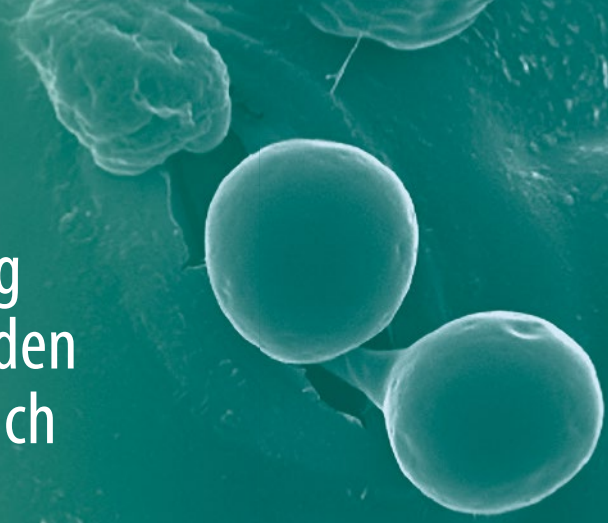

Helmut König
Gottfried Unden
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Editors

Biology of Microorganisms on Grapes, in Must and in Wine

Second Edition

Springer

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Foreword for the Second Edition

The field of wine microbiology has seen significant progress, both in scientific theory and practical application, since the publication of this book's first edition in 2009. Modifying Bill Clinton's famous dictum of his successful presidential campaign in 1996, we could say 'It's the microbes—stupid'.

In fact, the recent enormous progress that has been made using 'next-generation' DNA sequencing techniques in order to characterise microbes on the deep community scale has begun to stimulate important research in the field of wine microbiology. Spearheaded in particular by the team surrounding David Mills at UC Davis, USA, efforts have begun to characterise the metagenome of not only different vineyard sites but also various grape varieties and even vintages, and these are revealing distinct patterns among the fungi and bacteria present. 'We demonstrated that grape-associated microbial biogeography is non-randomly associated with regional, varietal, and climatic factors across multiscale viticultural zones. This poses a paradigm shift in our understanding of food and agricultural systems beyond grape and wine production, wherein patterning of whole microbial communities associated with agricultural products may associate with downstream quality characteristics' (Bokulich et al. PNAS 2013). This even caused the headline in *The New York Times*' Science section 'Microbes may add something special to wines' in 2013, while 2015 saw the inception of the new EU project Microwine (www.microwine.eu). Its aim is to apply 'next-generation' DNA sequencing in order to profile a range of vine and wine-related metagenomics communities which may or may not have beneficial effects on wine quality. Combining state-of-the-art DNA sequencing, metabolomics of volatile and non-volatile wine constituents and evaluation of their sensory relevance for human consumption will shed more light on the still poorly understood impact of microbial diversity and dynamics during grape production and winemaking on final wine quality. Wine estates around the world have started to investigate their individual 'terroir' of microorganisms in their vineyards and cellars in order to gain more knowledge that will help them shape their wines more individually but also to develop a unique selling proposition in the global 220€ billion wine business.

In the decade since the publication of the first issue, the wine industry has experienced a paradigm shift with regard to its appreciation of yeast and bacteria which were traditionally viewed as the facilitators of desired alcoholic and malolactic fermentation and in some cases also as the source of unwanted deterioration of wine due to microbial-derived off-flavours and faults. Current and ongoing research, however, goes beyond these paths, investigating the role of single yeast strains and bacteria or interacting communities of both in enhancing the varietal character of wines. Liberating aroma compounds such as monoterpenes, C_{13}-norisoprenoids or powerful thiols from their non-volatile and thus odour-inactive glycosylated or cysteinylated precursors, certain yeast and bacteria strains can strongly contribute to more pronounced and attractive wines.

According to the idea that 'sensory diversity is the new synonym for wine quality', which I postulated during a conference discussing the merits of terroir for wine in Australia in 2016, the wine industry tries to broaden the range of microbial genera used for winemaking. Non-saccharomyces yeast strains and malolactic bacteria besides *Oenococcus oeni* are selected, tested and implemented in the winemaking process for several reasons: first of all, to increase the sensory diversity of wine in order to cope with changing consumer behaviour and new food items to be paired with wine and, possibly most importantly, to attract new wine drinkers from countries and cultures which hitherto have not had much interaction with wine. However, other approaches look at the contribution of wine-derived microbes to support the grapevine and its fruit in its fight against fungal diseases and other enemies, as well as to stimulate different ripening patterns in the grapes.

Despite all of these modern developments and opportunities, we need to bear in mind that it is the alcoholic fermentation which facilitates the fundamental change of a fruit juice commodity to one of the oldest and most valuable beverages on earth. However, alcohol is not only a great preservative against an armada of spoilage organisms; it is also the cause of severe disease and addiction for millions of people. Taking this global concern seriously, the wine industry tries to lower the alcohol exposure of individuals by nudging consumers towards more moderate wine consumption (http://www.wineinmoderation.eu), but also by developing technical and microbial solutions to reduce the alcoholic strength of wine. A survey of consumers has clearly shown that they prefer low ethanol-producing yeast to technological means of reducing alcohol. Thus, one focus of wine microbiology in the future will be the selection and breeding of new yeast strains which will accomplish both: making wines of outstanding quality that are authentic expressions of grape varieties, winemaking styles and geographic heritage, but also of lower alcohol content. This attempt will be vital in safeguarding the future and growing appreciation of wine as the cultural drink for celebrations, pairing a great diversity of cuisines around the world and being a wonderful source of delight and inspiration.

This excellent compilation by world-famous experts in the broad field of wine microbiology and related fields of biochemistry, analytical chemistry and technology provides a valuable basis for these studies at the very horizon of international wine research.

Neustadt, Germany Ulrich Fischer
2017

Foreword of the First Edition

The ancient beverage wine is the result of the fermentation of grape must. This naturally and fairly stable product has been and is being used by many human societies as a common or enjoyable beverage, as an important means to improve the quality of drinking water in historical times, as a therapeutical agent and as a religious symbol.

During the last centuries, wine has become an object of scientific interest. In this respect, different periods may be observed. At first, simple observations were recorded, and subsequently, the chemical basis and the involvement of microorganisms were elucidated. At a later stage, the scientific work led to the analysis of the many minor and trace compounds in wine, the detection and understanding of the biochemical reactions and processes, the diversity of microorganisms involved and the range of their various activities. In recent years, the focus shifted to the genetic basis of the microorganisms and the molecular aspects of the cells, including metabolism, membrane transport and regulation. These different stages of wine research were determined by the scientific methods that were known and available at the respective time.

The recent 'molecular' approach is based on the analysis of the genetic code and has led to significant results that were not even imaginable a few decades ago. This new wealth of information is being presented in the *Biology of Microorganisms on Grapes, in Must and in Wine*. The editors were lucky in obtaining the cooperation of many specialists in the various fields. This joint international effort has resulted in a comprehensive book presenting our present-day knowledge of a specialised group of organisms that are adapted to the very selective habitat of wine. The various contributions of the book have the character of reviews and contain an extensive bibliography, mainly of the actual scientific papers.

I sincerely wish the editors and the authors that the presented book will be widely received by the scientific community and will be frequently used as a welcome source of information and a helpful means for further work on the

microorganisms of wine. Furthermore, understanding the intricate microbiological and biochemical processes during the fermentation should be helpful in the production of wine.

Mainz, Germany
June 2008

Ferdinand Radler

Preface of the Second Edition

The first edition about grape- and wine-associated microorganisms appeared 8 years ago. In the last two decades, the background information about microbes colonising grapes and those being involved in the conversion of must into wine increased strongly. The current knowledge was compiled in 27 chapters of the first edition that focused on the systematics, physiology, biochemistry and genetics of the corresponding grape- and wine-related microbes.

The concept of the first edition, which was well received by the community of wine scientists as well as other oenophiles from different areas, was also maintained in the second edition. The chapters of the first edition were updated, and some novel aspects were considered. New aspects concern microorganisms which infect vine and the characterisation of microbial enzymes as well as their application during winemaking. Moreover, a new chapter gives a survey of the variety of state-of-the-art enzymes for winemaking, which are of fungal origin showing additional potential for improvement of taste and enhancement of aroma.

The large progress made in the last decade can be seen in the increasing knowledge in the field of molecular biological characterisation and genetics of the bacteria, yeast and fungi associated with vine and wine. The recently acquired knowledge and the application of the corresponding techniques allow the rapid quantitative determination of mixtures of lactic and acetic acid bacteria as well as yeasts without prior cultivation. A combination of DNA fingerprinting methods and mass spectrometry has been successfully applied to identify microorganism down to the strain level.

For most important wine-related microorganisms, the genome sequences are known. This knowledge can be used for an application-oriented inventory-taking of the physiological and especially enzymatic potential of certain bacterial and yeast species. It also supports the selection of strains with very specific properties in respect to an enhanced wine quality such as improved sensory profiles, reduction of disturbing compounds, health benefits and the generation of hybrids. Worldwide, the production of genetically modified organism (GMO), except two strains, is not accepted yet. However, the generation of hybrid strains containing parts of the genome and hence some advances of different yeast species or strains is possible.

These hybrids can be found naturally or are produced by protoplast fusion. Yeast hybrids are already available on the market. They allow winemaking under more sophisticated conditions.

Enhanced experience has also been acquired in the parallel or successive application not only of the classical wine yeast *S. cerevisiae* and other *Saccharomyces* species but especially in certain combinations with different so-called wild yeasts which enable the production of more complex wine aromas or the partial imitation of a spontaneous fermentation.

Such a broad range of topics combined in 27 chapters of one book was only possible because of the contributions of different wine scientists around the world. We thank all authors for offering their experience in the special fields of the microbiology of winemaking. We are also grateful to the publisher Springer, especially to Mrs. Man-Thi Tran, Mr. Srinivasan Manavalan and Ms. Mahalakshmi Rajendran, for accompanying the production process of this book.

We wish that this book offers inspiring information for the community of oenophiles which includes students, scientists and winemakers about the tiny creatures which transform the must into wine.

Mainz, Germany
March 2017

Helmut König
Gottfried Unden
Jürgen Fröhlich

Preface of the First Edition

Ce sont les microbes qui ont le dernier mot
(Louis Pasteur)

Archaeology, genetics, ancient literature studies (*Epic of Gilgamesh*, ca. 2000 BC), paleobotany and linguistics point to the Neolithic period (ca. 8000 BC) as the time when domestic grape growing (*Vitis vinifera vinifera*) and winemaking began, most probably in Transcaucasia (McGovern 2003). For ages wine has been an essential part of the gracious, cultured and religious way of life.

Starting at the heartlands of the Middle East, winemaking techniques have been empirically improved since Neolithic times, expanding into experimental and scientific viticulture and oenology in our days. Despite these long traditions in winemaking, it was only in 1857 that significant contributions of Louis Pasteur on alcoholic and lactic acid fermentation, as well as on acetic acid formation, proved that the conversion of grape juice into wine was a microbiological and not a purely chemical process.

Up to now, bounteous knowledge about winemaking techniques and procedures has been accumulated, which was already found in several books about wine microbiology, biotechnology and laboratory practices. Especially in the last two decades, our knowledge about the role of microbes and their application as starter culture has been greatly increased.

Therefore, the aim of this book is to focus on the ecological and biological aspects of the wine-associated microbiota, starting from grape-colonising to wine-spoiling microbes. Purely technical aspects of winemaking are not a subject of this publication.

Growth in the must and wine habitat is limited by low pH values and high ethanol concentrations. Therefore, only acid- and ethanol-tolerant microbial groups can grow in grape juice, must and wine, which include lactic acid and acetic acid bacteria, yeasts and fungi. The most important species for winemaking are *Saccharomyces cerevisiae* and *Oenococcus oeni*, which perform the ethanol and malolactic fermentation, respectively. These two species are also applied as starter cultures.

However, the diverse other microorganisms growing on grapes and must have a significant influence on wine quality.

The book begins with the description of the diversity of wine-related microorganisms, followed by an outline of their primary and energy metabolism. Subsequently, important aspects of the secondary metabolism are dealt with, since these activities have an impact on wine quality and off-flavour formation. Then chapters about stimulating and inhibitory growth factors follow. This knowledge is helpful for the growth management of different microbial species. During the last 20 years, significant developments have been made in the application of the consolidated findings of molecular biology for the rapid and real-time identification of certain species in mixed microbial populations of must. Basic knowledge was acquired about the functioning of regulatory cellular networks, leading to a better understanding of the phenotypic behaviour of the microbes in general and especially of the starter cultures as well as of stimulatory and inhibitory cell-cell interactions during winemaking. In the last part of the book, a compilation of some modern methods rounds off the chapters.

This broad range of topics about the biology of the microbes involved in the vinification process could be provided in one book only because of the input of many experts from different wine-growing countries. We thank all the authors for offering their experience and contributions. Finally, we express our special thanks to Springer for agreeing to publish this book about wine microbes.

We hope that this publication will help winemakers as well as scientists and students of oenology to improve their understanding of microbial processes during the conversion of must to wine.

Mainz, Germany
June 2008

Helmut König
Gottfried Unden
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Part I
Diversity of Microorganisms

Chapter 1
Lactic Acid Bacteria

Helmut König and Jürgen Fröhlich

1.1 Introduction

In 1873, 10 years after L. Pasteur studied lactic acid fermentation (between 1857 and 1863), the first pure culture of a lactic acid bacterium (LAB) ("*Bacterium lactis*") was obtained by J. Lister. Starter cultures for cheese and sour milk production were introduced in 1890, while fermented food has been used by man for more than 5000 years (Schlegel 1999; Stiles and Holzapfel 1997). The first monograph by S. Orla-Jensen appeared in 1919. A typical lactic acid bacterium grown under standard conditions is aerotolerant, acid tolerant, organotrophic, and a strictly fermentative rod or coccus, producing lactic acid as a major end product. It lacks cytochromes and is unable to synthesize porphyrins. Its features can vary under certain conditions. Catalase and cytochromes may be formed in the presence of hemes and lactic acid can be further metabolized, resulting in lower lactic acid concentrations. Cell division occurs in one plane, except pediococci. The cells are usually nonmotile. They have a requirement for complex growth factors such as vitamins and amino acids. An unequivocal definition of LAB is not possible (Axelsson 2004).

Lactic acid bacteria are characterized by the production of lactic acid as a major catabolic end product from glucose. Some bacilli, such as *Actinomyces israeli* and bifidobacteria, can form lactic acid as a major end product, but these bacteria have rarely or never been isolated from must and wine. The DNA of LAB has a G+C

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Table 1.1 Current taxonomic outline of lactic acid bacteria[a] of the order "Lactobacillales" in the *Clostridium* branch

Family	Genus	Species from must and wine
I. "Aerococcaceae"	I. *Abiotrophia* II. *Aerococcus* III. *Dolosicoccus* IV. *Eremococcus* V. *Facklamia* VI. *Globicatella* VII. *Ignavigranum*	
II. "Carnobacteriaceae"	I. *Alkalibacterium* II. *Allofustis* III. *Alloiococcus* IV. *Atopobacter* V. *Atopococcus* VI. *Atopostipes* VII. *Carnobacterium* VIII. *Desemzia* IX. *Dolosigranulum* X. *Granulicatella* XI. *Isobaculum* XII. *Marinilactibacillus* XIII. *Trichococcus*	
III. "Enterococcaceae"	I. *Enterococcus* II. *Melissococcus* III. *Tetragenococcus* IV. *Vagococcus*	*E. faecium*
IV. Lactobacillaceae	I. *Lactobacillus*[b]	*Lb. brevis*, *Lb. buchneri*, *Lb. casei*, *Lb. curvatus*, *Lb. delbrueckii*, *Lb. diolivorans*, *Lb. fermentum*, *Lb. florum*, *Lb. fructivorans*, *Lb. hilgardii*, *Lb. jensenii*, *Lb. kunkeei*, *Lb. mali*, *Lb. nagelii*, *Lb. oeni*, *Lb. paracasei*, *Lb. plantarum*, *Lb. vini*
	II. *Paralactobacillus*	
	III. *Pediococcus*	*P. damnosus, P. inopinatus, P. parvulus*, *P. pentosaceus*
V. "Leuconostocaceae"	I. *Leuconostoc* II. *Oenococcus* III. *Weissella*	*Lc. mesenteroides* *O. oeni* *W. paramesenteroides*
VI. Streptococcaceae	I. *Lactococcus*[b] II. *Lactovum* III. *Streptococcus*	

[a]Garrity (2005), Vos et al. (2009), Whitman (2016), DSMZ (2016b)

[b]Species of *Enterococcus* and *Lactococcus* (*Lcc. lactis*) have been found on grapes (Bae et al. 2006; Nisiotou et al. 2015). *Enterococcus faecium* was identified in fermenting must (Pérez-Martín et al. 2014). Species of these two genera are not further considered here. In addition, *Lb. graminis* (Nisiotou et al. 2015) and *W. uvarum* (Nisiotou et al. 2014) have been isolated from grapes

Table 1.2 Differential characteristics of the wine-related lactic acid genera

Genus	Morphology from Glc	Carbohydrate fermentation[a]	Lactic acid isomer
Lactobacillus	Rods, coccobacilli cells single or in chains	homo- or heterofermentative, facultatively heterofermentative	D, L, DL
Leuconostoc[b]	Spherical or lenticular cells in pairs or chains	heterofermentative	D
Oenococcus[b]	Spherical or lenticular cells in pairs or chains	heterofermentative	D
Pediococcus	Spherical cells, pairs or tetrads	homofermentative or facultatively heterofermentative[c]	DL, L
Weissella	Spherical, lenticular, irregular cells	heterofermentative	D, DL

[a]Nonlimiting concentration of glucose and growth factors, but oxygen limitation
[b]Differentiation of wine-related species of *Leuconostoc* and *Oenococcus* cf. Table 1.4
[c]Facultatively heterofermentative species: *P. pentosaceus*, *P. acidilactici*, *P. claussenii*

content below 55 mol%. LAB are grouped into the *Clostridium* branch of gram-positive bacteria possessing a relationship to the bacilli, while *Bifidobacterium* belongs to the Actinomycetes. They are grouped in one order and six families. From the 33 described genera, only 26 species belonging to six genera have been isolated from must and wine (Table 1.1).

The homofermentative species produce lactic acid (<85%) as the sole end product, while the heterofermentative species produce lactic acid, CO_2 and ethanol/acetate from glucose. At least half of the end product carbon is lactate. Heterofermentative LAB utilizes the pentose phosphate pathway, alternatively referred to as the phosphoketolase or phosphogluconate pathway. Homofermentative wine-related LAB include pediococci and group I lactobacilli. Obligate heterofermentative wine-related LAB include *Leuconostoc*, *Oenococcus*, *Weissella* and group III lactobacilli (Tables 1.2, 1.3, 1.4 and 1.5).

Our present knowledge about LAB in general (Carr et al. 1975; Wood and Holzapfel 1995; Holzapfel and Wood 1998; Wood 1999; Wood and Warner 2003; Salminen et al. 2004; Lahtinen et al. 2012) and their activities on grape or in must and wine (Fleet 1993; Dittrich and Großmann 2005, 2011; Ribéreau-Gayon et al. 2006a, b; Fugelsang and Edwards 2007) has been compiled in several books. Here we concentrate mainly on lactic acid bacteria found in fermenting must and wine.

1.2 Ecology

In general, LAB occur in habitats with a rich nutrition supply. They occur on decomposing plant material and fruits, in dairy products, fermented meat and fish, beets, potatoes, mash, sauerkraut, sourdough, pickled vegetables, silage,

Table 1.3 Differential characteristics of wine-related species of the genus *Lactobacillus*

Characteristics	*Lb. brevis*	*Lb. buchneri*	*Lb. casei*[a]	*Lb. curvatus*	*Lb. delbrueckii*[b]	*Lb. diolivorans*	*Lb. fermentum*	*Lb. fructivorans*[c]
Phylogenetic group	I	A	D	G	C	A	F	A
Fermentation mode	III	III	II	II	I	III	III	III
Mol% G+C	44–47	44–46	45–47	42–44	49–51	40	52–54	38–41
Murein type	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	n.d.	Orn-D-Asp	Lys-D-Asp
Teichoic acid	glycerol	n.d.	n.d.	n.d.	n.d.	n.d.	ribitol or	n.d.
Lactic acid	DL	DL	L	DL	D	n.d.	DL	DL
Growth at 15/45 °C	+/–	+/–	+/–	+/–	–/+	+/–	–/+	+/–
NH_3 from Arg	+	+	n.d.	n.d.	d	n.d.	+	+
Fermentation of								
Amygdalin	n.d.	n.d.	+	–	+	–	n.d.	n.d.
L-Arabinose	+	+	–	–	n.d.	+	d	–
Cellobiose	–	–	+	+	d	–	d	–
Esculin	d	d	+	+	n.d.	n.d.	–	–
Galactose	d	d	n.d.	n.d.	d	+	+	–
Gluconate	n.d.	n.d.	+	+	n.d.	+	n.d.	n.d.
Lactose	n.d.	n.d.	n.d.	n.d.	+	–	n.d.	n.d.
Maltose	+	+	n.d.	n.d.	+	+	+	d
Mannitol	n.d.	n.d.	+	+	–	–	n.d.	n.d.
D-Mannose	–	–	n.d.	n.d.	+	–	w	–
Melezitose	–	+	+	–	n.d.	–	–	–
Melibiose	+	+	–	–	–	+	+	–
D-Raffinose	d	d	–	–	–	w	+	–
Ribose	+	+	+	–	n.d.	+	+	w
Salicin	n.d.	n.d.	n.d.	n.d.	+	–		n.d.
Sorbitol	n.d.	n.d.	+	–	n.d.	–	n.d.	n.d.
Sucrose	d	d	+	+	+	–	+	d

Trehalose	–	–	n.d.	n.d.	+	n.d.	d	–
D-Xylose	d	d	–	–	n.d.	+	d	–
Characteristics	*Lb. hilgardii*[c]	*Lb. jensenii*	*Lb. kunkeei*	*Lb. mali*	*Lb. nagelii*	*Lb. paracasei*[d]	*Lb. plantarum*[e]	*Lb. vini*
Phylogenetic group	A	C	B	H	H	D	E	H
Fermentation mode	III	II	III	I	I	II	II	I
Mol% G+C	39–41	35–37	n.d.	32–34	n.d.	45–47	44–46	39
Murein type	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	mDAP direct	mDAP direct	Lys-D-Asp	mDAP direct	Lys-D-Asp
Glycerol teichoic acid	glycerol	n.d.	n.d.	n.d.	n.d.	n.d.	ribitol or	n.d.
Lactic acid	DL	D	L	L	DL	L	DL	DL
Gowth at 15/45 °C	+/–	–/+	+/–	+/ n.d.	+/+	+/n.d.	–/+	–/+
NH_3 from Arg	+	+	+	n.d.	–	n.d.	–	–
Fermentation of								
Amygdalin	n.d.	+	–	n.d.	+	+	+	+
Arabinose	–	n.d.	–	–	–	–	d	+
Cellobiose	–	+	–	+	+	+	+	+
Esculin	–	n.d.	–	n.d.	n.d.	+	+	+
Galactose	d	+	–	n.d.	+	n.d.	n.d.	–
Gluconate	n.d.	n.d.	–	n.d.	–	+	+	–
Lactose	n.d.	–	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Maltose	+	d	–	–	+	n.d.	n.d.	+
Mannitol	n.d.	d	+	+	+	+	+	–
D-Mannose	–	+	–	n.d.	+	n.d.	n.d.	+
Melezitose	d	–	–	n.d.	–	+	+	–
Melibiose	–	n.d.	–	n.d.	–	–	+	–
D-Raffinose	–	–	w	n.d.	–	–	+	–

(continued)

Table 1.3 (continued)

Characteristics	*Lb. hilgardii*[c]	*Lb. jensenii*	*Lb. kunkeei*	*Lb. mali*	*Lb. nagelii*	*Lb. paracasei*[d]	*Lb. plantarum*[e]	*Lb. vini*
Ribose	+	n.d.	–	n.d.	–	+	+	+
Salicin	n.d.	+	n.d.	n.d.	+	n.d.	n.d.	n.d.
Sorbitol	n.d.	n.d.	–	+	+	d	+	–
Sucrose	d	+	+	n.d.	+	+	+	+
Trehalose	–	+	–	n.d.	+	n.d.	n.d.	+
D-Xylose	+	n.d.	–	n.d.	–	–	d	–

+, ≥90% of the strains are positive; −, ≥90% of the strains are negative; *d* 11–89% of the strains are positive; *w* weak positive reaction (Hammes and Vogel 1995). Three phylogentic groups (Hammes and Vogel 1995; Schleifer and Ludwig 1995a, b) were described in 1995 (group A: *Lb. delbrueckii* group; group B: *Lb. casei-Pediococcus* group; group C: *Leuconostoc* group). Eight years later Hammes and Hertel (2003) described seven phylogenetic groups, which were modified by Dellaglio and Felis (2005) and Felis and Dellaglio (2007) (wine-related species are given in brackets): A. *Lb. buchneri* group (group a: *Lb. buchneri*, *Lb. diolivorans*, *Lb. hilgardii*; group b: *Lb. fructivorans*). B. *Lb. kunkeei* group (*Lb. kunkeei*). C. *Lb. delbrueckii* group (*Lb. delbruechii*, *Lb. jensenii*). D. *Lb. casei* group (group a: *Lb. casei*, *Lb. paracasei*). E. *Lb. plantarum* group (group a: *Lb. plantarum*). F. *Lb. reuteri* group (group a: *Lb. fermentum*). G. *Lb. sakei* group (*Lb. curvatus*). H. *Lb. salivarius* group (*Lb. mali*, *Lb. nagelii, Lb. vini*). I. *Lb. brevis* group (*Lb. brevis*). Definition of the fermentative groups (Kandler and Weiss 1986; Hammes and Vogel 1995; Schleifer and Ludwig 1995a, b): Group I: Obligately homofermentative lactobacilli. Hexoses are almost exclusively (>85%) fermented to lactic acid by the Embden–Meyerhof–Parnas pathway (EMP). The organisms possess a fructose-1.6-bisphosphate aldolase, but lack a phosphoketolase. Gluconate or pentoses are not fermented. Group II: Facultatively heterofermentative lactobacilli. Hexoses are almost exclusively fermented to lactic acid by the Embden–Meyerhof–Parnas pathway (EMP). The species possess both a fructose-1.6-bisphosphate aldolase and a phosphoketolase. Consequently, the species can ferment hexoses and pentoses as well as gluconate. In the presence of glucose the enzymes of the phosphogluconate pathway are repressed. Group III: Obligately heterofermentative lactobacilli. Hexoses are fermented by the phosphogluconate pathway yielding lactic acid, ethanol/acetic acid and CO_2 in nearly equimolar amounts. Pentoses are fermented by the same pathway

[a]Formation of acetate and formate from lactate or pyruvate, or acetate and CO_2 in the presence of oxidants

[b]Subsp. *Lactis*

[c]High tolerance to ethanol and acidity

[d]Subsp. Paracasei

[e]Nitrate reduction, presence of pseudocatalase

n.d. No data given

The characteristics of the newly described species *Lb. florum* (Endo et al. 2010) and *Lb. oeni* (Mañes-Lázaro et al. 2009) are summarized in Sect. 1.7.1

Table 1.4 Differential characteristics of wine-related species of the genera *Leuconostoc*, *Oenococcus* and *Weissella*

Characteristics	*Lc. mesenteroides*	*O. oeni*	*W. paramesenteroides*
Acid from sucrose	+	–	+
Dextran formation	+	–	–
Growth below pH 3.5	–	+	n.d.
Growth in 10% ethanol	–	+	n.d.
NAD^+-dependent Glc-6-P-DH	+	–	n.d.
Murein type	Lys-Ser-Ala_2	Lys-Ser_2, Lys-Ala-Ser	Lys-Ser-Ala_2, Lys-Ala_2

n.d. Data not given

Table 1.5 Differential characteristics of wine-related species of the genus *Pediococcus*

Characteristics	*P. damnosus*	*P. inopinatus*	*P. parvulus*	*P. pentosaceus*
Mol% G+C	37–42	39–40	40.5–41.6	35–39
Growth at/in				
35 °C	–	+	+	+
6% NaCl	–	+	+	+
pH 8.0	–	–	–	+
Arginine hydrolysis	–	–	–	+
Acid from				
Arabinose	–	–	–	+

Pedicocci can be identified by multiplex PCR (Pfannebecker and Fröhlich 2008)

beverages, plants, water, juices, sewage and in cavities (mouth, genital, intestinal and respiratory tract) of human and animals. They are part of the healthy microbiota of the human gut. Apart from dental caries, lactobacilli are generally considered apathogenic. *Lb. plantarum* could be associated with endocarditis, septicemia and abscesses. Some species are applied as starter cultures for food fermentation. Because of the acidification they prevent food spoilage and growth of pathogenic microorganisms (Hammes et al. 1991). Some LAB are employed as probiotics, which are potentially beneficial bacterial cells to the gut ecosystem of humans and other animals (Tannock 2005). *O. oeni* strains induced strain-specific cytokine patterns measureable immunomodulatory potential (Foligné et al. 2010).

Lactic acid bacteria can also be found on grapes, in grape must and wine, as well as beer. Undamaged grapes contain $<10^3$ CFU per g and the initial titer in must is low (Lafon-Lafourcade et al. 1983). Because of the acidic conditions (pH: 3.0–3.5) grape must provides a suitable natural habitat only for a few microbial groups which are acid tolerant such as LAB, acetic acid bacteria and yeasts. While many microbes are inhibited by ethanol concentrations above 4 vol%, ethanol tolerant species survive in young wine or wine. Besides yeasts, some *Lactobacillus* species (e.g. *Lb. hilgardii*) and *Oenococcus oeni* can grow at higher ethanol concentrations. While only a few LAB species of the genera *Lactobacillus* (*Lb.*), *Leuconostoc* (*Lc.*), *Pediococcus* (*P.*), *Oenococcus* (*O.*) and *Weissella* (*W.*) (Tables 1.1 and 1.2) and the

acetic acid genera *Acetobacter*, *Gluconobacter* and *Gluconoacetobacter* can grow in must and wine, more than 90 yeast species have been found. Malolactic fermentation by lactic acid bacteria is occasionally desirable during vinification, but they can also produce several off-flavours in wine. The genera *Carnobacterium*, *Streptococcus* and *Bifidobacterium* have not been isolated from must and wine, but sometimes also species of the genus *Enterococcus* (*E. faecium*) could be detected in wine (Pérez-Martín et al. 2014).

Detailed investigations of the grape associated bacteria have been undertaken (Jackson 2008). Species of the lactic acid genera *Lactobacillus* (*Lb. casei*, *Lb. hilgardii*, *Lb. kefiri*, *Lb. kunkeei*, *Lb. lindneri*, *Lb. mali*, *Lb. plantarum*), *Weissella paramesenteroides*, *Enterococcus* (*E. avium*, *E. durans*, *E. faecium*, *E. hermanniensis*), *Lactococcus lactis* and infrequently species of the acetic acid genera *Asaia* and *Gluconobacter* as well as grampositive genera *Bacillus* and *Staphylococcus* have been identified in enrichment cultures from undamaged or damaged grapes of the varieties (Cabernet Sauvignon, Chardonnay, Pinot Noir, Sauvignon Blanc, Semillion, Shiraz, Tyrian) in Australia (Bae et al. 2006). Vineyard- and winery-associated lactic acid bacteria (LAB) from the Greek wine growing regions Peza and Nemea revealed that *Pediococcus pentosaceus* and *Lb. graminis* dominated the grape microbiota and *Lb. plantarum* the fermenting must (Nisiotou et al. 2015). Species of the genera *Enterococcus* and *Lactococcus* are not further considered here.

1.3 Phenotypic and Phylogenetic Relationship

The classification of LAB is largely based on morphology (rods, cocci, tetrads), mode of glucose fermentation, substrate spectrum, growth at different temperatures (15 and 45 °C), configuration of lactic acid produced, ability to grow at high salt concentrations (6.5% NaCl; 18% NaCl), and acid, alkaline or ethanol tolerance, as well as fatty acid composition and cell wall composition, lactic acid isomers from glucose, behaviour against oxygen (anaerobic or microaerophilic growth), arginine hydrolysis, acetoin formation, bile tolerance, type of hemolysis, production of extracellular polysaccharides, growth factor requirement, presence of certain enzymes, growth characteristics in milk, serological typing, murein, teichoic acid, menaquinone type, fatty acid composition, electrophoretic mobility of the lactate dehydrogenases, DNA base composition, PCR-based fingerprinting techniques (SAPD-PCR; Pfannebecker and Fröhlich 2008; Sebastian et al. 2011; Petri et al. 2013), restriction analysis (Ze-Ze et al. 2000), restriction fragment length polymorphism (PCR-RFLP) analysis of 16S ribosomal RNA (rRNA) genes (Ilabaca et al. 2014), 16S-ARDRA (Rodas et al. 2003), DNA–DNA homology, soluble protein pattern, 16S rDNA and gene sequencing (e.g. *recA*) (Axelsson 2004), multilocus sequence typing (MLST) and pulsed field gel electrophoresis analysis (PFGE) (González-Arenzana et al. 2014), quantitative PCR (Cho et al. 2011), marker-targeted quantitative PCR (Solieri and Giudici 2010), amplification of 16S rRNA

gene restriction with the endonuclease FseI (Marques et al. 2010), real-time PCR (Kántor et al. 2016), fluorescence in situ hybridization (FISH; Hirschhäuser et al. 2005), mass spectrometry (Napoli et al. 2014; Petri et al. 2015), multiplex PCR (Pfannebecker and Fröhlich 2008; Petri et al. 2013) and complete genome comparison (GGDC - The Genome-to-Genome Distance Calculator; DSMZ 2016d). qPCR after propidium monoazide treatment of samples is a rapid tool to enumerate *O. oeni* viable cells with intact membranes in must and wine (Vendrame et al. 2013).

The genera and species of lactic acid bacteria occurring in must and wine can be differentiated by phenotypic features (Tables 1.2, 1.3, 1.4 and 1.5). The species can be identified by the API 50 CHL identification system (Bio-Mérieux) or the Biolog Microbial Identification System (Biolog, Inc.) (Testa et al. 2014).

The first taxonomic outline given by Orla-Jensen (1919) is still of some importance. Based on physiological features Kandler and Weiss (1986) divided the genus *Lactobacillus* into the three groups (1) obligate homofermenters, (2) faculative heterofermenters and (3) obligate heterofermenters (Table 1.3). The phylogenetic relationship has been revealed by rRNA sequencing (Fig. 1.1; Collins et al. 1990, 1991, 1993; Martinez-Murcia and Collins 1990; Dicks et al. 1995). According to the 16S rDNA analysis Collins et al. (1990, 1991, 1993) divided the genus *Lactobacillus* into three groups. Group I contains obligate homofermentative species and

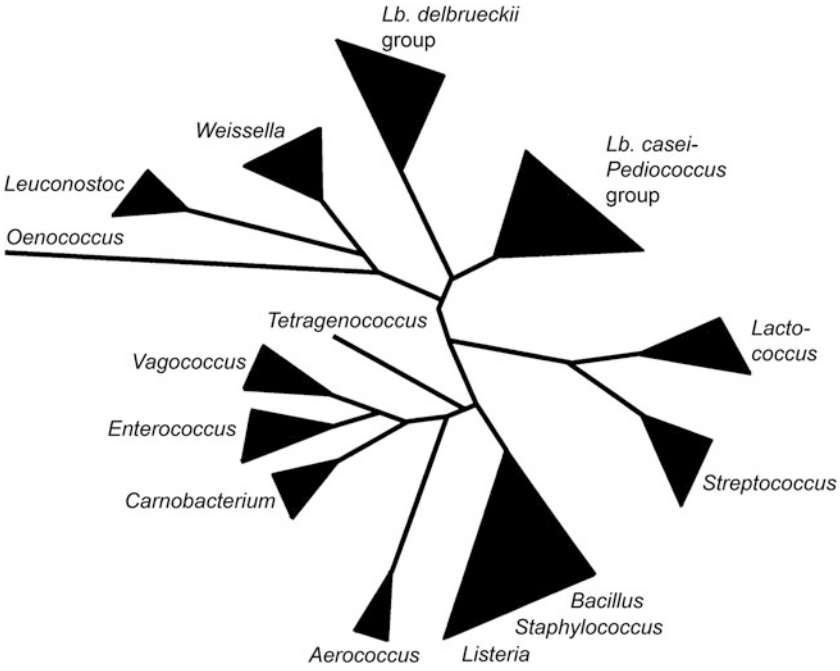

Fig. 1.1 Schematic unrooted phylogenetic tree of lactic acid bacteria and related genera (Axelsson 2004; with permission of the author and the publisher)

facultatively heterofermentative species. Group II contains more than 30 *Lactobacillus* species and five pediococcal species. The wine-related facultative heterofermenters *Lb. casei* and the obligate heterofermenters *Lb. brevis*, *Lb. buchneri* and *Lb. fermentum* belong to this group. Group III contains the genus *Weissella,* the leuconostocs (*Lc. mesenteroides*) and *O. oeni*. Schleifer and Ludwig (1995a, b) proposed the phylogenetic groups (1) *Lb. acidophilus* group, (2) *Lb. salivarius* group, (3) *Lb. reuteri* group (*Lb. fermentum*), (4) *Lb. buchneri* group (*Lb. buchneri*, *Lb. fructovorans*, *Lb. hilgardii*) and (5) *Lb. plantarum* group.

The *Leuconostoc* group can be clearly separated from other lactobacilli (Collins et al. 1991; Schleifer and Ludwig 1995a, b). The wine-related species *Lc. mesenteroides* forms a subgroup of the obligately heterofermentative *Leuconostoc* group. *Lc. oenos* was placed in the separate genus *Oenococcus* (Dicks et al. 1995) consisting of the three species *O. oeni* and *O. kitahareae* (Endo and Okada 2006) as well as *O. alcoholitolerans* (Badotti et al. 2015). *O. kitahareae* was isolated from a composting distilled shochu residue. It does not grow at acidic conditions (pH 3.0–3.5) of must and lacks the ability to perform malic acid degradation. *O. alcoholitolerans* thrived in an ethanol production plant in Brazil.

Hammes and Hertel (2003) described seven phylogenetic groups, which were modified by Dellaglio and Felis (2005) (cf. Table 1.3).

Today, the lactic acid bacteria are members of the domain Bacteria, where they are assigned to the phylum Firmicutes, the class Bacilli and the order Lactobacillases (Table 1.1) (Garrity 2005); Vos et al. 2009; Whitman 2016).

1.4 Physiology

Carbohydrates are used as carbon and energy source by a homofermentative or heterofermentative pathway. Fructophilic species have been described (Endo and Okada 2008; Mtshali et al. 2012). Sugars or oligosaccharides taken up by the phosphotransferase system (PTS, e.g. lactose: *Lb. casei*) or the permease system. Homofermentation of hexoses procedes via the Embden-Meyerhof-Parnas pathway, while heterofermentation is performed via the 6-*P*-gluconate/phosphoketolase pathway resulting in lactate, acetate/ethanol and CO_2 as endproducts or the Bifidus pathway (*Bifidobacterium*). Pentoses are fermented by 6-phosphogluconate/phosphoketolase pathway leading to lactic acid and acetic acid/ethanol. Some lactobacilli such as *Lb. salivarius* (Raibaud et al. 1973) or *Lb. vini* (Rodas et al. 2006) can ferment pentoses homofermentatively. Some strains can produce acetate, ethanol and formate from pyruvate under low substrate concentrations and strictly anaerobic conditions (Hammes and Vogel 1995). Lactic acid bacteria form D(−) or L(+) lactic acid or a racemic mixture of lactic acid isomers (Kandler 1983).

The Embden–Meyerhof–Parnas pathway is used by lactobacilli (group I and II; Table 1.3) and pediococci, while group III of lactobacilli, leuconostocs and oenococci use the 6-phosphogluconate/phosphoketolase pathway (other designations: pentose phosphate pathway, pentose phosphoketolase pathway, hexose

monophosphate pathway). Changes in the end product composition can be influenced by environmental factors. Depending on the growth conditions the end products of homofermenters can be changed largely. In addition to glucose, the hexoses mannose, fructose and galactose may be fermented after isomerisation and/or phosphorylation. Galactose is used via the tagatose pathway by e.g. *Lb. casei*.

Under anaerobic conditions pyruvate can be metabolized by *Lb. casei* to formate and acetate/ethanol (pyruvate formate lyase system) under glucose limitation. End produts are lactate, acetate, formate and ethanol (mixed acid fermentation). Under aerobic conditions *Lb. plantarum* can convert pyruvate to CO_2 and acetyl phosphate with a pyruvate oxidase (Sedewitz et al. 1984).

Flavin-containing enzymes such as NADH:H_2O_2 oxidase and NADH:H_2O oxidase (Condon 1987) can occur in lactic acid bacteria. Oxygen acts as external electron acceptor. Oxygen-dependent glycerol fermentation by *P. pentosaceus* and mannitol fermentation of *Lb. casei* are examples. An oxygen-dependent lactate metabolism has been proposed for *Lb. plantarum* involving NAD^+-dependent and/or NAD^+-independent lactate dehydrogenase, a pyruvate oxidase and an acetate kinase (Murphy et al. 1985). The defense system against in vitro oxidative stress includes the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging ability, reactive oxygen species (ROS) scavenging ability, iron ion chelation (FE), glutathione system, ferric reducing ability of plasma (FRAP), reduction activity (RA), inhibition of ascorbic oxidation (TAA), and linoleic acid oxidation (TLA) abilities (Su et al. 2015).

Lactobacilli interact with oxygen. Some lactic acid bacteria use high intracellular manganese concentration for protection against superoxide (30–35 mM; Archibald 1986). Theobald et al. (2005) found a growth stimulation of *O. oeni* at concentrations of 68 μM or 34 mM manganese in the growth medium. In some strains 34 mM manganese could replace tomato juice. Other compounds are also stimulatory for oenococci (Theobald et al. 2007a, b).

Flavin-containing enzymes such as NADH:H_2O_2 oxidase and NADH:H_2O oxidase (Condon 1987) can occur in lactic acid bacteria. Oxygen acts as external electron acceptor. Oxygen-dependent glycerol fermentation by *P. pentosaceus* and mannitol fermentation of *Lb. casei* are examples. An oxygen-dependent lactate metabolism has been proposed for *Lb. plantarum* involving NAD^+-dependent and/or NAD^+-independent lactate dehydrogenase, a pyruvate oxidase and an acetate kinase (Murphy et al. 1985).

Citrate can lead to diacetyl/acetoin formation if the excess of pyruvate is reduced to lactic acid. Oxaloacetate can also function as electron acceptor leading to succinic acid formation when *Lb. plantarum* was grown on mannitol (Chen and McFeeters 1986). *Lb. brevis* and *Lb. buchneri* can use glycerol as electron acceptor in an anaerobic cofermentation with glucose leading to lactate, acetate, CO_2 and 1.3-propandiol (Schütz and Radler 1984a, b). Fructose can be fermented via the 6-phosphocluconate/phosphoketolase pathway and function as electron acceptor to yield mannitol by *Lb. brevis* (Eltz and Vandemark 1960). Malic acid can be used as sole energy source by *Lb. casei* yielding acetate, ethanol and CO_2 or it can be

converted to L-lactate and CO_2 (malolactic fermentation) by e.g. *O. oeni* (Radler 1975). The biosynthesis of amino acids in lactic acid bacteria is limited. Some have peptidases and can hydrolyse proteins. Lactic acid bacteria can also perform chemical cell communication (Nakayama and Sonomoto 2002).

Adaptation of lactobacilli to harsh environmental conditions concern: synthesis of heat-shock proteins, key enzymes of glycolytic pathways, the glutamate decarboxylase system, homoeostasis of intracellular pH, alkalization of the external environment, DNA and protein damage repair, changes in cell membrane composition, changes in cytosolic and surface-located proteins, the fatty acid contents of the cytoplasmic membrane, cell wall biosynthesis, transport of peptides, coenzyme levels and membrane H+-ATPase (Hussain et al. 2013).

1.5 Genetics

The genome size of lactic acid bacteria varies (Morelli et al. 2004). The total genome of about 211 species/strains of the genera *Lactobacillus* (genome size: 1.27765–4.87232 Mb), *Leuconostoc* (genome size: 1.63897–2.29809 Mb), *Oenococcus* (genome size: 1.15038–1.84224 Mb), *Pediococcus* (genome size: 1.76496–2.50947 Mb) and *Weissella* (genome size: 1.33444–2.57773 Mb) is available, including all wine-relevant species (Table 1.1; Makarova et al. 2006; https://www.ncbi.nlm.nih.gov/genome, February 2017). For example, the genome of *Lb. paracasei* ATCC 334 consists of 2.17 Mb (Ferrero et al. 1996) and that of *Lb. plantarum* CCM 1904 of 3.4 Mb (Chevallier et al. 1994). Genome sequences of *O. oeni* strains have been determined (Jara and Romero 2015).

Lactic acid bacteria (LAB) have about 2000 genes in average. They have lost ca. 1000 genes during separation from the ancestral Bacilli during evolution. The lost genes coded for sporulation, cofactors, heme cytochromes and catalase. LAB have also acquired about 86 new genes by gene duplication and horizontal gene transfer regarding e.g. murein and B12 biosynthesis, novel functions of genes coding usually for antibiotic resistance, phage defense mechanisms and IS elements (Morelli et al. 2012).

Lactic acid bacteria possess circular as well as linear plasmids associated with carbohydrate fermentation and proteinase activities, bacteriocin production, phage defense mechanisms, and antibiotic resistance mechanisms (Morelli et al. 2004, 2012).

Phages have been found with the wine-related species of *Lactobacillus* (*Lb. casei*, *Lb. fermentum*, *Lb. plantarum*,), *Leuconostoc* (*Lc. mesenteroides*) and *Oenococcus* (*O. oeni*) (Josephsen and Neve 2004). They can cause stuck malolactic fermentation (Poblet-Icart et al. 1998).

1.6 Activities in Must and Wine

Lactic acid bacteria are involved in food and feed fermentation and preservation as well as food digestion in the intestinal tracts of humans and animals. Due to their tolerance against ethanol and acidic conditions, LAB can grow in must. Generally they are inhibited at ethanol concentrations above 8 vol%, but *O. oeni* tolerates 14 vol% and *Lb. brevis*, *Lb. fructivorans* and *Lb. hilgardii* can be found even in fortified wines up to an ethanol concentration of 20 vol%. Slime-producing strains of *P. damnosus* grow up to 12 vol% of ethanol. Lactic acid bacteria isolated from wine grow between 15 and 45 °C in the laboratory with an optimal growth range between 20 and 37 °C. Best growth in must during malolactic fermentation is obtained around 20 °C. During the first days of must fermentation the CFU of LAB increases from 10^2 to 10^4–10^5 ml^{-1}. After the alcoholic fermentation and during the malic acid fermentation, the cell number can reach a titer of 10^7–10^8 CFU per ml (Ribérau-Gayan et al. 2006a, b). The titer of different lactic acid species during alcoholic fermentation has been determined by Lonvaud-Funel et al. (1991): *O. oeni*, 3.4×10^6 (day 13, alcohol content: 18 vol%); *Lc. mesenteroides*, 9.6×10^4 (day 6, alcohol content: 9 vol%); *P. damnosus*, 3.8×10^4 (day 3, alcohol content: 7 vol%); *Lb. hilgardii*, 8.0×10^4 (day 3, alcohol content: 7 vol%); *Lb. brevis*, 2.0×10^4 (day 3, alcohol content: 7 vol%) and *Lb. plantarum*, 2.0×10^4 (day 3, alcohol content: 7 vol%).

Lactic acid bacteria gain their energy mainly from sugar fermentation. They use both main hexoses of the wine, glucose and fructose, as energy and carbon source. In this respect they are competitors of the ethanol producing yeast *Saccharomyces cerevisiae*. The heterofermentative LAB in wine can also use the pentoses (arabinose, xylose, ribose), which occur in minor concentrations in wine.

Lactic acid bacteria also metabolize the three main acids of must: tartrate, malate and citrate. Citrate is converted to lactate, acetic acid, CO_2 and acetoin. Malate is converted to L-lactate and CO_2 (malolactic fermentation). Especially in northern countries, where must can have high acidity, the biological reduction with starter cultures of *O. oeni* is an important step in vinification. The malolactic enzyme has been found in many lactic acid bacteria occurring in wine (e.g. *Lb. casei*, *Lb. brevis*, *Lb. buchneri*, *Lb. delbruechii*, *Lb. hilgardii*, *Lb. plantarum*, *Lc. mesenteroides, and O. oeni*). *O. oeni* is applied for reduction of the malic acid content because of its high tolerance against ethanol and acidity. Indigenous *P. damnosus* strains were found to perform malolactic fermentation into Albariño and Caiño wines (Spain) without negative effects on the wine (Juega et al. 2014). Malolactic fermentation and the use of sugars can lead to a more stable wine. *Lb. plantarum* could be an alternative species to *O. oeni* for performing malolactic fermentation (Bravo-Ferrada et al. 2013). Tartrate can be converted to lactate, acetate and CO_2 by the homofermentative lactic acid bacterium *Lb. plantarum* and to acetate and CO_2 or fumaric acid (succinic acid) by the heterofermentative lactic acid bacterium *Lb. brevis* (Radler and Yannissis 1972).

Lactic acid bacteria produce different biogenic amines. *O. oeni*, *P. cerevisiae* and *Lb. hilgardii* (Landete et al. 2005; Mangani et al. 2005; Kaschak et al. 2009; Sebastian et al. 2011; Christ et al. 2012) are examples of producers of biogenic amines. The most important is histamine, which is produced by decarboxylation of histidine. The COST Action 917 (2000–2001) of the EU "Biologically active amines in food" suggested prescriptive limits for histamine (e.g. France: 8 mg l^{-1}, Germany: 2 mg l^{-1}) in wines. Biogenic amines can cause health problems (Coton et al. 1998) and sensory defects in wine (Lehtonen 1996; Palacios et al. 2004). From arginine, ammonium is liberated by heterofermentative species such as *Lb. higardii* and *O. oeni*, but also by facultatively heterofermentative species like *Lb. plantarum*. The highest citrulline production in Malbec wine could be correlated with its lower concentrations of glucose, fructose, citric and phenolic acid than the other wines. Therefore, a wine with lower concentration of these sugars and acids could be dangerous due to the formation of ethyl carbamate precursors. The degradation if arginine proceeds via citrulline that forms with ethanol the carcinogen ethyl carbamate. Phenolic compounds could decrease the arginine consumption (protocatechuic acid, gallic acid) or increase (quercetin, rutin, catechin, caffeic acid, vanillic acids). Arginine deiminase activity was stimulated by rutin, quercetin, caffeic acid and vanillic, while gallic acid and protocatechuic acids inhibited this enzyme activity (Alberto et al. 2012; Araque et al. 2016). Nuclear magnetic resonance (NMR) spectroscopy is a tool to follow the transformation of histidine into histaminol and into histamine during alcoholic and malolactic fermentations and consequently to select suitable strains for malolactic fermentation (López-Rituerto et al. 2013). On the other hand biogenic amines such as histamine, tyramine, and putrescine can be degraded by lactic acid bacteria (e.g. *Lb. plantarum*, *P. acidilactici*) (Callejón et al. 2014), which is also true for some yeasts (Bäumlisberger et al. 2015). Strains of *Lb. plantarum* were selected because of their ability to degrade putrescine and tyramine (Capozzi et al. 2012). Although at different extent, 25% of the LAB especially *Lactobacillus* and *Pediococcus* strains were able to degrade histamine, 18% tyramine and 18% putrescine, whereas none of the commercial malolactic starter cultures or type strains were able to degrade any of the tested amines. The application of some lactic acid bacteria could be a promising strategy to reduce biogenic amines in wine (García-Ruiz et al. 2011a).

Lactic acid bacteria have an influence on the flavour of wine, because they can produce acetic acid, diacetyl, acetoin, 2,3-butandiol, ethyl lactate, diethyl succinate and acrolein. The ability of wine lactobacilli to accumulate 3-hydroxypropionaldehyde (3-HPA), a precursor of acrolein, from glycerol in the fermentation media was demonstrated (Bauer et al. 2010). Lactic acid bacteria can also cause a decrease in colour up to 30%. In German wines 1.08 g acetic acid per l white wine or 1.20 g acetic acid per l red wine are the upper limits for acetic acid, while e.g. "Beerenauslese" (German quality distinction) can even have higher concentrations. The natural value is 0.3–0.4 g l^{-1} and it becomes sensory-significant at concentrations above 0.6 g l^{-1}. Aerobic acetic acid bacteria, facultatively anaerobic heterotrophic lactic acid bacteria, yeast under difficult fermentation conditions and *Botrytis cinerea* on infected grapes are the potential producers.

Fructose is reduced to mannitol or converted to erythrol and acetate. Heterofermentative lactic acid bacteria can produce higher concentrations of acetic acid (>0.6 g l^{-1}), especially in the absence of pantothenic acid (Richter et al. 2001). Lactic acid bacteria can convert sorbic acid, which is used because of its antifungal properties, to 2-ethoxy-3.5-hexadiene (geranium-like odour) (Crowel and Guymon 1975). Glycerol is converted to propandiol-1.3 or allylalcohol and acrolein leading to bitterness (Schütz and Radler 1984a, b). Off-flavour is produced by *O. oeni* from cysteine and methionine. Cysteine is transformed into hydrogen sulfide or 2-sulfanyl ethanol and methionine into dimethyl disulfide, propan-1-ol, and 3-(methasulfanyl) propionic acid. They increase the complexity of the bouquet. The latter has an earthy, red-berry fruit flavour (Ribéreau-Gayon et al. 2006a, b). Lactic acid bacteria may produce a smell reminiscent of mice (mousiness). Species of *Lactobacillus* such as *Lb. brevis*, *Lb. hilgardii* and *Lb. fermentum* produce 2-acetyltetrahydropyridine (perception threshold: 1.6 ng l^{-1}) from ethanol and lysine (Heresztyn 1986). Also 2-acetyl-1-pyrroline and 2-ethyltetrahydropyridine can contribute to this off-flavour (Costello and Henschke 2002). Ethyl carbamate is produced from urea and ethanol by *O. oeni* and *Lb. hilgardii* (Uthurry et al. 2006; Arena et al. 2013), which probably is carcinogenic. Lactic acid bacteria possess esterases for the synthesis and hydrolysis of esters (Sumby et al. 2013). *Lb. plantarum* posseses arylesterase which showed high hydrolytic activity on phenyl acetate and lower activity on other relevant wine aroma compounds (Esteban-Torres et al. 2014). Commercial strains of *Oenococcus oeni* and *Lb. plantar*um synthesize flavour active fatty acid ethyl esters with the aid of an acyl coenzyme A: alcohol acyltransferase (AcoAAAT) activity and a reverse esterase activity leading to an increased ethyl ester content of wine (Costello et al. 2013). The polyphenol flavan-3-ol was metabolized by *Lb. plantarum* to phenylpropionic acids (Barroso et al. 2014). In general, flavonols and stilbenes showed the greatest inhibitory effects among wine polyphenols on *O. oeni*, *Lb. hilgardii* and *P. pentosaceus* (García-Ruiz et al. 2011b). The proteome of *Oenococcus oeni* was studied to get hints about metabolic activities that can modify the taste and aromatic properties of wine (Mohedano et al. 2014). *Lb. plantarum* converted p-coumaric acid to volatile phenolic compound 4-vinylphenol under wine related conditions (Fras et al. 2014), reactions described earlier to be performed by intestinal bacteria of termites Kuhnigk et al. 1994). Hydroxycinnamic acids stimulated the production of the volatile phenolic compound 4-vinylphenol from p-coumaric acid by the LAB test strains *Lb. plantarum*, *Lb. collinoides* and *P. pentosaceus* (Silva et al. 2011). Isolates belonging to the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Enterococcus* exhibited intracellular esterase activities using p-nitrophenyl octanoate as test compound. The esterase activity was decreased by increasing ethanol concentrations (Pérez-Martín et al. 2013).

Polysaccharide production (Claus 2007) leads to graisse of the must, which causes problems during filtration. *O. oeni* synthesizes homo- and heteropolysaccharides which are important for the adaptation to the wine environment, but also may influence the wine structure (Dimopoulou et al. 2012). *P. damnosus* increases viscosity. It produces a glucose homopolymer. The

repeating unit is a β-1.3 linked glucose disaccharide carrying a β-1.2 linked glucose site group [3)-β-D-Glc*p*-(1.3)-[β-D-Glc*p*-(1.2)]-β-D-Glc*p*-(1] (Llaubères et al. 1990; Dueñas et al. 2003). The viscosity, which is influenced by many factors such as the ethanol concentration and temperature, becomes apparent at 10^7 colony forming units. A lytic enzyme for the hydrolysis of the slime produced by *P. parvulus* has been described (Blättel et al. 2011). β-D-Glucosidase activity occurred intracellularly in lactic acid bacteria (Mesas et al. 2012; Pérez-Martín et al. 2012). The application with lysozyme and β-glucanase leads to an improved treatment against glycan producing strains strains (Coulon et al. 2012). Of course, some phenolic compounds are inhibitory for lysozyme (Guzzo et al. 2011). When the S-layer was removed, the corresponding *Lb. hilgardii* B706 cells became more sensitive to bacteriolytic enzymes and some wine-related stress conditions (Dohm et al. 2011).

Lactic acid disease occurs at higher sugar concentrations when lactic acid bacteria grow during ethanolic fermentation at higher pH values and low nitrogen concentrations. Higher amounts of acetic acid can be produced, which hampers the activities of yeast. Most often, LAB do not multiply or disappear during alcoholic fermentation, except oenococci, which resist at low cell levels. It was found that fatty acids (hexanoic, octanoic and decanoic acid) liberated by growing yeast have a negative effect on bacterial growth (Lonvaud-Funel et al. 1988). Oenococci can grow during the stationary/death phase of the yeasts after alcoholic fermentation, when released cell constituents of yeasts stimulate bacterial growth. In this stage oenococci have an influence on yeast lysis by producing glycosidases and proteases.

The degradation of sugars and acids contributes to the microbial stabilisation of wine by removing carbon and energy substrates. Low concentrations of diacetyl increase the aromatic complexity. If the concentration of volatile acids increases 1 g l^{-1} the lactic disease becomes apparent, which can lead to a stuck alcoholic fermentation.

Lactic acid bacteria potentially produce antimicrobial components (Rammelberg and Radler 1990; Blom and Mörtvedt 1991) such as acetic acid, higher concentrations of carbon dioxide, hydrogen peroxide, diacetyl, pyroglutamic acid and bacteriocins, which inhibit the growth of other bacterial and yeast species. The production of bacteriocins by wine lactobacilli and *L. mesenteroides* is important for the production of wine aroma and combating other spoilage lactobacilli or controlling the malolactic fermentation (Du Toit et al. 2011; Dündar et al. 2016). Brevicin from *Lb. brevis* inhibits growth of *O. oeni* and *P. damnosus* (Rammelberg and Radler 1990). The malolactic fermentation and the consumption of nutrients (hexoses and pentoses) as well as the production of bacteriocines (De Vuyst and Vandamme 1994) lead to a stabilization of wine. Compared to *O. oeni* *Lb. plantarum* possesses more genes encoding for glycosidases, proteases, esterases, phenolic acid decarboxylases and citrate lyases and bacteriocins (plantaricins).

