologues of FaeA are only present in some *Aspergilli* (Benoit et al. 2008), while FaeB orthologues can be found in many filamentous ascomycetes. Gene duplication appears to be a common phenomenon for feruloyl esterase encoding genes. *A. terreus* contains two homologues of both FaeA and FaeB, while *A. oryzae* contains three homologues of FaeB (Benoit et al. 2008). A third family of feruloyl esterases, with members from both ascomycete and basidiomycete fungi and similarity to acetyl xylan esterases, contains characterised enzymes of *Neurospora crassa* and *Penicillium funiculosum*, but so far not from the *Aspergillus* species analysed in this study (Benoit et al. 2008). Finally, a new family of putative feruloyl esterases was identified that contains two *A. niger* genes and one from *A. oryzae*, but contains no functionally characterized members.

Glucuronyl esterases are able to break the ester linkage between glucuronic acid residues of xylan and lignin (Duranova et al. 2009a). The enzyme and gene have been described for *Trichoderma reesei* (Li et al. 2008), but are also present in several *Aspergilli* (Duranova et al. 2009b). The

Table 16.2. Xylan-related function in the genomes of *A. niger*, *A. nidulans* and *A. oryzae*. Numbers in brackets are for the JGI *A. niger* genome sequence that differs in a small number of genes from the DSM *A. niger* genome sequence

Enzyme	<i>A. niger</i>	<i>A. nidulans</i>	<i>A. oryzae</i>
Endoxylanase (GH10, GH11)	5	5	8
β -Xylosidase (GH3, GH43)	7(8)	11	15
α -Arabinofuranosidase (GH43, GH51, GH54)	4	7	7
Arabinoxylan arabinofuranose -hydrolase (GH62)	1	2	2
α -Glucuronidase (GH67, GH115)	1	2	5
Total	18(19)	27	37

gene encoding this enzyme appears to be an ancient gene, as it is present in both ascomycetes and basidiomycetes (Duranova et al. 2009a).

However, it has been subject to frequent gene duplication and gene loss events, suggesting that the biotope of the fungus strongly determines the need for this enzyme. Of the seven *Aspergilli* analysed (*A. niger*, *A. nidulans*, *A. oryzae*, *A. fumigatus*, *A. terreus*, *A. flavus*, *N. fisherii*) only *A. terreus* and *A. fumigatus* contain this gene. In contrast, the basidiomycetes *Coprinus cinereus* and *Phanerochaete chrysosporium* both contain multiple copies.

C. Presence of Xylan-Related Genes in Genomes of *Aspergillus*

Based on the annotation of the *Aspergillus* genomes (Coutinho et al. 2009), total numbers of xylan related genes are summarized in Table 16.2. This analysis shows that the *A. oryzae* has the largest set of xylan degrading functions, which is visible for each of the individual activities. *A. niger* has the smallest set of respective activities from the *Aspergilli* studied while related *A. nidulans* has an intermediate number.

IV. Cellulose and Xyloglucan Degrading and Modifying Enzymes

A. Description of Cellulose and Xyloglucan Active Enzymes

The biodegradation of cellulose requires the combined action of three classes of enzymes: $\beta(1\rightarrow4)$ -endoglucanases (EC 3.2.1.4), $\beta(1\rightarrow4)$ -exoglucanases

or $\beta(1\rightarrow4)$ -cellobiohydrolases (EC 3.2.1.91) and β -glucosidase (EC 3.2.1.21). Endoglucanases cleave the internal $\beta(1\rightarrow4)$ linkages in the glucose chains and have been purified from many *Aspergilli* as well as *Trichoderma* (e.g. Bagga et al. 1990; Vidmar et al. 1984). They are in general small proteins with a molecular mass of 20–40 kDa and are cut mainly in the amorphous regions of cellulose. The endoglucanases cloned so far from *Aspergillus* and *Trichoderma* spp. have been classified to glycoside hydrolase (GH) families 5, 12, 61 and 74. Members from GH12 and GH74 are active on xyloglucan only or on xyloglucan and cellulose. These enzymes have been identified in both *Aspergillus* and *Trichoderma* (Grishutin et al. 2004; Hasper et al. 2002) and kinetic differences between the different endoglucanase families with respect to xyloglucan hydrolysis have been reported (Ibatullin et al. 2008).

Cellobiohydrolases release cellobiose from the terminal ends of cellulose. Most fungi produce two types of cellobiohydrolases which release cellobiose from either the reducing or the non-reducing end.

β -Glucosidase, GH family 3, hydrolyses cellobiose to glucose, supplying the fungus with an easily metabolisable carbon source. Many fungi have been reported to contain multiple β -glucosidases with a large variety in molecular masses (e.g. Bagga et al. 1990; Witte and Wartenberg 1989). All the cellulolytic enzymes hydrolyse the substrate via a retaining mechanism.

In addition to the activities described above, hydrolysis of xyloglucan also requires accessory enzymes such as α -xylosidase (GH31), α -fucosidase (GH29), α -arabinofuranosidase (GH51, 54) and β -galactosidase (GH1, 2, 35). Genes encoding all these enzymes have been identified in *Aspergilli* (Coutinho et al. 2009; de Vries et al. 2005b).

B. Presence of Cellulose and Xyloglucan-Related Genes in Genomes of *Aspergillus*

Based on the annotation of the *Aspergillus* genomes (Coutinho et al. 2009), total numbers of cellulose and xyloglucan related genes are summarised in Table 16.3. Similar to the xylan-related functions, *A. oryzae* also has the largest set of cellulose/xyloglucan degrading functions,

Table 16.3. Cellulose- and xyloglucan-related function in the genomes of *A. niger*, *A. nidulans* and *A. oryzae*. Numbers in brackets are for the JGI *A. niger* genome sequence that differs in a small number of genes from the DSM *A. niger* genome sequence

Enzyme	<i>A. niger</i>	<i>A. nidulans</i>	<i>A. oryzae</i>
Endoglucanase (GH5, GH45, GH61)	11	12	12
β -Glucosidase (GH1, GH3)	15	17	21
Cellobiohydrolase (GH6, GH7)	4	5	4
Xyloglucan-active endoglucanase (GH12, GH74)	5	3	4
α -Arabinofuranosidase (GH43, GH51, GH54)	4	7	7
α -Xylosidase (GH31)	0	1	1
α -Fucosidase (GH29, GH95)	3	3	3
Total	42	48	52

while *A. niger* again has the smallest set and *A. nidulans* an intermediate number. However, *A. niger* has the largest number of putative xyloglucan-active endoglucanases, but appears to lack the α -xylosidase.

V. Regulation of (Hemi-)Cellulolytic Gene Expression

Aspergillus only produces the whole spectrum of xylan degrading enzymes during growth in the presence of xylan or xylose. This suggests a general system of regulation for the genes encoding xylanolytic enzymes. This regulatory system has been studied in detail in *A. niger*. Promoter deletion constructs of the *A. tubingensis xlnA* gene, a gene absent in *A. niger* N402, were used to identify a specific region involved in xylan specific induction of *xlnA* (de Graaff et al. 1994). Using this region in combination with a reporter system, the xylanolytic transcriptional activator encoding gene was cloned from *A. niger* (van Peij et al. 1998b). This gene, *xlnR*, encodes a protein with a zinc binuclear DNA binding domain common to transcription regulators of the GAL4 family (Marmorstein and Harrison 1994). A comparison

of xylanolytic promoters in combination with DNaseI footprinting resulted in identification of the XlnR binding site, 5'-GGCTAA-3', of which the second G was demonstrated to be essential by mutational analysis (van Peij et al. 1998b). Promoter analysis of *A. niger aguA*, encoding α -glucuronidase, demonstrated that the functional XlnR binding site in its promoter was GGCTAG, while the GGCTAA site that was also present was not active. This study therefore proposed a new consensus binding site for XlnR, namely GGCTAR (de Vries et al. 2002b).

A detailed expression analysis was performed in *A. niger* using a wild-type strain, a XlnR-negative mutant and a *xlnR* multicopy strain (van Peij et al. 1998a). The expression of genes encoding two endoxylanases (*xlnB* and *xlnC*), a β -xylosidase (*xlnD*), an acetyl xylan esterase (*axeA*), an arabinoxylan arabinofuranohydrolase (*axhA*), an α -glucuronidase (*aguA*) and a feruloyl esterase (*faeA*) were all under the control of XlnR, demonstrating that XlnR activates the complete xylanolytic spectrum of *A. niger*. Studies into functional domains of *A. niger* XlnR identified a C-terminal putative coiled coil region that is involved in nuclear import and indications for regulatory domains in the C-terminal part of XlnR (Hasper et al. 2004).

Although both *AxhA* and *AbfB* are able to release L-arabinose residues from arabinoxylan, the regulation of gene expression of these two L-arabinose releasing enzymes differ. Expression of *abfB* is strongly induced by L-arabitol and L-arabinose, whereas *axhA* expression is strongly induced by xylan and much less by L-arabitol, L-arabinose and D-xylose (Gielkens et al. 1997). The presence of a L-arabinose related regulator was first suggested using UV mutants that were disturbed in the expression of L-arabinose catabolic genes (de Groot et al. 2003). Later studies showed that *axhA* expression is under control of XlnR and *abfB* expression is mainly under control of the arabinolytic regulator AraR (Battaglia et al. 2010). XlnR has no or very little influence on the expression of *abfB*, while expression of *axhA* is only moderately influenced by AraR. Thus, the genes that release L-arabinose from arabinoxylan are either under control of XlnR or AraR. It has been shown that XlnR and AraR are highly interactive regulators involved in not only pentose release, but also pentose metabolism (Battaglia et al. 2010).

Introduction of multiple copies of one of the target genes of XlnR resulted in a decreased

expression of the other xylanolytic genes (Gielkens et al. 1999a; Kitamoto et al. 1998; van Peij 1999). This indicates a delicate balance between the production of XlnR and the number of XlnR target genes.

Although most xylanolytic genes seem to be exclusively activated by XlnR, expression of the feruloyl esterase A encoding gene (*faeA*) can also be activated by aromatic compounds, with a conserved substitution of the benzene ring (de Vries and Visser 1999). The presence of a methoxy group at C3, a hydroxy group at C4 and a non-substituted C5 was required for significant *faeA* expression levels, but the nature of the aliphatic group at C1 was of little importance. Although this system was also active in the XlnR-negative mutant, a positive interaction with XlnR was observed during growth on a combination of xylose and ferulic acid (de Vries and Visser 1999). These studies demonstrate that expression of *faeA* is controlled by both XlnR and a non-identified ferulic acid related regulator. In contrast, XlnR does not affect the expression of the second feruloyl esterase from *A. niger*, *faeB*. This gene is only expressed in the presence of aromatic compounds, which are only partly the same as those that induce expression of *faeA* (de Vries et al. 2002a).

Production of cellulolytic enzymes by *Aspergilli* has been observed using cellulose, cellobiose, glucose and xylose (Ali and Sayed 1992) as carbon source. From these compounds, cellulose, cellobiose and glucose are most likely specific for the cellulolytic genes, whereas xylose also induces the production of xylanolytic genes. The xylanolytic transcriptional activator XlnR/Xyr1 appears to be a key factor in the regulation of both hemi-cellulolytic and cellulolytic genes in both *Aspergillus* and *Trichoderma*. Analysis of the spectrum of genes regulated by XlnR in *A. niger* revealed that not only xylanolytic genes, but also two genes encoding endoglucanases (*eglA*, *eglB*; van Peij et al. 1998a) and two cellobiohydrolase encoding genes (*cbhA*, *cbhB*; Gielkens et al. 1999a) were regulated by this protein, as well as a gene encoding a xyloglucan active endoglucanase (*eglC*; Hasper et al. 2002).

A. oryzae XlnR (AoxlnR) also mediates both xylanolytic genes (*xynF1*, *xynG1*, *xynG2*, *xylA*) and cellulolytic genes (*celA*, *celB*, *celC*, *celD*; Marui et al. 2002a, b) in response to the presence of xylose and xylan, but also in response to cellobiose and cellulose (Marui et al. 2002b). The endoglucanase gene (*eglA*) of *A. nidulans* and the cellobiohydrolase C gene (*celC*) of *A. oryzae* showed a cellulose/cellobiose responsive expression independently of XlnR (Endo et al. 2008; Marui et al. 2002a). A conserved region, referred to as the cellulose responsive element (CeRE, CCGTACCTTTTAGGA), has been found in *A. oryzae*

eglA promoter by mutational analysis (Endo et al. 2008). This element leads to XlnR-independent induction and is partially conserved in other *A. oryzae* and *A. nidulans* cellulase promoters.

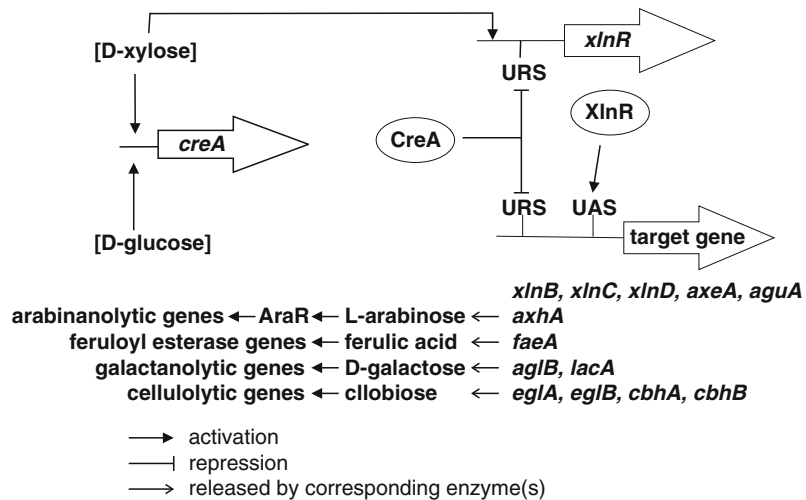
In *T. reesei*, the xylanolytic and cellulolytic system is also under regulation of Xyr1, the orthologue of *Aspergillus* XlnR (Stricker et al. 2008). Although Xyr1 has a similar function as XlnR there are some clear differences in the regulation mechanism. In contrast to *Aspergilli*, in which D-xylose has been suggested to be the main inducer of both the xylanolytic and cellulolytic system, a diversity of inducers have been reported in *T. reesei*, including D-xylose, xylobiose, sophorose and lactose. Analysis of the binding site of Xyr1 demonstrated that, in addition to GGCTAA, other sites are functional resulting in a consensus site GGC(A/T)₃ (Furukawa et al. 2009). No AraR orthologue is present in *T. reesei* (Battaglia et al. 2010), but part of the genes involved in L-arabinose release and metabolism are influenced by Xyr1 (Akel et al. 2009). The xylanolytic and cellulolytic regulatory system involves two fine-tuning regulators in *T. reesei*: AceI and AceII (Stricker et al. 2008). AceI orthologues are commonly present in fungi, including the *Aspergilli* (de Vries et al., unpublished data) and the *A. nidulans* orthologue (StzA) has been shown to be involved in abiotic stress response (O'Neil et al. 2002). However, an orthologue for AceII cannot be found in *Aspergillus niger* (Stricker et al. 2008).

A comparative transcriptome analysis of *A. niger*, *A. nidulans* and *A. oryzae* identified 22 xylose-induced genes that were conserved in all the three *Aspergilli* (Andersen et al. 2008). Transcriptional analysis of a *xlnR* overexpression strain identified 75 xylose-induced genes in *A. oryzae* (Noguchi et al. 2009). In addition to 32 glycoside hydrolases, these genes also included MFS transporters, pentose catabolic pathway genes and other putative metabolic genes.

Additional studies demonstrated the involvement of XlnR in, not only the xylanolytic and cellulolytic enzyme system, but also in the expression of an α -galactosidase (*aglB*; de Vries et al. 1999a) and β -galactosidase encoding genes (*lacA*, *bga1*; de Vries et al. 1999a; Stricker et al. 2007).

The carbon catabolite repressor CreA (Dowzer and Kelly 1991) is of major importance for the expression of genes encoding cell wall degrading enzymes (e.g. see Ruijter and Visser 1997). In the presence of glucose this protein represses the expression of these genes and in *A. nidulans* it was shown that CreA represses xylanolytic genes

Fig. 16.1. Model for the influence of XlnR and CreA on the expression of *A. niger* genes encoding cell wall degrading enzymes. URS Upstream repressing sequence, UAS upstream activating sequence. Partially based on Gielkens (1999) and Van Peij (1999)



indirectly or directly in presence of glucose (Tamayo et al. 2008). Analysis of the promoter regions of xylanolytic genes revealed the presence of the CreA binding sequence (SYGGRG; Kulmburg et al. 1993) in all these genes, including *xlnR*. Generally, the expression level of xylanolytic genes is higher on xylan than on xylose (e.g. de Vries et al. 1998), suggesting that a high xylose concentration might also cause repression.

A previous study demonstrated that the expression of xylanolytic genes on xylose indeed decreases with increasing xylose concentration as a result of CreA-mediated repression (de Vries et al. 1999b). The major level of transcription regulation for (hemi-)cellulolytic genes is thus formed by two regulatory proteins, the activator XlnR and the repressor CreA, supplemented by a number of more specific regulatory systems. In *T. reesei*, Cre1 (the orthologue of CreA) is also responsible for carbon catabolite repression. Some cellulolytic and xylanolytic genes have been shown to be Cre1-dependent, while others are Cre1-independent (Stricker et al. 2008).

Based on these data a model can be proposed for the regulation of (hemi-)cellulose degradation in *Aspergilli* (Fig. 16.1). During growth of *A. niger* in the presence of arabinoxylan monomeric xylose, already present in the substrate or released by endoxylanase B and β -xylosidase present at low constitutive levels, results in activation of XlnR. XlnR subsequently activates expression of (hemi-)cellulolytic genes (de Vries et al. 1999a; Gielkens et al. 1999b; van Peij et al. 1998a). However, high concentrations of monomeric xylose also cause CreA-mediated repression, resulting in reduced expression of these genes (de Vries et al. 1999b).

The presence of glucose results in a strong CreA effect, largely preventing the expression of (hemi-)cellulolytic genes (e.g. de Graaff et al. 1994; de Vries et al. 1998).

When XlnR activates the expression of (hemi-)cellulolytic genes, corresponding enzymes release arabinose, cellobiose, ferulic acid and galactose. These compounds are on their turn responsible for the expression of genes encoding arabinolytic enzymes (Battaglia et al. 2010), cellulolytic enzymes (Ali and Sayed 1992), feruloyl esterases (de Vries et al. 2002a; de Vries and Visser 1999) and galactanolytic enzymes (de Vries et al. 1999a), respectively. CreA is also involved in the repression of these genes and has therefore several levels of control.

VI. Applications of (Hemi-)Celluloses

Xylan degrading enzymes have found ample applications in many different industrial food and non-food processes. One of the major areas is the baking industry. By (partly) degrading the arabinoxylan moieties in dough, this fraction is solubilised. As a result of this, the quality of the dough is improved and an increase in bread volume is obtained (Maat et al. 1992; Petit-Benvegnen et al. 1998; Poutanen 1997).

Xylan degrading enzymes are also of major importance to the pulp and paper industry where they are used during bio-bleaching. A prerequisite for their use is that these enzyme preparations are free of cellulases, since cellulose

degradation would result in a reduction of the quality of the pulp. Bio-bleaching of pulps has become an interesting alternative to chemical bleaching using chlorine (Viikari et al. 1994). By enzymatically degrading the lignin-hemicellulose complexes present in the pulp, the cellulose fibres remain intact. Additionally, bio-bleaching strongly reduces the environmental problems caused by bleaching using chlorine. So far, mainly endoxylanase has been applied for bio-bleaching of pulps (Viikari et al. 1994), but other xylan degrading enzymes have an additive effect in this process (Kantelinen et al. 1988).

Xylan degrading enzymes are also applied in the production of animal feed. By partially degrading the biomass in animal feed a higher feed conversion efficiency is obtained (Bedford and Classen 1992; van Paridon et al. 1992). Oligosaccharides are increasingly applied as functional food additives or as alternative sweeteners with beneficial properties. Using defined xylan degrading enzymes, xylose, xylobiose and specific xylo-oligosaccharides can be produced from arabinoxylan containing plant cell wall preparations (Biely et al. 1991; Pellerin et al. 1991; Puls et al. 1988). Xylan degrading enzymes are also applied for the clarification of juices (Zeikus et al. 1991), although not to the extent of the pectin degrading enzymes. The most recent application of xylanases is in pre-treatment of plant biomass for bio-fuel production. In this application, complete hydrolysis of the biomass is required and xylan degrading enzymes play an important role in this process together with cellulases (Tabka et al. 2006).

Cellulases in general have found widespread application in industrial processes. For instance they are used in washing powders to reduce pilling and to give clothes a new look by removing loose cellulose fibers. Also, certain cellulases are used in the paper industry at various stages of the production process. However, for each industrial process a typical cellulase of certain origin is most suitable. In the detergent industry one of the critical parameters is the alkaline tolerance of the cellulases.

An *Aspergillus* endoglucanase is used to reduce the viscosity of the wort during beer making which improves the filtration of the beer and moreover, improves the digestibility of barley when used as feed-stuff (Madrid et al 1996).

A. niger FaeA was used on oilseed flax straw, resulting in a very low kappa number (directly proportional to lignin content), a beneficial effect on pulp brightness and a recovery of phenolic compounds of interest (Sigoillot et al. 2005; Tapin et al. 2006). Feruloyl esterases are able to

release phenolics compounds such as ferulic, *p*-coumaric, caffeic and sinapic acids from the plant cell wall. These phenolics compounds are widely distributed in the plant kingdom and have been receiving increasing attention with regard to applications in the food, health, cosmetic and pharmaceutical industries (Kroon and Williamson 1999). In particular ferulic acid can perform several biological functions, such as UV absorber, anti-oxidant (Kikuzaki et al. 2002) and anti-inflammatory (Murakami et al. 2002) activity. It is one of the major anti-oxidant constituents in beer (Maillard and Berset 1995), while its occurrence in orange juice is responsible for the off-flavour formation during storage (Peleg et al. 1992).

Endomannanases and β -mannosidases are used in laundry detergents, poultry feeds, coffee processing and together with xylanases in the paper and pulp industry (Sachslehner et al. 2000; Schafer et al. 2002; Wong and Saddler 1993; Wu et al. 2005). α -Galactosidases are used to improve the gelling properties of galactomannans for both the food and pharmaceutical industry (Brillouet and Joseleau 1987; Critchley 1987), while they are also used to reduce raffinose concentrations in soybean milk (Mulimani and Ramalingam 1995), cowpea meal (Somari and Balogh 1995) and sugar beet syrup (Ganter et al. 1988).

A major new application field is the use of (hemi-)cellulases in bio-fuel production. Conversion of plant biomass to fermentable sugars can be performed chemically (e.g. acid treatment) but for practical and environmental reasons a strong preference is developing for enzymatic conversion (in combination with mild heat or acid treatments; Margeot et al. 2009; Slade et al. 2009; Wilson 2009).

VII. Conclusions and Prospects

The availability of fungal genome sequences offers new opportunities for a more rational approach to study and apply fungal (hemi-)cellulases. Our understanding of the variety of enzymes that can be produced by fungi has increased significantly and the available selection of enzymes for applications has grown exponentially and will continue to grow to a near limitless number. The challenge is to match the increase in gene data with biochemical support of the differences in substrate specificity, product profiles and physical characteristics, such as temperature and pH optimum. More detailed biochemical or functional data will likely enable

the establishment of subfamilies of specific enzyme activities that are similar in their action.

As shown in this chapter, even among relatively closely related species such as the *Aspergilli*, significant differences in the carbohydrate degrading genome content were observed. These changes likely reflect adaptations to their particular biotopes. Detailed analysis of additional species and a good description of the natural biotopes of the individual species may reveal correlations between the composition of the substrates found in the biotope and the CAZy genes in the genomes of the different species. In addition, these comparisons will allow a fast selection of the best organism used for mining for specific enzyme activities.

Efficient production of single enzymes and defined enzyme mixtures can best be achieved by strain improvement of *Aspergillus* or *Trichoderma*, considering the long history of use of these enzyme production systems in industry. Use of closely related species of the traditional industrial hosts that have improved properties with respect to enzyme production is another option.

An example of this is *Aspergillus vadensis*, a close relative of *A. niger* that unlike *A. niger* does not acidify the medium and does not produce extracellular proteases (de Vries et al. 2004, 2005a). This strain is particularly useful for the production of homologous and heterologous proteins that suffered from degradation in *A. niger*. This property of *A. vadensis* has already been demonstrated for *A. niger* FaeB (Alberto et al. 2009) and for a glucuronyl esterase from *Phanerochaete chrysosporium* (Duranova et al. 2009b).

The introduction of multiple copies of the gene(s) encoding the desired enzyme(s) will increase their production and simplify purification. Applying constitutive promoters in combination with these genes will enable production of the enzyme(s) under conditions in which the production of other enzymes is repressed.

Genetic manipulation of regulatory systems will also have a large effect on the production of the enzymes. Introduction of multiple copies or disruption of genes encoding regulatory factors will either increase or decrease the production of enzymes influenced by these factors. Application of more specific regulatory systems for strain improvement depends on the isolation of the regulatory genes, the characterisation of the corresponding proteins and the identification of the target genes. This will require efficient screening

systems, using for instance specific reporter systems or DNA-chip technology.

Site-directed mutagenesis can be used to introduce specific changes in the genes resulting in the production of enzymes with altered features (e.g. thermostability, pH profile). Gene shuffling can be applied to combine desired properties from different enzymes, creating new and better enzymes.

With the availability of good bioinformatics tools for genome mining, detailed knowledge about the action of the enzymes, efficient systems for the production of pure enzymes and specific enzyme mixtures and techniques to design new enzymes, new and approved applications for polysaccharide degrading enzymes will rapidly be developed.

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17 Fungi and Their Enzymes for Pitch Control in the Pulp and Paper Industry

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I. Introduction

Traditionally, the use of biotechnology has been mainly confined to fermentation processes in the food and beverage industry and in the pharmaceutical sector. Over the past decades, however, biotechnology has gained ground from chemical processes in the manufacturing of a variety of industrial products, including pulp and paper (Skals et al. 2008). During recent years, the number of applications of enzymes in pulp and paper manufacturing has steadily grown, and several have reached or are approaching commercial use. These include enzyme-aided bleaching with xylanases, direct delignification with oxidative enzymes, refining with cellulases, pitch reduction with lipases, freeness enhancement with cellulases and hemicellulases as well as enzymatic slime control (Bajpai 1999, 2006). In addition to enzymes, microbial treatments have the potential

to increase pulping efficiency, reduce pitch problems, and enhance process water re-use.

In the present chapter, we focus on an important aspect of pulp and paper manufacturing, namely pitch control, which is the first example where biotechnology provided successful solutions in this industrial sector. To determine the practical potential of a biological treatment for pitch control, it is imperative to understand the heterogeneity of lipophilic extractives in wood and pulp, to determine which classes of lipids are important in pitch deposition, and to assess the effectiveness of biological agents in reducing and removing many in all troublesome lipid classes. With this purpose, an overview of the pitch problems in the pulp and paper industry is first provided, followed by a description of the state of the art and the current research on biotechnological solutions to pitch problems, including the use of fungal strains for the pretreatment of wood chips prior to pulping and fungal enzymes for the direct treatment of pulps. Finally, some concluding remarks and future trends in this field are included.

II. Pitch Problems in Pulp and Paper Manufacturing

Lipophilic wood extractives, i.e. the non-polar extractable fraction from wood often referred to as wood resin (Fig. 17.1A), cause so-called pitch deposits during pulp and paper manufacturing processes (Fig. 17.1B). Pitch deposition is a serious problem in the pulp and paper industry since it is responsible for reduced production levels, higher equipment maintenance costs, higher operating costs, and an increased incidence of defects in the finished products, which reduces quality and benefits (Back and Allen 2000).

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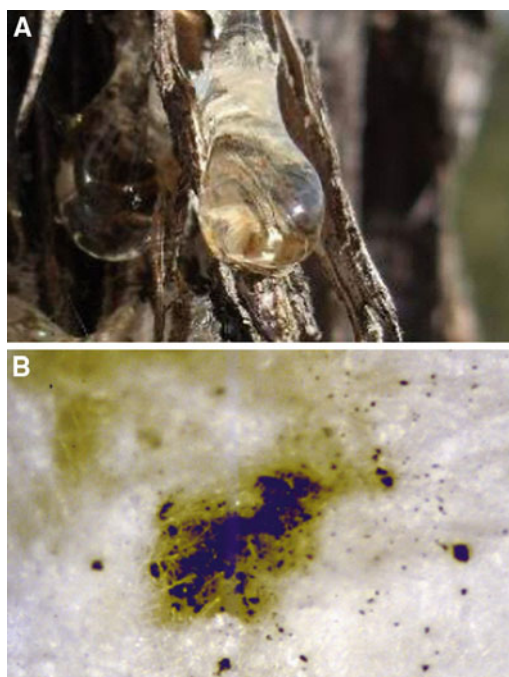


Fig. 17.1. Resin exudate on a conifer tree (A) and pitch deposit in a TCF pulp (B); courtesy of M.J. Ortega and J. Romero, respectively

Furthermore, process effluents containing wood extractives may also be toxic and harmful to the environment (Leach and Thakore 1976; Liss et al. 1997).

A. Resin in Plant Raw Materials

Lipophilic extractives comprise a variety of different compounds including alkanes, fatty alcohols, fatty acids, resin acids, sterols, other terpenoids, conjugated sterols (as esters and glycosides), triglycerides, and waxes (Fig. 17.2). The content and composition of resin vary considerably between the different angiosperm and gymnosperm species (Fig. 17.3) and even between different parts of the same plant. Some differences are caused by the growing conditions, the age of the tree, and other genetic and environmental factors.

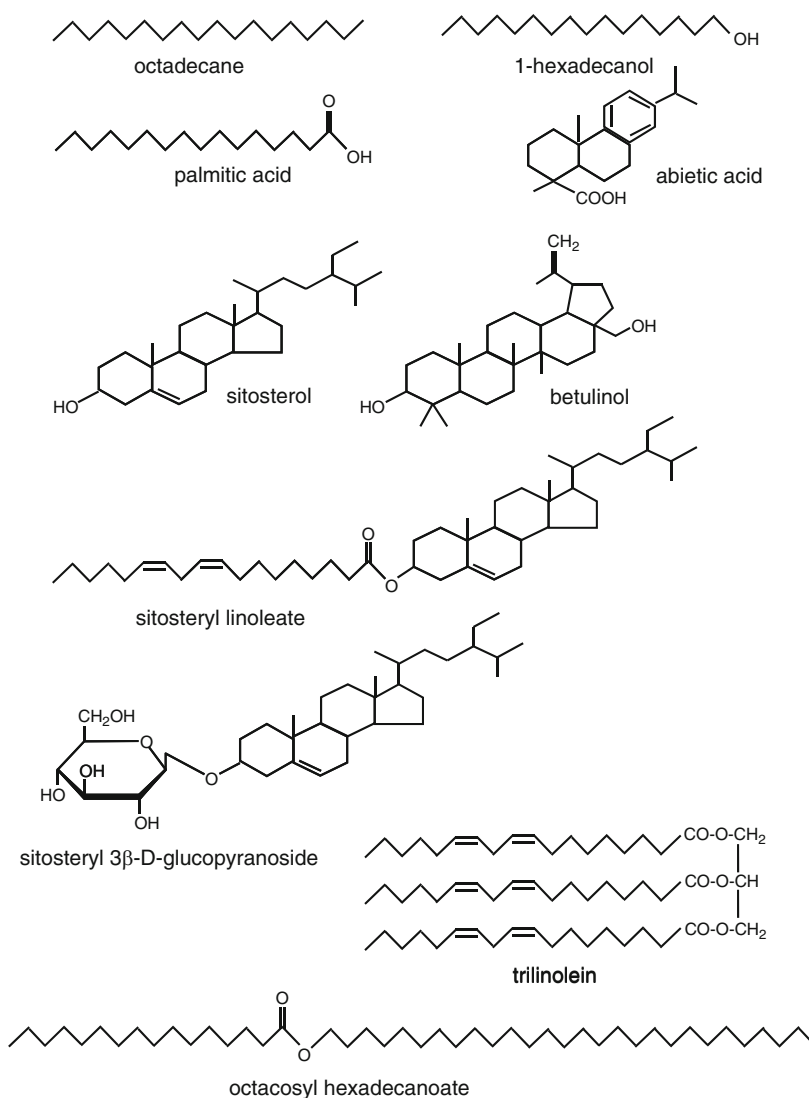
The resin components of softwoods (gymnosperms) commonly used in the pulp and paper industry, such as Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*), have been extensively studied (Ekman and Holmbom 2000). Triglycerides, resin acids, and fatty acids represent a high percentage of Scots pine extractives.

Norway spruce contains similarly high amounts of triglycerides, resin acids, and steroids. All these lipophilic compounds have been traditionally associated with pitch problems in the paper-making process (Back and Allen 2000; Ekman and Holmbom 2000).

Among hardwood trees (woody angiosperms), silver birch (*Betula pendula*) and trembling aspen (*Populus tremuloides*) have been traditionally used for paper pulp production and, therefore, their wood resin have been thoroughly studied (Ekman and Holmbom 2000). A significantly higher proportion of sterols and other unsaponifiable lipids are reported in birch and aspen compared to softwoods, although triglycerides predominate in both species. Resin acids present in all kinds of softwood are not found in hardwoods. Sterol esters and waxes (Chen et al. 1995) and triterpenols and saturated fatty acids (Bergelin et al. 2005) are reported to be at the origin of pitch deposits in aspen and birch, respectively. New pieces of information on the composition of lipophilic extractives have come from eucalypt wood (*Eucalyptus globulus* and related species), during the past ten years due to the increasing use of this fast-growing hardwood tree by the paper pulp industry. Free and conjugated sterols (in the form of fatty-acid esters and glycosides) are the main lipophilic components of eucalypt wood, together with other steroids and free fatty acids (Freire et al. 2002; Gutiérrez et al. 1999a, 2001; Rencoret et al. 2007), being also responsible for pitch deposition (del Río et al. 1998, 1999, 2000; Freire et al. 2005; Gutiérrez et al. 2001b, c; Silvestre et al. 1999).

The chemical composition of lipophilic extractives from non-woody species used by the pulp and paper industry has not been as extensively studied as that of woody species, although knowledge on straw-like materials has accumulated during recent years (del Río and Gutiérrez 2006; Gutiérrez and del Río 2003a, b; Morrison and Akin 2001; Sun and Sun 2001). Fatty acids, free and esterified sterols, triglycerides, and waxes are the predominant lipophilic compounds in wheat-straw, which is an important raw material for pulp and paper manufacturing in China because of the shortage of forest resources and the extensive supply of wheat-straw (Sun and Sun 2001). Alkanes, fatty alcohols and fatty acids, aldehydes, sterols, and waxes are the major lipid compounds identified in non-woody plants such as flax, hemp, kenaf, sisal, and abaca, which are used in developed countries for the production of high-quality pulps for specialty papers. In particular, fatty alcohols, alkanes, and sterols are among the compounds responsible for pitch deposits formed during pulping of non-woody plants (Gutiérrez and del Río 2005).

Fig. 17.2. Chemical structures of selected compounds representing the main classes of lipophilic extractives found in woody and non-woody plants, including alkanes (octadecane), fatty alcohols (1-hexadecanol), fatty acids (palmitic acid), resin acids (abietic acid), sterols (sitosterol), triterpenols (betulinol), sterol esters (sitosteryl linoleate), sterol glycosides (sitosteryl 3 β -D-glucopyranoside), triglycerides (trilinolein), and waxes (octacosyl hexadecanoate)



B. Resin in Pulp and Paper

The lipophilic extractives present in the raw materials can cause pitch problems along the entire pulp and paper manufacturing processes (pulping, bleaching, paper machine processing). The nature and severity of pitch problems depend not only on the raw materials used, but also on the industrial processes of pulping and bleaching applied at the mill. In this way, mechanical pulping often applied to softwoods for manufacturing newspaper and similar pulps, slightly affects the

composition of extractives since no chemicals acting on lignin are added. Therefore, the corresponding pulp extractives show a composition similar to that found in natural softwood. In contrast, chemical pulping, including kraft cooking that provides over 50% of the total paper pulp, modifies the extractives composition to different extents depending on the raw material. In this way, an important fraction of pitch-forming compounds in softwoods is removed during kraft cooking, since it consists of triglycerides that are completely saponified under the alkaline

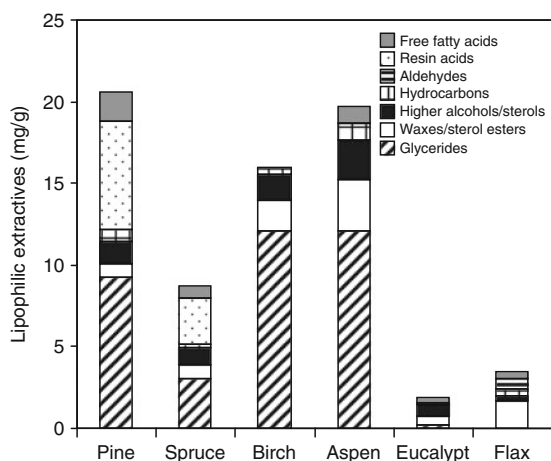


Fig. 17.3. Abundances of main classes of lipophilic extractives in several softwoods (pine, spruce), hardwoods (birch, aspen, eucalypt) and a non-woody plant (flax). Data from Fengel and Wegener (1984), Gutiérrez et al. (1999a) and Gutiérrez and del Río (2003a, b)

conditions used and the fatty and resin acids are dissolved (Fengel and Wegener 1984). The sterol and triterpenol esters, especially abundant in many hardwoods including eucalypt and birch, are saponified more slowly than the glycerol esters (Bergelin and Holmbom 2003; Gutiérrez et al. 2001b). The remaining free and esterified sterols and triterpenols do not form soluble soaps as free acids do and, therefore, have a tendency to deposit.

The wood extractives remaining in the unbleached pulp are carried over to the bleach plant, where they react with the bleaching agents used (Jansson et al. 1995). Pulp bleaching technology radically changed in the 1990s, and the previously used chlorine was replaced and new bleaching sequences were introduced. The use of totally chlorine-free (TCF) bleaching in place of elementary chlorine-free (ECF) bleaching has increased the severity of pitch problems due to the lower reactivity with pulp lipids (Gutiérrez et al. 2001b). This applies to most unsaturated sterols and triterpenoids as well as fatty acids, which are strongly modified by chlorine dioxide (ClO_2) used in ECF bleaching but remain practically unaltered by oxygen and hydrogen peroxide used in TCF bleaching sequences (Bergelin and Holmbom 2003; Freire et al. 2005, 2006; Gutiérrez et al. 2001b). Pitch colloidal particles “surviving” the action of bleaching chemicals, can coalesce and deposit in the pulp or the equipment, resulting in low quality pulp and causing the shutdown of pulp mill operations. The residual resin in the pulp is at the origin of pitch problems in the paper mill, including spots and holes in the paper, sheet breaks, and technical shutdowns of the paper machine

(Allen 2000a). Moreover, in the paper mills as well as in the pulp mill, the degree of system closure can have a considerable impact on the propensity to pitch problems. Thus, when white waters recirculate, there is a tendency for the dissolved and dispersed resins to increase in concentration which, in turn, increase the tendency for the formation of certain kinds of pitch (Allen 2000b).

Because the nature of pitch problems can be very different depending on the raw material and the process used, different physicochemical methods to control pitch in pulp and paper mills are used (Allen 2000a, b). These include adsorption or dispersion of the pitch particles with chemicals in the pulping and papermaking processes by adding alum, talc, ionic or non-ionic dispersants, cationic polymers, and other types of additives. In addition to chemical solutions, biological methods to control pitch problems have also been developed, as described below.

III. From “Natural” to “Controlled” Seasoning of Wood

Traditional methods to control pitch problems include deliberate storage of logs and/or wood chips in the woodyard before pulping (natural “seasoning”). The outside storage of pulpwood was introduced in the 1920s for whole logs and in the early 1950s for wood chips (smaller pieces of chopped wood) (Farrell et al. 1997). This method was the direct consequence of the need to stockpile wood as inventory to mill and to season wood, which resulted in decreased resin deposition. During wood storage, the content of extractives decreases since some of them are subject of hydrolytic or oxidative transformations by plant enzymes as well as by the action of wood-colonizing microorganisms. The reactions of wood resin components during storage have been studied for several pulpwood species including spruce, pine, birch, aspen, and eucalypt (Ekman 2000; Gutiérrez et al. 1998; Silvério et al. 2008). However, prolonged storage causes a decrease in the pulp brightness and yield due to the uncontrolled action of microorganisms. Therefore, the industrial practice today does not include overlong log or chip storage times. In addition, polymerization of unsaturated wood resin components are also reported to occur during seasoning

and studies with model compounds have been performed (Ekman 2000; Sitholé et al. 2009). As an alternative, the use of special fungi to accelerate and control the seasoning of wood has been considered. The ability to colonize lignified plant materials is a characteristic of wood-decay fungi, including white-rot, brown-rot, soft-rot, and sapstain species (Martínez et al. 2005). Among these eco-physiological groups, selected sapstain and white-rot fungi have particularly been considered for the control of pitch, as explained below. In addition, wood treatment with bacteria to remove lipophilic extractives has also been suggested and studied in the laboratory (Burnes et al. 2000; Kallioinen et al. 2003).

A. Sapstain Fungi

A small number of ascomycetes, so-called staining fungi, colonize wood through parenchymatic rays and resin canals. They cause the discoloration of sapwood tissues, due to the presence of melanin-like pigments in the fungal hyphae (Zimmerman et al. 1995), but only a limited degradation that mainly affects extractives and water-soluble components (Martínez et al. 2005). Since most lipophilic compounds involved in the formation of pitch deposits are concentrated in wood rays and resin canals, the sapstain fungi were the first candidates for the biological control of pitch (Brush et al. 1994; Farrell et al. 1993). Sapstain, also called blue stain, is caused by different pioneer colonizers, such as *Ophiostoma*, *Ceratocystis*, *Leptographium* or *Sphaeropsis* species, which utilize fatty acids, triglycerides, simple carbohydrates, and other components of the sapwood (Farrell et al. 1993). In addition, these fungi can rapidly colonize non-sterile wood chips. Research on the biotechnological application of sapstain fungi for wood depitching has mainly focused on *Ophiostoma piliferum* strains, a dark-colored ascomycete (Fig. 17.4A) widely distributed in forests and in chip piles. These research efforts led to the commercialization of an albino strain (Cartapip 97) of this fungus (Fig. 17.4B) for pitch reduction in wood chip piles without causing any staining effect. This and other colorless strains were obtained by classic mating approaches (followed by single ascospore isolation) and neither mutagenesis nor genetic engineering methods were applied. As in the case of other sapstain

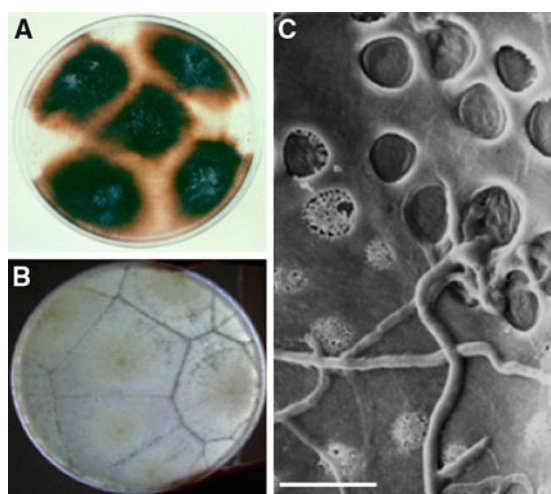


Fig. 17.4. Wild (A) and albino (B) *Ophiostoma piliferum* strains, and typical growth of the albino fungal strain through wood vessels as revealed by scanning electron microscopy (C; bar 10 μ m); courtesy of R. Farrell

fungi, their growth mainly proceeds through wood vessels and rays (Fig. 17.4C), removing the extractives that accumulate in these structural elements.

Cartapip 97 was originally marketed by Sandoz Chemicals/Clariant Corporation and has been used by the pulp and paper industry for the past 15 years. A 90% decrease of triglycerides causing pitch problems in the manufacture of mechanical and acidic sulfite pulps was reported after Cartapip treatment of pine wood (Farrell et al. 1993). The capabilities of some *Ophiostoma* species and other sapstain fungi in removing different classes of lipophilic extractives were assessed for both softwoods (Table 17.1) and hardwoods (Table 17.2). Among others, the effectiveness of these fungi to degrade triglycerides and free fatty acids was demonstrated in several independent studies using different types of wood (Breuil et al. 1998; Brush et al. 1994; Dorado et al. 2000a; Gutiérrez et al. 1999b; Josefsson et al. 2006; Martínez-Íñigo et al. 1999; Rocheleau et al. 1999). A higher degradation rate was observed for Scots pine than for Norway spruce extractives, which was attributed to differences in the degradation of triglycerides (Josefsson et al. 2006). Some studies reported a low efficiency in the removal of sterol esters (Chen et al. 1994; Josefsson et al. 2006) whereas other reports showed a significant conversion of these compounds by sapstain fungi (Gutiérrez et al. 1999b; Martínez-Íñigo et al. 1999). However, the Cartapip strain and related fungi seem to be unable to efficiently remove free sterols from hardwoods and resin acids from softwoods (Chen et al. 1994; Dorado et al. 2000a; Gutiérrez et al. 1999b; Martínez-Íñigo et al. 1999), even though, a significant decrease in free sterols (up to 60%) was reported by Su et al. (2004) to be caused by some newly screened fungal strains.

Table 17.1. Degradation of main lipid classes in *Pinus sylvestris* sapwood and heartwood during the growth of two white-rot (*Bjerkandera* sp. and *Funalia trogii*) and two sapstain fungi (*Ophiostoma ainoae* and *Ceratocystis allantospora*) compared to the lipid content of a control wood (from Martínez-Iñigo et al. 1999)

	Control (mg/g)	Lipid degradation (%)			
		<i>Bjerkandera</i> sp.	<i>F. trogii</i>	<i>O. ainoae</i>	<i>C. allantospora</i>
Sapwood					
Fatty acids	3.96	97	95	78	88
Resin acids	8.92	90	81	0	24
Sitosterol	0.16	69	50	0 ^a	0 ^c
Waxes	1.60	100	100	93	94
Sterol esters	1.20	100	100	100	79
Triglycerides	7.30	100	100	100	100
Heartwood					
Fatty acids	2.15	87	38	26	45
Resin acids	31.09	50	0	0	19
Sitosterol	0.27	18	41	0 ^b	0 ^d
Waxes	0.43	74	44	67	12
Sterol esters	0.48	62	58	58	17
Triglycerides	1.02	80	100	100	60

^aThe amount was increased by 1.20 mg/g wood

^bThe amount was increased by 0.27 mg/g wood

^cThe amount was increased by 0.43 mg/g wood

^dThe amount was increased by 0.19 mg/g wood

Table 17.2. Degradation of main lipid classes in *Eucalyptus globulus* wood by two white-rot (*Phlebia radiata* and *Ceriporiopsis subvermispota*) and two sapstain fungi (*Ophiostoma valdivianum* and *O. piliferum*) compared to the lipid content of a control wood (from Gutiérrez et al. 1999b)

	Control (mg/100 g)	Lipid degradation (%)			
		<i>P. radiata</i>	<i>C. subvermispota</i>	<i>O. valdivianum</i>	<i>O. piliferum</i>
Fatty acids	17.2	90	97	8	47
Sterols	41.8	96	98	-55	-82
Sterol esters	33.6	100	100	45	78
Triglycerides	8.6	100	100	25	100

In addition to pitch reduction, albino strains of *O. piliferum* and related species exert a biocontrol effect, preventing sapstain and other wood-rot fungi from growing on logs and wood chips (Held et al. 2003). This is due to the early appearance of *O. piliferum* as a wood-colonizer (pioneer species) and particularly applies to freshly cut wood, where not only the growth of late wood-colonizers (such as white-rot fungi) is repressed but also other sapstain species.

When Cartapip 97 is used as a biocontrol agent, it has the tradename Sylvanex. In 2008, the current owners of this technology, Parrac Ltd, improved the production and storage properties of this product, and they are now announcing the re-launching of Cartapip 97/Sylvanex 97 worldwide. Generally, this technology can refer to any albino strain of the genus *Ophiostoma* that is able to

decrease the resin content resulting in the maintenance of brightness levels during transportation and storage of wood prior to pulping (Farrell 2007). An albino strain of *Ceratocystis resinifera* (named Kasper) has also been successfully tested for its ability to prevent discoloration of spruce sapwood (Morin et al. 2006).

B. White-Rot Fungi

White-rot fungi are the predominant degraders of lignin in nature (see Chapter 15). Some species preferentially degrade lignin and hemicelluloses over cellulose (“selective degraders”), whereas other species degrade all wood components simultaneously (“simultaneous degraders”). The ability of white-rot fungi to attack all major cell-wall components, including lignin, cellulose,

and hemicelluloses has been well characterized (Blanchette 1995; Martínez et al. 2005). In addition, there are reports on the degradation of non-structural components in woody tissues, such as wood extractives, by certain white-rot fungi (above all by species causing a selective degradation pattern).

The ability of white-rot fungi in removing lipophilic extractives was assessed both for softwoods and hardwoods (Tables 17.1 and 17.2, respectively). A comprehensive study reported on the fungal degradation of lipophilic extractives in sapwood and heartwood from Scots pine (Martínez-Íñigo et al. 1999). Triglycerides, fatty acids, sterol esters, and waxes in pine sapwood were almost completely removed by the white-rot fungi assayed (Table 17.1) and even sterols and resin acids were extensively degraded.

This work furthermore showed that the fungal degradation of heartwood extractives was not only limited by the degradative ability of the fungi, but also by the inhibitory effect exerted by the extractives themselves. Thus, the white-rot fungus *Funalia trogii* was strongly inhibited in heartwood, whereas a *Bjerkandera* sp. strain showed a high tolerance towards the toxic extractives and was the most efficient fungus in degrading extractives in Scots pine wood. Resin acids were already previously reported to cause the inhibition of wood-inhabiting fungi including white-rotters (Eberhardt et al. 1994). In another study, several white-rot fungi were tested for the removal and detoxification of extractives from Scots pine sapwood and the time course of extractives degradation by two pre-selected fungi, namely *Bjerkandera* sp. and *Trametes versicolor*, was monitored (Dorado et al. 2000b, 2001). The authors showed a fungal removal up to 90% of most lipophilic extractives which was accompanied by a 7- to 17-fold reduction in toxicity in the Microtox bioassay. Further studies were performed by these authors with *T. versicolor* to evaluate the effects of fungal treatment on spruce chips at laboratory scale, in terms of pulp and paper quality, thermo-mechanical pulping (TMP) parameters, and effluent toxicity (van Beek et al. 2007). Several studies on the removal of lipophilic extractives from *E. globulus* wood showed that a few white-rot fungi are able to remove up to 100% of the main extractives present in this wood.

These studies started with the screening of a large number of fungal species (21 ascomycetes, 33 basidiomycetes, 19 conidial fungi) including strains isolated from eucalypt wood (Martínez et al. 1999). Different patterns of lipophilic extractives degradation were analyzed (Gutiérrez et al. 1999b) and several white-rot basidiomycetes, including *Phlebia* and *Ceriporiopsis* species, were selected. They efficiently removed up to 100% both of free and esterified sterols (Table 17.2), which are particularly abundant in eucalypt wood. In contrast, several ascomycetous fungi including *Cartapip* decreased the sterol ester content but

did not convert free sterols. The time-course of fungal removal of these compounds was followed to optimize the treatment time for selected fungal species, namely *Phlebia radiata*, *Ceriporiopsis subvermispota*, *Bjerkandera adusta*, and *Pleurotus pulmonarius* (Martínez-Íñigo et al. 2000). Kraft pulping and TCF bleaching of eucalypt wood chips treated with these four basidiomycetes and subsequent papermaking from the pulps obtained confirmed their potential for pitch biocontrol (Gutiérrez et al. 2000). Resin removal by wood chip treatment with white-rot fungi can have additional benefits in biomechanical and biochemical pulping due to the partial removal of lignin (Akhtar et al. 2000; Bajpai et al. 2001).

IV. Pitch Biocontrol with Fungal Enzymes

Enzymes offer an environmentally benign and efficient alternative to chemical reagents in many industrial applications. Based on mechanism, the majority of the commercial enzymes are hydrolases (EC 3), while oxidoreductases (EC 1) account for a miniscule share (Xu 2005). This is in contrast to the widespread occurrence of oxidoreductases in nature. The gap between a vast natural repertoire of oxidoreductases and their limited commercial application creates the space for developing more oxidoreductase-based biocatalysts. Concerning pitch biocontrol, an enzymatic method using hydrolytic enzymes (lipases) to treat the pulp was the first example world-wide, in which an enzyme was successfully applied in the papermaking process. More recently, the high potential of certain oxidative enzymes for the removal of lipophilic compounds has been shown. Both established applications based on hydrolases and emerging applications based on oxidoreductases have a promising potential for pitch biocontrol in pulp and paper manufacturing and are therefore discussed below.

A. Hydrolytic Enzymes

Hydrolases are involved in the catalytic breakdown of glycosidic bonds in polysaccharides, the main constituents of plant biomass, and they are also active on peptide and ester bonds in proteins and lipids, respectively. Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) form a group of well known hydrolases, whose natural function is the hydrolysis of esters formed from glycerol and

long-chain fatty acids. Their enzymology and structure is discussed in several review papers, some of which also provide overviews of the wide range of industrial applications of these enzymes (Bornscheuer et al. 2002; Hasan et al. 2006). Lipases commercialized by Novozymes A/S (Bagsvaerd, Denmark) under the trade name Resinase A2X were successfully applied at mill scale for the enzymatic control of pitch in mechanical softwood pulping in Japan in the early 1990s (Fujita et al. 1991, 1992; Hata et al. 1996; Matsukura et al. 1990). This enzymatic pitch control technology was initially developed in the 1980s by Jujo Paper Company (later Nippon Paper) in conjunction with the Japanese office of Novozymes and is still nowadays in use. In Europe, pilot-scale trials for pitch control in softwood sulfite pulp using Resinase also provided promising results (Fischer and Messner 1992a, b; Fischer et al. 1993), and more recently lipase has been successfully assayed at the mill-scale for stone-ground wood pulping of Northern spruce wood in Finland within the frame of a European Union (EU) project (www.irnase.csic.es/projects/pitch). In addition to Resinase, other industrial lipases were also investigated for the enzymatic control of pitch (Gutiérrez et al. 2001a).

In December 1999, Nanping Paper Mill in China started the world's fastest newsprint paper machine at this time, whose main pulp supply came from ground-wood and TMP pulp, using local Chinese red pine (*Pinus massoniana*) as raw material. Due to the very high content of lipophilic extractives of this pine species, conventional pitch control programs were ineffective in preventing frequent pitch outbreaks on various parts of the paper machine during start-up. In March 2000, the mill conducted a trial with a formulated pitch control treatment, which contained a variety of Novozymes enzymes including lipase products. The efforts focused not only on the operation of the paper machine, but also on the pulp mills where the pitch has usually its highest concentration. Finally, the pitch outbreaks were successfully controlled by the use of enzyme cocktails and lipase-based treatments (Chen et al. 2001).

During the past years, knowledge on the structural basis of lipase activity has strongly increased, including information on the active site and the characteristic lid region that regulates the access of lipophilic substrates in Resinase and other lipases (Fig. 17.5A). In this context, Novozymes A/S, within the frame of an EU project (www.irnase.csic.es/projects/pitch), developed a

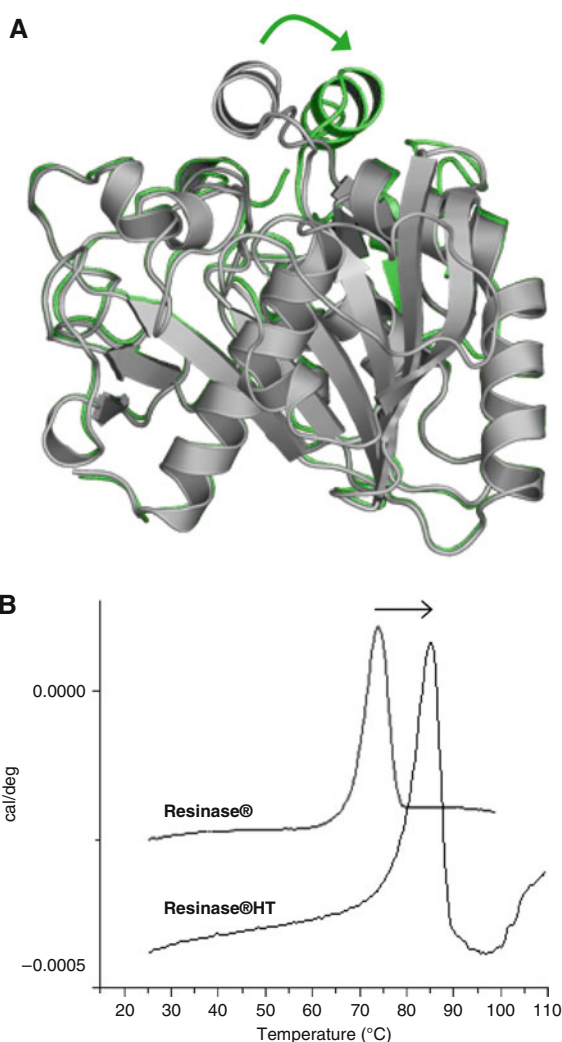


Fig. 17.5. Commercial lipase (Resinase from Novozymes A/S). **A** Scheme of the molecular structure showing the superimposition of the closed lid (gray) and open lid (green) conformations of the molecule to illustrate the opening of this mobile element (arrow) enabling the entrance of triglyceride substrates to the active site (the position of the different helices and β -sheets is also shown). **B** Differential scanning calorimetry showing the thermal stabilization (arrow) of the enzyme to obtain a thermostable variant (Resinase HT) by directed evolution (including random mutagenesis coupled to high throughput screening) within the framework of an EU project (www.irnase.csic.es/projects/pitch; courtesy of K. Borch, unpublished data)

Resinase variant that tolerates a 15 °C higher temperature than the wild-type enzyme (Fig. 17.5B). This was achieved by directed evolution techniques (i.e. random mutagenesis coupled to

Table 17.3. Effect of enzymatic treatments of unbleached TMP with recombinant sterol esterase from *M. albomyces* (rSTE1) and lipase (Resinase A2X) on the lipophilic extractives of the TMP water compared with corresponding controls (1 and 2) without enzyme supplementation (from Kontkanen et al. 2006b)

	Lipid content (mg/l)			
	Control 1	rSTE1	Control 2	Resinase A2X
Fatty acids	6.1	11.7	6.2	18.7
Resin acids	7.7	7.1	8.5	8.9
Sterols	1.0	1.5	0.9	1.1
Sterol esters	9.9	8.1	10.7	10.8
Triglycerides	17.0	2.5	14.8	0.0

high-throughput screening to incorporate the best mutations). The optimum temperature range of this Resinase HT is 70–85 °C. Currently, Resinase A2X and Resinase HT are used in mills from USA, Canada, China, Japan, and other countries in the Far East (where wood species with high resin content are handled). Very recently, it was shown that combining a novel biosurfactant with a lipase can reduce the concentration of a broad range of extractives in softwood TMP pulps (Dubé et al. 2008). Lipase treatments were shown to successfully reduce the triglycerides in pulps, but besides triglycerides, other disturbing compounds such as free and esterified sterols, resin acids, fatty alcohols, alkanes, etc. are also responsible for pitch problems. Thus, sterol esterases (sterol-ester acylhydrolases, E.C. 3.1.1.13), which hydrolyze fatty acid esters of sterols, were also suggested for pitch control (Calero-Rueda et al. 2004; Kontkanen et al. 2006c).

Sterol esters are often at the origin of pitch deposits, taking into account their abundance in some kinds of wood as well as their tackiness and tendency to aggregate. In addition, some enzymes classified as lipases were reported to catalyze the hydrolysis of sterol esters (Gao and Breuil 1998; Kontkanen et al. 2004). Sterol esterases have a broad substrate specificity and are capable of hydrolyzing different triglycerides and *p*-nitrophenyl esters in addition to sterol esters. These enzymes are produced in mammalian tissues and by several fungi and bacteria (Panda and Gowrishankar 2005). Fungal sterol esterases from *Melanocarpus albomyces* (Kontkanen et al. 2006a, c) and *Ophiostoma piceae* (Calero-Rueda et al. 2002, 2009) have recently been characterized from the biochemical and molecular points of view. The ability to hydrolyze sterol esters is considered to be a particular characteristic of this group of enzymes, together with the lack of the above-mentioned mobile lid in their molecular structure, as found in typical lipases (Calero-Rueda et al. 2009). These fungal esterases were tested for treating a model pitch preparation simulat-

ing the TMP (Kontkanen et al. 2006c) and eucalypt pulp resin (Calero-Rueda et al. 2004). The treatments revealed that both sterol esters and triglycerides were hydrolyzed in the presence of suitable detergents, whereas only triglycerides were cleaved in their absence.

Fungal sterol esterases can be produced by heterologous expression in industrial hosts and the recombinant enzymes have been characterized (Calero-Rueda et al. 2009; Kontkanen et al. 2006a) and immobilized to increase their enzyme stability (Torres et al. 2008). The effects of a recombinant sterol esterase from *M. albomyces* on the extractives of TMP water (Table 17.3) as well as on the properties of derived paper sheets were evaluated and compared with Resinase A2X (Kontkanen et al. 2006b). Although high hydrolysis rates were reported for treatments of model pitch mixtures with sterol esterases (Calero-Rueda et al. 2004; Kontkanen et al. 2006c), only up to 18% of real sterol esters were hydrolyzed (Table 17.3).

Concerning the use of sterol esterases for pitch biocontrol, it is worth mentioning that the hydrolysis increases the amount of free sterols, which have been shown to increase the viscosity and deposition tendency of resin (Qin et al. 2003, 2004). Therefore, the complete hydrolysis of sterol esters would not be advantageous for the papermaking process (Kontkanen et al. 2006b; Qin et al. 2004). The above results show that resin deposition control by enzymatic treatments should be evaluated carefully before any large-scale application, since degradation of certain compounds may result in more unstable wood resin dispersions (Qin et al. 2004). Little is known on the enzymes involved in enzymatic degradation of sterols and resin acids despite reports of microbial degradation of these compounds, as mentioned above (Gutiérrez et al. 1999b, 2001a; Hata et al. 1998; Liss et al. 1997; Marsheck et al. 1972; Martínez-Íñigo et al. 2000). Therefore, new enzymes acting on a broader range of substrates are being investigated, including oxidoreductases, as described below.

B. Oxidative Enzymes

Oxidoreductases are widely distributed among microbial, plant, and animal organisms. They catalyze the exchange of electrons or redox equivalents between donor and acceptor molecules, in reactions involving electron transfer, proton abstraction, hydrogen extraction, hydride transfer, oxygen insertion, or other key steps (Xu 2005). Oxidoreductases have been objects of interest in the pulp and paper industry with the aim of developing environmentally sound technologies for pulp delignification and bleaching. Among them, laccases (EC 1.10.3.2) constitute a group of oxidative enzymes which has been paid more and more attention in the recent years (Mayer and Staples 2002; Riva 2006; Rodríguez Couto and Toca Herrera 2006; Widsten and Kandelbauer 2008). Laccases are metalloenzymes containing four catalytic coppers in their molecular structure (Fig. 17.6). Due to the redox potential of the copper centers, the direct action of laccases is in principle limited to phenolic structures that only represent a small percentage in lignin

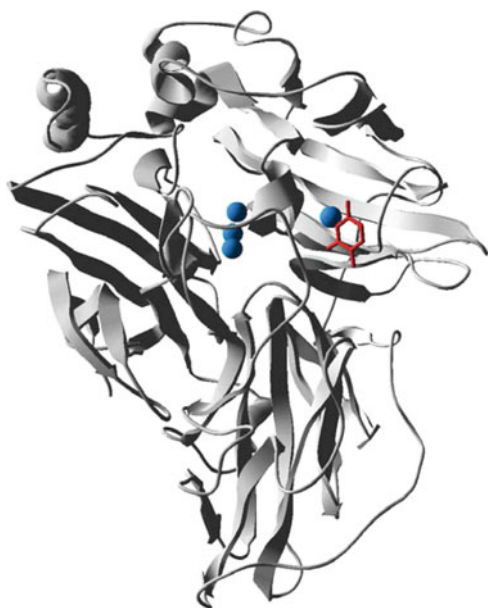


Fig. 17.6. Scheme of the molecular structure of a high redox-potential laccase, from the basidiomycete *Trametes versicolor*, in complex with an aromatic molecule (red) that occupies a position close to copper 1 (blue sphere, right) directly involved in electron abstraction from the substrate. The different helices and β -sheets are shown, as well as the position of the three other copper ions involved in catalysis (from PDB entry 1KYA)

(5–15%). Therefore, high-redox-potential lignin and manganese peroxidases were more intensively studied in the beginning (about 30 years ago), regarding both lignin biodegradation and the development of biotechnological applications (Paice et al. 1995).

However, the interest in laccases as industrial biocatalysts strongly increased after discovering the effect of some synthetic compounds acting as redox mediators, including 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Bourbonnais and Paice 1990) and 1-hydroxybenzotriazole (HBT; Call 1994). They expanded the action of laccase to non-phenolic substrates and increased their potential to degrade lignin and, therefore, their potential for pulp bleaching (Bourbonnais and Paice 1996; Camarero et al. 2004; Ibarra et al. 2007; Poppius-Levlin et al. 1999; Sealey et al. 1999). In addition to its action on phenolic compounds, some reactivity of laccase (e.g. from a *Trametes* species) was reported on polyunsaturated fatty acids (20% decrease after 4 h treatment), conjugated resin acids (29% decrease), and trilinolein (20–35% reduction within 3 h; Karlsson et al. 2001; Zhang et al. 2002). In the reaction of laccase with trilinolein, the predominant oxidation products were monohydroperoxides, bis-hydroperoxides, and epoxides. Likewise, a >30% decrease of lipophilic extractives present in softwood pulp (TMP pulping) and derived process water was reported (Buchert et al. 2002; Dubé et al. 2008; Zhang et al. 2000, 2005).

Recently, the high efficiency of laccase-mediator systems for the removal of lipophilic extractives present in pulps from different origins was described for the first time, regardless of the particular pulping process, the raw materials, or the chemical nature of the compounds to be degraded (Gutiérrez et al. 2006b); and a respective international patent application was submitted (Gutiérrez et al. 2008a). In these studies, the laccase from the basidiomycete *Pycnoporus cinnabarinus* was used in the presence of the mediator HBT and very efficiently removed free and conjugated sterols (95–100% decrease) from eucalypt kraft pulp (Fig. 17.7), triglycerides, resin acids, and sterols (65–100% decrease) from spruce (TMP pulp), and fatty alcohols, alkanes, and sterols (40–100% decrease) from flax soda pulp (Table 17.4). The removal of lipids by the laccase-HBT couple resulted in the formation of several oxidized derivatives that were absent or just present in traces in the original pulps. The total lipid content of the pulps decreased significantly, and the most problematic compounds were completely removed. In another study, this enzymatic treatment was applied as an additional stage of an industrial-type TCF sequence for bleaching

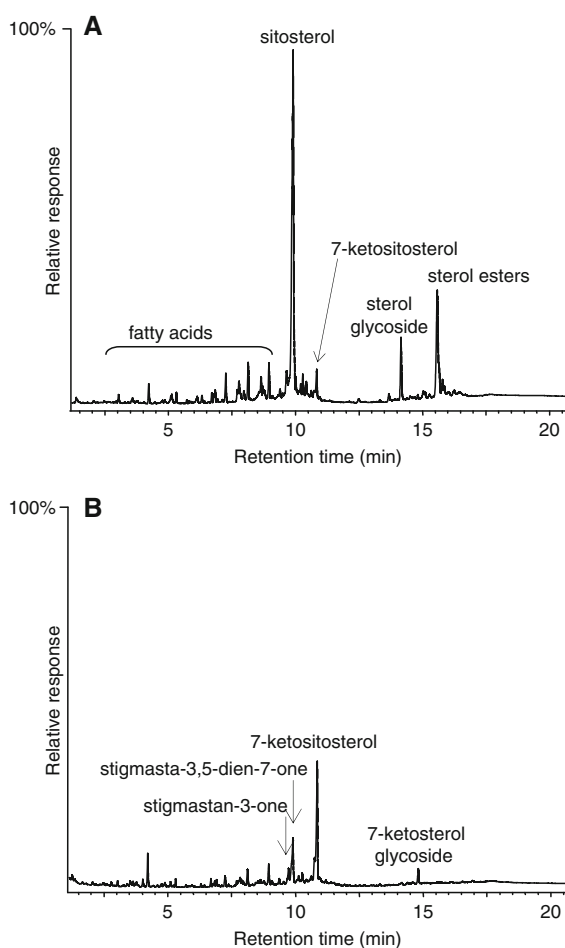


Fig. 17.7. Gas chromatography analysis of lipophilic compounds in eucalypt kraft pulp before (A) and after (B) laccase-HBT treatment (adapted from Gutiérrez et al. 2006a)

eucalypt kraft pulp and resulted in the complete removal of free and conjugated sitosterol (Gutiérrez et al. 2006a). Moreover, pulp brightness was improved due to the simultaneous removal of lignin by the laccase-mediator treatment.

In another recent study, the laccase-mediator system was optimized for the removal of free sterols from *E. globulus* pulp using a factorial design (Valls et al. 2009; Fig. 17.8). Different operation conditions following a three-variable (laccase dose, mediator dose, reaction time) sequential statistical plan were tested. The decrease in the pulp's sterol content was related to the decrease in kappa number and to the brightness increase, as well as to the initial increase in some oxidation products of sitosterol (namely 7-ketositosterol and stigmasta-3,5-dien-7-one, whose abundance decreased again at the end of the treatment). The increase in reaction time from 1 h to 5 h strongly reduced the sterol content, while no more sterols were eliminated during the next to hours (5–7 h). Increasing the laccase dose from 1 U/g to 20 U/g of pulp produced a high reduction in pulp sterols, whereas an increase in the mediator (HBT) dose (from 0.5% to 2.5% of pulp weight) had only a slight influence on the removal of sterols. In this study, it was also demonstrated that sterols were more sensitive to a laccase-mediator treatment (practically 100% of sterols were removed) than to a chlorine dioxide stage (54% of sterols removal).

Further investigations into the chemistry of the reactions of the laccase-mediator system were carried out with the main lipophilic extractives present in hardwood, softwood, and non-woody paper pulps (including alkanes, fatty alcohols, fatty acids, resin acids, free sterols, sterol esters, triglycerides). The reaction products were identified and quantified during the enzymatic treatment to better understand the degradation patterns in different pulps (Molina et al. 2008).

Table 17.4. Effect of the enzymatic treatment of several pulps with the laccase-HBT system on the lipophilic pulp extractives (from Gutiérrez et al. 2006b). *tr* Traces, *nd* not determined

	Lipid content (mg/kg)					
	Eucalypt kraft pulp		Spruce TMP pulp		Flax soda/AQ pulp	
	Control	Treated	Control	Treated	Control	Treated
Fatty acids	48.5	27.9	116.1	68.3	nd	nd
Resin acids	–	–	1278.4	400.9	–	–
Fatty alcohols	–	–	18.6	14.9	121.3	49.7
Alkanes	–	–	–	–	12.8	8.0
Steroid hydrocarbons	14.2	4.9	–	–	–	–
Free sterols	140.3	5.5	120.5	41.4	81.5	–
Steroid ketones	9.0	97.2	7.0	67.0	2.4	19.1
Sterol glycosides	17.1	–	45.0	–	14.0	–
Oxidized sterol glycosides	–	6.9	–	tr	–	5.4
Waxes	–	–	–	–	2.6	0.6
Sterol esters	95.4	–	1274.6	218.3	–	–
Triglycerides	–	–	1982.5	151.2	–	–

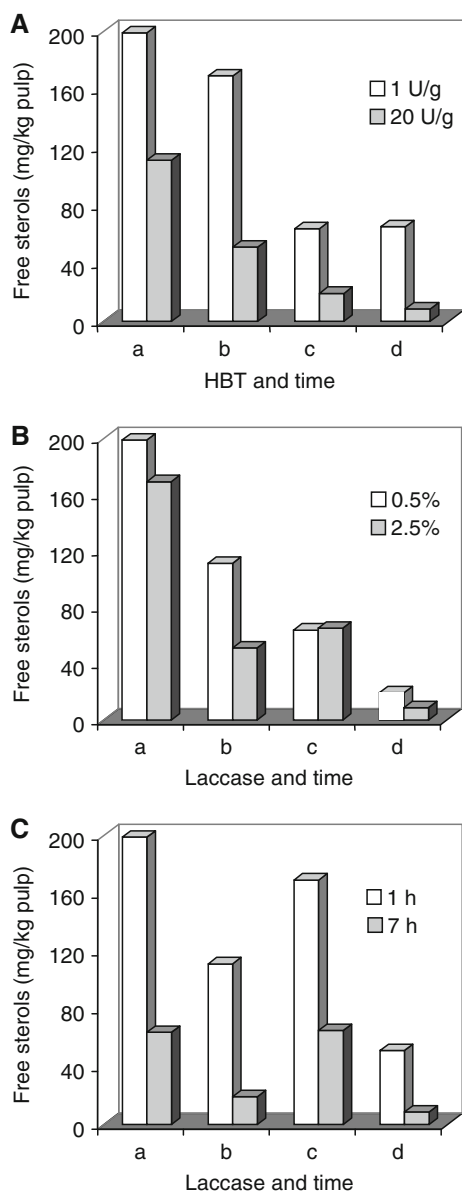


Fig. 17.8. Effect of three treatment parameters on sterol removal during the enzymatic treatment of eucalypt pulp with the laccase-HBT system, investigated using a factorial design. **A** Effect of laccase dose under four different combinations (*a–d*) of the two other variables (*a* 0.5% HBT, 1 h reaction time; *b* 2.5% HBT, 1 h reaction time; *c* 0.5% HBT, 7 h reaction time; *d* 2.5% HBT, 7 h reaction time). **B** Effect of mediator (HBT) dose under four different combinations (*a–d*) of the two other variables (*a* 1 U g⁻¹ laccase dose, 1 h reaction time; *b* 20 U g⁻¹ laccase dose, 1 h reaction time; *c* 1 U g⁻¹ laccase dose, 7 h reaction time; *d* 20 U g⁻¹ laccase dose, 7 h reaction time). **C** Effect of reaction time under four different combinations (*a–d*) of the two other variables (*a* 1 U g⁻¹ laccase dose, 0.5% HBT; *b* 20 U g⁻¹ laccase dose, 0.5% HBT; *c* 1 U g⁻¹ laccase dose, 2.5% HBT; *d* 20 U g⁻¹ laccase dose, 2.5% HBT). Adapted from Valls et al. (2009)

These studies showed that a 60–100% decrease of the initial amount of unsaturated compounds (e.g. abietic acid, trilinolein, linoleic and oleic acids, sitosterol, cholesteryl palmitate, oleate, linoleate) was achieved at the end of a 2-h laccase-HBT treatment (Fig. 17.9). Likewise, a decrease of 20–40% of these unsaturated lipids was observed after treatment with laccase alone, except in the case of abietic acid (95% decrease) and in the case of cholesteryl palmitate and sitosterol (not affected).

The latter study confirmed the finding that laccase alone can decrease the concentration of some unsaturated lipids (Karlsson et al. 2001; Zhang et al. 2002). However, the most rapid and extensive lipid modification was obtained with the laccase-mediator system. Model unsaturated lipids were largely oxidized and the dominant products detected were epoxy- and hydroxyfatty acids (Fig. 17.10A), as well as free and esterified 7-ketosterols and steroid ketones were formed from sterols and sterol esters (Figs. 17.10B and 17.11, respectively). The enzymatic reaction on sterol esters largely depended on the nature of the fatty-acyl moiety, i.e. oxidation of saturated fatty-acid esters started at the sterol moiety (Fig. 17.11A), whereas the initial attack on unsaturated fatty-acid esters occurred on the bonds (Fig. 17.11B, C). In the case of sterol linoleate (Fig. 17.11C), breakdown of the fatty acid chain proceeds via release of the so-called core aldehydes. Interestingly, no reaction products of abietic acid, trilinolein and linoleic acid were detectable despite their high reactivity and rapid disappearance in the reaction solution. Saturated lipids were not susceptible to the laccase-HBT systems, although some of them decreased when the laccase-mediator reactions were carried out in the presence of unsaturated lipids, suggesting the participation of intermediary lipid peroxyl radicals.

Since some shortcomings of HBT and related synthetic mediators (e.g. high costs, toxicity) hamper their industrial application, the search for natural compounds that could act as laccase mediators has been forced over recent years. The existence of fungal metabolites acting as redox mediators had already been suggested 15 years ago (Eggert et al. 1996; Gutiérrez et al. 1994). In this context, it was recently demonstrated by Gutiérrez et al. (2007) that three cost-effective phenolic compounds related to lignin can act as laccase mediators for the removal of lipophilic compounds from paper pulp within the frame of a TCF sequence. These natural mediators were first described as an alternative to synthetic mediators in paper pulp delignification (Camarero et al. 2007).

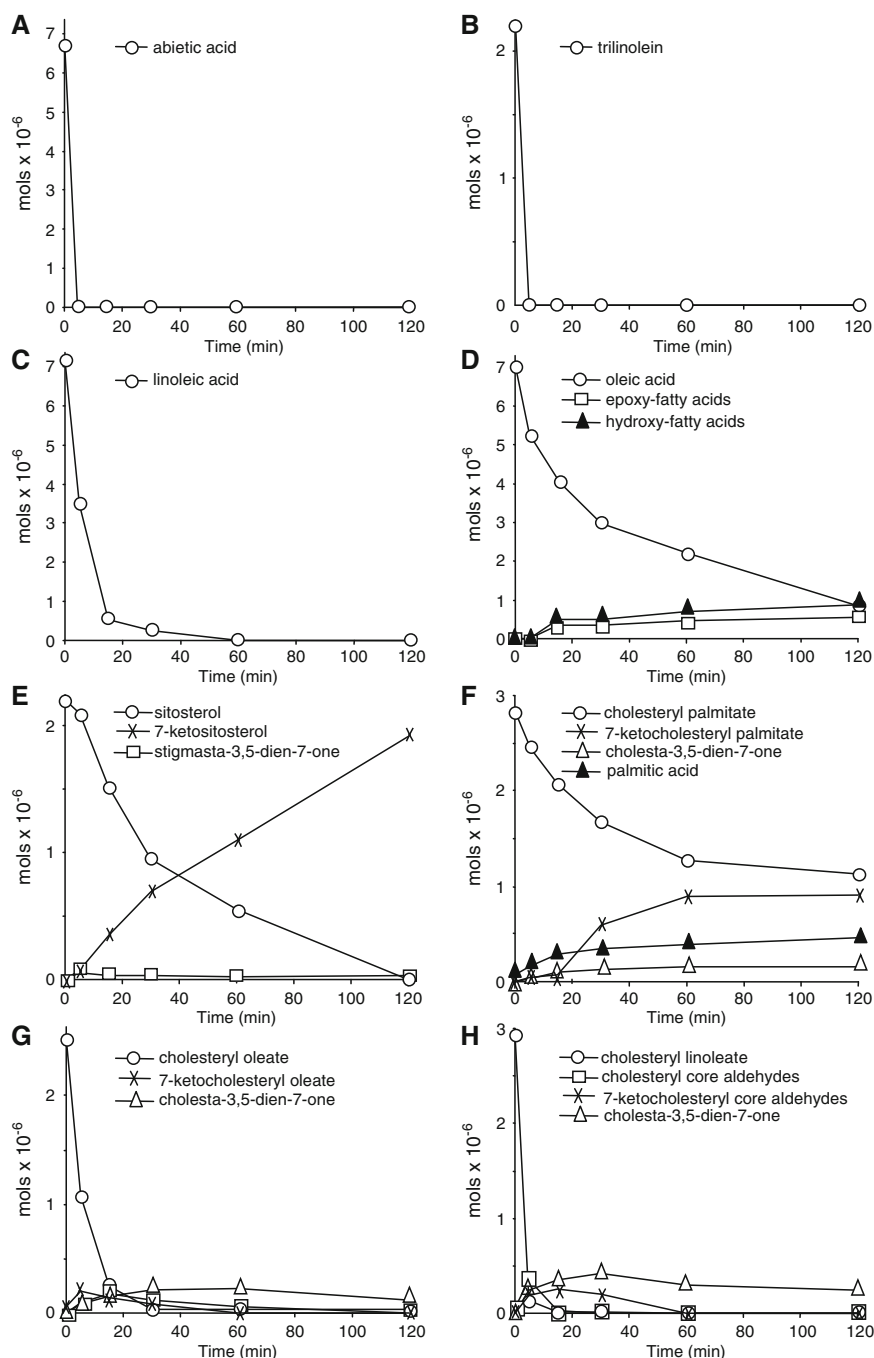
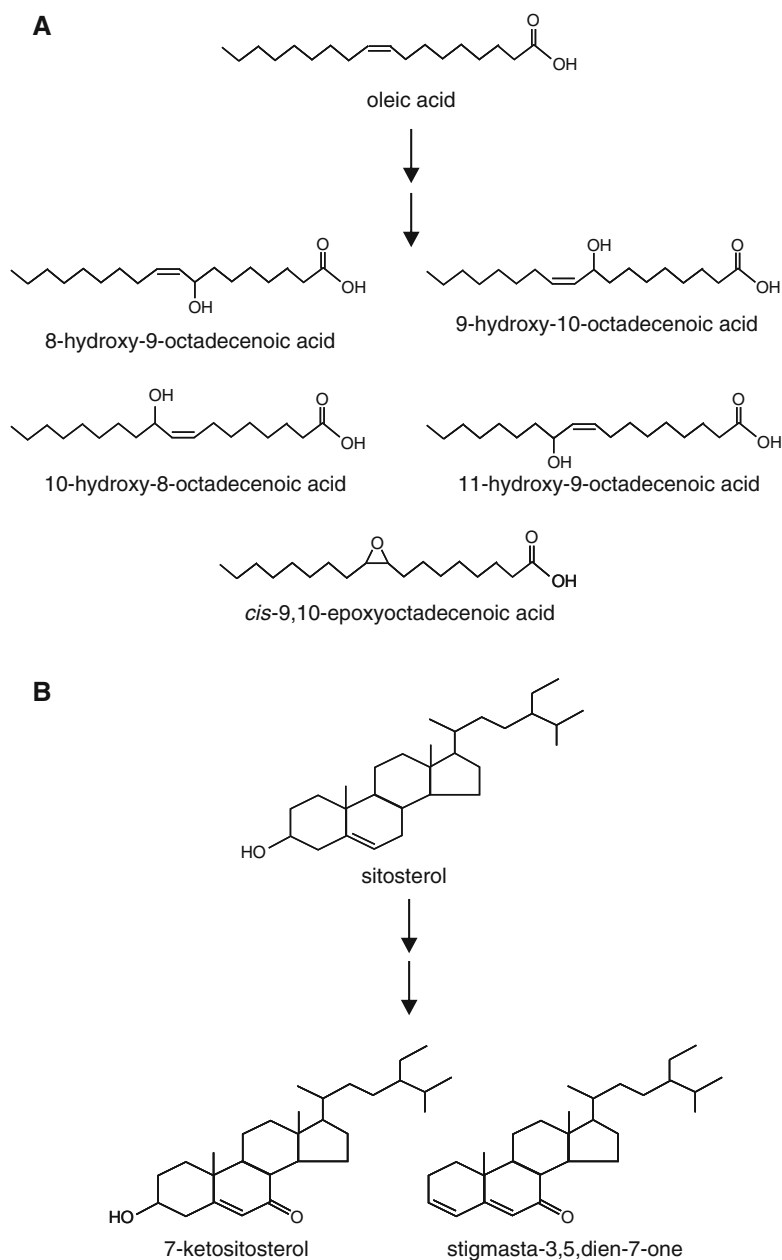


Fig. 17.9. Removal of eight model lipids and formation of oxidation products during laccase-HBT reactions: abietic acid (A), trilinolein (B), linoleic acid (C), oleic acid (D), sitosterol (E), cholesteryl palmitate (F), cholesteryl oleate (G) and cholesteryl linoleate (H). Adapted from Molina et al. (2008)

In the study mentioned above, unbleached eucalypt kraft pulp was treated with a fungal laccase in the presence of syringaldehyde, acetosyringone, and *p*-coumaric acid as phenolic mediators followed by a hydrogen peroxide stage

(Table 17.5). The enzymatic treatment in the presence of syringaldehyde caused the highest removal (>90%) of free and conjugated sitosterol, similar to that attained with HBT, followed by acetosyringone (>60% removal), whereas

Fig. 17.10. Main oxidation products identified by GC-MS during the reaction of oleic acid (A) and sitosterol (B) with laccase-HBT. Data from Molina et al. (2008)



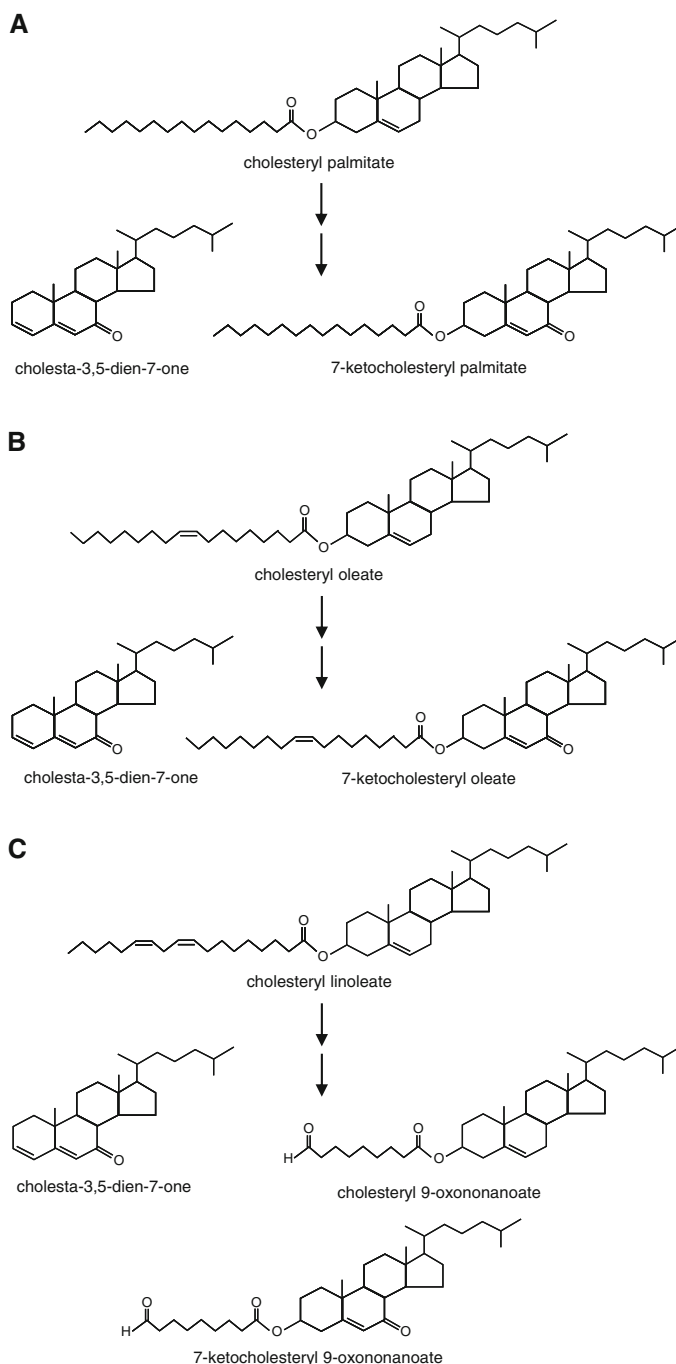
p-coumaric acid was barely effective. Pulp brightness was also improved (57–66% ISO brightness) by the laccase-mediator and subsequent peroxide treatment. The use of natural compounds as laccase mediators could make these enzymatic treatments more feasible to be applied in the pulp and paper sector. However, more knowledge is needed before this enzymatic treatment for the (simultaneous) removal of pulp pitch and lignin will be considered as a serious proposition to be implemented in the respective processes.