Analysis with DNA microarrays and proteomic techniques revealed that genes associated with the amino acid, the malate and the citrate metabolism, the synthesis

of certain cell wall proteins were up, but genes related to carbohydrate metabolism were down regulated under wine making conditions. In addition, the thioredoxin and glutathione systems played an adaptive function for life (Margalef-Català et al. 2016).

During incubation with proteins and polypeptides obtained from Cabernet Sauvignon and Syrah wines *O. oeni* excreted a proteolytic activity. The produced peptides enhanced the beneficial biological activities in respect to antioxidant and antihypertensive status of the wine (Apud et al. 2013a, b). *O. oeni* could give additional value to wine because of the bioactive peptides from yeast autolysates with multifunctional beneficial activity released as consequence of its proteolytic activity (Aredes Fernández et al. 2011).

The viability of the cells of *O. oeni* is increased when microcolonies are formed. *O. oeni* forms microcolonies on stainless steel and oak chip surfaces with extracellular substances (Bastard et al. 2016). Cell in biofilms possessed increased tolerance to wine stress, and performed effective malolactic activities. Biofilm of *O. oeni* can modulate the wood-wine transfer of volatile aromatic compounds and influence the aging process by decreasing furfural, guaiacol, and eugenol. Most likely, the biofilms consists of polysaccharides, because *O. oeni* produces cell-linked exopolysaccharides (EPS) consisting of glucose, galactose and rhamnose as well as soluble β-glucan and soluble dextran or levan polymers (Dimopoulou et al. 2016). In addition, heat shock proteins contribute to stress reduction under wine conditions. Beside polysaccharide formation heat shock proteins play a role in acid tolerance. Darsonval et al. (2015) applied the antisense RNA approach to revealed the function of the small heat stress protein (HSP) Lo18 of *O. oeni*. They found that Lo18 is involved in heat and acid tolerance, which was explained by its membrane-protective role. The heat shock protein Hsp20 is over-expressed (Olguín et al. 2015; Costantini et al. 2015). Nevertheless, high ethanol concentrations in wine have an effect on metabolite transport as well as cell wall and membrane biogenesis.

The development of certain bacterial and yeast starter cultures for wines with special features is a continuous challenge (du Toit et al. 2011; Sumby et al. 2014). Multicolor capillary electrophoresis was performed to derive genotypic and phenotypic characters from fragment length analysis (FLA) profiles (Claisse and Lonvaud-Funel 2014). To improve strain selection a typing scheme for *O. oeni* using multiple-locus variable number of tandem repeat analysis was developed (Claisse and Lonvaud-Funel 2012). In this context it is desirable to find links between *O. oeni* metabolism, genomic diversity and wine sensory attributes (Bartowsky and Borneman 2011). The genomic diversity is well known among *O. oeni* strains, which possess variations in the starter-culture efficiency.

Some undesirable lactic acid bacteria from wine samples have other positive features. A *P. parvulus* strain that was isolated from Douro wines was able to degrade the prominent mycotoxin Ochratoxin A (OTA) (Abrunhosa et al. 2014) and *P. pentosaceus* exhibited a potential as probiotic (García-Ruiz et al. 2014). Also some unwanted compounds such as copper can be adsorbed of by wine-relevant lactobacilli. About 0.5–1.0 μg copper per ml could be removed from wine samples, which is sufficient enough to lower critical copper concentrations. The highest

binding capacity of the tested lactic acid bacteria was found with *Lb. buchneri* DSM 20057 with a maximum of 46.17 µg copper bound per mg cell in deionized water. (Schut et al. 2011).

1.7 Characteristics of Genera and Species of Wine-Related Lactic Acid Bacteria

1.7.1 Genus Lactobacillus

Lactobacillus is one of the most important genus involved in food microbiology and human nutrition, owing to their role in food and feed production and preservation, as well as their probiotic properties. In October 2016 this genus contained in total 189 validly described species (DSMZ 2016a). In addition, several species consist of well characterized subspecies. *Lactobacillus* species live widespread in fermentable material. Lactobacilli contribute to the flavour of fermented food by the production of diacetyl, H_2S and amines. They play a role in the production as well in the spoilage of food (sauerkraut, silage, dairy and meat as well as fish products) and beverages (beer, wine, juices) (Kandler and Weiss 1986; Hammes et al. 1991).

Lactobacilli are straight gram-positive non-motile or rarely motile rods (e.g. *Lb. mali*), with a form sometimes like coccobacilli. Chains are commonly formed. The tendency towards chain formation varies between species and even strains. It depends on the growth phase and the pH of the medium. The length and curvature of the rods depend on the composition of the medium and the oxygen tension. Peritrichous flagellation occurs only in a few species, which is lost during growth in artificial media. They are aciduric or acidophilic. The maximum for growth pH is about 7.2.

The murein sacculi possess various peptidoglycan types (Lys-D-Asp, m-Dpm-direct, Orn-D-Asp, Lys-Ala, Lys-Ala_2, Lys-Ala-Ser, Lys-Ser-Ala_2) of group A (DSMZ 2016c). Polysaccharides are often observed. Membrane-bound teichoic acids are present in all species and cell wall-bound teichoic acids in some species (Schleifer and Kandler 1972).

The G+C content of the DNA ranges from 32 to 53 mol%.

Lactobacilli are strict fermenters. They can tolerate oxygen or live anaerobic. They have complex nutritional requirements for carbohydrates, amino acids, peptides, fatty acids, nucleic acid derivatives, vitamins and minerals.

Some species possess a pseudocatalase and some strains can take up porphorinoids and then exhibit catalase, nitrite reductase and cytochrome activities.

They gain energy by homofermentative or heterofermentative carbohydrate fermentation in the absence or presence of oxygen. An energy source is also the conversion of carbamyl phosphate to CO_2 and NH_3 during arginine degradation. They possess flavine-containing oxidases and peroxidases to carry out an oxidation

with O_2 as the final electron acceptor. The pathways of sugar fermentation are the Embden-Meyerhof pathway converting 1 mol hexose to 2 mol lactic acid (homolactic fermentation) and the phosphoketolase pathway (heterolactic fermentation) resulting in 1 mol lactic acid, ethanol/acetate and CO_2. Pyruvate produced during hexose fermentation may be converted to lactate, but also to other products such as diacetyl or acetic acid, ethanol and formate/CO_2. In the presence of oxygen, lactate can be converted to pyruvate and consequently to acetic acid and CO_2 or acetate and formate. The conversion of glycerol to 1,3-propanediol with glucose serving as electron donor was observed in *Lb. brevis* isolated from wine (Schütz and Radler 1984a, b). The homofermentative species possess an FDP aldolase, while the heterofermentative species have a phosphoketolase. The facultative heterofermenters possess an inducible phosphoketolase. Heterofermentative species can also use pentoses as substrate. Some homofermenters use pentoses homofermentatively (Rodas et al. 2006). Strains of *Lactobacillus kunkeei* turned out to be fructophilic lactic acid bacteria (Endo et al. 2012).

Sucrose is also used for the formation of dextrans with the help of dextran sucrase. Fructose can serve as electron acceptor and mannitol is formed by heterofermentative species. Monomeric sugars and saccharides are taken up by permeases or the phosphotransferase system. They are split inside the cell by glycosidases. Galactose-6-phosphate from lactose phosphate is fermented via the tagatose-6-phosphate pathway (Kandler 1983). Several organic acids such as citric acid, tartaric acid or malic acid are degraded (Radler 1975). Several amino acids are decarboxylated to biogenic amines.

Depending on the stereospecificity of the lactate dehydrogenase or the presence of an inducible lactate racemase lactate may have the D(−) or L(+) configuration. The lactate dehydrogenases can differ with respect to electrophoretic mobility and kinetic properties. Some enzymes are allosteric with FDP and Mn^{2+} as effectors.

Plasmids linked to drug resistance or lactose metabolism are often found (Smiley and Fryder 1978). Double-stranded DNA phages have been isolated (Sozzi et al. 1981) and lysogeny is widespread (Yokokura et al. 1974). Strains producing bacteriocins (lactocins) have been found among the homo- and heterofermentative species (Tagg et al. 1976). Several serological groups have been designed. From the species in must, *Lb. plantarum* belongs to group D (antigen: ribitol teichoic acid), *Lb. fermentum* to group F and *Lb. brevis* to group E (Archibald and Coapes 1971).

The complete genome of 173 *Lactobacillus*-species/strains has been sequenced; it includes all the wine related species of the genus *Lactobacillus* (http://www.ncbi.nlm.nih.gov/genome, February 2017).

Some characteristics of the species are compiled in Table 1.3. A combination of physiological and biochemical as well as molecular tests are required for the unambiguous identification of *Lactobacillus* species (Pot et al. 1994; Hammes and Vogel 1995). The validly published species of the genus *Lactobacillus* have been assigned to nine groups (cf. Table 1.3) (Yang and Woese 1989; Collins et al. 1991; Hammes et al. 1991; Hammes and Vogel 1995; Dellaglio and Felis 2005). Out of 189 validly described species, eighteen species have been found in must and wine (Table 1.3) (Ribéreau-Gayon et al. 2006a, b; Fugelsang and Edwards 2007).

The type species is *Lb. delbrueckii* DSM 20074^T.

Lb. brevis
Morphology: Rods. 0.7–1.0 μm × 2.0–4.0 μm. Single or chains.
Isolation: Milk, cheese, sauerkraut, sourdough, silage, cow manure, mouth, intestinal tract of humans and rats, grape must/wine.
Type strain: DSM 20054.

Lb. buchneri
Morphology: Rods. 0.7–1.0 μm × 2.0–4.0 μm. Single or short chains.
Characteristics: As described for *Lb. brevis* except the additional fermentation of melezitose and the distinct electrophoretic behaviour of L-LDH and D-LDH.
Isolation: Milk, cheese, plant material and human mouth, grape must/wine.
Type strain: DSM 20057.

Lb. casei
Morphology: Rods. 0.7–1.1 μm × 2.0–4.0 μm.
Isolation: Milk, cheese, dairy products, sour dough, cow dung, silage, human intestinal tract, mouth and vagina, sewage, grape must/wine.
Type strain: DSM 20011.

Lb. cellobiosus
→ *Lb. fermentum*.

Lb. curvatus
Morphology: Bean-shaped rods. 0.7–0.9 μm × 1.0–2.0 μm. Pairs, short chains or close rings. Sometimes motile.
Characteristics: LDH is activated by FDP and Mn^{2+}. Lactic acid racemase.
Isolation: Cow dung, milk, silage, sauerkraut, dough, meat products, grape must/wine.
Type strain: DSM 20019 (subsp. *curvatus*).

Lb. delbrueckii
Morphology: Rods. 0.5–0.8 μm × 2.0–9.0 μm. Single or in short chains.
Isolation: Milk, cheese, yeast, grain mash, grape must/wine.
Type strain: DSM 20072 (subsp. *lactis*).

Lb. diolivorans
Morphology: Rods. 1.0 μm × 2.0–10.0 μm. Single, pairs and short chains.
Isolation: Maize silage, grape must/wine.
Type strain: DSM 14421.

Lb. fermentum
Morphology: Rods. Diameter 0.5–0.9 μm, length variable. Single or pairs.
Isolation: Yeast, milk products, sourdough, fermenting plant material, manure, sewage, mouth and faeces of man, grape must/wine.
Type strain: DSM 20052.

Lb. florum
Morphology: Rods. 0.8 μm × 1.5–7 μm. Single, pairs, chain.
Characteristics: Catalase negative, except in the presence of sheep blood. Heterofermentative. Production of D,L-lactic acid, ethanol and acetic acid from D-glucose. Nitrate not reduced. Acid production only from D-glucose and D-fructose out of 49 tested sugars. Fructophilic. No acid production from L-arabinose, D-arabitol, *N*-acetylglucosamine, maltose, ribose, D-arabinose, L-arabitol, adonitol, amygdalin, arbutin, cellobiose, dulcitol, aesculin, erythritol, D-fucose, L-fucose, β-gentiobiose, 2- and 5-ketogluconate, methyl α-D-glucoside, glycerol, glycogen, inositol, inulin, D-lyxose, D-mannose, methyl α-D-mannoside, melezitose, raffinose, rhamnose, sucrose, salicin, starch, sorbitol, L-sorbose, D-tagatose, trehalose, turanose, xylitol, L-xylose, methyl β-xyloside, D-galactose, lactose, mannitol, melibiose or D-xylose. No dextran production from sucrose. Growth at 300 g D-fructose per l, between pH 4.0–8.0, in the presence of 5% (w/v) NaCl and at 15 °C, but not at 45 °C. Pyruvate stimulatory. Murein lacks meso-diaminopimelic acid. DNA G+C content: 42 mol%.
Isolation: South African peony and bietou flowers, grape, wine
Type strain: DSM 22689

Lb. fructivorans
Morphology: Rods. 0.5–0.8 μm × 1.5–4.0 μm (occasionally 20 μm). Single, pairs, chains or long curved filaments.
Isolation: Spoiled mayonnaise, salad dressing, vinegar preserves, spoiled sake, dessert wine and aperitifs.
Type strain: DSM 20203.

Lb. heterohiochii
→ *Lb. fructivorans*.

Lb. hilgardii
Morphology: Rods. 0.5–0.8 μm × 2.0–4.0 μm. Single, short chains or long filaments.
Isolation: Wine samples.
Type strain: DSM 20176.

Lb. jensenii
Morphology: Rods. 0.6–0.8 μm × 2.0–4.0 μm. Single or short chains.
Isolation: Human vaginal discharge and blood clot, grape must/wine.
Type strain: DSM 20557.

Lb. kunkeei
Morphology: Rod. 0.5 μm × 1.0–1.5 μm.
Characteristics: Week catalase activity.
Isolation: Commercial grape wine undergoing a sluggish/stuck alcoholic fermentation.
Type strain: DSM 12361.

Lb. leichmannii
→ *Lb. delbrueckii* subsp. *lactis*

Lb. mali
Morphology: Slender rods. 0.6 μm × 1.8–4.2 μm, Single, in pairs, palisades and irregular clumps.
Characteristics: Motile by a few peritrichous flagella. Pseudocatalase activity in MRS medium containing 0.1% glucose. Menaquinones with predominantly eight or nine isoprene residues.
Isolation: Apple juice, cider and wine must.
Type strain: DSM 20444.

Lb. nagelli
Morphology: Rods. 0.5 μm × 1.0–1.5 μm.
Characteristics: Nitrate reduction.
Isolation: Partially fermented wine with sluggish alcoholic fermentation.
Type strain: DSM 13675.

Lb. oeni
Morphology: Rods, 0.63–0.92 μm × 1.38–3.41 μm, single, pairs, chains
Characteristics: motile, catalase negative, growth between 15 and 45 °C and pH 4.5–8.0, no growth at 5 °C and pH 3.3. Heterofermentative. Transformation of L-malic acid into L-lactic acid. Gluconate or ribose not fermented. L-Lactate formed from hexoses. Ammonia is not produced from arginine. Fructose not reduced to mannitol. Exopolysaccharide production from sucrose. Acid produced from N-acetylglucosamine, fructose, glucose, mannose, mannitol, sorbitol, L-sorbose, methyl a-D-glucoside and trehalose. No acid production from adonitol, amygdalin, D- or L-arabinose, D- or L-arabitol, arbutin, cellobiose, dulcitol, erythritol, D- or L-fucose, galactose, gluconate, 2-or 5-ketogluconate, glycogen, inositol, inulin, D-lyxose, lactose, maltose, melezitose, melibiose, raffinose, rhamnose, ribose, starch, sucrose, D-tagatose, turanose, xylitol, D- or L-xylose, methyl a-D-mannoside or methyl bxyloside. Aesculin not hydrolysed. Variable usage of glycerol, salicin and gentiobiose. Murein contains D-meso-diaminopimelic acid. DNA G+C content 37.17 mol%.
Isolation: Bobal grape wine
Type strain: DSM 19972

Lb. paracasei
Morphology: Rods. 0.8–1.0 μm × 2.0–4.0 μm. Single or chains.
Isolation: Dairy products, silage, humans, clinical sources, grape must/wine.
Type strain: DSM 5622 (subsp. *paracasei*).

Lb. plantarum
Morphology: Rods. 0.9–1.2 μm × 3.0–8.0 μm. Single, pairs or short chains.
Characteristics: Nitrate can be reduced under glucose limitation and a pH above 6.0. A pseudocatalase may be produced especially under glucose limitation. A ribitol or glycerol teichoic acid can be present in the cell walls.

Isolation: Dairy products, silage, sauerkraut, pickled vegetables, sourdough, cow dung, human mouth, intestinal tract and stool, sewage and grape must. Type strain: DSM 20174.

Lb. trichodes
→ *Lb. fructivorans*.

Lb. vermiforme
→ *Lb. hilgardii*.

Lb. vini
Morphology: Rods. 0.49–0.82 μm × 1.36–2.8 μm. Single, in pairs or in short chains. Motile.
Characteristics: Uses ribore and arabinose homofermentatively. Catalase-negative. Exopolysaccharide is not produced from sucrose.
Isolation: Fermenting grape must.
Type strain: DSM 20605.

Lb. yamanashiensis
→ *Lb. mali*

1.7.2 Genus Leuconostoc

Leuconostocs thrive on plants and sometimes in milk, milk products, meat, sugar cane and other fermented food products. One species, *Lc. mesenteroides,* has been isolated from must. It is nonhemolytic and nonpathogenic to plants and animals (Garvie 1986a). *Leuconostocs* are heterofermentative cocci producing only D-lactic acid from glucose and are unable to produce ammonia from arginine (Björkroth and Holzapfel 2006).

Leuconostocs form spherical or lenticular cells, pairs or chains. The peptidoglycan belongs to type A. The interpeptide bridge of the peptidoglycan consists of Lys-Ser-Ala_2 or Lys-Ala_2.

Sugars are fermented by the 6-P-gluconate/phosphoketolase pathway with D-lactic acid, ethanol/acetate and CO_2 as end products. NAD^+ or $NADP^+$ will serve as coenzyme of the glucose-6-phosphate dehydrogenase. During malolactic fermentation malate is degraded to L-lactate and CO_2. Cells are nonproteolytic. Nitrate is not reduced.

Cells grow in a glucose medium as elongated cocci. Cells are found singly or in pairs, and form short to medium length chains. On solid media, cells form short rods.

Leuconostocs share many features with the heterofermentative lactobacilli (Dellaglio et al. 1995).

Dextrans, which are of industrial importance, are produced by leuconoctocs, especially *Lc. mesenteroides,* from sucrose as substrate.

Leuconostoc species were divided by Garvie (1960) into six different groups according to the fermentation of 19 carbohydrates. Electrophoretic mobilities of enzymes e.g. LDHs, cell protein pattern, cellular fatty acids, DNA base composition and DNA homology are applied for differentiation of the species (Dellaglio et al. 1995). Citrate metabolisms of *Lc. mesenteroides* subsp. *mesenteroides* might be plasmid linked (Cavin et al. 1988). No other phenotypic features were found to be coded on plasmids, while plasmids of *Lactobacillus* and *Pediococcus* code for sugar utilisation, proteinase, nisin, bacteriocins production, drug resistance, slime formation, arginine hydrolysis and bacteriophage resistance (Dellaglio et al. 1995).

Leuconostocs play a role in the organoleptic quality and texture of food such as milk, butter, cheese, meat and wine. *Leuconostocs* can also spoil food, but they often contribute to the flavour of dairy products due to the production of diacetyl from citrate. These strains are used as starter cultures, for e.g., buttermilk and cheese production. They produce gas from glucose, which can change the texture of fermented food. Due to their slow growth and acidification properties, they represent a minor percentage of the LAB in food. They can become predominant when antibiotic agents are present. They can influence the organoleptic behavior of wine.

Lc. mesenteroides subsp. *mesenteroides* (Wibowo et al. 1985), *Lc. mesenteroides* subsp. *dextranicus* (Björkroth and Holzapfel 2006) and *Lc. mesenteroides* subsp. *cremoris* (Yurdugul and Bozoglu 2002) have been isolated from grape must during alcoholic fermentation.

The G+C content of the DNA ranges between 37 and 41 mol%.

The genus *Leuconostoc* contains in total: 13 species (July 2016; DSMZ 2016a). Some species contain well characterized subspecies. Only three subspecies of *Lc. mesenteroides* play a role in must and wine. Some characteristics are compiled in Table 1.4.

The type species is *Lc. mesenteroides* DSM 20343^T.

Lc. mesenteroides* subsp. *cremoris
Morphology: Like *Lc. mesenteroides* subsp. *mesenteroides*, occur often in long chains.
Characteristics: No dextran formation from sucrose.
Isolation: Milk, fermented milk products, grape must/wine.
Type strain: DSM 20346

Lc. mesenteroides* subsp. *dextranicus
Morphology: Like *Lc. mesenteroides* subsp. *mesenteroides*.
Characteristics: Dextran formation to a lesser extent than *Lc. mesenteroides* subsp. *mesenteroides*.
Isolation: Plant material, meat, milk, dairy products, grape must/wine.
Type strain: DSM 203484

Lc. mesenteroides* subsp. *mesenteroides
Morphology: Coccoid cells in milk, elongated cocci in glucose containing culture media. Single, pairs, short to medium chains. Often rod-shaped on solid media.

Characteristics: Production of excess of exopolysaccharides (dextran) from sucrose. Phages have been described (Sozzi et al. 1978).
Isolation: Silage, fermenting olives, sugar milling plants, meat, milk, dairy products, grape must/wine.
Type strain: DSM 20343

1.7.3 Genus Oenococcus

Oenococci have been isolated from must and wine (Garvie 1986a). They form spherical or lenticular cells, pairs or chains. Murein belongs to type A (DSMZ 2016c). The interpeptide bridge contains Lys-Ala-Ser or Lys-Ser-Ser. Only NAD^+ will serve as coenzyme of the glucose-6-phosphate dehydrogenase (Björkroth and Holzapfel 2006). Petri et al. (2015) applied MALDI-TOF-MS and nested SAPD-PCR for the discrimination of *Oenococcus oeni* isolates at the strain level.

Oenococci have been separated from the genus *Leuconostoc* by 16S rDNA sequence analysis (Fig. 1.1; Dicks et al. 1995; Schleifer and Ludwig 1995a, b). Only three species *O. oeni* (Dicks et al. 1995), *O. kitahareae* (Endo and Okada 2006) and *O. alcoholitolerans* (Badotti et al. 2015) have been described (DSMZ 2016a), and can easily be distinguished. *O. kitaharae* (type strain: DSM 17330^T) has been isolated from a composting distilled shochu residue. L-Malate is not decarboxylated to L-lactate and CO_2 in the presence of fermentable sugars. Cells do not grow below pH 4.5 and in 10% ethanol. Growth is not stimulated by tomato juice. The DNA G+C content ranges from 41 to 43 mol%. *O. kitaharae* possess several functions in cellular defence (bacteriocins, antimicrobials, restriction-modification systems), which are lacking in *Oenococcus oeni* living in must with fewer competitive microbes (Borneman et al. 2012). *O. alcoholitolerans* was isolated from an ethanol production plant in Brazil. Distinctive phenotypic characteristics are the ability to metabolise sucrose but not trehalose (Badotti et al. 2015). The usage of glucose, cellobiose, trehalose, and mannose was demonstrated (Jamal et al. 2013).

O. oeni can grow at pH 3.0 and 10% ethanol. Many strains of *O. oeni* can even grow at 14% of ethanol (Bordas et al. 2013). Heat shock proteins and special membrane lipids are produced under these environmental conditions (Coucheney et al. 2005). Changes in the expression level of the geranylgeranyl pyrophosphate synthase gene was detected under ethanol stress (Cafaro et al. 2014b). Vigentini et al. (2016) isolated *O. oeni* strains from wineries of the Aosta Valley developing at 10 °C in Petit Rouge wine. These strains can be used for performing malolactic acid fermentation (MLF) in cold climate territories.

The DNA homology with other lactic acid genera is relatively low with a certain relationship to the genera *Leuconostoc* and *Weissella* (Stiles and Holzapfel 1997). The distinct pylogenetic position (Fig. 1.1) because of the quite different 16S rDNA sequence may indicate a quick evolving rRNA in the genus *Oenococcus* (Yang and Woese 1989), which could not be approved by a comparison of the gene sequences

of the DNA-dependent RNA-polymerases (Morse et al. 1996). Oenococci can be distinguished from less acid tolerant *Leuconostoc* species by using saccharose, lactose and maltose as substrate (Garvie 1986a).

It is important to use selected strains for wine making under special conditions, because some features are expressed at strain level. Insertion sequences (IS) could be one of the reasons for genotypic and phenotypic variants of oenococci (El Gharniti et al. 2012). The whole genome of different strains of *O. oeni* was performed, which allowed to define the invariant and variable DNA regions between the strains. Genetic variation in amino acid and sugar metabolism was a common feature (Capozzi et al. 2014; Lamontanara et al. 2014; Sternes and Borneman 2016). Protein expression profiling of *Oenococcus oeni* from Aglianico wine allowed to analyze the cellular pathways (Cafaro et al. 2014a). Mohedano et al. (2014) identified 152 unique proteins were identified *in O. oeni*.

O. oeni can use the hexoses glucose and fructose, while not all strains use trehalose (Garvie 1986a). L-arginine can be degraded to carbon dioxide, ammonia and ornithine. *O. oeni* can perform a malolactic fermentation (Caspritz and Radler 1983), which is also found in the genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus*. The malolactic fermentation leads to a membrane potential and a proton gradient. With the aid of an F_1F_0 ATPase energy can be gained (Poolman et al. 1991).

Oenococci were able to synthesize capsular heteropolysaccharides made of glucose, galactose and rhamnose, β-glucans and homopolysaccharide (α-glucan or β-fructan) (Dimopoulou et al. 2014)

Oenococci exhibit a high mutability due to the lack of the mismatch repair genes *mutS* and *mutL* (Marcobal et al. 2008), which may facilitate the formation of strains. Specific methods for the rapid detection or differentiation of *O. oeni* strains in must and wine samples have been developed (Kelly et al. 1993; Viti et al. 1996; Zavaleta et al. 1997; Fröhlich 2002; Fröhlich and König 2004; Larisika et al. 2008).

O. oeni strains can contain bacteriophages (Doria et al. 2013; Jaomanjaka et al. 2013) and plasmides (Favier et al. 2012).

The type species is *O. oeni* DSM 20252^T.

O. oeni
Morphology: Spherical, lenticular cells in pairs or chains.
Characteristics: Growth below pH 3.0 and 10% ethanol.
Isolation: must/wine.
Type strain: DSM 20252^T.

1.7.4 Genus Pediococcus

Pediococci occur on plant material, fruits and in fermented food. They are non-pathogenic to plants and animals. Cells are spherical and never elongated as it is the case with leuconostocs and oenococci. The cell size is 0.36–1.43 μm in diameter.

Cell division occurs in two directions in a single plane. Short chains by pairs of cells or tetrads are formed (Garvie 1986b). Tetrad-forming homofermentative LABs in wine are pediococci. Pediococci are nonmotile and do not form spores or capsules (Simpson and Tachuchi 1995). The murein belongs to type A with an interpeptide bridge consisting of L-Lys-Ala-Asp (Holzapfel et al. 2003).

Glucose is fermented by the Embden–Meyerhof–Parnas pathway to DL or L-lactate. A wide range of carbohydrates is used such as hexoses, pentoses, disaccharides, trisaccharides and polymers such as starch. All wine-related species grow only in the presence of carbohydrates. The PTS system is used for glucose transport. Species producing DL-lactate possess an L- and D-LDH. Pyruvate can be converted mainly by *P. damnosus* to acetoin/diacetyl. *P. pentosaceus* and *P. damnosus* can degrade malate. They are nonproteolytic and nitrate is not reduced. Pediococci are catalase negative. Some strains of *P. pentosaceus* produce pseudocatalase. Pediococci do not reduce nitrate.

The G+C content of the DNA ranges from 34 to 44 mol%.

Pediococci can have plasmids, which code for production of bacteriocins or fermentation of carbohydrates. *P. pentosaceus* has three different plasmids for the fermentation of raffinose, melibiose and sucrose.

Pediococci are involved in beer spoilage (*P. damnosus*) and cause off-flavour in wine by production of diacetyl. *P. halophilus*, which has not been found in must/wine, is used to prepare soya sauce. Pediococci are used as starter culture in cheese production, silage and sausage production (*P. acidilactici*; *P. pentosaceus*). They play a role in cheese ripening. Pediococci (*P. acidilactici*; *P. pentosaceus*) can produce bacteriocins (pediocin) which can prevent meat spoilage. *P. damnosus* is a major spoilage organism in beer manufacturing, since it may produce diacetyl resulting in a buttery taste.

The species are differentiated by their range of sugar fermentation, hydrolysis of arginine, growth at different pH levels (4.5, 7.0), the configuration of lactic acid produced (Axelsson 2004) and ribotyping (Satokari et al. 2000). *P. pentosaceus* produces a nonheme pseudocatalase (Engesser and Hammes 1994).

The genus *Pediococcus* contains 11 species (July 2016; DSMZ 2016a). Four species have been found in must or wine (*P. damnosus*, *P. inopinatus*, *P. parvulus*, *P. pentosaceus*). Some characteristics of the species are compiled in Table 1.5).

The type species is *P. damnosus* DSM 20331^T.

P. damnosus
Morphology: Tetrades.
Characteristics: Ribose not fermented, arginine not hydrolysed. No growth at pH 8 or 35 °C. DL-lactic acid produced from glucose.
Isolation: Beer and wine.
Type strain: DSM 20331

P. inopinatus
Morphology: Tetrades
Characteristics: *P. parvulus* and *P. inopinatus* can be distinguished by the electrophoretic mobility of the L- and D-LDHs.

Isolation: Fermenting vegetables, beer, wine.
Type strain: DSM 20285

P. parvulus
Morphology: Tetrades, 0.7 μm × 1.1 μm in diameter. Single, pairs, tetrads, irregular clusters.
Characteristics: Grows at pH 4.5. Lactose, starch and pentoses not utilized. Arginine not hydrolysed. DL-lactic acid produced from glucose.
Isolation: Plant material, sauerkraut, fermented vegetables, fermented beans, beer, cider and wine.
Type strain: DSM 20332

P. pentosaceus
Morphology: Tetrades.
Characteristics: Pentoses and maltose fermented. Arginine is hydrolysed. Growth up to 45 °C. Used for the inoculation of semi-dry sausage, cucumber, green bean or soya milk fermentations and silage. Some strains produce pediocins. Isolation: Plant material and wine.
Type strain: DSM 20336

1.7.5 Genus Weissella

Based on rDNA analysis *Lc. paramesenteroides* ("*Lc. paramesenteroides* group") was reclassified as *W. paramesenteroides*. Five *heterofermentative lactobacilli* (*Lb. confusus*, *Lb. halotolerans*, *Lb. kandleri*, *Lb. minor*, *Lb. viridescens)* were also assigned to the genus *Weissella* (Collins et al. 1993; Björkroth and Holzapfel 2006). Weissellas are spherical, lenticular or irregular rods. They are heterofermentative species, which produce D, L-lactic acid, while *W. paramesenteroides* forms D-lactic acid from glucose. They have been isolated from food and meat. Weissellas produce greenish oxidized porphyrins in meat products by H_2O_2 accumulation. The genus *Weissella* contained 21 validly described species (July 2016, DSMZ 2016a). *W. paramesenteroides* is the only species of this genus isolated from must/wine.

The type species is *W. viridescens* DSM 20410^T.

W. paramesenteroides
Morphology: Sperical, lenticular
Characteristics: Pseudocatalase may be produced in the presence of low glucose content.
Isolation: must/wine, fresh vegetables, sausages
Type strain: DSM 20288^T

1.8 Conclusions

Lactic acid bacteria are widespread in habitats with complex nutritional supply such as plant material or fruit juice as well as animals. They influence the aroma, the quality, the consistency and safety of food. Since the 1900s, the production of fermented food and consequently the demand for starter cultures of lactic acid bacteria has been largely increased (Mäyrä-Mäkinen and Bigret 2004). They play an important role in the fermentation of sugar-containing food. Because of the acid formation and production of inhibitory components, they contribute to the preservation of food. On the other hand, they can produce off-flavour (e.g. diacetyl) and cause ropiness by exopolysaccharide production.

Especially in northern wine growing regions, grapes can contain high amounts of acid with unfavourable organoleptic properties. So far, mainly *O. oeni* and sometimes *Lb. plantarum* are used as starter cultures for wine making to reduce the malic acid content.

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Chapter 2
Acetic Acid Bacteria

José Manuel Guillamón and Albert Mas

2.1 Introduction

Acetic acid bacteria (AAB) are considered one of the most common wine spoilage microorganisms and a threat for the oenologists. Their ability to transform most of the sugars and alcohols into organic acids produces easily the transformation of glucose into gluconic acid in damaged grapes and ethanol or glycerol into acetic acid or dihydroxyacetone in wines. As a result of their strictly aerobic metabolism and high dependence to oxygen, acetic acid bacteria population is highly reduced during the must fermentation, with only few strains able to survive. However, wine aeration and oxygen exposure during oenological practices after alcoholic fermentation can activate their metabolism and increase their population with risks of acetic acid production. Inappropriate long-term wine storage and bottling conditions may also activate the acetic acid production. Good cellar practices such as high hygiene, microbiological control, oxygen restriction and reduction of porous surfaces reduce considerably the risks of wine spoilage by acetic acid bacteria.

Acetic acid bacteria (AAB) are a group of microorganisms included in the *Acetobacteraceae* family that have a very unique characteristic of oxidising the alcohol into acetic acid, and this differential capacity originates their name. However, this metabolic ability derives in a high capacity of quick oxidation of alcohols and sugars yielding the corresponding organic acids, which can easily accumulate

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in the media. This feature makes AAB a especial group to be used in biotechnological applications such as the production of ascorbic acid (vitamin C) or cellulose (Deppenmeier et al. 2002). In the food industry, AAB are being used as main participants in the production of several foods and beverages, such as vinegar, cocoa, kombucha and other similar fermented beverages. However, their presence and activity can easily derive into spoilage of other foods or beverages such as wine, beer, sweet drinks and fruits.

In the environment, AAB occur in sugary elements, such as fruits or flowers. Additionally, naturally spoiled fruits, which might be partially fermented into alcohols, are an excellent medium for the proliferation of some AAB due to their tolerance to ethanol and the transformation into acetic acid, both compounds highly restrictive for the proliferation of other microorganisms. Thus, they are especially abundant in the man-made environments where alcohol is produced.

As the AAB are specialised in rapid oxidation of sugars or alcohols, oxygen availability plays a pivotal role in their growth and activity. Their metabolic activity and growth is especially enhanced when oxygen is present or specifically added (e.g. in vinegar production). Their optimal pH is 5.5–6.3 (De Ley et al. 1984); however, they can survive and grow in the pH of the wine, which is normally around 3.0–4.0 although it can be still lower (Du Toit and Pretorius 2002). In fact, acid resistance can be induced by prolonged and gradual exposure to low pH (Kösebalan and Özingen 1992). Finally, the optimal growth temperature is 25–30 °C (De Ley et al. 1984), yet some strains can grow very slowly at 10 °C (Joyeux et al. 1984a), and some others can develop to higher temperatures, which is a feature of interest in the production of vinegars (Chen et al. 2016; Matsushita et al. 2016; Mounir et al. 2016).

2.2 From Grapes to Wine: An Adverse Environment

Yeasts, bacteria and filamentous fungi all contribute to the microbial ecology of wine production and the chemical composition of wine, although yeasts have the dominating influence because of their role in conducting the alcoholic fermentation (Fleet 1993). Many factors affect the microbial ecology of wine production, of which the chemical composition of the grape juice and the fermentation processes are the most significant. In complex microbial ecosystems, containing mixtures of different species and strains, there is the possibility that interactions between microorganisms will occur and that this will also determine the final ecology (Drysdale and Fleet 1989a; Ribereau-Gayon et al. 2000).

It is commonly known that grape juice presents extreme conditions for the growth of microorganisms, such as a low pH and a high sugar concentration. Overall, during the alcoholic fermentation, this sugar content is transformed into ethanol by yeasts, meaning an additional restriction for the development of microorganisms. Such environmental changes are responsible for the differences in the microbial ecology throughout the process, where tolerance to high concentrations

of ethanol and low pH will be the main factors that select species occurrence in wine ecosystems (Fleet 1993). Additionally, the coexistence of different microorganisms in the medium generates competition for the nutrients. Thus, early growth of yeasts in grape juice decreases the nutrient content, making the resulting wine less favourable as an environment for any further microbial growth. Moreover, such growth releases to the media different metabolites, some of which could be toxic to other species. Another factor that affects the development of some microorganisms is the carbon dioxide production that strips off oxygen, thereby limiting the growth of the aerobic species, such as AAB. Therefore, if a vigorous onset of alcoholic fermentation by yeasts (or *Saccharomyces cerevisiae*) occurred, non-*Saccharomyces* yeasts and bacteria would show little growth (González et al. 2005). However, if yeast growth is delayed, various species of lactic acid bacteria and AAB may grow, inhibit the growth of yeast and cause sluggish or stuck fermentations (Ribereau-Gayon et al. 2000; Fleet 2001).

The interaction among the different wine microorganisms may or may not favour a particular microbial group. For example, when the large amount of yeast biomass produced during fermentation dies, the autolysis releases to the media amino acids and vitamins which may encourage the growth of AAB and lactic acid bacteria species later in the process (Fleet 2001). Another concept that must be considered is quorum sensing as a mechanism by which microbial cells communicate with each other and regulate population growth. It is therefore evident that the development of microorganisms during the winemaking process depends on different parameters, such as the aforementioned microbial interactions, and also the media composition and the oenological practices.

A parameter that could affect the development of microorganisms during alcoholic fermentation is temperature. Winemakers have developed a preference towards fermenting white and rosé wines at controlled temperatures (between 13 and 18 °C) in order to enhance the production and retention of flavour volatiles (Llauradó et al. 2002); while in red wine fermentations, temperature is less controlled and able to reach temperature values of 25–30 °C. It is known that fermentation temperature will affect the rate of yeast growth and, consequently, the duration of fermentation (Torija et al. 2003). Therefore, a delay of the fermentative yeast growth, which will occur in low temperature fermentation, could help the development of indigenous oxidative yeasts and cause a sluggish fermentation (Llauradó et al. 2002). In any case, the wine bacterial population is not very psychrotolerant, and low temperature of fermentation should not be a parameter boosting its growth.

Finally, the addition of sulphur dioxide to grape juice and wine is a commonplace winemaking practice in order to control oxidation reactions and prevent the growth of indigenous microflora, such as indigenous non-*Saccharomyces* yeasts or AAB and lactic acid bacteria. The antimicrobial effectiveness of the SO_2 is highly dependent on the pH and on the presence of reactive molecules which can bind to SO_2. With a lower pH in the must and wine, there will be more SO_2 in free molecular form, which is the active form against microorganisms (Ribereau-Gayon et al. 2000). However, there are some AAB not very sensitive to this

antimicrobial compound and strains that are highly resistant (Du Toit et al. 2005; González et al. 2005).

2.3 Isolation and Taxonomy

The physiological differences among microorganisms made it possible to develop differential culture media for isolating AAB whose carbon source is glucose, mannitol, ethanol, etc. Some of these media can also incorporate $CaCO_3$ or bromocresol green as acid indicators (Swings and De Ley 1981; De Ley et al. 1984). Culture media are usually supplemented with pimaricin or similar antibiotics in the agar plates to prevent yeasts and molds from growing and with penicillin to eliminate Gram-positive acidophilic bacteria such as lactic acid bacteria (Ruiz et al. 2000).

Some of the most widely used culture media are GYC [5% D-glucose, 1% yeast extract, 0.5% $CaCO_3$ and 2% agar (w/v)] and YPM [2.5% mannitol, 0.5% yeast extract, 0.3% peptone and 2% agar (w/v)]. Plates must be incubated for between 2 and 4 days at 28 °C under aerobic conditions. These culture media are suitable for wine samples (Bartowsky et al. 2003; Du Toit and Lambrechts 2002).

Nevertheless, some studies show that it is difficult to culture this bacterial group from some industrial samples, especially those originated in extreme media, such as vinegar (Sokollek et al. 1998). This problem has been partially solved by introducing a double agar layer [0.5% agar in the lower layer and 1% agar in the upper layer (w/v)] into the cultures and media containing ethanol and acetic acid in an attempt to simulate the atmosphere of the acetification tanks, such as AE medium (Entani et al. 1985). However, culturing AAB is still a limitation to proper studies of this group of microorganisms that is a critical point for ecological studies. Thus, culture-independent molecular techniques are being developed to solve this problem.

The identification of AAB has gone in parallel with the changes in taxonomy and AAB classification. Since *Mycoderma* was first described by Persoon in 1822 and observed by Pasteur, Hansen and Beijerinck in the nineteenth century, the general consensus throughout the last part of the twentieth century is that there were two AAB genera: *Gluconobacter* and *Acetobacter*. The keys to the taxonomy of bacteria have been traditionally collected in Bergey's Manual of Systematic Bacteriology. In the 1984 edition (De Ley et al. 1984), it included such molecular techniques as fatty acid composition, soluble protein electrophoresis, percentage of G+C content and DNA-DNA hybridisation.

The taxonomy of AAB microorganisms, initially based on morphological and physiological criteria, has been continuously varied and reoriented, largely because of the application of molecular techniques. The most common techniques are:

– DNA–DNA hybridisation: from a taxonomic point of view, this is the most widely used technique for describing new species within bacterial groups. The

technique measures the degree of similarity between the genomes of different species.
- Percent base ratio determination: this was one of the first molecular tools to be used in bacterial taxonomy. It calculates the percentage of G+C in a bacterial genome. Bergey's Manual of Systematic Bacteriology (De Ley et al. 1984) included these values to differentiate among *Acetobacteraceae* species.
- 16S rDNA sequence analysis: the 16S rDNA gene is a highly preserved region with small changes that characterise different species. Ribosomal genes are compared in most taxonomical studies of bacteria. However, the differences in 16S rDNA sequences are very limited, and some species have few nucleotide pairs of difference.

However, the new approaches in AAB taxonomy have a polyphasic approach, combining some physiological features and full genome sequencing (Cleenwerk and De Vos 2008). The continuous reduction in prices of full genome sequencing has made this the preferred option for taxonomic identification.

The *Acetobacteraceae* family is no exception, and it has been deeply reorganised in parallel with easiness for full taxonomic analysis. AAB are considered a lineage within the *Acetobacteraceae* family, which is characterised by the ability to produce acetic acid, although some of them are very weak producers. Several new AAB genera have been added to the two traditional genera mentioned above: *Acidomonas*, *Gluconacetobacter*, *Asaia*, *Kozakia*, *Saccharibacter*, *Swminathania*, *Neoasaia*, *Granulibacter*, *Tantichaorenia*, *Commensalibacter*, *Ameyamaea*, *Neokomagataea*, *Komagataeibacter*, *Endobacter*, *Swingsia*, *Nguyenibacter and Bombella* (an updated 2016 list can be seen in Table 2.1). From 1992 until 2014, over 70 novel species of acetic acid bacteria have been described, with the highest number, over 20, in the genus *Gluconacetobacter* (Trček and Barja 2015). However, the deepest change has been incorporated with the description of new genus *Komagataeibacter*, which is the result of a revision of the previous genus *Gluconacetobacter* in two (Yamada et al. 2012). As expected, some species have also been renamed (particularly some species of *Acetobacter* which were assigned to the *Gluconacetobacter* and *Komagataeibacter* genera).

2.4 Molecular Techniques for Routine Identification of AAB

The main objective of microbial classification is to identify an isolated microorganism up to the species level. However, discriminating or typing the different strains or genotypes of a species is gaining an increase importance from an industrial point of view. Not all the strains of a species have the same ability to oxidise ethanol into acetic acid. Therefore, it is important to be able to determine how well each technique can discriminate among strains and to know how many species or strains are involved.

Table 2.1 Species of Acetic acid bacteria Genera (number of species/number of sequenced species)

Acetobacter (25/16)	*Asaia (8/4)*	*Gluconacetobacter (11/1)*	*Kozakia (1/1)*
A. aceti	***As. astilbis***	*Ga. aggeris*	***K. baliensis***
A. cerevisiae	***As. bogorensis***	*Ga. asukensis*	
A. cibinongenesis	*As. krungthepensis*	*Ga. azotocaptans*	***Neoasaia (1/0)***
A. estuniensis	*As. lannensis*	***Ga. diazotrophicus***	*N. chiangmaiensis*
A. fabarum	***As. platycodi***	*Ga. entanii*	
A. farinalis	***As. prunellae***	*Ga. johannae*	**Nguyenibacter (1/0)**
A. ghanensis	*As. siamensis*	*Ga. liquefaciens*	*N. valangensis*
A. indonesiensis	*As. spathodeae*	*Ga. sacchari*	
A. lambici		*Ga. takamatsuzukensis*	***Saccharibacter* (1/1)**
A. lovaniensis	***Bombella (1/0)***	*Ga. tumulicola*	***Sa. floricola***
A. malorum	*B. intestini*	*Ga. tumulosoli*	
A. nitrogenifigens			***Swaminathania* (1/0)**
A. oeni	***Endobacter (1/0)***	***Granulibacter (1/1)***	*S. salitolerans*
A. okinawensis	*E. medicaginis*	***Gr. bethesdensis***	
A. orientalis			***Swingsia* (1/0)**
A. orleaniensis	***Gluconobacter (14/7)***	***Komagataeibacter (14/8)***	*S. samuiensis*
A. papayae	***G. albidus***	***Km. europaeus***	
A. pasteurianus	*G. cerevisiae*	***Km. hansenii***	***Tanticharoenia* (1/1)**
A. peroxydans	***G. cerinus***	***Km. intermedius***	***T. sakaeratensis***
A. persici	***G. frateurii***	***Km. kakiaceti***	
A. pomorum	***G. japonicus***	*Km. kombuchae*	**Non-validated names**
A. senegalensis	*G. kanchanaburiensis*	*Km. maltaceti*	***Commensalibacter (2/2)***
A. sicerae	*G. kondonii*	***Km. medellinensis***	***C. intestini***
A. syzygii	*G. nephelii*	*Km. nataicola*	***C. papalotli***
A. tropicalis	***G. oxydans***	***Km. oboediens***	
	G. roseus	***Km. rhaeticus***	***Gluconobacter (1/1)***
Acidomonas (1/1)	*G. sphaericus*	*Km. saccharivorans*	***G. morbifer***
Ac. methanolica	***G. thailandicus***	*Km. sucrofermentans*	
	G. uchimurae	*Km. swingsii*	***Neokomagatea (2/0)***
Ameyamaea (1/0)	*G. wancherniae*	***Km. xylinus***	*Nk. tanensis*
Am. chiangmaiensis			*Nk. thailandica*

Data obtained from www.bacterio.net and www.ncbi.nlm.nih.gov/genome/browse/ retrieved on September 28, 2016
In Bold: sequenced species

Depending on the degree of polymorphism provided by the various molecular markers, they are more suitable for interspecific or for intraspecific discrimination. Therefore, we divided the molecular techniques into two main groups: those that can discriminate up to species level and those that can discriminate up to strain level. Currently, the only acceptable classification is the one based on sequencing.

However, in ecological studies, when a large number of samples and isolates have to be determined, a first screening technique is very helpful in order to associate all the isolates to certain groups, and then representative DNAs of these groups could be sequenced and properly identified.

Species Level

- PCR-RFLP of the rDNA 16S: this technique is appropriate for differentiating and grouping microorganisms on the basis of their phylogenetic relationships (Poblet et al. 2000; Ruiz et al. 2000). In eubacterial DNA, the rRNA loci include 16S, 23S and 5S rRNA genes, which are separated by internally transcribed spacer (ITS) regions. The technique consists of amplifying the 16S rDNA region and then digesting the amplified fragment with different restriction enzymes (Fig. 2.1) (Guillamon et al. 2002; González et al. 2006a). However, nowadays, this technique can be complemented by sequencing the amplified fragment making it possible to characterise almost all the AAB species, although the high similarity of some AAB species in this gene can produce some mistakes or undefinition (Valera et al. 2011)
- PCR-RFLP of the 16S-23S rDNA internally transcribed spacer (ITS): this technique consists of amplifying a region of the ITS (here it spans the 16S and 23S rRNA genes) and then digesting the amplified products with different restriction endonucleases (Sievers et al. 1996; Ruiz et al. 2000; Trcek and Teuber 2002; González et al. 2006a; Prieto et al. 2007). The sequences and lengths of the 16S-23S ITS region varies considerably among the species, and this region also

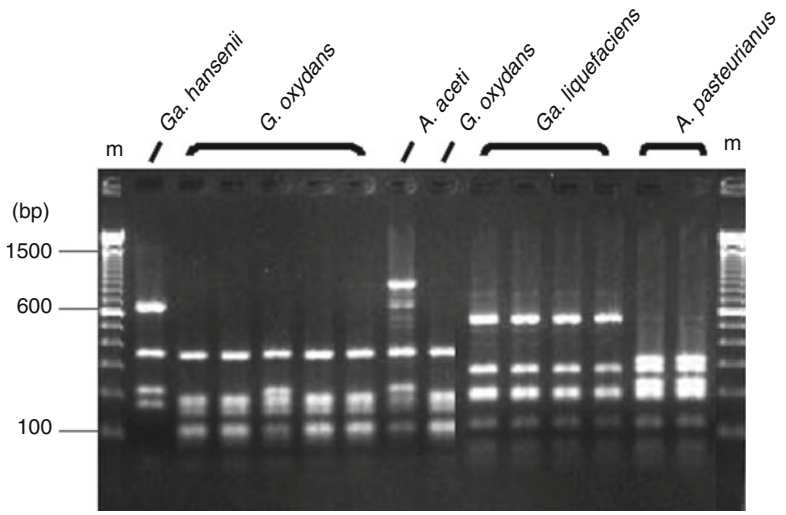

Fig. 2.1 *Taq*I restriction patterns obtained after the amplification of 16S rDNA of different acetic acid bacteria strains isolated throughout the alcoholic fermentation. All the strains belonging to the same species showed the same restriction pattern. Size in bp (*left*) of 100 bp ladder (Gibco-BRL), used as markers (m), are indicated

contains conserved sequences with functional roles such as tRNA genes and antitermination sequences (Sievers et al. 1996). In other bacterial groups, intergenic sequences are known to have higher variability than functional sequences, and they make it possible to distinguish below the species level. However, in AAB, the results obtained by Ruiz et al. (2000) and Trcek and Teuber (2002) only differentiated up to species level. It is more resolutive than the previous one, and it has been considered as an alternative to the undefinition that could be generated due to high similarity of 16S rDNA (González and Mas 2011; Valera et al. 2011).

- PCR-RFLP of the 16S-23S-5S sequences: this technique consists, as in the two previous ones, of amplifying part of the ribosomal DNA; in this case the region compressed by the 16S, 23S and 5S rDNA genes, generating an amplified product of around 4500 bp. This is then digested using *Rsa*I as a restriction endonuclease (Gullo et al. 2006). The results obtained were similar to the previous techniques, although with higher and more resolutive polymorphism. However, the main limitation of this technique results from the amplification of such a long fragment.
- Denaturing gradient gel electrophoresis (DGGE): DGGE separation of bacterial DNA amplicons is a common method used to characterise microbial communities from specific environmental niches. This technique has been used to study the AAB population in wine (Cocolin et al. 2000; Andorrà et al. 2008; Takahashi et al. 2014) and vinegar (De Vero et al. 2006; Yetiman and Kesmen 2015). It does not require the microorganisms to be isolated. The most commonly used genes for the DGGE method are 16S and 23S rDNA because they are species specific. The band pattern obtained is indicative of the number of different species present in a sample. Each individual band can be recovered and used for sequencing, which can be an additional tool for species identification. A main limitation of this technique is that minor species are hardly detected, especially when other species constituted an overwhelming majority.
- Real-time PCR: this technique identifies and enumerates bacterial species without culturing. It has been successfully used to enumerate total populations of AAB in both wines and vinegars (González et al. 2006b) and for the enumeration of *Gluconobacter* and *Gluconacetobacter* species in soft drinks (Gammon et al. 2007). A clear advantage of this technique is its specificity to detect a specific family group, genera or species (Torija et al. 2010; Valera et al. 2013).
- PQQ-dependent alcohol dehydrogenase gene targeting: this technique has been used to detect both generic AAB and specifically *A. aceti* from cider vinegar (Trcek 2005). The variable and conserved segments in partial *adhA* sequences allows the construction of generic PCR set of primers for all the AAB species and a specific PCR primer for the detection of *A. aceti*. The author claimed that the analysis of partial *adhA* sequences showed that this region was more discriminative for AAB species than 16S rRNA gene but less than 16S-23S rRNA intergenic regions.
- Fluorescence in situ hybridisation (FISH): this technique has been used to detect *Ga. sacchari* (Franke et al. 1999) and other wine-related microorganisms such as lactic acid bacteria (Blasco et al. 2003). FISH directly identifies and quantifies bacterial species at microscopic level without previous cultivation. It consists of DNA fluorescent labelled probes that will specifically hybridise each of the

species or genera. The high content of different binding compounds in wine or vinegar can quench fluorescence and also limit the resolution.

- Metagenomics and techniques based on massive sequencing: this technique is currently being applied to determine the complete ecology of several niches and processes, including grape, wine and vinegar production. It is a very powerful technique in the sense that can detect a large number of sequences in a single run. However, the large number of data generated implies some processing restrictions that are dealt with bioinformatics. However, the groupings of the different techniques are based on sequence similarities, and it is very difficult to achieve species level. The sequences are grouped in operational taxonomic units (OTUs) that not necessarily are equal to species or even genus. Furthermore, the design of the primers used has to exclude some sequences originated in prokaryote DNA from eukaryotes (chloroplasts or mitochondria), which can generate some biases. It has been used for grape, wine and vinegar population analysis (Bokulich et al. 2012, 2014, 2015; Portillo et al. 2016; Portillo and Mas 2016; Valera et al. 2015; Trček et al. 2016)
- Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS): this is an alternative method based on protein profile, instead of DNA polymorphism, obtained by MALDI-TOF MS from intact bacteria. This technique has been successfully applied to differentiate AAB, among different genera, species and strains of the same species (Andrés-Barrao et al. 2013; Wieme et al. 2014). This method has the advantage that requires less sample manipulation and is very suitable for routine identification of a large number of samples (Trček and Barja 2015).

Strain Level

- Random amplified polymorphic DNA–PCR (RAPD–PCR): the RAPD fingerprint amplifies the genomic DNA with a single primer of arbitrary sequence, 9 or 10 bases in length, which hybridise with sufficient affinity to chromosomal DNA sequences at low annealing temperatures so that they can be used to initiate the amplification of bacterial genome regions. The amplification is followed by agarose gel electrophoresis, which yields a band pattern that should be a characteristic of the particular bacterial strain. The technique was initially used with AAB by Trcek et al. (1997) in spirit vinegar and later by Nanda et al. (2001) to characterise rice vinegar AAB. Bartowsky et al. (2003) and Prieto et al. (2007) also used this technique to differentiate strains in spoiled wines.
- Enterobacterial repetitive intergenic consensus–PCR (ERIC–PCR) and repetitive extragenic palindromic–PCR (REP–PCR): ERIC and REP elements have been described as consensus sequences derived from highly conserved palindromic inverted repeat regions found in enteric bacteria. However, these sequences seem to be widely distributed in the genomes of various bacterial groups. The amplification of the sequences between these repetitive elements has generated DNA fingerprints of several microbial species. ERIC–PCR has already been used by Nanda et al. (2001) to identify AAB strains isolated from vinegar and more recently by Wu et al. (2010) to genotype 21 AAB strains

isolated from Chinese cereal vinegars. Both techniques have been applied to AAB in wines (González et al. 2004) and used to follow the AAB population dynamics before and during alcoholic fermentation (González et al. 2005).

- A similar technique based on repetitive elements for genomic fingerprinting has been proposed by De Vuyst et al. (2006) using $(GTG)_5$ primers. These noncoding sequences are present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria. This study reported a good discrimination method with a high degree of polymorphism in AAB.

2.5 AAB Ecology During Winemaking

2.5.1 AAB in Grapes and Musts

As the grapes mature the amount of sugars (glucose and fructose) increases and improves the chances for AAB growth. In healthy grapes, the predominant species is *G. oxydans*, and the most common populations are around 10^2–10^5 cfu ml^{-1} (Joyeux et al. 1984a; Du Toit and Lambrechts 2002; González et al. 2005; Renouf et al. 2005; Prieto et al. 2007). *Acetobacter* species have also been isolated from unspoiled grapes, albeit in very low amounts (Du Toit and Lambrechts 2002; González et al. 2004; Prieto et al. 2007). On the other hand, damaged grapes contain larger AAB populations (Barbe et al. 2001), mainly belonging to the *Acetobacter* species (*A. aceti* and *A. pasteurianus*). In these conditions, the sugars released from the spoiled grapes can be metabolised by yeasts into ethanol, which is a preferred carbon source of the *Acetobacter* species that overgrow *Gluconobacter* (Joyeux et al. 1984b; Grossman and Becker 1984; González et al. 2005).

However, the description of new AAB species has increased the number of species isolated from this substrate, and the extension of new molecular methods has extended the number of genera and species identified in wines. This can be applied to the "traditional" genera already described, most likely due to previous identification as some of the known species. For instance, in the *Gluconobacter* genus, probably previously identified as *G. oxydans*, has been now described: *G. albidus*, *G. cerinus*, *G. frateurii*, *G. japonicus* and *G. thailandicus* (Mateo et al. 2014; Navarro et al. 2013; Valera et al. 2011). In the *Acetobacter* genus, a new species initially described in wine, *A. oeni*, was considered a main spoiling microorganism in Portuguese wine (Silva et al. 2006), although other species have been also described in musts from all over the world: *A. cerevisiae*, *A. malorum*, *A. orleanensis*, *A. syzygii* and *A. tropicalis* (Barata et al. 2012; Mateo et al. 2014; Prieto et al. 2007; Valera et al. 2011). And within the *Gluconacetobacter* or *Komagateibacter* genera, which can also be considered as traditionally associated to grapes or wines, *Ga. liquefaciens*, *Km. hansenii*, *Km. saccharivorans*, and *Km. intermedius* have been also described (Valera et al. 2011; González et al. 2005; Barata et al. 2012; Du Toit and Lambrechts 2002). However, some species

belonging to the new genera have been also described: *Asaia siamensis* was described both in Australia and during Tempranillo malolactic fermentation (Bae et al. 2006; Ruiz et al. 2010; Mateo et al. 2014), *Asaia lannaensis* and Amayamea chiangmaiensis from Australian grapes (Mateo et al. 2014) and finally *Kozakia baliensis* was also isolated in musts from Tarragona (Spain) (Navarro et al. 2013).