Finally, the use of lipoxygenases (EC 1.13.11.12), a class of non-heme iron-containing dioxygenases

which catalyze the oxygenation of unsaturated fatty acids and their esters, was suggested for pitch control in softwood TMP pulps (Zhang et al. 2007).

Lipoxygenases are ubiquitous in plants, mammals, and fungi. Despite extensive studies on their biochemical and molecular properties (Brash 1999; Saam et al. 2007), this group of enzymes has not yet been introduced into industrial processes. Nevertheless, the specific activity of lipoxygenases to oxidize linoleic acid makes them interesting for applications in the papermaking process. In the work of Zhang et al. (2007), the lipophilic extractives content of

Fig. 17.11. Main oxidation products identified by GC-MS during the reaction of cholesteryl palmitate (A), cholesteryl oleate (B) and cholesteryl linoleate (C) with laccase-HBT. Data from Molina et al. (2008)



TMP pulp samples was reduced by more than 25% after a 2-h treatment with soybean lipoxygenase. The activity of lipoxygenase towards wood extractives was determined by using a mixture extracted from TMP and the enzyme was found to exhibit a significant activity towards all these wood extractives. However, it turned out that some of the extractives (such as resin acids) and lignin fragments

have inhibitory effects on lipoxygenase-catalyzed reactions with linoleic acid. An earlier study published in a patent by Novozymes had suggested the possibility of using lipoxygenases to reduce a model wood "pitch" mixture (Borch et al. 2003). Further studies on lipoxygenases for pitch biocontrol are currently under investigation (Nguyen et al. 2007).

Table 17.5. Effect of three “natural” mediators on the removal of lipids during laccase treatment of eucalypt pulp, followed by an H₂O₂ stage, compared with the synthetic mediator HBT and controls without laccase as well as with laccase but without mediator (from Gutiérrez et al. 2007). SAD Syringaldehyde, ACS acetosyringone, PCA *p*-coumaric acid

	Lipid content (mg/kg)					
	Control	Laccase	SAD	ACS	PCA	HBT
<i>Total free sterols</i>	273	280	25	113	230	27
Sitosterol	222	226	19	83	186	14
Stigmastanol	42	44	4	25	37	12
Fucosterol	9	10	2	5	7	1
<i>Total oxidized sterols</i>	32	53	7	10	43	69
Stigmastan-3-one	0	0	0	1	0	2
Stigmasta-3,5-dien-7-one	4	4	2	7	8	17
7 α -Hydroxysitosterol	7	13	1	1	6	8
7 β -Hydroxysitosterol	12	21	1	1	9	4
Sitostanetriol	3	5	2	0	13	5
7-Oxositosterol	5	10	1	1	7	34
<i>Total sterol glycosides</i>	27	27	2	12	24	5
Sitosteryl 3 β -D-glucopyranoside	27	27	2	12	24	2
7-Oxositosteryl-3 β -D-glucopyranoside	0	0	0	0	0	2
<i>Sterol esters</i>	90	57	7	43	57	2
<i>Steroid hydrocarbons</i>	22	21	3	11	15	8
<i>Total</i>	444	438	45	189	369	110

V. Summary and Conclusions

Wood lipids surviving the pulping and bleaching processes have a high tendency to form pitch deposits, resulting in economic losses and operational troubles during pulp and paper manufacturing. The impact of these pitch problems has increased during recent years due to the latest developments in the sector, such as the closure of water circuits and the use of TCF bleaching sequences. In particular fatty acids, fatty alcohols, resin acids, hydrocarbons, steroids, triterpenoids, and triglycerides are among these lipophilic wood extractives forming pitch deposits. Therefore, clean biotechnological technologies capable of modifying these compounds are highly desirable for reducing these problems. In the 1990s, two biotechnological applications for pitch control were successfully developed and applied at full mill-scale in various parts of the world. The fungal method for pitch control using an albino strain of the ascomycete *Ophiostoma piliferum* (a sapstain fungus) was the first successful example of using a living organism in the pulp and paper manufacturing process (particularly in mechanical softwood pulping). Likewise, the enzymatic method for pitch control using lipase was the first successful example of the use of an isolated enzyme in the papermaking process. In both cases, triglycerides – representing

the main cause of pitch problems – are preferably removed. Since then, work has focused on finding biotechnological solutions to get rid of other lipophilic extractives, such as free and esterified sterols, as well as resin acids.

Biotechnological processes based on selective white-rot fungi would have certain advantages compared to sapstain fungi. The latter easily degrade some lipophilic extractives, such as triglycerides and fatty acids, and they can even hydrolyze sterol esters; however, free sterols, triterpenols and resin acids are too recalcitrant for an efficient degradation by sapstain fungi, and better removals have been obtained with white-rot basidiomycetes. In addition to the advantages directly resulting from the removal of wood extractives, wood chip pretreatment with certain white-rot fungi can also decrease the amount of lignin, a process known as biopulping, enabling substantial savings in the energy required for obtaining mechanical pulps. The applicability of white-rot fungi in wood pulping will greatly depend on their effects on pulp yield and properties. Thus fungal attack of cellulose in pulp fibers would be highly undesirable as it causes a reduction in yield and pulp strength properties. Therefore, these biodepitching/biopulping treatments need to be applied under controlled conditions (including treatment duration) to attain

a maximal removal of lipids and lignin with a minimal deterioration of fibers and hydrolysis of cellulose. Moreover, an energy-intensive pretreatment of wood chips (such as steaming for partial sterilization) is often required for successful colonization by white-rot fungi, while sapstain fungi are characterized by their ability to colonize fresh wood without the need of any pretreatment.

The use of enzymes to remove extractives from pulp has clear advantages compared to the use of fungal inocula, such as shorter treatment times and higher specificity. Commercial lipases are successful in the hydrolysis of triglycerides in softwood pulps and are currently used in different types of mills. Recently, some promising results have been reported in the use of oxidative enzymes, particularly laccases in combination with suitable redox mediators. They are effective towards several lipophilic extractives, such as fatty acids, resin acids, free and conjugated sterols, and triglycerides. High-redox-potential laccases are characteristic for white-rot fungi and, as in the case of the latter, a double benefit can be obtained from their application on pulps in the presence of redox mediators. Laccase-mediator systems both permit the effective removal of pulp lipids (decreasing the pitch problem) and improve pulp delignification (resulting in a higher brightness and stability) using the same enzymatic preparation. The costs and environmental concerns associated with the use of synthetic mediators (such as NOH-type compounds) could be overcome in the future by using natural mediators, e.g. lignin-related phenolics obtained from easily available sources (such as pulping liquors), along with reasonably priced enzymes produced by genetically engineered organisms.

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18 Biosorption of Metals

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I. Introduction

There are many interactions between living cells and metals. Essential metals must be taken up into the cells, and they must be stored at their destinations. When the metals are present in the medium in too low a concentration, they must be accumulated. If the ambient concentration is too high, even essential metals become toxic and mechanisms of detoxification must ensure survival of the cell. Non-essential metals may enter cells by several ways and cause damage to their metabolism. Therefore, these metals have to be detoxified.

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All living cells have developed mechanisms by which they take up, store, detoxify or dispose of metals. Surprisingly, some species have been found which are able to concentrate metals to an extent which far exceeds necessary concentrations for the cells (Gabriel et al. 1994, 1997; Muraledharand et al. 1995; Pillichshammer et al. 1995; Michelot et al. 1998). Even non-essential metals are concentrated by these organisms to a tremendously high extent. This property has attracted the attention of many researchers looking for a cheap way of concentrating metals from dilute solutions of various origins. This chapter deals with the use of fungal biomass for the removal of metals from solution. We refer to the literature since 1990, as the older literature has been reviewed exhaustively by Volesky (1990). It is not the aim of this chapter to give a detailed description of the physicochemical process of sorption, or to discuss in detail the mechanisms by which intracellular metal concentrations are regulated. The physicochemistry of sorption has been discussed extensively by Volesky (1990, 1994), the industrial use of biosorption as well as different types of biosorbents by Volesky and Holan (1995) and by Vieira and Volesky (2000), metal cation uptake by yeast by Blackwell et al. (1995), metal transport in *Sacharomyces cerevisiae* by Eide (1998) and the metal dependent regulation of genes by Winge et al.(1998). More general reviews on the interaction of fungi with toxic metals have been given by Gadd (1993) and Gray (1998). A review describing biosorption of heavy metals by *S. cerevisiae* was published by Wang and Chen (2006).

One must clearly differentiate between the well regulated process of metal uptake (Eide 1998; Pena et al. 1998) and the mere binding of metals to structures at the cell which can be exploited for metal binding. We use the term biosorption to describe the latter process, regardless of whether the underlying mechanisms depend on regulated

uptake and storage of metals by living cells or on mere binding of metals to certain binding sites on the surface of cells.

Although this chapter deals only with the use of fungal biomass, one should be aware that biosorption can of course also be carried out using other organisms. There have been many examples in the literature where bacteria or algae have been used successfully for metal removal.

A. Metal Resistance

Metal resistance should not be confused with biosorption. The field of microbial (mainly bacterial) heavy metal resistance has been reviewed by Nies (1999). Several copper resistance mechanisms have been reviewed by Cervantes and Guitierrez-Corona (1994), including copper binding at the cell wall, reduced uptake and extra- or intracellular chelation. The importance of metal transport for metal resistance in yeast has been highlighted by the finding of Li and Kaplan (1998) that increased metal sensitivity can be caused a defect in high affinity iron uptake leading to the expression of transporters with lower specificity.

There are fungi that have an intrinsically high resistance against heavy metals or that give rise to mutants which can tolerate high metal concentrations. These strains, however, often show poor sorption properties as they accumulate by far lower amounts of metal than sensitive strains (Whiteside and Plocke 1992; Wunderlich et al. 1995; Rama Rao et al. 1997; Sajani and Mohan 1997).

Zafar et al. (2007) isolated filamentous fungi from metal contaminated soil and compared their metal resistance and metal accumulation properties. They found little, if any, correlation between metal tolerance and biosorption properties. Therefore, looking for good biosorbents among resistant strains may be misleading.

B. Practical Reasons for Using Biosorption

There are several reasons for studying biosorption. First, there is an increasing demand of pure water for the world's population, especially in developing countries. The water supply, however, is endangered by toxification of surface and groundwater. A major source of this toxicity is the still-increasing amount of metal-contaminated water that is spilled into the environment. Treatment of wastewater and

the purification of water for human purposes by conventional means is costly.

In many countries, the technology of wastewater treatment or water purification has to be simple, as there is a lack of skilled labor to safely perform more sophisticated technologies such as chemical precipitation. In these countries the use of biomass for the removal of metal ions promises to be a cheap alternative.

A second reason is the fact that a lot of fungal biomass is produced as a byproduct of fermentation aimed at the production of ethanol, antibiotics, organic acid etc. This biomass would have to be disposed of some way, creating a new waste problem, or alternatively, it could be used as a biosorbent. A good example for this rationale is the work by Murugesan et al. (2006) who used pretreated waste tea fungal biomass for the removal of As(V) and As(III) from ground water.

A third reason is the fact that some metals are of a rather high value so that the recovery of them is an asset. Naturally occurring metal-bearing minerals could be solubilized using a soil fungus like *Aspergillus niger* (Sayer et al. 1997).

In the past decades many researchers have shown that fungal biomass either produced solely for the purpose of biosorption or as a waste product from fermentation can be used to remove metal ions from solution. Biosorption rates of up to 25% of bound metal per biomass dry weight have been reported for the mycelia of *Rhizopus* and *Absidia* (Volesky and Holan 1995). The highest biosorption capacity we have found in the literature was more than 800 mg lead per gram biomass of *Candida albicans* (Baysal et al. 2009).

II. Structures Involved in Heavy Metal Binding

The first barrier a metal ion entering the cell encounters is the cell wall. Depending on the species and the growth conditions the cell wall contains a number of potential binding sites for metal ions.

A. Cell Wall

In most of the studies on biosorption, adsorbed metals were mainly localized within the cell wall,

as could be shown by analysing cellular subfractions (Ono et al. 1988; Brady and Duncan 1994a, b; Yazgan and Özcengiz 1994; Krantz-Rülcker et al. 1995; Gorovoi and Kosyakov 1996; Zhou 1999) electron microscopy (Meyer and Wallis 1997; Mogollon et al. 1998) or X-ray analysis (Ariff et al. 1999).

Some exceptional observations have been made: Volesky and May-Phillips (1995) found that uranium was present at *Saccharomyces cerevisiae* as fine needle-shaped crystals not only in the cell wall but also within the cell. In the thermotolerant yeast *Kluyveromyces marxianus*, the deposition of uranium crystals in the outer layer as well as within the cells was observed (Bustard et al. 1997). Cadmium also was found within the vacuole of living *S. cerevisiae* cells, which showed a higher cadmium accumulation capacity than dead cells (Volesky et al. 1992). In the case of Ag^+ biosorption by *Aspergillus niger* and *Mucor rouxii* precipitation of colloidal silver at the cell surface was observed (Mullen et al. 1992). Riordan et al. (1997) found that both sorption and precipitation in the cell wall contributed to the removal of uranium by residual brewery yeast.

Riordan and McHale (1998) used residual brewery yeast for lead biosorption. They found that not only sorption but also precipitation of the metal occurred at the cell wall. The same phenomenon occurred in biosorption experiment with *Rhizopus arrhizus* (Naja et al. 2005) depending on the concentration of lead in the solution. Precipitation of Cr(VI) and Ni(II) was also observed by electron microscopy in biosorption experiments with a yeast hybrid between *Candida tropicalis* and *C. lipolytica* at higher metal concentrations (Yin et al. 2008).

There are only few examples of studies where the nature or localization of the binding sites have been elucidated. Many studies show time-course experiments of metal sorption which give a good indication of the kinetics of the process; however, the determination of the underlying thermodynamics is rather difficult. When the kinetics and thermodynamics of binding were investigated, the process followed in most cases pseudo-second-order kinetics and the binding could be described by Langmuir or Freundlich isotherms, for examples see: Han et al. (2006), Ertugay and Bayhan (2008), Ozsoy et al. (2008). Pseudo-first-order kinetics was observed only in a few cases when one partner was in strong excess (Akhtar et al. 2008). In some cases the binding reaction was shown to be exothermic and spontaneous (Ozer and Ozer 2003).

In experiments with mixtures of metals often competition of the metals for the binding sites occurred with some metals being better bound

than the others (Han et al. 2006; Li and Yuan 2006; Tsekova et al. 2007).

A detailed study on the biosorption of lead with *Rhizopus arrhizus* using a metal-based titration technique showed that the sorption process was a combination of several successive sorption reactions on different binding sites with different selectivities. Not only the association coefficient of the binding sites with protons or other counterions was important, but also the spatial distribution of the binding groups was important (Naja et al. 2005). Chen and Wang (2007a) analysed the metal ionic characteristics on their biosorption properties for ten ions and *Saccharomyces cerevisiae*. They derived an equation that might predict the biosorption capacity of a metal ion on *Saccharomyces*.

B. Chemical Nature of the Binding Groups at the Cell Wall

The precise nature of the groups responsible for metal binding has been intensively investigated for a long time. Using chemical modification, the carboxyl, hydroxyl and amino groups of the cell wall carbohydrates, proteins and chitin/chitosan were identified as the major binding determinants (Brady and Duncan 1993; Ashkenazy et al. 1997).

Using *Penicillium chrysogenum* Sarret et al. (1999) showed that lead was bound to carboxyl and phosphoryl groups within the cell wall. The carboxyl groups had a high affinity for lead but were present only in low amounts, whereas the phosphoryl groups had a lower affinity but were more abundant.

For uranium Guibal et al. (1995) found by using infrared spectroscopy that amino-groups were predominantly responsible for binding, at least in *Aspergillus niger*, *Penicillium chrysogenum* and *Mucor miehei*. Zn biosorption by *Rhizopus arrhizus* occurred mostly at chitin and chitosan (Zhou 1999).

Krantz-Rülcker et al. (1995) analysed the influence of heavy metal binding to fungal cell wall on the enzymatic hydrolysis of these walls. They found a strong reduction in hydrolysis, mostly due to a reduction of glucanolytic activity which they described as a consequence of metal binding by these substrates.

The role of cell wall proteins in Cu adsorption by *Saccharomyces cerevisiae* was analysed by Brady et al. (1994a, b). They found that the outer layer of mannan-protein is more important for Cu-binding. Upon accumulation of Cu^{2+} , a loss of K^+

and Mg^{2+} from the cells occurred (Brady and Duncan 1994a).

For *Aspergillus niger*, it has been demonstrated that mostly the carboxyl and amino-groups are involved in the binding of heavy metals (Akhtar et al. 1995; Kapoor and Viraraghavan 1997). The production of extracellular polymeric substances may also influence biosorption, as was shown for *Aureobasidium pullulans* by Suh et al. (1999a, b). The glycoproteins of the cell wall may have a strong influence on metal biosorption as was shown by Breierova et al (2002) for the sorption of Cd onto *Saccharomyces cerevisiae* and *Hansenula anomala*. The glycoproteins of *H. anomala* bound almost 90% of the Cd, the ones of *S. cerevisiae* only 6% of the total bound Cd.

C. Uptake of Metals into the Cell

Metals that have entered into the cell can be bound within the cytoplasm or sequestered in the vacuole. This latter process has been analysed in detail, since it is closely linked to heavy metal resistance. Much work has been carried out in *Saccharomyces cerevisiae*, *Candida glabrata* and *Schizosaccharomyces pombe* regarding the nature of chelators within the cell, their regulation and their transport into the vacuole (Wemmie et al. 1994; Li et al. 1997).

In *Kluyveromyces marxianus*, Yazgan and Özcengiz (1994) found a small, intracellular metal-binding protein that was synthesized upon treatment of the cells by silver or cadmium. Sajani and Mohan (1998) found a cobalt-binding glycoprotein in cobalt-resistant mutants of *Neurospora crassa* consisting mainly of cysteine, glutamic acid, aspartic acid and glutamic acid, but also small amounts of aromatic amino acids. This strain could be used for biosorption of cobalt (Rama Rao et al. 1996). Phytochelatins, the small cadmium-binding peptides produced by nearly all plants and several yeasts have been used for detection and detoxification of heavy metals by Satufoka et al. (1999). An *Aspergillus niger* strain that was highly resistant to Ni(II) was able to precipitate the metal in the cell wall and within the cell in the form of nickel oxalate dehydrate (Magyarosy et al. 2002). This process was depending on metabolic energy.

D. Dependence on Metabolic Energy

Uptake of metals into the cells requires metabolic energy since transport is often coupled to ATP hydrolysis or the presence of an electrochemical gradient. During the biosorption process, cells must stay alive and therefore nutrients have to be added. This would render a biosorption pro-

cess based on metal uptake by living cells quite difficult. Therefore, most studies have used dead biomass.

III. Technology of Biosorption

A. Retention of Biomass and Mixing with the Solution

In order to remove metals from a solution, the biosorbent must be able to be mixed with the liquid phase and also be retained in a filter or separated from the water by other means after the metals have been bound. There are several examples of the successful immobilisation of fungal biomass prior to use as biosorbents. In most cases the biosorbents could be regenerated by elution of the metals (Brady and Duncan 1994; Brady et al. 1995a, 1999; Wilhelmi and Duncan 1995; Bustard and McHale 1997; Kapoor and Viraraghavan 1998a; Aloysius et al. 1999).

Wales and Sagar (1990) prepared filters consisting of paper, textile fibers and mycelia of *Mucor mucedo* or *Rhizomucor miehei* that had previously been treated with hydroxide. The filters showed good performance, although the flow rates of thicker filters were reduced. Brady et al. (1994a) used hollow fiber modules for cross-flow micro-filtration with *Saccharomyces cerevisiae* biomass. Zhao and Duncan (1997) used formaldehyde cross-linked yeasts for the removal of metals from aqueous solutions.

Very high accumulation rates were reached in sequential systems, especially when mixtures of metals were used. An alternative to filtration might be flotation, as demonstrated by Matis et al. (1996), or the use of a series of continuous flow stirred bioreactors (Stoll and Duncan 1997). White and Gadd (1990) used air-lift bioreactors to improve mixing when removing thorium by *Rhizopus arrhizus* or *Aspergillus niger* biomass. Many studies were carried out using well defined metal solutions. Chhikara and Dhankar (2008), however, could remove Cr(VI) ions from an industrial electroplating effluent with *A. niger* biomass that had been treated with H_2SO_4 or NaOH and subsequently immobilized in alginate beads. The biosorbent could be re-used at least five times. Fungi isolated from a tanning effluent were used to remove Cr(III) from this effluent with good results (Prigione et al. 2009). Salts that are present in the wastewater may pose serious problems as Aksu

and Balibek (2007) observed in biosorption experiments with dead *Rhizopus arrhizus* and Cr (VI). Salts may compete for the binding sites.

B. Pretreatment of the Biomass to Improve Biosorption

Chemical pretreatment of the fungal material by acetone (Ashkenazy et al. 1997), strong alkali (Luef et al. 1990; Akhtar et al. 1995), acid (Huang and Huang 1996), surfactants (Loukidou et al. 2003) or polyethylenimine (Deng and Ting 2005) resulted in increased performance.

Lu and Wilkins (1996) treated *Saccharomyces cerevisiae* cells by heating at 70–90 °C in 0.75 M NaOH. The resulting biosorbent had high biosorption capacity over a wide pH range. Pretreatment is however not always beneficial, as Kapoor and Viraraghavan (1997, 1998a, b, 1999) demonstrated. When they pretreated *Aspergillus niger* biomass by boiling it in NaOH, it showed an increased capacity to remove Pb, Cd and Cu, but for the removal of Ni, live biomass was superior. The alkali-treated biomass was superior to activated charcoal and could be regenerated at least five times. The polyethylenimine-treated *Penicillium chrysogenum* (Deng and Ting 2005) cells had a strong increase in biosorption capacity for Cr(VI), due to the high number of amine groups, a part of the metal was reduced to Cr(III) and chromium aggregates were found on the surface by scanning electron microscopy.

High-voltage electric pulses were used to increase the biosorption of uranium by *Kluyveromyces marxianus* (Donnellan et al. 1995) or yeast-material from a whiskey distillery (Bustard et al. 1998). Using this treatment, the capacity of the yeast biomass from the distillery was improved from 170 mg uranium per gram dry weight to 275 mg uranium per gram dry weight. Methylation of yeast cells was used in order to increase the biosorption of Cr(VI) and As(III) by Seki et al (2005).

When cells fermented specifically for biosorption are used, optimization of the fermentation parameters such as culture time and addition of additives may be helpful.

Simmons and Singleton (1996) could improve the capacity of an industrial strain of *Saccharomyces cerevisiae* to accumulate Ag^+ by adding L-cysteine to the medium. Fourest and Roux (1992) and Fourest et al. (1994) showed that the pretreatment of mycelial biomass with calcium together with pH control during the adsorption process increased biosorption capacity. Immobilization of the cells was often carried out in Ca-alginate beads (Akhtar et al. 2009; Bishnoy et al. 2007).

IV. Binding of Heavy Metals by Mycorrhizal Fungi

The question whether mycorrhizal fungi can protect plants against heavy metals was in dispute for a long time. However, in the last years many studies have shown a beneficial effect of mycorrhizal fungi on the ability of plants to cope with high metal concentrations (Rivera-Becerril et al. 1992; Galli et al. 1994; Tam 1995; Ricken and Hoefner 1996; Krzmaric et al. 2009; Liang et al. 2009; Redon et al. 2009).

A different effect was described by Galli et al. (1995). It has been shown that mycorrhizal fungi can accumulate heavy metals and that isolates obtained from contaminated soils are often more resistant to metals than reference strains (for a review, see Galli et al. 1994).

Changes in the heavy metal concentration have an effect on the diversity of mycorrhizal fungi (Del Val et al. 1999) as well as on the abundance of the fungi. Intermediate concentrations seem to enhance the diversity. The mechanisms of metal resistance of the fungi are discussed by Courbot et al. (2004), Bellion et al. (2006) and Hildebrandt et al. (2007). Gomalero et al. (2009) show the beneficial role of bacteria and fungi on plant growth under metal stress and discuss how these findings might be used to improve phytoremediation.

The uptake of heavy metals by plants and the protection conferred to them by mycorrhizal fungi seems to depend strongly on the plant, the soil and its nutritional status as well as the fungus (Rizzo et al. 1992; Shetty et al. 1994; Leyval et al. 1997; Jentschke et al. 1998).

V. Examples of Heavy Metal Removal by Fungal Biomass

A. Overview of Fungal Species in Biosorption Experiments

Numerous papers deal with the application of fungi which are specialists in metal biosorption. In order to give the reader an overview of this field, we have compiled the various organisms together with the absorbed metals and the relevant references in Table 18.1.

Table 18.1. Examples of heavy metals removed by fungal biomass

Fungus	Metals	Reference
<i>Absidia orchis</i>	Pb	Holan and Volesky (1995)
<i>Agaricus macrosporus</i>	Cu, Cd, Hg, Pb, Zn	Melgar et al. (2007)
<i>Aspergillus carbonarius</i>	Cu, Cr	Al-Asheh and Duvnjak (1995)
<i>A. flavus</i>	Cu, Pb	Akar and Tunali (2006)
<i>A. fumigatus</i>	U	Bhainsa and D'Souza (1999)
<i>A. fumigatus</i>	Au, Ag, Cu	Gomes and Linardi (1996)
<i>A. nidulans</i>	Cd	Plaza et al. (1996)
<i>A. niger</i>	Cd, Cu, La, Ag	Mullen et al. (1992)
<i>A. niger</i>	Cd, Cu, Zn, Ni, Co	Akthar et al. (1995)
<i>A. niger</i>	Pb, Cd, Cu	Kapoor and Viaraghavan (1999)
<i>A. niger</i>	Ag	Akthar et al. (1995)
<i>A. niger</i>	Pb	Meyer and Wallis (1997)
<i>A. niger</i>	Au, Ag, Cu	Gomes et al. (1996)
<i>A. niger</i>	Zn	Luef et al. (1990)
<i>A. niger</i>	Cu	Mukhopadhyay et al. (2007)
<i>A. oryzae</i>	Cu	Huang (1996)
<i>A. parasiticus</i>	Pb	Akar et al. (2007)
<i>Botrytis cinera</i>	Zn	Tunali and Akar (2006)
<i>Candida</i> sp.	Ni	Kambe-Honjoh et al. (1997)
<i>C. tropicalis</i>	Cu	Junghans and Straube (1991)
<i>Cladosporium cladosporioides</i>	Ag, Au, Cu, Cd	Pethkar et al. (2001)
<i>Claviceps paspali</i>	Zn	Luef et al. (1990)
<i>Fomitopsis pinicola</i>	Cd	Gabriel et al. (1996)
<i>Funalia trogii</i>	Hg, Cd, Zn	Arica et al. (2004)
<i>Fusarium flocciferum</i>	Cu, Cd, Ni	Delgado et al. (1998)
<i>Kluyveromyces marxianus</i>	U	Bustard et al. (1997)
<i>Mucor dimorphosporus</i>	Cu, Sr	Koronelli et al. (1999)
<i>M. miehei</i>	Cr	Tobin and Roux (1998)
<i>M. rouxii</i>	Cd, Cu, La, Ag	Mullen et al. (1992)
<i>M. rouxii</i>	Pb, Cd, Ni, Zn	Yan and Viaraghavan (2003)
<i>Neurospora crassa</i>	Co	Rama Rao et al. (1996)
<i>Penicillium chrysogenum</i>	Pb	Niu et al. (1993)
<i>P. chrysogenum</i>	Zn	Luef et al. (1990)
<i>P. chrysogenum</i>	Cd	Holan and Volesky (1995)
<i>P. chrysogenum</i>	Zn, Cu, Ni	Bakkaloglu et al. (1998)
<i>P. chrysogenum</i>	As(V)	Loukidou (2003)
<i>P. simplicissimum</i>	Cu, Pb	Li et al. (2008)
<i>Phanerochaete chrysosporium</i>	Hg	Saglam et al. (1999)
<i>Ph. chrysosporium</i>	Cu, Cr(III), Cd, Ni, Pb	Yetis et al. (1998)
<i>Ph. chrysosporium</i>	Cu	Sing and Yu (1998)
<i>Ph. chrysosporium</i>	Pb, Cr(VI), Cd, Ni	Dey et al. (1995)
<i>Ph. chrysosporium</i>	Cd, Cu, Pb, Mn, Ni, Co	Falih (1997)
<i>Ph. chrysosporium</i>	Hg	Dhawale et al. (1995)
<i>Ph. chrysosporium</i>	Cd, Cu, Pb	Say et al. (2001)
<i>Ph. chrysosporium</i>	Cu, Pb, Cd	Gopal et al. (2002)
<i>Ph. chrysosporium</i>	Cd, Cu	Pakshirajan and Swaminathan (2009)
<i>Pleurotus sajor-caju</i>	Cd	Cihangir and Saglam (1999)
<i>Polyporus versicolor</i>	Cu, Cr(III), Cd, Ni, Pb	Yetis et al. (1998)
<i>Rhizopus arrhizus</i>	U	Tsezos et al. (1997)
<i>R. arrhizus</i>	U, Pu, Am, Ce, Pm, Eu, Zr	Dhami et al. (1998)
<i>R. arrhizus</i>	Cu, Zn	Sag et al. (1998a)
<i>R. arrhizus</i>	Cr(VI), Fe(III), Cu	Sag et al. (1998b)
<i>R. arrhizus</i>	Cr(VI)	Merrin et al. (1998)
<i>R. arrhizus</i>	Cd	Yin et al. (1999)
<i>R. arrhizus</i>	Cr(VI)	Prakasham et al. (1999)
<i>R. arrhizus</i>	FeCN ₃	Aksu et al. (1999)
<i>R. arrhizus</i>	Pb	Naja et al. (2005)
<i>R. nigricans</i>	Cr(VI)	Bai and Abraham (1998)
<i>R. nigricans</i>	Cr(VI)	Bai and Abraham (2001)

continued

Table 18.1. continued

Fungus	Metals	Reference
<i>R. oligosporus</i>	U, Cu, Cd, Zn	Castro et al. (1992)
<i>R. oligosporus</i>	Pb	Ariff et al. (1999)
<i>R. oligosporus</i>	Cd	Aloysius et al. (1999)
<i>R. oligosporus</i>	Cu	Ozsoy et al. (2008)
<i>R. oryzae</i>	Cu	Huang (1996)
<i>R. oryzae</i>	Cu	Bhainsa and D'Souza (2008)
<i>Rhodotorula glutinis</i>	Pb	Cho and Kim (2003)
<i>Saccharomyces cerevisiae</i>	Cu, Co, Cd	Brady and Duncan (1994)
<i>S. cerevisiae</i>	Pb	Riordan and McHale (1998)
<i>S. cerevisiae</i>	U	Bustard et al. (1998)
<i>S. cerevisiae</i>	Ag	Simmons and Singleton (1996)
<i>S. cerevisiae</i>	U	Omar et al. (1996)
<i>S. cerevisiae</i>	Ag	Korenevskii et al. (1999)
<i>S. cerevisiae</i>	Ag	Bustard and McHale (1998)
<i>S. cerevisiae</i>	Cu, Ag	Simmons et al. (1995)
<i>S. cerevisiae</i>	Se (IV, VI), Sb (III, V)	Perez-Corona et al. (1997)
<i>S. cerevisiae</i>	Sr	Avery and Tobin (1992)
<i>S. cerevisiae</i>	Cr (VI)	Krauter et al. (1996)
<i>S. cerevisiae</i>	La, Co, Mn, UO ₂ , Pb, Ag, Zn, Cd, Cr ²⁺	Ben Omar et al. (1997)
<i>S. cerevisiae</i>	Pb	Suh et al. (1999a, b)
<i>S. cerevisiae</i>	Cu, Pb	Han et al. (2006)
<i>S. cerevisiae</i>	Mn	Parvathi et al. (2007)
<i>S. cerevisiae</i>	Zn	Chen and Wang (2007)
<i>Schizophyllum commune</i>	Cd	Lilly et al. (1992)
<i>Streptovercillium cinnamoneum</i>	Pb, Zn	Puranik and Paknikar (1997)
<i>Talaromyces emersonii</i>	U	Bengtsson et al. (1995)
<i>Trametes versicolor</i>	Cd	Gabriel et al. (1996b)
<i>Tra. versicolor</i>	Pb, Cu, Zn	Bayramoaylu et al. (2003)
<i>Trichoderma harzianum</i>	U	Akhtar et al. (2009)
<i>Tri. viride</i>	Cr(VI)	Bishnoi et al. (2007)
<i>Tri. viride</i>	Fe, Co, Cu, Zn, Cd, Pb	Sanna et al. (1997)
<i>Tri. conglobatum</i>	U	Akira and Takashi (1993)

B. Levels of Accumulation Obtained for Selected Metals and Fungi

The main parameter of interest in biosorption of metals is the respective capacity of various organisms. In order to give examples of the biosorption capacity, Table 18.2 summarizes the amounts of metals adsorbed to fungal biomass that were found in experiments with different fungal species aimed at an industrial process.

C. Use of Biosorption for Analytical Purposes

Although research has forced into biosorption, it has only rarely been exploited for analytical measurements.

Madrid and Camara (1997) described the use of biosorption for analytical purposes as a procedure for pre-concentration and speciation. A solid-phase extraction

system based on immobilized *Penicillium italicum* cells was described by Mendil et al. (2008) for the concentration of Cu, Cd, Pb, Mn, Fe, Ni and Co prior to determination by atomic absorption spectroscopy.

VI. Conclusions

It has been demonstrated that fungal biomass, either as a fermentation waste product, or fermented directly for the purpose of biosorption, can be used effectively for the removal of metals from solutions. Within the past decade, many publications have simply shown that a given fungus is able to adsorb a certain amount of a given metal. However, in a number of publications the mechanisms underlying this process have been analysed in greater detail and the contribution of the different groups in the cell wall have been determined in some cases. Once the binding sites and

Table 18.2. Heavy metal adsorption capacity by selected fungi

Metal	Fungus	Concentration (mg/g)	Reference
Arsenic	<i>Inonotus hispidus</i>	52	Sari and Tuzen (2009)
Cadmium	<i>Rhizopus oligosporus</i>	34	Aloysius et al. (1999)
	<i>A. niger</i>	92	Akhtar et al. (1995)
	<i>Saccharomyces cerevisiae</i> (live cells)	70	Volesky et al. (1992)
Chromium	<i>Trichoderma viride</i>	16	Bishnoi et al. (2007)
Copper	<i>A. niger</i>	108	Akhtar et al. (1995)
Lead	<i>R. oligosporus</i>	126	Ariff et al. (1999)
	<i>S. cerevisiae</i>	127	Riordan and McHale (1998)
	<i>Candida albicans</i>	800	Baysal et al. (2009)
Nickel	<i>A. niger</i>	41	Akhtar et al. (1995)
Silver	<i>A. niger</i>	100	Akhtar et al. (1995)
Thorium	<i>A. fumigatus</i>	370	Bhainsa and D'Souza (2009)
Uranium	<i>S. cerevisiae</i>	275	Bustard et al. (1998)
	<i>Talaromyces emersonii</i>	280	Bengtsson et al. (1995)
	<i>A. fumigatus</i>	423	Bhainsa and D'Souza (1999)
Zinc	<i>Rhizopus arrhizus</i>	14	Zhou (1999)
	<i>A. niger</i>	67	Akhtar et al. (1995)
Zirconium	<i>C. tropicalis</i>	180	Akhtar et al. (2008)

their characteristics are known, gene technology might be used to optimize them for the removal of a specific metal. To achieve this goal, a lot will have to be learned about the structure of the cell wall. One problem here is the fact that the species for which extensive molecular tools are available, i.e. *Saccharomyces cerevisiae*, is not a good biosorbent agent as it does not accumulate many metals and cannot form a filter like the filamentous fungi.

Another field where much research is still needed refers to a method of converting the biomass into filters that can be used for a prolonged period of time. There are, however, some studies that show that the process is indeed quite promising.

The economy of biosorption processes is governed not only by the cost of the process but also by the cost of alternative procedures. It should be borne in mind that the cheapest solution to pollution is often still dilution. From a practical point of view, the economy of the process should be analysed in more depth taking into account current and upcoming legislation. These economic considerations are still a field of dispute, as some authors (Gadd and White 1993; Kratochvil and Volesky 1998) regard biosorption as highly cost-effective whereas others (Lovely and Coates 1997) state that it is still mainly a field of research without much industrial relevance. Atkinson et al. (1998) compared the conventional method of

chemical precipitation with a biosorption process carried out in a pilot plant and found the conventional process to be more economical. So there is still a lot of work to do on the implementation of biosorption into industrial processes.

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Recent Developments and New Strategies

19 Bioherbicides

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I. Introduction

A considerable number of plant pathogens have been studied for their possible use as bioherbicides for the control of weeds. Only a few have proven adequately virulent to control weed species and to compete with commercial chemical herbicides. The majority of plant-pathogenic bacteria and fungi evaluated for the control of weeds cause inconsequential disease symptoms or reductions in a weed population. This observed suppression is not adequate to limit expansion of the weed population, much less eradicate it.