Nevertheless, the main changes in the grape ecology of AAB have been developed with the next-generation sequencing (NGS). In general all the reports using NGS on grapes have detected *Gluconobacter* in grapes, sometimes as the major species (Portillo and Mas 2016; Bokulich et al. 2012, 2014; Piao et al. 2015) although sometimes in very small proportions (Bokulich et al. 2014; Zarraonaindia et al. 2015) or even absent (Portillo et al. 2016). Instead, only Perazzolli et al. (2014) detected *Acetobacter* in grapes. Probably the strong differences in the presence of these different AAB genera in grapes has to do more with the analytical technique used (pyrosequencing, Illumina, Ion Torrent) or even the primers used for the amplification.

Grape processing in the cellar (pressing, pumping, racking, etc.) may contaminate must since there is contact with cellar equipment, which contains resident AAB and will increase their population, mostly made up of *Acetobacter* species (González et al. 2005). However, in the literature, it is possible to find some exceptions to this ubiquitous presence of AAB. Subden et al. (2003) were not able to find AAB among the bacteria isolated from icewine musts. Curiously the predominant species isolated from this substrate was *Pantoea agglomerans*, which had never been reported as a contaminant in grape musts. Bokulich et al. (2013) analysed all the equipments and soils of a cellar by NGS, finding only a significant amount of *Gluconacetobacter* in the crusher.

Most of the studies on AAB in winemaking have focused on the evolution of species during the process. We also typed the different AAB isolates from grapes to wine at strain level (González et al. 2005). We found important strain diversity in grapes (calculated as the percentage of different strains in the total isolates analysed), which ranged from 45% to 70%. A few of these grape strains were continuously isolated throughout the alcoholic fermentation. Prieto et al. (2007) also typed isolates from Chilean grapes, confirming the high diversity of AAB strains in grapes, in particular among the *G. oxydans* isolates.

2.5.2 AAB During Fermentation

Studies concerning the evolution of AAB species along wine fermentations have established certain general trends (Table 2.2). *G. oxydans* is usually the dominant species in fresh must and the initial stages of fermentation and is rarely isolated from wines, while *A. aceti* is a major strain in the final stages of fermentation (Joyeux et al. 1984a; Drysdale and Fleet 1988; González et al. 2005). However, we have also found G. *oxydans*, *Ga. liquefaciens* and *Ga. hansenii* in higher percentages as well as *A. pasteurianus* in the final stages of fermentation (González et al. 2004), or more recently, *A. oeni* has been proposed as a new species isolated in wine (Silva et al. 2006). Thus, this pattern of species evolution seems somewhat reductionist and may depend on multiple oenological

Table 2.2 Summary of publications that indicate the presence of AAB on grapes, musts and wines

Origin	Grapes/must	Alcoholic fermentation	Source
White wine, Semillon botrytised grapes. Bordeaux, France	*G. oxydans* *A. pasteurianus*	*A. aceti* *A. pasteurianus*	Joyeux et al. (1984b)
Red wine Cabernet Sauvignon. Bordeaux, France	*G. oxydans*	*G. oxydans* *A. pasteurianus*	Joyeux et al. (1984b)
Botrytised grapes. Bordeaux, France. 1995–1997	*Gluconobacter sp.* *A. aceti* *A. pasteurianus*		Barbe et al. (2001)
Cabernet Sauvignon. South Africa. 1998–1999	*G. oxydans* *A. pasteurianus*	*G. oxydans* *A. aceti* *A. pasteurianus* *Ga. liquefaciens* *Km. hansenii*	Du Toit and Lambrechts (2002)
Bottled red wine, Australia		*A. pasteurianus*	Bartowsky et al. (2003)
Red Grenache. Tarragona, Spain. 2001	*G. oxydans*	*A. aceti* *Ga. liquefaciens* *Km.* (*Ga*) *hansenii*	González et al. (2004)
Red Grenache. Tarragona. Spain. 2002	*G. oxydans* *A. aceti*	*A. aceti*	González et al. (2005)
Spoiled red wine. Portugal		*A. oeni*	Silva et al. (2006)
Undamaged Semillon grapes. New south Wales, Australia. 2004	*As. siamensis*		Bae et al. (2006)
Spoiled wines. Austria		*A. tropicalis*	Silhavy and Mandl (2006)
Different grape varieties. Chile. 2004	*G. oxydans* *A. cerevisiae*		Prieto et al. (2007)
Malolactic fermentation of Tempranillo wines. La Rioja, Spain		*As. siamensis* *G. oxydans*	Ruiz et al. (2010)
Rotten grapes. Lisbon, Portugal. 2007	*A. malorum* *A. orleanensis* *A. syzygii* *G. oxydans* *Km. hansenii* *Km. intermedius* *Km. saccharivorans*	*A. aceti* *A. cerevisiae* *A. malorum* *A. tropìcalis* *G. oxydans* *Km. europaeus* *Km. hansenii* *Km. intermedius* *Km. saccharivorans*	Barata et al. (2012)
Sound grapes. Canary Islands. Spain. 2009	*A. cerevisiae* *A. malorum* *A. pasterurianus* *A. tropicalis* *G. japonicus* *G. thailandicus* *Km. saccharivorans*		Valera et al. (2011)

(continued)

Table 2.2 (continued)

Origin	Grapes/must	Alcoholic fermentation	Source
Different grape varieties. Tarragona, Spain	*A. cerevisiae* *A. malorum* *A. pasteurianus* *G. albidus* *G. cerinus* *G. oxydans* *G. japonicus* *G. thailandicus* *Ko. baliensis*		Navarro et al. (2013)
Sound and spoiled grapes. Adelaide Hills, Australia. 2011	*A. malorum* *As. siamensis* *As. lannaensis* *Am. chiangmaiensis* *G. oxydans* *G. albidus* *G. cerinus* *G. frateurii*		Mateo et al. (2014)

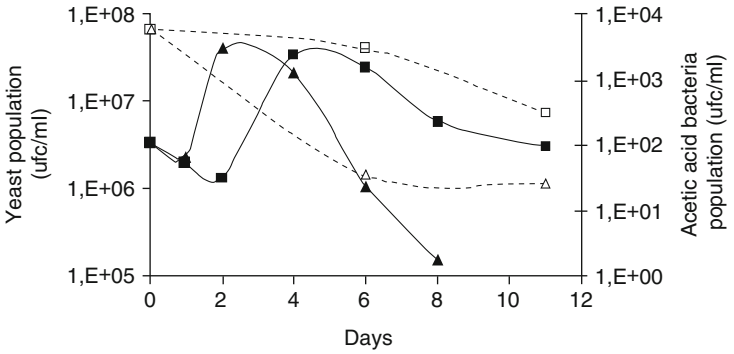

Fig. 2.2 Comparison of yeasts (*solid line* and *solid symbol*) and acetic acid bacteria populations (*dotted line* and *open symbol*) in inoculated (*filled triangle*) and spontaneous (*filled square*) wine fermentations

factors such as SO_2, pH, ethanol, low temperature and yeast inoculation. All these factors have been reported as inhibitors of AAB growth, yet they can also modify the species distribution during the process. For instance, different studies have suggested that *A. pasteurianus* is more resistant to SO_2 (Du Toit and Lambrechts 2002), ethanol (De Ley et al. 1984) and low temperature than *A. aceti*. Otherwise, inoculation with high population of yeasts, which is a common practice in winemaking, will produce a rapid onset of alcoholic fermentation and a concomitant decrease of the AAB population (Fig. 2.2) (Guillamon et al. 2002). However little is known about the impact of these oenological factors or the interactions with other wine microorganisms on the selection and evolution of the AAB species during wine fermentation.

The growth of AAB during alcoholic fermentation is also linked to the number of bacteria and yeast in the must at the start of the fermentation (Watanabe and Lino 1984). The initial population of AAB, before the alcoholic fermentation starts, may determine the number of cells surviving during and after fermentation (Du Toit and Pretorius 2002). If AAB grow significantly during the initial stages of alcoholic fermentation, it may become stuck or sluggish, which might enhance the growth of AAB during wine storage, with a corresponding reduction in the quality of the wines (Joyeux et al. 1984b).

Even less is known about the AAB development during malolactic fermentation and their interaction with lactic acid bacteria (the main microorganisms during this process). Joyeux et al. (1984a) reported constant cells counts of AAB of approximately 10^2–10^3 cfu ml^{-1}, consisting mainly of *A. pasteurianus*, throughout malolactic fermentation. Conversely we detected a major increase in the AAB population up to approximately 10^6 cfu ml^{-1} during this process, *A. aceti* being the main species found in this environment (Fig. 2.3) (Guillamon et al. 2002). This increase in the AAB population did not interfere with the simultaneous development of the lactic acid bacteria population up to cells densities of approximately 10^8 cfu ml^{-1}. A possible synergic mechanism between both bacterial groups may emerge from this result.

All these concepts related to the presence of AAB in winemaking with the predominance of *Acetobacter* during the alcoholic fermentation and the apparent decline of *Gluconobacter* during fermentation associated to increased sensitivity to ethanol have been strongly challenged by the NGS studies. No significant quantities of DNA from *Acetobacter* have been found in those studies, whereas relevant presence of *Gluconobacter* during the alcoholic fermentation and even increasing at the end has been reported (Portillo and Mas 2016; Piao et al. 2015; Bokulich et al. 2015). More studies should be undertaken to clarify if this apparent controversy between the classical culture-dependent studies and the NGS ones is due to methodological aspects or if it is indeed an error induced by the lack of specificity and "broad" view of NGS.

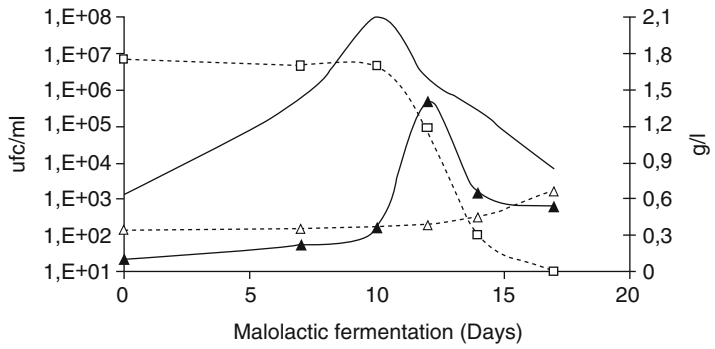

Fig. 2.3 Acetic acid bacteria (*filled triangle* with *solid line*) and lactic acid bacteria (*filled square* with *solid line*) growth during a malolactic fermentation. Malic acid consumption (*open square* with *dotted line*) and acetic acid (*open triangle* with *dotted line*) production are also indicated

In our studies on typing AAB strains and monitoring strain evolution during alcoholic fermentations, we were able to conclude: (1) the origin of the strains isolated during wine fermentation are both the grape and wine cellar environment; (2) the high strain diversity detected at the beginning of the process decreased significantly during the final stages of the process. The anaerobic conditions and ethanol increasing concentrations clearly selected the most resistant strains; (3) regardless of the degree of genotype diversity, there were clear dominant genotypes in all stages (González et al. 2004, 2005).

2.5.3 AAB During Ageing and Wine Maturation

Once the alcoholic fermentation has finished the pumping over and racking of wine may stimulate the growth of AAB and can lead to populations of up to 10^8 cells ml^{-1} (Joyeux et al. 1984b; Drysdale and Fleet 1985), owing to the intake of oxygen during these operations. During storage and ageing, the main species found belong to *Acetobacter* (*A. aceti* and *A. pasteurianus*). AAB have been isolated from the top, middle and bottom of the tanks and barrels, suggesting that AAB can actually survive under the semi-anaerobic conditions occurring in wine containers (Du Toit et al. 2005). This can be explained by the ability of AAB to use such compounds as quinones and reducible dyes as electron acceptors (Du Toit and Pretorius 2002). The number of bacteria usually decreases drastically after bottling, because of the relatively anaerobic conditions present within a bottle. However, excessive aeration during bottling can increase the number of AAB (Millet and Lonvaud-Funel 2000). Furthermore, the bottle position during storage, poor storage conditions or spoiled corks may facilitate AAB growth. In fact, wine spoilage in the bottle by AAB has also been reported, mostly due to *A. pasteurianus* (Bartowsky et al. 2003). It should be pointed out that the number of AAB in wine after fermentation can be underestimated, because the counting of colonies grown in solid media does not take into account the VBNC status (Millet and Lonvaud-Funel 2000).

2.6 Acetic Acid Bacteria and Wine Spoilage

The presence and growth of acetic acid bacteria has generally been related to wine spoilage, mostly by increasing the acetic acid and, thus, the volatile acidity. However, the changes introduced by acetic acid bacteria in wine depend on the process stage involved.

Grape and must: the overall effect of the acetic acid bacteria growth in the grapes is considered as acid rot that can sometimes involve other microorganisms such as fungi like *Botrytis cinerea* (Barbe et al. 2001). In the grape or must, the main carbon

source used by AAB is glucose, which is readily oxidised to gluconic acid. In fact, gluconic acid in oenology is considered as an indicator of *Botrytis* infection, although it seems clear that most of the gluconic acid is produced by the AAB associated with the *Botrytis* infection (Barbe et al. 2001). Also, fructose can be oxidised to oxofructose, although glucose is preferred as a substrate. The production of gluconic acid and oxofructose is important not only because of the organoleptic changes that might take place but also because of the binding and reduction of free SO_2. This will result in the need for a higher SO_2 dosage (Barbe et al. 2001; Du Toit and Pretorius 2002).

Although this is the main change induced by AAB in grapes, there are further changes relevant to oenology. The production of cellulose as a result of sugar metabolism (Kouda et al. 1997) can result in the production of fibres that can affect grape must and wine filterability (Drysdale and Fleet 1988).

Wine: probably the best-known transformation of AAB in general is the transformation of ethanol into acetic acid, which gives the group its name. Thus, during wine production, this will be the main carbon source (Drysdale and Fleet 1988; Du Toit and Pretorius 2002). However, the AAB population decreases during winemaking due to the anaerobic conditions exerted by yeast metabolism and produces only limited amounts of acetic acid, although in some cases high enough to be noticeable to consumers (0.8 g l^{-1}, vinegary taint). However, not all the acetic acid found in wines is due to AAB since yeasts and lactic acid bacteria can also produce it. Thus, even low AAB population counts can significantly affect the final quality of wines as they are strong acetic acid producers that will add to what is produced by other microorganisms. After alcoholic fermentation, even when ethanol concentrations of 5–10% are toxic for AAB, some strains are able to survive in very high ethanol concentrations of up to 15% (De Ley et al. 1984). The production of acetic acid by AAB requires oxygen, and it is directly related to oenological practices that may produce an increase in dissolved oxygen (aeration, pumping over, fining, etc.).

During the transformation of alcohol into acetic acid, both acetaldehyde (Drysdale and Fleet 1989a) and ethyl acetate (as a result of yeast and AAB alcohol acetyl transferease activity) (Plata et al. 2005) are produced, which are also noticeable in the final wines due to their low perception threshold (Drysdale and Fleet 1989a). Beyond the effect of acetaldehyde upon aroma and taste, acetaldehyde is the most reactive species to bind SO_2 and therefore reduce its free form (Ribereau-Gayon et al. 2000).

After ethanol, glycerol is also a main alcoholic fermentation product and can be a substrate for AAB oxidation. The resulting product is dihydroxyacetone, which does not give the mouth the smoothness of glycerol and also binds free SO_2. Other wine minority compounds, such as organic acids, can be used as an oxidisable substrate. The levels of malic, tartaric and citric acids decrease after wine AAB contamination (Drysdale and Fleet 1989b). All these changes affect the sensory perception of the final wine.

2.7 Conclusion: Prevention of Wine Spoilage and Oenological Practices

Although oxygen availability is low during winemaking, AAB can survive such conditions (Drysdale and Fleet 1989a), either cultivable or VBNC status (Millet and Funnel 2002). Thus an AAB risk-free oenological practice is impossible, although some practices will reduce the risks by minimisation of the AAB population or by limitation of its metabolism and activity. Among these, it is important to pay special attention to:

- Control of grape production, aimed at obtaining an appropriate acidic pH (Holt et al. 1994), and maintenance of that low pH during wine production and ageing. Although AAB survive at wine and grape pH of 3–4, their populations are reduced after lowering pH (Joyeux et al. 1984a; Du Toit and Lambrechts 2002). This low pH also favours the presence of SO_2 in free form (Ribereau-Gayon et al. 2000).
- Healthy grape status and care during handling and pressing. The presence of AAB, rotten or damaged grapes should be avoided as much as possible. Also, entrance to the cellar should be well controlled and quick, as this stage can be an excellent source for winery-resident AAB (González et al. 2005). At this stage, SO_2 addition, cold settling and clarification could be highly recommended practices in order to reduce the population size and prevent unwanted microorganisms (Ribereau-Gayon et al. 2000). However, it has to be emphasised that AAB could survive high SO_2 concentrations (Du Toit et al. 2005).
- A quick start of fermentation is advisable as this will produce both ethanol and CO_2 that can reduce the AAB population and metabolic activity. Thus, inoculations with ADWY or equivalent practices are advisable to ensure this quick start (Guillamon et al. 2002).
- A well-controlled process of aeration or oxygenation. The O_2 need for AAB growth has already been discussed, yet some oxygen supply is needed for ageing, while other oxygen supply is unavoidable in certain oenological practices (pumping over, racking, bottling, etc.). The current microoxygenation practice may enhance the growth of AAB; however Perez-Magariño et al. (2007) reported that low levels of oxygen supply do not affect volatile acidity. During ageing in wooden barrels, some oxygen penetrates through the wood, enough to maintain a population of viable AAB (Millet and Lonvaud-Funnel 2002). Pumping over and bottling can be an additional source of oxygen. Thus, filtering through 0.45 μm mesh prior to bottling will prevent the presence of AAB in bottled wine, although this drastic filtration may imply an important loss of compounds that are important for the quality and aroma of the wines.
- The optimal growing temperatures of AAB are between 25 and 35 °C, with variations according to strains and species. Lowering storage temperatures to 10–15 °C will inhibit growth to a large extent (Joyeux et al. 1984a).
- Finally, good cellar hygiene practices are a must. The risk of AAB contamination in the cellars is very high as demonstrated by the incorporation of different

AAB strains that can be considered as cellar resident in grape musts (González et al. 2005). Low alcohol content and low-pH wines ageing in barrels have the highest risk of AAB spoilage in cellars, since AAB develop well in porous solid materials like wood. Regular practices such as equipment and barrel sanitization with SO_2 or hot water could be very effective in keeping AAB counts low.

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Chapter 3
Yeasts

Linda F. Bisson, C.M. Lucy Joseph, and Paola Domizio

3.1 Introduction

Wine is the end product of the fermentative activity of yeast and bacteria. The microbiota of grape juice fermentation can vary significantly as over 40 genera and 100 different species of yeast have been isolated from grapes or wine (Table 3.1). Although the genera listed are commonly identified in surveys of grape mycobiota, some yeast species are more universally found than others, and numerous factors impact the composition of the yeast microbial community of grapes and their persistence during fermentation. *Saccharomyces cerevisiae* is the primary agent responsible for the conversion of grape sugars into alcohol but other yeast, collectively known as non-*Saccharomyces* yeast, and bacteria may also contribute to the aroma and flavor profile of the wine. Thus interspecies as well as intraspecies diversity plays an important role in the evolution of wine composition.

There are two basic types of wine production practices with respect to management of the microbial populations: autochthonous and inoculated. In autochthonous fermentations (also known as native or uninoculated), there is no deliberate addition of pure cultures of any microorganism. The microbiota resident in the vineyard and on winery surfaces conducts the conversion of grape juice into wine. Autochthonous fermentations are believed to display more complexity in aroma and

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Table 3.1 Yeast species associated with grapes and wine[a]

Species name[b]	Synonyms	Source
Aureobasidium pullulans		Grape
Brettanomyces bruxellensis	*Dekkera bruxellensis*	Wine
Bulleromyces albus		Grape
Candida albicans		Grape
C. apicola		Grape
C. azyma		Grape
C. boidinii		Grape
C. bombi		Grape
C. cidri		Grape
C. fermentati		Grape
C. hellenica	*Zygoascus meyerae*	Grape
C. intermedia		Grape
C. metapsilosis		Grape
C. oleophila		Grape
C. parapsilosis		Grape
C. pomicola		Grape
C. sake		Grape
C. stellata		Grape, must, wine
Citeromyces matritensis	*Candida globosa*	Grape
Cryptococcus laurentii		Grape
C. magnus		Grape
Curvibasidium pallidicorallinum		Grape
Cystobasidium minuta	*Rhodotorula minuta*	Grape
C. slooffiae	*Rhodotorula slooffiae*	Grape
Debaryomyces hansenii	*Candida famata*	Grape, must
Filobasidium oeirense *F. wieringae*	*Cryptococcus oeirensis* *C. wieringae*	Grape Grape
Hanseniaspora clermontiae		Grape, must, wine
H. guilliermondii	*Kloeckera apis*	Grape, must
H. meyeri		
H. occidentalis	*Kloeckera javanica*	Grape
H. opuntiae		Grape
H. osmophila	*Kloeckera corticis*	Grape
H. thailandica		Grape
H. uvarum	*Kloeckera apiculata*	Grape, must
H. valbyensis	*Kloeckera japonica*	Grape, must
H. vineae	*Kloeckera africana*	Grape, must, wine

(continued)

Table 3.1 (continued)

Species name[b]	Synonyms	Source
Hyphopichia burtonii	*Pichia burtonii*	Grape
Issatchenkia hanoiensis		
Kabatiella microsticta	*Aureobasidium microstictum*	Grape
Kazachstania unispora	*Saccharomyces unisporus*; *S. delbrueckii*	Grape
Kluyveromyces hubeiensis		Grape, must
K. lactis		Grape, must
Lachancea kluyveri	*Saccharomyces kluyveri*	Grape
L. thermotolerans	*Kluyveromyces thermotolerans*	Grape, must
Lipomyces lipofer		Grape
L. tetrasporus		Grape
Metschnikowia andauensis		Grape
M. chrysoperlae		Grape
M. fructicola		Grape
M. pulcherrima	*Candida pulcherrima*	Grape, must
M. viticola	*C. kofuensis*	Grape
Meyerozyma caribbica	*Pichia caribbica*, *Candida fermentati*	Grape
M. guilliermondii	*Candida guilliermondii*, *Pichia guilliermondii*	Grape, must, wine
Millerozyma farinosa	*Saccharomyces farinosa*, *Zygosaccharomyces farinosus*, *Pichia farinosa*	Grape
Naganishia albida	*Cryptococcus albidus*	Grape
N. bhutanensis *N. globosa*	*C. bhutanensis* *C. saitoi*	Grape Grape
Nakazawaea ishiwadae	*Candida ishiwadae*	Grape
Papiliotrema flavescens	*Cryptococcus flavescens*	Grape
P. fuscus	*Auriculibuller fuscus*	Grape
P. nemorosus	*Cryptococcus nemorosus*	Grape
P. terrestris	*Cryptococcus terrestris*	Grape
Pichia fermentans	*Candida lambica*	Grape, must, wine
P. kluyveri	*Hansenula kluyveri*	Grape, must
P. kudriavzevii	*Issatchenkia orientalis*	Grape, must
P. manshurica		Wine
P. membranifaciens	*Saccharomyces membranifaciens*, *Debaryomyces membranifaciens*	Wine
P. terricola	*Issatchenkia terricola*	
P. occidentalis	*Candida sorbosa*, *Issatchenkia occidentalis*	Grape, must

(continued)

Table 3.1 (continued)

Species name[b]	Synonyms	Source
Pseudozyma hubeiensis		Grape
Rhodosporidium babjevae		Grape
R. kratochvilovae	*Rhodotorula kratochvilovae*	Grape
R. toruloides	*R. glutinis*	Grape
Rhodotorula bacarum		Grape
R. fujisanensis	*Candida fujisanensis*	Grape
R. mucilaginosa	*Torulopsis mucilaginosa*	Grape
R. nothofagi		Grape
Saccharomyces bayanus		Grape, must, wine
S. cerevisiae		Grape, must, wine
S. pastorianus		Grape, must, Wine
S. uvarum		Grape, must, wine
Saccharomycodes ludwigii	*Saccharomyces ludwigii*	Grape, must, wine
Schizosaccharomyces japonicus		Grape
S. pombe		Grape, must, wine
Sporidiobolus salmonicolor		Grape
S. pararoseus	*Sporobolomyces japonica*	Grape
Sporobolomyces carnicolor		Grape
S. coprosmae		Grape
S. longiusculus		Grape
S. nylandii		Grape
S. roseus		Grape
S. oryzicola		Grape
Starmerella bacillaris	*Candida stellata*[c], *C. zemplinina*	Grape, must, wine
S. bombicola	*C. bombicola*	Grape
Torulaspora delbrueckii	*Candida colliculosa*, *Zygosaccharomyces globiformis*	Grape, must
Tremella globispora		Grape
Vishniacozyma foliicola	*Cryptococcus foliicola*	Grape
V. canescens	*C. carnescens*	Grape
V. tephrensis	*C. tephrensis*	Grape
Wickerhamomyces anomalus	*Hansenula anomala*, *Pichia anomala*	Grape, must
Yarrowia lipolytica		Grape
Zygoascus hellenicus		Grape
Z. meyerae		Grape

(continued)

Table 3.1 (continued)

Species name[b]	Synonyms	Source
Zygosaccharomyces bailii		Grape, must, juice concentrate, wine
Zygotorulaspora florentina	*Zygosaccharomyces florentinus*	Grape

[a]As reported in: Boulton et al. (1996), Renouf et al. (2007), Jolly et al. (2013), Byrsch-Herzberg and Seidel (2015), Drożdż et al. (2015), Setati et al. (2015), Boynton and Greig (2016), Garofalo et al. (2016), Jara et al. (2016), Rossouw and Bauer (2016), Villalba et al. (2016)
[b]Current accepted name as listed in MycoBank: http://www.mycobank.org/
[c]Many strains of *C. zemplinina* were misidentified as *C. stellat*a in the literature resulting in some confusion (Csoma and Sipiczki 2008). *C. zemplinina* is now classified as *S. bacillaris* but in older literature it was sometimes identified as *C. stellata*

mouthfeel characters than those conducted with a less rich and complex microbiota (Boynton and Greig 2016).

In contrast, in many wine production regions, the grape juice or must is immediately inoculated with a commercial strain of *S. cerevisiae*. There are two main reasons for this practice. First, addition of high population numbers of *S. cerevisiae* assures rapid dominance of the fermentation thereby minimizing the contribution of the non-*Saccharomyces* yeast and bacteria. This is desired if the resident biota will negatively impact the progression of the fermentation or lead to off-character formation or the formation of undesirable compounds from a health perspective, such as the biogenic amines. Second, inoculation is also used in styles that wish to accent the fruit component of the wine aroma and flavor profile and minimize that of the wild microbiota.

These diverse styles of winemaking require different fermentation management strategies and sanitation practices. The characteristics of autochthonous fermentations derive from the interspecies richness of the environment, while inoculated fermentations rely more on intraspecies diversity within *S. cerevisiae* for complexity and distinction. Intermediate styles are also employed such as creation of an autochthonous starter culture for the inoculation of native fermentations or partial native fermentations, those that will be inoculated with a commercial strain of *S. cerevisiae* at some specific point after the initiation of fermentation by the non-*Saccharomyces* yeasts.

Use of pure cultures in winemaking arose only within the last 70 years of the 7000-year history of wine production (Jolly et al. 2013). It is becoming more common to also inoculate with non-*Saccharomyces* yeast to obtain the positive characters associated with some of these yeasts while minimizing the risk of spoilage (Ciani and Comitini 2015; Englezos et al. 2016b; Liu et al. 2016; Lleixa et al. 2016; Medina et al. 2016; Padilla et al. 2016b). Commercial preparations of the non-*Saccharomyces* yeast *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Pichia kluyveri*, and *Metschnikowia pulcherrima* are available for use in wine production. These practices aim to have the best of both worlds, the complexity that accompanies a diverse microbial community with a reduced risk for off-character formation or arrest of the alcoholic fermentation.

The microbiota associated with grapes is impacted by several factors: climate, topography, geographic location of the vineyard, soil composition, farming practices, the varietal used, resident and transient insect vectors, bird and animal vectors, human activity and adjacencies, and time of harvest (Longo et al. 1991; Bagheri et al. 2015; Setati et al. 2015; Padilla et al. 2016a; Robinson et al. 2016). Grapes can harbor both beneficial and detrimental populations with respect to wine quality. Beneficial microbiota are those that contribute positive aroma or mouthfeel characteristics and that do not interfere with the completion of fermentation by *S. cerevisiae*. Detrimental effects of grape bacteria include arrest of yeast fermentation at undesired residual sugar concentrations and the production of off or unwanted characters in the wine.

Research has shown the importance of microbial activity to the "terroir" of many regions and vineyards (Bokulich et al. 2014, 2016a; Cappozzi et al. 2015; Belda et al. 2016a; Francesca et al. 2016). Terroir simply put is the expression of place (or site) and time (or vintage) largely associated with old world winegrowing regions. That the microorganisms present strongly contribute to the signature characters of regional terroir is not surprising. However, newer wine-producing regions are focused more on the expression of the characters of the grape in the finished wine, and such wine styles reflect the grape variety and dominant microbial aroma signatures are considered unwelcome or outright spoilage. What is a desired microbial contribution in one region may be undesirable in another. Both styles of wine production need to consider the roles of the spectrum of yeast and bacteria present in creation of the final product whether or not that role is to be amplified or diminished. Given this important dichotomy of styles, this review will consider diversity of both *Saccharomyces* and non-*Saccharomyces* yeast in the production of grape wine.

3.2 Non-*Saccharomyces* Strain Diversity

Yeast nomenclature is in a state of flux as more of the 1500 known yeast species are sequenced and new species are discovered (Jolly et al. 2013; Kurtzman et al. 2015). Classical yeast taxonomy divided isolates into teleomorphs (sexual cycle observed under laboratory conditions) and anamorphs (no observed sexual cycle). DNA sequence comparisons have demonstrated the capriciousness of such designations, and efforts are underway to eliminate this type of taxonomic identification. Comparative DNA sequence analysis now underpins yeast taxonomy, and many historical genera have been completely reclassified as a result of these analyses. The current dynamic nature of yeast taxonomy poses a challenge to the field of wine microbiology since many studies on the same organism might use different taxonomic terminology. Table 3.1 presents the current list of yeast species that have been isolated from grapes or wine with preference for the "current name" nomenclature of MycoBank (http://www.mycobank.org) and also shows other commonly used names for the species in the wine literature.

3.2.1 *Assessment of Non*-Saccharomyces *Yeast Diversity*

The assessment of yeast diversity in complex environments such as the surface of grape or crushed grape must is challenging. Recovery of organisms from the surface of fruit can be problematic depending upon the nature of the attachment formed. In some cases, qualitative information may be all that is desired, and selective media or defined molecular probes can be used to determine presence or absence of species of interest. Profiling the diversity of the microbial community present is significantly more difficult especially if information on relative numbers within the community is desired. Minor species within the ecosystem may have a large impact depending upon their metabolism and ability to either negatively or positively affect other members of the community.

Cultivation and non-cultivation methodologies have been used to assess the composition of microbial communities on grapes, each with their own biases and limitations (Tamang et al. 2016). Both types of methods suffer from an inability to detect relatively small subpopulations within the community. Further, it is simply not possible to culture yeast from an environment without imposing a selection based on type of medium used or temperature of growth. Rapidly proliferating organisms can inhibit growth of other species on laboratory media or simply outgrow more slowly growing members of the community preventing their detection. Non-culture-based methodologies often detect dead as well as live cell populations and, depending upon the method chosen, may amplify populations for detection in nonuniform ways thereby misrepresenting the community structure of the original environment. Small subpopulations may have a dramatic impact on wine aromatic composition given that many wine aroma impact compounds have low human thresholds of detection.

Differential media, such as the selection for use of lysine as sole nitrogen source or resistance to cycloheximide, are often used to discriminate between *Saccharomyces* and non-*Saccharomyces* yeasts (Fleet 1993; Boulton et al. 1996; Egli et al. 1998; Ganga and Martinez 2004; Renouf et al. 2006a, b). Use of nonselective media combined with colony morphology analyses can aid in strain identification but is rarely definitive (Pallmann et al. 2001). Nonselective media can also be used for mass colony isolation and identification using molecular tools but these methods are time consuming unless high-throughput techniques are employed. Many different molecular techniques have been used for yeast species identification including polymerase chain reaction (PCR) of the 26s ribosomal DNA (Kurtzman and Robnett 1998) and sequencing of the fragment or PCR combined with restriction enzyme digestion of internal transcribed spacers (ITS) from the 5.8s ribosomal DNA (Guillamon et al. 1998). These techniques still contain the bias inherent in the requirement for initial plating and isolation of the organism to be identified. Another rapid and high-throughput method that has been used extensively in medical fields is the use of matrix-assisted laser desorption/ionization–time-of-flight (MALDI–TOF) mass spectrometry (Bille et al. 2012). This method typically calls for the isolation of organisms prior to identification but we have used it

successfully to identify heavy blooms of organisms in wine (data not published). While the technique appears to be rapid and robust, the instrumentation is expensive and the lasers are prone to failure.

Molecular techniques such as PCR combined with denaturing gradient gel electrophoresis (DGGE) (Cocolin et al. 2000) and quantitative PCR (q-PCR) (Phister and Mills 2003) have also been employed to evaluate the ecological succession of microbes during fermentations and to identify spoilage organisms in wine. Newer direct DNA sampling methods are becoming the norm to profile microbial communities of grapes, winery surfaces, and wine (Prakitchaiwattana et al. 2004; Bokulich et al. 2016b; Boynton and Greig 2016; Portillo and Mas 2016). In this case total DNA from the sample is purified and processed to enable sequence analysis of fragments obtained. The frequency with which a specific fragment is observed can be used to determine relative proportions of microbes in the environment. Improvements to DNA sequencing technology have extended these analyses now enabling unprecedented community profiling or metagenomics. Next-generation sequencing technologies rely on nanotechnology for fragment sequencing and have high accuracy and quantitative capability depending upon the precise methodology employed (Bokulich et al. 2012, 2016b). Non-DNA methods such as FT-IR are also being used to both characterize the metabolic activity of the community as well as to identify organisms present (Grangeteau et al. 2016), and the combination of this technology with next-generation sequencing tools will provide information on organisms present and their relative metabolic activities and can help address issues with viable but non-culturable organisms in the ecosystem (Wang et al. 2015a; Grangeteau et al. 2016).

3.2.2 *Factors Affecting Non*-Saccharomyces *Diversity: Presence in Vineyard*

The range of yeast species present on grapes has been extensively examined in vineyards worldwide (Barnett et al. 1972; Davenport 1974; Goto and Yokotsuka 1977; Sapis-Domercq et al. 1977; Bureau et al. 1982; Rosini et al. 1982; Parish and Carroll 1985; Yanagida et al. 1992; Martini et al. 1996; Sabate et al. 2002; Mercado et al. 2004; Prakitchaiwattana et al. 2004; Combina et al. 2005; Raspor et al. 2006; Nisiotou and Nychas 2007; Renouf et al. 2007; Börlin et al. 2009; Garijo et al. 2011; Barata et al. 2012; Byrsch-Herzberg and Seidel 2015; Drożdż et al. 2015; Setati et al. 2015; Vigentini et al. 2015; Capece et al. 2016; Garofalo et al. 2016; Jara et al. 2016) and previous reviews have covered this topic (Fleet 1993; Kunkee and Bisson 1993; Fleet et al. 2002; Bisson 2012; Jolly et al. 2013; Cappozzi et al. 2015). In general, these studies show that yeast, both *Saccharomyces* and non-*Saccharomyces* species, represent a minor population on the surface of the fruit when compared to filamentous fungi and bacteria. Yeast have been estimated to be present at a concentration of 3×10^5 yeast cells/cm^2 following aggressive

washing of the berry surface (Rosini et al. 1982). Other studies report a range of 10^4 –10^6 yeast cells/cm^2 on the grape surface (Fleet et al. 2002). *S. cerevisiae* is often a minor resident among the minor resident yeast or not found at all (Boulton et al. 1996).

The yeast species comprising the highest relative population numbers on the surface of grapes change during ripening and follow a consistent pattern of early dominance by the basidiomycetous yeasts, *Aureobasidium*, *Cryptococcus*, *Rhodosporidium*, and *Rhodotorula*, giving way to the ascomycetous yeast, particularly *Hanseniaspora*, *Metschnikowia*, and *Candida*, as the fruit matures regardless of the location of the vineyard (Kunkee and Bisson 1993; Fleet et al. 2002; Jolly et al. 2013; Boynton and Greig 2016). Many factors in addition to stage of ripening have been identified that impact the presence and numbers of yeasts on the surface of grape. The presence of specific yeast genera depends upon regional and climactic influences, the grape variety, disease pressure and level of damage of the grapes, and vineyard practices (Barbe et al. 2001; Bagheri et al. 2015; Setati et al. 2015; García et al. 2016; Padilla et al. 2016a). The location of the sample within the vineyard can impact the species identified, so broad sampling across vineyards is needed to obtain information on the diversity of yeasts present (Garofalo et al. 2016), and species isolated vary by latitude and relative humidity (Jara et al. 2016). Studies across vintages demonstrate that the vintage, and presumably climate thereof, have more of an impact on yeast diversity of grape than the geographic location of the vineyard (Vigentini et al. 2015). One study demonstrated that summer temperature patterns were predictive of yeast populations at harvest (Robinson et al. 2016).

The three principal ascomycete genera found on grapes, *Hanseniaspora uvarum* (anamorph: *Kloeckera apiculata*) and *Metschnikowia pulcherrima* (anamorph: *Candida pulcherrima*) and *Candida stellata*, vary in relative and absolute numbers across different vineyard sites. In some reports *Hanseniaspora* is the dominant species (Beltran et al. 2002; Combina et al. 2005; Hierro et al. 2006) and in others it is *Candida* (Torija et al. 2001; Clemente-Jimenez et al. 2004) or *Starmerella bacillaris* (*Candida zemplinina*/*Candida stellata*) (Setati et al. 2015; Masneuf-Pomarede et al. 2016). *Candida* strains have been shown to be able to complete the alcoholic fermentation in some cases (Clemente-Jimenez et al. 2004; Setati et al. 2015). Several of the *Candida stellata* isolates from wine have been subsequently identified as *Candida zemplinina* (Csoma and Sipiczki 2008) now known as *Starmerella bacillaris* (Masneuf-Pomarede et al. 2016). In one study of grapes from cooler climates (Yanagida et al. 1992), the basidiomycetes *Cryptococcus* and *Rhodotorula* dominated in number over the ascomycete yeasts. In another the dimorphic fungus, *Aureobasidium,* was found as the dominant yeast on grape surfaces in addition to *Cryptococcus*, followed by *Rhodotorula* and *Rhodosporidium*, depending upon the grape variety (Prakitchaiwattana et al. 2004). The spectrum of yeast strains identified varies by the identification methodology with the major species present in both next-generation and cultivation analyses (Setati et al. 2015). However, some minor species were only detected by

sequencing and some only by cultivation (Setati et al. 2015). The method of analysis can impact the diversity of species found in the vineyard.

The microbiota of the grape surface can be influenced by physical damage mediated by insects, birds, or invasive fungal species, or as a consequence of berry aging and dehydration (Parish and Carroll 1985; Fleet et al. 2002; Prakitchaiwattana et al. 2004; Barata et al. 2008). Fermentative organisms dominate in rot situations including wild vineyard species of *Saccharomyces*. The amount of natural seepage varies with different grape varieties and the tightness of the clusters, and some studies have seen a correlation of variety and biodiversity of the fruit surface (Yanagida et al. 1992).

Other yeasts may also be found on grape surfaces although they are not as universal. *Saccharomyces* can be detected, but is present on grape surfaces at very low levels (Martini et al. 1996; Prakitchaiwattana et al. 2004; Ivey et al. 2013), and in some studies has been undetectable (Combina et al. 2005; Raspor et al. 2006). *Saccharomyces* is more commonly isolated from heavily damaged grapes (Mortimer and Polsinelli 1999). Yeast populations on the surface of the fruit are also impacted by the presence of other microbiota. Species interactions are complex and vary from cross-feeding and mutualism to competition to directed inhibition based on killer factor or other toxin production (Ivey et al. 2013; Bisson and Walker 2015; Ramakrishnan et al. 2016; Villalba et al. 2016).

3.2.3 *Factors Affecting Non-*Saccharomyces *Diversity: Impact of Fermentation Management on Persistence*

Yeast species present on the surface of the grape comprise the initial microbiota of the juice in combination with winery surface biota transferred to the juice by passage through winery equipment. Over 20 yeast genera have been identified from fermenting must (Vezinhet et al. 1992; Versavaud et al. 1995; Cavalieri et al. 1998; Sabate et al. 1998; Sipiczki 2002, 2006; Schuller et al. 2005; Renouf et al. 2007; Valero et al. 2007) with the fermentative yeasts persisting longer than the obligate aerobes. Immediately upon crushing of the fruit the dynamics of the environment changes, oxygen becomes depleted due to continued microbial activity and enzymatic and chemical reactions that consume molecular oxygen creating conditions hostile particularly to filamentous fungi. The low pH of the juice (pH 3.2–3.8) is nonpermissive for many bacteria and the microbial community dynamics become altered. The osmolarity increases due to sugar release, which is also inhibitory to many organisms. All of these factors narrow the spectrum of culturable organisms present, although next-generation sequencing analysis suggest a persistence of diversity. Numerous studies have categorized the changes and persistence of non-*Saccharomyces* strains during inoculated and uninoculated fermentations (Vezinhet et al. 1992; Querol et al. 1994; Schutz and Gafner 1994; Constanti et al. 1997; Gutierrez et al. 1997, 1999; Van der Westhuizen et al. 2000b;

Torija et al. 2001; Beltran et al. 2002; Van Keulen et al. 2003; Hierro et al. 2006; Renouf et al. 2006b; Xufre et al. 2006).

Yeast population dynamics are impacted by winemaking conditions. Use of antimicrobial agents dimethyldicarbonate (DMDC), lysozyme, sulfur dioxide (SO_2), or treatments (high temperature, ozonation) will impact the microbial biota. Sulfur dioxide does not show a significant effect on the wild fermentative yeast species present (Henick-Kling et al. 1998). Other studies have observed a slight effect of SO_2 in a decrease in yeast cell numbers (Egli et al. 1998). In contrast, the basidiomycetous yeasts seem to show a greater sensitivity to SO_2, with a reported up to 90% decrease of these yeasts (Rementeria et al. 2003).

A common practice in red winemaking is to hold the must at a low temperature retarding the onset of fermentation, enabling release of water-soluble pigments and enhanced color formation. This holding of must at low temperatures or "cold soak" impacts the relative composition of the yeast community enriching for yeast species tolerant of low temperatures (Fleet and Heard 1993). The presence of these yeasts can then influence the metabolic behavior of the principle agent of the yeast fermentation, *Saccharomyces*, as well as directly contributing aroma impact compounds to the wine. The precise temperature of the cold soak was shown to impact yeast populations differentially with slightly warmer temperatures, 14 °C favoring *Hanseniaspora* and *Candida* over *Saccharomyces*, while *Saccharomyces* was more dominant at 8 °C (Maturano et al. 2015). Other practices, such as aeration of the fermentation, nutrient additions, and temperature of fermentation will likewise impact the yeast microbial community differentially.

The yeast populations found on winery surfaces, equipment, and in winery air have also been investigated (Martini 2003; Ciani et al. 2004; Mercado et al. 2004; Renouf et al. 2007; Garijo et al. 2008, 2009; Santamaria et al. 2008; Blanco et al. 2011; Haas et al. 2010; González-Arenzana et al. 2012; Ocón et al. 2013). Winery microbiota are a significant source of pre-fermentation microbial activity in juice and must (Fleet and Heard 1993; Renouf et al. 2007). As crush progresses juices may pick up 10^3–10^4 cells/mL of *S. cerevisiae* which represents a striking alteration of the juice microbial community (Boulton et al. 1996). Analysis of the surfaces of barrels indicated high numbers of *Saccharomyces*, with *Candida*, *Cryptococcus*, and *Brettanomyces* also commonly present, although in lower concentrations (Renouf et al. 2006b, 2007). Sanitation practices can therefore have a dramatic effect on the organisms present during fermentation by either allowing or restricting the buildup of high populations on equipment and in tanks. Wineries with poorer sanitation practices had higher levels of the fermentative yeasts presumably because these yeasts had colonized winery equipment (Regueiro et al. 1993).

The diversity of yeast seen at the onset of fermentation quickly decreases as *S. cerevisiae* populations become dominant. *S. cerevisiae* is generally believed to have evolved to dominate batch fermentations of substrates containing high concentrations of hexoses (Cray et al. 2013; Ivey et al. 2013; Williams et al. 2015). This yeast employs several strategies to enable domination, depletion of nutrients, particularly of nitrogen sources and molecular oxygen, narrowing of the environmental niche through the production of ethanol, carbon dioxide, and heat from

fermentation and in reduction of the redox potential of the juice, and also produces a wide variety of inhibitory compounds: fatty acids, antimicrobial peptides, and less well-characterized components (Boulton et al. 1996; Bisson and Walker 2015; Williams et al. 2015; Albergaria and Arneborg 2016; Ramakrishnan et al. 2016). *S. cerevisiae* can grow at lower redox potentials than most yeast (Visser et al. 1990), and the rapid deployment of glycolysis enables rapid growth and alteration of the environmental niche. We consider narrowing of the niche as distinct from inhibition as the niche is narrowed for *S. cerevisiae* as well increasing the toxicity of various stress factors in the environment (Ramakrishnan et al. 2016). Inhibition on the other had is targeted toward other species present. Thus the diversity of the grape surface initially present in wine diminishes under wine production conditions due to the creation of nonpermissive or narrowed niche conditions and the inoculation by organisms present on winery surfaces and equipment.

3.2.4 *Impact of Non-*Saccharomyces *Strains on Wine Flavor, Aroma, and Mouthfeel*

The non-*Saccharomyces* yeasts have been shown to release a wide range of hydrolytic enzymes interacting with grape precursor compounds and impacting the sensorial and structural features of the wine (Lema et al. 1996; Fernández et al. 2000; Strauss et al. 2001; Hernández-Orte et al. 2008; Belda et al. 2015; Padilla et al. 2016b). Some of these enzymatic activities are aroma related, such as β-glucosidase, β-lyase, esterase, and alcohol acetyltransferase.

The β-glucosidase enzymes allow the release of monoterpenes, present in grapes as glycosylated flavorless precursors. Monoterpenes are important compounds determining the flavor of grapes and wine. The yeasts belonging to the genera *Brettanomyces*, *Candida*, *Debaryomyces*, *Hanseniaspora*/*Kloeckera*, *Kluyveromyces*, *Issatchenkia*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomycodes*, *Schizosaccharomyces*, *Torulaspora*, *Wickerhamomyces*, and *Zygosaccharomyces* have been shown to produce β-glucosidase in amounts that are species and strain dependent, and with varying degrees of activity and different catalytic activities with respect to grape aroma glycosides (Rosi et al. 1994; Gueguen et al. 1996; Charoenchai et al. 1997; McMahon et al. 1999; Yanai and Sato 1999; Fernández et al. 2000; Manzanares et al. 2000; Mendes Ferreira et al. 2001; Strauss et al. 2001; Spagna et al. 2002; Cordero Otero et al. 2003; Fernández-González et al. 2003; Wallecha and Mishra 2003; Rodríguez et al. 2004, 2007; Arévalo Villena et al. 2005, 2007; González-Pombo et al. 2008, 2011; Hernández-Orte et al. 2008; Swangkeaw et al. 2009, 2011; Comitini et al. 2011; Domizio et al. 2011; Sadoudi et al. 2012; Cordero-Bueso et al. 2013; López et al. 2014; Sabel et al. 2014; Belda et al. 2015; Hu et al. 2016a; Polizzotto et al. 2016). The activity of these enzymes has been assessed primarily using artificial substrate. Some studies have shown the enzymatic activity either hydrolyzes a glycoside extract from grape

must, as for the yeasts *Debaryomyces hansenii*, *Hanseniaspora uvarum*, *Kloeckera apiculata*, and *Rhodotorula mucilaginosa* (Rosi et al. 1994; Yanai and Sato 1999; Mendes Ferreira et al. 2001; Fernández-González et al. 2003; Hu et al. 2016b) or releases terpenols after addition to must or wine, as for the yeasts *Hanseniaspora* sp., *Hanseniaspora uvarum*, and *Pichia anomala* (Swangkeaw et al. 2009; Hu et al. 2016a).

In contrast, the enzyme β-lyase permits the release of volatile thiols from grape precursor conjugated to cysteine or glutathione and is responsible of the varietal aroma enhancement related to nuances of box tree, passion fruit, grape fruit, and citrus. Only a few strains have been screened for this activity, specifically strains of *M. pulcherrima*, *T. delbrueckii*, and *K. marxianus* have shown high β-lyase activity (Anfang et al. 2009; Zott et al. 2011; Belda et al. 2016b; Renault et al. 2016).

Different spectrums of esters may be produced by non-*Saccharomyces* yeast as well. Esters are primarily responsible for wine fruitiness, and their level in wine is determined by the balance between the esterase enzymes responsible for their cleavage and alcohol acetyltransferase enzymes promoting their synthesis (Fukuda et al. 1998). The production of the ester compounds is species and strain dependent but several yeasts (*Candida*, *Hansenula*, *Pichia*, *Hanseniaspora*, *Rhodotorula*, *T. delbrueckii*, *K. gamospora*) have been generally recognized as high producers (Padilla et al. 2016b). Although most non-*Saccharomyces* yeasts can produce high amounts of ethyl acetate (Moreira et al. 2008; Andorrà et al. 2010) which has a solvent-like aroma at levels higher than 150 mg L^{-1}, several non-*Saccharomyces* wine yeasts are also high producers of other interesting esters compounds, such as isoamyl acetate (banana-like aroma) and 2-phenylethyl acetate (roselike aroma). The yeasts *Hanseniaspora guilliermondii* and *Hanseniaspora osmophila* have shown high production of 2-phenylethyl acetate (Rojas et al. 2001, 2003; Moreira et al. 2008; Viana et al. 2008).

Other interesting ester compounds, such as isoeugenyl phenylacetate (spicy, clove-like aroma), phenethyl propionate (roselike aroma), and isobornyl acetate (complexly woody, camphorous, piney, and herbal with citrus nuances), have been shown to be produced by some non-*Saccharomyces* yeasts (Whitener et al. 2015).

During the alcoholic fermentation, other important aromatic compounds, such as the higher alcohol, are produced. These compounds contribute to the wine aromatic complexity when present at concentration below 300 mg/L. Different levels of higher alcohols are produced by non-*Saccharomyces* yeasts and in most of the case are strain dependent (Pretorius and Lambrechts 2000). Yeasts that are low producer of higher alcohols such as those belonging to the species *Hanseniaspora*, *Pichia* (Rojas et al. 2003; Moreira et al. 2008; Viana et al. 2008), and *Zygosaccharomyces* (Romano and Suzzi 1993) are of particular interest. Among the higher alcohols, a positive contribution derives from 2-phenylethyl alcohol, (floral, roselike aroma) that is produced at high levels by *M. pulcherrima*, *P. fermentans* (Clemente-Jimenez et al. 2004), *L. thermotolerans* (Whitener et al. 2015), *Torulaspora delbrueckii* (Herraiz et al. 1990; Renault et al. 2009), and *Starmerella bacillaris* (*C. zemplinina*) (Andorrà et al. 2010).

Non-*Saccharomyces* yeasts may produce compounds that either mask or enhance varietal characters. Compounds such as benzyl alcohol and other benzenoid derivatives can impart a generic floral or fruitiness to wine that increases perception of the grape aroma compounds (Martin et al. 2016).

Besides aroma compounds, non-*Saccharomyces* yeasts have been shown to produce compounds impacting wine mouthfeel. Polysaccharides, in particular mannoproteins, can impact sensorial features either directly or through their effect on some other wine compounds improving the mouthfeel and fullness, decreasing astringency, adding complexity and aromatic persistence, and increasing sweetness and roundness (Vidal et al. 2004; Carvalho et al. 2006; Chalier et al. 2007; Juega et al. 2012). The yeasts *Candida*, *Hanseniaspora*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Pichia*, *Saccharomycodes*, *Schizosaccharomyces*, *Torulaspora delbrueckii*, and *Zygosaccharomyces* have all been shown to release high quantities of polysaccharides during the alcoholic fermentation. Wide biodiversity for this characteristic was seen within the genera *Hanseniaspora* and *Zygosaccharomyces* (Romani et al. 2010; Comitini et al. 2011; Domizio et al. 2011, 2014). The yeasts belonging to the genera *Schizosaccharomyces* have shown the highest release of polysaccharides in the media (Domizio et al. 2017).

Despite glycerol being viscous in its pure form, it does not affect perceived viscosity (Noble and Bursick 1984; Gawel and Waters 2008). Instead it is responsible for the sweetness of red and white wine (Noble and Bursick 1984; Hufnagel and Hofmann 2008). The strain *Starmerella bacillaris* (*C. zemplinina*) has been the subject of many studies because of its ability to release high quantities of glycerol (Ciani and Picciotti 1995; Ciani and Maccarelli 1998; Ciani and Ferraro 1998; Soden et al. 2000; Romani et al. 2010; Englezos et al. 2015; Polizzotto et al. 2016). Other yeasts belonging to the genera *Kluyveromyces*, *Saccharomycodes*, and Schizo*saccharomyces*, have also been shown to produce high quantities of glycerol (Romani et al. 2010).

High ethanol concentration may modify various sensory attributes decreasing acidity sensations, increasing hotness and bitterness perceptions, influencing the volatility of important aroma compounds, and masking the perception of some aroma compounds as well (Robinson et al. 2009; Frost et al. 2015). Consequently some of the features typical of non-*Saccharomyces* yeast, such as the low fermentation efficiency, reduced ethanol yield, and respiro-fermentative metabolism, have resulted in their reevaluation for the possible reduction of the alcohol concentration in wine (Gonzalez et al. 2013; Quirós et al. 2014; Contreras et al. 2015b; Morales et al. 2015; Canonico et al. 2016; Ciani et al. 2016b; Englezos et al. 2016a; Röcker et al. 2016; Rossouw and Bauer 2016; Varela et al. 2016). These features make the co-fermentation of the non-*Saccharomyces* yeast with *Saccharomyces* mandatory to ensure complete sugar fermentation. The yeast *Starmerella bacillaris* (*C. zemplinina*) has a very low ethanol yield making it the most promising yeast candidate to reduce the ethanol content (Magyar and Tóth 2011; Di Maio et al. 2012; Canonico et al. 2016; Englezos et al. 2016a). However, other non-*Saccharomyces* yeasts have been evaluated as possible candidates to reduce wine ethanol content, such as strains belonging to the species *C. stellata*,

H. osmophila, *H. uvarum*, *M. pulcherrima*, *Pichia kudriavzevii*, *Schizosaccharomyces malidevorans*, *T. delbrueckii*, *Zygosaccharomyces sapae*, *Zygosaccharomyces bailii*, and *Zygosaccharomyces bisporus* (Gobbi et al. 2014; Contreras et al. 2014, 2015a,b; Curtin and Varela 2014; Quirós et al. 2014; Canonico et al. 2016).

The impact of the non-*Saccharomyces* strains is dependent upon strain-specific interactions with the *Saccharomyces* strains present (Ciani et al. 2010, 2016a; Ciani and Comitini 2015; Wang et al. 2015b, 2016; Albergaria and Arneborg 2016). Although a thorough understanding of the mechanisms involved in the interactions is still a long way off, several biotic (microorganisms, killer factors, grape variety, etc.) and abiotic factors (pH, temperature, ethanol, osmotic pressure, nitrogen, molecular sulfur dioxide, etc.) have been reported as influencing the degree of competition between the yeasts carrying out the fermentation process.

As discussed above, some aroma compounds and other metabolites can be used as markers for some yeasts belonging to a specific genera, species, or even strain. However, because of the different types of interactions, *Saccharomyces* yeasts can modulate the expression of some of the oenological traits of the non-*Saccharomyces* yeasts and the resulting product may not reflect the specific features of the yeast in the pure culture fermentation. The different types of interactions will determine different effects on metabolite levels: additive effects (production or reduction of a metabolite whose level is influenced by the persistence of the yeast producer), synergistic effects (exchange of metabolites between yeasts or enhancement of levels), and negative effects (reduction in the level of metabolites) have been reported (Ciani and Comitini 2015). To take advantage of the specific potential of the different non-*Saccharomyces* strains, mixed inocula with *Saccharomyces*/non-*Saccharomyces* has been explored as an approach to increase the wine aroma complexity. Pure fermentations carried out with non-*Saccharomyces* are not typically feasible at the winery scale, mainly due to their low competitiveness with respect to *Saccharomyces* yeasts. Hundreds of different strains of non-*Saccharomyces* yeasts are presents in any single vineyard (Barata et al. 2012), and an appropriate yeast selection program and their inoculum together with *S. cerevisiae* could allow producers to obtain wine with unique organoleptic characteristics reflecting and strengthening the "terroir concept" and allowing, to a certain extent, the replication of a microbial fingerprint for a given viticultural area.

3.3 Diversity of *Saccharomyces*

Yeast strain diversity arises as a consequence of mutation, genome assortment during sporulation, movement of transposable elements, introgressions, horizontal or lateral gene transfer, and formation of hybrid strains (Marsit and Dequin 2015). Mutations giving rise to a single nucleotide polymorphism or insertions/deletions may accumulate during vegetative or mitotic growth and can become expressed

following meiosis and subsequent mating of progeny cells in a process termed "genome renewal" and restoration of a homozygous state (Mortimer et al. 1994). The yeast life cycle of accumulation of heterozygosities in the diploid phase, followed by sporulation and either self-crossing or outcrossing, facilitates rapid adaptation to novel environments as well as enhancing fitness for an existing environment (Magwene 2014). Since many vineyard isolates are homozygous diploids, mating-type switching or self-fertilization appears to be an important mechanism of sexual reproduction in wine populations of *S. cerevisiae* (Cubillos et al. 2009). Phenotypic variation can be due to differences in chromosome structure or region adjacencies, gene copy number, expression or mRNA abundance, modification of protein structure or functionality, altered structure, organization, responsiveness, or signaling among cellular networks (Gasch et al. 2015; Peter and Schacherer 2016). Strain phenotypic diversity may also be due to differences in mitochondrial genomes (Wolters et al. 2015). Horizontal gene transfer among mitochondrial DNA elements in *Saccharomyces* species has also been observed (Peris et al. 2015). In addition to genomic diversity, *S. cerevisiae* has recently been shown to harbor a host of prion or prion-like elements that will lead to variation in population phenotypes (Garcia and Jarosz 2014; Halfmann and Lindquist 2010; Halfmann et al. 2010, 2012). Prions are heritable self-perpetuating protein structures or complexes that confer novel phenotypes to yeast cells and that have been found in wine strains (Brown and Lindquist 2009; Jarosz et al. 2014; Walker et al. 2016). Prion expression has been shown to vary across *S. cerevisiae* lineages (Kelley et al. 2014) and may impact surveys of phenotypes among wine strains.

Whether one believes *S. cerevisiae* displays high or low species diversity depends upon the type of analysis conducted as well as the definition of diverse. Some methodologies compare similarities of genomic composition, while others assess the number and frequency of differences. Analysis of the population genomics of commercial, winery, and vineyard isolates in comparison to non-wine isolates of *S. cerevisiae* indicates a high degree of relatedness among the wine strains regardless of the analytical methodology used (Liti et al. 2006, 2009; Legras et al. 2007; Schacherer et al. 2007, 2009; Knight and Goddard 2015).

3.3.1 Methods of Analysis

The above discussion highlights the importance of the type of experiment conducted to assess strain differences. Several different methodologies have been applied to the assessment of population diversity in *S. cerevisiae* (reviewed in Bisson 2012; Gasch et al. 2015). Direct comprehensive genomic sequence comparisons have been conducted as having partial sequence analysis of multiple loci (Liti et al. 2006, 2009; Borneman et al. 2008, 2016; Tsai et al. 2008). Microarray karyotyping and SNP assessment have been performed (Winzeler et al. 2003; Dunn et al. 2005; Gresham et al. 2006; Schacherer et al. 2009) as well. Microsatellite genotyping of global populations of *S. cerevisiae* was used to define lineages and

domestication events (Legras et al. 2007). Transcirptome or targeted gene expression comparisons have also been conducted to assess strain variation (Townsend et al. 2003; Fay et al. 2004). Strains that are undistinguishable from each other by gross genomic or mitochondrial DNA profiling may carry mutations leading to changes in important enological phenotypes, particularly if the genetic differences are targeted to high impact genes (such as transcription factors) or genes involved in flavor modification or production. Non-DNA-based methodologies such as FT-IR profiling have also been used to differentiate yeast strains (Grangeteau et al. 2016) as has MALDI–TOF mass spectrometry (Moothoo-Padayachie et al. 2013).

3.3.2 Genomic Diversity

S. cerevisiae and *S. bayanus* have both been reported capable of dominating and conducting the alcoholic fermentation, with *S. cerevisiae* being the more prevalent (Sipiczki 2002). *S. pastorianus* is occasionally found but hybrids of this yeast are more common (Naumov 1996). Sequence comparisons between *S. cerevisiae* and *S. bayanus* indicate approximately 80% and 74% identity of coding and noncoding sequences, respectively (Cliften et al. 2003). Several studies have reported significant genetic diversity among wine strain isolates of both *S. cerevisiae* (Schutz and Gafner 1994; Versavaud et al. 1995; Baleiras Couto et al. 1996; Briones et al. 1996; Sabate et al. 1998; Khan et al. 2000; Van der Westhuizen et al. 2000a, b; Lopes et al. 2002; Gallego et al. 2005; Schuller et al. 2005; Valero et al. 2006) and *S. bayanus* (Sipiczki 2002). *S. uvarum* isolated from grapes and wine shows similar diversity. Eighty-nine distinct genotypes were found among 108 isolates of this strain via microsatellite analysis (Masneuf-Pomarede et al. 2016). Strains of *S. uvarum* showing introgressions of *S. eubayanus* DNA have also been isolated from winery environments and display enhanced ability to conduct low-temperature fermentations (Zhang et al. 2015).

Comparative sequence analysis of nearly 100 commercial strains of *S. cerevisiae* against a larger backdrop of wild isolates suggests a high degree of genetic similarity and inbreeding (Borneman et al. 2016). In this study the commercial strains formed a highly related clade with limited diversity when compared to the global pool of *S. cerevisiae* (Borneman et al. 2016). These findings suggest that the phenotypes desired in commercial strains leads to a selection for genetic commonality. Other studies have reached similar conclusions regarding a "domestication fingerprint" of wine strains that reflects the association of wine yeast with human activities (Almeida et al. 2015; Eberlein et al. 2015; Marsit and Dequin 2015). Greater diversity was seen among vineyard and winery resident strains than was observed among commercial isolates (Börlin et al. 2009; Knight and Goddard 2015; Capece et al. 2016; Rossouw and Bauer 2016). Analyses of *S. cerevisiae* from human-associated and nonassociated environments displayed high divergence across *S. cerevisiae* with human-associated strains generally showing less diversity (Wang et al. 2012). These investigations indicate that wine strains appear to have

derived from a single common ancestor, and, although some geographically isolated lineages can be observed, there is a strong influence of human migration patterns on yeast population diversity (Legras et al. 2007; Liti et al. 2009; Schacherer et al. 2009). Roughly 28% of the over 600 wine and vineyard isolates examined in one study were found to be homozygous suggesting that sporulation and self-diploidization occur in the wild (Legras et al. 2007) confirming the genome renewal hypothesis (Mortimer et al. 1994).

The Borneman et al. (2016) study evaluated strain diversity by comparative whole-genome sequencing which may be biased toward identifying similarities across the genome. Other studies that report high diversity have assessed yeast strain differences such as screens for single nucleotide polymorphisms (SNPs) or locations of repetitive or microsatellite DNA sequences. Analysis of both phenotypic and metabolomic profile differences across strains indicates that phenotypes can vary dramatically due to the presence of a small number of SNPs and insertion/deletions (Cubillos 2016; Franco-Duarte et al. 2016). More global analyses of the presence of SNPs suggest that they exist across populations of *Saccharomyces* with a frequency of approximately 2.8 SNPs per kilobase of DNA (Schacherer et al. 2009). However, Borneman et al. (2008) found a SNP frequency of 1 per 150 base pairs or roughly 7 SNPs per kilobase in the sequence comparison of a wine strain AWRI1631 to the laboratory strain S288c. The frequency of SNPs depends upon chromosomal location and is found less frequently in genes located near the centromere as compared to genes located in subtelomeric regions (Schacherer et al. 2009). DNA deletions also occur but are less frequently found in essential genes (Schacherer et al. 2009) likely due to negative selective pressure.

Regulatory region or noncoding polymorphisms have been shown to lead to gene expression variation across strains (Salinas et al. 2016). In addition, metabolic flux analyses have shown variation across several non-glycolytic carbon pathways in *S. cerevisiae* (Nidelet et al. 2016). Subtle differences in sequence of a single glucose transporter, the *HXT3* gene, resulted in dramatic differences in the fermentative abilities of wine strains of *S. cerevisiae* (Zuchowska et al. 2015). Thus although genetically highly related, wine strains display key phenotypic variabilities that impact fermentation performance as well as the spectrum of end products produced during fermentation. That variation that arises naturally in populations of wine strains of *S. cerevisiae* has been demonstrated in adaptive evolution experiments (Franco-Duarte et al. 2015; Mangado et al. 2015). Wild isolates of *S. cerevisiae* tend to show less heterozygosity than domesticated strains suggesting little to no outcrossing in these populations (Magwene 2014).

This diversity across *Saccharomyces* species has a functional effect impacting the spectrum of aroma impact compounds produced in the wines. In one study 1600 isolates of *S. cerevisiae* were obtained from 54 spontaneous fermentations and 297 unique strains were identified (Schuller et al. 2005). In a more limited investigation, 13 out of 16 isolates (81%) were determined to be unique strains (Baleiras Couto et al. 1996). Higher ratios of unique genotypes have also been found, 87.5 % (Valero et al. 2007), 81–91% (Gallego et al. 2005), and 91–96% (Schuller et al. 2005). Significant strain diversity can exist within the same vineyard environment,

suggesting the importance of localized conditions for the selection of genetically modified strains or, alternately, the existence of local factors driving genetic change such as exposure to ultraviolet light.

Studies of strain diversity across fermentation have reveled two broad types of profiles with respect to the numbers of different strains found in an individual tank. In some cases one or a few strains dominate throughout fermentation (Versavaud et al. 1995). This is typically seen in cases where commercial strains are used as inocula as these strains are selected in part for their ability to dominate early and throughout the fermentation. In other cases, particularly in autochthonous fermentations, different strains have been shown to be dominant at different stages of the fermentation (Sabate et al. 1998) and in some cases several different strains of *Saccharomyces* appear to be simultaneously present in equivalently high numbers (Vezinhet et al. 1992; Torija et al. 2001). Presumably, the biodiversity of wine strains in the environment results in these different patterns of dominance across fermentation. Assessment of strain diversity across vintages has shown that different strains are present each year (Gutierrez et al. 1999; Schuller et al. 2005).

Commercial and native yeast isolates both display greater genomic and genetic instability as compared to commonly used laboratory strains (Ambrona et al. 2005). Aberrations in the number of some chromosomes are more common in wild strain populations (Bakalinsky and Snow 1990) as is the existence of introgressions (small regions of DNA transferred via illicit hybridization with other species) and lateral gene transfer (transfer of genetic material across kingdom as well as species barriers). Wild strains also display higher levels of structural chromosomal rearrangements, heterozygosity, and karyotype instability (Longo and Vezinhet 1993; Izquierdo Canas et al. 1997; Codon et al. 1998; Hughes et al. 2000; Johnston et al. 2000; Mortimer 2000; Oshiro and Winzeler 2000; Carro and Pina 2001; Myers et al. 2004; Landry et al. 2006a, b). The dynamic nature of the genome likely poses a distinct advantage in the environment, as extensive diversity is observed among native isolates from the same site (Hauser et al. 2001; Landry and Aubin-Horth 2014).

3.3.3 Saccharomyces *Hybrid Strain Diversity*

Comparative karyotype analysis and sequence analysis of the *MET2* gene in wine isolates demonstrated that these yeast stains were genetic hybrids of *S. cerevisiae* and *S. bayanus* (Masneuf et al. 1998). Subsequently restriction analysis of multiple loci revealed wine strain hybrids of *S. cerevisiae*, *S. bayanus*, and *S. kudriavzevii* (González et al. 2006) indicating that hybrid formation in the wild was more common than previously thought. A low prezygotic barrier exists among *Saccharomyces* species enabling hybrid formation (Lopandic et al. 2016). Since these yeasts can occupy the same local environment (are sympatric), it is not surprising that hybrids may form. *S. kudriavzevii* was isolated originally from decaying leaves in Japan (Naumov et al. 2000) but has subsequently been shown to occupy a more

diverse habitat and hybrids between *S. kudriavzevii* and *S. cerevisiae* have been widely found in Europe (González et al. 2007, 2008; Gangl et al. 2009; Erny et al. 2012; Peris et al. 2012a, 2016). The European parental *S. kudriavzevii* strain was first found on oak bark in Portugal and in the similar niches to *S. cerevisiae* and *S. paradoxus* (Sampaio and Gonçalves 2008). It was thought that the hybrids between *S. cerevisiae* and *S. kudriavzevii* arose in non-winery environments and carried some selective advantage for fermentations. Genomic sequencing of the yeast South African commercial strain of the VIN7 demonstrated that it is an allotriploid hybrid carrying a diploid set of chromosomes from *S. cerevisiae* and a haploid set from *S. kudriavzevii* (Borneman et al. 2012). Thus these species are commonly found in similar habitats and able to engage in interspecies mating.

The genomic characteristics of *S. cerevisiae*/*S. kudriavzevii* hybrids varies and ranges from diploids to triploids and tetraploids (2*n*, 3, and 4*n* respectively) (Erny et al. 2012) suggesting that illicit mating may occur between haploids, between a haploid and a diploid, or between diploids. These differences suggest that multiple independent hybridization events have occurred across wine regions. Multiple hybridization events between the initial hybrids and the original parents also seem to occur (Erny et al. 2012). At least six independent hybridization events for the 26 strains examined in detail were reported by Peris et al. (2012b). The hybrid genomes are often unstable and genetic changes include chromosomal loss, chromosomal rearrangements, and gene loss (Belloch et al. 2009; Peris et al. 2012a, b, c).

Isolation of independently arising hybrids is commonly observed in wine-producing regions suggesting that these hybrids have characteristics that are advantageous in wine fermentation. The fermentation characteristics of *S. cerevisiae*/*S. kudriavzevii* hybrids have compared against each other and to the parental strains (Gamero et al. 2011, 2013; Combina et al. 2012; Tronchoni et al. 2012; Peris et al. 2016). *S. kudriavzevii* has been shown to be more cryotolerant than *S. cerevisiae* growing well at temperatures below the range that supports growth of *S. cerevisiae* (Noé Arroyo-López et al. 2011; Combina et al. 2012; Peris et al. 2012a; Gamero et al. 2013), but not competitive in this environment (Noé Arroyo-López et al. 2011). Hybrid strains in general retain the ability to grow at low temperatures in combination with the fermentative capacity of *S. cerevisiae*. Low-temperature fermentation may therefore impose a selection for these hybrids under commercial conditions. The mechanism of cold temperature tolerance of the hybrids has been investigated. The lipid composition of the *S. cerevisiae*/*S. kudriavzevii* hybrids is more similar to *S. kudriavzevii* than *S. cerevisiae* potentially explaining the lower temperature tolerance of these strains (Tronchoni et al. 2012).

The effect of hybrid genomes on wine flavor and aroma profiles has also been investigated. A diversity of aroma profiles was found among native *S. kudriavzevii* isolates (Peris et al. 2016) suggesting that different hybrids may express different profiles depending upon the genetic composition of the parental strains. Hybrid strains display novel aroma profiles both with respect to aromatic metabolites synthesized and the enzymatic and chemical modification of grape compounds (Gamero et al. 2011). Temperature of fermentation impacted the spectrum of

compounds produced, and non-hybrid strains were thought to have a more appealing aroma profile at higher temperatures (Gamero et al. 2013). Analysis of regulation of aroma production indicated that these hybrids have unique regulatory mechanisms for aroma production not easily predicted from the parental strains (Combina et al. 2012).

Smaller regions of non-species DNA can also be found in wine strains of *S. cerevisiae*. These strains may have arisen from an original hybridization event followed by outcrossing against *S. cerevisiae* and chromosomal loss (introgression) or from the uptake of DNA from the environment and insertion in the genome (horizontal or lateral gene transfer). Sequence analysis of the commercial strain EC1118 revealed that this strain had undergone three independent introgression events. This strain carries three large sectors of genetic information not typically found in other wine strains of *S cerevisiae*. These introgressions carried a total of 34 functional genes and have resulted in the expression of unique phenotypic traits (Novo et al. 2009; Dequin and Casaregola 2011). The FOT gene, two oligopeptide transporters found within an introgression derived from *Torulasopora microellipsoides*, have been shown to increase fitness during fermentation and impact fermentation characteristics of the strain from comparison of wild-type and knockout mutations for these genes (Marsit et al. 2016).

Although hybrid strains are commonly found among vineyard and winery isolates, the niche in which these interspecific hybridizations occurred was unclear especially given the frequencies at which intraspecific hybridization was observed. A recent analysis of yeast associated with the wasp intestine (Stefanini et al. 2012) provided important evidence of the potential origins of interspecific hybrids. Viable strains of *Saccharomyces* have been isolated from wasps, and wasps along with other insects are thought to play an important role in dispersal of *Saccharomyces* in the wild and transit between ecosystems (Goddard et al. 2010; Stefanini et al. 2012; Dapporto et al. 2016).

In order to determine if interspecies hybrids could form in wasp intestines, wasps were fed five genetically distinct strains of *Saccharomyces cerevisiae* and yeast subsequently harvested from the wasp intestinal tract after 4 months (Stefanini et al. 2016). Genetic profiling of the recovered yeast indicated that sporulation and mating had occurred within the wasp and intraspecific hybridization occurs within this ecosystem. Further, these authors demonstrated that introduction of *S. paradoxus* and *S. cerevisiae* to wasp intestines enabled recovery of intraspecific hybrids of these two yeast. Interestingly *S. paradoxus* was not recoverable from wasp intestines other than as a hybrid with *S. cerevisiae*. Intraspecific hybrids of *S. cerevisiae* × *S. uvarum* and *S. cerevisiae* × *S. paradoxus* were isolated from wasps trapped in nature (Stefanini et al. 2016), suggesting that intraspecific hybridization can commonly occur in the wasp intestine. Hybrids appeared in general to be better able to adapt to the intestine or survive passage through the intestine than either of the parental strains (Stefanini et al. 2016). These findings explain the mechanism of formation, presence and persistence of intraspecific hybrids in the ecosystem, and the transmission of hybrids to winemaking environments.

3.4 Conclusions

Diversity of the non-*Saccharomyces* yeasts associated with wine production as well as diversity within *S. cerevisiae* has been well documented in analyses of grape and wine mycobiota. This microbial complexity impacts the spectrum of flavor, aroma, and mouthfeel characters of wine in multiple ways. The discovery of the existence of natural intraspecific hybrids and chromosomal introgressions further amplifies our knowledge of variability across strains of *Saccharomyces*. The richness of the genetic composition of wine ecosystems will enable the isolation and development of strains possessing unique properties and phenotypes and lead to further expansion of fermentation management practices for the controlled use of alternative yeasts in wine production.

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Chapter 4
Fungi of Grapes

Hanns-Heinz Kassemeyer

4.1 Introduction

Grapevine can be attacked by a number of fungi and fungus-like organisms which affect the berries and cause loss of quality and influence the taste of the wine. Due to attack by pathogens, the infected plant tissue is destroyed and necrotization occurs. When large areas of the canopy are affected by grapevine diseases, the assimilation capacity of the vine is reduced, and as a result the berry quality decreases. Aside from leaves, most grapevine pathogens also infect inflorescences, clusters and berries so that the yield can be reduced. Berry infections result in decay of fruit tissue; however specific effects on berry quality depend on the ripening stage at which the infection occurs. Some pathogens directly destroy the fruit tissue enzymatically; others impede ripening, and a number of fungi produce off flavours or mycotoxins. Grapevine diseases can spread rapidly under favourable conditions and cause more or less severe epidemics. To avoid loss of quality and yield, the pathogens have to be controlled by appropriated culture techniques and targeted application of fungicides. Besides the pathogenic fungi causing grapevine diseases, berries are also colonized by ubiquitous epiphytic fungi which use sugar and amino acids leaking out of berries as nutrient source. In general grapevine pathogens can be subdivided into main pathogens of high economical importance which are pre-dominant, like downy mildew (*Plasmopara viticola*), powdery mildew (*Erysiphe necator*) and bunch rot (*Botrytis cinerea*) and those which occur only locally or temporarily. Moreover other important grapevine diseases are caused by wood decaying fungi which pre-dominantly attack the trunk and canes (Fischer and Kassemeyer 2003). In the present chapter such fungi and oomycetes are regarded

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which colonize grapevine berries and consequently may influence the must and wine. All fungi reported to colonize grapevine berries are listed in Table 4.1; however some of them can be regarded as harmless epiphytes, others actually as antagonists of pathogenic fungi, e.g. *Trichoderma* and *Ulocladium* (Schoene and Köhl 1999; Li et al. 2003). Some of the fungi produce mycotoxins (Table 4.2; cf. Chap. 7) which are more or less human toxic, and some may release compounds which are toxic to yeasts. In addition numerous fungi colonizing the berry surface during different stage of berry development and ripening have been identified (Table 4.3).

4.2 Peronosporomycetes

4.2.1 Plasmopara viticola *(Berk. and Curt.) Berl. and De Toni: Grapevine Downy Mildew*

General Aspects Downy mildew is the most serious disease of grapevine, particularly in warm and humid climates. The pathogen is indigenous on wild grapevine species, e.g. *Vitis aestivalis* in the south-east of the USA. The European cultivars of *Vitis vinifera* first came in contact with this pathogen roughly around 1878 where first symptoms were found in the Bordeaux region. Due to high susceptibility of European cultivars, grapevine downy mildews spread within a few years and caused a pandemic in the viticultural regions of the whole of Europe. To date grape downy mildew occurs in all viticultural regions that are warm and wet during the vegetative growth of vine (e.g. Europe, Eastern part of North America, New Zealand, China and Japan). The absence of rainfall in spring and summer limits the spread of the disease in certain areas (e.g. Australia, California and Chile).

The disease affects all green parts of the vine, particularly leaves, inflorescences and young berries. Depending on grape cultivar and leaf age, at the end of the incubation period, lesions get yellowish and oily, expressing so-called oil spots. After a damp night, sporulation occurs on the lower leaf surface visible as dense, white patches. Later on the sporulation sites become necrotic, and severely infected leaves generally drop. Such defoliation reduces sugar accumulation in berries and decreases frost hardiness of shoots and overwintering buds. Inflorescences and clusters with young berries are highly susceptible which finally turn brown, dry up, and drop. Although berries become less susceptible as they mature, infection of the rachis can spread into older berries which turn into a dry brown rot, without sporulation (Wilcox et al. 2015). Late cluster infections resulting in shriveled berries with a brown-rose discoloration can cause an off-flavour of the wine if they reach in the harvested grapes (Bleyer unpublished communication).

Taxonomy The causal agent of grapevine downy mildew, *Plasmopara viticola* (Berk. and Curt.) Berl. and De Toni, belongs to the Oomycetes and according to

Table 4.1 Fungi taxa detected on grapevine clusters and berries (Serra et al. 2006; Uyovbisere et al. 2007; Whitelaw-Weckert et al. 2007); the taxonomy of the ascomycetes is according to Gams et al. (1998) and Mc Laughlin et al. (2001)

Kingdom	Class	Subclass	Order	Genus	Species
Chromista	Peronosporomycetes		Peronosporales	*Plasmopara*	*P. viticola* (Berk. and Curt.) Berl. and De Toni
Mycota	Zygomycetes		Mucorales	*Cunninghamella*	*C. spec*. Matr.
				Mucor	*M. mucedo* Fresen
					M. hiemalis Wehmer
					M. piriformis A. Fisch
				Rhizopus	*R. stolonifer* (Ehrenb.) Lind.
				Syncephalastrum	*S. spec*. J. Schröt.
	Ascomycetes	Plectomycetes	Eurotiales	*Aspergillus*	*A. aculeatus* Iizuka
					A. alliaceus Thumb and Church
					A. auricomus Saito
					A. candidus Link
					A. carbonarius Bainier
					"*A. ibericus*"
					A. carneus Blochwitz
					A. clavatus Desm.
					A. flavipes Thom and Church
					A. flavus Link
					A. fumigatus Fresen
					A. japonicus Saito
					A. niger aggregate Tiegh.
					A. ochreaceus G. Wilh.
					A. ostinianus Wehmer
					A. terreus Thom
					A. ustus Thom and Church
					A. versicolour Tirab.
					A. wentii Wehmer

(continued)

Table 4.1 (continued)

Kingdom	Class	Subclass	Order	Genus	Species
				Emericella	*E. spec*. Berk.
				Eurotium	*E. amstelodami* L. Mangin
					E. chevalieri L. Mangin
				Paecilomyces	*Paecilomyces variotii* Bain
				Penicillium	*P. aurantiogriseum* Dierckx
					P. bilaiae Chalabuda
					P. brevicompactum Dierckx
					P. canescens Sopp
					P. chrysogenum Thom
					P. citrinum Thom
					P. corylophilum Dierckx
					P. crustosum Thom
					P. echinulatum Fassatiova
					P. expansum Link
					P. fellutanum Biourge
					P. funiculosum Thom
					P. glabrum/spinulosum
					P. griseofulvum Dierckx
					P. implicatum Biourge
					P. janczewskii K.M. Zalessky
					P. miczynskii Zelaski
					P. minioluteum Dierckx
					P. novae-zeelandiae J.F.M. Beyma
					P. olsonii Bainier and Sartory
					P. oxalicum Currie and Thom
					P. pinopilum Hedgcock

					P. purpurogenum Stoll
					P. raistrickii G. Sm.
					P. restrictum J.C. Gilman and E.V. Abott
					P. roqueforti Thom
					P. rugulosum Thom
					P. sclerotiorum van Beyma
					P. simplicissimum Thom
					P. solitum Westling R.
					P. thomii Maire
					P. variabile Sopp
					P. verruculosum Peyronel
					P. waksmannii Zalski
			Onygenales	*Histoplasma*	*H. spec.* Darling
		Pyrenomycetes	Hypocreales	*Acremonium*	*A. spec.* Link
				Beauveria	*B. spec.* Vuill.
				Fusarium	*F. spec.* Link
				Gliocladium	*G. spec.* Corda
				Trichoderma	*T. spec.* Pers
				Trichothecium	*T. roseum* (Pers.) Link
			Diaporthales	*Coniella*	*C. petrakii* B. Sutton
				Phomopsis	*Ph. viticola* (Sacc.) Sacc
			Sordariales	*Arthrinium*	*A. spec.* Kunze
				Chaetomium	*Ch. spec.* Kunze
				Chrysonilia	*C. spec.* Arx
				Neurospora	*N. spec.* Shear and Dodge
			Xylariales	*Pestalotiopsis*	*P. spec.* Steyeart
				Truncatella	*T. spec.* Steyeart
			Erysiphales	*Erysiphe*	*Erysiphe necator* Schwein.

(continued)

Table 4.1 (continued)

Kingdom	Class	Subclass	Order	Genus	Species
		Loculoascomycetes	Dothideales	*Aureobasidium*	*A. spec*. Viala and Boyer
				Guignardia	*G. bidwellii* (Ellis) Viala and Ravaz
			Pleosporales	*Alternaria*	*A. alternata* (Fr.: Fr.) Keissler
				Curvularia	*C. spec*. Boedijn
				Drechslera	*D. spec*. S. Ito
				Epicoccum	*E. spec*. Link
				Periconia	*P. spec*. Tode ex Fr.
				Phoma	*Ph. spec*. Sacc.
				Pithomyces	*P. spec*. Ellis
				Stemphylium	*S. spec*. Wallr.
				Ulocladium	*U. atrum* Pers.
			Capnodiales	*Cladosporium*	*C. herbarum* (Pers.) Link
			Myrangiales	*Elsinoë*	*E. ampelina* Shear
		Discomycetes	Helotiales	*Botrytis*	*B. cinerea* Pers.: Fr.
				Pseudopezicula	*P. tracheiphila* (Müll.-Thurg.) Korf and Zhuang

Table 4.2 Taxa producing mycotoxins considered relevant for human health (Serra et al. 2005)

Taxon	Mycotoxin
Aspergillus ochraceus	Ochratoxins
Aspergillus alliaceus	
Aspergillus niger aggregate	
Aspergillus carbonarius	
Penicillium verrucosum	
Trichothecium roseum	Trichothecene
Penicillium expansum	Patulin

Table 4.3 Taxa identified on grapes in Portugal at different phenological stages (Serra et al. 2005)

	Phenological stage of the berry colonization		
Taxon	Pea size	Veraison	Harvest
Acremoniella Sacc.	X	X	X
Acremonium Link	X	X	X
Arthrinium Kunze.	X	X	0
Aspergillus Fr.:Fr.	X	X	X
Aureobasidium Viala and Boyer	X	X	X
Beauveria Vuill.	X	0	0
Chaetomium Kunze	X	X	0
Chrysonilia Arx	0	X	X
Cunninghamella Matr.	X	X	X
Curvularia Boedijn	X	X	X
Drechslera S. Ito	X	X	X
Emericella Berk.	X	X	X
Epicoccum Link	X	X	X
Eurotium Link: Fr.	X	X	X
Fusarium Link	X	X	X
Geotrichum Link: Fr.	X	0	0
Gliocladium Corda	X	X	X
Histoplasma Darling	X	0	0
Neurospora Shear and Dodge	0	X	X
Nigrospora Zimm.	0	X	X
Periconia Tode ex Fr.	X	0	0
Pestalotiopsis Steyeart	X	X	0
Phoma Sacc.	X	X	X
Pithomyces Ellis	X	X	X
Rhizopus Ehrenb.	X	X	X
Scytalidium Pesante	X	X	0
Stemphylium Wallr.	X	X	X
Syncephalastrum J. Schröt.	X	0	X
Trichoderma Pers.	X	X	X
Truncatella Steyeart	X	0	0
Ulocladium Preuss	X	X	X

current taxonomy is a member of the class of Peronosporomycetes (Dick 2002). This class is different from the kingdom of true fungi (Mycota) and is a part of Chromista, a kingdom which comprises heterogeneous microorganisms among others the autotrophic Chrysophyceae (golden algae) and Bacillariophyceae (diatoms). Like all Chromista, the cell wall of *P. viticola* consists of glucans, and biflagellated zoospores are formed. Within the Peronosporomycetes, *P. viticola* shows some primary characteristic of Chromista; among others, parts of the life cycle of the organism are bound to water. Like *P. viticola*, majority of Peronosporomycetes are plant pathogens such as the causal agent of potato late blight, *Phytophthora infestans*.

Biology and Epidemiology *P. viticola* is a biotrophic pathogen strongly adapted to members of the genus *Vitis*. It develops in the intercellular space within the colonized host tissues in the form of tubular, coenocytic hyphae, developing globular haustoria. The haustorium penetrates the cell wall and invaginates the outer membrane to take nutrients from the host cell. Asexual reproduction occurs by formation of lemon-shaped sporangia formed on branched sporangiophores emerging from the stomata during humid nights (Rumbolz et al. 2002). Each sporangium gives rise to four to ten biflagellate zoospores which are released as soon as the sporangium is incubated in water for a certain time (Kiefer et al. 2002). Asexual sporangiam developed zoospores as well vegetative hyphae are diploid.

Sexual reproduction begins in the summer by developing of gametangia. In the male antheridium as well as in the female oogonium, meiosis runs and the haploid nucleus of the antheridium fuse with that of the oogonium forming a diploid oospore. *P. viticola* is heterothallic and therefore fertilization occurs only between two different mating types (Wong et al. 2001). The thick-walled oospore overwinter in fallen leaves becomes mature in spring and germinates in free water forming a primary sporangium, which produces 30–60 zoospores. Germination occurs during the vegetation period from spring to midsummer as soon as temperatures reach 10 °C and rainfall ensures required wetness (Hill 1989). From the primary sporangium, the zoospores are dispersed during intensive rainfall.

The released zoospores both from oospores and asexual sporangia swim within a water film covering the surface of the host plant after precipitation and dew and attach around the stomata. They shed their flagella and encyst forming a cell wall (Riemann et al. 2002). Subsequently an infection tube emerges from each encysted spore (Fig. 4.1) which penetrates the stoma and forms a sub-stomatal vesicle in the sub-stomatal cavity where it dilates into a primary hypha (Kiefer et al. 2002). Under optimal conditions, the period from the release of zoospores to penetration is less than 90 min. From the sub-stomatal vesicle, a hyphae grows in the intercellular space of the host tissue. The primary hypha branches and forms a mycelium that colonizes the host tissue (Unger et al. 2007). The period from infection to first appearance of oil spots—the incubation period—depends on temperature and humidity. In general sporulation takes place at the end of the incubation period, in the first night when conditions for sporulation mentioned above occur (Rumbolz et al. 2002). Under favourable conditions, incubation period is very short, and

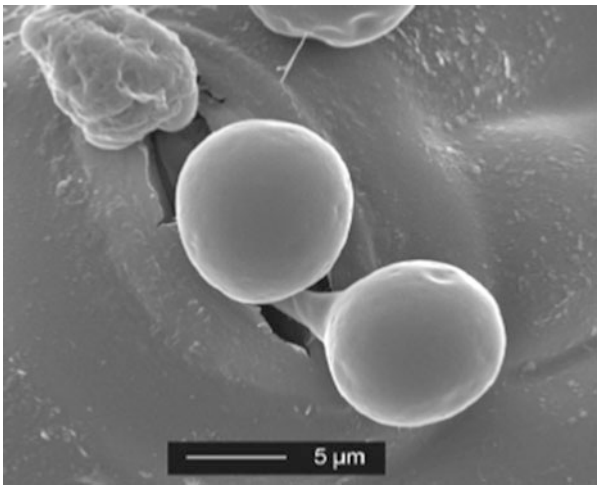

Fig. 4.1 Encysted zoospores with a penetration peg from *Plasmopara viticola* attached at a stoma; low-temperature scanning electron microscopy (Kassemeyer H.-H. and Düggelin M., University of Basel)

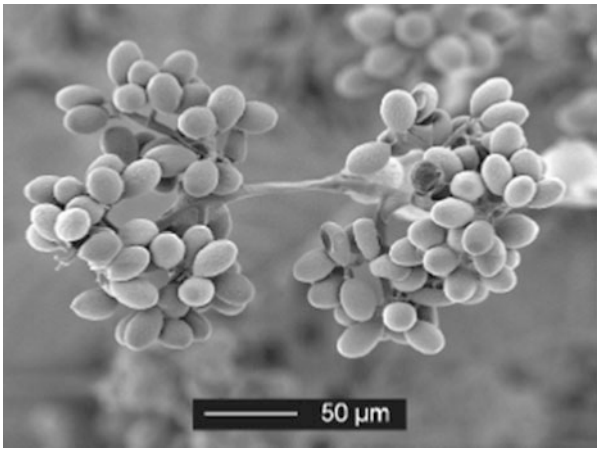

Fig. 4.2 Sporangiophore with sporangia from *Plasmopara viticola*; low-temperature scanning electron microscopy (Kassemeyer H.-H., Boso S. and Düggelin M., University of Basel)

P. viticola is able to sporulate 3–4 days after infection. As soon as the host tissue is totally colonized by the mycelium of *P. viticola*, sporulation takes place. Sporulation requires 95–100% relative humidity and at least 4 h of darkness at temperatures >12.5 °C; the optimal temperature for sporulation is 18–22 °C, and therefore an outbreak of the disease, visible in the morning after a warm and damp night, yields maximum sporangia. Induction of sporulation is influenced by the photoperiod and sporangiophores and sporangia differentiate only during the night (Rumbolz et al. 2002). At the beginning of sporulation, a secondary sub-stomatal vesicle is formed from which hyphae grow out of the stoma. The emerging hyphae branch and form typical sporangiophores (Fig. 4.2). Finally, sporangia develop at the tips of the branches, and around seven hours after the beginning of sporulation, mature sporangia are present. Immediately after formation, sporangia are detached from

sporangiophores and spread by wind. Successful infection conditions can be calculated using the relation between temperature and duration of leaf wetness (Huber et al. 2003).

4.3 Ascomycetes

4.3.1 Erysiphe necator *Schwein. (emend.* Uncinula necator *(Schw.) Burr) (Erysiphales): Grapevine Powdery Mildew*

General Aspects Grapevine powdery mildew occurs worldwide in all viticultural regions and causes severe losses of yield and quality especially in warm and dry weather conditions. This grapevine disease was introduced from North America and detected first in Europe in the middle of the nineteenth century. The disease spread within a short time in Europe and gave rise to economically relevant epidemics. After bud burst, first symptoms are visible as white or grey powdery patches on leaves and shoot tips between the three- and six-leaf stages on leaves. These "flagshoots" strike on highly susceptible cultivars such as Chardonnay, Cabernet Sauvignon, Carignane, Portugieser and Vernatsch (Trollinger); they occur less pronounced also on the vast majority of European cultivars. Young leaves, inflorescences, flowers and young berries are highly susceptible; however older leaves and berries up to the veraison are also infected (Ficke et al. 2002). Young leaves and berries can be totally covered with white powdery patches, whereas on older leaves small colonies occur on the upper leaf side. Infected leaves remain green over a longer period, but the assimilation efficiency of the leaves is reduced. Shoot tips, inflorescences and young clusters are also covered with whitish or greyish patches. Infected inflorescences become curled and necrotize. Shoots become stunted and leaves appear yellowed. As a result of impeded growth of the berry skin on infected berries from the pea-sized stage, cracking and splitting occur. The splits are entrance ports for secondary invaders such as acetate acid producing yeasts and bacteria (Fig. 4.3). Fully expanded berries can be colonized by the pathogen up to the beginning of veraison (Wilcox et al. 2015). Berries with these late infections ripen, but the mycelium of powdery mildew can affect wine quality by its mouldy taste. Additionally the pathogen penetrates the berry skin and facilitates infections by bunch rot.

Taxonomy The agent causing grapevine powdery mildew, *Erysiphe necator* Schwein. [emend. *Uncinula necator* (Schw.) Burr], is an Ascomycete belonging to the Erysiphales which comprise a broad range of plant pathogens (Bélanger et al. 2002). As in all Ascomycetes, the cell wall of *E. necator* consists of chitin, a polymer of *N*-acetylglucosamine.

Fig. 4.3 Berry infection by *Erysiphe necator* with splitting of the berry skin

Biology and Epidemiology Like all powdery mildew fungi, *E. necator* is a biotrophic fungus with limited spectrum of host plants, infecting only grapevine (*Vitis*) species. The fungus grows epiphytically on the epidermis of green plant tissue forming a dense white mycelium. *E. necator* overwinters as hyphae hidden in the buds or as ascospores in fruit bodies (Rügner et al. 2002; Rumbolz and Gubler 2005). Both overwintered hyphae and ascospores act as primary inoculum. During the formation of winter buds in spring, hyphae colonize the inner bud scales and remain dormant up to the following spring. After bud burst, overwintered hyphae colonize young leaves and shoots forming more or less striking "flagshoots". The powdery cover of this "flagshoots" pre-dominantly consists of conidiophores with chains of conidia (Pearson and Goheen 1988; Agrios 1997). The ascospores are formed after karyogamy in ascogenous hyphae, during dry and warm weather in late summer and autumn. *E. necator* is heterothallic, and two different mating types have to combine for sexual reproduction. The ascospores are located in asci which are embedded in chasmothecia (cleistothecia). These possess hooked appendices responsible for the attachment of the fruit bodies at the bark of canes and trunks during the winter. In the spring during rainfall, the chasmothecia open, and by means of a special mechanism, the ascospores are ejected out of the asci. Ascospores and conidia attach actively on the surface of host plants and germinate under optimal temperatures between 20 and 27 °C within 4 h (Rumbolz et al. 2000). No water is necessary for germination, but higher humidity favours this process. The germ tube forms an appressorium which strengthens the attachment of the pathogen on the host epidermis (Fig. 4.4). Beneath the appressorium, a penetration peg penetrates enzymatically the cuticle and epidermis cell wall (Rumbolz et al. 2000). At the tip of the penetration peg, a lobed haustorium is formed which invaginates the epidermis cell and deprive nutrients from the host. As soon as nutrient uptake is ensured, a second hypha emerges from the conidia and colonization of the host surface commences. Temperatures ranging from 18 to 28 °C promote hyphae growth and mycelium formation. Within 5–6 days after infection, a dense mycelium is formed, and conidiophores evolve projecting at a right angle

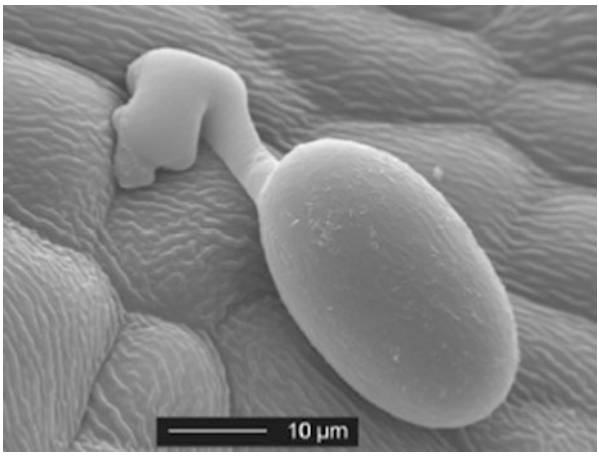

Fig. 4.4 Germinated conidia from *Erysiphe necator* on the surface of a grapevine leaf; low temperature scanning electron microscopy (Rumbolz, J., Kassemeyer H.-H., Düggelin M. and R. Guggenheim, University of Basel)

from the host surface. From a basal cell in the conidiophore, conidia develop and are cut off permanently. Conidia are adapted to transport by wind and spread over long distances. High temperatures and humid nights are favourable for the production of high amount of conidia. In most cases, the epidemic starts in spring from ascospore infection or "flagshoots" when three to six leaves are unfolded. Under warm and dry conditions, disease incidence and severity increase up to berry set, due to high susceptibility of young leaves, inflorescences and young berries (Ficke et al. 2002).

4.3.2 Botrytis cinerea *Pers.:Fr. (Helotiales): Botrytis Bunch Rot*

General Aspects *Botrytis cinerea* is a plant pathogen of economical importance causing rot in a broad range of crops, fruits and ornamental plants. In viticulture *B. cinerea* may cause both serious loss and enhancement of quality. Injury and profit, respectively, depend not only on the stage of ripening in which berries are infected but also on weather conditions. Under dry and warm conditions, infections of ripe berries may raise the quality especially of white cultivars. In this case berry ingredients are concentrated due to the perforation of the berry skin by the fungus. In addition *B. cinerea* produces gluconic acid which confers a pronounced tastiness to the wine. Consequently late infections of mature berries facilitate the production of dessert wines like "Trockenbeerenauslesen", "Sauternes" and "Tokay". On the other hand, berry infection at an early stage of ripening and during long-lasting wetness of the clusters reduces the quality due to berry decay. Infestation of clusters with berry moth enhances bunch rot because the feeding sites of the larvae on berries set entrance ports for *B. cinerea*. At the beginning of infection by *B. cinerea*, berries from white varieties become light-coloured from pinkish to light brown;

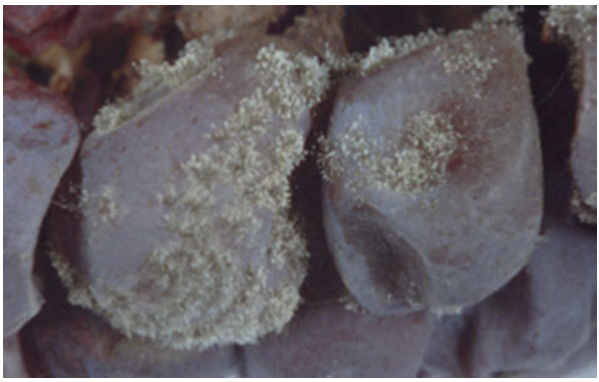

Fig. 4.5 Bunch rot caused by *Botrytis cinerea*; conidiophore emerges from pores and cracks in the rotten berry skin and forms a *grey* pad

those from red variety changes from red to purple. Later on a light grey mycelium occurs on the surface (Fig. 4.5), and in a proceeded infection stage, berries become brownish and rotten (Wilcox et al. 2015). On infected berries, *B. cinerea* produces high amounts of laccases which oxidate the anthocyanes and flavonoids to brown oxidation products. Laccases are very stable and can pass over in must and wine, and as a result, wine becomes brownish and red wines especially lose their characteristic red colour.

Taxonomy The teleomorph of *Botrytis cinerea* Pers.:Fr., *Botryotinia fuckeliana* (de Bary) Whetzel, is a member of the Helotiales (Ascomycetes). *B. cinerea* occurs mainly in its anamorph form, whereas the teleomorph *B. fuckeliana* is very rare (Gams et al. 1998; Elad et al. 2004).

Biology and Epidemiology *B. cinerea* is an ubiquitous fungus and has a broad range of host plants. The fungus can live saprophytically on organic debris and produce sclerotia as long-term survival form. *B. cinerea* overwinters both as mycelium and as sclerotium on canes and leaf litter on the ground. The conidia produced on sclerotia during periods with raising temperatures in the early spring are considered the main source of primary inoculum. Conidia are short-lived propagules during the season and are spread by wind, rain and also insects. On the host plant surface, the conidia germinate 1–3 h after inoculation forming various penetration structures. In the presence of sugar, the germ tubes of *B. cinerea* forms a multilobed appressorium (Elad et al. 2004; Leroch et al. 2013). To penetrate the host tissue, *B. cinerea* prefers wounds and natural openings, e.g. specialized structures of flowers on which sugar and other nutrient are available (Keller et al. 2003; Viret et al. 2004; Kretschmer et al. 2007). When spores germinate on floral tissue of inflorescences or later in the season on ripening berries, *B. cinerea* can change from saprophytic to necrotrophic lifestyle. The fungus expresses a set of enzymes such as lipases, cutinases and pectinases that enables the pathogen to penetrate the epidermis of the host tissue. The penetration of the host cuticle by *B. cinerea* mediated by cytolytic enzymes triggers a programmed cell death in the epidermis and the underlying cells before they are invaded by hyphae (Shlezinger et al. 2011,

2012). Effector proteins of *B. cinerea* acting as pathogenicity factors and the induction of the programmed cell death facilitate invasion and are essential for successful infection (Nassr and Barakat 2013). So the pathogen is able to complete its disease and life cycle (Elad et al. 2004). Flowers are susceptible to infection because the receptacle constitutes natural openings and provides sugar that facilitates flower colonization by the pathogen (Keller et al. 2003; Viret et al. 2004). Increasing susceptibility of ripening berries relies on several factors: (1) host defence, e.g. expression of stilbenes, weakens with ongoing ripening; (2) amount of fungistatic protoanthocyanidins reduces after veraison; and (3) structure of the cuticle and epidermis changes with advanced seed maturation and micro-cracks occur which allow the leakage of sugars (Kretschmer et al. 2007). Conidia germination, germ tube growth, penetration and colonization of the host tissue are crucial processes of the infection cycle. Conidia germination and infections occur under high humidity (>94% relative humidity) even on dry berries; however long wetness period favours development of *B. cinerea* and increases disease incidence. At 20–24 °C and humid conditions, a germ tube arises within four to eight hours, and under this condition hyphae grow up to 4 mm per day (Fig. 4.6). After penetration of the host tissue, hyphae grow, and after branching, a dense grey mycelium is formed in which conidiophores with conidia develop (Pearson and Goheen 1988; Agrios 1997). Conidia germination and growth of mycelium and conidia formation also occur at lower temperatures up to 5 °C; however infection and development of the pathogen are delayed. Epidemics with severe infections and high disease incidence arise under continuing rainfall after veraison.

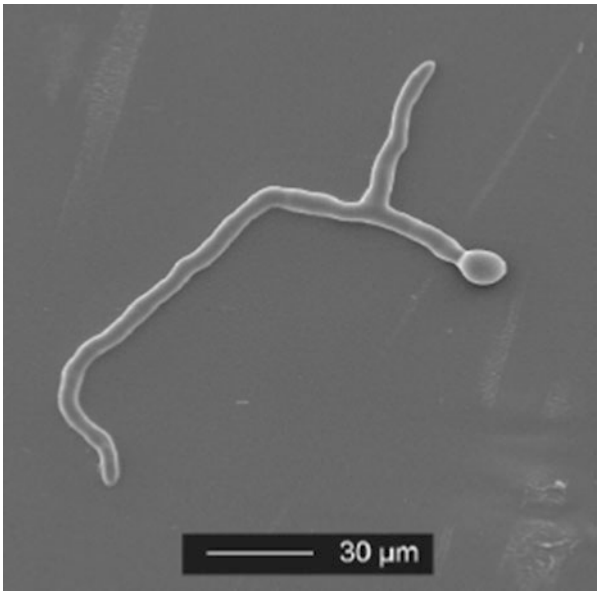

Fig. 4.6 First stage of development of *Botrytis cinerea* 17 h after inoculation, low-temperature scanning electron microscopy (Jäger, B., Jacków, J., Kassemeyer H.-H. and Düggelin M., University of Basel)

4.3.3 Pseudopezicula tracheiphila *(Müll.-Thurg.) Korf and Zhuang (Helotiales): Rotbrenner*

General Aspects Rotbrenner is locally confined and occurs primarily in warm vineyards with stony soil. In some areas the disease results in severe losses depending on temperature and humidity in spring, whereas in others it occurs only sporadically or not at all. Lesions on leaves are initially yellow on white and bright red to reddish brown on red cultivars. Subsequently a reddish brown necrosis develops in the centre of the lesion, leaving only a thin margin of yellow or red tissue between the necrotic and green areas of the leaf. The lesions are typically confined to the major veins and the edge of the leaf and are several centimetres wide. Early infections occur on the first to the sixth leaf of young shoots, resulting in minor losses. Later infections attack leaves up to the 10th or 12th position on the shoot which results in severe defoliation. In addition, fungus attacks inflorescences and berries causing them to rot and dry out (Mohr 2005).

Taxonomy The causing fungus of Rotbrenner, *Pseudopezicula tracheiphila* (Müll.-Thurg.) Korf and Zhuang (syn. *Pseudopeziza tracheiphila* Müll.-Thurg.), belongs to its teleomorph *Phialophora tracheiphila* (Sacc. and Sacc.) Korf to the Helotiales (Ascomycetes) (Korf et al. 1986).

Biology and Epidemiology The source of inoculum of the disease in spring is ascospores which are formed sexually in asci. *P. tracheiphila* appears to be composed of two mating types which exhibit a bipolar heterothallic mating system. Apothecia formed primarily on fallen leaves in the spring and hold the asci with the ascospore. Apothecia may also develop on current-season infected leaves in late summer or fall. Depending on weather conditions, apothecia with mature ascospores may be present throughout the season (Pearson et al. 1991). The primordia of the apothecia mature as soon as the temperature rises at the end of winter. Apothecia development requires sufficient wetness of fallen leaves. Under wet and warm conditions, ascospores are released already before bud burst. Heavy rainfall and prolonged surface wetness favour infection and lead to severe disease. Young leaves are susceptible after they reach a width of about 5 cm, but the probability of infections increases from the six-leaf stage. After an incubation period of 2–4 weeks, the fungus invades the vascular elements of infected leaves, causing symptom development (Reiss et al. 1997). The fungus remains latent if it is unable to invade the vessel elements, in which case it can be isolated from green leaves showing no symptoms. Conditions required for fungus to invade the vascular system are not well understood; however, soil conditions and water supply that place the vine under temporary stress appear to be important factors. Disease incidence and severity depend on the abundance of apothecia on fallen leaves on the ground of the vineyards and on released ascospores. Monitoring of the ascospore release by means of spore traps enables forecast of the disease situation. On malt agar, the anamorph may be formed, with hyaline, septate, short conidiophores that are coarser than vegetative hyphae. Conidiogenous cells are monophialidic and

lageniform, with well-defined but thin-walled collarettes. Conidia are ellipsoid, hyaline, and unicellular. Hyphae grow in a characteristic sine-wave pattern that, when observed in the vessel elements of diseased tissue, are considered diagnostic.

A disease very similar to Rotbrenner, called angular leaf scorch, has been described in New York State (Pearson et al. 1988). The fungus causing angular leaf scorch in North America produces smaller apothecia than *P. tracheiphila*, and its broadly clavate asci has four spores in contrast to the eight-spored European fungus. The American counterpart has been described as a distinct species, *P. tetraspora* Korf, Pearson and Zhuang (anamorph *Phialophora*-type).

4.3.4 Phomopsis viticola *(Sacc.) Sacc (Diaporthales): Phomopsis Cane and Leaf Spot*

General Aspect Phomopsis cane and leaf spot first observed in 1935 in California are also widespread in Europe for more than 50 years. Actual loss of quality due to the disease in most years is insignificant. However in rainy spring years, severe infections occur and cause lesions on shoots. In addition shoot infections affect the formation of basal buds, and in consequence in the following year, buds on the base of the canes especially do not sprout. Repeated infections affect the fertility of the basal parts of the canes and shorten life span of the vine. Under cool and rainy conditions during berry ripening, berry infections occasionally occur. The first symptoms on shoots are dark brown to black spots on the shoot base visible from the three- to six-leaf stage. The spots elongate, and the cortex cracks due to secondary growth of the shoots. Large numbers of spots at the shoot base become scabby and black. Heavy infected shoots can be dwarfed and may die. During winter, infected canes bleach and black pustules occur. Cluster infections show black spots on the rachis. However these lesions become inactivate in the course of cluster development. Rarely, rainfall in autumn reactivates the lesions and cause berry infections. Infected berries show brown spots which enlarge quickly and cause a bunch rot.

Taxonomy *Phomopsis viticola* (Sacc.) Sacc. (*Sphaeropsis viticola* Cooke) belongs to the Diaporthales (Ascomycetes). The teleomorph *Diaporthe* according to current knowledge is very rare in viticulture (Agrios 1997; Gams et al. 1998).

Biology and Epidemiology *Ph. viticola* overwinters on infected canes, and black pustules on bleached canes occurring during dormancy are pycnidia (Fig. 4.7) where pycniospores develop. Generally infections occur in spring as soon as pycniospores mature in the pycnidia and green shoots sprout. During rainfall pycniospores emerge in large quantities from the pycnidia embedded in vermiform cirri. Pycniospores (Fig. 4.8) are dispersed by splashing raindrops onto the sprouting shoots, and infections occur when water remains on the green host tissue for a longer time. Prolonged wetness of sprouts and young shoots favour infections

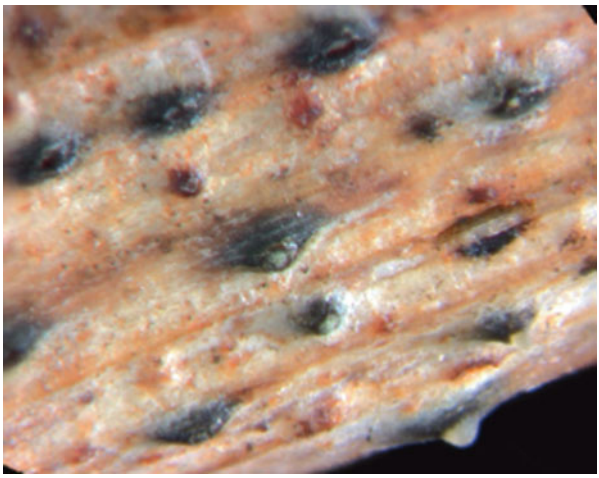

Fig. 4.7 Cane with *Phomopsis viticola* pycnidia

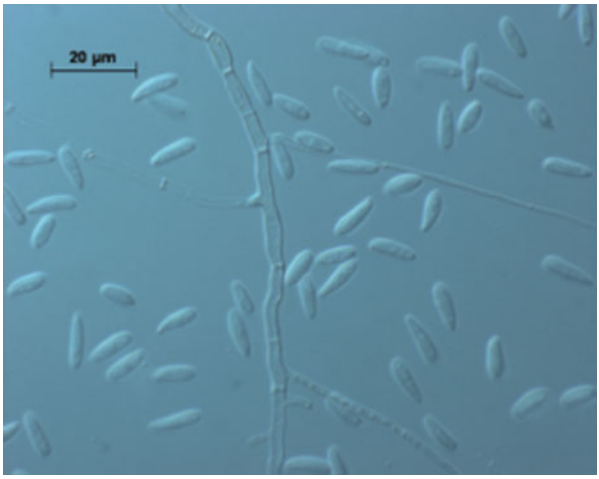

Fig. 4.8 Pycniospores from *Phomopsis viticola* and hyphae with characteristic septae; differential interference contrast (×63)

by *Ph. viticola* in particular from bud break up to the six-leaf stage. The number of basal buds affected by fungus varies according to frequency of rainfall and wetness of the host surface. After infection, mycelium grows in the infected host tissue but mainly shoots and buds are colonized. During summer *Ph. viticola* is less active, but in wet autumn, mycelium may be reactivated and berry infections may occur (Agrios 1997; Mohr 2005; Wilcox et al. 2015).

4.3.5 Elsinoë ampelina *Shear (Myringiales): Anthracnose (Schwarzer Brenner)*

General Aspects Anthracnose was widespread in earlier times in European viticulture and before downy mildew was identified as the most dangerous of grapevine disease. Due to regular application of fungicides, anthracnose occurs only sporadically under very humid conditions in untreated vineyards. Infected shoots show light brown spots with black-violaceous edges. Black circular lesions occur on the leaves that necrotize and over time give rise to small holes like a shotgun effect. Affected berries show sunken circular lesions with black-violaceous edges ("bird's eyes") which crack and finally decay. Infections of the rachis cause necrosis of the cluster with "bird's eyes" on the stems (Mohr 2005). Shoots and leaf infections reduce the vigour of vine, yield and quality and shorten the life span of the plant. Decayed berries have to be removed because they can influence the quality of must and wine (Magarey et al. 1993; Sosnowski et al. 2007).

Taxonomy The causing fungus of the anthracnose, *Elsinoë ampelina* Shear (syn. *Gloeosporium ampelophagum* (Pass) Sacc., *Ramularia ampelophagum* Pass., *Sphaceloma ampelinum* de Bary), is a member of the Elsinoaceae family which comprises ten genera (Gams et al. 1998). Elsinoaceae and Myrangiaceae belong together to the order of Myrangiales which is a member of the larger class of the Dothideomycetes (Ascomycetes).

Biology and Epidemiology E. ampelina overwinters as sclerotia on the canes which are formed in the autumn at lesions on shoots. The sclerotia develop stromata on which under humid conditions shell-like acervuli with conidia emerge in the spring (Agrios 1997). The conidia are covered with a gelatinous layer and provide primary inoculum at the beginning of the vegetation period. Conidia propagation is favoured by rainfall, and for conidia germination, wetness of the host surface for 12 h is necessary. At times fruiting bodies with asci and ascospores develop on the lesion. The propagules are transported during rainfall over a short distance; thus the disease initiates on more ore less widespread spots within the vineyards (Brook 1992).

4.3.6 Guignardia bidwellii *(Ellis) Viala and Ravaz (Dothideales): Black Rot*

General Aspects Black rot originated from North America and has been in Europe for nearly 30 years. The disease occurs particularly in abandoned vineyards and also on resistant cultivars which are not treated with fungicides. To date, severe epidemics caused by black rot are restricted to some viticulture regions, but single symptomatic berries are prevalent in particular on laterals. Typical symptoms on leaves are light brown necrotic lesions with black edges up to 10 mm in diameter. Within the necrotic spots, black dots are barely visible to the naked eye. On shoots,

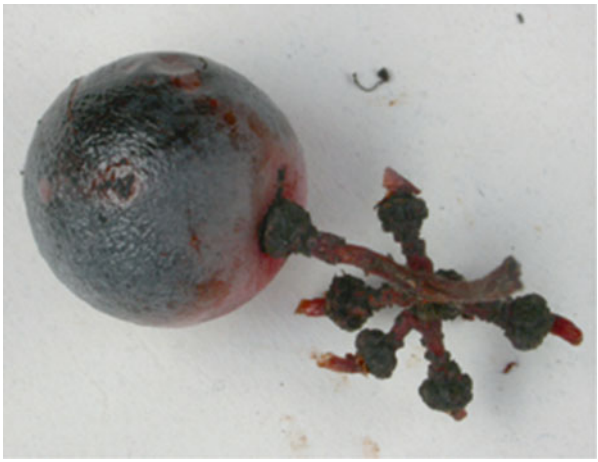

Fig. 4.9 Berry affected by black rot (*Guignardia bidwellii*)

petiols and the rachis black sunken lesions appear. Infected young berries primarily show pale spots which enlarge to concentric red-brown lesions. Within a few days, the affected berry gets blue-black and is covered with black pustules (Fig. 4.9). The berries finally wrinkle and dry but remain as mummies fixed on the rachis. Frequently originated from some infected berries, the whole cluster can be infected. High infestations of black rot defoliate the canopy and as a result decrease the quality of grapes seriously. Cluster infections have an effect on yield and berry quality, and affected grapes are not suitable for wine production (Pearson and Goheen 1988; Mohr 2005; Buckel et al. 2013).

Taxonomy Black rot is caused by *Guignardia bidwellii* (Ellis) Viala and Ravaz (syn. *Greeneria uvicola* (Berk. and M.A. Curtis) Punith., *Botryosphaeria bidwellii* (Ellis) Petr.) which belongs to the Dothideales an order within the Dothideomycetes (Ascomycetes) comprising some other plant pathogens such as *Ascochyta*, *Didymella*, *Botryosphaeria* and *Phoma* (Agrios 1997; Gams et al. 1998).