History details plant disease epidemics that have devastated a plant species and impacted human populations that are dependent upon that plant species. However, closer evaluation of these

epidemics reveal that they were often confined to a single region, radiated from a single or a few focal points, often were vectored by an insect, and affected a plant population with minimal genetic diversity (i.e. a monoculture). The classic historical example is late blight of potatoes caused by the phytopathogenic fungus-like protist *Phytophthora infestans*². This disease destroyed a large percentage of the potato crop in Ireland in 1845 and 1846 precipitating the death of nearly one-quarter of the Irish population and mass emigration to the United States (Rossman 2008). In the current decade, pine trees (*Pinus* spp.) throughout the northwestern United States and Canada have been devastated by the pine bark beetle (*Dendroctonus ponderosae*)/*Ophiostoma* complex (Rice et al. 2008). The severity of this pandemic has been intensified both by a drought which weakens the trees and by relatively warm winters (i.e. global warming) which increases the survival of the beetle.

Traditionally, weed biocontrol projects have been based on the premise that, given due diligence, natural biological control agents will be found that are capable of devastating a weed population. This strategy has proven successful in rare instances. However, more often than not, the weed population continues to expand despite the pathogen. Successful weeds have survival characteristics not commonly found in cultivated monocultures. For example, weed seed germination is indeterminate. At any given time, there could be un-germinated seed in the soil, seedlings, as well as maturing plants. If a pathogen can only attack one growth stage of the weed, the other stages will survive and produce new seed.

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²*Phytophthora infestans* is not a true fungus (Eumycota) and belongs to the Oomycetes classified under a different kingdom altogether: Chromalveolata (formerly Stramenopila; previously Chromista).

Few pathogens can overcome all defense and escape mechanisms of a weed population. The search for biocontrol pathogens is further problematical because biocontrol fungi and bacteria must meet standards for host specificity, virulence and ease of production and application. In the previous edition of this book, we proposed that the effectiveness of bioherbicides could be significantly enhanced by selecting plant pathogenic fungi or bacteria that overproduced and excreted an inhibitory amino acid. Nowadays this technology is effectively being applied to plant pathogenic fungi for control of the parasitic plants witchweed (*Striga* spp.) and broomrape (*Orobancha* spp., *Phelipanche* spp.).

II. The Paradigm

The general definition of biocontrol is utilization of living organisms (bacteria, fungi, nematodes or insects) to control plant pests (weeds, insects, nematodes or diseases). There are two different approaches to biological control: classical and inundative. The theory of classical biocontrol is that exotic plants thrive in their invaded environment because they have escaped natural enemies (insects and pathogens) in their native environment. The expansion and survival of the exotic weeds can be suppressed by introduction and establishment of natural enemies from the plant's place of origin. Classical biological control agents are typically insects or species of rust fungi. In contrast, the inundative or bioherbicides approach to biocontrol relies on the annual application of plant pathogenic fungi or bacteria to a weed species. Residual impact beyond the season of application is not expected.

A. Classical Approach

The classical approach to biocontrol involves finding a pathogen that exists in the region where the weed evolved. After extensive testing for efficacy and host specificity, the pathogen is introduced into the area where the weed has become a problem. Australian, New Zealand, Canada and the United States federal and state governments fund and employ researchers in

foreign countries to collect and test insects and pathogens for their possible introduction as biocontrol agents. With such introductions, they are generally hoped to colonize and spread on their own, as one time introductions. Exemplary of this classical approach – in the case of fungi – would be the rust diseases, and in fact most successful biocontrol projects have been with introduced rusts. These are public domain releases for rangeland and public land weeds. Biological control of rush skeleton weed (*Chondrilla juncea*) with the rust *Puccinia chondrillina* is an example of classical biocontrol with successes in both Australia (Cullen et al. 1973) and the western United States (Emge et al. 1981). Unfortunately, only one biotype of the weed was severely attacked, allowing two other resistant types to fill the void. As with chemical control, resistance to biocontrol agents can be problematic.

B. Bioherbicide Approach

The bioherbicides or augmentative approach is used when an endemic pathogen is found that can attack an endemic or introduced weed. In these cases, the pathogen can be produced in high amounts and applied as an herbicide at the appropriate time and season of the weed development. Incorporated necessarily into this strategy are pathogen production in greenhouses or by fermentation, preservation, the use of adjuvants or particle formulations, and the development of specific instructions for time of application, safety, and efficacy. Note that these steps can be by nature rather expensive compared to the classical approach. An example of such agents are Collego (*Colletotrichum gloeosporides* f. sp. *aeschynomene*), for control of northern jointvetch in rice and soybean (Bowers 1986). Demand for this bioherbicide has wavered possibly due to the availability of herbicide-resistant varieties of rice and soybean. However, the pathogen *C. gloeosporioides* f. sp. *aeschynomene* (previously Collego), has been re-registered as of March 2006 under the commercial name LockDown for use on rice in Arkansas, Louisiana, and Mississippi (Yandoc-Ables et al. 2006).

Early work in this field was based on the dictums that biocontrol agents needed to be host specific, easily

produced, and effective in reducing the weed below economic thresholds. The scope of most current research projects in biocontrol are focused on weeds that cannot be controlled by herbicides because of environmental or economical reasons. Water weeds, rangeland weeds, parasitic plants, and weeds invading ecological preserves and national parks and forested areas are now the main foci of biological control researchers. Given the recent advances in genomics and plant metabolomics, there surely will be some breakthroughs that will enable biocontrols to compete with chemical herbicides (Rector 2008). At present, however, the advantages of chemical herbicides are their broad target spectra, rapid knockdown, and general reliability despite their perceived or real threats to the environment or to public safety. Their use can be coupled with genetically modified crops that have been transformed to resistance to particular herbicides, making chemical herbicides even more useful on the farm.

Biological herbicides are very different from chemical herbicides. Microbial biological agents are living organisms with natural biological constraints. They have evolved to be dependent on the survival of the very host that we ask them to destroy. An apparent equilibrium thus exists between host and parasite, and pathogen virulence seems to be naturally moderated sufficiently to avoid host elimination (Gressel 2001). However, biological agents have some advantages. They are natural and have evolved to survive from year to year. They may even be more cost effective in terms of research and application costs than chemical herbicides. They are complex multigenic systems, and are not as easily countered by a single mutation as is often the case with chemical herbicides. Their host specificity gives them safety attributes that are uncommon in chemical herbicides, although conversely this specificity is inconvenient in the case where there is a multiplicity of weeds to control. They can be reiteratively genetically selected and manipulated for higher virulence and thus earlier knockdown, and improvements in pathogenicity, survivability and safety can be added onto existing agents. Strains can be marked biochemically or genetically so that their efficacy and survival can be verified in a molecular version of Koch's postulates (Hintz et al. 2001; Boss et al. 2007; Cipriani et al. 2009). Their activity, presence, and dissemination can be followed and verified through cycles of infection in the field, providing needed data for registration for field release.

Canada and New Zealand have very active biological control research and development

programs. The emphasis in New Zealand is on classical biocontrol, whereas the emphasis in Canada is on mycoherbicides. Recent mycoherbicides development in Canada are described below.

Currently, there are two bioherbicides commercially available in Canada. Interestingly, these mycoherbicides are not host-specific but their survival and mobility in the environment are limited. *Chondrostereum purpureum* (Chontrol) was registered for the commercial control of trees and shrubs along rights of ways in 2002 (Becker et al. 2005). This saprophytic fungus is applied directly to wounded trees or stumps, preventing re-growth (Becker et al. 1999; Harper et al. 1999; Pitt et al. 1999). This bioherbicide is registered under the trade name Chontrol in Canada and the United States and under the trade name BioChon in the Netherlands. The fungus has a very broad host range but very low rates of dispersal from a treated plant to a non-treated tree. Successful commercialization of this product partially resulted from public demand for biological alternatives to chemicals and lack of competition from conventional pesticides. The Canadian forestry sector has been under ever increasing pressure to move away from conventional herbicides. However, there were few alternatives for vegetation management. Since the price point for Chontrol was similar to that of the alternative measures, it has become a successful technology.

Sclerotinia minor (Sarritor) was registered for the commercial and domestic control of dandelions in turf-grass in 2007 (Abu-Dieheh and Watson 2006; 2007). Again, this fungus kills many broadleaved plants but not grasses. Importantly, the mycelia of this fungus do not survive in the turf grass environment longer than ten days (Abu-Dieheh and Watson 2006). The rapid adoption of Sarritor by end-users benefited from the provincial bans of conventional herbicide in all urban areas of Quebec and Ontario. The bans effectively removed the cheaper conventional herbicides and left few effective weed management alternative options.

The fungus, *Phoma macrostoma*, is being developed to control a number of broadleaved weeds in turf, cereal crops, and reforestation nurseries (Zhou et al. 2004, 2005). This potential bioherbicide is under evaluation by Agriculture and Agri-Food Canada.

The modern keys to successful development of classical biocontrol agents and bioherbicides and acquisition of appropriate registration are:

1. Understanding of the life history of the specific pathogens.
2. Understanding the genetic variability of the target weed.
3. Selection of the best isolate(s) based on the characterization of naturally occurring populations in the intended area of application.

4. Determination of the environmental fate and impact of release, and efficacy based on intended patterns of use.
5. Development of a production system which allows for scale-up of a quality:
 - a. developing a fermentation strategy for product quantity and shelf life,
 - b. controlling the product identity, titer and efficacy,
 - c. controlling product purity and safety,
 - d. keeping down the costs for growth media and formulation components,
 - e. integrating the new product into existing application methods of the end-user, f. pricing of the product for the end-user.
6. Continuance of strain improvement based on field results.

C. Genetic Enhancement

The third approach to biological control of weeds is genetic enhancement of biocontrol agents. This approach can involve selection of desired traits or genetic modification. The justifications for this approach are centered about the following hypotheses:

1. Biological control agents are not as virulent as they might be, and the more virulent they are made, the more likely they will be able to reduce weed populations with a cost effectiveness that exceeds that of conventional chemical herbicides.
2. Plant pathogens can be genetically altered so that they are more virulent. The pathogens ability to survive or disperse in the environment might be increased or decreased.
 - a. All plants, including parasitic weeds, are inhibited by one or more amino acids, and fungi can be selected to produce specific amino acids, greatly enhancing their virulence. This work is reviewed in a recent paper (Sands and Pilgeram 2009).
 - b. Several genes have been isolated from plant pathogenic fungi or bacteria that can increase the virulence of a biocontrol agent. For example the NEP1 (necrosis and ethylene-inducing protein 1) gene from *Fusarium oxysporum* f. sp. *erythroxyli* (Bailey 1995) increases the virulence of a number of different biocontrol fungi including *Pleospora*

papaveracea for control of opium poppy (Bailey et al. 2000), and *Colletotrichum coccoodes* for control of velvetleaf (Amsellem et al. 2002) and *Fusarium oxysporum* for control of *Orobanche* (Meir et al. 2009a).

- c. Plants can be impaired physiologically by naturally occurring plant specific compounds such as ethylene, abscisic acid, indole acetic acid, oxalic acid, gibberellins, jasmonic acid and kinetins that are known to be produced by plant pathogens. Different species of plants react in diverse ways to such compounds. Selected production or overproduction of these metabolites by biocontrol agents might provide “new weapons” for the development of bioherbicides and increase their efficacy (Bacon et al. 1996; Cohen et al. 2002; Rector 2008; de Zelicourt et al. 2009; Meir et al. 2009a, b).
3. The survival of biocontrol agents in the environment can be monitored using genetic techniques. These same techniques facilitate differentiation of biocontrol agents from endemic agents.

III. Virulence Enhancement

Several laboratories, in the past decade, have approached the problem of virulence enhancement of biocontrol agents. One such approach is the “Frenching model” where a natural field disease is caused by microbes in the soil or rhizosphere, which produce the inhibitory amino acids isoleucine.

James Oglethorpe first described the “Frenching disease” of tobacco in the American colony of Georgia in the seventeenth century. Affected seedlings showed symptoms of chlorosis, wilting, stunting, and leaf distortion, yet none of the bacteria or fungi recovered from the symptomatic seedlings was known to be a pathogen in the classical sense of invading tissues. Steinberg demonstrated that isoleucine would produce similar physiological symptoms in tobacco (Steinberg 1946). In 1950, he proposed that Frenching disease of tobacco was actually a physiological disorder or disease syndrome caused by saprophytic rhizosphere bacteria [*Pseudomonas fluorescens* Mig. or *Bacillus subtilis* (Ehrenberg) Cohn], with the unusual trait of isoleucine excretion (Steinberg 1950). Frenching symptoms resulted from the uptake of the free isoleucine into the tobacco plant. Isoleucine was suggested to inhibit acetohydroxyacid synthase (AHAS), the first enzyme of the branched-chain pathway for biosynthesis of leucine, valine and isoleucine, and of pantothenic acid (vitamin B5).

A. Direct Application of Amino Acids as Herbicides

A straightforward approach to the use of amino acid inhibition to control weeds is direct application of inhibitory amino acids (Table 19.1). This approach has been effective for control of emerged Canada thistle, red bromegrass, kudzu, and cannabis. The effect of amino acid application on the viability of the weed seed bank or underground structures such as tubers and root networks needs to be determined in a variety of climates and soils. The persistence of the applied amino acid in the soil profile has not yet been evaluated. The assumption is that the amino acid will rapidly be degraded or assimilated by the soil microflora. The rate of degradation or assimilation will depend upon the microbial community, the soil matrix and environmental conditions such as temperature and moisture. To date, this use of amino acids for weed control has not been put into practice.

Many homeowners and neighborhood organizations restrict the use of chemical pesticides in order to minimize exposure. Organic producers only have limited tools to control weed infestations, especially in post-emergent crops. Amino acids are green and non-toxic. Direct application of an inhibitory amino acid may provide economic and safe control of specific weeds, minimizing the application of synthetic herbicides.

B. Amino Acid Excretion

The amino acid synthesis pathways in plants and microbes have multiple amino acid end-products

Table 19.1. Effect of different L-amino acid concentrations on the germination of stimulated *Orobancha ramosa* seeds (Vurro et al. 2006)

Amino acid concentration (mM)	Germination (%) ^a		
	Methionine	Lysine	Arginine
4.0	0.0	0.0	0.0
2.0	24.3	10.3	0.0
1.5	42.3	38.3	0.0
1.0	70.3	81.0 ^a	0.0
0.5	85.7 ^a	89.7 ^a	77.3 ^a
Control	84.7 ^a	84.7 ^a	84.7 ^a

^aValues within a column with no letters in common differ significantly at $P = 0.05$

[aromatic amino acids (tryptophan, phenylalanine, tyrosine), branched-chain amino acids (valine, leucine, isoleucine), aspartate-derived amino acids (lysine, threonine, methionine)]. Such pathways are generally regulated by feedback inhibition of a controlling enzyme by one or more of the amino acid end-products. For example, isoleucine is synthesized from threonine via α -ketobutyrate, and leucine and valine are synthesized from pyruvate. The first common enzyme in the convergent pathway is acetohydroxyacid synthase (AHAS). In tobacco, AHAS is feedback-inhibited by leucine and valine (Forlani et al. 1994). Thus, an excess of valine results in feedback inhibition of AHAS, resulting in a shortage of leucine and isoleucine, curtailing protein biosynthesis. Incidentally, AHAS is a key target for several different herbicides including sulfonureas, imidazolinones, triazolopyrimidines, and pyrimidinyl oxybenzoates. Herbicide resistance can result from alterations in AHAS. Such mutations can often effect the sensitivity of the enzyme to feedback inhibition. For example, in chlorsulfuron-resistant prickly lettuce (*Lactuca serriola*), AHAS has reduced sensitivity to feedback inhibition by valine, leucine, and isoleucine (Eberlein et al. 1997).

We have demonstrated that amino acid overproduction can be used to enhance the virulence of host-specific formae speciales of *Fusarium* (Tiourebaev et al 2001) as well as a pathovar of *Pseudomonas syringae* (Tiourebaev et al. 2000). *Fusarium oxysporum* is a candidate pathogen for virulence enhancement because its formae speciales are very host-specific, the fungus survives in the soil for many years, and the pathogen is easy to culture and to manipulate. *Fusarium oxysporum* can grow on minimal media without amino acids, implying that it is capable of synthesizing all 20 protein amino acids. We have reported the isolation and characterization of valine-excreting mutants of *F. oxysporum* forma specialis *canabina* (Tiourebaev et al. 2000, 2001). Valine-overproducing variants of this biocontrol agent showed increased virulence to cannabis plants. Although the virulence was greatly enhanced in these cases, the host-range of these variants was not altered. This proof of concept does not yet extend to biocontrol of parasitic plants.

C. Molecular Virulence Enhancement

Transgenes encoding auxin production have been introduced into *Orobancha*-specific strains of *Fusarium*. The transgenic fungi have significantly

higher virulence than the wild-type strains (Cohen et al., 2002). This increased efficacy still does not completely control *Orobanch*e or restore yield. The NEP1 gene from *F. oxysporum* f. sp. *erythroxyli* (Bailey 1995) increases the virulence of a number of different biocontrol fungi, including *Pleospora papaveracea* for control of opium poppy (Bailey et al. 2000) and *C. coccodes* for control of velvetleaf (Amsellem et al. 2002).

NEP1 transformants of several species of *Orobanch*e-borne strains of *Fusarium* have been evaluated for enhanced control of *Orobanch*e (Meir et al. 2009a). The virulence of a strain of *Fusarium arthrosporioides* was enhanced but the virulence of strains of *F. oxysporum* was unaffected. The authors proposed that the native NEP1 gene in the *Orobanch*e-borne strains of *F. oxysporum* and the NEP1 transgene actually silenced each other.

Transgenic and non-transgenic tools to enhance virulence could be easily combined. The biosafety aspects of using transgenically hypervirulent biocontrol agents are specifically addressed in Gressel (2001).

As researchers, our options for strategies of biological control of weeds are relatively open, at least for experimentation:

1. We can select and/or engineer pathogens that are unusually virulent.
2. We can find a way to constrain broad host range pathogens either by auxotrophy (conditional mutants that require an externally applied adjuvant such as an amino acid) and thus only survive in its presence (however, as shown by work with *Sclerotinia* and *Phoma*, broad host range may be limited by lack of survival or lack of dissemination).
3. We might discover a new class of soil-borne biocontrol agents that specifically attacks and depletes the weed seed bank in the soil.
4. We can improve delivery and formulation.

IV. Biological Control of *Striga* and *Orobanch*e

Given the strength but complexity of biological control, we and many others have previously suggested that it might be expeditious to focus our efforts on biocontrol of a few targeted weeds. The value of this approach has been elegantly demonstrated in research to elucidate the biology and

genetics of model organisms such as *Escherichia coli* and *Arabidopsis thaliana*. Many world scientists have responded to this need for collaboration and are actively working to develop bioherbicides for control of the parasitic weeds, witchweed (*Striga* spp.) and broomrape (*Orobanch*e spp., *Phelipanche* spp. (Rubiales et al. 2009a). Broomrape control strategies have focused on agronomic practices (Rubiales et al. 2009a, b), the use of resistant crops (Pérez-de-Luque et al. 2009), chemical control (Gressel 2009; Hershenhorn et al. 2009), and biological control with strains of the pathogenic fungus *Fusarium oxysporum* (Hershenhorn et al. 2009; Kohlschmid et al. 2009). Similar strategies are being developed for the control of *Striga*, including agronomic, host resistance (Badu-Apraku and Yallou 2009; Ibikunle et al. 2009), chemical (Gressel 2009; Kanampiu et al. 2009; Tuinstra et al. 2009) and biological methods (Magani et al. 2009).

Witchweed (*Striga hermontheca*) is a parasitic weed that attacks and greatly reduces production and yields of maize, Sorghum, millet, and sugar cane in sub-Saharan Africa. (Table 19.2). Effective management of *Striga* is limited by the persistence and fecundity of the weed as well as by the nature of the plant/parasite interaction (Pérez-de-Luque et al. 2009).

Striga seed is viable in the soil for at least ten years. The dormant seed germinates in response to specific chemicals exuded from the roots of a host crop. The *Striga* germ tube grows toward the host and eventually attaches to the host root. After attachment, the *Striga* penetrates into the vascular

Table 19.2. Sub-Sahara African countries with the highest food production losses due to *Striga* (losses include sorghum, millets, maize). Compiled by A.B. Obilana, from NARS documents, reports, and personal records. Source: Gressel et al. (2004)

Country	Yield loss (%)	Yield loss ($\times 1000$ t)
Burkina Faso	35–40	710–820
Eritrea	20–60	30–90
Ghana	35	170
Kenya	35–40	50–60
Mali	40	580
Mozambique	35	40
Niger	40–50	930–1160
Nigeria	35	3750
Sudan	30	1230
Tanzania	up to 90	550
Togo	35	70
Total/mean	39–45	8110–8520

system of the crop, stealing water, photosynthates, and minerals. This diversion limits crop yield and is one of the chief causes of decreased agricultural productivity across the African continent. Conventional weed management methods are not particularly effective on *Striga* (Oswald 2005; Hearne 2009). First, the bulk of the damage to a crop plant is done long before the *Striga* plant emerges from the soil and is accessible to herbicides or to mechanical pulling. Second, the *Striga* plant is physically attached to the crop plant and there are few herbicides available that selectively attack the *Striga*. Furthermore, such herbicides are far beyond the means of subsistence farmers or rural communities. Finally, each mature *Striga* plant produces thousands of seeds that remain viable in the soil for several years.

Crop rotation is one of the most effective ways to reduce *Striga* infestations and increase maize yields (Oswald and Ransom 2001). However, land use intensification and increasing cereal monocropping, with little or no use of purchased external inputs, have exacerbated the *S. hermonthica* problem in sub-Saharan Africa.

Strains of *Fusarium oxysporum* have been reported to specifically attack *Striga hermonthica*, and to be somewhat effective in the field (Ciotola et al. 2000; Yonli et al. 2004; Marley and Shebayan 2005; Elzein et al. 2006, 2009; Zahran et al. 2008a, b, c; Magani et al. 2009; Venne et al. 2009). The inadequacies of this biocontrol agent in its present genetic unaltered genetic state are that it is too delayed in its attack, it does not seriously deplete the soil bank of *Striga* seed, it is not easily or economically applied at the subsistence farmer level, and there is no data on its long-term survival or long-term efficacy. In proposed altered states this pathogen could have great potential if:

1. Virulence is enhanced to the level where less inoculum and much earlier *Striga* mortality are the result.
2. The soil seed bank is attacked sufficiently.
3. There is enough genetic diversity in improved strains to counter new resistance responses from *Striga*.
4. The altered strains are clearly marked allowing soil survival and efficacy data to be generated for presentation to regulatory agencies.
5. The primary inoculum of effective strains of the biocontrol agent is available to subsistence farmers who are enabled with methods to grow their own secondary inoculum.

Broomrape (*Orobancha ramosa*) is a parasitic plant related to *Striga*. This parasitic weed is

also attacked by a specific forma specialis of *F. oxysporum* (Amsellem et al. 2001; Andolfi et al. 2005; Bouizgarne et al. 2006; Hershenhorn et al. 2009; Kohlschmid et al. 2009; Muller-Stover et al. 2009; Pérez-de-Luque et al. 2009). *Fusarium oxysporum* has similar inadequacies as a biocontrol agent on broomrape as it does with *Striga*:

1. It needs sufficient virulence to knockdown the germinated seedlings.
2. It would need to destroy the seed bank.
3. It would also need to fulfill the diversity, traceability, and on-farm production requirements.

V. Alternative Approaches for Enhancement of Virulence and for Improved Control of Parasitic Plants

A. *Fusarium* as a Model System

Fusarium oxysporum strains are attractive as biocontrol agents because they are highly virulent and their host range is usually limited to one plant species. Furthermore, the fungus is persistent in the soil for long periods of time and is able saprophytically to colonize the roots of many non-host plants. *Fusarium* mutants can readily be obtained and characterized and *Fusarium* also produces other pathogenicity factors: jasmonic acid (Miersch et al. 1999), hydrolytic enzymes (DiPietro and Roncero 1996), necrosis peptides (Bailey 1995), fusaric acid (Bacon et al. 1996; Zonno and Vurro 1999; Bouizgarne et al. 2006), and other plant hormones (Tsavkelova et al. 2006). For this reason, these pathogens are viewed as “Trojan horses” able to carry enough new “warrior genes into the battle” cumulatively to tilt the playing field against parasitic plants.

B. Seed Germination

Preconditioned *Striga* and *Orobancha* seed germination is stimulated by plant metabolites produced by the host plant (Babiker et al. 1993; Humphrey and Beale 2006). In the absence of a susceptible host plant, seed germination of parasitic plants is suicidal. Trap crops and germination stimulants have been utilized to reduce the

Striga seed bank in infested soils (Babiker et al. 1993). Fungal metabolites can also impact *Striga* and *Orobanche* seed germination.

Trichothecenes produced by *Fusarium solani* inhibit *Striga* germination (Dhanapal and Struik 1996), and metabolites from *Fusarium* spp. inhibit *Orobanche* germination (Zonno and Vurro 2002; Vurro et al. 2009). Certain fungal metabolites also stimulate plant germination, including jasmonic acid (Yoneyama et al. 1998; Andolfi et al. 2005) and ethylene (Berner et al. 1999). Many formae speciales of *F. oxysporum* and numerous other fungi produce some ethylene (Swart and Kamerbeek 1976). *Fusarium oxysporum* f. sp. *tulipae* produces 1000-fold higher levels of ethylene than most *Fusaria*, and the level of ethylene production of this pathogen was correlated with tulip virulence. Ethylene production by pathogens of *Striga* or *Orobanche* could stimulate weed germination and be correlated with weed control. It remains to be seen how much ethylene could be produced and whether or not it can be tolerated by the crop plant. *Orobanche* seed germination is less sensitive to ethylene stimulation than *Striga* seed germination. Thus, this approach may be limited to biocontrol of *Striga*. The impact of the described plant regulators on growth and development of parasitic plants is unknown.

C. Innovations in Delivery Systems of Biocontrol Agents

There is a great need to make biological control research relevant to field situations. Too often research stops short of practical and efficacious delivery to the field. There are some strong features of the chosen pathogen, *F. oxysporum*, which will enable it to be produced and disseminated effectively. It is host specific but often an aggressive colonizer of non-host seedling roots. In recent work, the authors used non-host seeds as a carrier system to disseminate *F. oxysporum* because of the cost constraints of producing large quantities of spores in culture. The production of spores in the rhizosphere of non-host seedlings greatly decreased field application costs. The depth and size of the carrier root system effectively distributed the *F. oxysporum* throughout the soil profile.

In the case of *Striga* in Africa, it is speculated that an aggressive *Striga* pathogen applied to maize or Sorghum seeds might well suppress other crop pathogens, including *Fusarium graminearum*, a problem now requiring expensive seed treatments. If seed treatments are still used, it would be important to ensure that biocontrol pathogens

are resistant to the fungicides or herbicides. Inoculum will be most useful if it can be cheaply delivered, possibly prepared on an agricultural waste product such as bagasse (produced by sugar cane processors), and if it can be recycled through several seasons by composting, passage through animal wastes, survival in the rhizosphere of alternate crops, etc.

D. *Striga* and *Orobanche* Resistant Crop Plants

There are ongoing efforts to develop maize varieties, which are resistant to *Striga* (Badu-Apraku and Yallou 2009; Venne et al. 2009) or resistant to herbicides that could then be used to control *Striga* (Kanampiu et al. 2003; De Groote et al. 2008; Gressel 2009). Herbicide-resistance technology has only been applied to a limited number of maize and Sorghum varieties. The impact of these efforts on sustenance agriculture may be minimal as herbicide resistant germplasm and seed tend to be expensive to develop and usually proprietary. Subsistence farmers need both short-term and longer-term strategies that allow effective production of susceptible crops in *Striga*-infested land. Thus, for sustained *Striga* control and management, it is imperative to foster new integrated approaches including biotechnological solutions, with concerted resource mobilization, wider strategic partnerships, novel multidisciplinary linkages, and participatory approaches with farmers.

VI. Summary and Outlook

Control of parasitic plants represents one of the greatest enigmas for modern agriculture. Is it possible to kill a parasitic plant that is so physically and biochemically attached and associated with its host plant without also inhibiting the host as well? The more diverse the approaches that can be integrated into parasitic weed control, the greater will be the chance of staying ahead of the counter mutation and adaption that can be expected to follow. One strong technological approach is presented here, specifically the use of amino acid-excreting mutants of *Fusarium oxysporum* or other similar pathogens. It would not be wise to deploy this Frenching technology alone, as the seed banks of *Striga* and *Orobanche* could provide sufficient numbers of mutants to overcome the temporary advantage afforded by this singular

approach. This lesson has been learned many times over in the field of plant pathology, and only when a multiplicity of approaches has been combined will it be possible to rest. Such a multiplicity of approaches will most likely be achieved by a spirit of collaboration among a number of laboratories, with each contributing in their special areas of expertise. It is hard to envision a more urgent and pressing goal than that of biological control of *Orobanche* and *Striga*.

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20 Genomic Approaches for Identification of the Biopolymer Degrading Enzyme Network of *Aspergillus niger*

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I. Introduction

A. Fungal Genomes

The first full genome of a filamentous fungus, *Neurospora crassa*, was released in 2003 (Galagan et al. 2003). Currently, more than 70 genomes of filamentous fungi have been sequenced, annotated and deposited in public databases, including model and pathogenic species, and species which are significant as platforms for bioindustry and bioenergy. Considering the extensive impact of filamentous fungi on many facets of human welfare, it is expected that in the near future many more will follow. Fungal genomes are relatively small in size (30–80 Mb), with a high coding density (37–61%) and a relatively simple gene structure (Galagan et al. 2005) which makes them a promising target for de novo sequencing with pyrosequencing technologies at vastly lower costs compared to the traditional Sanger method.

The large increase of available fungal genome sequences and the high conservation of their genomic content have allowed the comparison of different fungi on a meta-species level. This has led to high resolution reconstructions of fungal phylogenies, between more than 40 species using entire genomes providing new insights into genome structure and evolution (Fitzpatrick et al. 2006; Robbertse et al. 2006; Sugiyama et al. 2006).

Filamentous fungi share many biological characteristics and processes with the complex higher eukaryotes, including multicellularity, development and differentiation programs and intercellular signaling. Their experimental tractability makes them useful model systems to study the complexity of the eukaryotic cell.

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B. *Aspergillus niger*

Within the phylum of ascomycetous fungi, the aspergilli, form a large and diverse group of filamentous fungi with more than 300 different species identified (NCBI Taxonomy browser, <http://www.ncbi.nlm.nih.gov>). They occur worldwide, mostly as saprophytic, soil-borne molds or phytopathogens, although some species are allergenic or opportunistic human pathogens. A number of aspergilli, notably *A. niger*, produce important metabolites, such as citric acid and are excellent producers of a wide range of industrially important extracellular enzymes.

One of the companies that is using *A. niger* as a platform for enzyme production, the Dutch biotechnology company DSM (actually Dutch State Mines) has sequenced and publically released the complete genome of *A. niger* CBS 513.88, the ancestor of a range of enzyme production strains. The genome sequence was completed in October 2001. During the sequencing phase, a structured BAC approach was used which resulted in a high quality DNA sequence of 8× coverage organized in 19 supercontigs with an assembled genome size of 33.9 Mb and captures approximately 99% of the estimated 37 Mb genomic content of this fungus. The genome sequence of *A. niger* was annotated by the German company Biomax using an automatic gene annotation phase followed by a manual correction and verification phase. Strong function predictions were made for almost half of the more than 14000 open reading frames identified. The annotated *A. niger* CBS513.88 genome sequence was publicly released in 2007 (Pel et al. 2007), and later that year complemented with a draft assembly of the genome of strain ATCC1015 released by the DOE Joint Genome Institute (JGI). ATCC1015 is considered to be a wild-type strain and a typical producer of citric acid.

A metabolic network of *A. niger* was reconstructed based on the cross comparison of the available *A. niger* genome sequences and other filamentous fungi, including *A. nidulans*, *A. oryzae*, *A. fumigatus*, *A. flavus*, *Fusarium graminearum* and *Neurospora crassa*, and the yeast *Saccharomyces cerevisiae* (Sun et al. 2007). Network analyses revealed that *A. niger* possesses a significant number of enzymatic activities involved in the production of secondary metabolites. Secondary metabolites are considered to be important for niche specialization and their production is frequently found to be associated with sporulation processes. In addition, degradation pathways of different substrates like pectin, starch, cellulose and other polymeric

carbohydrates have lineage specific expansions unique to *A. niger* (Pel et al. 2007). For CBS513.88 in total more than 1000 unique *A. niger* genes were found. Many of these unique genes are the result of gene duplication events, suggesting that exploiting gene duplications is a key strategy for *A. niger* to adapt to different environmental conditions.

This chapter aims to review the insights into the repertoire of carbohydrate modifying enzymes as gained from the genome sequences of *A. niger* and other industrially applied filamentous fungi. Partially, this chapter will be a reflection of the lessons learned in a project on carbohydrate modifying enzymes from *A. niger*, called CarbNet.

This project, which was a collaboration between several Dutch universities and knowledge institutes, had access to the complete genome sequence of *A. niger* CBS 513.88, before it was publicly available. During the CarbNet project, several other *Aspergillus* genomes became publicly available, allowing comparative genomics studies. In this chapter, several examples are discussed of genomic approaches to the identification and functional characterization of enzymes involved in the metabolism of three industrially relevant model carbohydrate substrates: starch, fructan and pectin.

Full genome sequences of filamentous fungi have revealed the presence of a large number of genes encoding transcriptional regulators. Compared to yeasts such as *S. cerevisiae*, *Candida albicans* and *Yarrowia lipolytica*, in particular the number of transcription factors belonging to the Zn(II)₂Cys₆ family has dramatically expanded in the fungi. In the second part of this review, several approaches to identify transcriptional regulators involved in carbon catabolism are described and their prospects discussed.

II. Biopolymer Degradation by *Aspergillus niger*

A. Starch and Glycogen

1. General Remarks

Starch is an abundantly available carbon source for saprophytic fungi. It is the main storage polysaccharide in plants, present in the roots, tubers, seeds or fruits (Peters 2006). Starch also constitutes a major component of the human diet in the

form of rice, wheat, maize and potato. Products derived from starch are used for various applications in the food and non-food industry, for example, as a thickener in sauces, anti staling agents in baking and in the production of paper and biodegradable plastics (Taggart 2004).

Starch is composed of two types of molecules: amylose and amylopectin. Amylose is an unbranched chain of α -(1,4)-linked glucose residues, which generally has a degree of polymerization (DP) between 250 and 5000. Amylopectin is also mainly composed of α -(1,4)-linked glucose residues, but approximately 3–5% of the glucose moieties is additionally linked with an α -(1,6)-bond, creating a branched molecule with a DP of 10000–100000. In general, starch is composed of 15–30% amylose and 70–85% amylopectin, but the exact composition depends on the source (Robyt 1998). Glycogen is the equivalent polysaccharide storage compound in bacteria, fungi and animals. Glycogen is a highly branched molecule, with 7–12% α -(1,6)-linked glucose molecules, and is therefore more accessible for enzymatic degradation than starch.

2. Genomics of Enzymes Acting on Starch

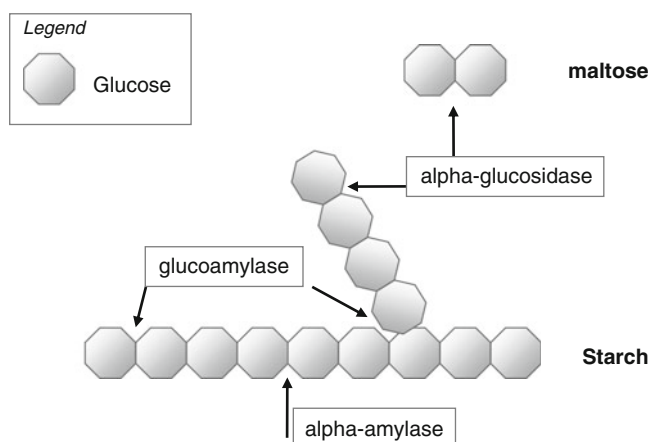
Degradation of starch by heterotrophic organisms is performed by the concerted action of different enzymes, including α -amylases, α -glucosidases and glucoamylases (Fig. 20.1). Amylases hydrolyse the internal α -(1,4)-glycosidic bonds in starch, glycogen and maltooligosaccharides [α -(1,4)-linked glucose oligomers] producing shorter maltooligosaccharides and maltose. Glucoamylases release

β -glucose from the non-reducing end of maltooligosaccharides, while α -glucosidase releases α -glucose from the non-reducing end. These hydrolysing enzymes are classified in different families according to the classification of glycoside hydrolase (GH) enzymes, which is based on sequence similarity of the proteins, as presented in the carbohydrate active enzymes database (<http://www.cazy.org/>; Cantarel et al. 2009). Glucoamylases are grouped in family GH15 and most α -glucosidases group in family GH31. The majority of the α -amylases belong to the large family GH13, which contains enzymes either hydrolysing or forming α -(1,4)- or α -(1,6)-glycosidic bonds (Stam et al. 2006). In general, family GH13 enzymes can be recognized by the presence of four highly conserved amino acid regions, which contain most of the amino acids present in the active site (MacGregor et al. 2001).

Within these regions, seven amino acids important for catalytic activity and binding and positioning of the substrate are generally conserved. Classification of enzymes up to GH (sub)family level is relatively easy, because the GH classification is based on sequence homology, and this classification provides some idea of enzymatic activity and functionality of the enzyme. However, some families, notably GH13 containing the amylases, are very large and contain many enzymes with related but to some extent differing enzymatic activities, which cannot always be predicted based on the amino acid sequence.

In *A. niger*, a number of extracellular, starch degrading enzymes had already been characterized. These enzymes included an acid amylase (Boel et al. 1990), the almost identical proteins AmyA/ AmyB identified from *A. niger* var. *awamori*

Fig. 20.1. Schematic model of the enzymatic activities involved in starch degradation by fungi



(Korman et al. 1990), glucoamylase GlAa (Boel et al. 1984) and α -glucosidase AgdA (den Herder et al. 1992; Nakamura et al. 1997). However, annotation of the genome revealed no less than 16 enzymes from the GH13 family, including the three above-mentioned amylases (Yuan et al. 2008b). Detailed sequence comparisons showed that two GH13 enzymes appear to be involved in glycogen synthesis and degradation, as could be deduced from their homology with known eukaryotic enzymes. Three novel GH13 enzymes were annotated as putative α -amylases, but lacked some of the amino acids highly conserved in classical α -amylases. None of the homologous proteins, which appeared to be present in other fungal genomes, appeared to be characterized. Production and biochemical characterization of such proteins were necessary to determine the activity of these hypothetical enzymes beyond doubt.

To circumvent the problem of purifying an enzyme with unknown activity, addition of a tag to a homologously expressed protein was considered for detection and purification. In the literature, a number of examples for the use of purification tags, such as His-tags for secreted protein in filamentous fungi, have been described (Karlsson et al. 2001; Bergquist et al. 2002; Kusumoto et al. 2008). However, in some cases this resulted in partial proteolysis of the His-tagged protein (Karlsson et al. 2001; Kontkanen et al. 2009). As an alternative we considered the use of specific fungal carbohydrate binding modules (CBM) as purification tags. Recent reviews on the different CBMs have already suggested their use as purification tools (Machovic and Janacek 2006; Shoseyov et al. 2006). As the substrate matrices of specific CBM proteins are widely available and cheap, this approach also allows development of large-scale processes based on the purification tools.

Previously, the use of CBM1 (cellulose binding domain) was described to generate hybrid proteins with specific binding characteristics. The presence of a CBM1 domain fused to a specific protein was shown to result in affinity to cellulose containing matrices (Limón et al. 2001; Hägglund et al. 2003; Limón et al. 2004; Voutilainen et al. 2009). However, in several cases the binding obtained with this type of domains is not reversible (Carrard and Linder 1999) which significantly limits the use of these domains as purification tools. Moreover, most filamentous fungi contain multiple endogenous proteins carrying CBM1

domains, and also several other domains with affinity to cellulose and related β -glucans, including sepharose, xylan, chitin (and galactan). Consequently, selective purification of a single recombinant CBM1 containing protein is not readily feasible using these matrices.

As a starting point to evaluate the potential use of alternative CBM domains in *A. niger* (and other industrial fungi) we mined the available genomes of *A. niger*, *A. oryzae* and *T. reesei* for the presence of CBM domains based on the CaZy systematic (Cantarel et al. 2009). In the CaZy website 53 different CBM domains have been described. As seen in Table 20.1 only a limited number of CBM domains interacting with plant derived carbohydrates have been described for yeasts and filamentous fungi. In yeasts only CBM43 and CBM52 domain have been identified, which are always linked to GH72 glucanoyl transglycosylase and GH81 β -1,3-glucanase activity domains. Also in filamentous fungi, most known CBM domains are linked to only a single family of proteins. In the case of the CBM20 (starch binding domain) both amylases (GH13) and related glucoamylases (GH15) have been identified with this domain. A clear exception from this apparent one-CBM-one-GH rule is observed for CBM1 (cellulose binding domain). In *Aspergilli* this domain occurs in a wide variety of proteins from very different GH classes. In *Trichoderma* and related genera, it even occurs on completely different non-GH type of proteins. In Table 20.1 only the examples for the three listed species are given, but in other genera such as *Podospora* and *Magnaporthe* an even wider variety of proteins including lipases, esterases and chitinases with CBM1 domains have been described.