Biology and Epidemiology *G. bidwellii* overwinters mainly in the mummified clusters and berries remaining on the shoot and also on infected canes. Asci with ascospore develop in perithecia on infected berries in spring. The ascospore are ejected actively from the asci during low rainfall and spread by wind. For ascospore germinate, prolonged wetness of host surface is necessary. All young green grapevine tissue including shoots, inflorescences and berries may be infected. At the beginning, infections are hard to detect, but with progressed development of the fungus, necrotic spots are visible, and finally necrotic lesions occur. Within necrotic lesions on leaves, shoots and berries, pycnidia with pycniospores develop during the season. Pycniospores are released during rainfall and cause infections on berries. In late summer the sexual cycle initiates on infected berries, and perithecia are formed which overwinter on the infected mummified berries and clusters (Jermini and Gessler 1996; Hoffman et al. 2002; Longland and Sutton 2008; Ullrich et al. 2009; Molitor et al. 2012).

4.3.7 Penicillium expansum *Link (Eurotiales): Green Mould*

General Aspects Green mould is a secondary disease on mature berries, after wounding or bunch rot infections. Green mould occurs in warm and humid years when berries enter into ripening stage precociously. Recently incidence of green mould increases may be due to high temperature in summer and frequent precipitation during berry ripening. White pads occur on the edges of wounds and cracks which enlarge and change to glaucous (Fig. 4.10). Infected berries soften and change colour from olive green to light brown. In an advanced stage of infection, berries decay and shrink under dry conditions. Due to the squeezing of berries and related wounds, closed bunches are more frequently affected by green mould. These clusters show nests inside with decayed berries (Mohr 2005). Therefore cultivars with close bunches are more susceptible than those with loose bunches. Besides in years with high berry set resulting in dense clusters, green mould occurs more frequently. From single infected berries, the whole cluster may be affected causing mummified clusters covered with green mould. Green mould produce mycotoxins (Abrunhosa et al. 2001; La Guerche et al. 2004; Serra et al. 2006; Pardo et al. 2006), for example, patulin which is however degraded during fermentation and by sulphurization. Berries affected by green mould have an off-flavour, and even a small amount of infected berries add a mouldy taste to the wine.

Taxonomy The causal agent of green mould is mainly *Penicillium expansum* Link; other species of *Penicillium* can also be detected on affected berries (Serra and Peterson 2007). The genus *Penicillium* is regarded as a member of the Deuteromycotina, but according to current taxonomy, it belongs to the Eurotiales (Ascomycetes) (Agrios 1997; Gams et al. 1998; Mc Laughlin et al. 2001).

Biology and Epidemiology *P. expansum* is ubiquitous and propagates by conidia which are formed abundantly on conidiophores. The conidiophores of *P. expansum* consist of two asymmetric branches with a number of flask-shaped phialides at the tip of each branch. Phialides are conidiogenous cells which produce masses of

Fig. 4.10 Berry infected by *Penicillium expansum*, the fungus colonizes pores in the epidermis on which nutrients leak from the berry

conidia in short intervals under humid and warm conditions. The phialides appear as clusters on each tip of the conidiophore, and the conidia are formed in chains on each phialide. Conidiophores with the mass of conidia are visible as white to glaucous pad on infected berries (Gams et al. 1998). *P. expansum* is a typical airborne pathogen, and the long-living conidia are transported by wind. In consequence conidia are released even by a gentle movement while removing infected clusters. Conidia germinate on wet surface of berries as soon as a sugary medium is available. Possibly vigorous berry development due to high amount of water supply and high temperatures causes micro-cracks in the berry skin and consequently sugar runs off the slow berries. The temperature range of *P. expansum* for conidia germination, growth of the mycelium and sporulation is relatively broad, but optimal development of the fungus occurs at 25 °C and high humidity. Under cool and dry conditions, *P. expansum* is rare even on berries whose skin is not intact. Slow and consequently late ripening cultivars and those with a strong epidermis are less susceptible to colonization by *P. expansum*.

4.3.8 Aspergillus spec. *(Eurotiales): Aspergillus Rot*

General Aspects Aspergillus rot is widespread on substrates containing a disposable source for carbohydrates such as mono- and polysaccharides. Rot is common in crops and fruits and contaminates also sugary and starchy foods. At present aspergillus rot occurs on grapevine particularly in warm climate (Leong et al. 2007). The symptoms of aspergillus rot are visible as soon as sugar leaks from ripening berries after the beginning of veraison. The surface of infected berries is covered by a black mould, and the berries decay. Aspergillus rot produces ochratoxins (Samson et al. 2004; Pardo et al. 2006) and contaminates must and wine with this mycotoxin suspected to be carcinogenic. For this reason, clusters affected with aspergillus rot have to be sorted at harvest.

Taxonomy Aspergillus rot is caused by different members of the genus *Aspergillus* which are widely distributed worldwide. On grapes particularly *A. alliaceus* Thom and Church, *A. carbonarius* (Bainier) Thom, *A. niger* aggregate Tiegh. and *A. ochraceus* G. Wilh. occur. Totally the genus *Aspergillus* comprises more than 200 taxa including species with numerous sub-species and is a genetically heterogeneous group. Therefore the current taxonomy can change in the course of new findings on the phylogeny of this group. Some teleomorphs associated with *Aspergillus* are known, for instance, *Emericella* Berk and Br. and *Eurotium* Link and allow integration in the Eurotiales (Mc Laughlin et al. 2001).

Biology and Epidemiology *Aspergillus* species sporulate asexually by forming conidia without fruit bodies. Conidia develop on conidiophores which are sometimes aggregated and visible as a black powdery pad. The unbranched conidiophores terminate in vesicle on which phialides arise (Gams et al. 1998; Domsch et al. 1980). At the tip of the flask-shaped phialides, conidia develop in chains

which are spread by wind. The optimum temperature range for development of *Aspergillus* is 17–42°; minimum temperature for growth is 11–13 °C (Ferrari et al. 2016).

4.3.9 Coniella petrakii *B. Sutton (Diaporthales): White Rot*

General Aspects White rot occurs sporadically in southern viticultural regions, while in cool and moderate climate viticulture, the disease appears very rarely. Wounds, mainly from hail, favour infections by white rot. Above all damages appear on affected rootstocks showing brown spots. Rootstocks infected by white rot are not suitable for grafting and may disseminate the disease. Infected berries become yellowish, shrink and have brown pustules. Due to the development of the pustules on the berry skin, the cuticle detaches from the epidermis, and as a result the berry becomes pale. Clusters affected by white rot should be sorted at harvest because otherwise they may influence the quality of must and wine. In most cases white rot is controlled by regular treatments against downy mildew and bunch rot.

Taxonomy *Coniella petrakii* B. Sutton is the causal agent of white rot and belongs to the order of Diaporthales (Ascomycetes) (Tiedemann 1985).

Biology and Epidemiology The mycelium of *C. petrakii* is frequently septated and abundantly branched. Globose and ostiolate pycnidia are formed by a stroma below the cuticle. The elliptical or ovate-shaped pycniospores are single celled and light brown and arise from a basal stroma in the pycnidia from the pycnidial wall (Sutton and Waterston 1966; Locci and Quaroni 1972; Tiedemann 1985). *C. petrakii* is soil borne, and splash events are necessary to transport the propagules onto the host surface (Aragno 1973). High temperatures between 24 and 27 °C favour conidia development and infection. After infection of wounded host tissue, the incubation period varies from 3 to 8 days (Bisiach 1988). Masses of pycnidia forming pycniospores arise from the berry surface and overwinter on the ground and are source of inoculum over years. High temperature and simultaneous wetness of the host surface necessary for successful infections exclude in most cases, infestation in cool or moderate climate viticulture.

4.3.10 Alternaria alternata *(Fr.: Fr.) Keissler (Pleosporales): Alternaria Rot*

General Aspects Alternaria rot is ubiquitous and distributed worldwide. A number of fruit and crops may be affected and also foodstuffs and organic material like textiles, leather and paper. Alternaria rot causes merely marginal losses of berry quality in viticulture and colonizes mainly ripe berries with leaked sugar. Colonized

berries show a black smut on the surface. Only occasionally injured berries are infected. In this case Alternaria rot raises a mouldy taste of grapes and wine and produces mycotoxins. Therefore infected clusters have to be sorted at harvest.

Taxonomy To the taxon *Alternaria* belongs to numerous species of which *Alternaria alternata* (Fr.: Fr.) Keissler is most common on grapevine. The genus *Lewia* is described as teleomorph for *Alternaria* (Pleosporales, Ascomycetes) (Gams et al. 1998; Mc Laughlin et al. 2001). Recently, a new *Alternaria* species (*A. viniferae* sp. Nov) has been described on grapevine cultivars in China (Tao et al. 2014).

Biology and Epidemiology The conidiophores of *Alternaria alternata* produce pale to medium brown conidia in long, often branched chains. The brown to olive-green conidia have transverse and longitudinal septae and a cylindrical or short conical beak (Samson and Reenen-Hoekstra 1988). The fungus has a saprophytic lifestyle and prefers a sugary substrate, but occasionally it becomes parasitic. For setting an infection, high relative humidity is necessary (98–100%). Under these conditions, the germination peg of the conidia is able to penetrate the epidermis directly. Therefore, frequent rain in late summer and autumn is favourable for the infection process (Hewitt 1988; Valero et al. 2007). In general, endophytic *Alternaria* species are typical members of the grapevine microbiome (Polizzotto et al. 2012; Pinto et al. 2014).

4.3.11 Cladosporium herbarum *(Pers.) Link (Capnodiales): Cladosporium Rot*

General Aspects Cladosporium rot is widespread and very common in temperate regions on dead or dying plant substrates and other organic matter. Cladosporium rot is typically a post-harvest disease on fruits and crops. Late harvested grapes and table grapes may be infested by the rot, and berries can decay (Swett et al. 2016). No major mycotoxins of concern are produced (Frisvad 1988; Northolt and Soentoro 1988); however volatile organic compounds are accumulate conferring a mouldy off-flavour to the affected clusters.

Taxonomy The genus *Cladosporium* comprises numerous species of which some are the most common indoor and outdoor moulds. On grapevine clusters and berries mainly *C. herbarum* (Pers.) Link occurs (Whitelaw-Weckert et al. 2007). According to its teleomorph *Davidiella tassiana* (De Not.) Crous and U. Braun (emend. *Mycosphaerella tassiana* (de Not.) Johanson), the fungus belongs to the Capnodiales (Ascomycetes) (Gams et al. 1998; Mc Laughlin et al. 2001).

Biology and Epidemiology Colonies of *C. herbarum* are velvety-powdery, and the colour is olivaceous to brown due to the abundant mass of conidia formed on the mycelium. Smooth-walled conidiophores have terminal and intercalary swellings.

At the tip of the conidiophores, one-, two- or more-celled conidia are formed in simple or sometimes branched chains. The conidia are ellipsoidal to cylindrical and pigmented. They germinate on moist surfaces and lesions are black and circular ranging from several millimetres in diameter to up to two-thirds of the berry surface. If the lesion turns olivaceous, sporulation starts and conidiophores with numerous conidia are present. The fungus has a broad temperature range (4–30 °C); the optimum lies between 20 and 24 °C (Hewitt 1988; Whitelaw-Weckert et al. 2007; Lorenzini and Zapparoli 2015).

4.3.12 Trichothecium roseum *(Pers.) Link (Hypocreales): Pink Rot*

General Aspects Pink rot occurs under high relative humidity and rain on berries after veraison. Normally the rot is associated with wounds and may occur on berries infected by *Botrytis* cinerea which acts as a primary invader. Infected berries show white to pink pads, shrink and decay. Expended infections cause mummified clusters covered by a pinkish mycelium. While harvesting, rotten parts of the clusters should be sorted out as mycotoxins such as crotoxin, trichothecene and roseotoxin may be produced (Frisvad 1988). Additionally the rot causes an unsavoury and bitter taste in affected parts of the cluster and can be responsible for off-flavour in the wine.

Taxonomy *Trichothecium roseum* (Pers.) Link belongs to the order of Hypocreales (Ascomycetes); however the current Taxonomy is *incertae sedis* (Gams et al. 1998; Mc Laughlin et al. 2001).

Biology and Epidemiology On berry surfaces, pinkish erect, unbranched conidiophores arise from the mycelium. Conidiophores are often septate near the base and more or less rough-walled. At the apex, chains of ellipsoidal to pyriform conidia develop by retrogressive division (Gams et al. 1998). Young conidia are aseptate, and when they mature, one septum in the middle of the conidia is formed. After conidia removal from the conidiophore, an obliquely truncate basal scar is present where the conidia have been inserted into the conidiophore. The fungus develops also at lower temperature, but optimal conidia germination and hyphae growth occur at 25 °C (Samson and Reenen-Hoekstra 1988).

4.4 Zygomycetes

4.4.1 Rhizopus stolonifer *(Ehrenb.) Lind. (Mucorales): Rhizopus Rot*

General Aspects Rhizopus rot is common on soft fruits, more abundant in warm, humid climates than in cool climate viticulture. In several fruits and crops such as strawberry, tomato, cucumber and table grapes, Rhizopus rot causes a soft rot during transport and storage (Hallmann et al. 2007). Rhizopus rot also affects bread and is known as black bread mould.

Taxonomy The causal agent *Rhizopus stolonifer* (Ehrenb.) Lind is a member of Mucorales which belong to the Zygomycetes a phylum of the kingdom of Mycota distinct from the Ascomycetes (Mc Laughlin et al. 2001).

Biology and Epidemiology *R. stolonifer* is heterothallic and sexual reproduction occurs exclusively when opposite mating types fuse (Schipper and Stalpes 1980). The young mycelium is whitish, becoming greyish-brown due to brownish sporangiophores and brown-black sporangia. Sporangiophores stand alone or in groups of usually 3–4. They are extremely tall, often over 20 mm high, erect and unbranched. At the opposite side of the globose sporangia branched rhizoids are obvious, a typical formation of *R. stolonifer* among most other *Rhizopus* species (e.g. *R. oryzae*). The columella is of globose to ovoid shape. The sporangiospores are irregularly formed (polygonal, globose, ovoid) with numerous striations on the spore surface (Samson and Reenen-Hoekstra 1988). Rhizopus rot is not restricted to berry infection alone. Under humid weather conditions, the fungus may spread to other berries in a cluster, causing a bunch rot (Hewitt 1988).

4.4.2 Mucor Spp. (Mucorales): Mucor Rot

General Aspects In general Mucor rot occurs as a post-harvest disease on fruits including grapes (Hallmann et al. 2007). The rot is very rare on grapes for wine production. Recent characterizations of the grapevine microbiome revealed a notable frequency of occurrence of *Mucor fragilis* in the microbial community on grapes (Setati et al. 2015).

Taxonomy The four species *Mucor mucedo* Fresen, *M. hiemalis* Wehmer, Mucor fragilis (Tode) Traverso and *M. piriformis* A. Fisch. are characteristic species of the Mucorales (Zygomycetes) (Mc Laughlin et al. 2001).

Biology and Epidemiology The thallus of *Mucor* spp. is white or coloured. The sporangiophores end in a globose sporangium in which spores are formed. The wall of the sporangiophore bursts for spore release (Gams et al. 1998). *Mucor* spp. can even grow and develop at cool storage conditions, In the case of *M. hiemalis*, the

optimum temperature for growth and sporulation is 5–25 °C (Samson and Reenen-Hoekstra 1988).

4.5 Conclusions

The main grapevine diseases affecting yield and quality of clusters and berries are powdery and downy mildew. Both diseases are caused by biotrophic pathogens which can infect and parasite only intact tissue with vigorous living cells. Hence, the susceptibility of the berries decreases during the development and ripening. Nevertheless, cluster infestations by downy and powdery mildew can occur up to the stage of veraison causing severe berry damage and off-flavour. The majority of the other fungi infecting clusters and berries cause berry rot and need mono- and oligosaccharides for spore germination and formation of a mycelium which is at disposal in the substrate. Consequently berry rot is favoured by sugar leaking from the ripening berries. In addition all these fungi develop at high temperature with an optimum from 22 to 24 °C. Precocious veraison and consequently early onset of sugar import in the berry under high temperature in the summer particularly provide optimal conditions for berry infection. Susceptibility of berries to fungi is also triggered by the structure of the berry skin. An intact berry skin with a dense layer of epicuticular waxes and a compact cuticle provide a constitutive barrier against invaders. On the other hand, a weak skin is permeable for both the infection structures of the invading fungus and sugars from inside the berries. The structure of the berry skin depends on numerous factors—among others on the weather conditions, water supply and nitrogen uptake of the vine. Warm and humid conditions favouring the development of fungi may also reduce reinforcement of the berry skin. These interactions between susceptibility of berries for fungi and the epidermal tissue including the cuticle have to be noted for the control of berry rot. Another aspect of berry colonizing fungi is the off-flavour and the production of mycotoxins which can devastate the yield of the affected vineyard. To raise the quality and to avoid remarkable economical loss, all measures have to be taken to control fungi colonizing berries and clusters. An important prerequisite for that is the knowledge of the biology, biochemistry and epidemiology of fungi.

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Chapter 5
Viruses of Wine-Associated Yeasts and Bacteria

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5.1 Introduction

Stuck and sluggish fermentations are still a major problem for winemakers. While stuck fermentations can usually be characterized by high residual sugar contents at the end of the alcoholic fermentation, sluggish fermentations are accompanied by a low rate of sugar utilization. In both scenarios, malfermentations can be caused by a variety of factors, most of which lead to a decrease in the metabolism of the fermenting yeast strain, associated by a decrease in biomass production, cell viability, and/or fermentation rate (Bisson 1999). One such factor potentially causing a variety of oenological problems during wine fermentation is the production of toxic proteins, so-called killer toxins, by certain killer yeasts. Soon after the discovery of toxin-secreting killer strains in the wine yeast *S. cerevisiae* (Bevan and Makower 1963), it became evident that killer yeasts and killer toxins can cause severe stuck fermentations, particularly under conditions when yeast starter cultures become suppressed by wild-type killer strains present on the grapes (Bussey

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1981; Young 1987; Heard and Fleet 1987; Vagnoli et al. 1993; Perez et al. 2001; Medina et al. 1997; Santos et al. 2011; de Ullivarri et al. 2014).

Bacteriophage attack of food-fermenting bacteria has always been a major cause of economic losses, particularly in the dairy industry (Sanders 1987; Everson 1991; Garneau and Moineau 2011; Samson and Moineau 2013). Research on phages and phage–host interactions in lactic acid bacteria (LAB) has thus developed, with the ultimate goal of preventing phage-induced lysis of starter strains (Mahony and van Sinderen 2015). In the wine industry, the LAB bacterium *Oenococcus oeni* (formerly *Leuconostoc oenos*) is the organism of choice to promote malolactic fermentation (MLF), a process of major importance for the oenological properties of most wines (Henick-Kling 1993; Bartowsky and Borneman 2011; Betteridge et al. 2015). The economical importance of MLF and of its favored promoting agent (*O. oeni*), combined with the experience coming from the dairy industry, has prompted the research of bacteriophages—in this case, oenophages—as a potential cause of MLF failure.

In the following sections, we will focus first on the killer phenotype in yeast and its frequent association with cytoplasmic persisting double-stranded (ds)RNA viruses and then move to the state-of-the-art research on phages infecting *Oenococcus*.

5.2 Killer Yeasts and Wine Fermentation

Negative effects of a particular killer yeast on wine fermentation have meanwhile been repeatedly reported and demonstrated to critically depend on one or more of three major factors: (a) initial ratio of killer to killer-sensitive yeast strains in the must and at the commencement of fermentation, (b) toxin sensitivity of the fermenting yeast strain, and (c) presence of protein adsorbing additives such as bentonite (Petering et al. 1991; Radler and Schmitt 1987; Carrau et al. 1993; Van Vuuren and Wingfield 1986; Van Vuuren and Jacobs 1992). Spoilage yeasts, including toxin-secreting as well as non-killer strains, occur spontaneously during wine fermentation and compete with commercial starter cultures, in particular if the starter yeast is susceptible to killer toxins (Jacobs and Van Vuuren 1991; Shimizu 1993; Musmanno et al. 1999; Perrone et al. 2013). Vice versa, killer toxin-secreting *S. cerevisiae* strains with desirable enological properties have been employed as starter culture to improve wine quality by preventing outgrowth of spoilage yeasts during the early onset of fermentation (Seki et al. 1985; Ciani and Fatichenti 2001; Du Toit and Pretorius 2000; Marquina et al. 2002; Schmitt and Breinig 2002; Comitini et al. 2004; Golubev 2006; Schmitt and Schernikau 1997). In this respect, not only killer yeasts but also purified killer toxins have been considered as efficient strategy to prevent, or at least control, the undesired growth of wine spoilage yeasts including various strains and species of the genera *Dekkera*/*Brettanomyces*, *Pichia*, *Zygosaccharomyces* and *Candida* (Santos et al. 2011; de Ullivarri et al. 2014).

5.3 Viral Infections Can Cause a Killer Phenotype in Yeast

Although initially discovered in brewery strains of *Saccharomyces cerevisiae* as determinant of the killer phenomenon, cytoplasmic inherited dsRNA viruses are not restricted to strains of brewer's and baker's yeast but can be frequently found in different yeast genera (Wickner 1996; Schmitt and Breinig 2002). Among these, killer strains of *S. cerevisiae*, *Zygosaccharomyces bailii*, *Hanseniaspora uvarum*, *Debaryomyces hansenii* and *Ustilago maydis* are best studied. Characteristic of all killer yeasts is the secretion of protein toxins that are lethal to sensitive (susceptible) strains of different species and genera. Toxicity and cell killing are usually achieved in a receptor-mediated process, requiring initial toxin binding to components of the yeast cell wall (such as β-1,6-D-glucans, α-1,3-mannoproteins, or chitin) and subsequent toxin transfer to a particular plasma membrane receptor. Depending on the toxin's mode of action, final lethality can be caused by, e.g., plasma membrane damage, cell cycle arrest, and/or inhibition of DNA synthesis (Magliani et al. 1997; Bruenn 2005; Schmitt and Breinig 2006).

In the yeasts *S. cerevisiae*, *Z. bailii*, and *H. uvarum*, as well as in the corn smut fungus *U. maydis*, the killer phenotype is associated with a non-Mendelian inheritance caused by an infection with cytoplasmic persisting dsRNA viruses of the *Totiviridae* family which spread vertically by cell-to-cell mating or heterokaryon formation (Bruenn 2005). Diploid yeasts formed by mating of a killer with a sensitive strain are likewise killers, as are all haploid progeny of subsequent meiosis. In contrast, virus-free yeast strains are usually sensitive non-killers, while yeasts containing two types of dsRNA viruses (ScV-L-A and ScV-M) are killers (see below). Sensitive strains survive mating with killers and cytoplasmic mixing of the dsRNA viruses during zygosis accounts for the inheritance pattern during meiosis. Since an extracellular spread of yeast viruses is largely hampered by the rigid yeast and fungal cell wall barrier, mycoviruses have adopted a strategy of transmission via mating and hyphal fusion which frequently occurs in nature, making an extracellular route of spread dispensable. While some mycoviruses are associated with adverse phenotypic effects on the fungus (like La France disease in *Agaricus bisporus*, plaque formation in *Penicillium*, and hypovirulence in *Endothia*), M-dsRNA containing yeast "killer" viruses are responsible for a killer phenotype that is based on the secretion of a polypeptide (killer toxin) that is lethal to a variety of sensitive yeasts and fungi. With the exception of toxin-secreting strains of *Z. bailii*, killer toxin production is usually associated with a specific immunity component that protects a killer yeast against its own toxin (Schmitt and Neuhausen 1994; Breinig et al. 2006).

5.3.1 *dsRNA Viruses and Killer Phenotype Expression in Wine Yeast*

On the basis of killing profiles and the lack of cross-immunity, four virally encoded killer types have so far been identified and characterized in *S. cerevisiae*: K1, K2, K28, and Klus (Schmitt and Breinig 2006; Rodríguez-Cousino et al. 2011). Each of them produces a specific killer toxin and a self-protecting immunity component. Killer phenotype expression correlates with the presence of two dsRNA genomes that stably persist in high copy numbers in the cytoplasm of the infected host: a larger 4.6 kb dsRNA of the helper virus (ScV-L-A) and one of four smaller toxin-coding satellite dsRNAs which are separately encapsidated (ScV-M1, ScV-M2, ScV-M28, and ScV-Mlus). In vivo, ScV-L-A does not confer a phenotype nor does it lead to host cell lysis or cell growth slowing. While the killer phenotype can be transmitted by transfection of yeast cell spheroplasts with purified ScV-L-A and ScV-M (El-Sherbeini and Bostian 1987), extracellular virus transmission occurs rarely in nature, if at all. The survival strategy adopted by these dsRNA viruses appears to be a balanced host interaction, resulting in stable maintenance, little if any growth disadvantage, and vertical transmission. Mechanisms of exiting and entering the host cell through its rigid cell wall are rendered unnecessary by efficient horizontal transmission during frequent zygosis events in yeast. Acquisition of a toxin-encoding M satellite dsRNA provides positive selection, as virus-free segregants are killed.

As summarized in Table 5.1, the linear dsRNA genome of ScV-L-A contains two open reading frames (ORF) on its (+) strand RNA: ORF1 encodes the major capsid protein Gag necessary for encapsidation and viral particle structure, and the second gene (ORF2) represents the RNA-dependent RNA polymerase (RDRP) which is expressed as a Gag-Pol fusion protein by a −1 ribosomal frameshift event (Wickner 1996). In contrast to L-A, each M-dsRNA genome contains a single open reading frame encoding a preprotoxin (pptox) that represents the unprocessed precursor of the mature and secreted toxin which also confers functional immunity. As each M-dsRNA genome depends on the coexistence of ScV-L-A for stable maintenance and replication, ScV-M killer viruses resemble classical satellites of ScV-L-A. Although coexistence of multiple M-dsRNAs with different killer specificities in a single cell is excluded at the replicative level, this limitation can be bypassed by introducing cDNA copies of K2 and K28 pptox genes into a natural K1 killer, resulting in triple killers that stably produce three different killer toxins at a time and simultaneously express multiple immunity (Schmitt and Schernikau 1997).

Table 5.1 Double-stranded (ds)RNA viruses involved in killer phenotype expression in yeast

dsRNA virus	Virus host	dsRNA (kb)	Encoded protein(s)
ScV-L-A	*Saccharomyces cerevisiae*	L-A (4.6)	Gag, major capsid protein; Gag-Pol, RDRP[a]
ScV-M1	*Saccharomyces cerevisiae*	M1 (1.6)	K1 preprotoxin
ScV-M2	*Saccharomyces cerevisiae*	M2 (1.5)	K2 preprotoxin
ScV-M28	*Saccharomyces cerevisiae*	M28 (1.8)	K28 preprotoxin
ScV-Mlus	*Saccharomyces cerevisiae*	Mlus (2.1)	Klus preprotoxin
UmV-P1	*Ustilago maydis*	M-P1 (1.4)	KP1 preprotoxin
UmV-P4	*Ustilago maydis*	M-P4 (1.0)	KP4 toxin
UmV-P6	*Ustilago maydis*	M-P6 (1.2)	KP6 preprotoxin
HuV-L	*Hanseniaspora uvarum*	L-Hu (4.6)	Gag, major capsid protein; Gag-Pol, RDRP[a]
HuV-M	*Hanseniaspora uvarum*	M-Hu (1.0)	KT470 toxin precursor
ZbV-L	*Zygosaccharomyces bailii*	L-Zb (4.6)	Gag, major capsid protein; Gag-Pol, RDRP[a]
ZbV-M	*Zygosaccharomyces bailii*	M-Zb (2.1)	Zygocin preprotoxin

[a]*RDRP* RNA-dependent RNA polymerase

5.3.2 Viral Preprotoxin Processing and Toxin Maturation

In virus-infected killer yeasts, the toxin-encoding M(+)ssRNA transcript is translated into a preprotoxin (pptox) that is posttranslationally imported into the secretory pathway for further processing, maturation, and toxin secretion. Intracellular pptox processing in killer strains of *S. cerevisiae* (K1, K2, K28, Klus), *Z. bailii* (zygocin), and *U. maydis* (KP4) has been intensively studied and seems mechanistically conserved. In K28 killers, originally isolated from grape (Pfeiffer and Radler 1982), the pptox precursor is processed into a α/β heterodimeric protein whose subunits are covalently linked by a single disulfide bond. As secretory protein, the unprocessed toxin precursor contains an N-terminal signal peptide for pptox import into the ER lumen, followed by the toxin subunits α (10.5 kDa) and β (11.0 kDa) separated by an intervening *N*-glycosylated γ sequence. During passage through the secretory pathway, the toxin precursor is enzymatically processed in a way that is highly homologous to prohormone conversion in mammalian cells. In a late Golgi compartment, the *N*-glycosylated γ-sequence of K28 pptox is removed by the furin-like endopeptidase Kex2p, and biologically active K28 is secreted as 21 kDa heterodimer whose β-C-terminus carries a four-amino acid motif (HDEL) and

potential ER-targeting signal which is generated by carboxypeptidase cleavage (Kex1p) in a late Golgi compartment (Schmitt and Breinig 2006).

5.3.3 *Endocytosis and Intracellular Toxin Transport*

In contrast to most viral yeast killer toxins that kill by disrupting cytoplasmic membrane function, K28 enters a cell by receptor-mediated endocytosis (Eisfeld et al. 2000). Once it has reached an early endosomal compartment, the toxin travels the secretory pathway in reverse, subsequently translocates into the cytosol, and finally kills in the nucleus. Essential and sufficient for this retrograde toxin transport is the HDEL motif at the C-terminus of K28-β which normally functions as ER retention signal to prevent escape of soluble ER residents from the secretory pathway. In yeast and higher eukaryotes, proteins that entered the ER lumen and carry such a C-terminal motif are recognized by a membrane-bound H/KDEL receptor, Erd2p in yeast, which ensures their recycling from the Golgi back to the ER. In case of K28, this sequence allows retrograde transport from endosomes via Golgi to the ER, from where the toxin retrotranslocates into the cytosol to finally kill in the nucleus (Fig. 5.1). Interestingly, endocytotic uptake and retrograde

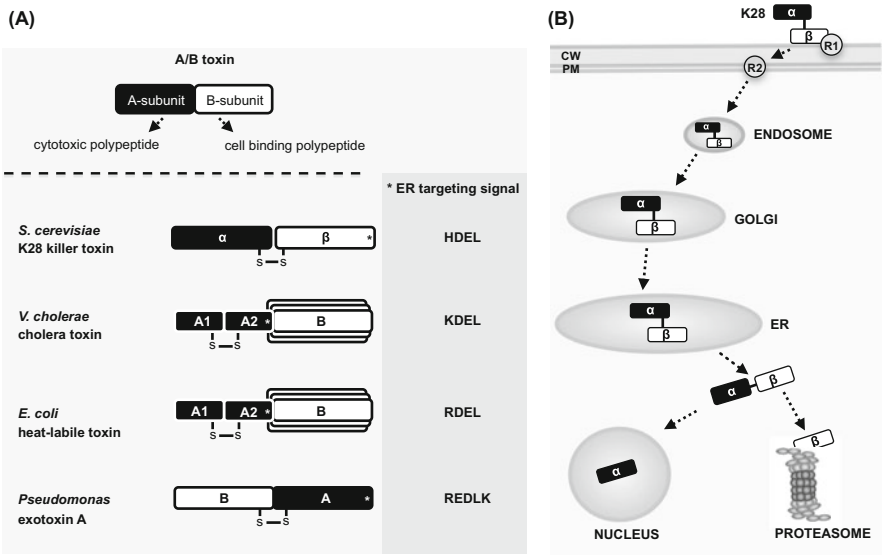


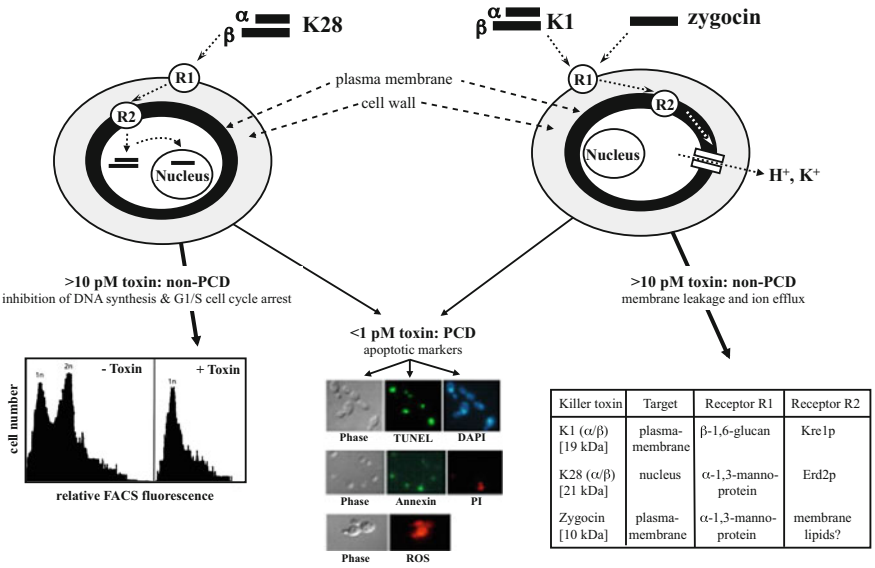
Fig. 5.1 (**a**) Schematic outline of the general structure of microbial and viral A/B protein toxins containing a carboxyterminal H/KDEL-like motif and potential ER-targeting signal. (**b**) Intoxication of a sensitive yeast cell by killer toxin K28 via receptor-mediated endocytosis, backward transport through the secretory pathway and final killing in the nucleus [R1, cell wall receptor of K28; R2, plasma membrane receptor parasitized for K28 uptake; adapted from Becker et al. (2016)]

transport are common strategies of microbial A/B toxins including *Pseudomonas* exotoxin A, *E. coli* heat-labile toxin (HLT), and yeast killer toxin K28. Each of these toxins contains a putative ER-targeting signal at one of the polypeptide chain (s) which has recently been shown to be of major importance for toxin entry into a target cell (Becker et al. 2016). In this respect, a major difference between the yeast toxin (K28) and bacterial A/B toxins is that K28 itself is produced and secreted by a eukaryotic (yeast) cell, and therefore the C-terminal ER-targeting signal in the toxin precursor is initially masked by a terminal arginine residue to ensure pptox passage through the early secretory pathway. Once the toxin has reached a late Golgi compartment, Kex1p cleavage removes the β-C-terminal arginine and thereby uncovers the HDEL motif for subsequent host cell entry and intracellular transport.

After ER exit and entry into the cytosol, the toxin dissociates into its subunit components: β is ubiquitinated and proteasomally degraded, while α enters the nucleus and causes cell death (Fig. 5.1). It is assumed that ER exit of the toxin is mediated by the Sec61 complex in the ER membrane; besides being the major protein import channel in the ER, Sec61p is also part of a protein quality control system that removes not correctly folded/assembled proteins from the secretory pathway and initiates their proteasomal degradation in the cytosol. Although the precise molecular mechanism by which killer toxin K28 retrotranslocates from the ER is still puzzling, it occurs independent of ubiquitination and proteasome activity and does not require classical components of the ER-associated protein degradation (ERAD) machinery (Heiligenstein et al. 2006).

5.3.4 K28 Affects DNA Synthesis and Cell Cycle Progression and Induces Apoptosis

Because of its low molecular weight, the cytotoxic α-subunit of K28 can enter the nucleus by passive diffusion; however extension of α by a classical nuclear localization sequence (NLS) significantly enhances its in vivo toxicity. Within the nucleus, the toxin interacts with host cell proteins of essential function in cell cycle control and initiation of DNA synthesis. Thus, as K28 targets evolutionary highly conserved proteins of basic cell functions, toxin resistance due to mutations in essential chromosomal yeast genes hardly occurs in vivo, indicating that the toxin has developed an amazing "smart" strategy to penetrate and kill a cell. Most interestingly, while higher toxin concentrations (10 pM or higher) cause necrotic cell killing via cell cycle arrest and inhibition of DNA synthesis, treatment with low doses of killer toxins (<1 pM) results in an apoptotic cell death (Fig. 5.2). As toxin concentration is usually low in the natural environment and habitat of yeasts, toxin-induced apoptosis can be assumed to be the major mechanism by which killer yeasts outcompete and kill other yeasts in their natural environment (Reiter et al. 2005). Furthermore, as apoptosis is also important in the pathogenesis of virus infections in mammals, it is not surprising that yeast killer viruses can also induce a



Killer toxin	Target	Receptor R1	Receptor R2
K1 (α/β) [19 kDa]	plasma-membrane	β-1,6-glucan	Kre1p
K28 (α/β) [21 kDa]	nucleus	α-1,3-manno-protein	Erd2p
Zygocin [10 kDa]	plasma-membrane	α-1,3-manno-protein	membrane lipids?

Fig. 5.2 Receptor-mediated toxicity of the viral yeast killer toxins K1, K28, and zygocin. Killing of a sensitive yeast is envisaged in a two-step process involving initial toxin binding to receptors within the cell wall (R1) and the cytoplasmic membrane (R2). After interaction with the plasma membrane, ionophoric toxins such as K1 and zygocin disrupt membrane function, while K28 enters cells by endocytosis and diffuses into the nucleus to cause cell death (note that the cell surface receptors R1 and R2 are different in all three toxins; see also *table inset*). At high toxin doses (>10 pM), sensitive cells arrest in the cell cycle with pre-replicated DNA (1*n*; *left panel*), while cells treated with K28 in concentrations <1 pM respond with apoptosis, as shown by typical apoptotic markers such as chromosomal DNA fragmentation (TUNEL-positive cells), accumulation of reactive oxygen species (ROS), and phosphatidylserine exposure at the external surface of the plasma membrane detected by annexin V staining (*right panel*)

programed suicide pathway in noninfected yeast (Ivanovska and Hardwick 2005). Although virally encoded killer toxins were shown to be primarily responsible for this phenomenon, yeast killer viruses are not solely responsible for triggering a cell death pathway in yeast.

5.3.5 *Lethality of Membrane-Damaging Killer Toxins*

Yeast killer toxins kill sensitive yeasts in a receptor-mediated fashion by interacting with receptors at the level of the cell wall and the cytoplasmic membrane. The initial step involves rapid toxin binding to a primary receptor within the mannoprotein or glucan fraction of the cell wall. In the second step, the toxin translocates to the plasma membrane and interacts with a secondary receptor (Fig. 5.2). To date, only the membrane receptors for killer toxins K1 (Kre1p) and K28 (Erd2p) have been identified; for other yeast killer toxins, the precise binding

sites in the plasma membrane are still unknown. Once bound to the membrane, killer toxins K1 and zygocin disrupt cytoplasmic membrane function by forming cation-selective ion channels, while K28 enters a cell and kills by inhibiting DNA synthesis and arresting cells at the G1/S boundary of the cell cycle (Fig. 5.2).

Ion channel formation in yeast membranes induced by killer toxin K1 was initially demonstrated using patch-clamping techniques (Martinac et al. 1990). However, this observation is inconsistent with the complete resistance seen in immune yeast cell spheroplasts, and, so far, receptor-independent channels have not been observed, neither in yeast membranes nor in *Xenopus laevis* oocytes. Similar to K1 and probably K2 as well (Orentaite et al. 2016), zygocin represents a membrane-damaging killer toxin which is naturally secreted as monomeric and non-glycosylated protein by the osmotolerant spoilage yeast *Zygosaccharomyces bailii*. Compared to most other killer toxins of yeast, zygocin shows an unusual broad killing spectrum and is equally active against phytopathogenic and human pathogenic yeasts, including *Candida albicans*, *C. glabrata*, *C. tropicalis*, and *Sporothrix schenckii*. Since even filamentous fungi such as *Fusarium oxysporum* and *Colletotrichum graminicola* are likewise killed by the toxin, zygocin bears a significant antimycotic potential. Similar to K1, zygocin disrupts plasma membrane integrity and causes rapid cell killing. Its ionophoric mode of action has been reinforced by in silico sequence analysis, identifying a stretch of potential α-helical conformation that forms an amphipathic structure characteristic for membrane-disturbing antimicrobial peptides such as alamethicin, melittin, and dermaseptin. In addition, this feature is accompanied by a transmembrane helix at the C-terminus of zygocin which is predicted to favor a membrane permeabilizing potential, not by activating endogenous ion channels but rather by forming pores after toxin oligomerization. It is assumed that the hydrophobic part in its amphipathic α-helix is responsible for toxin binding to a target cell. The postulated model of zygocin action resembles that of human α-defensins. In analogy to alamethicin, toxicity of zygocin is probably mediated by insertion of its α-helix into the plasma membrane, a process solely driven by the natural transmembrane potential of the energized yeast and fungal plasma membrane. Thus, toxicity of zygocin portrays the lethal mechanism of antimicrobial peptides that are produced by virtually all higher eukaryotes. Mechanisms of resistance against antimicrobial peptides are rare and often limited to changes in the composition of the cytoplasmic membrane. In major contrast to mammalian cells, the outer leaflet of the plasma membrane of yeasts and fungi is enriched in negatively charged lipids. Due to the cationic net charge of antimicrobial peptides (including zygocin), an affinity to these lipids facilitates toxin adsorption to the target membrane. Consistent with that, deletion of chromosomal yeast genes whose products affect plasma membrane lipid composition (such as *PDR16* and *PDR17*) causes a dramatic decrease in zygocin sensitivity because toxin binding to the plasma membrane is largely prevented. In contrast to K1, a zygocin-specific membrane receptor is not required for in vivo toxicity, as the physicochemical properties of zygocin allow efficient plasma membrane interaction independent of a membrane receptor or docking protein (Weiler and Schmitt 2005).

5.4 Toxin Immunity Ensures Self-Protection in Killer Yeast

How killer yeasts protect themselves against their own secreted toxin remained a mystery for many decades. In killer yeast, functional immunity is essential for survival as the toxins often target and inhibit central eukaryotic cell functions. This is in major contrast to bacterial toxins such as cholera toxin and Shiga toxins which selectively kill eukaryotes, thus making immunity dispensable in a toxin-secreting prokaryote. In the case of killer toxin K28, the mechanism of protecting toxin immunity was uncovered (Breinig et al. 2006). While in an immune K28 killer yeast, the toxin is likewise taken up and transported to the ER, just like in a sensitive yeast cell, the internalized toxin forms a high-affinity complex with the pptox precursor in the cytosol. Within this complex, K28 is selectively ubiquitinated and targeted to proteasomes, while part of the pptox moiety escapes degradation and is imported into the ER for secretion of active killer toxin. This simple and highly efficient mechanism ensures that a killer yeast is fully protected against the lethal action of its own toxin. With the exception of K28, the molecular mechanism (s) of toxin immunity in other killer yeasts is still unknown.

5.5 Phages in Wine and Malolactic Conversion

The presence of phage particles in wine was first evidenced by Sozzi et al. (1976) through electron microscopy analysis of Suisse white wine samples. A few years later, several papers by the same and other groups reported the isolation of phages from wine with undesirable MLF properties, which were capable of lysing different strains of *O. oeni* (Sozzi et al. 1982; Gnaegi and Sozzi 1983; Gnaegi et al. 1984; Davis et al. 1985; Henick-Kling et al. 1986a, b). However, the real impact of phages on MLF and growth of *O. oeni* in the wine environment is somewhat controversial. On the one hand, some authors argued that irregular MLF and the consequential development of undesirable LAB strains (e.g., *Pediococcus* sp.) could be the result of phage attack against *O. oeni*. On the other hand, it appeared that the ability of oenophages to lyse *O. oeni* in wine and perturb MLF was greatly dependent both on the type of phage and the bacterial strain, which responded differently to the properties of the wine, such as the pH and the composition in SO_2, ethanol, bentonite, and phenolic compounds (Davis et al. 1985; Henick-Kling et al. 1986b). In any case, it appears that phage attack is not a critical problem in winemaking (Poblet-Icart et al. 1998), contrary to the scenario found in the dairy industry.

5.6 General Properties of Oenophages

Following the pioneer studies referred to above, oenophage research shifted toward the study of lysogeny in *O. oeni* and the molecular characterization of the isolated phages. A large number of phages were recovered upon treatment of potential *O. oeni* lysogenic strains with mitomycin C (see next section). These phages were subsequently characterized not only with respect to their morphology and lytic spectrum but also through the analysis of virion structural proteins and phage DNA (restriction patterns and DNA homology). Although the data collected from these studies allowed the definition of phage genetic groups according to their relatedness (e.g., see Nel et al. 1987; Arendt and Hammes 1992; Santos et al. 1996), the general picture that emerged was a close relationship between the isolated oenophages with respect to morphology, DNA sequence, and structural proteins.

All isolated oenophages belong to the *Siphoviridae* family (Ackermann 2005), whose virions are characterized by the presence of an icosahedral nucleocapsid (containing a double-stranded DNA genome) attached to a long, noncontractile tail. Morphologically, all phages are of the morphotype B1 (Bradley 1967; Ackermann and DuBow 1987). Frequently, a base plate can be distinguished in the tail extremity distal to the phage head (Fig. 5.3). The reported capsid diameters vary between 33 and 75 nm, and the tails are 179–308 nm long and 6–15 nm wide. Baseplates can be up to 24 nm in diameter. The vast majority of the studied oenophages seems to be of temperate nature, i.e., they are phages that can either replicate through the lytic pathway or be propagated as prophages integrated in the host chromosome. Some temperate oenophages might have become virulent as a result of point-mutational events (Arendt et al. 1990). The oenophage genome size

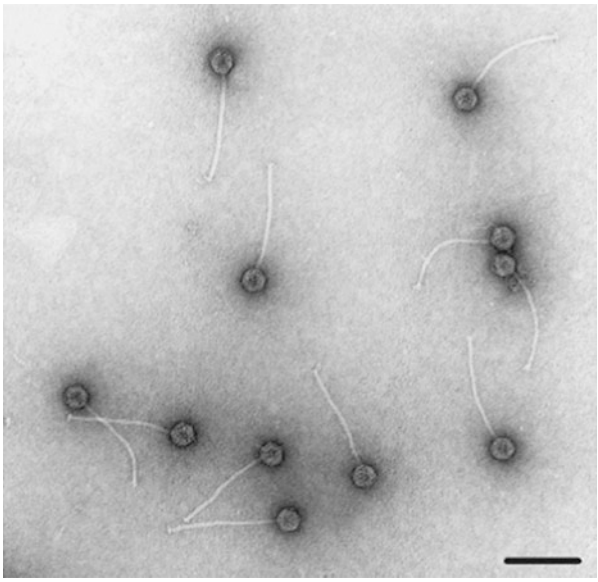


Fig. 5.3 Transmission electron microscopy photograph of negatively stained *O. oeni* phage fOg30. Magnification ×60,000; scale bar represents 170 nm

has been shown to vary between 28 and 43 kb, and the DNA extremities typically present cohesive ends (*cos*, Becker and Murialdo 1990).

5.7 Oenophage–Host Interactions

A number of studies have revealed that lysogeny is widespread among *O. oeni*. Two independent groups (Arendt et al. 1991; Cavin et al. 1991) reported the induction of prophages from *O. oeni* lysogenic strains upon addition of mitomycin C (MitC). The lysogeny incidence was as high as 63%, and indicator strains were found for 17 of the induced phages (Arendt et al. 1991). This work suggested, for the first time, that spontaneous induction of prophages harbored by lysogenic strains could be a major source of bacteriophage contamination in wine. Subsequent studies have confirmed the high incidence of lysogeny in *O. oeni* (Arendt and Hammes 1992; Tenreiro et al. 1993; Poblet-Icart et al. 1998). Tenreiro et al. (1993) have examined lysogeny in 29 *O. oeni* strains, of which 22 were isolated from Portuguese wines. Phages could be detected in the supernatants of 19 different induced cultures (66% lysogeny). In a following study, the Portuguese group further characterized 17 of the original isolated oenophages, and lysogenization of a phage-cured derivative of *O. oeni* strain PSU-1 was achieved with 16 phages (Santos et al. 1996). More recent studies, which include complete genome sequence data of several *O. oeni* strains, further demonstrated the high prevalence of lysogeny in this species and suggested that oenophages have a major role in *O. oeni* genome diversity, dynamics, and evolution (Zé-Zé et al. 1998; Bon et al. 2009; Borneman et al. 2012; Doria et al. 2013; Jaomanjaka et al. 2013).

In addition to lysogeny, some reports have indicated that pseudolysogeny may also occur in *O. oeni*. In pseudolysogeny, cells harbor unintegrated copies of phage DNA in a so-called carrier state, without being lysed significantly. In contrast to true lysogeny, serial subculturing of single colonies frequently results in the loss of contaminating phages from pseudolysogens. Studies conducted by Arendt et al. (1990, 1991) suggested strongly that *O. oeni* strain 58 N harbored a phage in such a carrier state. This unstable phage–host interaction might explain the lack of superinfection immunity of the original strains from which phages were initially isolated, the relatively high spontaneous induction of phages (10^3–10^5 pfu per ml of culture supernatant), and the emergence of virulent oenophages (Arendt et al. 1990; Santos et al. 1996). Moreover, it was also observed later that infection of strain ML34-C10 with phage fOg44 at high multiplicities favored the isolation of lysogens carrying, at the same time, integrated and unintegrated forms of phage DNA (Type I lysogens, Parreira et al. 1999). Type I lysogens spontaneously released phages in relatively high titers and exhibited a pattern of sensitivity to various oenophages that differed from strains carrying a stable prophage (type II lysogens). It was proposed that type I lysogens could result from cytoplasmic maintenance of unintegrated phage DNA following simultaneous penetration of several genomic

copies. Type I strains would thus resemble phage-carrying strains, with the exception that, in this case, prophage DNA was also detected.

5.8 Sequencing and Functional Genomics

The first report on the partial nucleotide sequence of a phage infecting *O. oeni* was published by Sutherland et al. (1994), which sequenced an *Eco*RI–*Hind*III DNA fragment with 3.2 kb from the phage L10 genome. At the time, it was not possible to ascertain the function of the proteins encoded by this DNA fragment. Presently, with the increasing number of phage sequences deposited in databases, we can now predict that the sequenced region harbors genes involved in phage morphogenesis.

The nucleotide sequence of a central 5.2 kb *Eco*RI DNA fragment of the phage ϕ10 MC genome was also partially determined. Analysis of the sequence (3.992 kb) allowed the recognition of the elements involved in site-specific integration (the integrase gene and the *attP* site, Gindreau et al. 1997) and the genes mediating lysis of the host cell at the end of the lytic cycle (*lys*, encoding a peptidoglycan hydrolase and *P163*, coding for a putative holin; Gindreau and Lonvaud-Funel 1999).

Santos et al. (1998) have mapped the restriction sites of six enzymes in six oenophage genomes and performed a comparative analysis of whole phage DNA. Phages were separated into two distinct groups (α and β), based on restriction site conservation and DNA–DNA cross-hybridization results. In spite of the heterogeneity in the restriction site profiles, hybridization results clearly evidenced homology between α and β phages in the central part of their genomes. This study has proven to be a useful starting point for studying specific regions of the phage DNA. A particular phage (fOg44, β-group) was studied in more detail through the determination of the nucleotide sequence of the central and of the *cos*-containing genomic regions (see next section).

5.8.1 General Outline of Oenophage Genome Organization

The nucleotide sequence of two regions of the phage fOg44 genome (Fig. 5.4a) was determined (Parreira et al. 1999; São-José 2002): a 7.810-kb segment spanning the *cos* site (Fig. 5.4b, Acc. Number AJ421942) and a 10.858-kb central fragment (Fig. 5.4c, Acc. Number AJ421943). The total sequence covered about 50% of the fOg44 genome. Figure 5.4 summarizes the relevant data emerging from the analysis of the two sequenced regions.

The region encompassing the *cos* site of the fOg44 genome (Fig. 5.4b) carries genes involved in DNA metabolism and packaging and head morphogenesis. Of the genes located upstream the *cos* site, we highlight *orf74* (*orf* stands for open reading frame), encoding a putative glutaredoxin-like protein, and *orf176*, which codes for an HNH-type endonuclease. These proteins are thought to be involved in the

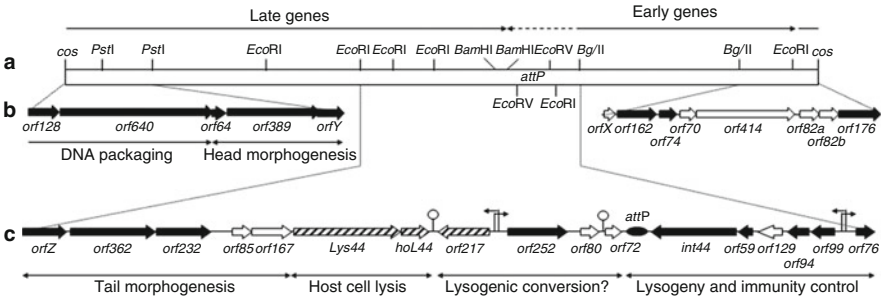


Fig. 5.4 Gene organization in two genomic regions of fOg44. (**a**) The fOg44 genome is represented by a *white rectangle*, and the position of relevant restriction sites is indicated. The *thin arrows* above the map evidence the regions that are early and late-transcribed during the phage lytic cycle (São-José 2002). The *dashed arrow* indicates a genome segment showing a low level of transcription (harboring *orf217* to *orf59*). (**b**) The *arrows* represent the *orfs* identified by nucleotide sequence analysis of the *cos*-containing region. The *orfs X* and *Y* denote 5′ and 3′ truncated open reading frames, respectively. The *orfs* to which a putative function could be attributed based on homology searches and available literature are colored in *black*. Those coding for putative products of unknown function are depicted in *white*. Gene clusters putatively involved in a particular function are identified below the corresponding *orfs*. (**c**) Organization of *orfs* identified in the genome central region. *Black* and *white arrows* as in (**b**). *orfZ* denote a 5′ truncated open reading frame. *Slashed arrows* indicate genes whose homology-based predicted functions have been confirmed experimentally. Putative transcription terminators and divergent transcriptional promoters are depicted as *hairpins* and *bent arrows*, respectively. Gene sets involved in specific processes are indicated below the corresponding *orfs*

maintenance of a deoxyribonucleotides pool for phage DNA synthesis (Gleason and Holmgren 1988) and in DNA packaging (Dalgaard et al. 1997), respectively. Downstream the *cos* site (Fig. 5.4b), we find the genes coding for the small (*orf128*) and large (*orf640*) subunits of the terminase oligomeric complex, which mediate phage genome maturation and encapsidation (Feiss 1986; Becker and Murialdo 1990). The putative products of *orf64*, *orf389*, and of the incomplete *orfY* correspond to the head–tail joining, portal, and ClpP proteins. In all studied phages, the portal protein forms a ring structure composed of 12 subunits in one vertex of the phage capsid, which serves as an entrance and exit port for the phage DNA (Bazinet and King 1985). The role of ClpP-type endopeptidases in maturation of the phage head has been experimentally shown for phage HK97 (Duda et al. 1995; Hendrix and Duda 1998).

The relative position of the *orfs* identified in the central region of fOg44 DNA (Fig. 5.4c) and the homology found for most of its encoded proteins allowed the identification of gene sets involved in specific functions: tail morphogenesis (based only in gene position), host cell lysis, lysogenic conversion, and lysogeny and immunity control.

The cluster defined by *orf217*, *orf252*, *orf80*, and *orf72* is not essential (at least under laboratory conditions) for the ability of fOg44 to complete its lytic cycle or to form lysogens (Parreira et al. 1999). The deduced product of *orf217* exhibits a central domain that is conserved in the TetR family of transcriptional repressors

(Ramos et al. 2005). LacZ-fusion experiments have shown that Orf217 represses both its own promoter and that of *orf252*, which encodes a putative permease of the TauE/SafE family probably involved in sulfite export (São-José et al. 2004; Weinitschke et al. 2007). The location of this module between the lysis and integration elements, its dispensability for the phage life cycle, and the putative function of some of its genes suggest that, like other "morons," it may confer selective advantage to the host bacteria when the phage is residing as prophage (Juhala et al. 2000; Desiere et al. 2001).

In the lysogeny and immunity control cluster, we find the elements involved in phage DNA integration in the host chromosome, i.e., the *attP* site and the *int44*gene (Parreira et al. 1999; São-José et al. 2004). The genes located upstream of *int44* regulate immunity and lysogeny functions (São-José 2002). In the prophage state, the putative products of *orf129* and *orf94* (a membrane protein and a metalloproteinase, respectively) most probably compose a system that excludes heteroimmune, superinfecting phages. *Orf99* is the λ-CI repressor-like protein regulating the lysis/lysogeny decision and conferring immunity to homoimmune phages when expressed from the prophage.

The central genomic region topologically equivalent to that spanning from *orf167* to *orf59* in fOg44 was sequenced in two other oenophages, fOgPSU1 (Acc. Number AJ629109) and fOg30 (Acc. Number AJ629110), and compared to the sequences of phages fOg44 and ϕ10 MC (São-José et al. 2004). fOgPSU1 and fOg44 share the same lysis cassette, whereas in the other two phages, the putative holin gene is replaced by *orf163*. With the exception of ϕ10 MC where the putative holin and integrase genes are separated exclusively by the *attP* site, several *orfs* (five in fOgPSU1 and six in fOg30) could be found between the lysis and integration regions, with *orf217*, *orf252*, and *orfr80* being common to the three fOg phages. The integration elements of fOg30 and fOg44 were almost identical at the nucleotide level and differed significantly with respect to the fOgPSU1 and the ϕ10 MC sequences. These differences are responsible for the targeting of different loci in the *O. oeni* chromosome for phage DNA integration (see next section).

The overall gene organization in the sequenced regions of oenophage genomes was found to closely follow that described for equivalent regions in dairy phages belonging to the "λ supergroup" of *Siphoviridae* (Brüssow and Desiere 2001). It is thus possible to envisage a general outline of oenophage genome organization, with functional gene clusters arranged from the left to the right side of the genome as follows: DNA packaging/head morphogenesis/head–tail joining/tail morphogenesis/host lysis/lysogenic conversion/lysogeny and immunity control/DNA replication and recombination/transcription regulation.

5.8.2 Multiple tRNA Loci as Sites for Phage DNA Integration in the Host Chromosome

The viral and bacterial DNA attachment sites (*attP* and *attB*, respectively), containing the identical core sequences that are involved in the site-specific recombination event that leads to phage DNA integration (Campbell 1962), were determined at the nucleotide sequence level initially for three oenophages. Phage ϕ10 MC integrates its genome at a $tRNA^{Leu}$ gene (Gindreau et al. 1997). It was shown later that phage fOg44 integrates its DNA at the 3′ end of an *O. oeni* $tRNA^{Glu}$ gene, whereas fOgPSU1 targets a gene for $tRNA^{Lys}$ (São-José et al. 2004). In all cases, the intact tRNA gene sequence was reconstituted upon phage DNA integration. When the available DNA sequences of fOg44 and ϕ10 MC were used for homology searches in the draft genome sequence of *O. oeni* strain PSU-1 (complete genome sequence published in 2005, Mills et al. 2005), two additional tRNA genes ($tRNA^{Glu}$ and $tRNA^{Leu}$) were found as potential integration sites (São-José et al. 2004). One of these sites may correspond to the secondary integration locus described by Santos et al. (1996). More recently, the integration of prophages and prophage remnants at tRNA loci was confirmed in a genomic comparison of eleven strains of *O. oeni*. A total of six different tRNAs were identified as targets for prophage integration (Borneman et al. 2012).