For three classes of CBM domains, CBM6 [galactan (Ichinose et al. 2005), CBM21 [starch (Lin et al. 2009)] and CBM38 [inulin (Moriyama and Ohta 2007)], the three industrial species listed in Table 20.1 have no representatives. Moreover, these domains are only represented in a very limited number of secreted proteins in fungal species. From Table 20.1, both inulin and starch binding domains appear as relatively favorable to use as a tag for homologous protein expression, while only few or none are present in native proteins and both substrates can easily be used for purification. For the suggested inulin binding domain CBM38, only very limited information is available regarding its functionality in fungi (Moriyama and Ohta

Table 20.1. Overview of fungal carbohydrate binding domains represented in selected fungal genomes

CBM domain ^a	Target	Associated enzyme/protein	<i>A. niger</i> ^b	<i>A. oryzae</i> ^b	<i>T. reesei</i> ^b	Yeasts/Other fungi ^c
CBM1	Beta-glycan (cellulose, xylan, chitin)	GH5: endoglucanase	2	1	2	
		GH6: endoglucanase	1	0	1	
		GH7: cellobiohydrolase	1	0	2	
		GH18: chitinase	0	0	3	
		GH45: endoglucanase	0	0	1	
		GH61: endoglucanase	3	2	1	
		GH74: xyloglucanase	1	0	1	
		CE5: acetyl xylan esterase	0	0	1	
		Swollenin	0	0	1	
		Cellulose-induced protein	0	0	1	
		GE: glucuronyl esterase	0	0	1	
CBM6	Beta-glycan	GH43: xylanase/xylosidase	0	0	0	<i>Magnaporthe</i> (2×), <i>Podospora</i> , <i>Gibberella</i>
CBM13	Galactan/mannan	GH27: alpha-galactosidase	1	1	0	
		Ricin-binding protein	0	1	0	
CBM20	Starch	GH13: amylase	1 (JGI)	0	0	
		GH15: glucoamylase	1	1	1	
CBM21	Starch	GH15: glucoamylase	0	0	0	<i>Rhizopus</i> , <i>Mucor</i>
		Glycogen-associated protein phosphatase subunit	1	1	1	4 (<i>Sac. cerevisiae</i>)
CBM24	Mutan (α -glucan)	GH71: alpha-glucanase	2 (JGI 3)	2	2	
CBM32	Lactose	Glyoxal oxidase	0	2	0	
CBM35	Xylan	GH43: xylanase/xylosidase	1	1	0	
CBM38	Inulin	GH32: inulinase	0	0	0	<i>Penicillium</i> , <i>Neurospora</i>
CBM42	Arabinoxylan	GH54: arabinofuranosidase	1	1	2	
CBM43	Beta-glucan	GH72: glucanosyltransferase	4 (JGI 5)	4	5	2 (<i>Sac. cerevisiae</i> , <i>Pichia</i> , <i>Sch. pombe</i>)
		Unknown ^d	–	1	–	
CBM52	Beta-glucan	GH81: beta-glucanase	0	0	0	1 (<i>Sch. pombe</i>)

^aThis table only lists fungal GH protein family members and related secreted proteins carrying CBM domains interacting with plant derived polymeric substrates, thus excluding chitin and related substrates.

^bGenome searches for *A. niger* and *A. oryzae* are based on sequences present in the CAZy database. These sequences are derived from NCBI. For *T. reesei* the genome sequence available from JGI is used. In a few cases where the *A. niger* data present in NCBI suggested evidence of wrongly annotated or incorrect sequences also the *A. niger* sequence present at JGI was mined. In three cases (CBM20, CBM24, CBM43) this resulted in the identification of additional CBM-containing proteins.

^cIn three cases (CBM6, CBM21, CBM38) the three listed fungal species or yeast species had no protein representatives carrying the respective CBM domain. For these cases, selected fungal species carrying GH protein family members with these domains are listed.

^dIn the *A. oryzae* genome search a CBM43 sequence was identified in a poorly annotated region precluding identification of the linked protein.

2007). Moreover, in all but one of the fungal proteins for which it was described, it was found within the catalytic domain of GH32 inulinase-like proteins. Only the *N. crassa* inulinase-like protein CAC28747 has an N-terminal CBM38 domain. Therefore, it was decided to use a fungal CBM20 starch binding domain as a detection and purification tool for the expression of a GH13 enzyme with unknown activity.

As a first step towards a versatile production system for CBM20-tagged proteins, a suitable CBM20-free fungal host strain was generated.

Therefore, a protease deficient *A. niger* strain already disrupted for the single endogenous glucoamylase GH15 (*glaA*) gene (MGG029; Conesa et al. 2000), was transformed with an acid amylase GH13 (*aamA*) deletion vector (Weenink et al. 2006). In contrast to the MGG029 parental strain, the resulting host strain MGG029 Δ aamA, showed severely reduced growth on media with starch as the sole carbon source indicating the proteins encoded by *glaA* and *aamA* together are responsible for starch utilization in our *A. niger* host strain.

Interestingly, one alternative actively transcribed gene encoding a secreted GH13 amylase, *amyC* was also found to be present in this strain (Yuan et al. 2008b). Apparently the encoded protein is unable to fully metabolize starch substrates. The used strain MGG029 clearly differs from *A. niger* strain CBS513.88 in which at least two other highly expressed amylase GH13 genes (*amyA*, *amyB*) are present. Genome comparison of the various available *Aspergillus niger* sequences suggest that these two copies of a gene actually invaded into the CBS513.88 genome by horizontal gene transfer from *A. oryzae* (unpublished data).

The CBM20 (starch binding domain) from *A. niger* GlaA was used as a tag for selective purification of one of the putative GH13 enzymes with aberrant conserved amino acid regions, as described above. The gene encoding this enzyme also encoded a C-terminal sequence predicting the addition of a glycosyl-phosphatidyl-inositol (GPI) anchor, as well as an N-terminal signal sequence for secretion.

GPI anchor structures, only found in eukaryotes, allow a protein to be linked to the cell wall or cell membrane via its complex structure of mannoses, glucosamines and a lipid component. The two homologous proteins in *A. niger* and orthologs in other filamentous fungi are in most cases also predicted to bear a GPI-anchor. The gene fragment encoding CBM20, including a linker region for flexibility, was cloned at the 3' end of the GH13 encoding gene, replacing the GPI-anchoring sequence. *A. niger* strain MGG029 Δ *aamA* was chosen as a host for expression to allow specific purification but also to minimize any effect of background amylase activity which could interfere with the enzyme's activity.

When several over-expression mutants were tested for expression of the hybrid gene, an antibody specific for the detection of the CBM20 was used to check expression levels, allowing easy identification of the best over-expression strain. Subsequently, a first concentration and purification step could be performed by binding the CBM20-tagged protein to starch granules, and subsequent release using a mild treatment (elevated pH, temperature, maltose competition; Lin et al. 2009). Further ResourceQ separation allowed complete purification of the protein. Biochemical and physiological investigation showed that the enzyme was not an amylase, but an glucanotransferase enzyme, probably with a function in cell wall α -glucan modification rather than starch degradation. In this specific case, annotation based on amino acid homology clearly failed, and expression and functional analysis was necessary to correctly annotate this

protein and its homologs. The use of a CBM20 as a tag allowed the identification and partial purification of the protein using homologous expression (van der Kaaij et al. 2007).

B. Pectin

1. General Remarks

Pectin is the name for a diverse group of complex heteropolymers present in the middle lamella of the cell wall of dicotyledonous plants. Within the cell wall, pectin forms a negatively charged, hydrophilic network that together with hemicellulose and some proteins gives compressive strength to primary walls. Pectin consists of a number of distinct polysaccharides, composed of different monosaccharides such as α -D-galacturonic acid in homogalacturonan (HG), β -D-xylose and galacturonyl residues in xylogalacturonan (XGA), and rhamnosyl and α -galactosyluronic acid residues with side chains of arabinose and galactose in rhamnogalacturonan I and II (RGI/II). Pectin accounts for about one-third of the total primary cell wall material (Carpita and Gibeaut 1993) and as such represents an important carbon source for saprophytic bacteria and fungi. Because of its complex and heterogeneous structure, the complete biodegradation of pectin requires the presence of many different enzymatic activities. Pectin is widely used as a functional food ingredient because of its gelling properties, and as a soluble fiber it was shown to have positive health effects.

2. Genomics of Enzymes Acting on Pectin

In noticeable contrast to the situation for starch and starch degrading enzymes, as many as 17 different monosaccharides are accounted as pectin constituents and at least 12 different enzymatic activities are needed to completely degrade pectin: these include hydrolase, lyase and esterase activities (Fig. 20.2). Although differing in their substrate specificity and mechanism of action, pectin hydrolase, lyase and esterase activities show a surprising lack of structural variation. Independent of their bacterial, fungal or plant origin all these enzymes share the same single stranded right handed parallel β -helix topology characteristic for the pectin lyase-like superfamily. In the CarbNet project various standard comparative genome mining tools were used

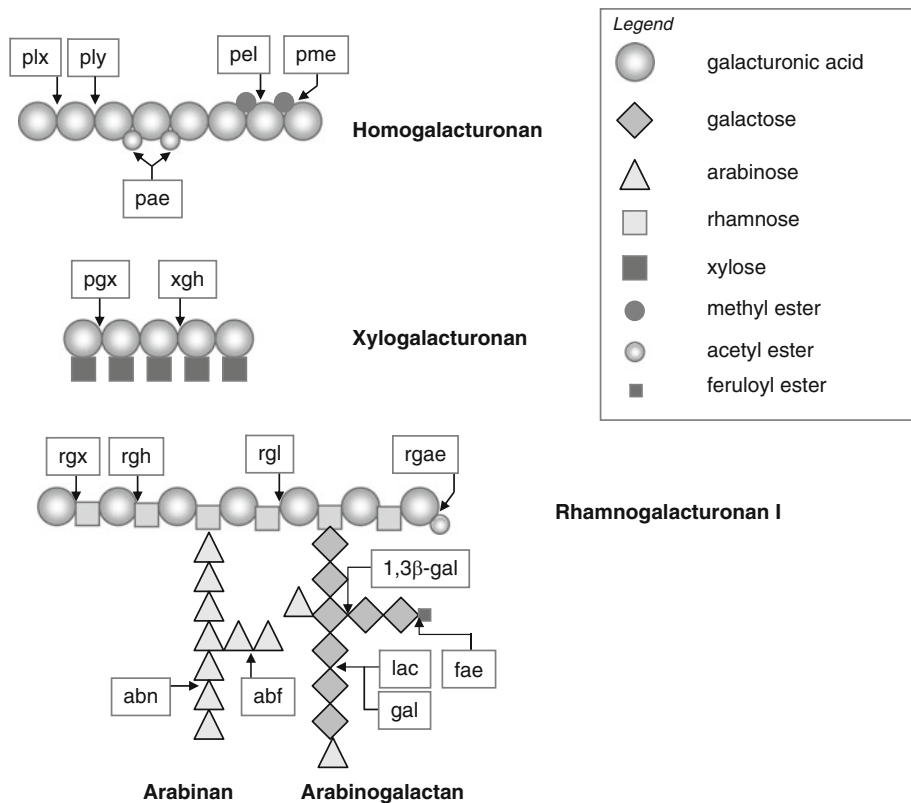


Fig. 20.2. A tentative model of the synergistic network of enzymatic activities involved in fungal pectin degradation. *Arrows* represent the (possible) points of attack of different functional groups of enzymatic activities. Note that the illustrated polymers are only a schematic representation of the major pectin components and do not depict definite molecular structures. *pgx* Exopolygalacturonase, *pga* exopolygalacturonase, *ply* pectate lyase, *pel* pectin lyase *pae*

pectin acetyl esterase, *pme* pectin methyl esterase, *rgx* exorhamnogalacturonase, *rgl* rhamnogalacturonan lyase, *rgae* rhamnogalacturonan acetyl esterase, *xgh* endoxylogalacturonidase, *rhg* rhamnogalacturonase, *gal* galactanase, *lac* galactosidase, *abf* arabinofuranosidase, *abn* arabinase, *fae* feruloyl esterase, *1,3-β-gal* 1,3-β-galactosidase. Adapted from Martens-Uzunova and Schaap (2009)

to unlock the pectinolytic potential of *A. niger* (Pel et al. 2007; Martens-Uzunova and Schaap 2009). This led to the identification of 60 loci predicted to encode pectinolytic activities from which almost half were previously described (Martens-Uzunova and Schaap 2009). Surprisingly, 12 new genes putatively encoding family 28 glycoside hydrolases (GH28) were identified.

A detailed analysis of the conservation of structural and catalytic residues between the sequences of all the members of GH28 distinguished two different subgroups of exo-activities – four genes were annotated as encoding exo-polygalacturonan hydrolases (PgaX, PgxA, PgxB, PgxC) and three genes as encoding exo-rhamnogalacturonan hydrolases (RgxA, RgxB, RgxC). Biochemical identification using polygalacturonic acid and xylogalacturonan

as substrates demonstrated that indeed PgxB and PgxC are exopolygalacturonases while PgxA is a novel exo-xylogalacturonan hydrolase. PgxC appeared to be an enzyme with reduced substrate specificity that remains unaffected in its activity when galacturonic acid residues of the substrate are substituted with xylose (Martens-Uzunova et al. 2006).

Mass spectrometry was used to assign a molecular function to the exo-rhamnogalacturonan hydrolases RgxA, RgxB and RgxC. Previously a rhamnogalacturonan galacturonohydrolase activity specifically removing terminal residues from rhamnogalacturonan 1 was characterized from *Aspergillus aculeatus* (Mutter et al. 1996) but the corresponding protein sequence was not determined. In order to identify a possible

A *niger* functional homolog, the corresponding *A. aculeatus* protein was subjected to *de novo* peptide sequencing. At least six peptides perfectly matched the *A. niger* RgxC sequence and, based on the sequence similarity between RgxA, RgxB and RgxC, all three were considered to be putative exo-rhamnogalacturonan hydrolases (Martens-Uzunova et al. 2006).

3. Strain Differences

The *pelE* gene encoding a pectin lyase (Harmsen et al. 1990) previously identified from *A. niger* CBS120.49 was not discovered in the released CBS 513.88 genome.

The corresponding locus is, however, present on chromosome IV of strain ATCC 1015, in a telomeric extension that is not covered by scaffold An02 (accession NT_166519) of the CBS 513.88 genome sequence. Likewise the locus of the *abfE* gene encodes a predicted additional α -L-arabinofuranosidase present in ATCC 1015. In strain CBS 513.88, this gene could be present in an estimated 57-kb sequence gap between scaffolds An10 (accession NT_166537) and An17 (accession NT_166532). While the existence of the *abfE* gene in strain CBS 513.88 could be confirmed by sequencing of PCR products, this strategy failed for the *pelE* gene.

4. Expression and Regulation of Pectinases

Genomics and comparative genomics tools can predict the potential dynamics of the living cell, but they are restricted explicitly to the static content encoded in DNA. Transcriptomics, as a way to study the entire populations of messenger RNA in a steady state manner can reveal the functional changes that the cell undergoes in response to the changes in environment and provides an inventory of functional genes. DNA microarray analysis of *A. niger* grown on several well-defined simple and complex pectin substrates showed that at least 48 of the 60 identified loci are transcriptionally active. Microarray results also demonstrated that the newly discovered exo-acting GH28 glycoside hydrolases mentioned above play a very prominent role in pectin degradation (Martens-Uzunova et al. 2006).

Compared to bacteria, the regulation of pectin degrading enzymes in fungi is still poorly understood, and a general transcriptional regulator for expression, as was identified for e.g., expression of genes encoding fungal starch and inulin hydrolyzing enzymes, so far has not been identified.

Previous systematic analysis of the promoter region of the *A. niger* polygalacturonase II gene revealed a domain essential for high expression levels of the corresponding protein and a less defined additional upstream repressor region (Bussink et al. 1992).

From transcriptomics data there are strong indications that galacturonic acid is a main inducer for some of the pectinolytic genes in *A. niger* (Martens-Uzunova and Schaap 2008). However, subsets of pectinolytic genes display distinct expression profiles either induced by different inducers (L-arabinose, L-rhamnose or ferulic acid; De Vries et al. 2002) or are expressed constitutively in a galacturonic acid independent manner, or during later growth stages on rhamnogalacturonan (Martens-Uzunova and Schaap 2009). Taken together this complexity may explain why a pectin-specific transcriptional regulator has until now remained elusive.

Transcriptome analysis of *Aspergillus niger* transfer cultures grown on galacturonic acid media further identified a highly correlating pectinolytic core set of genes encoding pectin degrading enzymes that clustered with four strongly induced genes encoding hypothetical proteins. Functional, genetic and biochemical analysis subsequently showed that three of the encoded hypothetical proteins now designated GaaA to GaaC are intracellular enzymes directly involved in galacturonic acid catabolism (Fig. 20.3). The *A. niger* *gaaA* and *gaaC* genes share a common promoter region and this feature appears to be strictly conserved in the genomes of plant cell wall degrading fungi from

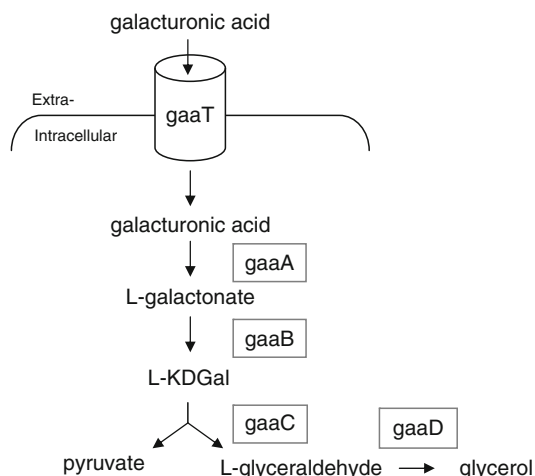


Fig. 20.3. Model of the import and subsequent utilization of galacturonic acid in *A. niger*. *gaaA* Galacturonic acid reductase, *gaaB* L-galactonate dehydratase, *gaaC* 2-keto-3-deoxy-L-galactonate aldolase, *gaaD* L-glyceraldehyde reductase

subphylum *Pezizomycotina*. Combined with the presence of homologs of the *gaabB* gene in the same set of fungi, this strongly suggests that a common D-galacturonic acid utilization pathway is operative in these species (Martens-Uzunova and Schaap 2008).

In summary the identification of the pectinolytic genes in the *A. niger* genome and the studies of their transcriptional control allowed us to propose a tentative model of the induction of the synergistic enzymatic network of this fungus that is involved in pectin degradation which is represented in Fig. 20.2. This network is comprised of more than 40 different enzymes that are induced in the presence of pectin and can degrade the different building blocks of this structurally complex polymer. A comparison of their transcriptional response on pectin vs. galacturonic acid demonstrates that galacturonic acid acts as a common inducer for only some of the activities from each of the major functional classes of pectinolytic enzymes of *A. niger*.

C. Inulin and Sucrose

1. General Remarks

Sucrose is the principal transport carbohydrate in most plants, and is also produced either as a carbohydrate reserve or in response to osmotic stress (Jouve et al. 2004). In human nutrition, sucrose (β -D-fructofuranosyl α -D-glucopyranoside) is an important sweetener and calorie source. Sucrose is also the substrate for the production of fructan polymers, both in bacteria, plants and fungi. The best known fructan polymer is inulin, which consists of β -(2,1)-linked fructose moieties with a terminal glucose residue. Inulin widely finds applications in the food industry due

to its probiotic properties and low caloric value (roughage).

2. Genomics of Enzymes Acting on Inulin and Sucrose

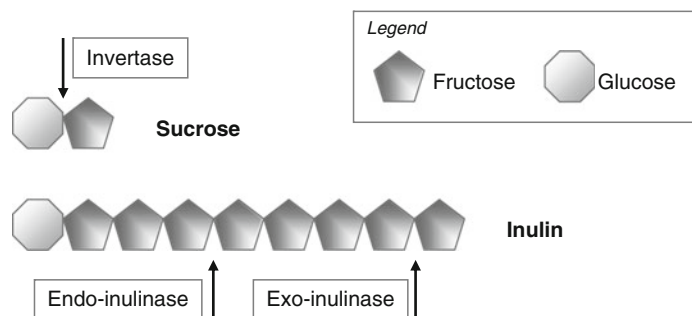
In fungi, both hydrolysis of sucrose and fructan, as well as production of fructo-oligosaccharides is performed by enzymes from the glycosyl hydrolase family 32 (GH32). Within this family, enzymes can be identified with invertase activity (hydrolysis of sucrose), endo- and exo-inulinase activity, and fructosyl transferase activity, i.e. the transfer of a fructose moiety from sucrose or a fructan to another fructan acceptor substrate (Fig. 20.4).

The enzymes in family GH32 share eight conserved domains, of which three are directly involved in catalysis. The active site is located in a five-folded β -propeller domain: the general function of the additional β -sandwich domain is still unclear. Although it is clear that invertase and inulinase enzymes in fungi are needed for the catabolism of their respective substrates, the function of fructo-oligosaccharide formation is less understood, although it has been observed in a variety of different species including *aspergilli* (Nguyen et al. 1999; Sangeetha et al. 2004).

In contrast to the situation for starch and pectin degrading proteins, mining of the *A. niger* genome did not identify any putative previously unknown, extracellular inulin degrading activities. Interestingly, in several cases the genes encoding GH32 inulinases or invertases were found in gene clusters also containing genes encoding specific regulatory proteins and sugar transporter proteins from the major facilitator superfamily (MFS).

These findings provided us with new ideas to identify pathway specific regulatory proteins, as further exempli-

Fig. 20.4. Schematic model of the enzymatic activities involved in inulin and sucrose degradation by fungi



fied in the next paragraph. In addition, genome mining for family GH32 enzymes identified two previously unknown enzymes, named SucB and SucC in *A. niger*, both lacking a N-terminal hydrophobic signal sequence for translocation into the endoplasmic reticulum and subsequent secretion (Yuan et al. 2006). Homologous enzymes, all predicted to be intracellular, could be identified in a variety of other filamentous fungi. Characterization of heterologously expressed SucB showed its invertase activity, as well as formation of small oligosaccharides from sucrose. Disruption of the gene in *A. niger* did not show pronounced effects on its growth on inulin or sucrose. The function of these intracellular GH32 enzymes, related to inulin or sucrose metabolism, is therefore still unclear.

III. Post-Genomic Approaches to Identify Transcriptional Regulators Involved in Carbon Source-Dependent Expression of Extracellular Hydrolases

A. Carbohydrate-Related Transcription Factors

Industrially important filamentous fungi such as *A. niger*, *A. oryzae*, and *Hypocrea jecorina* (*Trichoderma reesei*) have the ability to produce high amounts of enzymes to degrade the carbon sources available in their environment. The expression of extracellular hydrolases is often strongly controlled at the transcriptional level by the presence of the available carbon source. Pre-genomic approaches have identified several transcriptional regulators involved in carbon source specific gene expression. Following, we shortly discuss some of these pre-genomic approaches that have resulted in the identification of important transcription regulators and subsequently focus on post-genomic approaches to identify transcriptional regulators involved in carbon catabolism. Well studied examples of carbon source specific transcription factors in *A. niger* and *A. oryzae* are the Zn2Cys6 transcription factors AmyR and XlnR, involved in the regulation of amylolytic and xylanolytic genes, respectively.

The AmyR transcription factor was first identified in *A. oryzae* using two different genetic strategies (Petersen et al. 1999; Gomi et al. 2000). Deletion of the *amyR* gene results in strongly reduced growth on starch and maltose for *A. niger* (P.A. vanKuyk, personal communication) and reduced growth on starch for *A. oryzae* (Gomi et al. 2000).

Interestingly, deletion of the AmyR transcription factor in *A. oryzae* does not cause a growth defect on maltose, indicating alternative regulatory factors controlling the expression of α -glucosidase or maltose transporters in *A. oryzae* (Kobayashi et al. 2007).

The XlnR transcription factor has been isolated by complementing an *A. niger* mutant lacking xylanolytic activity (van Peij et al. 1998). XlnR and its function is conserved among aspergilli and also the XlnR homolog in *A. oryzae* has been identified (Marui et al. 2002). Deletion of the *xlnR* transcription factor in both *A. niger* and *A. oryzae* results in a strongly reduced expression of several hemicellulose degrading enzymes and strongly reduced growth on hemicellulose substrates. The XlnR transcription factor not only controls extracellular hemicellulose degradation, it also controls the expression of enzymes involved in intracellular D-xylose metabolism in *A. niger* (Hasper et al. 2000).

In *H. jecorina*, the XlnR homolog, *xyl1*, has also been shown to play a central role in the regulation of expression of hemicellulolytic and cellulolytic genes (Rauscher et al. 2006). Although the XlnR function as the main regulator in both *H. jecorina* as aspergilli is highly conserved, notable differences exist in the mechanisms by which the transcription factors are activated and fine-tuned (Stricker et al. 2008). Activation of the XlnR transcription factors is controlled by the same inducer molecule (D-xylose), but the induction mechanism of *H. jecorina* is more diverse as at least four inducer molecules have been described. Furthermore, additional transcription factors have been described in *H. jecorina* that are involved in the regulation. One of these factors, AceI, is a C2H2 type of transcription factor and acts as a repressor of cellulolytic and xylanolytic genes in *H. jecorina* (Aro et al. 2003). AceII encodes an additional Zn2Cys6 type of transcription factor involved in the regulation of cellulases and xylanases in *H. jecorina* (Aro et al. 2001).

Besides enzymes degrading starch, hemicellulose and cellulose, filamentous fungi have many more enzyme systems that enable them to degrade and utilize (complex) carbon sources such as pectin, arabinan, galactan, mannan, inulin etc. Extracellular enzymes involved in the degradation of these polymers have been described, but the transcriptional regulators involved in the coordinated expression are currently unknown or have recently been identified with the help of available genome sequences. Here, we further review some of the post-genomic approaches for the identification of these regulators.

B. Genome Mining for Transcription Factors

Until now, all known transcription factors directly involved in the regulation of carbon catabolism are members of the Zn(II)₂Cys₆ transcription factor family. This family of transcription factors is characterized by a highly conserved zinc-containing DNA binding domain almost exclusively located in the N-terminal part of the protein. Because of the strong conservation of the cysteine residues, as well as the spacing of the cysteines within the DNA binding domain, blast- or motif searches may be used to identify members of these families of transcription factors in the fungal genome. In *A. niger*, 296 Zn(II)₂Cys₆ domain transcription factors have been annotated (Pel et al. 2007). In *A. oryzae* and *H. jecorina* 187 and 191 Zn(II)₂Cys₆ proteins are annotated as Zn(II)₂Cys₆ transcription factors respectively (Kobayashi et al. 2007; <https://genome.jgi-psf.org/cgi-bin>).

These numbers do probably not represent all the Zn(II)₂Cys₆ transcription factors present in the genomes of these fungi. Detailed examination of Zn(II)₂Cys₆ transcription factors in the genome of *A. nidulans* showed that it is difficult to predict these factors because of the high occurrence of intron sequences within the DNA binding motif (Wortman et al. 2009). Further improvement of the gene models will lead to an increase in the number of annotated transcription factors containing these domains.

Table 20.2 summarizes the presence or absence of currently known transcriptional regulators in the carbon polymer catabolism of industrial important host fungi. From this small sample, it becomes already evident that the spectrum of transcription regulators is highly variable among different fungi. This conclusion is further substantiated when comparing the complete set of Zn(II)₂Cys₆ transcription factors (Wortman et al. 2009). In each species, a considerable num-

Table 20.2. Genome mining of currently known transcription factors involved in carbon catabolism in industrial fungal hosts. + Proteins were bi-directional best hits, indicating orthologous proteins. – No ortholog present in the genome

	AmyR	InuR	XlnR	AceII	CreA	PntR
<i>A. niger</i>	+	+	+	–	+	+
<i>A. oryzae</i>	+	+	+	–	+	+
<i>H. jecorina</i>	+	–	+	+	+	–

ber of these encoded proteins lack homologs in other, sometimes even closely related species.

C. Transcriptional Regulation of Transcription Factors on Their Substrate

Genome sequence information also allows the generation of DNA microarrays to perform genome-wide transcriptomics analysis. Affymetrix arrays have been developed for *A. niger* and access to these arrays is available via the Dutch biotech company DSM. In addition, *tri*-species arrays, containing probe sets of *A. niger*, *A. nidulans* and *A. oryzae* have become available recently (Andersen et al. 2008). *A. niger* microarray experiments have been performed to identify genes that are induced on maltose in *A. niger* (Yuan et al. 2008b). Interestingly, it was also noticed in this study that the expression of the AmyR transcription factor is induced in response to the growth on maltose compared to growth on xylose, indicating transcriptional regulation of the AmyR regulator itself.

The *amyR* promoter region contains two putative AmyR binding sites, allowing the possibility that AmyR induces its own expression. Thus, a possible way to identify transcriptional regulators responsible for the induction of genes encoding enzymes to break down the substrate, is to perform microarray analysis and subsequently analyse the expression of all putative transcription factors. It should be noted that there are also examples which show that the transcription factor is not transcriptionally induced under conditions that require its activity. For example, the XlnR transcription factor is constitutively low expressed under inducing (in the presence of D-xylose) conditions. Similarly, the expression of *xyr1* transcript in *H. jecorina* is not induced by any of its inducers (Mach-Aigner et al. 2008).

D. Clustering of Transcription Factors in the Genome with Target Genes

Some transcriptional regulators involved in carbohydrate catabolism are clustered in their genome with their target genes. This has been shown for the AmyR transcription factor both in *A. oryzae* and *A. niger*. In *S. cerevisiae* the Mal63 cluster involved in maltose utilization carries its own transcription factor (Chow et al. 1989). A similar cluster has been reported in *A. oryzae* (Hasegawa et al. 2008). The clustering

of transcription factors with putative targets genes has also been used to identify the transcription factor involved in the regulation of inulinolytic genes in *A. niger* (Yuan et al. 2008a).

Clustered with a predicted intracellular invertase (SucB) and a putative sugar transporter, a Zn(II)2Cys6 transcription factor (InuR) was identified. Deletion of the transcription factor resulted in a strain displaying a strong reduction in growth on inulin and sucrose. In addition, the expression of the inulinolytic gene and a putative transporter located in the cluster were not longer induced on sucrose in the *inuR* deletion mutant, indicating that InuR acts as a transcriptional activator of inulinolytic genes (Yuan et al. 2008a).

Thus, the availability of whole genome sequence is a useful tool to screen for potential gene clusters in order to identify transcriptional regulators involved in polysaccharide degradation.

E. Phylogenetic Analysis of Zn(II) Transcription Factors

Filamentous fungi have expanded numbers of Zn(II)2Cys6 transcription factors compared to *S. cerevisiae*. The reason for the increased number of Zn(II)2Cys6 transcription factors in their genomes compared to yeasts may reflect the more complex lifecycle of filamentous fungi and their versatility in producing secondary metabolites and hydrolytic enzymes. Zn(II)2Cys6 transcription factors are known to be involved in regulating a variety of different processes in the fungal cell, including regulation of amino acids biosynthesis pathways, secondary metabolite production, sexual reproduction and regulation of catabolic enzyme networks.

Interestingly, the transcription factors known to regulate the expression of amylolytic (AmyR) and inulinolytic (InuR) group together in a separate cluster when performing a phylogenetic analysis of full length Zn(II)2Cys6 proteins (Fig. 20.5A). The *A. niger* genome contains three AmyR-like Zn(II)2Cys6 transcription factors, including InuR. From the *A. oryzae* genome six Zn(II)2Cys6 transcription factors were identified that belong to this group of AmyR-like transcription factors. This group also includes transcription factor AO0900200035 that is highly homologous to the SugR regulator from *A. parasiticus* (Yu et al. 2000). Also included in this

phylogenetic subtree, is AO09103000131 which is suggested to encode a maltose regulator (Kobayashi et al. 2007). Searching the *H. jecorina* genome for transcription factors homologous to AmyR identified the putative AmyR homolog (55105) and four additional members, clustering in different branches of the AmyR/InuR cluster.

Several hypotheses can be deduced from this phylogenetic analysis. First, it is likely that transcription factors from different species, which show a high sequence similarity and cluster closely together in the phylogenetic tree (e.g. the AmyR subcluster), have a similar function. Conservation at the protein levels of the Zn(II)2Cys6 TF might also be coupled to conservation of the binding site of the Zn(II)2Cys6 TF. The putative binding site for the AoAmyR and AngAmyR is likely to be conserved between the species (Gomi et al. 2000; Yuan et al. 2008b). Interestingly, the predicted InuR binding in *A. niger* is also very similar to the *A. niger* AmyR binding site (Yuan et al. 2008a). Phylogenetic analyses may also reveal that some fungi lack certain transcription factors. For example, *H. jecorina* lacks the InuR homolog in its genome which fits with the absence of invertase and inulinase genes in the genome of this organism. The clustering of AmyR and InuR sequences further suggests that this whole sub-cluster of transcription factors might be involved in regulating enzyme networks related to carbon polymer catabolism. Finally, the presence of unique transcription factors within a phylogenetic subgroup might indicate unique enzyme networks that would correlate with certain growth characteristics of the fungus. A similar phylogenetic analysis was also performed for the XlnR transcription factor (Fig. 20.5B). Again, additional transcription factors were identified in the genomes of *A. niger*, *A. oryzae* and *H. jecorina* that show a high level of sequence similarity with the XlnR group. One of these factors, An04g08600, has been identified as an alternative pentose regulatory gene (PntR) (Nikolaev and Madrid 2007) and may be involved in regulation of arabinose and xylose regulated genes, confirming that phylogenetic analysis may indeed be useful to identify novel regulatory genes. *A. niger* seems to have expanded the number of XlnR related genes to three, whereas *A. oryzae* and *H. jecorina* have only one additional XlnR-like protein in their genome and are lacking a PntR homolog.

The *S. cerevisiae* Gal4 protein, involved in galactose inducible gene expression and considered to be the enigma of Zn(II)2Cys6 transcription factors, was also used for a BlastP analysis against the *A. niger*, *A. oryzae* and *H. jecorina* genomes. Again, transcription factors with a strong similarity to the ScGal4 protein were identified and used to construct a phylogenetic tree (Fig. 20.5C). For both *A. niger* and *A. oryzae*, four proteins were identified that show significant similarity to the ScGal4 protein whereas for *H. jecorina* only a single protein in this group was identified. Initial

deletion analysis of this specific gene Hj47479, has recently been reported (Guangtao et al. 2009). However, no phenotypic analysis has as yet been performed to confirm the function of this gene for galactose induced gene expression. Transcription factor An08g09370 from the GAL4 related sequence is clustered in the *A. niger* genome with putative sugar transporters and sugar/sugar alcohol dehydrogenase underscoring a possible role in carbon metabolism. Clearly, these phylogenetic analyses may help to identify interesting transcriptional regulators involved in carbon metabolism. Their particular role in this process in the various fungi will have to be examined experimentally in future research.

F. Novel Directions for Transcription Regulator Research

With the development of approaches allowing efficient targeted gene modifications using so-called ku70/ku80 disruptant strains in all three industrial important fungi (Takahashi et al. 2006; Meyer et al. 2007; Guangtao et al. 2009), approaches have become feasible to delete all genes encoding Zn(II)2Cys6 transcription factors. Such an approach was already carried out for *N. crassa* (Colot et al. 2006) and similar approaches are ongoing in *A. oryzae* (Ogawa et al. 2008). Such genome-wide deletion analyses should be combined with detailed phenotypic analysis of the obtained gene deletion mutants. Microlog GN2 panels (Biolog, Haywood, Calif., USA), a 96-well system to assay fungal growth on a variety of substrates, can be a very valuable method to assay phenotypes of deletion mutants (Singh 2009).

The genome sequences of filamentous fungi have revealed a large expansion in the number of Zn(II)2Cys6 transcription factors. Clearly, one of the future challenges will be to determine the function of these transcription factors. The approaches discussed above, such as the phylogenetic analysis of Zn(II)2Cys6 transcription factors, the inspection of neighboring proteins (which may hint to gene clusters) and the analysis of expression levels of the transcription factors under different growth conditions, are valuable approaches to discover new regulators involved in carbon metabolism. These approaches do not necessarily lead to the identification of the transcription factor regulating the expression of

enzymes required for the breakdown of a certain substrate.

Systematic gene deletion approaches in combination with detailed phenotypical analysis of the deletion mutants is certainly a very valuable starting point to further identify specific transcription factors regulating the expression of the extracellular enzymes as well as the sugar transporters that are required for the uptake of the released sugars. It is also anticipated that the efficient degradation of especially complex polymers, like those of the plant cell wall, is regulated by a complex regulatory network which is likely to include several transcription factors. It should be kept in mind that disrupting a single transcription factor might only affect the expression of a few enzymes involved in the degradation of the substrate, which may have only a limited effect on growth. An alternative approach might be to overexpress transcription factors and to perform expression analysis to identify possible target genes.

IV. Outlook

Many glycosyl hydrolase (GH) enzymes are relatively well described from a biochemical point of view, especially those enzymes with important biotechnological applications like the amylases and pectinases. However, advanced genome sequence analysis, often in combination with other techniques, is revealing enzymes with unexpected biochemical activities and physiological roles. Thus within the CarbNet project, a novel intracellular pathway for metabolizing galacturonic acid was identified using a combination of sequence analysis, transcriptomics and classical genetics. Similarly, a novel group of GH13 enzymes, previously annotated as amylases, was identified based on sequence anomalies. Targeted over-expression of two of these genes revealed unexpected glycosyl-transferase activity of the encoded proteins, probably related to cell wall maintenance.

In the course of the CarbNet investigations, many other glycosyl hydrolases have been annotated, but not yet functionally investigated. Examples are the members of the relatively small families GH62-L-arabinofuranosidase and GH74 xyloglucanases (Grishutin et al. 2004). As presented in this chapter, tools were developed to enhance genome mining and annotation, transcriptomics analysis and heterologous expression

of fungal proteins. In combination with the increasing availability of fungal genomes due to improved sequencing techniques, such tools will aid in the identification and characterization of novel glycosyl hydrolases and other enzymes with potential for application in biotechnology.

Moreover, these studies will also give a further insight in the complex regulatory mechanisms dealing with the conversion of complex carbohydrate substrates. Initial genome mining efforts, as described in the chapter, have already indicated that the regulatory repertoire governing complex substrate utilization can be expected to be much more complex than previously suggested. To study gene function in these complex processes, further improved gene modification tools and more high-throughput analysis tools, which have recently been developed, will be required.

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21 New Trends in Fungal Biooxidation

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I. Introduction

Biooxidations, i.e. the use of whole cells or isolated enzymes for the oxidative conversion of organic compounds and materials, are becoming more and more important in the industrial sector (Schmid and Urlacher 2007). Research in this direction is part of the rapidly developing field of White Biotechnology and is carried out not least against the background of sustainable development and the need to replace environmentally risky technologies by eco-friendly processes (Horvth and Anastas 2007). Whereas whole cells are already widely used as oxidative biocatalysts, such as in pharmaceutical biotransformations (e.g. steroid transformation by the ascomycetes *Curvularia lunata* or *Penicillium raistrickii*; Sedlaczek 1988; see also chapter 14), the application of

isolated enzymes is still limited to a few examples (e.g. glucose oxidase from *Aspergillus niger*; Wong et al. 2008). One reason for that is the cost-intensive production and laborious purification of enzymes which are mostly intracellular and sometimes membrane-bound proteins with low stability and complex co-factor requirements. Therefore, the use of extracellular oxidoreductases would offer several advantages: (1) they are more or less “digestion” enzymes secreted into the microenvironment of cells which facilitates their separation and subsequent purification, (2) they need merely cheap co-substrates such as dioxygen or hydrogen peroxide and (3) they are comparatively stable due to their action outside the protecting cell.

Filamentous fungi secrete a broad spectrum of biocatalysts which are involved, amongst others, in the degradation of recalcitrant biopolymers, the synthesis of melanins as well as the detoxification of plant ingredients, microbial metabolites and organopollutants (Plonka and Grabacka 2006). Since other chapters in this volume (see Chapters 15, 16, 22) deal with lignocellulose and organopollutant degradation, we focus here on novel secreted enzymes - peroxygenases and DyP-type peroxidases – with remarkable catalytic properties. Furthermore selected features of “classic” fungal oxidoreductases, such as laccase, tyrosinase and chloroperoxidase are discussed against the background of innovative recent developments in the field of enzyme application. Though most of the presented reactions still work merely at the laboratory scale, they possess a promising potential for industrial use. The scale-up of these reactions and their realization in industry will decisively depend on the enzymes’ availability. Therefore, the lowering of costs for enzyme production along with the improvement of enzyme properties by molecular tools will be key tasks of respective research in the years to come.

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II. Peroxidases

Peroxidases (EC 1.11.1.x) are secreted, microsomal or cytosolic enzymes found in all kingdoms of life, which use hydrogen peroxide (H_2O_2) or organic hydroperoxides (R-OOH) as co-substrates. Most of them are heme proteins with an exceptional broad substrate spectrum that includes various organic and inorganic compounds (Dunford 1999). Thus they catalyze, amongst others, oxidations resulting in the formation of free radicals (e.g. phenoxyl and aryl cation radicals), reactive cations (e.g. Mn^{3+}) or anions (e.g. OCl^-) which are involved in the destruction of lignin and humic substances, the oxidation of toxic compounds as well as in non-specific defense reactions (Ashby 2008; Hammel and Cullen 2008). In addition, a few peroxidases can perform two-electron oxidations along with oxygen transfer reactions and resemble in this respect monooxygenases (EC 1.13.x, EC 1.14.x; Klibanov et al. 1981; Ullrich and Hofrichter 2007). Due to their versatility and stability, peroxidases are of particular interest for industrial biooxidations (Conesa et al. 2002; Azevedo et al. 2003), which becomes not least evident by the large number of peroxidase patents having been applied for over the past years (Alvarado and Torres 2009).

A. Peroxygenases

Peroxygenase activities refer to the transfer of peroxide oxygen to substrate molecules. Though so far, no particular enzyme is classified as a peroxygenase as such in the Enzyme Nomenclature system (<http://www.chem.qmul.ac.uk/iubmb/enzyme/>), there are several natural and engineered biocatalysts which possess respective activities and are often addressed as peroxygenases (Ullrich and Hofrichter 2007). Examples are the membrane-bound caleosin proteins from plant seeds (Hanano et al. 2006; Partridge and Murphy 2009), the peroxide-dependent activities of cytosolic tyrosinase (Yamazaki et al. 2004), P450-dependent monooxygenases following the peroxide “shunt” pathway (Joo et al. 1999; Otey et al. 2006b) and the strong peroxygenase activities of secreted heme-thiolate peroxidases (Hofrichter and Ullrich 2006).

1. Chloroperoxidase

Animal and non-animal peroxidases (former plant peroxidases) are the largest superfamilies of heme peroxidases and the latter contains most of the fungal secretory peroxidases (class II), including the well investigated ligninolytic enzymes (see Chapter 15; Welinder et al. 1992; Passardi et al. 2007). However, there are at least two types of fungal heme peroxidases which do not fit into this classification system, namely dye-decolorizing peroxidases (DyPs, see Section II.B below) and chloroperoxidase (Passardi et al. 2007; Sugano et al. 2007; Pecyna et al. 2009). Chloroperoxidase (CPO; EC 1.11.1.10) reveals dramatic structural differences to “traditional” peroxidases, not least since it is a heme-thiolate protein while most other peroxidases belong to the heme-imidazole enzymes (Liu and Wang 2007). Heme-thiolate proteins bear a cysteine-ligated protoporphyrin IX as prosthetic group (axial ligand) and comprise, besides CPO, the numerous cytochrome P450 enzymes (P450s), nitric oxide synthase and a few sensing proteins (Omura 2005). While thousands of P450s from all kingdoms of life (archaea, bacteria, animals, plants, fungi) have been described (Ortiz de Montellano 2005), CPO produced by the ascomycetous sooty mold *Caldariomyces (Leptoxiphium) fumago* had been the only known heme-thiolate peroxidase for almost 50 years, until the authors of this chapter discovered related enzymes in a number of mushrooms (Hager et al. 1966; Ullrich et al. 2004; Anh et al. 2007). The main activity of CPO is the oxidation of halides (chloride, bromide, iodide; van Pee et al. 2006), but in the absence of the latter, also phenols and anilines can be converted via one-electron oxidation (Corbett and Chipko 1979; Carmichael et al. 1985). In addition, CPO is capable of oxygenating activated carbons in alicyclic and aliphatic double bonds via a peroxygenase mechanism mimicking the “shunt” pathway of P450s. The latter catalytic property and its heme-thiolate nature were the reasons for the characterization of CPO as a “heme peroxidase–cytochrome P450 functional hybrid” or a “janus enzyme” (Sundaramoorthy et al. 1995; Dunford 1999; Manoj and Hager 2008). One has to consider, however, that CPO shares no sequence homology at all with P450s, has a different molecular architecture and cannot oxygenate unactivated substrates like aromatic rings or alkanes (the typical substrates of P450s).

CPO has been thoroughly studied with respect to its application in organic synthesis and proven to be a useful and versatile biocatalyst (Burton 2003; Hofrichter and Ullrich 2006; Alvarado and Torres 2009). Thus, it catalyzes the introduction of oxo-functionalities into cycloalkene derivatives (e.g. 1,3-cyclohexadiene, 1,2-dihydronaphthalene, indene), oxidizes *p*-xylol into alcohol, aldehyde and carboxyl derivatives, styrene into styrene oxide and selectively sulfoxidizes organosulfur compounds such as thioanisole and dibenzothiophene (Geigert et al. 1986; Seelbach et al. 1997; van de Velde et al. 2001a, b; Manoj et al. 2000; Morgan et al. 2002; Sanfilippo and Nicolosi 2002; Sanfilippo

et al. 2004; Fig. 21.1). The latter reaction has gained attention by the petroleum industry since the removal of dibenzothiophene derivatives from crude oil is necessary because of environmental concerns and technical needs (Ayala et al. 2000).

A patent on the peroxidase mediated oxidative desulfurization of fossil fuels was published in 2002 and describes a two-step process, in the course of which a heme peroxidase (preferably CPO) oxidizes thiophenes and organosulfides to their corresponding sulfones. The latter can be separated in a second step, for example by distillation (Vazquez Duhalt et al. 2002). The authors reported, on the basis of gas chromatographic data, that most of the organosulfur compounds in a high-sulfur diesel oil

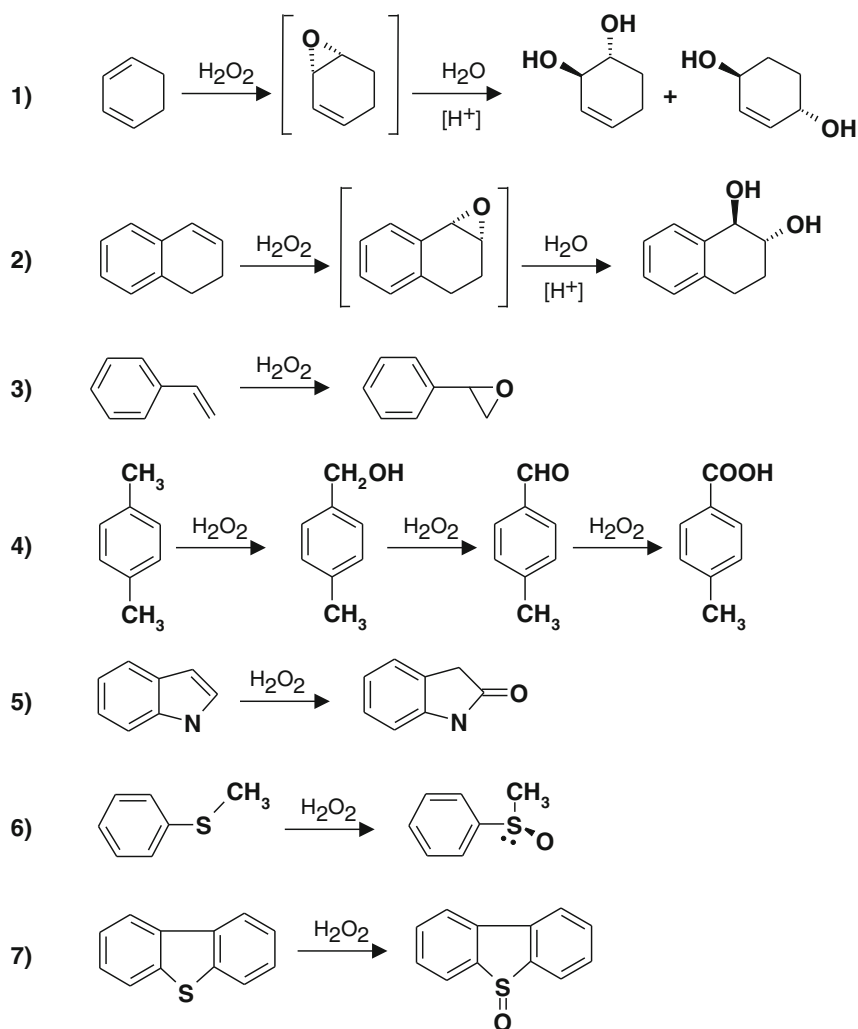


Fig. 21.1. Selected oxygen transfer reactions catalyzed by chloroperoxidase (CPO). 1 Epoxidation of 1,3-cyclohexadiene, 2 epoxidation of 1,2-dihydronaphthalene, 3 epoxidation of styrene, 4 stepwise hydroxylation of one methyl

group of *p*-xylol, 5 oxidation of indole, 6 enantioselective sulfoxidation of thioanisole into the (*R*)-sulfoxide, 7 sulfoxidation of dibenzothiophene (modified according to Hofrichter and Ullrich 2006)

(1.6% sulfur) were oxidized by CPO resulting in a considerable increase of the oil's boiling point. The biocatalytic reaction can be performed either in mixtures consisting of the fuel, an organic solvent miscible with water and a low amount of water or in a ternary micro-emulsion.

The halogenating activities of CPO – though eponymous for the enzyme – are less attractive to the organic chemist because the reactions follow a simple “swimming pool” chemistry, i.e. CPO oxidizes, for example, chloride (Cl^-) into hypochlorite (OCl^-) that in turn, acts as a diffusible halogenating species (Chen and van Pee 2008; Blasiak and Drennan 2009).

Yet, this unspecific halogenating activity has been the basis of an interesting approach that uses haloperoxidases (CPO and especially vanadium-dependent peroxidases) to disinfect surfaces on the basis of the formation of free hypochlorous acid that has a strong antimicrobial activity (Hansen et al. 2003). Vanadium-dependent haloperoxidases are widespread among marine algae (Phaeophyta, Rhodophyta) and animals but are also found in lichens (*Xanthoria parietina*) and filamentous fungi (*Curvularia inaequalis*; Littlechild 1999; Littlechild et al. 2009). The haloperoxidase of *C. inaequalis* is the biocatalyst of choice for the application in disinfectants (Hansen et al. 2003) and has successfully been engineered resulting in a 100-fold higher halogenating activity (Hasan et al. 2006). As CPO, vanadium haloperoxidases can catalyze oxygen transfer reactions, e.g. the sulfoxidation of thioanisole (Wu et al. 2008).

A problem that hampers the practical utility of CPO is its inherent activity to dismutate parts of H_2O_2 added, which leads to the formation of superoxide ($\text{O}_2^{\bullet-}$) and in turn, to heme bleaching and enzyme inactivation (van de Velde 2001a, b; Park and Clark 2006a). To overcome this shortcoming and to improve the stability of CPO, specific reaction devices and systems (e.g. using protecting antioxidants) have been developed or the enzyme has been immobilized on/in different carriers or chemically modified (Park and Clark 2006b; Liu and Wang 2007; Grey et al. 2008; de Hoog et al. 2009). Because several of these attempts have been successful, CPO may be introduced into special organic syntheses in the near future (Borole and Davison 2008; Jung et al. 2009; Zhang et al. 2009).

2. Mushroom Peroxygenases

A few years ago, a new type of heme-thiolate enzymes was discovered in the wood- and mulch-

colonizing agaric mushroom *Agrocybe aegerita*, which later turned out to be a true peroxygenase (*Agrocybe aegerita* Peroxygenase = AaP¹) transferring oxygen from peroxides to various organic substrates, including aromatic, heterocyclic and aliphatic compounds (Ullrich et al. 2004; Ullrich and Hofrichter 2005; Fig. 21.2). Later similar biocatalysts were found in other agaric basidiomycetes, e.g. *Coprinellus radians*, *Coprinopsis verticillata* (Anh et al. 2007) and *Marasmius rotula* (Gröbe et al. 2009; unpublished data). Due to their unique ability to epoxidize and hydroxylate aromatic rings by means of hydrogen peroxide – which sets mushroom peroxygenases apart from CPO – and following the designation “aromatic hydroxylase” used for some monooxygenases (Suske et al. 1997), these enzymes are also referred to as aromatic peroxygenases (APOs; Kluge et al. 2009; Pecyna et al. 2009). They have an enormous biotechnological potential, since selective oxo-functionalizations are among the most challenging and desired reactions in organic synthesis (Bernhardt 2006; Pecyna et al. 2008). Table 21.1 lists a representative selection of peroxygenase substrates and reactions, along with major and minor products formed in the course of APO catalysis.

Up to 200 mg/l peroxygenase protein can be produced by fungal wild-type strains in stirred-tank bioreactors or agitated culture flasks containing slurries of soybean meal and pepton (Ullrich et al. 2004; Gröbe et al. 2009; unpublished data). Since the enzymes are secreted by the fungus, they can be concentrated and purified by ultrafiltration of the culture liquid and subsequent fast protein liquid chromatography (FPLC) using different ion exchangers and chromatofocussing techniques (Ullrich et al. 2004, 2009). In purified form, aromatic peroxygenases (APOs) have a characteristic copper-red color (λ_{max} 415–420 nm) and are exceptionally stable; thus, they can be stored in the refrigerator for more than a year without losing any activity. Usually, APO proteins are heavily glycosylated (15–40% carbohydrates), have molecular masses between 33 and 46 kDa and they are mostly secreted in multiple forms differing in isoelectric points (pI 4.5–6.1; Ullrich et al. 2004, 2009; Anh et al. 2007). The UV-Vis spectra of resting peroxygenases are almost identical to the spectra of resting-state P450s, and the reduced CO-complex of APOs shows a characteristic shift of the Soret band towards 450 nm (Ullrich et al. 2004; Hofrichter and Ullrich 2006; Anh et al. 2007). The activity of APO is routinely measured by

¹In earlier publications AaP is also referred to as *Agrocybe aegerita* peroxidase, or haloperoxidase–peroxygase (e.g. Ullrich et al. 2004; Ullrich and Hofrichter 2005; Hofrichter and Ullrich 2006; Kluge et al. 2007)

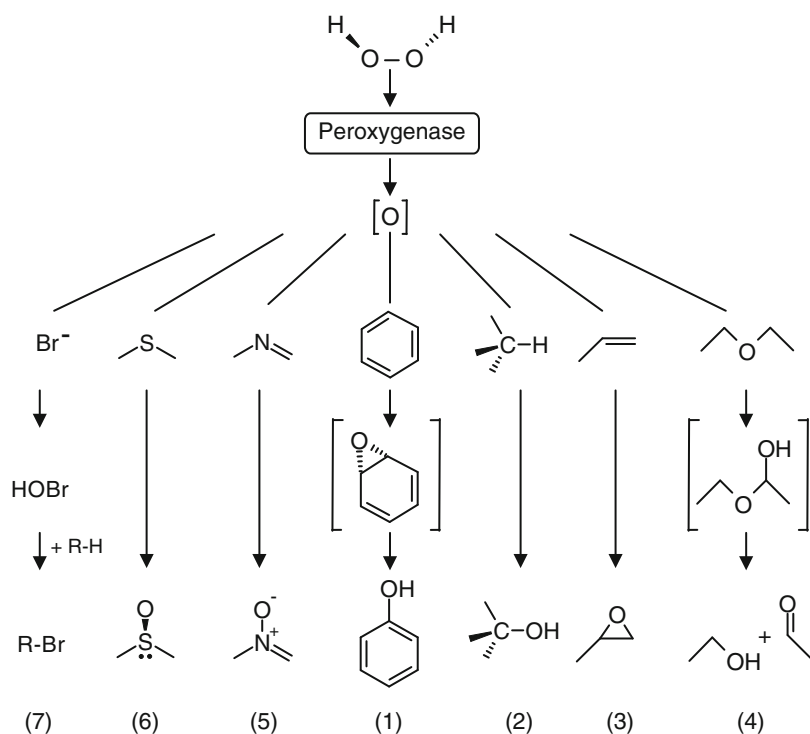


Fig. 21.2. Overview of oxygen transfer reactions catalyzed by mushroom peroxygenases. 1 Aromatic peroxyoxygenation via unstable epoxides to phenols, 2 hydroxylation of aliphatic carbon, 3 epoxidation of double bonds, 4 cleavage of ether bonds via unstable hemiacetals, 5 N-oxygenation, 6 sulfoxidation, 7 oxidation of bromide into hypobromite and subsequent bromination of organic compounds (R-H)

following the oxidation of veratryl alcohol to veratraldehyde at pH 7 (Ullrich et al. 2004). It is the same substrate used to assay lignin peroxidase (LiP; Tien and Kirk 1984), but while LiP abstracts one electron from the aromatic ring resulting in the formation of an aryl cation radical that undergoes C_α-deprotonation and a second electron abstraction to form the aldehyde (Lundell et al. 1993; Baciocchi et al. 2002), APO hydroxylates the benzylic carbon (C_α) to form a geminal alcohol (*gem*-diol) representing the hydrated form of veratraldehyde that is in equilibrium with the latter in aqueous solution (Fig. 21.3). APO can further oxidize veratraldehyde into veratric acid via an additional oxygen transfer (Fig. 21.3; Ullrich et al. 2004; Kinne et al. 2009; unpublished data). Mushroom peroxygenases oxidize also other aryl alkanols into the corresponding aldehydes, for example, benzyl, 4-methoxybenzyl and 4-hydroxy-3-methoxybenzyl alcohols into the flavoring agents benzaldehyde (almond flavor), anisaldehyde and vanillin (Ullrich et al. 2004).

Depending on the particular substrate and the reaction conditions, mushroom peroxygenases catalyze various reactions including oxygenations, one-electron abstractions and halogenations (Table 21.1, Fig. 21.2). In all cases, the APO enzyme needs merely hydrogen peroxide as co-substrate for function. Oxygen transfer reactions occur in a wide pH range (3–10) with the maximum around pH 7 for uncharged molecules. Toluene and naphthalene were the first substrates found

to undergo epoxidation and hydroxylation in the course of APO catalysis.

The toluene molecule is hydroxylated both at the aromatic ring and at the methyl group resulting in the formation of a mixture of *p*- and *o*-cresol, methylhydroquinone as well as benzyl alcohol, benzaldehyde and benzoic acid (ratio of aromatic:side chain hydroxylation = 1:2; Ullrich and Hofrichter 2005). Interestingly, the aromatic ring has been found to be no longer attacked by APO when alkyl benzenes with longer side chains are used (Kluge et al. 2009; unpublished data). Thus ethyl- and propylbenzene are exclusively hydroxylated by the peroxygenase of *A. aegerita* at the C_α-atom to form (*R*)-1-phenylethanol and (*R*)-1-phenylpropanol, respectively. The reaction is highly enantioselective with an enantiomeric excess of 95–99% for the (*R*)-isomers. Naphthalene is regioselectively epoxidized by different APO enzymes into naphthalene 1,2-oxide that spontaneously hydrolyzes into 1- and 2-naphthol (ratio 13:1; Anh et al. 2007; Kluge et al. 2007, 2009). Other polyaromatic compounds such as fluorene, anthracene, phenanthrene and pyrene as well as dibenzofuran were also found to be subject of APO-catalyzed oxygenation leading to mixtures of mono- and polyhydroxylated products (Table 21.1; Aranda et al. 2009b).

APO is also able to transfer oxygen to organic heteroatoms such as sulfur and nitrogen. For example, the heterocycle dibenzothiophene (DBT) was found to be oxidized either at the sulfur atom

Table 21.1. Selected substrates of aromatic peroxygenases (APO). In most cases, *Agrocybe aegerita* peroxygenase (AaP) was used. Modified according to Hofrichter et al. (2009)

Substrate	Major products	Minor products	Reaction type
Benzene	Hydroquinone	Phenol, <i>p</i> -benzoquinone	Ring hydroxylation (phenol oxidation)
Toluene ^a	Methylhydroquinone, benzyl alcohol	<i>o</i> -, <i>p</i> -Cresol, benzaldehyde, benzoic acid	Ring and side chain hydroxylation
Ethylbenzene ^a	(<i>R</i>)-1-Phenylethanol ^a	Acetophenone	Side chain/alkyl hydroxylation
Propylbenzene	(<i>R</i>)-1-Phenylpropanol	–	Side chain/alkyl hydroxylation
Styrene	Styrene oxide	Benzaldehyde	Side chain/alkene epoxidation
Pyridine	Pyridine <i>N</i> -oxide	–	<i>N</i> -Oxygenation
4-Chlorpyridine	4-Chlorpyridine <i>N</i> -oxide	–	<i>N</i> -Oxygenation
3-Methylpyridine	Nicotinic aldehyde	3-Methylpyridine <i>N</i> -oxide, nicotinic alcohol, nicotinic acid	<i>N</i> -Oxygenation, alkyl hydroxylation
Thioanisole	Thioanisole (<i>R</i>)-sulfoxide	Thioanisole sulfone	<i>S</i> -Oxygenation
Naphthalene ^a	Naphthalene 1,2-oxide, 1-naphthol ^a	2-Naphthol ^a , 1,4-naphthoquinone ^a	Ring epoxidation/hydroxylation
2-Methylnaphthalene	2-Naphthaldehyde	2-Naphthalene-methanol, mono- and dihydroxylated 2-methylnaphthalene	Ring and side chain hydroxylation
Fluorene	Monohydroxyfluorene derivatives, 9-hydroxyfluorene ^a	Di- and trihydroxyfluorene derivatives, 9-fluorenone ^a	Ring hydroxylation
Anthracene	Monohydroxyanthracene derivatives	Dihydroxyanthracene, anthraquinone	Ring hydroxylation
Phenanthrene	1-Hydroxyphenanthrene	2-, 3-Hydroxyphenanthrene	Ring hydroxylation
Pyrene	1-Hydroxypyrene	Dihydroxypyrene derivatives	Ring hydroxylation
Dibenzofuran (DBF)	3-Hydroxy-DBF	Dihydroxy-DBF derivatives	Ring hydroxylation
Flavone	4'-Hydroxyflavone	6-Hydroxyflavone	Ring hydroxylation
Lignin model dimer (LMD) ^b	Dealkylated LMDs (phenolic LMDs)	Fission products, keto-LMD	Ether cleavages, ring hydroxylation
Pisatin (<i>phytoalexin</i>)	6a-Hydroxymaackiain	–	Ether cleavage/demethylation
Dibenzothiophene (DBT)	Mono-, dihydroxy-DBT, DBT-sulfoxide	DBT-sulfone, tri- and dihydroxy-DBT	Ring hydroxylation, <i>S</i> -oxygenation
4-Nitrophenol ^a	4-Nitrocatechol ^a	Trihydroxy nitrobenzenes	Ring hydroxylation
<i>N</i> -Methylaniline	4-Hydroxy <i>N</i> -methylaniline	Aniline, 4-aminophenol	Ring hydroxylation, <i>N</i> -dealkylation
Acetanilide	Paracetamol	–	Ring hydroxylation
Propranolol	5-Hydroxypropranolol	(4-Hydroxypropranolol ^a , ^{only})	Ring hydroxylation
Phenoxypropionic acid (POPA)	4-OH-POPA	–	Ring hydroxylation
1,4-Dimethoxybenzene	4-Methoxyphenol	–	Ether cleavage
Methyl <i>p</i> -nitrobenzyl ether	<i>p</i> -Nitrobenzaldehyde	Methanol, formaldehyde	Ether cleavage
Tetrahydrofuran	1-Hydroxybutanal	–	Ether cleavage
Diisopropyl ether	Acetone	–	Ether cleavage
2,2'-dichlorodiethyl ether	Glyoxal	2-Chloroethanol	Ether cleavage, dechlorination
Vanillyl alcohol	Vanillin ^a	Vanillic acid ^a	Alcohol and aldehyde oxidation
Veratryl alcohol ^a	Veratraldehyde ^a	Veratric acid ^a , 4-hydroxybenzoic acid	Alcohol and aldehyde oxidation
Benzyl alcohol ^a	Benzaldehyde ^a	Benzoic acid ^a	Alcohol and aldehyde oxidation
Cyclohexane	Cyclohexanol	Cyclohexanone	Alicyclic ring hydroxylation

continued

Table 21.1. continued

Substrate	Major products	Minor products	Reaction type
<i>n</i> -Butane	Butanal	1-Butyl alcohol, butyric acid	<i>n</i> -Alkane hydroxylation
linoleic acid	(ω -2)-Hydroxylinoleic acid	(ω -1)-Hydroxylinoleic acid	Fatty acid/alkyl hydroxylation
2,2'-dichlorodiethyl ether	Glyoxal	2-Chloroethanol	Ether cleavage, dechlorination
Phenol (+ KBr) ^a	4-Bromophenol ^a , 2-bromophenol ^a	Dibromophenols	Halide oxidation, halogenation
Guaiacol ^a	Guaiacol dimer ^a	Polymerization products ^a	One electron oxidation (phenol oxidation)
ABTS ^{a,c}	ABTS cation radical ^a	Coupling products	One electron oxidation

^aSubstrates and products also oxidized and respectively formed by peroxygenases of *Coprinellus radians*, *Coprinopsis verticillata* and/or *Marasmius rotula*

^b1-(3,4-Dimethoxyphenyl)-1-oxo-2-(2-methoxyphenoxy)-1,3-dihydroxy-propane

^c2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

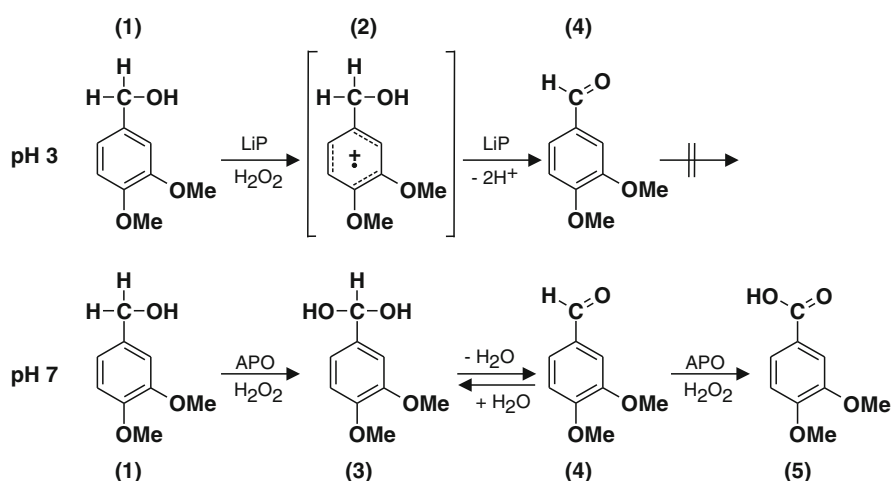


Fig. 21.3. Comparison of veratryl alcohol oxidation by lignin peroxidase (LiP; 1–3) and aromatic peroxygenase (APO; 1, 4, 5). Mechanisms are based on Lundell et al. (1993), ten Have and Franssen (2001), Ullrich et al. (2004) and Ullrich and Hofrichter (2005), as well as on unpub-

lished labeling studies with H₂¹⁸O₂ (M. Kinne, personal communication). 1 Veratryl alcohol, 2 aryl cation radical of veratryl alcohol, 3 veratraldehyde, 4 *gem*-diol of veratryl alcohol, 5 veratric acid

or at the benzene rings (Aranda et al. 2010); pyridine is exclusively attacked at the nitrogen (Ullrich et al. 2008).

Differences were observed in the product pattern between the peroxygenases of *Agrocybe aegerita* (AaP) and *Coprinellus radians* (CrP). While the former enzyme preferably hydroxylated the benzene rings of DBT, the latter preferred the heterocyclic sulfur (Aranda et al. 2010). In a similar reaction, APO enantioselectively oxidized the side chain of thioanisole into the corresponding (*R*)-sulfoxide with high efficiency (Horn 2009). Pyridine and its monohalogenated derivatives were oxidized by APO exclusively

at the heterocyclic nitrogen to form the respective pyridine *N*-oxides (Ullrich and Hofrichter 2007), while methylated pyridines were oxygenated both at the methyl group and at the ring nitrogen (Table 21.1). Three examples of pharmaceutically relevant compounds selectively hydroxylated by APO are propranolol, acetanilide and diclofenac; they were found to be converted into the effective β -blocker 5-hydroxypropranolol, the pain killers paracetamol and the human metabolite 4'-hydroxydiclofenac, respectively (Fig. 21.4, Table 21.1; Kinne et al. 2008, 2009a).

There are some APO-catalyzed reactions which proceed via unstable oxygenated intermediates and

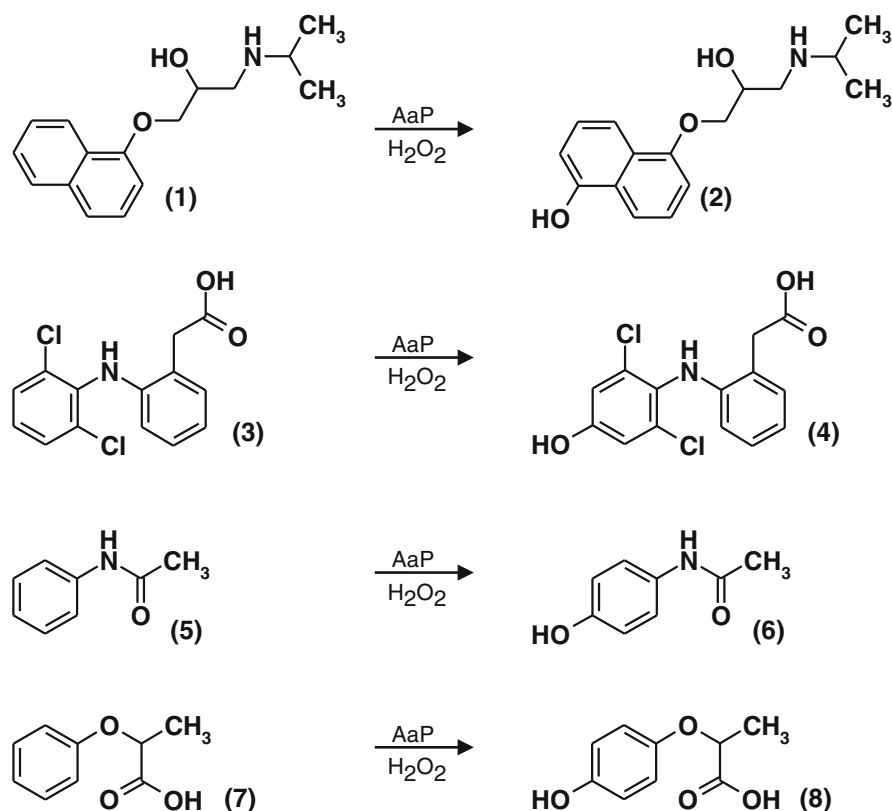


Fig. 21.4. Hydroxylation of drug and pesticide precursors by the aromatic peroxygenase of *Agroclybe aegerita* (AaP). Reactions were performed in the presence of ascorbic acid to prevent further oxidation of the phenols formed. 1 Propranolol (1-naphthalen-1-yloxy-3-[propan-2-ylamino]propan-2-ol), 2 5-hydroxypropranolol (β-blocker),

3 diclofenac (2-{2-[(2,6-dichlorophenyl)amino]phenyl}acetic acid; antirheumatic agent), 4 4'-hydroxydiclofenac (human metabolite of 3 formed in the liver), 5 acetanilide (N-phenylacetamid), 6 paracetamol (N-acetyl-4-aminophenol; painkiller), 7 2-phenoxypropionic acid, 8 (R)-2-(4-hydroxyphenoxy)propionic acid (pesticide)

lead to the cleavage of covalent bonds. An example is the scission of ether bonds that is initiated by the hydroxylation of one of the ether bond's adjacent carbon atoms. This reaction results in the formation of unstable hemiacetals that spontaneously hydrolyze into alcohols or phenols and aldehydes (Figs. 21.1, 21.5). Ether cleavage was found to take place between aromatic and aliphatic molecule parts (e.g. 1,4-dimethoxybenzene, non-phenolic β-O-4 lignin model dimers) but also in alicyclic and aliphatic ethers (e.g. tetrahydrofuran, dioxane, diisopropyl ether; Kinne et al. 2009b).

Using methyl *p*-nitrobenzyl ether and H₂¹⁸O₂ as substrates, the incorporation of labeled oxygen (¹⁸O) into an aldehyde (*p*-nitrobenzaldehyde: -CH¹⁸O) formed in the course of ether scission could be demonstrated for the first time (Kinne et al. 2009b; Fig. 21.5). Intramolecular isotope effects observed with deuterium-labeled aromatic ethers furthermore indicate an H-abstraction rebound

mechanism for oxygen insertion into ethers and aliphatic substrates (rebound mechanism). In contrast, the oxygenation of aromatic rings (e.g. naphthalene) by APO involves the oxidation of one of the π-bonds rather than direct insertion of oxygen into one of the aromatic C-H bonds (Ortiz de Montellano and de Voss 2005; Kinne et al. 2009b). APO catalyzes also one-electron oxidations of, for example, phenolic substrates. Aromatic oxygenation and phenol oxidation can consecutively occur when the former reaction introduces an OH-group into the substrate, which in turn, serves as target for the peroxidative activity. In such cases, further oxidation and polymerization of phenolic intermediates can be prevented by the radical scavenger ascorbic acid that re-reduces the phenoxy radicals formed (Aranda et al. 2010; Kinne et al. 2009a, b). One important future objective, that would improve the applicability of APOs considerably, will be the removal of their peroxidative activity by genetic engineering.

The catalytic cycle of APO combines elements of that of heme peroxidases with the peroxide “shunt” pathway of P450 monooxygenases

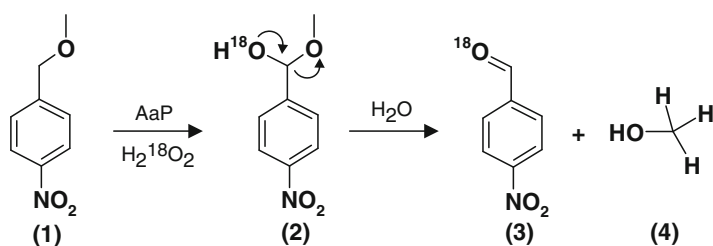


Fig. 21.5. APO-catalyzed ether cleavage and ^{18}O -incorporation into an aldehyde by the example of *p*-nitrobenzyl ether (1)²¹ fission by APO from *A. aegerita* (AaP). 2 Unstable hemiacetal formed by benzylic hydroxylation of (1). 3 *p*-Nitrobenzaldehyde ($-\text{CH}^{18}\text{O}$). 4 Methanol (according to Kinne et al. 2009b)

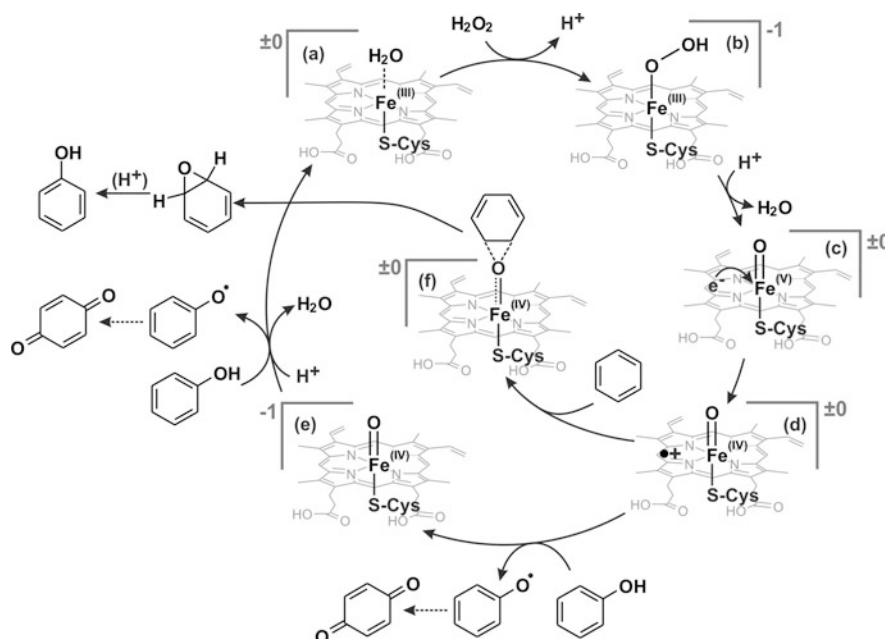


Fig. 21.6. Hypothetical catalytic cycle of aromatic peroxygenase by the example of benzene oxygenation (modified according to Ullrich and Hofrichter 2007)

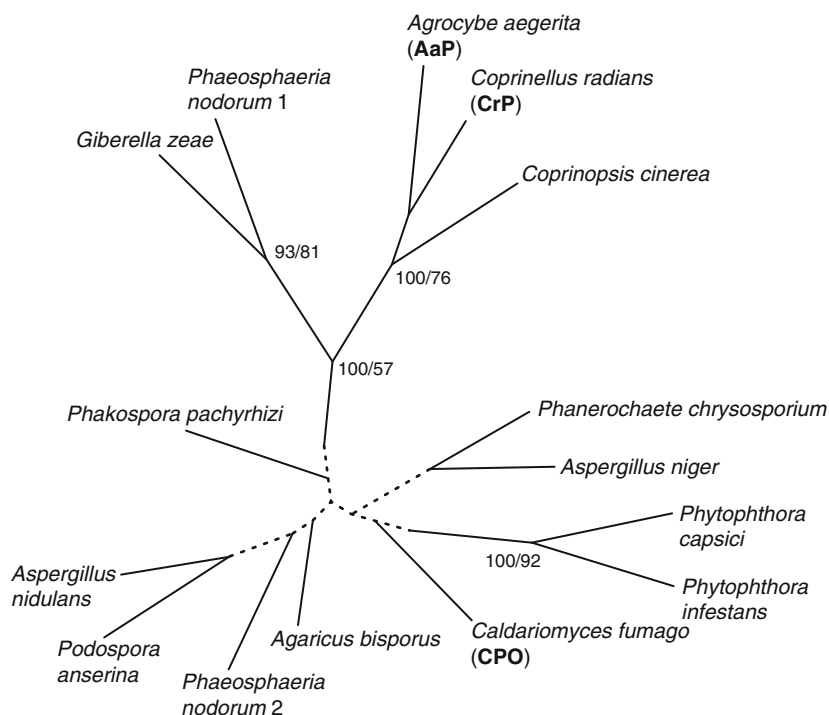
(Makris et al. 2005; Ullrich and Hofrichter 2007). Figure 21.6 proposes a catalytic cycle of APO by the example of benzene oxygenation and phenol oxidation; it is mainly based on the specific product patterns observed and on the comprehensive existing knowledge on cytochrome P450s and heme peroxidases (Dunford 1999; Ortiz de Montellano and de Voss 2005; Ullrich and Hofrichter 2007).

Briefly, the cycle may rapidly lead via a short-lived Compound 0 (hydroperoxo-ferric heme, $\text{Fe}^{3+}\text{-OOH}$) to the formation of Compound I, an oxo-ferryl porphyrin cation radical complex ($\text{Fe}^{4+}=\text{O}^{\bullet+}$). Compound I can react, depending on the substrate, in monooxygenase or peroxidase mode and can either transfer the ferryl oxygen to the substrate while receiving two electrons (e.g. benzene epoxidation) or can abstract twice – via Compound II – one electron from the substrate to form free radicals (e.g. phenoxy radical from phenol) and expel the ferryl oxygen

as water (Fig. 21.7). Which reaction takes place depends on the particular substrate, its binding site and the reaction conditions.

Up to now, two full genes of AaP (*apo1*, *apo2*) and a partial gene of CrP (*crp1*) have been sequenced. The sequences are 60–86% identical but show hardly any homology to any P450 or class II heme peroxidase. Solely, with CPO, they share some sequence identity (up to 27%) but just in the N-terminal part of the protein including the conserved heme-thiolate region; the C-terminal part of APO is totally different (Pecyna et al. 2009). Interestingly, various sequences of putative APO-like proteins have been found in data bases both on the genomic DNA and mRNA levels (similarity up to 75%), indicating a widespread occurrence of these enzymes in basidiomycetes, in ascomycetous fungi and even beyond that

Fig. 21.7. Preliminary unrooted phylogenetic tree of fungal peroxygenases, based on a multiple alignment of characterized peroxygenases of *Agrocybe aegerita* (AaP) and *Coprinellus radians* (CrP), as well as chloroperoxidase (CPO) and partial mRNA sequences of putative proteins from real fungi and fungi-like organisms found in databases (modified according to Pecyna et al. 2009)



(Fig. 21.8). Thus, mRNA sequences of two putative peroxygenases were found in oomycetes of the genus *Phytophthora*, which do not belong to the “true” fungi but represent “fungus-like” organisms closer related to the chromista (Fig. 21.7).

Crystallography and X-ray diffraction as well as heterologous expression of APOs in *Aspergillus* spp. are currently under investigation. First results in the former field confirm the structural similarity of *Agrocybe* APO to CPO around the active site (heme-thiolate region) but also reveal considerable differences in the heme channel and the amino acids there (Klaus Piontek, personal communication). The natural function of APO is still an open question. Due to their versatility, they could be involved in the non-specific oxidation and detoxification of plant ingredients (e.g. phytoalexins such as pisatin, see Table 21.1) and microbial metabolites or also in the conversion of lignin-derived compounds.

3. Artificial Peroxygenases (Peroxygenase Mimics)

Three lines are being currently followed to create artificial peroxygenases starting from cytochrome *c* (microperoxidase), soluble cytochrome P450

monooxygenases or class I/II heme peroxidases. The overall goal of this research is to develop peroxide-utilizing biocatalysts which resemble natural peroxygenases and transfer one oxygen atom from peroxide to a substrate (preferably less-activated aromatics and aliphatics).

Microperoxidase, an octapeptide with a heme prepared by partial digestion of cytochrome *c*, mostly originates from animal tissues (e.g. horse heart; Aron et al. 1986). Upon reaction with peroxides, it forms high-valency oxo-ferryl intermediates that are analogous to Compounds I and II and can take effect in peroxidase or peroxygenase modes (Veeger 2002). With respect to the latter mode, microperoxidase was shown to hydroxylate aniline, phenol and anthracene as well as to some extent naphthalene but not benzene (Dorovska-Taran et al. 1998; Marques 2007). Soluble P450s such as BM3 or P450_{cam} are stable enzymes of bacterial origin and can use H₂O₂ as co-substrate in place of NAD(P)H (Chefson and Auclair 2006; Hill et al. 2008; Rabe et al. 2008). Though this shunt pathway is just a side reaction, in the course of which the enzyme is directly converted by peroxide to form the hydroperoxo-ferryl state without the stepwise activation of dioxygen and electron requirement, it offers an opportunity to engineer self-sufficient “P450 peroxygenases” (Joo et al. 1999; Matsunaga et al. 2002). In fact, this approach led already to mutants of P450_{cam} and P450 BM3 with more than 20-fold increased peroxygenase activity towards substrates such as naphthalene, styrene or lauric acid (Joo et al. 1999; Cirino and

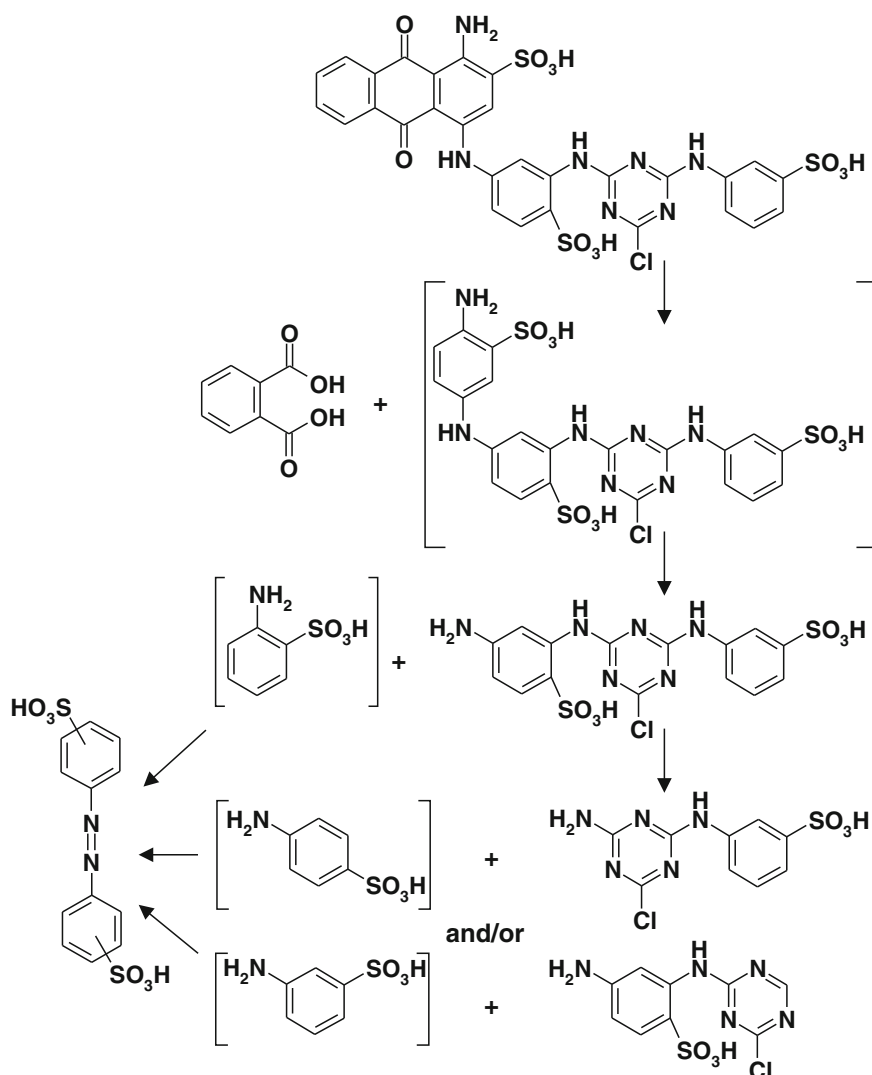


Fig. 21.8. Hypothetical mechanism of the oxidation and cleavage of Reactive Blue 5 by the dye-decolorizing peroxidase (DyP) of *Thanatopheros cucumeris* (modified according to Sugano 2009)

Arnold 2003), and an engineered P450 BM3 was successfully used to prepare pharmaceutically relevant human metabolites from the drug propranolol (Otey et al. 2006a).