5.8.3 The Lysis Region and a New Mechanism of Phage-Induced Host Lysis

Double-stranded DNA phages induce lysis of infected cells in order to release progeny virions. For more than 20 years, it was assumed that these phages promoted host cell lysis following the same basic mechanism, which was extensively studied for the *Escherichia coli* phage λ (for reviews, see Young et al. 2000; Young 2014). According to this mechanism, lysis is accomplished by the concerted action of at least two phage-encoded products, a peptidoglycan hydrolase (referred to as endolysin) and a small hydrophobic protein (holin) that forms holes in the cytoplasmic membrane at a precise scheduled time. The latter function was considered essential to allow access of the cytoplasm-accumulated endolysin to the cell wall at the correct time.

Interestingly, it was demonstrated that the fOg44 endolysin (Lys44) is endowed with a typical N-terminal signal peptide that mediates its export to the cell wall through the general bacterial secretion pathway (the Sec-system, São-José et al. 2000). All known oenophage endolysins share this property (São-José et al. 2004). Although not required for Lys44 export, the fOg44 holin was able to complement a nonsense mutation of the λ holin gene, proving its functionality (São-José et al. 2004). This result, associated to the fact that the Lys44 active form is detected about

half-way through the fOg44 latent period (São-José et al. 2000), strongly argued that lysis regulation in fOg44 differed substantially from that described for λ.

A lysis model was proposed (São-José et al. 2000), in which the activity of the exported endolysin would be restrained by a molecular mechanism dependent on the membrane proton motive force (pmf). Holin-mediated dissipation of the pmf at a defined time would then activate the secreted enzyme. This model has recently gained support through experiments showing that oenococcal and lactococcal cells are intrinsically resistant to Lys44 when added from without or expressed from within, respectively (Nascimento et al. 2008). Moreover, Lys44-mediated lysis of both cells could only be efficiently triggered upon addition of nisin, the only antimicrobial among those tested that promoted ion-nonspecific dissipation of the pmf, an event that should be undertaken by the fOg44 holin in the phage infection context (Nascimento et al. 2008). In addition to its function as a "hole" for the export of canonical endolysins, it has been currently assessed that the membrane-depolarizing action of the holin may be also necessary to render cells fully susceptible to the action of the lytic enzymes (Fernandes and São-José 2016).

Most interestingly, phages producing secreted endolysins were later revealed to be more common than initially suspected. Several phages infecting Gram-positive, Gram-negative, and mycobacterial hosts were shown or suggested to produce Sec-exported endolysins (São-José 2002; São-José et al. 2003, 2007; Catalão et al. 2013; Oliveira et al. 2013; Young 2014). The detection and identification of *O. oeni* bacteriophages based on endolysin sequences were recently reported through the use of randomly amplified polymorphic DNA analysis (Doria et al. 2013).

5.9 Conclusions

In spontaneous wine fermentations, infection with a killer toxin-producing yeast can cause pronounced malfermentation, accompanied by low sugar consumption, by high residual sugar content, and, eventually, by cell death of the desired wine yeast at the end of the process. Killer toxin-induced stuck fermentations can also occur under conditions when yeast starter cultures become suppressed by wild-type killer yeasts present on the grape. The inhibitory effects of a given killer yeast on the winemaking process largely depend on the initial ratio of killer to non-killer yeasts in the must, on toxin sensitivity of the fermenting yeast, as well as on the presence of protein adsorbers such as bentonite. However, since most of these factors cannot be tightly controlled during wine fermentation, toxin-secreting yeasts with desirable enological properties are considered as attractive starter culture to prevent outgrowth of spoilage yeasts during fermentation and, thereby, to improve and ensure final wine quality.

Reports on the failure of malolactic fermentation, possibly due to the presence of bacteriophages in the must, led to several lines of investigation on phages infecting *Oenococcus oeni*. Molecular biology studies have so far been focused mainly on the

central region of the genome of a few temperate phages, highlighting the elements involved in prophage integration, genes possibly involved in lysogenic conversion phenotypes, and genes essential for lysis of the host. Knowledge about the sequence of *int* and *attP* and the finding of several tRNA genes as potential sites for site-specific recombination may lead to the future construction of integrative vectors useful for the genetic manipulation of *Oenococcus*. A novel mechanism for bacterial cell lysis was discovered when studying lytic functions of oenophage fOg44, relying on secretion signals in the endolysin. Analogous mechanisms for holin-independent transport of the lytic enzyme were subsequently found in several other phage–host systems.

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Chapter 6
Yeast Mixtures and *Saccharomyces* Hybrids: Suitable Tools for Performing More Sophisticated Must Fermentations

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6.1 Introduction

Recent investigations provide convincing evidence that in regions between the Black Sea and the Caspian Sea and also along the later Silk Road, wine was made and traded several thousand years BC. Possibly, viniculture started between 6000 and 8000 BC (McGovern 2003; McGovern et al. 2004; Kupfer 2013, 2015). Viniculture spread via Asia Minor, Iran, Iraq, and Turkey and to Phoenicia, Egypt, Crete, and Greece. Up to 1000 BC, the wine production is then verifiable in Sicily, Italy, Morocco, southern France, Spain, and Portugal. At the latest 1000 AD wine was also produced in northern France, Germany, and Eastern Europe. Today, the Eurasian wild form of *Vitis vinifera* L. subsp. *sylvestris* grows from Spain to Central Asia. From this wild form, the monoecious cultivated form *Vitis vinifera* L. subsp. *vinifera* arose.

It can be concluded from the early development of viniculture that the yeast S*accharomyces cerevisiae* is one of the oldest domesticated organisms (Feldmann 2010). The wine producing techniques have been further developed empirically over generations. Despite this long time of wine production, our knowledge about the microbiological and biochemical background of the transformation of must into wine is relatively recent. Fundamental investigations about must transformation into wine were carried out at the end of the eighteenth and in the course of the nineteenth century by scientists such as Antoine Laurent de Lavoisier, Joseph Louis

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Gay-Lussac, Charles Cagniard-Latour, Theodor Schwann, Friedrich Traugott Kützing, and Louis Pasteur (Gay-Lussac 1815; Schwann 1837; Kützing 1837; Cagniard-Latour 1838; Pasteur 1861). In 1883 Emil Christian Hansen succeeded in obtaining the first pure yeast cultures, which were used for beer brewing. Seven years later, Hermann Mueller-Thurgau introduced yeast starter cultures for the winemaking process. Commercial liquid cultures of yeasts were developed as starter cultures for the inoculation of must in the 1930s.

The limiting factors in must and wine are, in particular, the low pH value of the must (<3.5) and the high ethanol content. As we know today, only three groups of ethanol- and acid-tolerant microorganisms are growing in fermenting must (cf. - Chaps. 1, 2, 3 and 23). These are yeasts, lactic acid bacteria, and acetic acid bacteria. More than 100 yeast species belonging to 49 genera have been isolated and characterized from grapes, must, and wine. *Saccharomyces cerevisiae* and the so-called wild yeasts (non-*Saccharomycetes*) play an important role for the specific sensory profile of wine. In addition, around 25 species of wine-related lactic acid bacteria were cultivated in the laboratory, which are assigned to the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, and *Weissella*. Acetic acid bacteria can also be detected on grapes, must, and wine. The 23 hitherto known species belong to the genera *Acetobacter*, *Ameyamaea*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, *Komagataeibacter*, and *Kozakia*.

Today a relatively broad knowledge exists about the diversity of wine-related microorganisms, there successive appearance, and their activities during ethanolic fermentation of must. Starter cultures such as *Saccharomyces cerevisiae* and *Oenococcus oeni* or *Lactobacillus plantarum* are available for the alcoholic and malolactic fermentation, respectively. Nevertheless, sluggish or stuck fermentations cause significant financial losses for winemakers each year. These observations stimulate the search for new procedures to circumvent the fermentation obstacles.

6.2 Principal Modes and Problems of the Alcoholic Fermentation

The risk of sluggish or stuck fermentations can be significantly reduced by the application of commercial yeast starter cultures when about hundred thousand cells per ml are added by the winemakers to start the *controlled fermentation*. Such cultures are offered by different companies such as Erbslöh, Lallemand, Hansen, or Begerow. Because of the high titer of the starter yeast cells, the wild yeasts can hardly develop, and the fermentation is completed relative reliably by the starter cultures. The risk of fermentation problems is greatly reduced, but the sensory profile is restricted and depends on the starter cultures used.

In the case of the *monitored fermentation*, the fermentation is started spontaneously, and selected yeast cultures are only added when fermentation problems are

observed. We have obtained good results with optimized yeast strains or yeast mixtures. They were selected from fermenting must in the same vineyard in previous years before their addition to sluggishly fermenting must in order to continue and finish the fermentation. This method produced complex wines which met the special sensory requirements of the winemaker.

In the case of *spontaneous fermentation*, the earliest form of the must fermentation, the yeast strains present in the cellar or on the grapes enter the must and start the fermentation. The wine yeast *Saccharomyces cerevisiae* is present only at a relatively low number of cells at the beginning of the fermentation. At this stage of the fermentation, there is usually a dominant mixture of the so-called wild yeasts. In this case, the indigenous wild yeasts, the classic wine yeast *Saccharomyces cerevisiae*, and the local bacterial strains are involved in the transformation of the must into wine. When the ethanol concentration reaches 4% (v/v), *Saccharomyces cerevisiae* can overgrow the wild yeast and bacterial populations in the must. The corresponding wines are often more complex and meet more likely the expectations of a particular terroir. The risk of fermentation problems, however, is increased compared to controlled and monitored fermentation.

In spite of the advantages of adding yeast starter cultures after grape pressing with regard to fermentation reliability, winemakers in the upper quality segment especially opt for spontaneous fermentation in order to produce complex wines with a characteristic sensory profile distinctive for a certain winery or terroir. The sensory profile is influenced by the grape variety and grape quality, the terroir (soil and climate), and the conditions in the wine cellar as well as fermentation management but also by the added or indigenous microbial flora. In the case of spontaneous fermentation, the bacteria and yeast composition in the fermenting must depend on the microorganisms on the grapes and the cellar. Without doubt, the risk of fermentation problems in the case of spontaneous fermentation is increased. The reasons for fermentation problems can be manifold: (a) seriously infected grapes, (b) low temperatures or temperature fluctuations, (c) presence of toxic substances (fungicides, killer toxins), (d) low ratio of glucose to fructose ($>$1:10), (d) nutrient deficiencies (vitamins, trace elements), (e) low ammonium concentration ($<$120 mg/l), (f) low pH ($<$3.0), and (f) other factors (e.g., ergosterol, biotin, polyphenol concentration).

Classical methods for remedying existing fermentation problems are (a) adjustment of the temperature to 20 °C, (b) addition of yeast nutrients (diammonium hydrogen phosphate), (c) increase of the pH value, and (d) an inoculation with yeast starter culture. However, these measures frequently lead to a change in the initially targeted sensory profile, which is incompatible with the conceptions of winemakers in the upper quality sector in respect to very special sensory characteristics.

6.3 Future-Oriented Aspects of Producing More Sophisticated Wines

6.3.1 Increased Use of Wild Yeasts and Mixtures of Yeast Strains

At the beginning of the fermentation, the wild yeasts (non-*Saccharomycetes*) occur far in the majority compared to the classical wine yeast *Saccharomyces cerevisiae*. However, they are more sensitive to sulfite and increasing ethanol concentrations. Some have a high oxygen requirement such as *Torulaspora delbrueckii* and *Lachancea* (*Kluyveromyces*) *thermotolerans*. A problem for the optimal growth of *Saccharomyces cerevisiae* is that the wild yeasts already consume partly the nutrient and growth-promoting substances such as sugars, trace elements, or vitamins. They can also often form acetic acid, which has a negative effect on the growth behavior of *Saccharomycetes*. From a concentration of 0.6 g l^{-1}, acetic acid is sensitively perceptible. German law prescribes upper limits of 1.08 g l^{-1} for white wine and 1.20 g l^{-1} for red wine. A maximum of 2.10 g l^{-1} is allowed for wine of individually selected overripe berries (Trockenbeerenauslese). Since ethanol is converted into the volatile compound acetic acid, ethanol oxidation leads to a reduction in the ethanol content of the wine. Wild yeasts also form polysaccharides, which have a positive influence on the mouthfeeling at a certain concentration but can also contribute to graisse. Furthermore, a series of esters which are responsible for various fruit aromas can be formed. When *Lachancea thermotolerans* and *Candida zemplinina* grow in must, the glycerol content can be increased, which also has an influence on the mouthfeeling. *Candida zemplinina* can also lower the concentration of the acetic acid produced in must with high sugar content. In addition, aromatics and colored substances (anthocyanins) from glycosylated compounds are released by representatives of the genera *Debaryomyces*, *Hansenula*, *Candida*, *Pichia*, and *Kloeckera*. Thus, in Chardonnay wines, after inoculation of the must with a mixture of *Debaryomyces pseudopolymorphus* and *Saccharomyces cerevisiae*, increased concentrations of terpenols such as citronellol, nerol, and geraniol can be detected. In a sample of a Sauvignon Blanc must, the content of sulfur-containing compounds such as 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexan-1-ol acetate (3MHA) was increased after the addition of *Candida zemplinina* and *Pichia kluyveri*. Thioles contribute to the specific aroma of Sauvignon Blanc wines. We were able to detect the enzymatic hydrolysis of a series of glycosylated aroma precursors by the yeast *Wickerhamomyces anomalus* (Sabel et al. 2014; Schwentke et al. 2014).

At the beginning of the alcoholic fermentation of the must, the so-called wild yeasts predominate. Representatives of the genera *Hanseniaspora*, *Rhodotorula*, *Pichia*, *Candida*, *Metschnikowia*, and *Cryptococcus* are frequently detected (cf. - Chap. 23). They can be divided into different groups:

(a) Aerobic growth: *Pichia*, *Debaryomyces*, *Rhodotorula*, and *Candida*
(b) Apiculate yeasts with low fermentative activity: *Hanseniaspora uvarum* (perfect form: *Kloeckera apiculata*), *H. guilliermondii* (perfect form: *Kloeckera apiculata* var. *apis*), and *Hanseniaspora occidentalis* (perfect form: *Kloeckera javanica*)
(c) Yeast species with somewhat increased fermentation activities: *Kluyveromyces marxianus*, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, and *Zygosaccharomyces bailii* (Jolly et al. 2014)

In order to realize certain desirable aromas in the wine, it may be sufficient to start the fermentation by adding individual wild yeast species and then to continue the fermentation with a starter culture of the yeast *Saccharomyces cerevisiae* in order to complete the fermentation. For example, supplier companies of the wine industry such as Hansen, Begerow, Lallemand, and Erbslöh already offer wild yeast cultures and also yeast mixtures. Thus Oenoferm wild and pure is a *Torulaspora delbrueckii* strain. Cultures may contain in addition to *Saccharomyces cerevisiae* wild yeasts (20–40%), for example *Kluyveromyces thermotolerans* or *Torulaspora delbrueckii*. Mixed cultures are offered by the company Hansen consisting of *Lachancea* (*Kluyveromyces*) *thermotolerans* (20%), *Torulaspora delbrueckii* (20%), and *Saccharomyces cerevisiae* (60%). The starter cultures Sihaferm PireNature of the company Begerow or Level 2 TD of the company Lallemand consist of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae*. At the beginning, the must is inoculated with the wild yeast, and *Saccharomyces cerevisiae* is added after the beginning of the fermentation. Viniflora® PRELUDE™ of Hansen is a *Torulaspora* starter culture, which is added to the must at concentrations of 20 g hl^{-1}, which is then kept at 7–10 °C for 4–7 days. When the ethanol concentration reaches 4–6 vol%, a *Saccharomyces* strain is added in the same amount after transfer of the fermenting must to a fermentation tank, and the temperature is increased. A biological acid decomposition with the lactic acid bacterium *Oenococcus oeni* can then be carried out as required.

6.3.2 Fermentation with Hybrid Yeasts

The growth of wine-related microorganisms occurs rather successively during spontaneous fermentation. At the beginning of the fermentation, the so-called wild yeasts (non-*Saccharomycetes*) are active. In the harvest years 2011 and 2012, we were able to follow the succession of the microorganisms in the course of the spontaneous fermentation of Riesling must in the winery Heymann-Löwenstein (lower Moselle, Germany). The succession of yeasts and bacteria was investigated (Christ et al. 2015). The wild yeasts in a wine cask without fermentation problems belonged to the genera/species *Candida pararugosa*, *Saccharomycetes* sp./*Pichia membranifaciens*, *Saccharomycopsis crateagensis*, *Candida boidinii*, *Saccharomycetes* sp., *Aureobasidium* sp., *Metschnikowia* sp.,

Metschnikowia chrysoperlae, *Cryptococcus flavescens*, *Candida zemplinina*, *Pichia kluyveri*, and *Hanseniaspora uvarum*. It was also observed that in some barrels, wild yeast species did survive at elevated levels of ethanol. For example, living cells of *Candida boidinii* were detected until the fermentation was complete. The genus *Saccharomyces* contains nine species (Blättel et al. 2013). The most interesting observation was that the fermentation was not started by the classical wine yeast *Saccharomyces cerevisiae* but rather by *Saccharomyces bayanus*. About 4 weeks after an observed stuck fermentation, the alcoholic fermentation was completed by the triple hybrid *Saccharomyces cerevisiae* × *Saccharomyces kudriavzevii* × *Saccharomyces bayanus*. This hybrid possessed genome sequences of the three mentioned *Saccharomyces* species. The triple hybrid yeast strain HL 78 was not added to the must but grew during the fermentation in the must. Therefore, strain HL 78 must have been present in low cell numbers after fermentation started. The importance of a triple hybrid yeast for the elimination of fermentation disorders in spontaneous fermentations has not yet been described. *Saccharomyces cerevisiae* could not grow in the investigated must because the cellar temperature in wine cellar was between 12 and 14 °C and the temperature in the wine cask reached only about 16 °C. Furthermore, the available ammonium nitrogen decreased in a short time after starting the fermentation from 120 mg to 40 mg l^{-1}. Depending on the yeast strain of *Saccharomyces cerevisiae*, 140–880 mg l^{-1} is required for optimal growth. The sugar uptake activity also decreases at low ammonium concentrations (Dittrich and Großmann 2005). *Saccharomyces bayanus* has the advantage compared to the classical wine yeast *Saccharomyces cerevisiae* that it grows better at low temperatures and low available ammonium concentrations. *Saccharomyces bayanus* and especially the triple hybrid strain can still cover their nitrogen requirements from amino acids or probably proteins, even if these occur only at relatively low concentrations and even in the absence of available free ammonium. We detected increased protease activities in the triple hybrid strain HL 78 by quantitative proteomics (Szopinska et al. 2016). In contrast to the usual starter culture *S. cerevisiae*, both isolates, *S. bayanus* strain HL 77 and the triple hybrid strain HL 78, could grow in the absence of ammonium when amino acids were present. However, the triple hybrid was able to consume glucose and especially fructose at lower amino acid concentrations. The fructophilic character of the triple hybrid strain correlated with an enhanced uptake of radiolabeled fructose compared to glucose (Zuchowska et al. 2015).

González et al. (2006) described the hybrid *S. cerevisiae* × *S. kudriavzevii* which could be advantageous for specific fermentation conditions due to the combined characteristics of both parents. The hybrid was described to be tolerant against high ethanol concentrations and osmolarity as exhibited by *S. cerevisiae* and also tolerant against cool temperatures as a feature of *S. kudriavzevii*. Several authors have isolated hybrids in Europe (Bradbury et al. 2006; González et al. 2008; Lopes et al. 2010), although the strains of *S. kudriavzevii* known so far have been isolated from decaying leaves in Japan (Naumov et al. 2000) and from oak bark samples in Portugal (Sampaio and Gonçalves 2008) but not yet from grapes or must.

6.4 Conclusions

Since the discovery of the winemaking, for a few thousand years, must has been fermented spontaneously without the knowledge of the microorganisms involved and their specific activities. Thus, of course, improvements in wine production were possible only on the basis of empirical findings. In the middle of the last century, spontaneous fermentations were largely replaced by the use of starter cultures by many winegrowers and cooperatives. In Central Europe, yeast cultures of *Saccharomyces cerevisiae* have been regularly used in fermentation since the 1980s of the last century.

With regard to the impact on wine quality, considerable progress has been made in wine growing and vinification in recent years due to the available improved scientific and practical knowledge. The use of modern methods allows a much defined control of the fermentation. The use of molecular biology identification methods and the analysis of nucleic acids have shown that different yeast strains occur in the different wine-growing regions which enable the use of region-specific starter cultures after isolation of pure cultures (Hirschhäuser et al. 2005; Sebastian et al. 2011; Röder et al. 2007; Petri et al. 2013). A started spontaneous fermentation and subsequently occurring fermentation problems can thus be remedied by terroir-specific yeast strains and passed on without having to accept a major change in the desired flavor profile. In addition a partial imitation of spontaneous fermentation is now possible by the use of wild yeasts.

On the basis of the known sequences of the total genome of several yeasts, strategies for the targeted genetic modification of yeasts can in principle be worked out or have already been described (Pretorius 2000; Chambers and Pretorius 2010) in order to produce yeast strains with certain desired properties. Genetically modified yeasts are not authorized for winemaking in Europe or Australia. The application of two genetically modified yeast strains (ML01 and 533EC) is generally recognized as safe (GRAS) in the USA. A further possibility to improve starter cultures of yeast strains for certain selected features is the evolutionary in vitro adaptation or the production of hybrids (König et al. 2013, König and Christ 2015). Oenoferm® X-treme (Erbslöh) is a GMO-free hybrid yeast, obtained from protoplast fusion of two different *Saccharomyces cerevisiae* strains. During the application of the "evolutionary in vitro adaptation," the culture conditions are changed slowly during several months. Thus, we were able to obtain a fructophilic yeast from a normal *Saccharomyces cerevisiae* isolate by slowly shifting the glucose/fructose ratio, which is about one to one in the fresh must, toward fructose.

Challenges to a future successful and innovative generation of winemakers were characterized by Jolly et al. (2014) as follows. The art of winemaking can be compared with an orchestra. The maestro is the winemaker who directs the orchestra. The different instrumentalists are represented by the different wine-relevant microorganisms such as wild yeasts and wine yeasts as well as bacterial species. The composition is then the finished wine. The primadonna assoluta is still the classic wine yeast *Saccharomyces cerevisiae*. However, as we have seen above, primadonnae altrae such as *Saccharomyces bayanus* or especially hybrid yeasts are

competing. Whereas the use of genetically modified yeasts is not permitted with two exceptions, the use of hybrids with different characteristics does not impede legal regulations. The importance of a triple hybrid yeast for the elimination of fermentation disorders in spontaneous fermentations has been described (Christ et al. 2015). Thus, the triple hybrid strain HL 78 is a suitable tool to overcome stuck fermentation without changing the fermentation conditions and the aroma profile desired by the selected winery. It has already been successfully used to restart stuck fermentation.

The art of winemaking of the future may consist in the simultaneous or sequential use of different strains of *Saccharomyces cerevisiae* as in the past, but also mixtures of different species of *Saccharomycetes* and non-*Saccharomycetes* will be applied. This will create different wine styles. If the fermentation is carried out with as few microbial species, this can be compared with a chamber orchestra (Jolly et al. 2014). The winemaker thus decides whether he makes his wine with a multispecies approach (symphonic orchestra), only with a few musicians (chamber orchestra) or even exclusively with a solist (*Saccharomyces cerevisiae*). The future belongs to the well-trained creative winemakers, who can read the musical notes of the time and stimulate the microbe orchestra to new sounds.

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Chapter 7
Secondary Metabolites of Fungal Vine Pathogens

Jochen Fischer and Eckhard Thines

7.1 Introduction

Fungi are highly diverse organisms in terms of their lifestyle and habitats. This high diversity is reflected by the structural diversity and the large number of metabolites produced. The so-called secondary metabolites have been described as compounds not required for either the vitality of an organism or primary metabolic processes (Karlovsky 2008). Such compounds have in the past been of enormous interest due to their potential as lead structures for human therapeutics and plant protectants. More than 50% of all the human therapeutics on the market today are either natural products or are structurally related to secondary metabolites of plant or microbial origin. Recent assumptions are indicating that more than half of 1500 compounds isolated between 1993 and 2001 had antimicrobial or antitumor activity (Keller et al. 2005). Even though many of these secondary metabolites were found to have beneficial biological activities, mycotoxins are deleterious due to their impact within the food chain. In many cases, bioactive low-molecular-weight secondary metabolites were found to be produced as structurally closely related compounds in distinct parts of the life cycle of the producing organism. The biosynthesis of these compounds is often correlated with a specific stage of morphological differentiation

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(Martín et al. 2014). Furthermore a restricted taxonomic distribution of such metabolites has inspired chemotaxonomy. Nevertheless investigations into natural product research concerning the biosynthetic potential of producing organisms are often hampered by the fact that production of these metabolites is sensitive to culture conditions (Weinberg 1983). Therefore, the metabolites produced in axenic culture do not match the spectrum of natural products produced within the environment.

A large number of environmental stimuli and abiotic stress factors have been found to induce the biosynthesis of secondary metabolites, such as draft, heat stress, light, availability of nutrients, and oxidative stress. Exposure to such factors, specifically, depletion of nutrients, is transduced via conserved signaling pathways to result in the regulation of secondary metabolite production (Netzker et al. 2015; Brakhage 2015; Viaud et al. 2016). Furthermore, oxidative stress has been found to be associated with the regulation of secondary metabolite biosynthesis in plant/fungus interactions (Pusztahelyi et al. 2015).

Even though fungal secondary metabolites are of large diversity, all structures are based on simple precursors of the intermediary metabolism, such as acetyl coenzyme A resulting in polyketides, polyols, terpenoids, steroids, or carotenoids. Further initial building blocks are shikimate, amino acids, and glucose. While shikimate is the precursor of aromatic compounds, amino acids are constituents of peptides or alkaloids. In contrast, glucose is required for the biosynthesis of glycosides and aminoglycosides (Zahner et al. 1983). Secondary metabolism was proposed to be a playground on which biochemical evolution is taking place continuously. Starting from given precursors from intermediary metabolism, the evolution of biochemical pathways can develop in all directions, provided that the metabolites are not toxic to the producing organism (Zahner et al. 1983). Within the environment the compounds of this playground may furthermore be of advantage in terms of competition or the exploitation of novel habitats. However, many compounds have been identified due to their biological activity toward other organisms resulting in a selection advantage. For example, in the competition for nutrients, secretion of compounds with antimicrobial activity appears to be of advantage for the producing organism since putative rivals are eliminated. In contrast no ecological function has been found for many microbial secondary metabolites. It appears likely that many of these compounds are of use for the producing organism in the environment, e.g., as constituents of chemical communication in microorganisms or plant-microbe interactions.

Phytopathogenic fungi are basically classified as necrotrophs, biotrophs, or hemibiotrophs depending on their lifestyle and the way they infect and colonize the host plant. They constitute one of the main infectious agents in plants, causing alterations during in metabolic processes or developmental stages. Since they exploit the host metabolism or secrete toxins killing the plant or at least parts of it, these fungi cause huge economic losses (Pusztahelyi et al. 2015). In necrotrophic fungi, plants or parts of it are killed prior to the successful colonization. Therefore, extracellular enzymes or toxins are secreted, or the metabolism of the host cell is reprogrammed. The toxins secreted can be classified into host-specific/host-selective toxins or non-host-specific/non-host-selective toxins depending on the

susceptibility of others than the host plants to the toxin. Most of the host-specific toxins identified to date were produced by *Alternaria* or *Cochliobolus* species (Condon et al. 2013; Akimitsu et al. 2014). Even though the production of host-specific toxins is limited to few taxa, the chemical diversity of the compounds produced is high, ranging from low-molecular-weight secondary metabolites to cyclic peptides (Pusztahelyi et al. 2015). In general, the production of host-specific toxins is believed to be crucial for virulence of producing fungi (Walton 1996; Horbach et al. 2011). In contrast, non-host-specific toxins do not appear to be essential for pathogenicity although they may contribute to pathogenicity. They have a broader range of susceptible plants, and symptom formation can often be observed on plant species not affected by the pathogenic fungus (Walton 1996). Biotrophic fungi are depending on living plant tissue which is exploited by specialized structures (haustoria) required for nutrient uptake, suppression of the host metabolism, and reprogramming of the host cell (Perfect and Green 2001). In contrast, Condon et al. (2013) suggested that promotion of disease by the secretion of, e.g., host-specific toxins and protein effectors overlaps in necrotrophs and hemibiotrophs.

However, no host-specific toxins have to date been described for fungal pathogens colonizing vines (Bruno and Sparapano 2006a, b; Andolfi et al. 2006, 2011). Whether secondary metabolites produced by necrotrophic or hemibiotrophic fungi contribute to virulence or pathogenicity has for many compounds not been addressed to a large extent. This chapter summarizes phytotoxic secondary metabolites identified from vine pathogenic fungi.

7.2 Secondary Metabolites from Fungi as Causal Agents for Grapevine Diseases

7.2.1 Grapevine Trunk Diseases

Due to the increasing disease incidence within the last decade, grapevine trunk diseases have become a major challenge for modern viticulture. These diseases result in significant damage to long-lasting plant structures and thereby to the loss of entire vines (Valtaud et al. 2009). Especially esca, *Eutypa*, and *Botryosphaeria* diebacks were found to be the major threats. Since efficient disease management strategies are lacking, these diseases have been within the focus of research approaches in order to elucidate the molecular basis of plant/pathogen interactions. For this purpose, secondary metabolites produced by fungi associated with these diseases have been intensively studied.

7.2.2 *Phytotoxic Secondary Metabolites Produced by Esca-Associated Fungi*

Esca is a devastating disease affecting grapevines all around the world induced by a complex of xylem-inhabiting fungi (Gómez et al. 2016). Since the disease symptoms are visible on the leaves, whereas the fungal pathogens are colonizing the trunk, it was suggested that secreted phytotoxic metabolites are of importance for disease development. Among the most abundant fungal species associated with this destructive disease are *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, and *Fomitiporia mediterranea*. The fungal strain *Phaeoacremonium aleophilum* was recently renamed as *Phaeoacremonium minimum* and with therefore be referred as such (Gramaje et al. 2015).

Phaeomoniella chlamydospora and *Phaeoacremonium minimum* (*Togninia minima*). *Phaeomoniella chlamydospora* is a mitosporic fungus growing yeast-like in culture and forming prominently darkened conidiophores in the basal part and subhyaline and straight conidia. Several secondary metabolites have been identified from cultures of the fungus. Phytotoxic activity was mainly described for the secondary metabolites scytalone (Fig. 7.1), isosclerone (Fig. 7.2), and pullulan (Fig. 7.3), a polysaccharide polymer of maltotriose units (Andolfi et al. 2011; Bertsch et al. 2013). The compounds were found to be produced in axenic culture by *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora* and have been extensively studied concerning the biological activity (Graniti et al. 2006). The pentaketides scytalone and isosclerone are shunt products of the dihydroxynaphthalene (DHN)-melanin biosynthesis pathway in fungi. Both compounds induced dramatic lesion formation/disease symptom formation in detached leaves, whereas their presence in infected tissue or disease symptoms showing parts of the plant remains to be demonstrated. In contrast, several other intermediates/shunt products of the DHN-melanin biosynthesis pathway, e.g., flavioline (Fig. 7.4), 2-hydroxyjuglone, and 1,3,8-trihydroxynaphthalene (Fig. 7.5), have been identified from cultures of *Phaeoacremonium minimum*, but none of the compounds induced disease symptom formation in pathogenicity assays (Abou-Mansour et al. 2004).

Furthermore, investigations into the mechanisms of both fungi revealed phytotoxic polypeptides secreted into the culture medium. Both polypeptide fractions with molecular masses ranging from 6 to 250 kDa triggered the death of grapevine cells in culture (Luini et al. 2010).

From submerged cultures of *Phaeoacremonium minimum*, phaeofuran A (Fig. 7.6) was isolated. The compound was exclusively found in the submerged culture of the fungus under laboratory conditions. Apart from the phytotoxic activity, the compound was found to have strong antimicrobial activity (Fischer et al., unpublished; Hill and Sutherland 2006).

In addition to phytotoxins, many phytopathogenic fungi secrete enzymes that degrade macromolecules of the host plant tissues. Valtaud et al. showed that *Phaeoacremonium minimum* possessed all of the extracellular enzyme activities

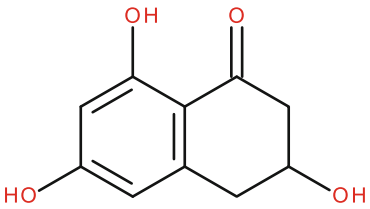


Fig. 7.1 Scytalone

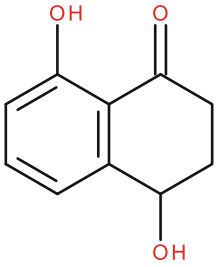


Fig. 7.2 Isosclerone

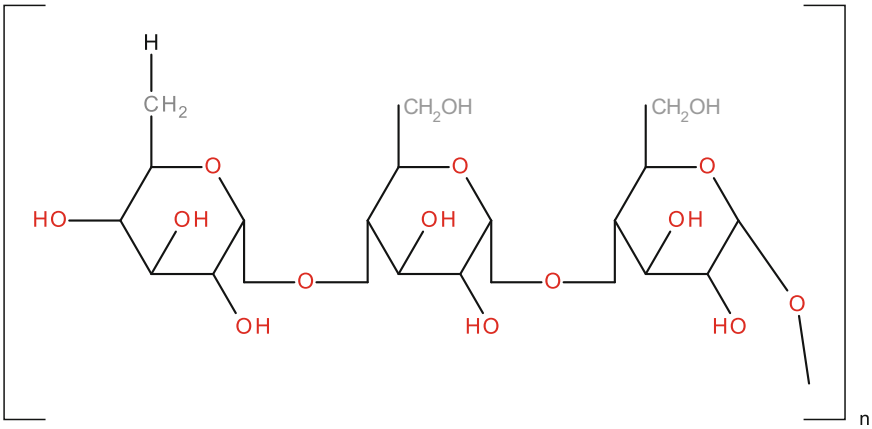


Fig. 7.3 Pullulan

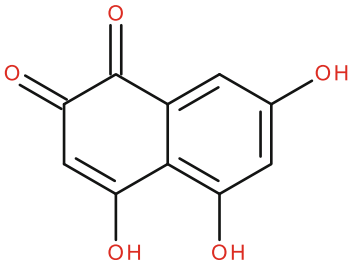


Fig. 7.4 Flavioline

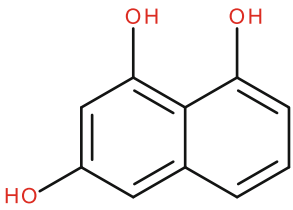


Fig. 7.5 1,3,8-Trihydroxynaphthalene

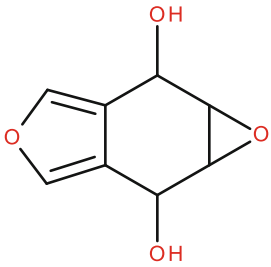


Fig. 7.6 Phaeofuran A

implicated in the degradation of polysaccharides, such as xylanase, *exo*- and *endo*-b-1,4-glucanase, and b-glucosidase. Additionally, lignin-degrading enzymatic activities were found in cultures of *Phaeoacremonium minimum*. In contrast, *Phaeomoniella chlamydospora* showed none of these enzyme activities (Bruno and Sparapano 2006b).

Fomitiporia mediterranea was described by Fischer (2002) as a new wood-decaying basidiomycete species associated with esca of grapevine in European wine-growing countries. Characters of the fruit body are essentially identical with those of the closely related species, *Fomitiporia punctata*. *Fomitiporia mediterranea* occurs not only on *Vitis vinifera* but also on a number of other hardwood genera, whereas it appears to be restricted to *Vitis vinifera* elsewhere in Europe.

From axenic cultures of *Fomitiporia mediterranea,* the secondary metabolites frustulosin (3,6-dihydroxy-2-(3-methyl-3-buten-1-in-1-yl)benzaldehyde; Fig. 7.7), 4-hydroxybenzaldehyde (Fig. 7.8), dihydroactinolide (Fig. 7.9), and 6-formyl-2,2-methyl-4-chromanone (Fig. 7.10) have been identified. However, none of the compounds have been linked directly to phytotoxicity or virulence in this esca-associated organism (Abou-Mansour et al. 2010; Andolfi et al. 2011).

Apart from secondary metabolites as putative determinants of virulence/pathogenicity, Bruno and Sparapano (2006a) identified a laccase in cultures of *Fomitiporia mediterranea*. Laccase is a polyphenol oxidase catalyzing the initial step in the mineralization of lignin. It is furthermore suggested that laccase produced by this white-rot fungus is of importance for the detoxification of ROS.

Botryosphaeria sp. (Diplodia seriata/Dothiorella viticola) Black dead arm (BDA) disease on vine is caused by the filamentous ascomycete *Botryosphaeria obtusa*. This fungus has a broad host range causing leaf spot, cankers, and black rot on many plant species. It colonizes the wood of the plant causing decline and eventually death. Since the BDA symptoms resemble those of esca, the diseases are easily confused. The frequency of disease symptom occurrence has increased

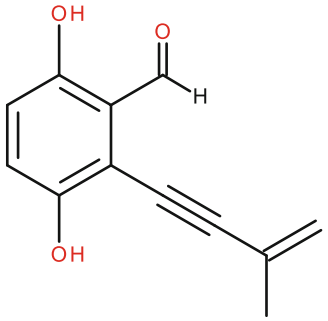


Fig. 7.7 Frustulosin (3,6-dihydroxy-2-(3-methyl-3-buten-1-in-1-yl)benzaldehyde)

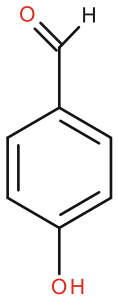


Fig. 7.8 4-Hydroxybenzaldehyde

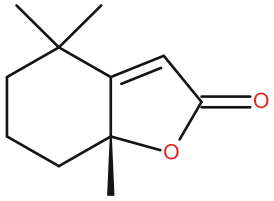


Fig. 7.9 Dihydroactinolide

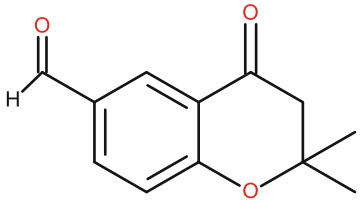


Fig. 7.10 6-Formyl-2,2-methyl-4-chromanone

considerably over the past decade since efficient treatment strategies are currently not available.

In order to identify phytotoxic secondary metabolites secreted by *Botryosphaeria obtusa*, bioassay-guided fractionation of culture filtrates led to the isolation of four dihydroisocoumarins: mellein (Fig. 7.11), 4-hydroxymellein, 2,7-dihydroxymellein, and the new 4,7-dihydroxymellein (Fig. 7.12). LC-UV-DAD-MS analysis of vine wood infected by *Botryosphaeria obtusa* revealed the

Fig. 7.11 Mellein

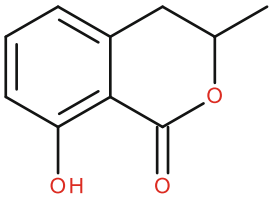
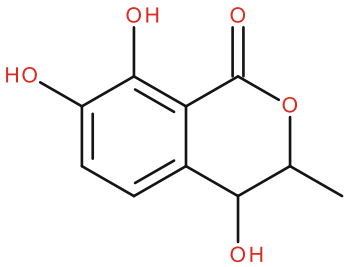


Fig. 7.12 4,7-Dihydroxymellein



presence of mellein *in planta*. Interestingly, the fungus that was found was also able to oxidize the phytoalexin δ-resveratrol into the dimer Δ-viniferin (Djoukeng et al. 2009; Bénard-Gellon et al. 2015).

From cultures of *Neofusicoccum parvum*, a fungus associated with *Botryosphaeria* dieback, Abou-Mansour et al. (2015) isolated a wide range of phytotoxic metabolites. The compounds isolated can be classified into four chemical families. Five metabolites, namely, (-)-terremutin (Fig. 7.13), (+)-terremutin hydrate, (+)-epi-sphaeropsidone (3)(-)-4-chloro-terremutin hydrate, and (+)-4-hydroxy-succinate-terremutin hydrate, belong to the family of dihydrotoluquinones. The structural class of epoxylactones is represented by two metabolites, namely, asperlin (Fig. 7.14) and dia-asperlin. (*R*)-(-)-mellein, (3*R*,4*R*)-4-hydroxymellein, (3*R*,4*S*)-4-hydroxymellein, and (*R*)(-)-3-hydroxymellein belong to the family of dihydroisocoumarins, while 6-methylsalicylic acid (Fig. 7.15) and 2-hydroxypropyl salicylic acid are hydroxybenzoic acids. The phytotoxic activity of the isolated metabolites was assessed via leaf disc assays and the expression of defense-related genes in *Vitis vinifera* cells cv. Chardonnay. Observations concerning the brown stripes of grapevine wood from plants showing *Botryosphaeria* dieback symptoms revealed phytotoxic activity for some of the isolated natural compounds. 3-hydroxymellein was found to be the most active compound, whereas the metabolites epi-sphaeropsidone (Fig. 7.16), terremutin, mellein, and dia-asperlin induced lesion formation/necrosis at higher concentrations (Phillips et al. 2007).

Eutypa lata (Eutypiose) Within vineyards throughout the world, Eutypiosis is an economically significant plant disease. The disease is caused by *Eutypa lata* (synonym: *Eutypa armeniacae*) an ascomycete infecting fresh pruning wounds upon suitable humidity/moisture on the vine, e.g., after rain. The fungus has a broad host specificity infecting and colonizing other hosts such as cherry trees, further *Prunus* species, as well as apples, pears, and walnuts. In France, several

Fig. 7.13 Terremutin

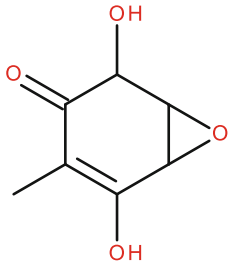


Fig. 7.14 Asperlin

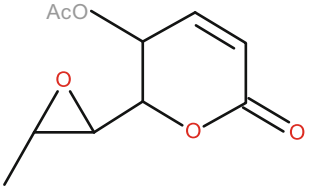


Fig. 7.15 6-Methylsalicylic acid

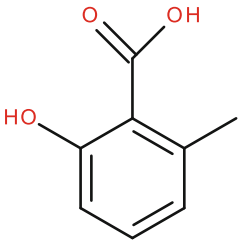
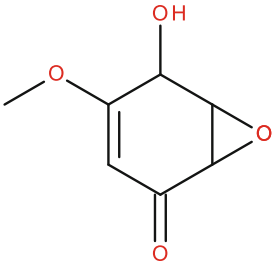
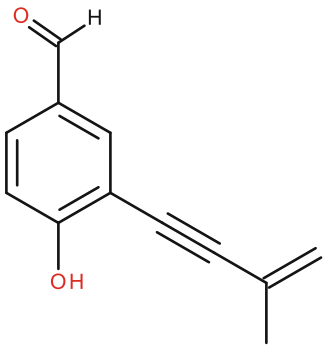


Fig. 7.16 Epi-sphaeropsidone



quality *Vitis vinifera* cultivars such as Cabernet Sauvignon were found to be very susceptible to this disease. The parasitic fungus colonizes the trunk and arms of old grapevines causing in particular foliar lesions. Dying arm disease symptoms are clearly visible in the vineyard at the beginning of summer. The development of leaves is impaired since yellowing and small necrotic patches appear upon unfolding. During disease development ultrastructural alterations of the leaf cells of the grapevine were observed by electron microscopy such as cytoplasmic lysis with plasma membrane detachment and complete chloroplast disorganization (Deswarte et al. 1994; Fallot et al. 1997).

Fig. 7.17 Eutypine



Several metabolites and derivatives of *Eutypa lata* were identified from axenic cultures of the fungus. Of the compounds identified, eutypine (Fig. 7.17) was found to be the metabolite with the most significant phytotoxic activity in leaf assays. Further natural products identified from cultures of the fungus are eutypinol (Fig. 7.18), *O*-methyleutypine, *O*-methyleutypinol, a eutypine carboxylic acid analogue, 3-(3,4-dihydroxy-3-methyl-1-butynyl)-4-hydroxybenzaldehyde, 2-(3,4-dihydroxy-3-methyl-1-butynyl)-4-hydroxymethyl-phenol, 3-(3,4-dihydroxy-3-methyl-1-butynyl)-4-hydroxybenzoic acid (Fig. 7.19), 2-iso-propenyl-5-formyl-benzofuran, siccayne (Fig. 7.20), eulatinol, and eulatachromene and its derivatives (Andolfi et al. 2011; Jiménez-Teja et al. 2006). Concerning their biosynthesis eutypine-like compounds appear to be of special interest since the characteristic triene moiety requires specific desaturases.

Phomopsis viticola The causal agent of *Phomopsis* dieback, *Phomopsis viticola* can infect all green parts of the grapevine, and its symptoms are present in all herbaceous organs, e.g., shoots, basal wood, leaves, stems, or fruits. Disease severity in *Phomopsis* cane and leaf spot is more significant in grape-growing regions characterized by a humid temperate climate through the growing season, and crop losses up to 30% have been reported. On young shoots the disease becomes visible in first internodes by the presence of small black spots developing into well-individualized blackish-brown crusts or brown lesions with strips. During the dormant season, canes show a white appearance with black points at internode zones. Blackish necrotic spots may also be encountered along the main and secondary veins as well as the petioles. Some leaf portions can also turn to yellow, pale green, and/or brown color. Severely infected leaves or leaves with heavily infected petioles may fall, whereas the fruits turn brown and wither, with mummies or shriveled berries close to harvest. Other associated fungi, like *Phomopsis theicola*, cause symptoms characterized by mortality of great parts of young plants. In the wood, particular sectoral necrosis and some punctuations of brown color are usually observed (Fontaine et al. 2016).

Phomopsis spp. strains have in the past intensively been studied concerning their secondary metabolite spectrum produced in axenic cultures. Five metabolites; four furanones, and cytosporone F, together with phomopsolide B (Fig. 7.21), two

Fig. 7.18 Eutypinol

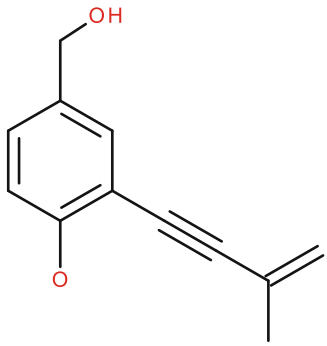


Fig. 7.19 3-(3,4-Dihydroxy-3-methyl-1-butynyl)-4-hydroxybenzoic acid

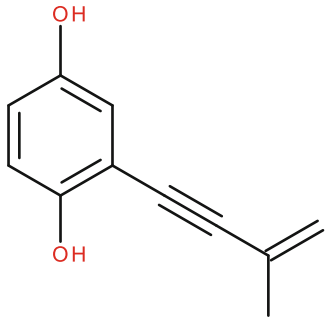
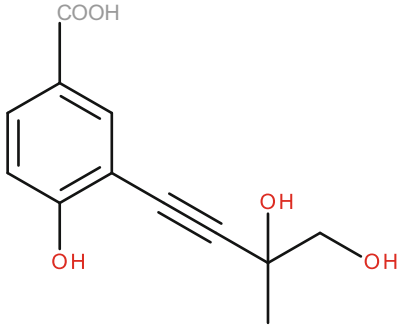
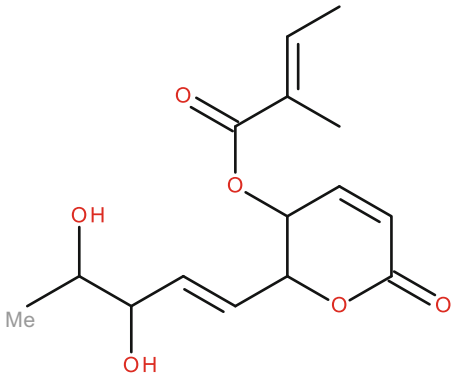


Fig. 7.20 Siccayne



xanthones, and three compounds previously described, were isolated from *Phomopsis viticola* strains (Corsaro et al. 1998). Biological assays on *Vitis vinifera* leaves and grape callus were assessed, and the antibacterial activity of the new isolated compounds was monitored. Phomopsolide B was found to be the most active natural product on vine leaves and in a callus assay. However, the compound is apparently degraded in grape leaves, and detoxification products were identified. Since the compounds were found *in planta*, it may be assumed that they are virulence factors. For the detection of the pathogen within plants, an LC-(API)-MS method was developed and used to identify metabolites when *Phomopsis*

Fig. 7.21 Phomopsolide B



sp. was grown on pruned grapevine (Abou-Mansour et al. 2007; Goddard et al. 2014).

Fusarium oxysporum Grapevine cuttings are often described as gateways for infections with soil or airborne pathogens, such as *Cylindrocarpon* spp., *Fusarium oxysporum*, *Phytophthora* spp., and *Rhizoctonia solani*. Significant damage has also been linked to the fungal species *Phomopsis viticola* and *Phaeoacremonium* sp. (Cruz et al. 2014). Infection of cuttings by these pathogenic organisms in nurseries may lead to the death of the plant.

F. oxysporum and *F. proliferatum* strains cultured in vitro produced beauvericin and fusaproliferin (Fig. 7.22). Apart from these natural products, Mikušová et al. (2013) identified and isolated further metabolites from these cultures, e.g., avenacein Y, apicidin, aurofusarin (Fig. 7.23), chlamydosporol, 2-amino-14, 16-dimethyloctadecan-3-ol, enniatin A (Fig. 7.24), enniatin A1, enniatin B2, enniatin B3, and equisetin. The cyclohexadepsipeptide mycotoxin was previously described as phytotoxic on potatoes. Whether the enniatins are phytotoxic to grapevines remains to be elucidated. The structural variants enniatins H and I were found to be phytotoxic on potato plants (Song et al. 2008).

Wounds caused by feeding of grape phylloxera on grape roots can become infected by a variety of fungi. For example, *Fusarium roseum*, *Fusarium oxysporum*, and *Pythium ultimum* are important in *Vitis vinifera cv*. Chardonnay wounds, whereas the moderately tolerant rootstock AXR#1 appears to be more susceptible to *Fusarium oxysporum* and *Cephalosporium* sp. (Waskiewicz et al. 2010).

Botrytis cinerea (gray mold, Grauschimmelfäule) The two major phytotoxins produced during the infection process by the gray mold fungus *Botrytis cinerea* are the sesquiterpene botrydial (Fig. 7.25) and the polyketide botcinic acid (Fig. 7.26). Both compounds were described as non-host-specific toxins. Botrydial is a phytotoxic sesquiterpene metabolite, for which the biosynthesis gene cluster has been characterized previously, and is secreted by the fungus. It was found that botrydial induces the HR on its hosts modulated by host signaling pathways

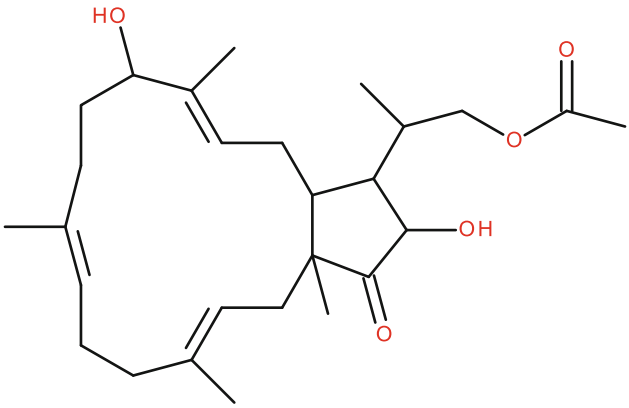


Fig. 7.22 Fusaproliferin

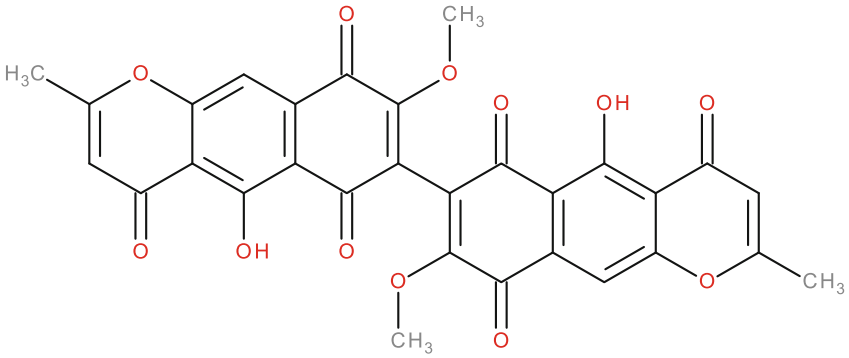


Fig. 7.23 Aurofusarin

mediated by salicylic acid and jasmonic acid. Further, the related compounds dihydrobotrydial (Fig. 7.27) and botrydienal (Fig. 7.28) were found to be produced in cultures of the fungus. In fungus-free culture filtrates, dihydrobotrydial was found to be the constituent giving phytotoxic effects on host plants (Rebordinos et al. 1996; Dalmais et al. 2011; Rossi et al. 2011).

Whereas cultivation of the fungus and isolation of secondary metabolites have led to the identification of about eight secondary metabolites, genome sequencing of *Botrytis cinerea* revealed a repertoire for approximately 40 natural products. It was suggested that the expression of gene cluster and biosynthesis of the secondary metabolites are regulated by biotic and abiotic environmental factors the fungus is exposed to. However, the modulation of secondary metabolism includes apparently a complex regulatory network. A strong correlation between the regulation of secondary metabolism and light-dependent development was demonstrated for *Botrytis cinerea* (Colmenares et al. 2002; Viaud et al. 2003; Gioti et al. 2006).

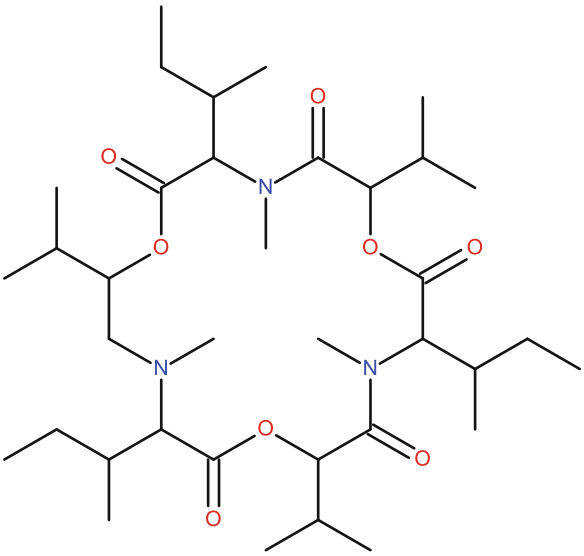


Fig. 7.24 Enniatin A

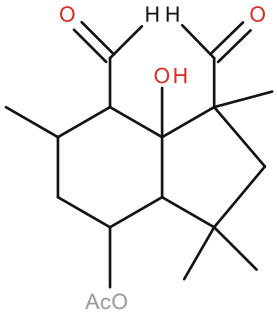


Fig. 7.25 Botrydial

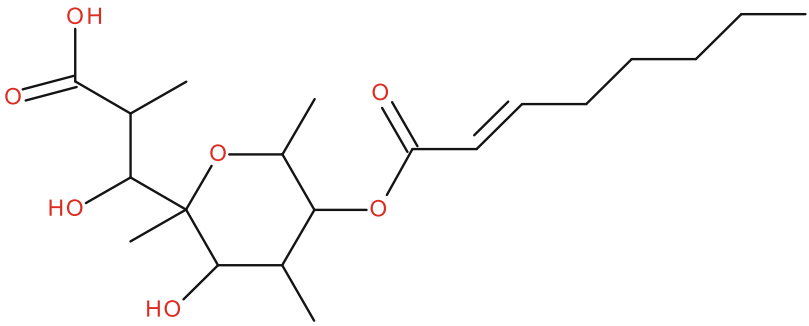


Fig. 7.26 Botcinic acid

Fig. 7.27 Dihydrobotrydial

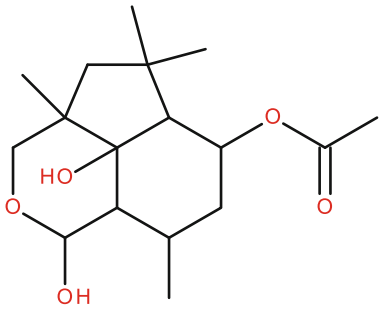
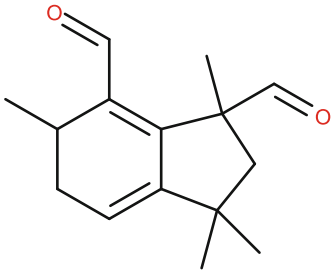


Fig. 7.28 Botrydienal



Botrydial is produced during plant infection (Deighton et al. 2001) and induces chlorosis and cell collapse, which seems to facilitate both penetration and colonization. Botrydial biosynthetic pathway genes are organized into a physical cluster, coregulated and overexpressed *in planta* as shown by macro-array studies (Choquer et al. 2007).

Plasmopara viticola (Peronospora, downy mildew, Falscher Mehltau) and Uncinula necator (Erysiphe necator, powdery mildew, Echter Mehltau) In 2016 powdery and downy mildew caused severe yield losses in German vineyards. The devastating diseases are caused by the phytopathogens *Plasmopara viticola* and *Uncinula necator* (Bettiga et al. 2013). Phytotoxic secondary metabolites from these organisms have not been published to date. It was described that due to the biotrophic lifestyle of *Plasmopara*, its genome is significantly reduced and lacks a wide range of enzymes linked to secondary metabolism (Diez-Navajas et al. 2008). For *Uncinula necator*, several volatiles have been identified from diseased grapes by GC-MS analysis. Whether these compounds, e.g., 1-octen-3-one (mushroom odor) and (*Z*)-1,5-octadien-3-one (geranium-leaf odor), are phytotoxic to the plant has not been assessed (Darriet et al. 2002; Spanu et al. 2010).

Pythium ultimum One important and ubiquitous plant pathogen taxon is the genus *Pythium*, an oomycete, fungal-like organism, which causes a variety of diseases including seed rots and damping-off; root, stem, and fruit rots; foliar blights; and postharvest decay. In South Africa *Phytophthora* and *Pythium* species were identified as the most common and widespread soilborne pathogens of grapevines, both in nurseries and established vineyards. In viticulture, changes in management

strategies during the past 30 years that were aimed at increasing yields and quality of grapes may have altered the incidence and species composition of *Pythium* and *Phytophthora* populations. Some evidence of this has been reported by Halleen and the diagnostic clinic of the Agricultural Research Council (ARC) at Stellenbosch in South Africa, where the frequency of isolation of *Pythium* species has increased steadily (Spies et al. 2011; Granett et al. 2015).

Rey et al. (2001) observed symptoms on tomato roots, characteristic for the activity of toxic compound(s) on host cells due to a *Pythium* sp. infection. Chemical analysis of the *Pythium* sp. filtrates demonstrated that indole-3-acetic acid (Fig. 7.29) and tryptophol (Fig. 7.30) were produced. The fact that *Pythium ultimum* transformed tryptamine and indole-3-acetaldehyde into indole-3-acetic acid and tryptophol confirms the existence of a tryptamine pathway within the fungus. These results support the hypothesis that auxins facilitate *Pythium* sp. infections. On the other hand, toxins and hydrolytic enzymes are likely involved in *P. ultimum* pathogenesis. Ichihara et al. (1985) isolated and characterized the phytotoxin, (3*R*,5 *Z*)-(-)-hydroxy-5-dodecenoic acid (Fig. 7.31), from the culture filtrate of *Pythium ultimum*, a fungal agent of black root disease in sugar beet. It remains to be demonstrated whether this phytotoxin is of importance for virulence in grapevines.

Guignardia bidwellii (black rot, Schwarzfäule) Black rot appears to be one of the most devastating diseases of grapes in North America. The causal agent of the disease occurring worldwide throughout humid viticultural production regions is the fungus *Guignardia bidwellii* (*anamorph: Phyllosticta ampelicida*; Rinaldi et al. 2017). The disease can result in crop losses ranging from 5% to 80%. In viticulture endorsed by integrated pest management programs, the disease is controlled by the application of modern and selective fungicides. However, disease outbreaks are regularly observed in organic viticulture in the areas around the Moselle and Nahe rivers and in the Middle Rhine Valley in Germany. It is believed that climate change, e.g., increased spring temperatures, contributes to the enhanced frequency of occurrence observed in Germany since 2002. The abandoned vineyards at the Moselle river ("Drieschen") are significant reservoirs for the fungus. All commercially important *Vitis vinifera* cultivars and most of the interspecies crosses are reported to be susceptible to the disease (Molitor et al. 2012; Buckel et al. 2013). Recently phenguignardic acid (Fig. 7.32) and guignardic acid (Fig. 7.33) were described as phytotoxic secondary metabolites from submerged cultures of the grape black rot fungus *Guignardia bidwellii*. The compounds phytotoxic activity was not limited to grapevine, whereas the plant appears to be more susceptible to the toxins in comparison to wheat, barley, or rice. These phytotoxic dioxolanones have been suggested to be synthesized from deaminated amino acids as precursors. However, the mechanism of linking the precursors together has not been addressed in detail.

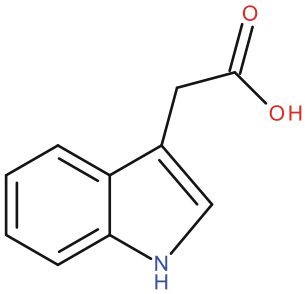


Fig. 7.29 Indole-3-acetic acid

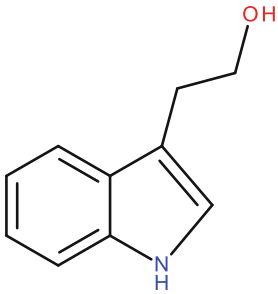


Fig. 7.30 Tryptophol

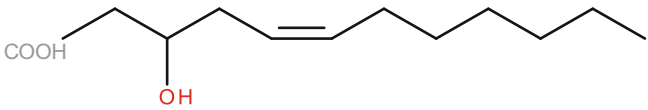


Fig. 7.31 (3*R*,5 *Z*)-(-)-Hydroxy-5-dodecenoic acid

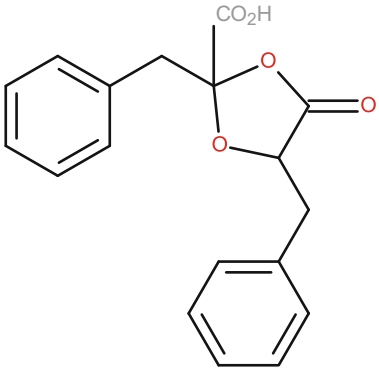
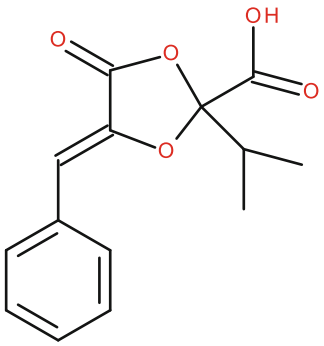


Fig. 7.32 Phenguignardic acid

Fig. 7.33 Guignardic acid



7.3 Concluding Remarks

Many pathogenic fungi associated with grapevine diseases were found to produce phytotoxic secondary metabolites. Genome sequencing of fungal organisms revealed that these strains produce limited numbers of secondary metabolites in axenic culture. The investigations into secondary metabolites being synthesized within plant/pathogen interactions are hampered by the fact that the culture conditions applied in the laboratories do obviously not match natural or environmental conditions. During host colonization secondary metabolite gene clusters might be activated which are silent under laboratory conditions. Considering the enormous potential for secondary metabolites identified by genome sequencing of fungal organisms and subsequent bioinformatic analysis, it becomes apparent that the secondary metabolites known to date from plant pathogenic fungi represent only the "tip of the iceberg". Many further secondary metabolites will be discovered in the future by improvements in analytical methods and, by e.g. epigenetic approaches. This knowledge will not only be of enormous value to further understand the development and virulence of grapevine pathogenic fungi; it will also contribute to investigations into chemical communication between fungi and host plants. A more profound knowledge concerning these molecular plant-microbe interactions will also contribute to the development of modern and selective plant protection strategies.

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Part II
Primary, Secondary and Energy Metabolism

Chapter 8
Carbohydrate Metabolism in Wine Yeasts

Rosaura Rodicio and Jürgen J. Heinisch

8.1 Introduction

The predominant feature in winemaking is the conversion of sugars contained in grape mashes or musts into ethanol, a task almost exclusively fulfilled by unicellular eukaryotes which divide by budding—the yeasts. Whereas several non-*Saccharomyces* yeast species are present in the early stages of fermentation, as outlined in Chaps. 3 and 6, *Saccharomyces cerevisiae* generally outgrows all other yeasts in the process of vinification and determines the principle quality of the end product (Bisson and Karpel 2010). Therefore, starter cultures of the wine yeast *S. cerevisiae* are generally employed in all large-scale wine production plants, as reviewed in Chap. 25. Nevertheless, the primary yeast microflora also contributes to ethanol production and, more importantly, to the aroma composition of the wine. Thus, their traditional perception as "spoilage yeasts" due to the production of acetate and other off-flavors is gradually changing (Jolly et al. 2014).

The enormous capacity of *S. cerevisiae* for alcoholic fermentation has triggered its use by mankind for millenniums, long before the glycolytic pathway and its subsequent reactions were elucidated (reviewed in Barnett 2003). Due to these applications, and driven by a huge arsenal of molecular genetic tools developed since the early 1980s, *S. cerevisiae* has evolved into the best studied eukaryotic organism. In this chapter we will therefore present data obtained from *S. cerevisiae*

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with a focus on the specific applications in wine fermentation. The interested reader is referred to several excellent reviews on yeast carbohydrate metabolism for further details (Fraenkel 1982; Gancedo and Serrano 1989; Broach 2012; Pretorius et al. 2012; Horak 2013; Compagno and Piskur 2014; Dashko et al. 2014).

8.2 Sugars in Wine Fermentations and Regulatory Principles

High sugar concentrations are found in the ripe grape, constituted primarily by equal amounts of glucose and fructose, with some sucrose also present. Despite the fact that these sugars are perfect substrates for all kinds of microorganisms, their access is shielded by the intact berry skin. Yeasts present on the grapes thus only thrive in and around local lesions caused by mechanical force, insects, or filamentous fungi. This drastically changes upon mashing of the grapes, when sugars are liberated at concentrations of approximately 110 g L^{-1} each of glucose and fructose.

In the first phase of must fermentations, or mash fermentations in the case of classical red wine production, starter cultures of *S. cerevisiae* are actively dividing while degrading these sugars. Glucose utilization starts right away and is slightly faster, so that the proportion of fructose increases as fermentation progresses (Fig. 8.1; Berthels et al. 2004). At the molecular level, the preferential degradation of glucose was explained by the features of both hexose transporters and sugar

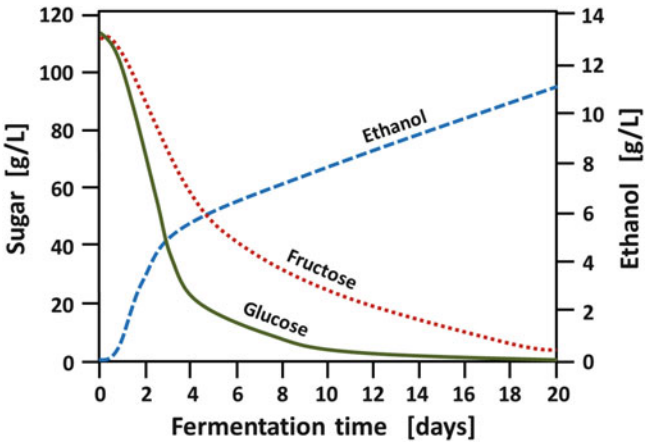

Fig. 8.1 Degradation of glucose and fructose in must. A typical kinetics of sugar degradation and alcohol production in must fermentations is presented. Note that significant differences from these idealized curves may occur depending on individual musts, yeast strains employed, and varying fermentation conditions such as temperature and pH (idealized and adapted from Berthels et al. 2004)

phosphorylating enzymes as detailed in Sect. 8.3. In addition, specific sensor proteins for glucose and fructose contained in the plasma membrane are also involved (Rolland et al. 2001). High fructose to glucose ratios have been suggested as one cause of stuck fermentations. Intentions to employ fructophilic non-*Saccharomyces* yeast species such as *Zygosaccharomyces bailii* and *Candida* species (Mills et al. 2002; Pina et al. 2004) may be contradicted by studies showing that 20 different yeast strains with varying abilities to use fructose as a sole carbon source did not show marked differences in simulations of wine fermentations (Liccioli et al. 2011).

In general, the rate of sugar catabolism decreases continuously in the course of fermentation. This phenomenon has been attributed to ethanol toxicity, declining transport of solutes, and a general lack of nutrients. In particular, ammonium and amino acids are consumed in the first 24–48 h, meaning that the majority of sugar fermentation in the later phases occurs under nitrogen starvation (Brice et al. 2014). Thus, in later stages of fermentations, a gradual increase in ethanol concentrations and a slow depletion of sugars and other nutrients is mediated by metabolism of nongrowing cells (Bauer and Pretorius 2000). Importantly, *S. cerevisiae* ferments sugars to ethanol and carbon dioxide even in the presence of oxygen. Thus, the "Pasteur effect" (a term coined by biochemists in the first half of the last century), commonly understood as the preference of respiration over fermentation under aerobic conditions, does not occur in the very yeast Pasteur was investigating (Lagunas 1981). Instead, *S. cerevisiae* channels most of the substrate into alcoholic fermentation provided sugar concentrations exceed approximately 2 g L^{-1}. Such a fermentative mode of metabolism despite the availability of oxygen is caused by glucose repression by the "Crabtree (or glucose) effect," and wine yeasts can be classified, accordingly (Table 8.1). Fermentative yeast metabolism in spite of oxygen availability probably originated after the divergence of the lineages of *Saccharomyces* and *Kluyveromyces* before the whole genome duplication event and after the loss of respiratory complex I (Hagman et al. 2013). One should bear in mind that such metabolic differences become vain soon after the onset of vigorous

Table 8.1 Physiological categories of wine yeasts

Mode of fermentation	
Crabtree-positive	Crabtree-negative
Saccharomyces cerevisiae	*Hanseniaspora uvarum*
Zygosaccharomyces bailii	*Pichia anomala*
Brettanomyces intermedius	*Candida utilis*
Torulopsis glabrata	*Hansenula neofermentans*
Hanseniaspora guilliermondii	*Kluyveromyces marxianus*[a]
Candida stellata	*Debaryomyces hansenii*
Metschnikowa pulcherrima	*Torulaspora delbrueckii*

[a]Note that several strains of *K. marxianus* are known for their high fermentative capacity and that the classification as Crabtree-negative may be subject to intraspecies variations (Jolly et al. 2014, and references therein).

fermentation, when in fact anaerobic conditions are achieved by the production of carbon dioxide and the shielding of musts from access to molecular oxygen.

8.3 Biochemistry and Physiology of Yeast Alcoholic Fermentation

Utilization of sugars as carbon and energy source first requires their uptake across the plasma membrane and the subsequent activation into a glycolytic intermediate. In this context, glucose-6-phosphate is of central importance and serves as a starting point for the distribution of carbohydrates (Fig. 8.2). Glycolysis was the first biochemical pathway to be elucidated. In fact, the experiments of Eduard Buchner in the late nineteenth century demonstrated the possibility of fermentation in cell-free yeast extracts and thus founded the science of biochemistry (Greek "enzymon" = "in yeast"). From then until the molecular cloning of the encoding genes in the 1980s, considerable detail has been gathered on the chain of reactions and their importance in yeast alcoholic fermentations. The most relevant information is summarized in Fig. 8.2 and Table 8.2, and in the following we will only

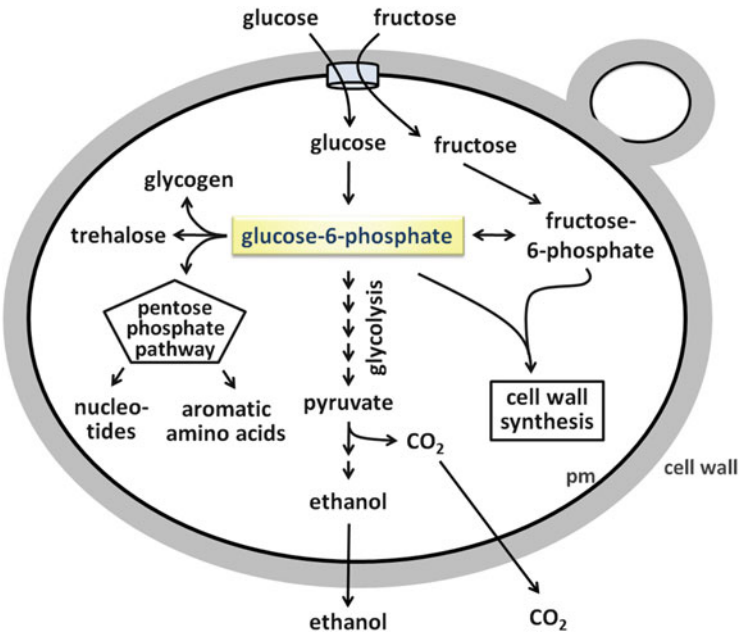

Fig. 8.2 Central role of glucose-6-phosphate in yeast sugar metabolism. A very simplified view of alcoholic fermentation is presented and the pathways for glucose-6-phosphate metabolism are highlighted. Synthesis and degradation of reserve carbohydrates is discussed in Chap. 16. *pm* plasma membrane

Table 8.2 Characteristics of sugar transporters in *Saccharomyces cerevisiae*

Hexose transporter	Glucose affinity[a]	Regulation by glucose[b] (Laboratory strains)	Expression during fermentation[c] (Wine strains)
Hxt1	Low	Induced by high [glucose]	Start of fermentation
Hxt2	Moderate	Induced by low [glucose] Repressed by high [glucose]	Lag phase
Hxt3	Low	Induced by high and low [glucose]	Throughout fermentation
Hxt4	Moderate	Induced by low [glucose] Repressed by high [glucose]	Induced during growth phase
Hxt5	Moderate high	Not regulated by glucose Regulated by growth rate	Not induced
Hxt6	High	Induced by low [glucose] Repressed by high [glucose]	Induced in stationary phase
Hxt7	High	Induced by low [glucose] Repressed by high [glucose]	Induced in stationary phase

[a]Reifenberger et al. (1997), Maier et al. (2002), Verwaal et al. (2002)
[b]Boles and Hollenberg (1997), Özcan and Johnston (1999)
[c]Luyten et al. (2002), Perez et al. (2005)

describe properties of the enzymes involved which are relevant to the yeast performance during vinification.

8.3.1 Hexose Transport

Early biochemical analyses indicated two basic types of hexose transport systems, so-called high-affinity and low-affinity transporters. Several carriers with intermediate affinities were described later on, which were shown to transport glucose, fructose, and mannose (Bisson and Fraenkel 1983; Özcan and Johnston 1999). The K_m for these transporters was shown to be lower for glucose than for fructose, while the V_{max} of fructose transport was higher than that for glucose. These differences in kinetic parameters have been correlated with the preferential degradation of glucose in wine fermentations (Bisson 1999; Berthels et al. 2008). It was also noted that transport does not need to be energized, but occurs by facilitated diffusion in *S. cerevisiae* (Lagunas 1993).

With the completion of the yeast genome sequencing project, it turned out that *S. cerevisiae* encodes at least 20 putative hexose transporters, which belong to the major facilitator superfamily. Though some of the encoded proteins may serve different functions, such as the glucose sensors Snf3 and Rgt2, multidrug transporters like Hxt9 and Hxt11, and polyol transporters comprising Hxt13 and Hxt15–Hxt17, the majority is capable of transporting glucose and fructose across the plasma membrane (reviewed in Bisson et al. 2016). A strain lacking seven of the genes (*hxt1–hxt7*) is not able to grow on glucose or fructose, anymore (Reifenberger et al. 1997). Growth of the mutant could be restored by reintroducing any one of the

encoding wild-type genes, which allowed functional in vivo studies of individual transporters. During vinification subsets of specific *HXT* genes are expressed in response to the external abundance of hexoses and enable *S. cerevisiae* to use sugars over a broad concentration range. Thus, the low-affinity transporters are produced at high glucose concentrations, whereas the high-affinity ones predominate when the sugar is scarce (Table 8.2; Özcan and Johnston 1999; Perez et al. 2005).

A mutant lacking the major hexose-transporter genes (*hxt1–hxt7*) was also obtained from a wine strain (Luyten et al. 2002). Expression of different transporter gene combinations in this genetic background suggested that Hxt3 plays a predominant role during must fermentations. The high-affinity carriers Hxt6 and Hxt7 are involved in hexose transport toward the end of fermentation, while Hxt1 may play a role only at the beginning. The general importance of hexose uptake was demonstrated by the combined overexpression of *HXT1* and *HXT7*, which led to an increased glucose uptake and a higher ethanol production (Kim et al. 2015; Rossi et al. 2010). Vice versa, the glucose flux could be redirected from alcoholic fermentation to respiration in a yeast strain producing a chimeric Hxt1/Hxt7 carrier (Henricsson et al. 2005). In genomic studies, several differences regarding hexose transporter genes and fermentative capacities have been found between different *S. cerevisiae* strains in the laboratory and strains of industrial use (Borneman et al. 2013).

With respect to the changing glucose/fructose ratios during fermentation, Guillaume et al. 2007 characterized a commercial strain with an *HXT3* allele leading to better fructose fermentation compared to other wine strains. This was recently confirmed by sequencing the *HXT3* genes in different *S. cerevisiae* strains and interspecies hybrids, where the mutant allele correlated with robust yeasts capable of restarting stuck fermentations (Zuchowska et al. 2015). Interestingly, the high-affinity fructose/proton symporter Fsy1, which is not present in laboratory *S. cerevisiae* strains, was found in the commercial wine yeast EC1118 and other strains. Its production is repressed by high concentrations of hexoses and thus may be important toward the end of fermentation (Galeote et al. 2010). Genetic variability was also observed for other hexose transporter genes (Zuchowska et al. 2015).

8.3.2 Glycolysis

Hexose uptake appears to be the primary step controlling the glycolytic rate in *S. cerevisiae*. The remaining enzymatic reactions are probably not rate limiting, since overproduction of key enzymes does not significantly increase the flux to ethanol, despite observations that simultaneous overexpression of all enzymes of the lower glycolytic pathway may have a positive effect (Peter Smits et al. 2000; Schaaff et al. 1989). In the following, we will briefly discuss the individual steps of glycolysis leading to the production of pyruvate in *S. cerevisiae* as the model yeast (Table 8.3).

Once inside the cell, glucose and fructose are phosphorylated by the action of kinases, which catalyze the first irreversible step of glycolysis (Entian and Barnett 1992).

Table 8.3 Genetic and biochemical features of enzymes involved in alcoholic fermentation in *S. cerevisiae*

Protein/Abbreviation	Genes	Structure	Effectors/ Coenzymes[a]	Mutants/Phenotypes[b]	Regulation[c]
Hexokinase1,2 Glucokinase Hxk1 Hxk2 Glk1	*HXK1* *HXK2* *GLK1*	Homodimer (2 × 54 kDa)	Hxk2: (−) trehalose-6-P	*hxk1Δ hxk2 Δ*: Fru^- *hxk1Δ hxk2Δ glk1Δ*: Glu^-, Fru^-	*HXK2*: only expressed on glucose; participates in glucose repression *HXK1*: not expressed on glucose *GLK1*: not expressed on glucose
Phosphoglucose isomerase Pgi1	*PGI1*	Homotetramer (2 × 61 kDa)		*pgiΔ*: Glu^-, Fru^-	Expressed on glucose and non-fermentable carbon sources
Phosphofructokinase Pfk[d]	*PFK1* + *PFK2*	Heterooctamer (4α × 108 kDa + 4β × 105 kDa)	(+) AMP, (+) Fru-2,6-bisphosphate (−) ATP	*pfk1Δ or pfk2Δ*: Glu^+ *pfk1Δ pfk2Δ*: Glu^-	Expressed on glucose and non-fermentable carbon sources
Fructose-1,6-bisphosphate aldolase Fba1	*FBA1*	Homodimer (2 × 40 kDa)		*fba1Δ*: Glu^-	Expressed on glucose and non-fermentable carbon sources
Triosephosphate isomerase Tpi1	*TPI1*	Homodimer (2 × 27 kDa)		*tpi1Δ*: Glu^-	Expressed on glucose and non-fermentable carbon sources
Glyeraldehyde-3-phosphate dehydrogenase GAPDH1-3	*TDH1* *TDH2* *TDH3*	Homotetramer (4 × 36 kDa)	NAD^+	*tdh2Δ or tdh3Δ*: Glu^+ *tdh2Δ tdh3Δ*: Glu^-	*TDH2, TDH3*: expressed in exponential phase *TDH1*: expressed in stationary phase
Phosphoglycerate kinase Pgk1	*PGK1*	Monomer (45 kDa)		*pgk1Δ*: Glu^-	Increased expression on glucose
Phosphoglycerate mutase Gpm1 Gpm2 Gpm3	*GPM1* *GPM2* *GPM3*	Homotetramer (*GPM1*: 4 × 28 kDa)	2,3-bisphosphoglycerate	*gpm1Δ*: Glu^- *gpm2Δ gpm3Δ*: Glu^+	*GPM1*: expressed on glucose and non-fermentable carbon sources Gpm2, Gpm3: no function in glycolysis

(continued)

Table 8.3 (continued)

Protein/Abbreviation	Genes	Structure	Effectors/ Coenzymes[a]	Mutants/Phenotypes[b]	Regulation[c]
Enolase Eno1 Eno2	*ENO1* *ENO2*	Homodimer (2 × 47 kDa)		*eno1Δ or eno2Δ*: Glu^+ *eno1Δ eno2Δ*: Glu^-	*ENO1*: expressed on glucose and non-fermentable carbon sources *ENO2*: strongly increased expression on glucose
Pyruvate kinase Pyk1 Pky2	*PYK1* *PYK2*	Homotetramer (4 × 55 kDa)	Pyk1: (+) Fru-1,6-bisP (−) ATP	*pyk1Δ*: Glu^- *pyk2Δ*: Glu^+	*PYK1*: increased expression on glucose *PYK2*: repressed by glucose
Pyruvate decarboxylase Pdc1 Pdc5 Pdc6	*PDC1* *PDC5* *PDC6*	Homotetramer (4 × 62 kDa)	TPP	*pdc1Δ, pdc5Δ or pdc6Δ*: Glu^+ *pdc1Δ pdc5Δ or pdc6Δ*: Glu^- in presence of antimycin A	*PDC1*: strongly increased expression on glucose *PDC1* and *PDC5* expression require the Pdc2 *PDC1, PDC5*: expression induced in *pdc1 Δ* mutants *PDC6*: repressed by glucose
Alcohol dehydrogenase Adh1-5	*ADH1* *ADH2* *ADH3* *ADH4* *ADH5*	Homo/hetero tetramer (4 × 37–41 kDa) Adh4: Homodimer (2 × 51 kDa)	NAD^+	*adh1Δ*: Glu^- in presence of antimycin A	*ADH1*: expressed on glucose and non-fermentable carbon sources *ADH2*: repressed by glucose Adh3-5: also participate in glucose fermentation

[a](+) indicates allosteric activation, (−) indicates allosteric inhibition; *TPP* thiamine pyrophosphate

[b]Glu^-, Fru^-: failure to grow on glucose or fructose as a sole carbon source, respectively

[c]The term "increased expression on glucose" refers to a more than twofold higher expression as compared to non-fermentable carbon sources; strongly increased expression indicates an at least fivefold increase

[d]Abbreviations for enzymes are usually according to the gene names. For Pfk we adopted a different approach, since the enzyme is a heterooctamer whose subunits are encoded by *PFK1* and *PFK2*. In the mammalian nomenclature, PFK1 designates the 6-phosphofructo-1-kinase, and PFK2 designates the 6-phosphofructo-2-kinase. To avoid misunderstandings, Pfk in this treaty designates the first and Pf2k the second enzyme. Isozymes of the latter in yeast are encoded by the genes *PFK26* and *PFK27*

Consequently, intracellular free sugar concentrations are below 10 mM (Bermejo et al. 2011). Three kinases, glucokinase (Glk1), hexokinase 1 (Hxk1), and hexokinase 2 (Hxk2), have been identified (Fig. 8.3, Table 8.3). Glucokinase uses glucose or mannose as substrates, whereas both hexokinases can phosphorylate either glucose, fructose, or

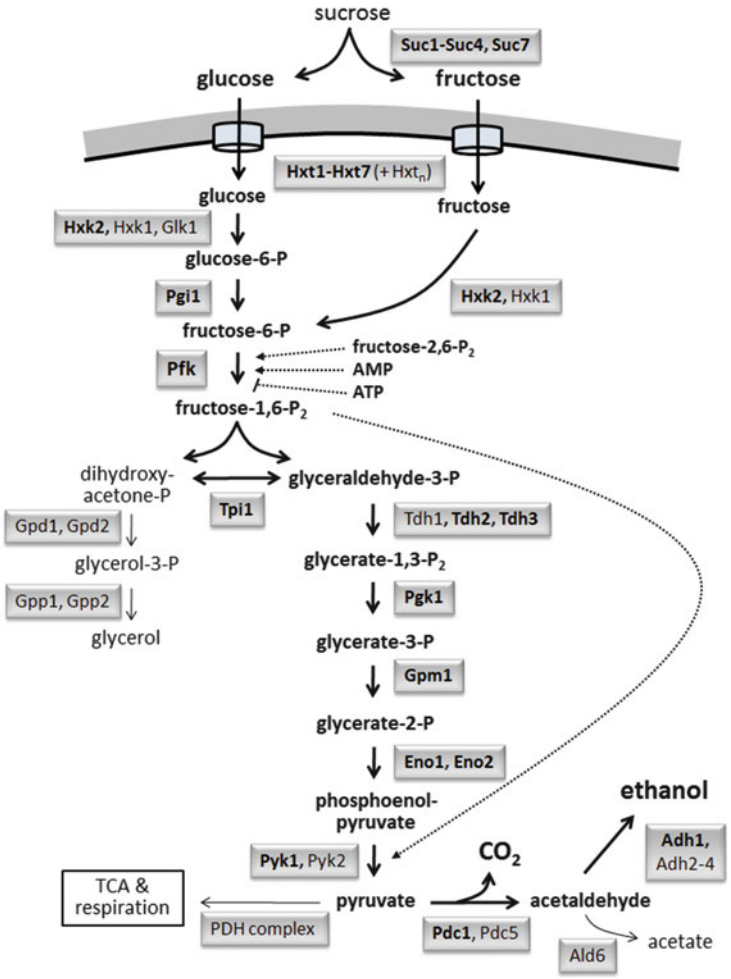

Fig. 8.3 Pathway of alcoholic fermentation in *S. cerevisiae*. The carbon flow from hexoses to ethanol is presented by the *solid black open arrows*. *Open arrows with thinner lines* indicate minor diversions from the main flux. *Filled arrows with doted lines* show positive regulatory mechanisms, *dotted lines ending in a bar* mark inhibitory effects. Full names and physiological characteristics of the enzymes designated here in the three letter code in *gray boxes* are given in Table 8.3. Enzymes and isozymes not expected to contribute significantly to hexose fermentation in wine are not in bold writing. *PDH* pyruvate dehydrogenase complex, *Pfk* heterooctameric phosphofructokinase (also see comment in Table 8.3); *TCA* tricarboxylic acid cycle. The pathways to glycerol and acetate are shown in more detail in Fig. 8.4

mannose. The three enzymes also differ in their kinetic parameters. Studies on the transcriptional regulation demonstrated that Hxk2 is the predominating isoform in cells growing on glucose and fructose, as found in the must (Moreno et al. 2005). After shifting the cells to a non-fermentable carbon source, *HXK2* expression ceases and *HXK1* and *GLK1* are rapidly derepressed.

Rossignol et al. (2003) monitored the transcriptome of a wine strain under production conditions. They found that in the first phase of fermentation, *HXK2* is highly expressed. In the second phase, when growth ceases but fermentation still proceeds, Hxk2 transcription decreases, and *HXK1* and *GLK1* become expressed, as observed for laboratory strains. These studies resolved the long-standing mystery of Hxk1 and Glk1 functions: as stated above, the fructose/glucose ratio rises at the end of must fermentations. Considering the kinetic parameters of the enzymes, a shift from Hxk2 to Hxk1 would be favorable, as the latter displays a higher V_{max} for fructose (Rossignol et al. 2003). On the other hand, the very high affinity of the glucokinase for glucose (with a K_m of 30 μM) would facilitate its utilization when glucose levels are very low. In contrast to the hexose transporter genes, little allelic variation which would affect sugar utilization was found in the hexokinase genes of wine yeast strains (Zuchowska et al. 2015).

Early studies on the regulation of yeast hexokinase indicated an inhibition by ATP. However, the data presented above show that Hxk2 is mainly active at high sugar concentrations such as found in the must, which suggests that ATP inhibition may not be important for the in vivo activity (Golbik et al. 2001). Rather, a potent allosteric inhibitor of Hxk2 has been described with trehalose-6-phosphate (Gancedo and Flores 2004), explaining why a deficiency in trehalose-6-phosphate synthase (Tps1) results in growth inhibition. However, this may also be attributed to the requirement of trehalose-6-phosphate for the inactivation of gluconeogenic enzymes (Deroover et al. 2016).

The reversible interconversion of glucose-6-phosphate and fructose-6-phosphate is performed by phosphoglucose isomerase, encoded by *PGI1*. Mutants in this gene grow on fructose, but are dependent on trace amounts of glucose in the medium (Aguilera 1986), underlining the importance of glucose-6-phosphate for feeding different routes of carbohydrate metabolism (Fig. 8.2).

The phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate is the first irreversible reaction specific for glycolysis (Heinisch and Hollenberg 1993). In *S. cerevisiae*, the heterooctameric enzyme phosphofructokinase consists of 4α- and 4β-subunits, encoded by *PFK1* and *PFK2*, respectively. In vitro activity can only be detected for the intact heterooctamer, yet each subunit is capable of catalysis in vivo, if the other one is missing, explaining why single deletion mutants in either one of the genes still grow on glucose (Arvanitidis and Heinisch 1994). Regarding its biochemistry, phosphofructokinase (Pfk) is a paradigm for allosteric regulation. A number of small molecules affect enzyme activity, with ATP being the most potent inhibitor and AMP and most of all fructose-2,6-bisphosphate serving as activators (reviewed in Kopperschläger and Heinisch 1997). Linking sugar metabolism with nitrogen availability in must fermentations, ammonium has been shown

to also stimulate Pfk activity in yeast, and all allosteric effectors are apparently sensed by each of the yeast Pfk subunits (Rodicio et al. 2000).

Fructose-1,6-bisphosphate produced is then reversibly cleaved by aldolase, encoded by *FBA1*, into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Schwelberger et al. 1989). Both triosephosphates are interchanged with the help of triosephosphate isomerase, encoded by *TPI1*, the most evolved enzyme known by biochemists (cited in Heinisch and Rodicio 1997). A lack of this enzyme causes accumulation of dihydroxyacetone phosphate which is then channeled into glycerol production (Compagno et al. 1996).

Further conversion of glyceraldehyde-3-phosphate through glycolysis depends on the availability of NAD^+ as an electron acceptor, which is regenerated from NADH in the last reaction of alcoholic fermentation. If the capacity for reoxidation by alcohol dehydrogenase is limited, NAD^+ can be regenerated either by respiration or by conversion of dihydroxyacetone phosphate to glycerol. This is the major source of glycerol present in wine, whose production may also be triggered by the osmotic stress during the primary stages of must fermentation (see Chap. 16).

All but the last of the following glycolytic reactions are also reversible: *S. cer*evisiae has three isozymes of glyceraldehyde-3-phosphate dehydrogenases, encoded by the genes *TDH1-TDH3*, to generate glycerate-1,3-bisphosphate with the addition of an inorganic phosphate and the production of NADH. More than half of the enzyme activity can be attributed to the *TDH3* gene product, whereas *TDH1* contributes only 10–15% of the total dehydrogenase activity (McAlister and Holland 1985a, b). Consequently, *tdh2 tdh3* double mutants fail to grow on glucose. *TDH1* expression studies indicate that a primary function is related to the oxidative stress response (Valadi et al. 2004).

The phosphoglycerate kinase Pgk1 catalyzes the first reaction to generate ATP in glycolysis. *PGK1* is the glycolytic gene most strongly expressed, and its promoter is frequently used for heterologous gene expression (Graham and Chambers 1997).

In the following, 3-phosphoglycerate is converted to 2-phosphoglycerate by the *GPM1*-encoded phosphoglycerate mutase (Rodicio et al. 1993). The yeast genome sequencing project revealed the presence of two further homologs, which are not involved in sugar metabolism (Heinisch et al. 1998).

Phosphoenolpyruvate (PEP) is produced by the enolase reaction. Two isozymes are encoded by *ENO1* and *ENO2*. Whereas expression of the first gene is constitutive, that of the second is induced 20-fold by the presence of sugars (Cohen et al. 1987). Thus, Eno2 predominates in early wine fermentation, gradually sharing substrate turnover with Eno1 toward the later phases.

The final step of glycolysis, mediated by pyruvate kinase, encoded by *PYK1*, generates the second ATP in the pathway and is essentially irreversible. Therefore, the enzyme serves as a second control point for glycolysis and is also allosterically regulated. Fructose-1,6-bisphosphate, produced by Pfk and the glycolytic metabolite found at highest concentrations, serves as a potent activator (Morris et al. 1986). Although Pyk1 is also phosphorylated by PKA, allosteric regulation exerts the major control (Xu et al. 2012). An alternative Pyk2 isozyme cannot confer fermentative growth in the absence of Pyk1 (Boles et al. 1997). Pyruvate kinase activity is

key to the subsequent carbon flux. Thus, decreasing pyruvate concentrations leads to rerouting into respiration (Pearce et al. 2001). This indicates that allosteric control at this step is not only important for the speed of fermentation but also for ethanol yield.

8.3.3 Pyruvate Decarboxylase and Alcohol Dehydrogenase

Ethanol and carbon dioxide are the major products of sugar fermentation by *S. cerevisiae* in the production of beverages and bread. They are generated in the two final steps of alcoholic fermentation, which are catalyzed by pyruvate decarboxylase and alcohol dehydrogenase, with the main isozymes encoded by *PDC1* and *ADH1*, respectively (Schmitt and Zimmermann 1982; Ciriacy 1975). Other homologs present in the yeast genome are of minor importance for fermentation (Table 8.3). Yeast also contains a mitochondrial pyruvate dehydrogenase complex, which can introduce pyruvate into respiration (Fig. 8.3) if medium sugar concentrations are low.

Taken together, glycolytic enzymes account for at least 30% of the total soluble protein in *S. cerevisiae* (Gancedo and Serrano 1989), consistent with transcriptome data indicating high level gene expression throughout alcoholic fermentation (Rossignol et al. 2003). However, glycolytic gene expression seems to be different when dried wine yeasts are prepared for fermentation. Thus, transcriptomic and proteomic analyses showed that upon inoculation of the must with rehydrated yeast, some mitochondrial enzymes involved in oxidative metabolism are induced. In fact, the shift from respiratory to fermentative metabolism occurs after 4 h of inoculation (Rossignol et al. 2006; Salvado et al. 2008).

8.3.4 Glycerol and Acetate as Fermentation By-Products

Glycerol is considered a valuable by-product of yeast alcoholic fermentation in wine production. It is primarily formed in the early phase of fermentation and remains stable throughout the process, since it is not consumed after sugar exhaustion under anaerobic conditions (Orozco et al. 2012). Glycerol originates from glycolysis at the level of triosephosphates (Fig. 8.4). Two glycerol-3-phosphate dehydrogenases, encoded by *GPD1* and *GPD2*, convert dihydroxyacetone phosphate to glycerol-3-phosphate, which is further processed by the irreversible reaction of the *GPP1* and *GPP2* encoded phosphatases. In contrast to ethanol, glycerol cannot diffuse through the plasma membrane and is excreted by the Fps1 transporter (Hohmann 2015). Vice versa, glycerol import could be mediated by a redundant pair of transporters, encoded by *GUP1* and *GUP2*, but the glycerol/proton symporter Stl1 may be more important in this respect (Ferreira et al. 2005).

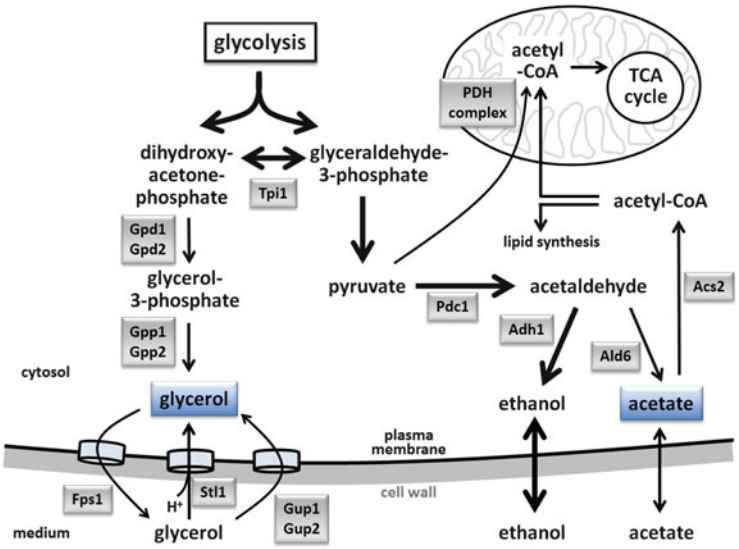

Fig. 8.4 Pathways to glycerol and acetate in *S. cerevisiae*. Glycerol originates from the glycolytic metabolite dihydroxyacetone phosphate and is exported by a special permease (Fps1). Ethanol is produced from pyruvate metabolism and is believed to freely diffuse through the plasma membrane. The "PDH bypass" ensures production of acetyl-CoA in the cytoplasm for lipid biosynthesis. *TCA* tricarbocylic acid cycle. See text for further details

Glycerol production is probably triggered by two main mechanisms in *S. cerevisiae*: (1) the initial lack of alcohol dehydrogenase, which causes an imbalance of reduction equivalents (Gancedo and Serrano 1989), and (2) the high initial sugar content in the must of 20%, which causes osmotic stress and induces the HOG signaling pathway (Tamas et al. 2003; see also Chap. 16). Depending on the wine, an increase in glycerol levels is desired, but wine strains constructed to this end frequently also generate more acetate (Zhao et al. 2015).

Acetate as the main component of volatile acidity adds a negative organoleptic property (Curiel et al. 2016). It originates from the acetaldehyde produced by the pyruvate decarboxylase reaction if used by aldehyde dehydrogenases (Fig. 8.4). These function together with acetyl-CoA synthetase (ACS) as an essential cytosolic PDH bypass and ensure availability of acetyl-CoA, e.g., for lipid biosynthesis (Remize et al. 2000). The cytosolic aldehyde dehydrogenase isoforms are encoded by *ALD2*, *ALD3*, and *ALD6*. *ALD4* and *ALD5* encode mitochondrial isoforms, which are employed when ethanol is the carbon source. Expression of *ALD2* and *ALD3*, encoding cytosolic isoforms, is glucose-repressed, leaving Ald6 as the relevant enzyme in fermentative acetate production (Saint-Prix et al. 2004). A limited capacity of the acetyl-CoA synthetase probably causes the acetate overflow (Van Urk et al. 1990). Of the two synthetase isoforms, encoded by *ACS1* and *ACS2*, only the second is expressed on glucose. A reduction in acetate produced during wine fermentation has been achieved in genetically engineered *S. cerevisiae* strains

lacking either Reg1 or Pdc1 (Curiel et al. 2016). Yeasts with reduced acetate production have also been obtained by genetic engineering and classical mutagenesis (Cordente et al. 2013, Ehsani et al. 2009).

Positive aroma compounds derived from yeast acetate metabolism (e.g., ethyl and isoamyl acetate) are produced by alcohol acetyltransferases, encoded by *ATF1* and *ATF2*, which localize to lipid particles (Lilly et al. 2006). However, perturbations in lipid and nitrogen metabolism have been shown to have a more prominent effect on ester production than overexpression of these genes (Rollero et al. 2016). Similarly, ethyl ester production by Eeb1 and Eth1 enzymes was also found to be regulated more by the availability of precursors, rather than at the level of gene expression (Saerens et al. 2008).

Two forms of reserve carbohydrate compounds have been described to naturally accumulate in yeasts: Glycogen (a polysaccharide composed of α-1,4-glucose chains branched by some α-1,6-linkages) and trehalose (a disaccharide of two α-1,1-linked glucose molecules). Their metabolism is reviewed in Chap. 16.

8.4 Regulation of Carbohydrate Metabolism in Yeasts: Glucose Signaling

Yeasts have evolved sophisticated mechanisms to appropriately adjust their metabolism to varying environmental conditions. Thus, *S. cerevisiae* has adapted best to the needs of alcoholic fermentation, where sugar concentrations constantly decrease, while those of ethanol rise. It can use glucose under all these conditions. The underlying signaling mechanisms have been extensively studied, are summarized in a simplified scheme in Fig. 8.5, and will be discussed in the following. Briefly, glucose represses the expression of genes encoding enzymes of the respiratory pathway and those required for the utilization of alternative sugars and gluconeogenesis through the SNF1 pathway, which is the main reason for the Crabtree effect. At the posttranslational level, glycolytic enzymes can be allosterically activated by small metabolites and enzymes of gluconeogenesis, respiration and sugar transporters may be inhibited and/or suffer proteolytic degradation, a process frequently mediated by the PKA/cAMP pathway. Transcription of some hexose transporter genes is also induced by the Snf3-Rgt2/Rgt1 signaling pathway and that of glycolytic genes by the transcriptional activators Gcr1/Gcr2. All these pathways may crosstalk among each other and with signaling of other nutrients such as the availability of nitrogen and phosphate (Gancedo 2008; Zaman et al. 2009).

For more details on what is summarized here in Fig. 8.5, the interested reader is referred to a number of excellent reviews (Rolland et al. 2002; Holsbeeks et al. 2004; Verstrepen et al. 2004; Santangelo 2006; Hedbacker and Carlson 2008; Broach 2012; Horak 2013; Pretorius et al. 2012; Kim et al. 2013; Dashko et al. 2014).

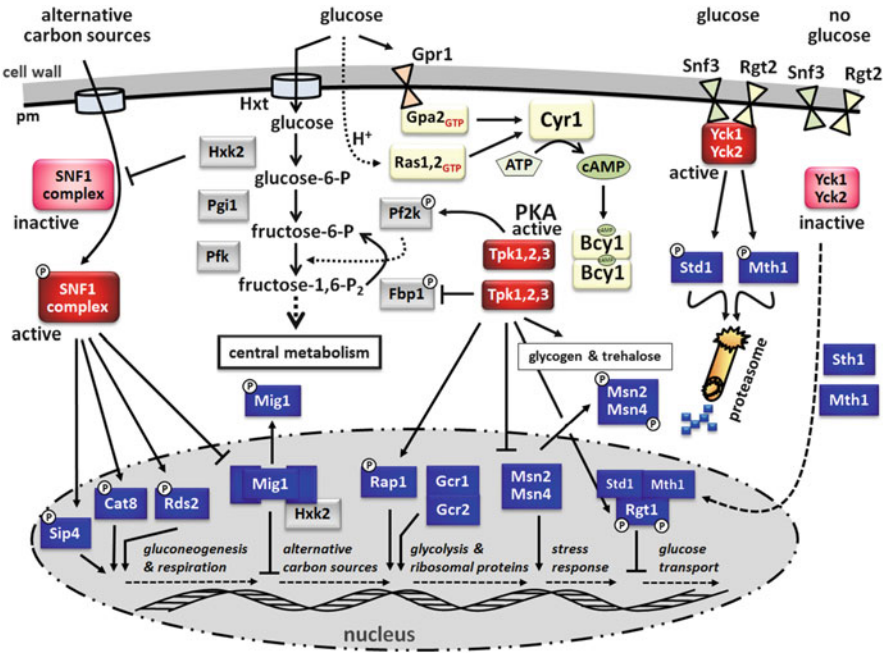

Fig. 8.5 Pathways of glucose signaling in *S. cerevisiae*. The three major glucose signaling pathways in *S. cerevisiae* are depicted in a simplified scheme. Enzymes of carbohydrate metabolism are depicted in *gray boxes*. Protein kinases are shown in *red*, regulatory enzymes in *yellow* and transcription factors in *blue boxes*. *Open arrows* indicate carbon flow, *closed arrows* depict activation in regulatory circuits, and *lines with bars* indicate repression. Phosphorylation of target proteins is shown by a *circled P*. *pm* plasma membrane. See Sect. 8.4 for a detailed description

8.4.1 The SNF1 Kinase Complex and Glucose Repression

Metabolism is tightly controlled at the transcriptional level by glucose. Since it was assumed that an intermediary metabolite would trigger this regulation, it was first called "carbon catabolite repression." In the early stages of must fermentation, transcription of genes whose products are involved in respiration, the utilization of alternative carbohydrates such as sucrose, and sugar transporters, is repressed by the high levels of glucose and fructose.

Derepression after sugar depletion depends on the activity of the heterotrimeric SNF1 kinase complex, the SNF1 upstream protein kinases Sak1, Tos3, or Elm1, the Reg1/Glu7 phosphatase, and the transcriptional repressor Mig1, which forms a complex including the hexokinase isozyme Hxk2 (Hedbacker and Carlson 2008). In this system, glucose repression requires hexose transport and phosphorylation, but no further metabolism, and Hxk2 has been proposed to act as an intracellular glucose sensor (Vega et al. 2016). The SNF1 complex is named after its α-catalytical subunit Snf1 (=Cat1). It forms a complex with the γ regulatory subunit Snf4 (=Cat3), which stimulates the kinase activity by blocking the auto-

inhibition of Snf1. The β-subunit in *S. cerevisiae* can be encoded by either of three genes, *SIP1*, *SIP2*, or *GAL83*, which mediate subcellular compartmentalization of the complex. The activated SNF1 complex phosphorylates several transcription factors besides Mig1, such as Cat8, Sip4, and Rds2, which regulate target gene expression. At high glucose concentrations, the SNF1 complex is rendered inactive by the Reg1-Glc7 phosphatase.

Mig1 is a member of a zinc finger family and is part of a complex which controls the utilization of alternative sugars such as galactose and sucrose, as well as various hexose transporter genes. On glucose medium, Mig1 forms a complex with the Hxk2 isozyme in the nucleus and represses the target gene promoters by recruiting the corepressor complex Ssn6/Tup1 (Horak 2013). Upon glucose limitation, Mig1 is phosphorylated by the SNF1 complex, which triggers its export into the cytoplasm and derepression of the target gene transcription. Mig1 also contributes to a minor extent in the repression of respiration and gluconeogenesis. When derepressed, the zinc finger transcription factors Cat8, Sip4, and Rds2 then activate expression of genes encoding gluconeogenic and glyoxylate cycle enzymes (left part of Fig. 8.5). Interestingly, *CAT8* expression is repressed by Mig1, whereas *SIP4* transcription is induced by Cat8.

Repression of respiration is also indirectly mediated by this system: a heteromultimeric complex consisting of Hap2-Hap5 binds to elements present in the respective target gene promoters and activates transcription. *HAP4* is expressed only in the absence of glucose, since it requires the transcriptional activator Rds2 (Turcotte et al. 2010; Broach 2012). Thus, respiration and gluconeogenesis become coordinately regulated by glucose.

8.4.2 Signaling Through the cAMP/PKA Pathway

Glucose addition to stationary cultures or cells grown on non-fermentable carbon sources results in a rapid and transient increase of cAMP, which is produced by the adenylate cyclase Cyr1, and triggers the activation of protein kinase A (PKA). PKA then phosphorylates a number of transcription factors and metabolic enzymes leading to metabolic adaptation and cellular growth. Transcriptome analyses indicate that the majority of metabolic changes observed upon glucose addition are mediated by the cAMP/PKA pathway and that SNF1 and Snf3/Rgt2 signaling are more specialized and have a limited target range (Zaman et al. 2009).

PKA is kept inactive by association of two Bcy1 inhibitory subunits with two catalytic subunits encoded by the redundant genes *TPK1*, *TPK2*, or *TPK3*. Binding of cAMP to the Bcy1 subunits promotes the dissociation of the tetramer, and the now catalytically active Tpk subunits phosphorylate their target proteins (Fig. 8.5, middle part). Extracellular glucose is mainly sensed through the Ras pathway, and changes in the membrane potential accompanied or intracellular acidification are probably responsible for adenylate cyclase activation (Broach 2012). Adenylate cyclase can also be activated through Gpr1-Gpa2 signaling, but this seems to be of minor importance, since

deletion mutants in this system do not affect the global transcriptional response upon shifting cells from glycerol medium to glucose (Zaman et al. 2009).

A central function of PKA is the inhibition of respiration and the promotion of fermentation by the phosphorylation of key metabolic enzymes. Thus, PKA triggers catabolite inactivation by glucose, i.e., it phosphorylates target enzymes of gluconeogenesis and respiration and marks them for proteolytic degradation. The most prominent example are fructose-1,6-bisphosphatase, encoded by *FBP1*, and the glyoxylate pathway enzymes isocitrate lyase and malate synthase. In contrast, Pf2k, the phosphofructo-2-kinase producing a potent activator of the glycolytic Pfk enzyme, is activated by PKA-dependent phosphorylation. Global proteolytic degradation of the gluconeogenic enzymes Fbp1 and malate dehydrogenase in the vacuole is also mediated by PKA (Hung et al. 2004). The latter proteins apparently can also be degraded in a cAMP-independent manner in the cytosol via the ubiquitin/proteasome system, which depends on glucose transport and Hxk2 activity, but is independent of Snf3/Rgt2 signaling described below (Horak 2013).

8.4.3 *Glucose Induction Mediated by the Snf3/Rgt2 Sensors Targets Sugar Transport*

Despite the high must sugar concentrations, intracellular glucose does not exceed 10 mM in *S. cerevisiae* wild-type cells (Bermejo et al. 2011). Regulation of the sugar transport across the plasma membrane is therefore crucial and has to be tightly controlled by the physiological state of the cells. Glucose promotes transcription of the genes *HXT1-HXT4* by the Snf3/Rgt2 signal transduction pathway which ultimately regulates the Rgt1 repressor (Fig. 8.5; Horak 2013). Snf3 and Rgt2 are hexose sensor proteins which detect glucose, fructose, and mannose (Dietvorst et al. 2010). While Snf3 is a high-affinity sensor required for the synthesis of high- and moderate-affinity transporters in low glucose, Rgt2 is a low-affinity sensor needed for the proper induction of *HXT1* (Özcan et al. 1998). The downstream target Rgt1 is a zinc cluster protein that binds to a consensus DNA sequence present in multiple repeats in most of the *HXT* gene promoters (Kim 2009). In the absence of glucose, Rgt1 represses *HXT* gene expression by recruiting the Ssn6/Tup1 corepressor into a complex with the corepressors Mth1 and Std1 (Roy et al. 2014). Expression of *HXT* genes is strongly upregulated in the transcriptome of a *mth1* deletion mutant but remains largely unaffected by a *std1* deletion. Thus, Mth1 may primarily maintain repression in the absence of glucose by preventing Rgt1 phosphorylation by PKA, while Std1 could repress *HXT* gene transcription during the transition to glucose exhaustion (Sabina and Johnston 2009; Broach 2012; Horak 2013).

When the sensors detect glucose, they change into an active conformation and transmit the signal to the protein kinases Yck1 and Yck2, which then phosphorylate the corepressors Mth1 and Std1 and cause their ubiquitin-mediated degradation by

proteasomes. In addition, *MTH1* transcription is downregulated by the SNF1/Mig1 pathway. Strains lacking Mth1 and Std1 display a cAMP/PKA-mediated hyperphosphorylation of Rgt1, which dissociates from the Ssn6/Tup1 complex, allowing expression of the *HXT* genes (Kim et al. 2013; Roy et al. 2014).

SNF1/Mig1 signaling not only contributes to *HXT* gene regulation through Mth1 as described above but also downregulates the genes for the Snf3 sensor and the moderate-affinity transporters Hxt2, Hxt4, Hxt6, and Hxt7 and possibly induces *HXT1* expression (Westholm et al. 2008). The latter gene is also transcriptionally activated by Gcr1, a factor previously described for activating most glycolytic genes and those encoding ribosomal proteins as described in the next paragraph (Kim et al. 2015). Finally, posttranslational control of hexose transporters is exerted for the high- and moderate-affinity transporters by endocytosis and vacuolar degradation at elevated glucose concentrations (Horak 2013). Likewise, the low-affinity transporters Hxt1 and Hxt3 are internalized and degraded upon glucose starvation, a process which depends on PKA activity (Roy et al. 2015).

8.4.4 *General Transcription Factors for High Level Expression of Glycolytic Genes*

Most yeast enzymes involved in glycolysis are also required for gluconeogenesis and are thus abundant under all growth conditions, even if ethanol is used as the sole carbon source (Fraenkel 1982). However, three steps are specific for glycolysis (Sect. 8.3.2), and some isozymes are only produced at high or low glucose concentrations, with their gene expression controlled accordingly (Table 8.3). This is exemplified by the first step of glycolysis. Only *HXK2* is transcribed at high sugar concentrations, and the transcriptional repressor Rgt1 governs repression of *HXK2* upon glucose depletion (Palomino et al. 2005). Expression of *GLK1* and *HXK1* is only activated when sugars become limited. Interestingly, Hxk2 is required for repression of *HXK1* and *GLK1* as well as for expression of its own gene on glucose (Moreno et al. 2005).

Most glycolytic genes contain binding sites for the general transcriptional factor Rap1, and the transcription activator Gcr1 in their promoters and *gcr1* mutants display low levels of all glycolytic enzymes (Sasaki et al. 2005). Rap1 and Gcr1 collaborate to stimulate transcription by binding directly to their promoter elements. A second protein, Gcr2, interacts with Gcr1 to enhance transcription of glycolytic genes. The Rap1/Gcr1 complex also regulates transcription of ribosomal genes but in a Gcr2 independent manner. Interestingly, Rap1 gets activated on high glucose concentrations by a cAMP/PKA-dependent phosphorylation. Overexpression of Gcr1 was shown to increase cellular growth and ethanol production. This was not associated to an increase of glycolytic enzymes, possibly due to the lack of excess Gcr2, but rather to an increase in Hxt1-mediated hexose transport and a higher abundance of ribosomal proteins (Kim et al. 2015).

8.5 Conclusions and Perspectives

As predicted in the last edition of this chapter, non-*Saccharomyces* yeasts have gained considerable interest in the past decade with regard to their use in mixed starter cultures in vinification (Varela 2016). In the context of control of their carbohydrate metabolism, attention has been focused mainly on the precursors of volatile and aromatic compounds to enhance flavor diversity, rather than on alcoholic fermentation (Fleet 2008; Jolly et al. 2014). An exception is *Kluyveromyces marxianus*, a thermotolerant yeast also found in must and capable of producing high amounts of ethanol. The fact that it is generally regarded as a Crabtree-negative yeast (Table 8.1) has been attributed to variations within the species (Radecka et al. 2015). It is a close relative of the milk yeast *Kluyveromyces lactis*, which is a true Crabtree-negative yeast and whose genetics has been extensively studied (Rodicio and Heinisch 2013). In addition, several other wine yeasts have been investigated for their production of desired compounds of primary sugar metabolism such as glycerol and undesired products such as acetate and acetoin (Gamero et al. 2016). A wine yeast commonly predominating the early phases of must fermentations is *Hanseniaspora uvarum*, previously called *Kloeckera apiculata* and giving the name to the "apiculate yeasts." It is known for its potent production of desirable ester compounds, but also for producing acetate. We have sequenced the genome and started to investigate its fermentative capacity by determining the specific activities of the enzymes involved. Pyruvate kinase appears to be a limiting step in the flow toward ethanol production in this yeast (Langenberg 2016 and unpublished results). The presence of non-*Saccharomyces* species in the early stages of fermentation also broadens the range of carbohydrates to be degraded. For example, cellobiose, trehalose, lactose, mannitol, ribose, xylose, and even xylitol can be used, and in some cases also fermented, by different wine yeast species (Barnett et al. 2000).

Another emerging issue for the improvement of wine quality is the study of evolution of *S. cerevisiae* strains and the use of adaptive evolution to obtain strains with desired features (Borneman et al. 2016). Combined with the progress in next-generation sequencing techniques and bioinformatic evaluation of transcriptome and proteome analyses, this has already yielded and will continue to yield exciting new insights. With this growing knowledge on wine strain diversity, it stands to hope that consumers will gradually accept the fact that there is no such thing as a "natural wine yeast" and become more open-minded to the use of genetically engineered yeast strains, specifically constructed to improve wine quality (Jolly et al. 2014).

Finally, beyond their importance for wine production, what we learn from the studies cited herein and the ones to come in the next years, will clearly lead to a better understanding of the regulatory circuits underlying yeast carbohydrate metabolism and the interactions between yeast species and lactic acid bacteria in all kinds of industrial applications, including the production of beer, bread, spirits, bioethanol, and next-generation biofuels.

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Chapter 9
Metabolism and Transport of Sugars and Organic Acids by Lactic Acid Bacteria from Wine and Must

Gottfried Unden, Tanja Eirich, and Hanno Richter

9.1 Introduction

Heterofermentative lactic acid bacteria (LAB) which are common in plant-associated environments are found also in grape must and wine. In this environment strains predominate which are adapted to the low pH and high alcohol contents. Must and wine harbour the strictly heterofermentative *Oenococcus oeni* (*O. oeni*), *Lactobacillus hilgardii* and *Lactobacillus brevis* (*Lb. brevis*) and the facultatively heterofermentative *Lactobacillus plantarum* (*Lb. plantarum*) and *Lactobacillus pentosus* (Rodas et al. 2005). In addition homofermentative lactic acid bacteria of the *Pediococcus* group are able to grow in wine and must but are normally found at low cell densities.