A suitable fungal target protein for molecular engineering is the peroxidase of the ink cap *Coprinopsis (Coprinus) cinerea*, a typical phenol-oxidizing heme-imidazole peroxidase functionally resembling horseradish peroxidase (HRP; Veitch 2004). The enzyme (CiP = *Coprinus cinereus* peroxidase) has been thoroughly studied and already successfully engineered with respect to its stability (Cherry et al. 1999; Conesa et al. 2002; Houborg et al. 2003a, b).

The actual objective of this enzyme engineering was the development of a peroxidase for use in washing powders. To this end, CiP was heterologously expressed in *Saccharomyces cerevisiae* and subjected to directed evolution combining different approaches (site-directed mutagenesis, error-prone PCR, homologous recombination, gene and *in vivo* shuffling). In this way, CiP mutants were obtained with a more than 100-times higher thermal and peroxide stability than the starting point enzyme (Cherry et al. 1999). The resulting peroxidase was found to be stable enough to be applied in washing powder formulations and can prevent dye transfer when washing colored with white laundry.

A CiP-like peroxidase was isolated from the imperfect soil-borne fungus *Arthromyces ramosus* (Shinmen et al. 1986). This wild-type enzyme (ArP = *Athromyces ramosus* peroxidase) has been commercialized as a fine chemical

and can be purchased, e.g. from Sigma–Aldrich. Since ArP was found to be 99% identical to CiP, it was later supposed that *A. ramosus* is an invalid species that could correspond to a *Coprinus* anamorph (Kjalke et al. 1992; Sawai-Hatanaka et al. 1995; Ruiz-Duenas et al. 2001).

Two years ago, a forward-looking patent was filed describing the transformation of CiP and HRP into peroxygenase mimics, which were found to be capable of selectively converting thioanisole and 2-(methylthio)naphthalene into the corresponding enantiopure sulfoxide isomers and epoxidizing the side chain of styrene (Smith and Ngo 2007). Both reactions are usually not catalyzed by wild-type CiP or HRP but by wild-type CPO and APO (see Section II.A). Modification of catalytic properties towards oxygen transfer was achieved by mutations at three amino acid positions.

Thus, the arginine at positions 38 and 50 (numbering according to the HRP and CiP sequences, respectively) was substituted by a smaller residue (e.g. alanine), the phenylalanine at positions 41 and 53 by a smaller neutral one (e.g. glycine or alanine) and the histidine at positions 42 and 54 by another polar residue (e.g. glutamic acid). These mutations altered the heme cavity in such a way that substrate molecules could enter the active site to come into direct contact with the ferryl oxygen of Compound I, which is a necessary prerequisite for efficient oxygen transfer (Groves 2005).

Current investigations in this field aim at the further extension of the substrate spectrum of CiP and HRP by creating new mutants which also peroxygenate less-activated substrates such as aromatic rings or alkyl substituents (Andrew Smith, personal communication). Together with chloroperoxidase and aromatic peroxygenases,

such engineered peroxidases could become a “toolbox” of robust biocatalysts for industrial oxy-functionalizations requiring merely cheap peroxide for their function.

B. Dye-Decolorizing Peroxidases

A second out-group of peroxidases identified in fungi (and later also bacteria) is known as dye-decolorizing peroxidases or, in short, DyP-type peroxidases (Kim and Shoda 1999; Zubieta et al. 2007a, b). They bleach different dyes (e.g. anthraquinone derivatives), cleave β -carotene (Fig. 21.9) and also oxidize typical peroxidase substrates such as ABTS or phenolics.

First indications for the existence of this new type of peroxidase were reported in 1995 within a screening for microorganisms decolorizing xenobiotic dyes (Kim et al. 1995). One fungal isolate – formerly named *Geotrichum candidum* Dec 1 and later assigned to the phytopathogenic basidiomycete *Thanatephorus cucumeris* (Sato et al. 2004) – was able to decolorize 18 types of reactive, acidic and dispersive dyes. A concentrated crude preparation of the fungus' culture liquid showed a 100-fold higher decolorizing activity in the presence of H_2O_2 than the original culture broth. In 1999, the responsible enzymes was purified, characterized and named DyP. It was found to be a glycosylated heme protein (17% sugars) with a Soret band at 406 nm, a molecular mass of 60 kDa and a pI of 3.8. Later it was shown that *T. cucumeris* produces several DyP isozymes, which are difficult to separate from each other (Kim and Shoda 1999). One *dyp* gene was cloned and the primary sequence was analyzed and heterologously expressed in *Aspergillus oryzae* and *Escherichia coli* (Sugano et al. 1999, 2000, 2007). Independent of these investigations, DyP-type enzymes have also been found and characterized in other fungi and bacteria over the past years.

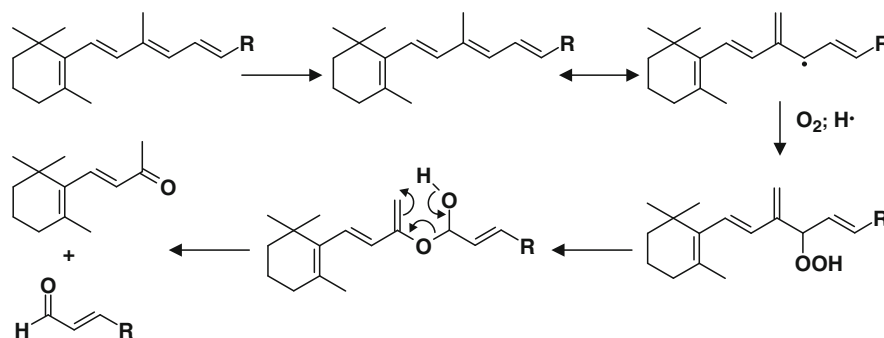


Fig. 21.9. Proposed mechanism for the oxidative cleavage of β,β -carotene into the fragrance compounds β -ionone and β -apo-10'-carotenal by DyP-type peroxidases (according to Zorn et al. 2003; Scheibner et al. 2008)

Structural comparison and sequence alignments of DyP-type peroxidases with representative members of all classes of the plant, bacterial and fungal peroxidases demonstrated that DyP-type peroxidases cannot be classified within either of these families (Sugano et al. 2007; Zubieta et al. 2007a; Scheibner et al. 2008). They show only little sequence similarity (0.5–5.0%) to classic fungal peroxidases such as lignin and manganese peroxidases or *Coprinopsis cinerea* peroxidase (CiP), and they lack the typical heme-binding region which is conserved in the whole plant peroxidase superfamily (one proximal histidine, one distal histidine, one essential arginine; Sugano et al. 1999; Faraco et al. 2007; Sugano 2009).

Though a number of peptide sequences of this new enzyme group are deposited in protein databases, only the data for seven characterized proteins and their respective genes (including DyP from *T. cucumeris*) are available: namely from the basidiomycetes *Termitomyces albuminosus* (TAP) and *Marasmius scorodoni*, (two enzymes: MsP1, MsP2) and the bacterial species *Bacteroides thetaiotaomicron* (BtDyP) and *Shewanella oneidensis* (TyrA; Kim and Shoda 1999; Sugano et al. 1999; Zubieta et al. 2007a, b; Scheibner et al. 2008). Furthermore, there is a recent report on DyP-like proteins in the jelly fungus *Auricularia auricularia-judae* (Liers et al. 2009). The gene of a DyP-type encoding sequence and the corresponding cDNA were identified on the basis of sequence homology analyses in *Pleurotus ostreatus* (Faraco et al. 2007); and a PSI-BLAST search revealed more than 400 proteins sharing homology with DyP (Sugano 2009).

A characteristic feature of all DyP-type peroxidases studied so far is their ability to oxidize synthetic high-redox potential dyes of the anthraquinone type which can hardly be oxidized by other peroxidases (Kim and Shoda 1999). This catalytic feature makes DyP-type peroxidases interesting for applications in the waste treatment sector (Syoda et al. 2006). Though the catalytic cycle of DyP and the cleavage of dyes are not fully understood yet, there are indications for a unique mechanism that includes, besides typical peroxidative reactions, the hydrolytic fission of the anthraquinone backbone (Sugano 2009; Sugano et al. 2009). The latter assumption is supported by the identification of phthalic acid as cleavage product of the recalcitrant dye Reactive blue 5 (Fig. 21.8). The high stability of DyPs is another remarkable property of these enzymes.

Pühse et al. (2009) reported that purified dimeric MsP1 (DyP-type peroxidase of *Marasmius scorodoni*) were exceptionally stable under both high pressure (up to 2500 bar; 250 MPa) and elevated temperatures (up to 70 °C). Thus remarkably, the activity of MsP1 towards ABTS and β -carotene (a substrate that is oxidized by MsP with high efficiency; Fig. 21.9) increased by a factor of two when the pressure was increased to 500 bar. As indicated by fluorescence and small-angle X-ray scattering (SAXS) data, the increased activity may result from slight structural changes stabilizing the transition state of the enzymatic reaction. MsP1 can be produced in high yields as recombinant protein in *E. coli* or *Aspergillus* spp. (Zelena et al. 2009b; Holger Zorn, personal communication) and there are recent patents and papers describing its usefulness in the food sector (e.g. bleaching of whey and other food products, preparation of isoprenoid flavors; Zorn et al. 2005; Fleischmann and Zorn 2008; Zelena et al. 2009a).

Owing to this marked pressure stability, DyP-type peroxidases may become valuable tools for industrial high-pressure applications (Pühse et al. 2009). In this context, the ability of two acid-stable DyP-like peroxidases from *Auricularia auricularia-judae* to cleave non-phenolic lignin model compounds (indicating a ligninolytic activity) may be of particular interest and sheds new light on the possible physiological function of these enzymes (Liers et al. 2009). The pre-treatment of natural lignocelluloses with MsP1 has already been shown to facilitate their subsequent saccharification by cellulases (Zorn et al. 2008).

III. Oxidases

Phenol oxidases (EC 1.10.3) are widespread biocatalysts which simply need dioxygen (O_2) as co-factor (terminal electron acceptor). Therefore and because of their high stability (most of them are extracellular or cell wall-associated proteins), they have become preferred subjects of enzyme-technological investigations. Two important representatives, laccase and tyrosinase, contain copper in their active sites and are produced by numerous fungi. A variety of potential applications of both enzymes have been reported in the literature (for the latest reviews, see Parvez et al. 2007; Mikolasch and Schauer 2009; Mikolasch et al. 2008). Here we focus on a few recent approaches to make use of them, namely in enzyme-based biofuel cells, organic synthesis and polymer modification.

A. Laccase

1. General Aspects

Together with ascorbate oxidase, bilirubin oxidase and ceruloplasmin, laccase belongs to the blue copper oxidases. It is a polyphenol oxidase (EC 1.10.3.2, *para*-benzenediol:dioxygen oxidoreductase) that contains four copper ions in the active site. Although the name laccase originated from the lacquer produced by the tree *Rhus verniciflua*, it is widespread in different branches of life such as fungi, bacteria and insects. The respective enzymes of these rather distinct organisms can considerably differ in their molecular structure but they are all referred to as laccases as long as they have a strong oxidase activity toward phenolic compounds (Nakamura and Go 2005). Numerous fungal species belonging to the basidiomycota and ascomycota have been found to produce laccase but seemingly for quite different purposes (lignin formation and degradation, melanin and dye synthesis, oxidative detoxification of phenolic plant ingredients). Laccases catalyze one-electron oxidations of various substrates preferably of phenolics and aromatic amines while transferring the abstracted electrons to dioxygen, which is thereby reduced to water (Sakurai and Kataoka 2007).

The particular reactions catalyzed depend – at least in part – on the redox potential of a laccase, and nowadays low-, medium- and high-redox potential laccases are distinguished with redox potentials of <500 mV, 500–700 mV and up to 790 mV, respectively (Morozova et al. 2007). The former are found in plants and bacteria, whereas medium- and high-redox potential laccases are exclusively produced by filamentous fungi (750–790 mV, the highest potentials, have been reported for the laccases of white-rot basidiomycetes such as *Trametes trogii*, *T. ochracea*, *T. villosa*, *Cerrena maxima*, *Pycnoporus cinnabarinus* and *P. sanguineus*; see also Chapters 13, 15).

The use of dioxygen (O₂) as a cheap and ubiquitously present co-factor has made laccases a preferred subject of enzyme technology-oriented research; comprehensive review articles dealing with different aspects of laccase function and application have been published over the past five years and emphasize their catalytic versatility and physical robustness (Mayer and Staples 2002; Baldrian 2006; Couto and Toca-Herrera 2007; Hilden et al. 2009). Lignin and lignocellulose modification and pulp bleaching was reviewed by Bajpai et al. (2006), waste water treatment and

organopollutant oxidation by Wesenberg et al. (2003) and Raghukumar et al. (2008); and a comprehensive patent survey was recently published by Kunamneni et al. (2008).

There are two already commercialized applications of laccase: the bleaching of denim to achieve the so-called “stone-washed effect” and the treatment of cork stoppers to remove the aromatic compounds which produce an unpleasant flavor in wine.

The respective products Denilite II and Suberase are distributed by Novozymes A/S and contain a high-redox potential laccase of the polyporous white-rot fungus *T. villosa* that was genetically engineered and optimized (regarding temperature and pH stability) and is heterologously produced in *Aspergillus oryzae* (Xu et al. 2007; see also Chapter 13). A second fungal laccase product for denim finishing, ECOSTONE LCC 10, is purchased by AB Enzymes (Darmstadt, Germany) and uses a genetically modified laccase from an ascomycete of the genus *Thielavia* (Paloheimo et al. 2006).

2. Biofuel Cells Using Laccase

Innovative fields of laccase applications include chemical synthesis, biofuel cells and biosensors. Biofuel cells based on purified enzymes or whole microbial cells are interesting future alternative sources of sustainable electrical energy (Davis and Higson 2007). They include two bioelectrocatalytic electrodes: one for the oxidation of organic compounds (e.g. sugars) – the actual fuel – at the anode and another for the reduction of dioxygen at the cathode. The latter can be achieved by laccase and there is a number of examples where different sugar dehydrogenases and oxidases (e.g. glucose, fructose or cellobiose dehydrogenase, glucose oxidase) have successfully been coupled with fungal laccase (mostly high-redox potential laccases from *Trametes* spp.; Kamitaka et al. 2007; Moehlenbrock and Minteer 2008; Amir et al. 2009).

There are two basic concepts of enzyme-based biofuel cells using either mediated or direct electron transfer between the enzymes and the electrodes. In the former case, low-molecular-weight redox-active compounds such as ABTS, synthetic dyes or ferrocenes are incorporated to mediate this transfer from the cathode to laccase (Palmore and Kim 1999; Chaubey and Malhotra 2002). The direct electron transfer reflects the latest development in this field, using enzymes immobilized on carbon nanotubes, Nafion

polymers, redox hydrogels or chitosan (Moehlenbrock and Minter 2008). In an interesting recent approach, laccase from *T. hirsuta* and cellobiose dehydrogenase from the ascomycete *Dichomera saubinetii* were adsorbed onto the surface of spectrographic graphite rods (Coman et al. 2008). Using these bio-electrodes, a membrane-, mediator- and cofactor-less glucose/oxygen biofuel cell was fabricated that produced an electrical current with a maximum power density of 5 W cm^{-2} (at 0.5 V cell voltage) in the presence of glucose and had an estimated half-life span of more than 38 h.

The principal scheme of a biofuel cell is shown in Fig. 21.10. Despite all scientific progress in this field, the incorporation of enzyme-based cells into common power-supply devices is still far from being implemented. Enzyme lifetimes, alternative fuel sources and power density issues still need to be addressed before they can be applied for commercial use. Respective concepts are being developed, but further testing and actual applications of biofuel methods are needed before coming to market (Pas 2007).

3. Laccase in Organic Synthesis and Polymer Chemistry

As a tool in organic synthesis, fungal laccases of different origin (*Pycnoporus cinnabarinus*, *Trametes versicolor*, *T. villosa*, *Myceliophthora thermophila*) have been shown to be powerful biocatalysts for coupling two different molecules to create new products in high yield (Mayer and Staples 2002; Mikolasch and Schauer 2009). In most cases, phenolic hydroxyl groups are the starting point of laccase attack, leading to reactive phenoxy radicals which can disproportionate to quinones, couple with itself to homodimers or with other molecules to form heterodimers. The latter comprise also non-laccase substrates (e.g. alcohols, amines, sulfinates) which are reaction partners as long as they are oxidizable by the phenoxy radicals formed first (Manda et al. 2006; Mikolasch and Schauer 2009). After longer reaction times, oligomers and finally humic substance-like polymers can be formed (Bollag 1983; Baldrian 2006). The coupling products

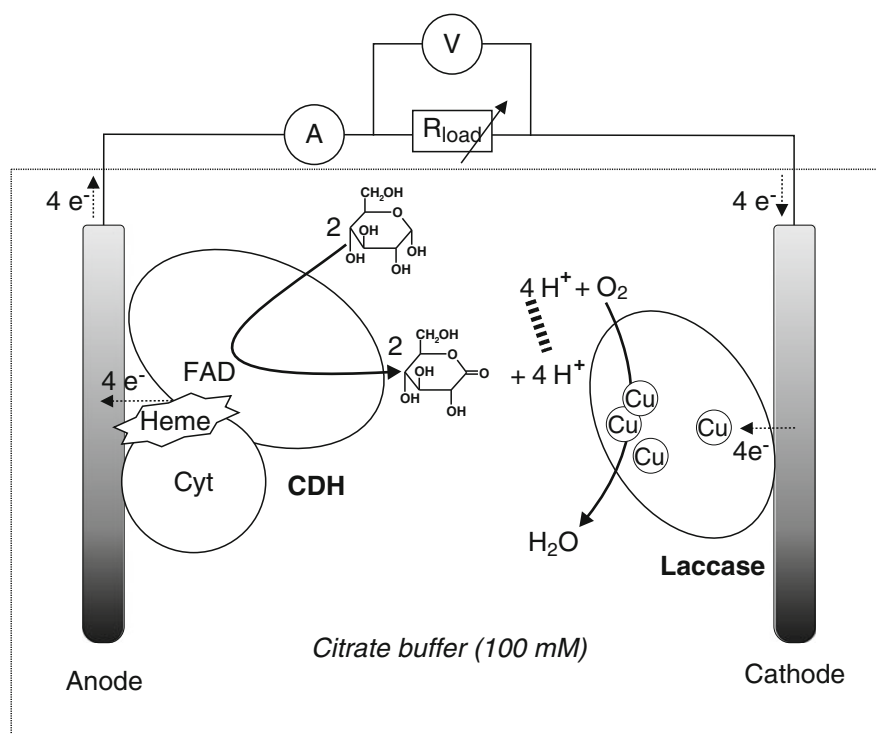


Fig. 21.10. Scheme of a biofuel cell with direct electron transfer. Fungal laccase is immobilized on the cathode surface, cellobiose dehydrogenase (CDH) on the anode (modified after Coman et al. 2008). CDH comprises a FAD and a cytochrome (Cyt) domain and oxidizes glucose

into gluconolactone. The electrons released are directly transferred via the heme of Cyt to the anode. Incoming electrons are transferred from the cathode via the copper centers of laccase to dioxygen that is reduced to water

formed contain new C–O, C–C, C–N and C–S bonds and among them are pharmaceutically interesting molecules (Mikolasch and Schauer 2009) (Fig. 21.11).

Thus, 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide and related compounds can be coupled with cephalosporin or penicillin derivatives followed by nuclear amination to form new bioactive hybrid molecules (Mikolasch et al. 2006, 2007). These C–N-coupled aminoquinonoid heterodimers consist of one molecule of the oxidized laccase substrate and one molecule of the β -lactam reaction partner and were found to inhibit the growth of several gram-positive bacterial strains, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant Enterococci. Other relevant derivatives of antibiotics are coupling products of the anti-neoplastic agents mitramycin and hydroquinone (Anyanwutaku et al. 1994), corollosporine (an antibiotic from the marine fungus *Corollospora maritima* that is active against *S. aureus* and *Bacillus subtilis*) and 2,5-dihydroxybenzoic acid derivatives (Mikolasch et al. 2008).

Laccase can be also used to derivatize amino acids such as glycine, tryptophan, cystein, lysin or phenylalanine in the presence of appropriate diphenols, mostly via C–N-coupling at the C_{α} -

carbon of the amino acid (Hahn et al. 2009). The resulting heterodimers, for example, formed by coupling of unprotected tryptophan or phenylalanine and hydroquinone derivatives, are interesting building blocks in drug development (Manda et al. 2006; Hahn et al. 2009).

Phenoxazinone derivatives, such as cinnabarinic acid or actinomycin, represent another interesting group of coupling products. The former compound is naturally found in fruiting bodies of the white-rot fungus *Pycnoporus cinnabarinus* and is responsible for its characteristic orange-red color (Cavill et al. 1953; Dias and Urban 2009). It acts as a strong antioxidant and was suggested to prevent oxidative damage in mammalian tissues (Christen et al. 1990).

Cinnabarinic acid can be synthesized from 3-hydroxyanthranilic acid using high-redox potential laccases from *P. cinnabarinus* or *Trametes versicolor* (Eggert et al. 1995; Osiadacz et al. 1999). Recently, novel phenoxazinone derivatives with improved water solubility have been prepared using sulfonated precursors (e.g. 2-amino-3-hydroxybenzenesulfonic acid) and *T. versicolor* laccase (Bruyneel et al. 2008, 2009; Fig. 21.11). In all these reactions, laccase

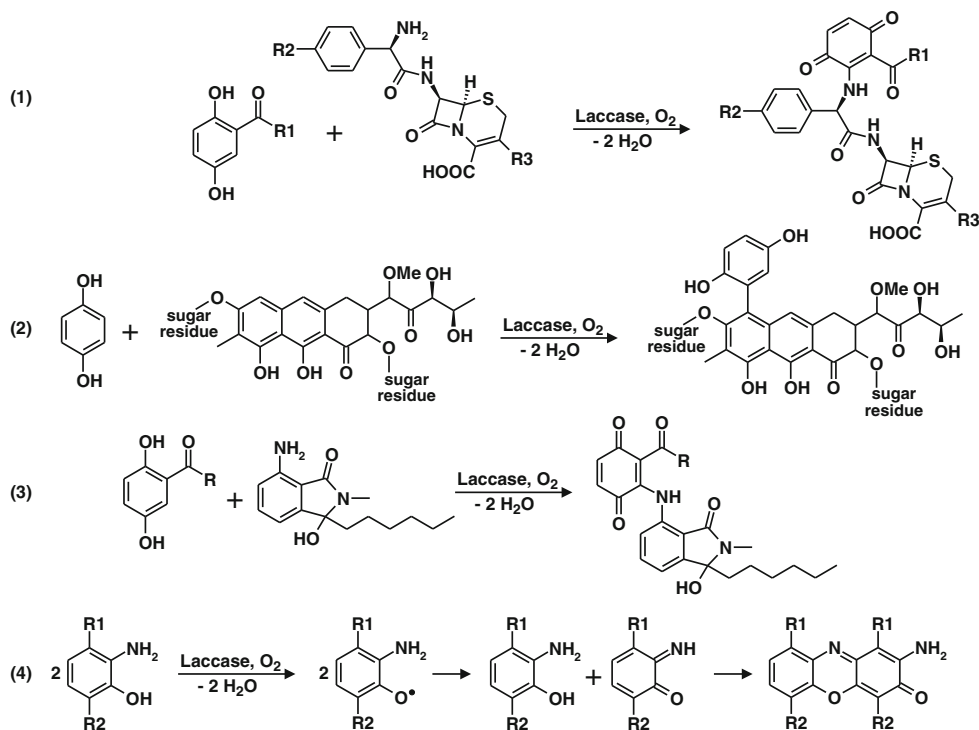


Fig. 21.11. Examples of laccase catalyzed coupling reactions leading to bioactive coupling products: 1 heterodimers of 2,5-dihydroxybenzoic acid derivatives and aminocephalosporins, 2 hydroquinone and mitramycin

as well as 3 2,5-dihydroxybenzoic acid derivatives and corollosporins; 4 homomolecular phenoxazinone derivatives formed by the coupling of aminophenolics (modified according to Mikolasch and Schauer 2009)

oxidizes the phenolic hydroxyl group of the substrate molecules into phenoxyl radicals, which disproportionate under the formation of reactive *o*-quinonimin compounds. The latter spontaneously react with the original substrate to form the final phenoxazone structure (Fig. 21.11). The main difference between the formation of phenoxazones and the reactions described before is that here two bonds, a C=N and a C-O, are formed in a one-step reaction involving two different molecules (Mikolasch and Schauer 2009).

The use of laccases (and also peroxidases) in polymer chemistry is a relative new field in enzyme technology but has already led to convincing findings. Excellent reviews dealing with different aspects of laccase-catalyzed polymerization of phenolic precursors and the modification and functionalization of polymers were published by Hüttermann et al. (2001), Widsten and Kandelbauer (2008) and Mikolasch and Schauer (2009). Respective research particularly focuses on the treatment of lignocellulosic materials or their individual components for the production of new compounded materials, fiberboards and adhesives in the absence of toxic chemicals like formaldehyde (Hüttermann et al. 1989; Jin et al. 1991; Widsten et al. 2004; Kudanga et al. 2008), as well as on the laccase-catalyzed polymerization of phenolic precursors into functional polymers (Mita et al. 2003; Uyama and Kobayashi 2003). The polymerized phenolics obtained in mixtures of aqueous-organic solvents have complex structures comprising phenylene and oxo-phenylene units linked via C-C and C-O bonds. The polymerization process can be controlled by the organic solvent and its concentration and the selection of appropriate phenolic precursors (e.g. hydroxybenzoic and syringic acid derivatives).

An interesting example is the laccase-based preparation of Oriental lacquer (urushi; Kobayashi et al. 2001). Urushi is a traditional biomaterial used in Japanese and Chinese arts and crafts, and it originates from the resinous sap (urushiol) of the Lacquer tree (*Toxicodendron vernicifluum*, formerly *Rhus verniciflua*, for which the first laccase was described; Yoshida 1883; Thurston 1994; Harigaya et al. 2007). The curing of urushiol-like precursors (mixtures of lipase-esterified phenols with primary alcohol side chain and unsaturated fatty acids) with laccase from *Pycnoporus coccineus* yielded an artificial urushi with a high hardness and a shining surface comparable to that of natural urushi coatings (Ikeda et al. 2001). A second example of an innovative polymeric product based on laccase is a wood adhesive obtained through a three-component system consisting of chitosan, phenolic compounds and laccase (Peshkova and Li 2003). The adhesion

mechanisms of this system were proposed to be similar to those of the highly effective adhesive proteins of mussels, which can form strong bonds on wet surfaces in a wide range of salinities and temperatures (Waite 1990; Silverman and Roberto 2007).

Laccase has also attracted the attention of food chemists and has been shown to intermolecularly cross-link whey and milk proteins (e.g. β -lactoglobulin, α -casein) directly or in suitable combination with ferulic acid and a *trans*-glutaminase (Faergemand et al. 1998; Steffensen et al. 2008). A comprehensive patent on the use of laccase in bakeries to improve the structure of dough and/or bread was filed by Novozymes A/S in 1993 and has been several times extended and up-dated (Si 2001).

B. Tyrosinase

Browning caused by tyrosinase was one of the first oxidative enzyme activities observed and already described more than 100 years ago, interestingly for fruiting bodies of the mushroom *Russula nigricans* (Bourquelot and Bertrand 1895). Though tyrosinase is also a copper-containing polyphenol oxidase, its active site is totally different from that of laccase and contains a di-nuclear copper center (Schoot-Uiterkamp and Mason 1973). Fungal tyrosinases are mostly cytosolic or cell wall-associated enzymes (van Gelder et al. 1997) and in dependence of the oxidation state of copper and the linking with dioxygen, their active site can exist in three different states: *deoxy*-, *oxy*- and *met*-tyrosinase (Halaouli et al. 2006). Most studies on fungal tyrosinase have been performed with enzyme preparations of the common white button mushroom (*Agaricus bisporus*), which can be purchased as a fine chemical (e.g. from Sigma-Aldrich).

Tyrosinase preferably oxidizes *ortho*-substituted diphenols (catecholase activity), and it has a second activity that hydroxylates *para*-substituted phenols (cresolase activity). Due to these two functions, tyrosinase was grouped into two enzyme subclasses: EC 1.10.3.1 and EC 1.14.18.1, i.e. it acts both as an oxidase and a monooxygenase (<http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/14/18/1.html>). In this way, the latter activity can form the substrate for the former one, which leads to a complex catalytic cycle (Cabanes et al. 2002). Two comprehensive reviews on fungal tyrosinases were published by Seo et al. (2003) and Halaouli et al. (2006). Among others, tyrosinases are involved in the

synthesis of melanins stabilizing the fungal cell wall. They are responsible for the first O_2 -consuming steps of the pathway: the hydroxylation of substituted phenols (e.g. L-tyrosine) to *o*-diphenols and their subsequent oxidation to *o*-diquinones (e.g. L-dopaquinone and L-dopapachrome); afterwards *o*-quinones polymerize non-enzymatically to the high-molecular-mass melanins (Henson et al. 1999). Besides tyrosine, fungi use also catechol and γ -glutaminy-3,4-dihydroxy benzene (basidiomycetes) as well as 1,8-dihydroxynaphthalene (ascomycetes) as precursors for melanin genesis (Halaouli et al. 2006). Both tyrosinases and laccases were proposed to be involved in the polymerization of these starting materials (Gómez and Nosanchuk 2003; Eisenman et al. 2009).

For almost two decades, studies on fungal tyrosinases have been motivated by the enzymatic browning phenomenon during the development and postharvest storage of fruits and vegetables

(Jolivet et al. 1998; Halaouli et al. 2006). A second important field of tyrosinase research concerns melanin synthesis in human skin and related aspects. Melanin plays a crucial protective role against photocarcinogenesis, but the formation of “abnormal” melanin pigmentation is a serious esthetic problem and requires cosmetic treatments (Priestley 1993; Kim and Uyama 2005). For all these reasons, comprehensive studies have been performed to find efficient natural and synthetic inhibitors of tyrosinases (reviewed by Parvez et al. 2007).

Further, there have been several attempts to make direct use of tyrosinase-catalyzed reactions in enzyme-technological applications (Fig. 21.12). An example is the production of antioxidant *o*-diphenols with beneficial properties as food

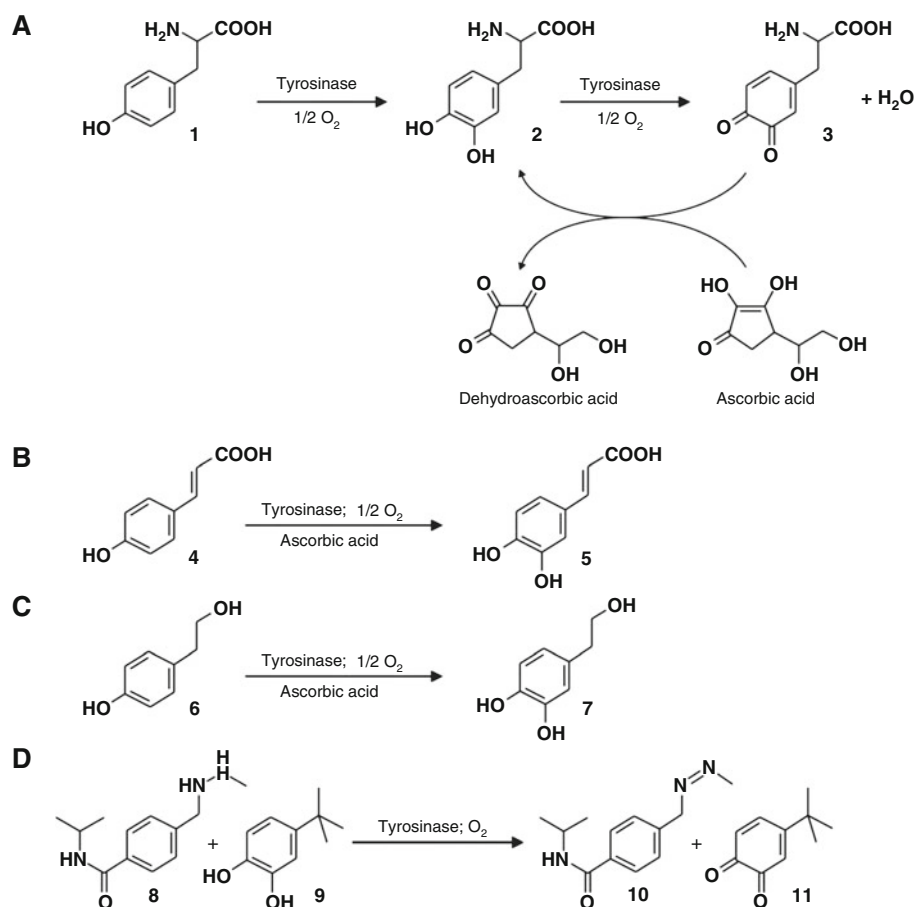


Fig. 21.12. Biotechnologically relevant hydroxylations catalyzed by fungal tyrosinase. **A** Formation of L-DOPA (2) from tyrosine (1); accumulation of *o*-dopaquinone (3) is prevented by ascorbic acid that re-reduces the respective semiquinone intermediates. **B** Hydroxylation of *p*-coumaric acid to caffeic acid. **C** Formation of the antioxidant

hydroxytyrosol (7) from tyrosol (6). **D** Indirect oxidation of procarbazine (8) in the presence of 4-*tert*-butylcatechol (9) into the pharmaceutically active metabolite azoprocarbazine (10) and 4-*tert*-butyl-*o*-benzoquinone (11). Modified according to Espin et al. (2001), Halaouli et al. (2006), Gasowska-Bajger and Wojtasek (2008)

additives or pharmaceutical drugs (Halaouli et al. 2006). Thus, the production of caffeic acid was already described in 1969 using a reaction mixture that contained *Agaricus bisporus* tyrosinase and *p*-coumaric acid as the substrate as well as ascorbic acid to prevent quinone formation (Sato 1969). Hydroxytyrosol is another strong antioxidant, naturally occurring in plant oils and fruits, which can be prepared by means of tyrosinase (Espin et al. 2001). Purified tyrosinases of *A. bisporus* and the tropical white-rot fungus *Pycnoporus sanguineus* were shown to be effective in the enzymatic synthesis of this compound from *p*-tyrosol, a monophenol found in agro-industrial byproducts from olive oil production (Espin et al. 2001, 2003; Halaouli et al. 2005). This type of enzymatic conversion may be an alternative to classic chemical synthesis when environmentally friendly, natural agents are required (e.g. as food additives), and it was also successfully tested for L-DOPA production using tyrosinases of *Aspergillus* spp. (Singh 1999; Haq et al. 2002; Fig. 21.12).

The indirect oxidation of the antitumor agent procarbazine with possible implication for the design of anti-melanoma products has recently reported (Gasowska-Bajger and Wojtasek 2008). In the presence of the phenolic substrates 4-*tert*-butylcatechol or *N*-acetyl-L-tyrosine, the dialkylhydrazine was oxidized into azoprocarbazine that is the first biologically active metabolite of this drug detectable during in vivo and in vitro studies (Fig. 21.12D).

Another field of tyrosinase research deals with new enzymatic technologies for modifying the structure and sensory properties of food products (meat and dairy products, bakery). Investigations in this direction (using not only tyrosinase but also laccase) have generated increasing interest with the development of products with low fat and low calories and a decrease in the use of emulsifiers and thickening agents. Thus, cross-linking of proteins may be useful in texturization and in the modification of softness, solubility, foaming and the emulsifying properties of food products (Selinheimo et al. 2008).

The tyrosinase of *P. sanguineus* was shown to be very effective in cross-linking casein. It is noteworthy that the use of 2 U ml⁻¹ pure tyrosinase was sufficient to perform the casein cross-linking, whereas the conjugation of gelatin to the polysaccharide chitosan (Chen et al. 2002) and the crosslinking of α -lactalbumin or β -lactoglobulin needed 30- to 160-fold higher enzyme activity, respectively

(Thalmann and Lötzbeyer 2002). The efficient formation of protein-oligosaccharide conjugates via hetero-cross-linking between tyrosine side chains of α -casein and phenolic acids of hydrolyzed oat spelt xylan was demonstrated in a study using tyrosinase from *Trichoderma reesei* and laccase from *Trametes hirsuta* (Selinheimo et al. 2007, 2008).

Finally, it has to be mentioned that immobilized *A. bisporus* tyrosinase can be used in biosensors for the detection of phenolic compounds or the organophosphorous pesticide dichlorvos (Svitel and Miertus 1998; Vidal et al. 2006). The low expression levels both of homologously or heterologously expressed tyrosinase is still the main bottleneck that impedes a wider application of this enzyme. Ongoing research on the design of suitable expression constructs for the production of extracellular tyrosinase may help to overcome this problem (Halaouli et al. 2006).

IV. Conclusions and Outlook

Over the past ten years, enormous progress has been made in the development of oxidative fungal biocatalysts. Regarding the expanding biocatalytic potential of peroxygenases, peroxidases and phenol oxidases, it is to expect that within the current decade, a break-through will come in this field and technological solutions will be found to allow the broad application of isolated oxidative enzymes on an industrial scale. The large-scale production of the envisaged enzymes as bulk biocatalysts at reasonable prices will be an indispensable prerequisite to achieve this goal. In the first place, the heterologous over-expression of peroxidases and oxidases in appropriate hosts will have to be realized. To this end, the whole machinery of modern molecular tools will have to be applied, including the rapidly developing facilities of genetic and protein engineering, molecular design and transcription factor analysis.

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22 Fungal Soil Bioremediation: Developments Towards Large-Scale Applications

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I. Introduction

The continually growing, worldwide use of hazardous compounds and the inevitable production of wastes and contamination feed the need of cleaning up our environment in order to provide healthier living conditions, especially in heavily inhabited areas. Spills of various chemicals, fires and other accidents pollute air, water and soil. The occurrence of recalcitrant and dangerous organic compounds especially in soil has led to the development of several bioremediation technologies (Cha et al. 1999; Dua et al. 2002; El Fantroussi and Agathos 2005; Jørgensen 2007). Particularly fungi have been found to be suitable microorganisms to modify, degrade or even mineralize xenobiotic compounds in soil. In the 1980s, Carl Cerniglia and co-workers had already studied the zygomycete *Cunninghamella elegans* extensively

(Cerniglia and Gibson 1979). This fungus seemed to be especially capable of degrading polycyclic aromatic hydrocarbons (PAH) by its intracellular system of cytochrome P450-dependent monooxygenases (Cerniglia et al. 1985). However, the use of an intracellular enzyme system in a soil environment can be rather limiting. With the discovery of new extracellular peroxidases and the characterization of a number of laccases (also in the 1980s), new possibilities for fungal bioremediation opened up (Kirk and Farrell 1987). Particularly, the model white-rot fungus (WRF) *Phanerochaete chrysosporium* was used to demonstrate the fungal ability to oxidize and degrade various organic pollutants (Paszczynski and Crawford 1995). Direct involvement of lignin peroxidase (LiP) and manganese peroxidase (MnP) in the degradation processes of PAH, pentachlorophenol (PCP) and trinitrotoluene (TNT) was demonstrated and later also confirmed in enzymatic in vitro experiments (Sack et al. 1997; Van Aken et al. 1999; Duran et al. 2002).

In the 1990s, promising results were obtained with different fungi, contaminants and remediation technologies, reviewed in a number of papers (Lamar et al. 1993; Scheibner et al. 1997; Novotny et al. 1999; Tuomela et al. 1999; Pointing 2001). Step by step, research provided answers concerning the particular degradation capabilities of fungi, the involvement of enzymes and biocatalytic reactions, the competitive behavior and survival strategies of fungi in soils and, finally, the whole soil bioremediation process (at least at the laboratory scale). The results were so promising that some pilot-scale and even large-scale trials were run (Davis et al. 1993), but maybe too fast, as it seems today. Though some promising strains were already available at that time, difficulties were ascertained during pilot-scale trials and these demonstrated a lack of knowledge to be really successful. Some of the trials did not provide positive results, which for a long time

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may have prevented further funding of this research by public or industrial sponsors. Even the few companies which were selling myco-remediation technology are no longer on the market. But why? To all appearances, the starting point has been good, as the following sections show. Still, although fungal bioremediation has been shown to be feasible and even economical, its development into a working technology is very expensive and laborious, not least due to extensively long periods of trials and pre-testing preceding the actual cleaning process. Furthermore, soils and soil contaminations are usually very heterogenic and, therefore, success may be difficult to prove. By the example of a current bioremediation trial, we will try to explain why fungal techniques can nevertheless be applied and to show how much time may be needed before proceeding with “true” pilot-scale studies.

II. Groups of Fungi Used in Bioremediation

Where to find a suitable fungus for soil bioremediation? There are many species in the two large taxonomic groups of ascomycetes and basidiomycetes (nowadays classified as phyla or subphyla) which have been shown to be capable of degrading various recalcitrant organic compounds, particularly using extracellular oxidoreductases such as laccase and/or peroxidases (Esposito and da Silva 1998; Pointing 2001).

But the fact that extracellular oxidoreductases may be useful for the degradation process does not really narrow down the choice of species. Still we could ask which would be suitable candidates: either filamentous moulds (producing sets of hydrolytic enzymes and specific intracellular oxidoreductases; Scheibner et al. 1997; Gramss et al. 1999; Veignie et al. 2004), or brown-rot fungi (with their unique capability to form reactive hydroxyl radicals in their micro-environment; Andersson et al. 2001; Purnomo et al. 2008). Two well-known examples are the brown-rot basidiomycete *Antrodia vaillantii* (Andersson et al. 2001) and the ascomycetous mould *Fusarium solani* (Veignie et al. 2004). Only a few studies have been published where a fungus and a bacterium were inoculated as a co-culture to clean contaminated soils (Boonchan et al. 2000; Šašek et al. 2003b; Wick et al. 2007) or where a fungus was inoculated after the bacterial activity had ceased (Garon et al. 2004; Baldi et al. 2007). In an interesting study by Wick et al. (2007), the bacterial species *Pseudomonas putida*, that actually degraded the target compounds, was found to

use a mycelium as a “fungal highway” to come into contact with the contaminated soil.

During the past three decades, most studies have focused on basidiomycetous wood-degrading fungi (white-rots), which have been shown to possess the best capabilities for soil bioremediation (reviewed by Pointing 2001). The reason for this is their production of extracellular peroxidases and laccases, both of which can efficiently degrade aromatic compounds, including xenobiotics (Hatakka 1994; Van Aken et al. 1999; Hofrichter 2002; Rodriguez et al. 2004). Most efficient white-rot fungi, however, are not easily grown into non-sterile soil regardless of being contaminated or not (Novotny et al. 1999; Valentin et al. 2009). There are some examples of fungi which have been shown to successfully invade non-sterile soil, e.g. *Bjerkandera* sp., *Pleurotus* sp. and *Trametes versicolor* (Leštan and Lamar 1996; Eichlerova et al. 2000; Schmidt et al. 2005). The main difficulty lies in the fact that white-rot fungi are primarily wood-decomposing organisms dwelling in compact timber or trunks, often without any contact to soil (Dix and Webster 1995). To overcome this problem, another eco-physiological group of basidiomycetes has been investigated during the recent decade, namely the litter-decomposing fungi (LDF; Steffen et al. 2002a). Many species of this group produce peroxidases and laccases similar to white-rot species (Hofrichter 2002; Steffen et al. 2002b). Consequently, they also cause the same type of white-rot but in soil litter (and not in wood), which becomes visible as bleached spots in leaf or needle heaps on the forest floor (Hintikka 1970). The basic idea of using these fungi is that they presumably possess a natural ability to survive in soil and can successfully compete with other soil micro-organisms. The combination of suitable extracellular enzymes with good survival skills in non-sterile soil makes LDF ideal candidates for soil bioremediation (Steffen et al. 2007; Valentin et al. 2009).

There are also wood-degrading fungi that grow typically in habitats close to soil, e.g. on fallen branches, in stumps or in subsurface roots. Such fungi could be classified as either wood-rotters or litter-decomposers, i.e. this fungal group overlaps the eco-physiological capabilities. It seems that these “intermediate” fungi also possess the capability to actively grow into soil (Valentin et al. 2009), which makes them an interesting group for bioremediation. Examples of such fungi are *Gymnopilus penetrans* and *G. luteopho-*

lius (syn. *Pholiota luteopholia*; Valentin et al. 2009; Winquist et al. 2009). Possibly also more common species, such as the edible mushroom *Stropharia rugosoannulata*, could be counted in this intermediate group. It preferably grows on straw or bark remains and its fruiting body production is triggered through soil contact. The fruiting bodies of this fungus are commercially sold (see Chapter 4) and also first trials to remediate soils contaminated with nitroaromatics were successfully run (Herre et al. 1998). The potential of this fungus to degrade different organopollutants has been demonstrated several times (Scheibner et al. 1997; Steffen et al. 2007) and today it is one of the most promising species for large-scale applications.

III. Soil Contaminants Degraded by Fungi

A wide range of organic contaminants can be treated with fungal technologies. In particular, research has focused on aromatic compounds, since they resemble elements of the lignin polymer (Pointing 2001). They are often recalcitrant, so-called persistent organic pollutants (POP). In soil, these contaminants are mostly present in "aged" form and typically adsorbed or bound to humic substances. Fungal tolerance to heavy metals has also been studied, as polluted environments typically contain several contaminants including both organopollutants and heavy metals. The following organic contaminants have intensively been studied.

A. Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) originate from petroleum, fuel oil, creosote and manufactured or natural gas. They have more than two aromatic rings and the more rings a molecule has the more recalcitrant and carcinogenic it is. PAH contamination of soil is often expressed as a sum of 16 PAH [naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenz(a,h)anthracene, benzo(g,h,i)perylene, indeno(1,2,3-cd)pyrene] defined by the United States Environmental Protection Agency (EPA). PAH with two rings (naphthalene) or three (acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene) are still relatively easy to

degrade by microbes (a number of bacteria can use them as carbon source). Thus, studies with fungi have mainly focused on compounds with more than three rings (Steffen et al. 2007). PAH are probably the most studied group of compounds concerning fungal conversion and degradation (Cerniglia 1997). The reason for this is that fungi possess both intracellular and extracellular enzymes which can effectively oxidize them.

B. Halo- and Nitroaromatic Compounds

Chlorinated compounds are omnipresent and can be found in high amounts, for example, in old sawmills in form of different chlorophenols. At present, the use of chlorophenols is prohibited in industrial countries but they may be still in use in developing countries. Pentachlorophenol (PCP) is the most persistent chlorophenol and thus the most widely studied one (Lamar et al. 1993; Tuomela et al. 1999). As chlorophenols contain only one aromatic ring, they are relatively easy to degrade (compared to complex chloroaromatics) in contaminated sites by processes of natural attenuation (Sims et al. 1999).

Polychlorinated dibenzo-*p*-dioxins and -furans (PCDD/F), which had been present in all chlorophenol-based wood preservatives as impurities (Kitunen et al. 1987), represent the most severe problem at sawmill sites today (Weber et al. 2008). PCDD/F molecules possess very stable structures and thus remain intact in the environment for centuries (Kitunen et al. 1987; Persson et al. 2008). However, it was observed that at least some white-rot fungi are able to degrade PCDD/F (Takada et al. 1996; Kamei and Kondo 2005). Recently, PCDD/F degradation by *Phlebia brevispora* was observed in a contaminated soil under slurry-state conditions (Kamei et al. 2009). Polychlorinated biphenyls (PCB) had been used for decades in transformers and capacitors and, although their use was banned already years ago, many sites are still being contaminated through the manufacturing process or inappropriate dumping. PCBs are stable compounds and the more chlorinated a molecule is, the more recalcitrant it is under aerobic conditions. Nevertheless, degradation even of highly chlorinated PCBs by white-rot fungi has been reported (Yadav et al. 1995; Kubatova et al. 2001).

The use of chlorinated compounds has substantially ceased globally due to new legislation. At present, halogenated compounds are still widely used in specific applications, e.g. polybrominated biphenyls (PBB), as flame retardants. Only lately, have their toxic potential and hazardous

environmental impact been realized and degradation studies successfully performed with different fungi (Uhnkov et al. 2009).

2,4,6-Trinitrotoluene (TNT) is one of the most widely occurring nitroaromatic compounds and has been used as an explosive for military purposes and industrial mining applications (e.g. mining) for more than 100 years. As a result, production and assembling sites of explosives and ammunition as well as numerous military training grounds and shooting ranges were contaminated with TNT and related products. Biodegradation of TNT by different groups of fungi was extensively studied at laboratory scale (Scheibner et al. 1997; Van Aken et al. 1999), and also a field trial using the litter-decomposer *S. rugosoannulata* was successfully performed (Herre et al. 1998).

C. Agrochemicals

Pesticides, which can be divided according to their target organisms into herbicides, insecticides, nematocides, acaricides, fungicides etc., are typically aromatic and/or heterocyclic molecules either persistent or non-persistent in nature. DDT is a well-known and infamous example of a persistent pesticide. Traces of DDT can be still found in industrialized countries in Europe and the United States, although its use was banned almost 30 years ago. Lignolytic fungi have been found to degrade various pesticides including DDT and other highly persistent compounds (Bending et al. 2002; Purnomo et al. 2008).

D. Endocrine Disrupters

Endocrine disrupters are natural hormones, such as estrogens, or compounds that mimic natural hormones, such as nonylphenol (NP) and parabens (Auriol et al. 2006). Very small concentrations of these compounds are harmful to the environment. The most common endocrine disrupter is the organometallic compound tributyltin (TBT), which is found especially in harbours where ships are treated with paints containing TBT as an antifouling agent (Antizar-Ladislao 2008). Bisphenol A (BPA) is used as a softener for plastics manufacturing and is found in domestic and industrial wastewaters. Both compounds

have been successfully degraded by white-rot fungi and other fungi, including aquatic hyphomycetes (Tsutsumi et al. 2001; Bernat and Długowski 2002; Junghanns et al. 2005).

E. Pharmaceuticals, Personal Care Products and Dyes

Pharmaceuticals and personal care products (PPCP) form a large group of compounds found in drugs and cosmetics. This group overlaps with endocrine disrupters but contains also antibiotics, anti-inflammatory drugs, lipid regulators, β -blockers, antiepileptic drugs, antidepressants etc. These compounds are typically aromatic and can therefore be degraded by lignolytic fungi (Marco-Urrea et al. 2009). So far there have been only a few studies (e.g. Carballa et al. 2005; Wen et al. 2009) as the topic emerged only lately. PPCP end up mostly in aqueous environments and they are mostly incompletely degraded in wastewater treatment plants (Lindqvist et al. 2005; Vieno et al. 2007). However, little is known about the environmental impact of these compounds and there is yet no legislation or regulation for their discharge.

Dyes used in the textile and leather industry often comprise aromatic and quinoide structures, as well as azo groups, and are therefore particularly persistent to microbial degradation. The treatment of wastewater from the textile industry by immobilized fungi or lignolytic enzymes was abundantly studied (e.g. Axelsson et al. 2006), recently also successfully in combination with ultrasound (Tauber et al. 2008). Dyes, however, are rarely found as contaminants in soil and are therefore not further considered here.

F. Other Contaminants

Among aliphatic pollutants, nitriles (including cyanide) and methyl *tert*-butyl ether (MTBE) are some of the most recalcitrant ones. MTBE was used for many years as a petrol additive and spread to the environment particularly from highways. Cyanide and nitriles can be found at former gasworks sites and in mining areas. A few degradation studies using these compounds and different micro-fungi (molds) were performed and the degradative enzymes identified (Hardison et al. 1997; Barclay et al. 1998; Woodward et al. 2008).

Many fungi are highly tolerant to heavy metals in their environment (Baldrian et al. 2000; Tuomela et al. 2005), which is a useful characteristic for fungi to be applied in bioremediation, as polluted sites are often concomitantly contaminated with heavy metals and organopollutants. Fungi have developed various mechanisms to detoxify heavy metals in their environment by influencing metal speciation and mobility (Gadd 2004). A targeted detoxification of respectively contaminated soils with fungal techniques has not yet been tried, mostly because the mycelia which have accumulated heavy metals are very difficult to separate from the soil.

IV. Methods

Since the capability of ligninolytic fungi to degrade environmental pollutants has been recognized, several attempts have been made to establish fungal technologies as an option for the remediation of contaminated soils. Some of the approaches have proceeded as far as field-scale studies (Table 22.1). However, due to the commercial interests of the large-scale operators, respective data and findings have not been published. Moreover, failed trials have generally not been reported in the literature and information is available only through personal communication.

Most of these studies have exploited WRF as degraders of soil contaminants. But in two field-scale trials, also LDF were used (Herre et al. 1998; Šašek et al. 2003a). Li et al. (2002) inoculated a contaminated soil with several fungal species at the same time, the WRF *P. chrysosporium* and three fungi of the indigenous soil mycoflora (non-basidiomycetous species). Rojas-Avelizapa et al. (2007) studied bio-stimulation by introducing straw into the soil, which specifically stimulated fungal growth. In one case, commercial mushroom compost for the production of the edible mushroom *Agaricus bisporus* was successfully used (Šašek et al. 2003a). Soils contaminated with petroleum hydrocarbons can also be cleaned up using indigenous soil fungi, which have to be isolated and accreted beforehand (Li et al. 2002; Rojas-Avelizapa et al. 2007). Conversions observed for the target compounds were always high and amounted sometimes up to 100% (Herre et al. 1998; EarthFaxEngineering 2008); but in some cases the degradation (especially of larger PAH molecules) was only moderate or even poor (Davis et al. 1993; Lamar et al. 1994; Li et al. 2002).

Substrates used for fungi in large-scale experiments varied in their composition and in the

applied soil-substrate ratio (Table 22.1). In all cases, inexpensive lignocellulosic substrates were used, often sawdust, straw or wood chips (Table 22.1). In some applications, the fresh substrate was introduced into the soil without pre-grown fungal mycelium but just together with the fungal inoculum (spores; Lamar et al. 1994; Axtell et al. 2000; Walter et al. 2005). Co-substrate amendments were sometimes used together with lignocelluloses, such as maize chaff or corn wastes, apple pomace, molasses or cereal brans (Leštan et al. 1996; Meysami and Baheri 2003; Walter et al. 2004; Ford et al. 2007). Also surfactants may be added to soils to enhance the solubility and thereby the bioavailability of contaminants and to increase generally the fungal activity (Axtell et al. 2000; Ford et al. 2007; Zhou et al. 2007). To this end, mostly the non-ionic surfactant Tween 80 has been used (Axtell et al. 2000; Ford et al. 2007; Zhou et al. 2007).

Large-scale trials are usually performed at an ambient temperature and thus in colder climates, degradation usually ceases during the winter time (e.g. in Scandinavia). In such cases, two or even three vegetation periods are needed to approach the necessary concentration of contaminants in the soil (Holroyd et al. 1994; Herre et al. 1998; Walter et al. 2005; EarthFaxEngineering 2008). In this context, it should be noted that – due to the microbial/fungal activity – the soil temperature may rise above the ambient temperature (“metabolic heating”; Holroyd et al. 1994; Hofrichter and Fritsche 1996). One interesting approach in treating soils is the use of ex situ slurry-phase reactors (Valentin et al. 2007; Kamei et al. 2009). In slurry, fungi can easily reach the contaminants and the system is rather homogeneous, but requires a large amount of water. Solid:liquid ratios reported are 1:10 in a study with a PAH-contaminated soil (Valentin et al. 2007) and up to 1:60 with a PCDD/F-contaminated soil (Kamei et al. 2009), which would mean that about 10–60 m³ water is needed for each ton of soil. This high water requirement might be the reason why no large-scale study has yet been performed in a slurry system.

The following example stands for the most recent effort to develop a fungal large-scale system for the application in bioremediation (see Sections IV.C, D). The development of this method was recently published by Valentin et al. (2009) and Winquist et al. (2009). The original concept was previously patented two years ago by Mäentausta and Steffen (2008). The basic idea is to use white- or brown-rot fungi on a lignocellulosic carrier material, such as wood chips or bark, placed into tubes made of mesh material (plastic

Table 22.1. Field-scale studies described in the literature

Fungus	Site	Contaminant (mg kg ⁻¹)	Amount of soil (t/m ³)	Co-substrate	Soil: substrate	Time substrate (d)	Degradation (%)	References
<i>Pleurotus ostreatus</i>	Wood treatment	PCDD/F (0.0168 TEQ ^a)	4 t	Sawdust	4:1	282	69 (TEQ ^a)	EarthFax Engineering, Inc.
<i>P. ostreatus</i>	Wood treatment	PCP (2300), lindane (13 000)	750 t	Sawdust-cottonseed hulls	21:4	730	99.9 (PCP)	EarthFax Engineering, Inc.
Indigenous fungi and bacteria	Oil drilling	Petroleum hydrocarbons (200–270 000 ^b)	4 t	Straw	97:3	180	100 (lindane) 94	Rojas-Avelizapa et al. (2007)
<i>Trametes versicolor</i>	Wood treatment	PCP (900)	0.55 m ³	Sawdust-cornmeal-starch + wood chips	3:2	518 (913)	94.4 (99.6)	Walter et al. (2005)
<i>Agaricus bisporus</i>	Gas works	PAH (630 ^c)	2.8 m ³	Standard mushroom compost	1:4	154	68.8 ^d	Šašek et al. (2003)
<i>Cunninghamella</i> sp. ^e	Oil drilling	Petroleum hydrocarbons (49 900 ^b)	32 m ³	Chicken excrement-micro-nutrients- rice husks + wheat bran	6:1	53	49 38 (aromatics)	Li et al. (2002)
<i>Fusarium</i> sp. ^e								
<i>Mucor</i> sp. ^e								
<i>Phanerochaete chrysosporium</i>								
<i>Pleurotus ostreatus</i>	Military	TNT (194)	9.2 m ³	Rye + cellulose fibre-“Spawn Mate”-gypsum-molasses- Tween 80	2:1	62	98	Axtell et al. (2000)
<i>Stropharia rugosoannulata</i>	Military	TNT (76)	0.3 m ³	Wheat straw	2:1	600	99.9	Herre et al. (1998)
<i>Ph. chrysosporium</i>	Wood treatment	Chlorophenols (200)	6400 m ³	Straw-wood chips	na ^f	570	95	Holroyd et al. (1994)
<i>Ph. chrysosporium</i>	Wood treatment	PCP (717), creosote (1210 ^c)	13 t	Grain-sawdust + wood chips	9:1	56	0-91 ^g (PAH) 67 (PCP, <i>P. chrys.</i>) 89 (PCP, <i>P. sor.</i>) 23 (PCP, <i>P. chrys.</i> + <i>T. hirs.</i>)	Lamar et al. (1994) Davis et al. (1993) Lamar et al. (1993)
<i>Ph. sordida</i>								
<i>T. hirsuta</i>								
<i>Ph. chrysosporium</i>	Pesticide storage	PCP (1–4435)	3 t	Wood chips + peat	18:1	46	86 (<i>P. chrys.</i>) 82 (<i>P. sor.</i>)	Lamar and Dietrich (1990)
<i>Ph. sordida</i>								

^aTEQ = toxic equivalent^bSum of total petroleum hydrocarbons (TPH)^cSum of 16 PAH compounds^dReduction of the sum of 16 PAH compounds^eIndigenous fungus enriched^fData not available^gReduction of various PAH compounds: the more rings the less degradation

or metal). Though the method was planned for the clean-up of contaminated soil, its first application was done during a project for the Finnish Funding Agency for Technology and Innovation (TEKES) with the aim to decrease the organic content of a dioxin-contaminated sawmill soil prior to combustion in an incinerator. The fungus was introduced into the soil without mixing any additional organic material into it and the developed mycelia were finally removed by composting.

A. Screening

To adequately address the potential of suitable fungal strains, screenings for various capabilities have to be performed. Available data often derive not only from one but from several screening experiments, in which the goal may be rather different and not exclusively related to bioremediation, for example enzyme screenings, or screenings for bio-pulping or pharmaceutical fungi. The method here discussed used specific screenings, as published by Valentin et al. (2009) and Steffen et al. (2007). Furthermore, information about some of the strains derived from screenings regarding either the degradation of contaminants or the production of suitable degradative enzymes was published by Steffen et al. (2003) and Hakala et al. (2004).

Since there is a huge number of fungal species and strains available in nature, choosing merely a few for screening can be difficult and challenging. Each screening test is finally based on an accessible culture collection, which is as large as possible. For the tests described here, fungal strains were obtained over almost a decade and some of them were isolated in remote and protected areas such as Lapland in the very north of Finland. The strains have been deposited in the Fungal Biotechnology Culture Collection (FBCC) of the University of Helsinki. There are currently around 2000 strains deposited there but, to make any reasonable screening, the number of fungal candidates has to be substantially reduced. As a result, only 25 strains were chosen and studied by Steffen et al. (2007) and, in a larger effort, 147 strains by Valentin et al. (2009). The selection criteria to choose the most suitable candidates out of a large number of fungal strains are mainly based on earlier publications containing information on the growth, enzyme activities or preferred media of fungi.

In summary there are three general possibilities to obtain the right fungus: just choosing a certain species from a well-known and already

proven active genus (e.g. *Stropharia*), isolating a new strain of a promising species (e.g. *S. coronilla*) or perhaps trying to get an original isolate from the contaminated site.

A number of different parameters can be used in a fungal screening for bioremediation purposes. In addition to the ability to degrade the target compound(s), a suitable fungus has to tolerate the contaminated material as such and, above all, it has to survive in non-sterile soil, since large-scale applications cannot make use of sterilization for feasibility reasons. As such, growth in non-sterile soil, i.e. successful competition with the indigenous microbes and survival there, is a natural selection criterion for fungi (Martens and Zadrazil 1998; Eichlerova et al. 2000; Tornberg et al. 2003).

Tolerance against secondary contaminations is also often needed, as the sites may contain several contaminants (e.g. organopollutants and heavy metals; see Section III.F). Heavy metals as such cannot be degraded and are therefore not the primary target but might hinder the growth of micro-organisms and the bioremediation process (Giller et al. 1998; Vaisvalavicius et al. 2006). Growth tests both in liquid culture and soil contaminated with lead (Pb) demonstrated differences between fungal species not only in growth, but also in their enzyme activities and finally revealed some species extremely tolerant to lead (Tuomela et al. 2005).

A careful choice of promising fungi according to their growth in soil, even when using those naturally dwelling in or close to soil (e.g. LDF), is generally useful, may simplify the further screening process and helps to save time and material. Both studies mentioned above (Steffen et al. 2007; Valentin et al. 2009) showed that only <20% of the pre-selected strains were able to grow into soil. Although soil may be very heterogenic, the organic matter content has only little influence on the growth capability (Valentin et al. 2009). Seemingly, each type of soils needs its own special fungus.

To remediate contaminated soil, fungi have to express and secrete certain enzymes which are capable of degrading the contaminant(s) and/or the organic matter more or less unspecifically. Therefore screening studies have often been performed by measuring the production of ligninolytic enzymes, namely laccase and peroxidases (mostly MnP; Hofrichter and Fritsche 1996; Gramss et al. 1999; Walter et al. 2003; Steffen

et al. 2007). The role of hydrolytic enzymes, such as xylanases, cellulases and esterases, in bioremediation is so far unclear and has never been used as a selection criterion.

One of the simpler screening methods uses agar plates containing polymeric dyes, such as Poly R-478 or other indicator substances (such as ABTS) and monitors either the disappearance of the dye's colour or the appearance of a coloured compound (in the case of ABTS a blue-green cation radical). The oxidation of these compounds was found to correlate to some extent with both the ligninolytic activity and the degradation of organopollutants (Hofrichter and Fritsche 1996; Wunch et al. 1997; Šašek et al. 1998; Walter et al. 2003). Plate tests were also used for the pre-evaluation of PAH-degrading fungi (Wunch et al. 1997), for the pre-selection of fungi for bio-pulping (Hakala et al. 2004) and for the selection of LDF strains with high ligninolytic activity (Steffen et al. 2000). Valentin et al. (2009) performed this type of plate test to further reduce the number of suitable candidates after growth experiments in soil. Five different plate tests were applied, revealing among others that brown-rot fungi do not show ligninolytic activities under these conditions. This finding was not surprising but the respective brown-rots served so to speak as "negative" controls. Since the production of lignin-, cellulose- and hemicellulose-degrading enzymes is important, especially for applications with the goal to decrease the amount of organic matter, tests using liquid cultures may help to evaluate the overall degradation potential of fungi. Assuming that the tested strains can grow in the chosen liquid medium, enzymatic activities of laccase, manganese peroxidase (MnP), lignin peroxidase (LiP), glucanase, mannanase and xylanases can be easily monitored by photometric assays. However, results cannot guarantee that fungi – secreting high enzyme titres – possess this ability also in soil. In contrast, fungi that degrade lignocellulose in nature slowly and incompletely would perform faintly in such tests and would be deselected.

Of course, the most important property of a fungus concerning its use in bioremediation is its degradative capability to degrade the target pollutant. This capability has been shown in general for many white-rot fungi and also LDF (Scheibner et al. 1997; Wunch et al. 1997; Gramss et al. 1999; Tornberg et al. 2003) and has been demonstrated also on the enzymatic level for isolated peroxidases and laccases (Roy-Arcand and Archibald 1991; Steffen et al. 2003; Rodriguez et al. 2004). In a few cases, brown-rot, ectomycorrhizal and micro-fungal species were included into the screening tests, but mostly with less promising results (Scheibner et al. 1997; Martens and Zadrzil 1998; Šašek et al. 1998; Gramss et al. 1999).

When confronted with a huge amount of contaminated soil, it is always advisable to make a

laboratory-scale test before the actual bioremediation trial, in the course of which selected fungal candidates should be checked for their ability to grow into the particular soil and to diminish the contamination within a given time. For applications with the goal to decrease the organic matter content, a cumulative CO₂ analysis at laboratory scale could be done (Valentin et al. 2009; Winquist et al. 2009). Before that, however, the inoculation issue needs to be solved.

B. Inoculation

In laboratory experiments, agar plates and slants as well as liquid cultures are routinely used for inoculation. However, even when non-sterile material is inoculated, these rich media can cause problems. Mostly, the remains of easily degradable sugars from these media trigger the development of fast-growing molds, preventing the slow-growing basidiomycetes to colonize the soil. Therefore specific carrier materials suitable for basidiomycetes should be used. Solid materials are seemingly the better choice, not least because they can be easily mixed with soil at different ratios. The solid carrier should not only host the fungus of choice but may also provide moisture and organic carbon, at least for some time. Lignocelluloses of different origin were found to be adequate for this purpose and a number of typical agricultural and forestry wastes have successfully been tested.

Typically, wood chips, sawdust, shredded straw, hemp, flax or the bark of different trees were tested as carriers for both WRF and LDF (Leštan et al. 1996; Herre et al. 1998; Meysami and Baheri 2003; Walter et al. 2004; Steffen et al. 2007). Some results indicated that the choice of substrate is species-specific (Steffen et al. 2007). Most wood-degrading fungi seemingly prefer wood shavings or chopped straw (Morgan et al. 1993; Boyle 1995; Walter et al. 2004), while litter decomposers, as well as a few wood-degraders, grow well in fresh pine bark (Steffen et al. 2007; Valentin et al. 2009; Winquist et al. 2009). The application of peat was also reported and it was used both as carbon source and carrier material in mixtures of different substrates (Lamar and Dietrich 1990; Meysami and Baheri 2003). Another interesting approach made use of pelleted substrates (Leštan and Lamar 1996; Leštan et al. 1996; Walter et al. 2004). Mixtures consisting of pressed sawdust, starch, corn meal and lignosulfonates turned out to be quite efficient and enabled an optimum fungal growth on the pellets (Leštan et al. 1996). Autoclaved and pelleted substrate was also coated with fungal material entrapped in sodium alginate and these coated pellets

could be inserted into soil as a robust fungal inoculum (Leštan and Lamar 1996).

Pine bark is particularly feasible for biotechnological applications as it is inexpensive and available in large quantities, at least in countries with pulp and timber production. It is decomposed much more slowly than other substrates due to the presence of cutin and suberin and has natural biocidal properties (Nichols-Orians 1991). The recalcitrance and antibiotic properties have an advantage for the basidiomycetes, since they prevent a rapid colonization by competing molds. If a suitable candidate fungus is able to grow on bark, it certainly will be the ideal carrier. It can provide carbon for a ligninolytic fungus over a long time, since the slow degradation process releases available carbon in a limited but continuous manner. Furthermore, bark material can store sufficient amounts of water over a prolonged period. All in all, it may provide a perfect shelter and basis in the harsh environment of heavily contaminated soils.

To produce sufficient amounts of pine-bark inocula, large incubation chambers are needed. At a laboratory scale, liquid cultures can be used to inoculate smaller amounts of bark, i.e. up to 1 kg. For larger-scale inoculation, this amount is introduced into a larger volume of bark. Inoculation from bark to bark has been found to work so well that for some species, even non-sterile (but wet) bark can be used. If the fungus should be given an advantage for growth on bark, it can be pre-steamed. For a pilot scale of 12 m³, an amount of only 84 kg of pre-inoculated bark was sufficient, which was pre-steamed, inoculated and pregrown in a stainless steel chamber of 2 m³ volume (Fig. 22.1) on plastic trays (Fig. 22.2).

The utilization of commercial mushroom compost (CMP) is also possible. CMP has already been successfully used in soil bioremediation and, in particular, CMP obtained after fructification (e.g. of *Agaricus bisporus*, *Pleurotus ostreatus*) is perfect, since it makes use of a waste material and hence mushroom production more feasible. Many strains of *P. ostreatus* not only produce tasty fruiting bodies but have also been shown to degrade organopollutants efficiently (Eggen and Majcherczyk 1998; Martens and Zadrazil 1998; Baldrian et al. 2000; Bhatt et al. 2002; Rodriguez et al. 2004). Thus the respective mushroom compost can be used as fresh material both for fruiting body production and remediation purpose (Eggen 1999; Šašek et al. 2003a) or applied as spent waste compost after the mushroom harvest (Eggen 1999; Lau et al. 2003; Law et al. 2003).



Fig. 22.1. Bark material, which was pre-steamed, inoculated and pre-grown in a stainless steel chamber of 2 m³ volume. It served as inoculum for fungal tubes



Fig. 22.2. Preparation of fungal tubes. The overgrown bark material was filled into trays of plastic mesh, which were afterwards closed to form a tube

According to Eggen (1999), spent mushroom compost of *P. ostreatus* was more efficient to remove PAH from soil than fresh compost, maybe due to ligninolytic enzymes still present there. The enzymes extractable from spent mushroom compost of *Agaricus bisporus* were successfully

investigated with respect to the oxidative conversion of phenolic compounds (Trejo-Hernandez et al. 2001).

A special method was designed to separate the fungal inoculum from the soil using mesh tubes (Fig. 22.3). Other techniques do not separate their material from the compost and inocula are just mixed with the contaminated soil (Lamar et al. 1994; Walter et al. 2005) or they can be added in layers between the soil fractions to be cleaned (Herre et al. 1998). There is still a discussion which is the best strategy to inoculate the contaminated soil: in some cases, mixing led to a weakening of the fungal inoculum (Zadrazil, personal communication); in other cases, mixing gave better results concerning fungal activity than adding inocula in layers (Eggen 1999; Schmidt et al. 2005). Various studies have been performed to optimize not only the substrate composition, but also the inoculum age, substrate:soil ratio, surfactant supplements and humidity of the soil (Leštan and Lamar 1996, 1999; Leštan et al. 1996; Eggen 1999; Meysami and Baheri 2003; Walter et al. 2004; Schmidt et al. 2005; Ford et al. 2007). However, as fungal strains, soil contaminations and numerous other factors were different in the experiments performed, it is difficult to draw general conclusions on the basis of the results obtained. It can only be deduced that fungi need certain lignocellulosic materials of a certain quality and size to “feel at home” and stay active. If this is not the case, their growth into the soil will be poor. Therefore pellets, layers of wood chips or bark-filled tubes represent adequate and applicable methods, and other factors are probably more or less case-specific.

C. Small-Scale Trials

The definition of small-scale may be sometimes controversial, therefore we use this expression here mainly for experiments in the laboratory and pilot experiments in the greenhouse. The pre-tests for fungal growth from bark into soil were performed in 150-ml glass bottles (Steffen et al. 2007), scaled-up to 250-ml bottles (Valentin et al. 2009) and finally ending up in 2-l bottles with up to 700 g of material (Winqvist et al. 2009). The advantage of using smaller bottles in the beginning is that more strains can be tested at the same time, which needs less space and material. It is advisable to use transparent glass bottles for that, since it helps to observe fungal growth into the soil. With larger bottles (e.g. containing 700 g of material), aeration problems can be solved and growth patterns are nicely monitored. At this stage, a large number of replicates is not necessary, since growth and CO₂ production were found to vary just slightly in 2-l bottles. The use of slurries for laboratory-scale bioremediation trials, however, turned out to be limited. Slurry reactors are limited in size and not suitable for basidiomycetes, mostly due to aeration and stirring problems. Since the tube method was actually developed for solid wastes, also all small-scale tests were carried out in a solid state.

The next logical step in scaling-up was to considerably increase the reactor size and volume. Therefore a small pilot-scale experiment was performed in a 0.56 m³ bioreactor containing up to 300 kg of soil (Table 22.2). In this context, it has to be noticed that the mass of the fungal bark inoculum was only 4% of the

Fig. 22.3. Completed fungal mesh tube with well-developed rhizomorphs of *Stropharia rugosoannulata*

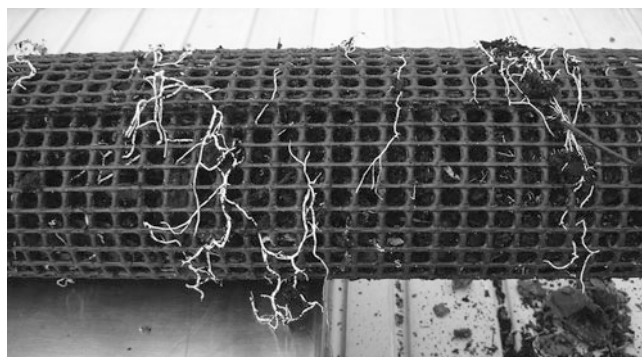


Table 22.2. Up-scaling of fungal bioremediation process. The inoculum substrate was pine bark

	Laboratory-scale	Pilot-scale	Field-scale
Number of strains	6	3	1
Type of soil (organic matter; %)	Sawmill (9%, 82%, 84%), shooting range (28%), landfill (14%)	Sawmill (82%), shooting range (28%), landfill (14%)	Landfill (14%), shooting range (28%)
Amount of soil	70–700 g	300 kg	7 t
Inoculum/soil, w/w	14:100	3:100	1:100
Aeration flow	70 l min ⁻¹	10 l min ⁻¹	1 l min ⁻¹
Temperature	21 °C	21 °C	Not monitored
Moisture control	Moist air, sealed bottles	Moist air, tarpaulin sheet	Moist air, tarpaulin sheet
Duration	96–180 days	6 months	6 months



Fig. 22.4. Radial growth of *Stropharia rugosoannulata* from two mesh tubes into the surrounding soil (window width 40 cm)

total mass. To follow the growth, a plexiglass window was installed whereby the radial fungal growth originating from two tubes could be followed (Fig. 22.4). After successful inoculation with the LDF *Stropharia rugosoannulata*, it took 60 days until the contaminated sawmill soil was thoroughly penetrated by the mycelium. In this experiment, it was possible to reduce the total carbon content by almost 10% within six months. Other fungal species were also tested in this reactor with promising results. Thus among others, *Phanerochaete velutina* turned out to be an exceptionally fast-growing species but reducing the organic carbon only by 5% and *Sphaerobolus stellatus*, an indigenous species isolated from sawmill area, most efficiently reduced the content of organic carbon (by about 20%) in six months (Winqvist et al. 2009).

D. Large-Scale Trials

The development and testing of the “fungal tube” method led to two larger pilot-scale trials in Finland, which are currently under investigation. Both piles work with a volume of 12 m³ (3 × 4 × 1 m) and approximately 7 t of soil (Fig. 22.5). Only 12 fungal tubes (3 m long, 10 cm in diameter, containing 7 kg bark each) are used in these piles. The inoculum:soil ratio is about 1:100. Each pile is equipped with an aeration tube system (Figs. 22.6, 22.7, Table 22.2) on the bottom and the contaminated soil is placed on it in two or three layers containing the fungal tubes (Figs. 22.5, 22.7). The aeration tubing can be connected to a compressor which actively blows air (1 l min⁻¹) through the pile to prevent overheating and to provide oxygen for the fungus. This aeration tubing can be arranged as a spiral (Fig. 22.6) or in a serpent. Additionally perforated tubes can be added to the pile to facilitate passive aeration. The fungus tube length for now has been adjusted to 3 m, because this size is still easy to handle (transport, weight etc.). Pre-inoculated pine bark is filled into the tubes made of plastic mesh (thickness 3–4 mm). Under these conditions, it can be estimated that bark and the fungal biomass amounts to approximately 1.2 mass% of the pile. The diameter of a filled tube is 8–10 cm and they can easily be placed and mounted by just one person. For certain applications, also shorter tubes have been tested. Moreover, a pile organized as illustrated in Fig. 22.7 can continuously operate as long as there is sufficient space on the site and, therefore, much larger volumes may be treated. To move and pile the soil, heavy machinery has been used (a front loader or digger). When dismantled,

Fig. 22.5. Pilot pile for the treatment of contaminated sawmill soil by the fungal tube technology. The pile works with a volume of 12 m^3 ($3 \times 4 \times 1 \text{ m}$) and finally contains 7 t of contaminated soil. Six fungal tubes are still visible, but are later covered with soil as well



Fig. 22.6. Spiral aeration system of a fungal pilot pile. It can be connected to a compressor for continuous active aeration

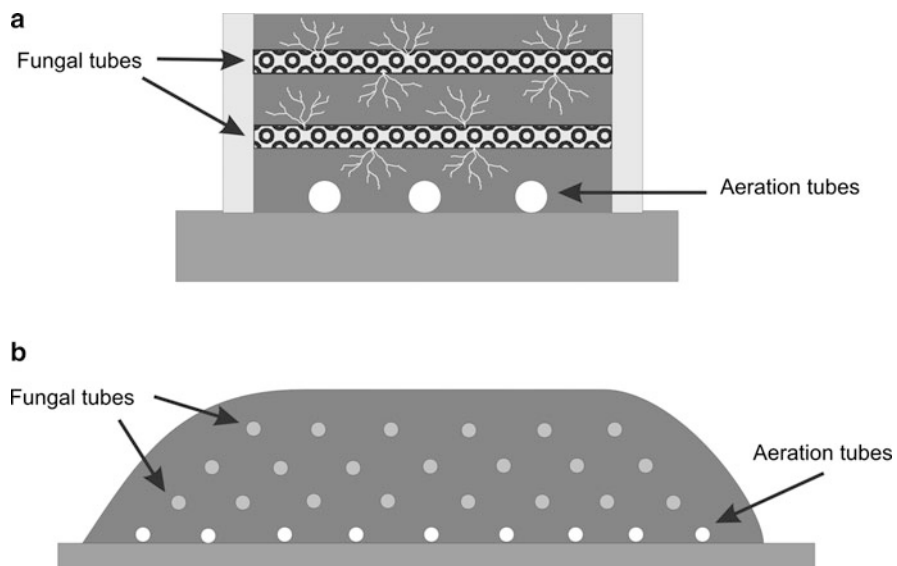


Fig. 22.7. Schematic cross-sections through a pile based on fungal tube technology

front loaders can take away the soil while releasing the tubes which are easy to collect. If necessary, an additional composting stage can be used to reduce the mycelial biomass left in the soil while simultaneously bacteria may finish the degradation process (e.g. in the case of contaminants pre-oxidized by fungi). The trials described here are brand-new and the treatment process is still going on (status summer 2009). Therefore, no final results about the organic matter removal or contaminants' degradation are available; however, a massive fungal penetration of the soil has already been observed after drill-probing.

V. Conclusions

Is fungal bioremediation really feasible? What are the actual drawbacks? For now, large-scale soil bioremediation utilizing fungi is still a more or less developing field and respective pilot trials at the level of "100 t" are missing. Not least, this depends also on the definition of large-scale. Currently one tends to say "larger" scale when meaning scales larger than laboratory or greenhouse trials. The approach of using a method like the fungal tube opens new opportunities to enlarge the scale to a reasonable level (of course, only if sufficient space is available). Are there any advantages in using fungi? In general, fungal techniques can be simple and thus also inexpensive. Even the technical part is not too difficult. However, if one tries to address this technique's true price, it would be rather high. Why? The main reason is the cost-intensive development stage (labour costs) but - for a chance of success - this stage is absolutely necessary before large-scale operation, in order to find the most suitable fungus for a particular soil-type and contamination. This takes time and costs money. There is no "one fits all" fungus available yet. In order to operate a successful remediation, one will have to develop and screen a "tool box" of suitable fungi, among which - at any time - about 12 suitable strains can be chosen from. Altogether, one can easily put 10 years of research into this field and still there would be no guarantee of success. Consequently, research in this area must exploit the knowledge and information of former studies and trials. Based on pre-existing knowledge including empirical and in parts non-published findings,

respective efforts may lead to the setting-up of true large-scale fungal bioremediation piles. The production of inocula will take a few weeks and the actual cleaning process several months, under good conditions. In this context, good conditions would simply mean a more or less adequate temperature and continuous aeration. If the pile is located outdoors, a normal growing season over the summer should be sufficient.

Some of these remarks may sound disappointing or even negative; however, a comparison with other available remediation methods argues for fungal techniques as an environmentally friendly alternative. Moreover, a total clean-up of a contaminated soil cannot be expected to be done by just one fungus. The goal will be also to provide an advantage for other soil microbes to further act on pre-oxidized organopollutants.

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