The growth of lactic acid bacteria in wine depends largely on sugars and organic acids which are present in grape must. Like most heterofermentative LAB, *O. oeni* is able to degrade hexoses, pentoses and other sugars from must, which can result in the excretion of undesirable products. The metabolic activities related to the degradation of organic acids, in particular of malate degradation which leads to deacidification of wine, are responsible for positive effects attributed to *O. oeni* in wine (Mayer 1974). *O. oeni* is also able of arginine fermentation by the arginine

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deiminase pathway which supports maintenance of the bacteria (Tonon and Lonvaud-Funel 2000; Tonon et al. 2001).

Grape must contains in addition to hexoses (glucose, fructose, galactose, mannose) considerable amounts of pentoses (arabinose, xylose, ribose, rhamnose). Glucose and fructose are the most abundant sugars and are present in an approximate 1:1 molar ratio. The concentrations depend on the ripeness and type of grapes but are typically $\geq$100 g L^{-1} for glucose and fructose, followed by much lower contents of galactose (<200 mg L^{-1}) and mannose (<50 mg L^{-1}) (Würdig and Woller 1989). Pentoses are derived from hydrolysis of plant cell wall pectins and are present in small amounts (L-arabinose, <1 g L^{-1}; D-xylose and D-ribose, each <0.1 g L^{-1}; L-rhamnose <0.4 g L^{-1}). Disaccharides (maltose, raffinose, trehalose) and oligosaccharides (stachyose) are found in small amounts (few mg L^{-1} each) (Würdig and Woller 1989). Excess hexoses are used also for the production of exopolysaccharides, such as dextran, levan and fructan by various groups of LAB (Cerning 1990). *Pediococcus damnosus* forms an extracellular polysaccharide (1,3:1,2-β-D-glucan) from glucose which increases the viscosity of wine (Llaubères et al. 1990). Many *O. oeni* strains contain α- or β-glycosidase enzymes (Grimaldi et al. 2005) that release the sugar component from glycoconjugates that are present in must. The sugar is either glucose or a disaccharide which can be substituted by other sugars. The sugars released by the exoenzymes can be used as substrates and are taken up by the bacteria. The aglycon released, including monoterpenes, C_{13}-norisoprenoids, benzene derivatives and aliphatic alcohols (Winterhalter and Skouroumounis 1997; Grimaldi et al. 2005), can have significant impact on the wine aroma. The aroma compounds as well as the glycosidases are of interest in winemaking (Grimaldi et al. 2005; Mesas et al. 2012).

Hexoses and pentoses are fermented by the heterofermentative LAB by the phosphoketolase (or oxidative pentose phosphate) pathway. The major products of glucose fermentation are D-lactate, ethanol and CO_2. Ethanol formation represents a limiting step in the heterofermentative hexose fermentation which can be overcome by modifications in the fermentation or by the use of external electron acceptors, such as fructose, O_2, pyruvate or citrate. The modified fermentation reactions produce also undesirable products like acetate or mannitol. The variations in the pathways and the biochemical background will be described.

Many heterofermentative LAB are able to metabolize organic acids which are common in grape must. Malate, citrate and pyruvate are degraded efficiently. Malate, L-tartrate, fumarate and pyruvate are metabolized without the need for co-substrates, whereas fermentation of citrate requires co-substrates, usually hexoses which serve as the electron donor. Organic acids like L-tartrate, fumarate or galacturonic acid are fermented only under specific conditions. L-Malate (<20 g L^{-1}) and L-tartrate (<10 g L^{-1}) are found in high concentrations. Concentrations of citrate (<300 mg L^{-1}), gluconic acid (<300 mg L^{-1}), galacturonic acid (<250 mg L^{-1}) and fumarate (traces) are much lower in must from healthy grapes (Würdig and Woller 1989; Dittrich and Großmann 2005).

9.2 Special Features of the Phosphoketolase Pathway of *O. oeni*: The Use of Alternative Reactions for NAD(P)H Reoxidation

9.2.1 Phosphoketolase Pathway and Limitation of Ethanol Formation

O. oeni is able to use glucose, fructose and ribose as the substrates for growth. In addition, various strains show growth on further hexoses (galactose, mannose), pentoses (xylose, arabinose) and disaccharides (trehalose, cellobiose, sucrose, melibiose) (Beelman et al. 1977; Garvie 1986; Zhang and Lovitt 2005). Growth on other oligosaccharides (lactose, maltose, and raffinose) has been observed, but this property is unstable and can be lost (Beelman et al. 1977). The sugars are fermented by the phosphoketolase pathway resulting in the formation of pyruvate (derived from glyceraldehyde-3P, or GAP) and acetyl-P (Fig. 9.1). From pentoses one NADH is produced per sugar during conversion to pyruvate, which is used as the acceptor for NADH reoxidation; the acetyl-P is converted to acetate. Hexoses are first oxidized to pentoses (Figs. 9.1 and 9.2), yielding two extra NAD(P)H, followed by one further NADH derived from pentose degradation to pyruvate. Formally, the latter NADH (equivalent to the NADH from GAP oxidation) is reoxidized by reduction of pyruvate, similar to growth on pentoses. The extra two NAD(P)H are loaded onto acetyl-P (or acetyl-CoA) with the formation of ethanol (ethanol pathway) instead of acetate. Shifting from pentoses to hexoses causes a drop in the growth rate by a factor of approximately three (Richter et al. 2003a; Zaunmüller et al. 2006), and a decrease in growth yields by a factor of about two in agreement with the lower ATP yield (two ATP/pentose versus one ATP/hexose).

The slow growth on glucose is caused by the low activity of the ethanol pathway (Maicas et al. 2002; Richter et al. 2001, 2003a) compared to high activities of the enzymes of the phosphoketolase pathway, e.g. glucose-6P dehydrogenase (Richter et al. 2001). The acetaldehyde dehydrogenase activity of the bifunctional acetaldehyde/ethanol dehydrogenase (AdhE) is low and becomes limiting under HSCoA (and acetyl-CoA) limitation. Shortage of D-pantothenate, an essential growth factor and precursor for HSCoA synthesis in *O. oeni* and other LAB (Garvie 1967; http://jgi.doe.gov/), reduces the HSCoA contents (Richter et al. 2001). The bacteria contain the enzymes for the conversion of D-pantothenate to HSCoA but not for the synthesis of D-pantothenate from central intermediates (Zaunmüller et al. 2006; http://jgi.doe.gov/). The ethanol pathway appears to be the first site responding to D-pantothenate limitation (Richter et al. 2001). 4′-O-(β-D-glucopyranosyl)-D-pantothenic acid, which is found in tomato and fruit juice, was suggested earlier as the source ('tomato juice growth factor') for synthesis (Amachi et al. 1970), but free D-pantothenate has the same biological activity (Zaunmüller et al. 2006). During growth on pentoses, when the ethanol pathway is not required, D-pantothenate depletion has no significant effect on the fermentation pattern.

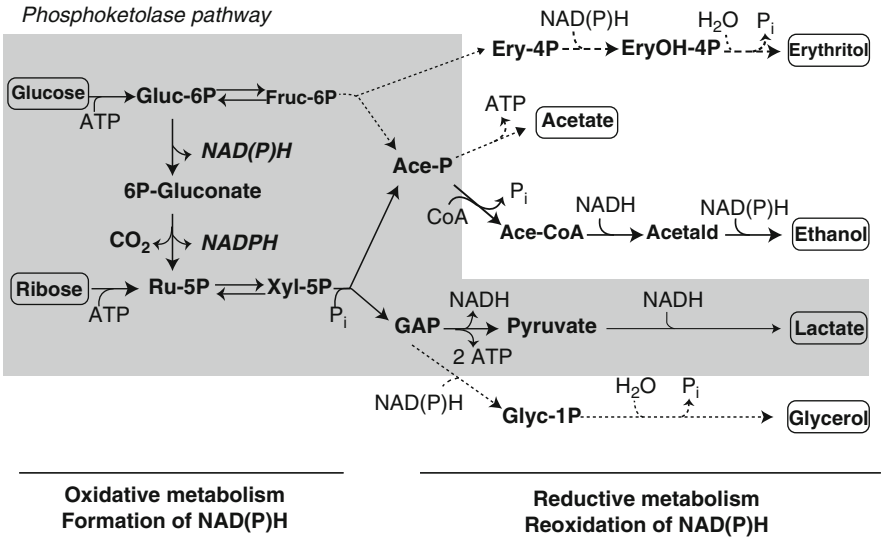

Fig. 9.1 Fermentation of hexoses and pentoses by the phosphoketolase (or oxidative pentose-P) pathway of *O. oeni*. The central phosphoketolase pathway and lactate formation (NAD(P)H reoxidation) which are constant parts of the metabolism are *boxed* (*grey* background). The major routes resulting in the formation of lactate and ethanol are shown with *solid lines*, alternative pathways resulting in the formation of erythritol, acetate and glycerol with *broken lines*. Phosphoketolase (Xfp, genes OEOE_1812 and OEOE_1183 of *O. oeni* PSU-1) cleaves pentose-5P (xylulose-5P) or fructose-5P. Acetaldehyde and ethanol dehydrogenase reactions are catalysed by the bifunctional AdhE enzyme (Koo et al. 2005). The genes encoding the enzymes of the phosphoketolase pathway and for ethanol and lactate formation are given by Mills et al. (2005), Zaunmüller et al. (2006) and in http://jgi.doe.gov/. The genome contains candidate genes for glycerol-1P dehydrogenase (gene OEOE_0562) and glycerol-1P phosphatase (gene OEOE_0563, annotated as Hpr kinase/phosphorylase). *Non-standard abbreviations*: *Ery-4P* erythrose-4 phosphate; *EryOH-4P* erythritol-4P; *Ace-P* acetylphosphate; *Ace-CoA* acetyl-CoA; *Acetald* acetaldehyde; *Glyc-1P* glycerol-1 phosphate

9.2.2 Endogenous Alternative Pathways for [H] Reoxidation

The limitation in the ethanol pathway results in a partial shift to alternative pathways for NAD(P)H reoxidation (Veiga-Da-Cunha et al. 1992, 1993; Richter et al. 2001) (Fig. 9.1). Part of the extra NAD(P)H is consumed by reduction of erythrose-4P to erythritol-4P and erythritol by *O. oeni* and other heterofermentative LAB (Veiga-Da-Cunha et al. 1993; Stolz et al. 1995; Richter et al. 2001). Erythrose-4P is derived from fructose-6P by phosphoketolase Xfp. Phosphoketolase Xfp of *O. oeni* and *Bifidobacterium* accepts fructose-6P in addition to xylulose-5P (Veiga-Da-Cunha et al. 1993; Meile et al. 2001; Mills et al. 2005; Yin et al. 2005). The activity of the erythritol pathway is low and does not increase the rate of glucose fermentation significantly. Under pantothenate limitation or in resting cells up to 0.2 mol, erythritol is formed per mol glucose. The enzymes (or corresponding structural genes) for the conversion of erythrose-4P to

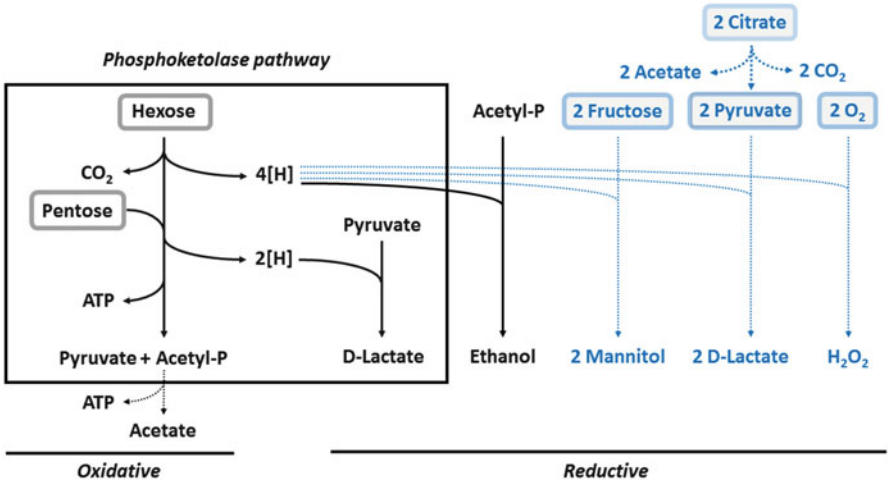

Fig. 9.2 Alternative routes for the reoxidation of NAD(P)H by *O. oeni* during growth on hexoses in the presence of external electron acceptors (fructose, pyruvate, citrate, O_2). The extra 2 NAD(P)H or 4 [H] which are derived from the oxidation of hexose to pentose in the phosphoketolase pathway can be transferred to the external acceptors (*blue*, *broken lines*). The oxidative (NAD(P)H producing) and reductive (NAD(P)H consuming) parts of metabolism are indicated. The following fermentation balances are found for growth on glucose (idealized reactions): (**i**) glucose → 1 lactate + 1 EtOH + 1 CO_2; (**ii**) glucose + 2 fructose → 1 lactate + 1 acetate + 2 mannitol + 1 CO_2; (**iii**) glucose + 2 pyruvate → 3 lactate + 1 acetate + 1 CO_2; (**iv**) glucose + 2 citrate → 3 lactate + 3 acetate + 3 CO_2; (**v**) glucose + 2 O_2 → 1 lactate + 1 acetate + 1 CO_2 + 2 H_2O_2

erythritol have not been identified in the genomes of *O. oeni* and *Leuconostoc mesenteroides* (*Lc. mesenteroides*) (Zaunmüller et al. 2006).

Glycerol is a further minor product of NAD(P)H reoxidation. It is obtained by reduction of GAP to glycerol-1P followed by dephosphorylation (Fig. 9.1) (Veiga-Da-Cunha et al. 1993). The genome of *O. oeni* contains candidate genes for glycerol-1P dehydrogenase and phosphatase (http://jgi.doe.gov/) (Fig. 9.1). Biochemically, the reactions for erythritol and glycerol formation are similar, and erythritol might be formed by the enzymes of the glycerol pathway.

When fructose is used as the substrate for growth, part of the fructose is used as an electron sink, resulting in mannitol formation (see following section).

9.2.3 Glucose Transport

The genome of *O. oeni* encodes 40 genes for secondary carriers, 8 complete phosphotransferase systems and 7 complete ABC transport systems that could be involved in the transport of sugar or sugar alcohols as concluded from sequence similarities (Mills et al. 2005; Unden and Zaunmüller 2009; Zaunmüller and Unden 2009). The large number of sugar transporters is supposed to reflect the preference

of *O. oeni* for sugars as the substrate for growth and the large variety of (minor) sugars present in must and wine, including the mixture of sugars released by glycosidases (see above).

O. oeni takes up glucose at a broad pH range from 4 to 9 with maxima at pH 5.5 and 7. Transporters 1574 and 0819 represent secondary transporters for glucose which were able to restore growth of hexose-deficient mutants of *B. subtilis* on glucose but not on fructose (Kim et al. 2011). Transport by the carriers was driven by the proton potential Δp. In addition a PTS phosphotransferase transport system (permease protein 0464) appeared to be responsible for the Δp-independent hexose transport at neutral and acidic pH. Expression of the genes is induced by glucose and fructose (Kim et al. 2011).

9.3 Modified Hexose Fermentation by the Use of External Electron Acceptors

9.3.1 The Use of External Electron Acceptors

Pyruvate, citrate, O_2 or fructose can be used by *O. oeni* as external acceptors for reoxidation of the extra NAD(P)H from hexose oxidation (Fig. 9.2) and replace then to various extents the ethanol pathway in NAD(P)H reoxidation. The external acceptors are more efficient electron acceptors than acetyl-CoA, and the acetyl-P is used then for ADP phosphorylation and excreted as acetate. As a consequence the ATP and molar growth yields increase by a factor of up to 2 per glucose (Zaunmüller et al. 2006). In addition, the growth rates increase by factors of 2–3 and approach those of ribose.

In this way, externally supplied pyruvate is reduced by the highly active lactate dehydrogenase to D-lactate (Nuraida et al. 1992; Richter et al. 2003a). Citrate is used in a similar way after its conversion to pyruvate by citrate lyase and oxaloacetate decarboxylase Mae (see Fig. 9.3) (Salou et al. 1994; Stolz et al. 1995; Hache et al. 1999). Molecular O_2 is reduced by an oxidase to H_2O_2 (Maicas et al. 2002) and fructose to mannitol (Salou et al. 1994; Richter et al. 2003a, b). During co-fermentation of fructose with glucose by *O. oeni*, fructose is used essentially as an electron acceptor and excluded from the phosphoketolase pathway, whereas glucose is channelled to the phosphoketolase pathway (Richter et al. 2003b).

When fructose is supplied as the only substrate, it is metabolized by the phosphoketolase pathway or by combined action of the phosphoketolase and mannitol pathway (Richter et al. 2003a, b). With limiting supply of fructose, most of the fructose is fermented by the phosphoketolase pathway similar to glucose. At high fructose concentrations and in resting cells, up to two thirds of the fructose are used as electron acceptor, and large amounts of mannitol are produced. Channelling of fructose in either pathway is regulated at the level of phosphoglucose isomerase (Richter et al. 2003b).

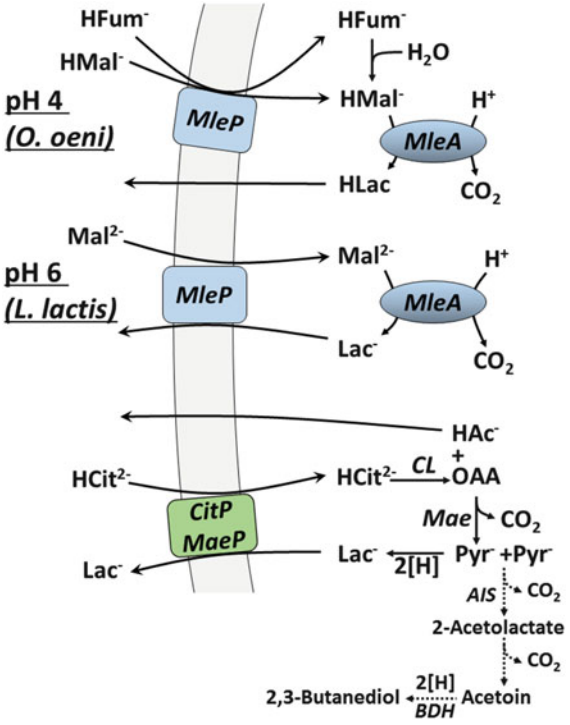

Fig. 9.3 Pathways and carriers for the fermentation of malate, citrate and fumarate by *O. oeni*, *Lc. mesenteroides* and *Lactobacillus lactis*. Important enzymes and carriers (*MleP* malate carrier; *MleA* malolactic enzyme; *CitP* or *MaeP* citrate/lactate antiporter; *Mae* oxaloacetate decarboxylase; *CL* citrate lyase; *AlS* acetolactate synthase; *BDH*, butanediol dehydrogenase) and intermediates (*HMal*$^-$ malate anion; *Mal*$^{2-}$ malate dianion; *Hlac* lactic acid; *HAc* acetic acid; *OAA* oxaloacetate; *Pyr*$^-$ pyruvate; *Lac*$^-$ lactate) are indicated. The pathway for fumarate fermentation (*broken lines*) is hypothetical. For details see text

Overall, growth of *O. oeni* and other heterofermentative LAB on hexoses is stimulated by the presence of electron acceptors which provide a bypass to NAD(P)H reoxidation by the limiting ethanol pathway (Richter et al. 2001; Maicas et al. 2002; Zaunmüller et al. 2006). The erythritol and glycerol pathways are of limited capacity, whereas the pathways using the external acceptors O_2, pyruvate, citrate and fructose have a much higher capacity and increase the growth rate of the bacteria significantly. The pathways for NAD(P)H reoxidation are cytoplasmic without involvement of electron transport or generation of a proton potential.

9.3.2 *Biotechnological Production of Polyols by Heterolactic Acid Bacteria*

The sugar alcohols mannitol and erythritol are widely used in food, pharmaceutical and chemical industry. Heterofermentative lactic acid bacteria like *Lc. mesenteroides or Lc. pseudomesenteroides*, close relatives of *O. oeni*, are good producers of the compounds, in particular mannitol (Otgonbayar et al. 2011; Saha and Racine 2011; Ortiz et al. 2013), and can serve as an alternative to industrial chemical production. The bacteria convert fructose from a glucose/fructose mixture

nearly completely to mannitol by the pathways discussed above when appropriate culture conditions are applied. Fructose can also be produced enzymatically in situ by mannitol dehydrogenase and NADH or NADPH which are regenerated enzymatically (Saha and Racine 2011; Hahn et al. 2003; Ortiz et al. 2013).

9.4 Fermentation of Organic Acids

Fermentation of organic acids plays an important role in the energy metabolism and physiology of heterofermentative LAB like *O. oeni* (Radler 1958, 1966; Radler and Brohl 1984; Stolz et al. 1995). From the organic acids present in grapes, citrate and malate are metabolized by many LAB, including *O. oeni* and *Lc. mesenteroides* but fumarate, tartrate and pyruvate only by a limited number. Citrate is used in co-fermentation with hexoses, whereas externally supplied malate, pyruvate and L-tartrate can be metabolized without the need for a co-substrate. However, pyruvate appears to be the only organic acid that supports substantial growth of *O. oeni* when it is present as the sole substrate.

9.4.1 Malate (or 'Malolactic') Fermentation

Fermentation of malate (L-malate → L-lactate + CO_2) by heterofermentative LAB is of physiological significance in wine and fruit juice which contain high amounts of this C_4-dicarboxylic acid. The malolactic enzyme (Caspritz and Radler 1983) catalyses the key reaction (L-malate → CO_2 + L-lactate). The free energy of the reaction is conserved by a chemiosmotic mechanism (Salema et al. 1996) which depends on an electrogenic malate transport (Poolman et al. 1991; Lolkema et al. 1995; Konings 2002) (Fig. 9.3). In *O. oeni* (growing around pH 4) the transport is catalysed by a carrier-mediated uptake of monoanionic malate versus a carrier-independent efflux of lactic acid. In *Lactococcus lactis* growing at less acidic conditions, the transport is mediated by the malate^{2-}/lactate^{-} antiporter. Both transport processes result in the net translocation of one charge per malate and energization of the membrane. In addition, one proton is consumed by the decarboxylation in the cytoplasm, generating a ΔpH. The proton motive force (1 H^+/malate) derived from both processes is used by the bacteria for maintenance of pH homeostasis and for the uptake of nutrients. Malolactic fermentation stimulates growth of the bacteria but is apparently not sufficient to support growth as the only energy source (Pilone and Kunkee 1976; Salema et al. 1996). The reaction results in raising the pH of the medium by conversion of a divalent to a monovalent carboxylic acid. The process is used in winemaking by applying starter cultures or by spontaneous fermentation of *O. oeni* in wine or must (Lonvaud-Funel 1999; Liu 2002; Coucheney et al. 2005; Mills et al. 2005; Moreno-Arribas and Polo 2005).

9.4.2 Citrate Fermentation

Many LAB including *O. oeni* and *Lc. mesenteroides* use citrate as an electron acceptor in co-metabolism with sugars. Sugars such as glucose, fructose, lactose or xylose provide NADH for the degradation of the citrate (citrate + 2 [H] → lactate + acetate + CO_2) (Drinan et al. 1976; Starrenburg and Hugenholtz 1991; Salou et al. 1994; Schmitt et al. 1997; Hache et al. 1999) (see Fig. 9.2). Some other LAB are able to grow on citrate as the sole substrate (Medina de Figueroa et al. 2000). Transport of citrate and of the products lactate and acetate plays an important role in citrate fermentation (Fig. 9.3). The secondary transporters CitP or MaeP catalyse an electrogenic precursor/product (Hcitrate^{2-}/lactate^{-}) exchange resulting in an electrochemical gradient over the membrane (Ramos et al. 1994; Marty-Teysset et al. 1995, 1996; Konings 2002). The electrochemical gradient is not sufficient to support growth of the bacteria on its own.

Citrate is cleaved by citrate lyase, and the resulting oxaloacetate is then decarboxylated to pyruvate by a cytoplasmic oxaloacetate decarboxylase which is related to (soluble) malate decarboxylase (Marty-Teysset et al. 1996; Sender et al. 2004; Mills et al. 2005). In the presence of sufficient NADH (e.g. from hexose oxidation), most of the pyruvate is reduced to lactate (Ramos et al. 1994) which drives the citrate/lactate antiport (Salou et al. 1994; Konings 2002). Part of the pyruvate is condensed and converted to acetoin and 2,3-butanediol (Fig. 9.3) (Ramos et al. 1994; Nielsen and Richelieu 1999). Chemical (non-enzymatic) oxidation of acetoin by O_2 yields diacetyl. The acetoin pathway is significant in LAB, when the bacteria are incubated with citrate in the absence of other carbon sources. Diacetyl, a flavour compound in products treated by LAB, is tolerated in wine only in low concentrations (Schmitt et al. 1997; Nielsen and Richelieu 1999; Bartowsky and Henschke 2004; Mills et al. 2005).

The *Cit* gene clusters of *Lc. mesenteroides* and *O. oeni* comprise genes for citrate lyase (*citDEF*), citrate lyase ligase (*citC*), oxaloacetate decarboxylase (*mae* gene) and the citrate carrier (*maeP* or *citP*) (Martin et al. 2000; Mills et al. 2005). The clusters contain in addition the *citX and citG* genes which are homologous to the corresponding genes of *Klebsiella* for the synthesis of the phosphoribosyl-dephospho-SCoA prosthetic group of citrate lyase (Schneider et al. 2002). Genes for acetolactate synthase and acetolactate decarboxylase are present in *O. oeni* and *Lc. mesenteroides* (Fig. 9.3).

9.4.3 Pyruvate Fermentation

Pyruvate can be used as an electron acceptor for NAD(P)H reoxidation but supports also growth of *O. oeni* and *Lc. mesenteroides* as the sole substrate (Wagner et al. 2005):

$$2\ \text{pyruvate} + H_2O \rightarrow \text{acetate} + CO_2 + \text{lactate}\left(\Delta G'_0 = -95.1\ \text{kJ/reaction}\right)$$

The growth rates by pyruvate fermentation are comparable to those for glucose fermentation. Pyruvate is decarboxylated by pyruvate dehydrogenase (PDH) to acetyl∼CoA and NADH. Acetyl∼CoA is used for ATP formation (via acetyl∼P), and the NADH is transferred by lactate dehydrogenase to a second molecule of pyruvate. *O. oeni* and *Lc. mesenteroides* contain a gram-positive type PDH which is annotated as acetoin/pyruvate dehydrogenase complex. PDH is regarded as a typical enzyme of aerobic metabolism, but alternative anaerobic enzymes like pyruvate: ferredoxin oxidoreductase, pyruvate decarboxylase and pyruvate formate lyase are not encoded by the bacteria (Wagner et al. 2005; http://jgi.doe.gov/). NADH produced by PDH is reoxidized by lactate dehydrogenase using a second molecule of pyruvate.

9.4.4 Fumarate Fermentation

The small amounts of fumarate present in wine or must are degraded by yeast after conversion to malate or succinate (Radler 1986). Fumarate inactivates LAB from wine, but malate-fermenting bacteria are able to degrade fumarate to lactate and CO_2 when incubated with the organic acid (Radler 1986). It was suggested that fumarate is hydrated by the bacteria to malate and decarboxylated by the malolactic enzyme to lactate and CO_2. The genome of *O. oeni* PSU-1 encodes a fumarase (gene OEOE_0029, http://jgi.doe.gov/); therefore all enzymes of the postulated pathway appear to be present in the bacterium. Fumarate uptake might be affected by the MleP transporter since DctA-, Dcu- or DauA-type C_4-dicarboxylate transporters are not encoded by *O. oeni*. See Fig. 9.3 for the hypothetical pathway.

9.4.5 L-Tartaric Acid Fermentation

L-Tartaric acid is degraded only under specific conditions after degradation of the other organic acids. Only few LAB are able to metabolize L-tartrate (Radler and Yannissis 1972), and tartrate degradation is found only in spoilt wine. *Lb. plantarum* degrades L-tartrate by a dehydratase to oxaloacetate which is decarboxylated to pyruvate (Fig. 9.4). In a reaction similar to pyruvate fermentation of *O. oeni*, half of the pyruvate is converted to acetate + CO_2, the other half to lactate. Tartrate fermentation does not support growth, although the pathway is supposed to allow ATP formation via acetyl-P. The genome of *Lb. plantarum* contains structural genes for L-tartrate dehydratase TtdAB, oxaloacetate/malate decarboxylase Mae and pyruvate dehydrogenase (http://img.jgi.doe.gov/ and

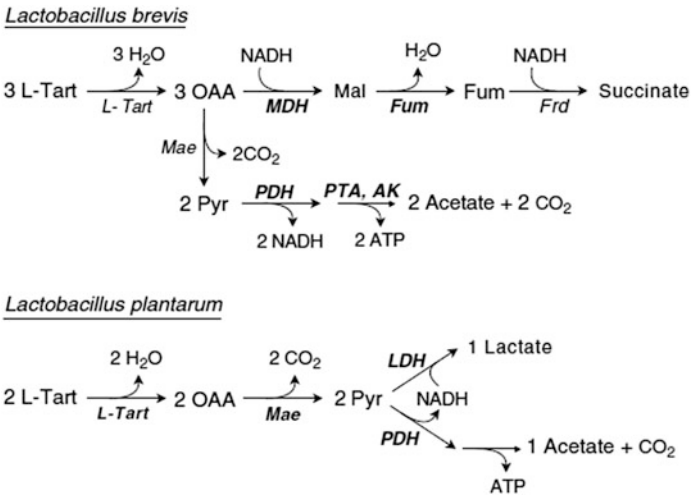

Fig. 9.4 Degradation of L-tartrate by *Lb. brevis* (3 L-tartrate → 1 succinate + 2 acetate + 4 CO_2) and *L. plantarum* (2 L-tartrate → acetate + lactate + 3 CO_2). *L-Tart* L-tartrate dehydratase, *MDH* malate DH, *Fum* fumarase, *Frd* fumarate reductase, *Mae* oxaloacetate decarboxylase, *PDH* pyruvate dehydrogenase. Enzymes for which the corresponding structural genes were not identified in the genomes of *Lb. brevis* and *Lb. plantarum* are printed in *normal type*, others in ***bold** type*. The enzymes and corresponding genes for *Lb. brevis* are *MDH* (LVIS_ 1406), *Fum* (LVIS_0714), *PDH* (genes LVIS_1407, 1408, 1409, 1410), *Pox* (LVIS_0313), phosphotransacetylase (LVIS_0674), and acetate kinase (LVIS_129, 1601, 1190) and for *Lb. plantarum* are *L-Tart* (LP_1090 and 1089 for *TtdAB*), *Mae* (LP_1105), *PDH* (LP_2151 to 2154), and *LDH* LP_2057 (D-lactate dehydrogenase). Abbreviations for substrates and intermediates: *L-Tart* T-tartrate, *OAA* oxaloacetate, *Mal* L-malate, *Fum* fumarate, *Pyr* pyruvate. Pathways according to Radler and Yannissis (1972)

Fig. 9.4). The pathway is different from L-tartrate fermentation found in bacteria such as *E. coli* which is linked to fumarate respiration (Kim and Unden 2007).

L-Tartrate fermentation by *Lb. brevis* and other heterofermentative *Lactobacillus* species occurs by a branched pathway (Radler and Yannissis 1972). Two thirds of the tartrate is fermented to acetate and CO_2 as described for *Lb. plantarum*. The residual L-tartrate is converted from oxaloacetate via malate and fumarate to succinate, similar to L-tartrate fermentation by *E. coli*. By combining a NADH-supplying and a NADH-consuming branch, the overall redox reactions are balanced (Fig. 9.4). The enzymes have not been demonstrated in detail for *Lb. brevis*, but the genome of *Lb. brevis* ATCC 367 contains structural genes for a fumarase (gene LVIS_0714), pyruvate dehydrogenase (genes LVIS_1407-1410), pyruvate oxidase (gene LVIS_0313) and a potential malate dehydrogenase (gene LVIS_1406 annotated as malate/lactate dehydrogenase). Genes for L-tartrate dehydratase, oxaloacetate decarboxylase and fumarate reductase have not been identified yet.

9.5 Conclusion

O. oeni and other lactic acid bacteria from wine are able to grow at the expense of sugars (hexoses and pentoses) using the phosphoketolase pathway. Genes, enzymes and important characteristics of the pathway are known, whereas the genes and (most of) the enzymes of erythritol formation are unknown. Fermentation of hexoses is limited by slow reoxidation of NAD(P)H in the ethanol branch. Consequently turnover of the hexoses is stimulated in the presence of substrates (fructose, citrate, pyruvate, O_2) which are able to reoxidize NAD(P)H, resulting in a shift of the fermentation pattern. *O. oeni* degrades also organic acids from wine or must (citrate, malate and pyruvate) by pathways for which enzymes and structural genes are known. The regulation underlying the use of alternative substrates (sugars, organic acids and electron acceptors) is largely unknown. Some heterofermentative lactic acid bacteria from wine metabolize L-tartrate and fumarate by reactions which have been characterized only in parts. The latter pathways appear to be strain-specific, and some of the postulated genes or enzymes were not identified in the genomes of all *O. oeni* strains (Borneman et al. 2012). Identification of the unknown genes, enzymes and carriers for the alternative substrates and regulation of the fermentation pathways remain an important goal for understanding energy metabolism of *O. oeni* (and other lactic acid bacteria from wine and must) and their adaptation to the physiological conditions for survival in wine.

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Chapter 10
Amino Acid Metabolisms and Production of Biogenic Amines and Ethyl Carbamate

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10.1 Introduction

In winemaking process, a wide range of volatile and non-volatile compounds originate from microbial catabolism of amino acids. Among these catabolites, biogenic amines, low molecular weight organic bases produced by decarboxylation of their respective free precursor amino acids, are receiving much attention in wine science because of their potential implication for human health. This chapter, after a brief overview on the occurrence of biogenic amines in wines, deals with the role played by yeasts and lactic acid bacteria in the formation and accumulation of these molecules during winemaking, giving emphasis to the most frequently found amines (histamine, tyramine and putrescine) and their physiological significance in bacterial cells. Moreover, the most suitable methods to detect biogenic amine-producing lactic acid bacteria or to quantify biogenic amine in wine as well as strategies to reduce biogenic amine content in wine are reported. Finally, a note on the formation of ethyl carbamate, a carcinogen compound originating in wine through a non-enzymatic reaction between ethanol and microbial catabolites containing a carbamyl group, is furnished.

The amino acid composition of a grape must is acknowledged to have noticeable implications with regard to quality and safety of the resulting wine, because both pleasant and unpleasant or even quite undesirable compounds can be produced by the microbiota participating in the winemaking process—yeasts and lactic acid bacteria (LAB)—especially as a consequence of their catabolic activity on the available amino acids. Yeasts and LAB differ both qualitatively and quantitatively

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in the utilization of assimilable nitrogen: while yeasts are able to utilize ammonia and amino acids, LAB synthesize amino acid only from organic nitrogen and possess peptidase activities. However, yeasts as well as LAB show a wide variability at species and strain level in terms of capability to utilize nitrogen sources (Kemsawasd et al. 2015; Remize et al. 2006). Microbial catabolism of amino acids mainly occurs through the activities of five groups of enzymes (i.e. aminotransferases, decarboxylases, dehydratases, lyases and deaminases) which, intracellularly, convert amino acids into a range of volatile and non-volatile compounds, such as α-keto acids, aldehydes, hydroxy acids, alcohols and amines, all playing an important role in determining the organoleptic qualities of wine. Among these catabolites from amino acids, the latter class of compounds, often referred to as biogenic amines (BA) to underline their main biological origin, is receiving much attention in wine science because of their potential implication for human health (Silla Santos 1996; EFSA 2011; Alvarez and Moreno-Arribas 2014).

BA are nitrogenous low molecular weight organic bases that can have an aliphatic, an aromatic or a heterocyclic structure and are widely present in foods, especially in fermented foods, mostly as a consequence of the decarboxylation of their respective free precursor amino acids, through the action of substrate-specific microbial decarboxylases. Hence, the amines histamine, tyramine, putrescine, cadaverine, 2-phenethylamine, agmatine and tryptamine originate from the precursor amino acids histidine, tyrosine, ornithine, lysine, phenylalanine, arginine and tryptophane, respectively.

Other amines, possibly present in fermented foods and wines, include the aliphatic volatile amines (methylamine, ethylamine and isoamylamine), which can be originated by the amination of nonnitrogenous compounds, such as aldehydes and ketones (Bauza et al. 1995), and the polyamines, spermine and spermidine, which can be produced from putrescine (1,4-diaminobutane), through methylation reactions involving *S*-adenosyl methionine.

As mentioned above, all these molecules have the potential to cause physiological distress in the human organism if ingested in relatively high concentration by sensitive people. More specifically, the heterocyclic amine histamine, the most toxic and studied biogenic amine, may induce headaches, hypotension, heart palpitation and cutaneous and gastrointestinal disorders; the aromatic amines, tyramine and 2-phenethylamine (a volatile amine), are known to cause migraines and hypertensive crises because they may originate the vasoconstrictor hormones noradrenaline and norephedrine; the polyamines (putrescine, agmatine, cadaverine, spermine and spermidine), although not toxic themselves, potentiate the effects of the toxic amines, being also able to inhibit enzymes such as the amino oxidases, catalysing the oxidative deamination of amines, constituting the main detoxifying system of BA in humans (ten Brink et al. 1990); the volatile monoamines, in spite of their poor physiological significance, are active as irritants and thus may negatively affect the sensorial profile of foods (Lehtonen 1996). Recently, the Biological Hazards (BIOHAZ) Panel of the European Food Safety Authority (EFSA) carried out a qualitative risk assessment of BA in fermented foods, using data from the

scientific literature, as well as from European Union-related surveys, reports and consumption data (EFSA 2011). The BIOHAZ Panel concluded that the present knowledge and data on toxicity of BA, individually and in combination(s), are limited and insufficient to carry out a quantitative risk assessment; nevertheless, histamine and tyramine are considered as the most toxic and particularly relevant biogenic amines for food safety. Based on the mean content of these amines, wine belongs to the fermented food categories showing the lowest values. However, it is to underline that ethanol, occurring in wine, is an inhibitor of amine oxidases, the enzymes that normally detoxify the BA in humans.

Fresh musts usually contain low levels of BA, almost entirely represented by spermidine and putrescine. This diamine that in plants is implicated in many physiological processes (Halász et al. 1994; Agudelo-Romero et al. 2013) is reported to be synthesized by the vine in response to stress conditions, such as a potassium deficiency in the vineyard soil, and then accumulated in the grapes.

Wines are usually characterized by a significantly higher content of BA than their respective fresh musts, red wines being generally characterized by BA content significantly higher than white wines (Table 10.1). The phenomenon was unambiguously ascribed to the fact that the winemaking process for red wine production usually includes a secondary transformation, widely known as malolactic fermentation (MLF) that does not occur or is not essential in white wine production. Consequently, BA presence in wine has been roughly considered as a consequence of MLF, but the matter also includes contradictory data.

In any case, it is without any doubt that BA formation in wine requires the presence of both precursor amino acids and microorganisms with amino acid decarboxylase activity, besides environmental conditions allowing microbial growth and enzyme activity.

Among the factors that have been suggested as favouring the abundance of amines in wine, some winemaking practices, such as nutrient addition and duration of wine contact with both grape skins and yeast lees, seem to play a major role because they can directly affect the content of the precursor amino acids of BA. However, it is worth mentioning that the amount of total amino acids in fresh grape musts generally is several dozen-fold higher than the total amount of amines in wines, so that it is difficult to imagine winemaking conditions where precursor amino acids are quite absent. Therefore, if the presence of available precursors is practically unavoidable, BA accumulation in wine is strongly related to the microbial ecology of wine fermentations, characterized by a complex mixture of different species and strains of yeasts and lactic acid bacteria (LAB).

This chapter will focus on the current knowledge on yeast and LAB capabilities to decarboxylate amino acids into BA and will give up to date information on accumulation of these molecules during winemaking, with an additional note on ethyl carbamate, a carcinogen compound originating in an alcoholic environment from precursors produced by microbial catabolism of amino acids.

Table 10.1 Concentration of the major biogenic amines found in wine, according to recent publications (values reported as range, minimum–maximum, or as mean ± SD)

Wine (origin)	n^a	Histamine (mg L^{-1})	Tyramine (mg L^{-1})	Putrescine (mg L^{-1})	References
White (Greece)	47	nd−5.95	nd−2.54	nd−3.22	Soufleros et al. (2007)
White (Greece)	17	0.34–1.13	nd–1.16	nd–9.07	Proestos et al. (2008)
Red (Greece)	45	nd−2.11	nd−3.65	nd−5.23	Soufleros et al. (2007)
Red (Greece)	15	0.98–1.65	nd–0.46	nd–2.70	Proestos et al. (2008)
Rosé (Greece)	8	nd−4.43	nd−1.64	nd−1.85	Soufleros et al. (2007)
White (Italy)	7	nd	nd–tr	1.48–2.48	Tuberoso et al. (2015)
White (Italy)	5	nd	nd–0.9	1.2–1.6	Manetta et al. (2016)
Red (Italy)	33	6.1 ± 5.3	3.5 ± 2.8	15.5 ± 17.5	Mangani et al. (2006)
Red (Italy)	8	tr–8.11	5.08–11.5	11.4–32.8	Tuberoso et al. (2015)
Red (Italy)	10	nd–1.02	0.67–1.97	3.76–11.13	Preti et al. (2015)
Red (Italy)	60	tr–3.16	0.22–34.99	0.84–25.40	Preti et al. (2016)
Red (Italy)	15	nd–0.9	nd–0.9	4.5–16.1	Manetta et al. (2016)
Red (Spain)	224	4.46 ± 5.7	3.13 ± 4.42	6.05 ± 10.6	Marcobal et al. (2006)
Red (Spain)	543	nd–25.1	–	–	Meléndez et al. (2016)
Red (Spain)	14	nd–18.7	1.1–17.8	7.6–35.7	Arrieta and Prats-Moya (2012)
Red (Spain)	36	nd–6.2	0.10–1.4	2.4–25.1	Martínez-Pinilla et al. (2013)
Red (Turkey)	30	nd–1.97	nd–0.29	nd	Anli et al. (2004)
Red (Spain)	6	2.75 ± 1.54	2.91 ± 1.92	9.59 ± 5.61	Romero et al. (2002)
Rosè (Spain)	7	1.81 ± 1.31	1.31 ± 0.71	6.04 ± 2.98	Romero et al. (2002)
Rosè (Spain)	49	nd–13.4	–	–	Meléndez et al. (2016)
White (Spain)	6	1.17 ± 0.99	0.48 ± 0.67	4.31 ± 4.21	Romero et al. (2002)
White (Spain)	92	nd–12.5	–	–	Meléndez et al. (2016)
Red (USA)	59	5.4 ± 5.6	1.3 ± 2.3	20.9 ± 32.3	Glória et al. (1998)
Red (France)	54	6.7 ± 13.5	3.7 ± 2.3	10.8 ± 6.7	Bauza et al. (1995)
Red (France)	84	nd–14.05	nd–12.35	3.71–48.72	Bach et al. (2012)

(continued)

Table 10.1 (continued)

Wine (origin)	n^a	Histamine (mg L^{-1})	Tyramine (mg L^{-1})	Putrescine (mg L^{-1})	References
Rosè (France)	15	22.0 ± 7.3	2.3 ± 1.7	2.5 ± 0.9	Bauza et al. (1995)
White (France)	15	3.7 ± 8.9	2.2 ± 1.4	1.9 ± 0.7	Bauza et al. (1995)

[a]Number of samples
nd not detectable, *tr* below the limit of quantification

10.2 Biogenic Amine Production by Wine Yeasts

Although yeasts have been long considered among the possible biological agents of BA formation in wine, their capability to decarboxylate precursor amino acids has been poorly investigated. If volatile amines, mainly synthesized from nonnitrogenous compounds, are excluded, the few available papers agree in indicating that BA production by wine yeasts is a practically negligible phenomenon, the concentration of most amines being at non-detectable or very low levels (Torrea and Ancin 2001; Caruso et al. 2002; Landete et al. 2007a). Nevertheless, some published results are, in some cases, contradictory.

According to the findings of Landete et al. (2007a), none of the studied yeast strains (belonging to different non-*Saccharomyces* species as well as to *Saccharomyces cerevisiae*) was able to produce at least one of the assayed amines (histamine, tyramine, 2-phenethylamine, putrescine, cadaverine and tryptamine), in both synthetic medium and grape must. On the contrary, according to a previous paper (Caruso et al. 2002), several strains of wine yeasts, assayed in sterilized grape must under laboratory conditions, showed a diffuse capability to produce 2-phenethylamine and agmatine, at concentrations variable within each species from non-detectable to more than 10 mg L^{-1}. In particular, both these amines were produced by strains of *Kloeckera apiculata*, *Metschnikowia pulcherrima*, *Brettanomyces bruxellensis* and *Saccharomyces cerevisiae*, whereas some strains of *Starmerella bacillaris*, synonym *Candida zemplinina* (strains reported as *Candida stellata* in the original paper), proved to be able to produce only agmatine. Moreover, also a commercial strain of *S. cerevisiae* was proved to be able to produce both histamine and tyramine in a Chardonnay fermentation (Medina et al. 2013). In spite of the contradictory data on BA-producing capability of wine yeasts, possible consequence of a high strain-dependent variability of this metabolic feature, agmatine formation deserves some comments. This polyamine should be produced by decarboxylation of arginine, one of the most abundant amino acids of grape musts and an important nitrogen source for yeast growth. However, in all known yeasts, arginine breakdown usually proceeds through the arginase pathway. According to this metabolic route, arginine, transported into the cell through specific and/or general amino acid permeases, is cleaved by arginase into ornithine and urea, which are further metabolized or, at least in the case of urea,

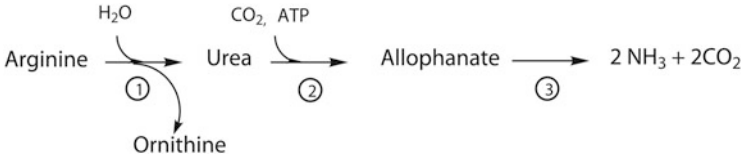

Fig. 10.1 Pathway of arginine breakdown by wine yeasts. (1) Arginase, (2) urea carboxylase and (3) allophanate hydrolase

excreted (Fig. 10.1). Hence, agmatine production from arginine should represent an alternative route for arginine catabolism in yeast cells, but no information is available on the regulatory mechanism of this metabolic pathway as well as on its physiological significance.

10.3 Biogenic Amine Production by Lactic Acid Bacteria

BA accumulation in wine was long considered as index of poor hygiene in the winery and/or the result of wine spoilage from strains of lactic acid bacteria belonging to the genera *Pediococcus*, *Lactobacillus* and *Leuconostoc*. Indeed, several strains of different species of these three genera, isolated from BA-containing wines, proved to be able to produce BA in the presence of their precursor amino acids (Landete et al. 2007a; Moreno-Arribas et al. 2003; Rosi et al. 2009; Henríquez-Aedo et al. 2016), *Pediococcus* strains being long considered among the major responsible for histamine accumulation in wine. However, it was soon demonstrated that also *Oenococcus oeni*, the bacterial species most commonly found in wines and most frequently associated with malolactic fermentation (MLF), was capable to decarboxylate histidine to histamine (Lonvaud-Funel and Joyeux 1994). Furthermore, several *O. oeni* strains, isolated from different Italian wines and assayed in synthetic media under laboratory conditions, demonstrated to be qualitatively and quantitatively variable in their capability to produce BA: more than 60% of the 44 assayed strains was able to produce histamine, at concentrations ranging from 1.0 to 33 mg L^{-1}, and about 16% showed the additional capability to form both putrescine and cadaverine, in variable relative proportions but with the constant prevalence of the former diamine (Guerrini et al. 2002). In the same study, no production of tyramine, spermine, spermidine or 2-phenethylamine was observed. More recently, *Enterococcus faecium* isolated from Italian wines was proved to be a strong tyramine producer (Coton et al. 2010; Capozzi et al. 2011), suggesting that tyramine biosynthesis might be a feature of this species (Ladero et al. 2012).

However, it is to underline that published results on the capability of wine lactic acid bacteria to produce individual amines continue to be contradictory and make the origin of BA complex. Most likely, the controversial results reflect a situation where the BA-producing capability is strain rather than species dependent and the

extent of BA accumulation is quite variable owing to the incidence of several factors that affect the concentration of precursor amino acids.

In any case, since histamine, tyramine and putrescine are the most abundant amines in wine, their formation by wine LAB will be examined in more details, with the preliminary remarks that a 100% correlation has been demonstrated between BA-producing capability of LAB strains in synthetic medium and in wine as well as between the strain ability to produce individual amines and the presence of the genes encoding the corresponding amino acid decarboxylases (Landete et al. 2007c; Coton et al. 2010).

Indeed, in the RESOLUTION OIV-OENO 449-2012, in order to assess the potential risk of BA accumulation in wine, molecular methods to detect BA-producing LAB in wine are described. These methods consist in detecting LAB that have the genes of amino acid decarboxylases and/or agmatine deiminase by targeting the suitable genes. In particular, polymerase chain reactions (PCR) have been performed either for detecting the presence of BA-producer strains (PCR end point) or for quantifying their concentrations (real-time PCR). Multiplex PCR have been also performed to detect the presence of several genes at the same time. Oligonucleotide primers for the detection of BA-producing LAB in wine are listed in Table 10.2. More recently, a fast and sensitive method to perform microdroplet-based multiplex PCR directly from wine and suitable for the simultaneous detection of bacterial genes involved in biogenic amine biosynthesis has been also developed (Sciancalepore et al. 2013).

The results obtained with these methods are not able to predict the final BA concentrations in wine but identify the risk of BA formation due to the presence of the decarboxylases and agmatine deiminase genes in the LAB population (Lucas et al. 2007). By assessing the potential risk of a BA accumulation in wine at an early stage of the winemaking, these methods can assist in managing the fermentation process in order to reduce the BA formation.

Table 10.2 Oligonucleotide primers for the detection of BA-producing LAB in wine reported in the RESOLUTION OIV-OENO 449-2012

Gene	Primers 5′ → 3′ sequence	References
Histamine decarboxylases	HDC3: GATGGTATTGTTTCKTATGA HDC4: CAAACACCAGCATCTTC	Coton and Coton (2005)
Tyramine decarboxylases	41: CAYGTNGAYGCNGCNTAYGGNGG 42: AYRTANCCCATYTTRTGNGGRTC	Marcobal et al. (2005)
	TD5: CAAATGGAAGAAGAAGTAGG TD2: ACATAGTCAACCATRTTGAA	Coton et al. (2004)
Putrescine decarboxylases	4: ATNGARTTNAGTTCRCAYTTYTCNGG 15: GGTAYTGTTYGAYCGGAAWAAWCAYAA	Marcobal et al. (2005)
	OdF: CATCAAGGTGGACAATATTTCCG OdR: CCGTTCAACAACTTGTTTGGCA	Granchi et al. (2006)
Agmatine deiminase	AGDIfor: GAACGACTAGCAGCTAGTTAT AGDIrev: CCAATAGCCGATACTACCTTG	Lucas et al. (2007)

10.3.1 Histamine Production

Oenococcus oeni is the dominant species during MLF of wines, and histamine increase occurs especially during and mostly after this process (Lonvaud-Funel 2001; Marcobal et al. 2006; Lucas et al. 2008a; Coton et al. 2010). Consequently, many authors have identified *O. oeni* as the main producer of histamine in wine (Coton et al. 1998a; Landete et al. 2005a; López et al. 2009; Lucas et al. 2008a). However, also other species of LAB (*Lactobacillus* and *Pediococcus* spp.) occurring in grape must and/or in wine are able to produce histamine (Costantini et al. 2006; Landete et al. 2005a, 2007a; Lucas et al. 2005), even if during and after MLF, these species are normally in lower concentrations than *O. oeni* (Lonvaud-Funel 2001). Moreover, histamine-producing *Lactobacillus parabuchneri* or *Lactobacillus rossiae* strains have been found as contaminating microbiota in yeast starter culture preparations (Costantini et al. 2009).

The capability to produce histamine by *Oenococcus oeni* has been extensively studied and currently still controversial (Garcia-Moruno and Muñoz 2012). The first isolation of a histamine-producing strain of *O. oeni* was described in 1994 by Lonvaud-Funel and Joyeux. Bacterial biomass from a wine containing BA was inoculated in a wine integrated or not with histidine. After 40 days, significant concentrations of histamine were found only in the wine integrated with histidine. From the biomass, various *O. oeni* isolates were found as histamine producers and *O. oeni* IOEB 9204 were selected, since it retained this ability after several subcultures. In the following years, various authors isolated *O. oeni* strains able to produce histamine from histidine in synthetic media, in wine-simulated medium or in wine (Del Campo et al. 2000; Guerrini et al. 2002; Landete et al. 2005a; Rosi et al. 2009). In these studies, histamine concentrations, quantified by HPLC, were usually in low concentrations (from 0.5 mg L^{-1} to more than 30 mg L^{-1}). More recently, López et al. (2009) reported that, after spontaneous MLF, a concentration of about 9 mg L^{-1} of histamine was detected and that only bacteria belonging to *O. oeni* species were isolated from the studied wines. These results suggested to the authors that *O. oeni* population was responsible for histamine production in wine. On the contrary, other authors came to the opposite conclusion because they were unable to isolate from wines *O. oeni* strains producing histamine in grape must, wine or synthetic media (Moreno-Arribas et al. 2003; Costantini et al. 2006). The reason for these contradictory results could be that BA production is associated with specific strains rather than with *O. oeni* species, as reported also for other bacteria (Garai et al. 2007).

Molecular methods for the detection of *O. oeni* histamine producers were initially based on PCR amplification of a partial sequence of the histidine decarboxylase encoding gene of *O. oeni* IOEB 9204 (Le Jeune et al. 1995). Later on, the complete gene and the flanking nucleotide sequences were located, identified and cloned (Coton et al. 1998b). In 2008, a 3800 nucleotide sequence of the *hdc* region of *O. oeni* IOEB 9204 was deposited (Gen Bank accession DQ132887) (Garcia-Moruno and Muñoz 2012). Comparing the deduced amino acid sequence of the

histidine decarboxylases encoded by the *hdc* genes of *O. oeni* IOEB 9204 with those of other LAB, a similarity of 99% with *Lactobacillus sakei* LTH 2076 (LSA) and *Lactobacillus hilgardii* IOEB 0006 (LHI), of 97% with *Lactobacillus buchneri* DSM 5987 (LBU) and of 80% with *Lactobacillus* 30a was found (Garcia-Moruno and Muñoz 2012). According to Lucas et al. (2008b), this high identity could be due to horizontal transfer of the histamine-producing pathway in LAB. In fact, a histamine-producing strain (*Lactobacillus hilgardii* IOEB 0006) proved to retain or to lose the ability to produce histamine, depending on the culture conditions (Lucas et al. 2005). Indeed, it was demonstrated that the *hdc* gene in this strain was located on an unstable 80-kb plasmid, suggesting an acceptable cause for the great variability of histamine-producing character among LAB. No studies on this topic have been carried out in *O. oeni*, but the existence of an unstable plasmid containing the *hdc* gene in this species could explain the contradictory results regarding some reference strains classified as BA producer by some authors and non-producer by others (Garcia-Moruno and Muñoz 2012). Therefore, further studies are needed to verify the existence or not of a plasmid containing the *hdc* gene also in *O. oeni* and, most of all, to evaluate the possibility of a horizontal transfer of this plasmid among different wine LAB species (i.e. from *Lactobacillus* spp. occurring in grape must fermentations to *O. oeni* population responsible of MLF). Nevertheless, Landete et al. (2006) demonstrated that in *Lactobacillus hilgardii* 464, *P. parvulus* P270 as well as in *O. oeni* 4042 strains, histamine production is similarly regulated by both histidine and histamine. Indeed, the expression of the *hdc* gene, encoding the histidine decarboxylase enzyme, is induced by the presence of the amino acid (the substrate of the enzyme) and repressed by the presence of the final product from the enzyme activity, the biogenic amine. Other low molecular weight compounds occurring usually in wine, such as malic and citric acids, seem to affect *hdc* expression, demonstrating that the gene is highly regulated. The histidine decarboxylase (HDC) enzyme from LAB strains, differently from other HDC enzymes from Gram-negative bacteria that require pyridoxal phosphate as cofactor, uses a covalently bound pyruvoyl moiety as a prosthetic group in the reaction (Coton et al. 1998b).

10.3.2 Tyramine Production

The ability to form tyramine is not a common feature of wine LAB. The character is highly variable among strains belonging to the genus *Lactobacillus* and very likely absent or quite rare within the species *O. oeni* and among strains of pediococci (Moreno-Arribas et al. 2000; Landete et al. 2007b). Among the lactobacilli of wine origin so far tested, this metabolic trait is held by most strains of the species *Lactobacillus brevis* and by some strains of *L. hilgardii*, the former species showing the highest tyramine-producing activity. Consequently, lactobacilli might be primarily responsible for high concentration of tyramine in wine. However, a strain of *O. oeni*, isolated from a spontaneous MLF, proved to be able to decarboxylate

tyrosine into tyramine in a model system (Gardini et al. 2005). In the same paper, emphasis was given to the fact that tyramine production by resting cells of the studied *O. oeni* strain is markedly affected by several variables (pH, arabinose and pyridoxal-5-phosphate concentrations, ethanol percentage and SO_2 level), so that it is possible that the actual capability of *O. oeni* to decarboxylate tyrosine is highly underestimated.

The tyrosine decarboxylase (TDC) enzyme from *Lactobacillus brevis* IOEB 9809 of wine origin proved to be a protein with two subunits of 70 kDa each, requiring pyridoxal phosphate as cofactor and showing maximum activity at pH 5.0 (Moreno-Arribas and Lonvaud-Funel 2001). In some cases, tyramine-producing capability has been found to be associated with a low but significant 2-phenethylamine production, as a confirmation that TDC is able to use also phenylalanine as a substrate for its activity. However, it has been also reported that only the best tyramine producers are able to generate both amines simultaneously (Moreno-Arribas et al. 2000).

10.3.3 Putrescine Production

In general, putrescine is the biogenic amine most abundantly found in wines, and putrescine-producing capability may be considered widespread among LAB strains of oenological interest (Moreno-Arribas et al. 2003). The ornithine decarboxylase gene *odc*, firstly sequenced from *Lactobacillus* sp. 30a (Hackert et al. 1994), has been later sequenced also from *O. oeni* (Marcobal et al. 2004). Many bacteria contain two forms of the decarboxylating enzyme (ODC): a constitutive enzyme, expressed when bacteria grow at neutral pH in minimal culture media, and a form induced mainly under low pH conditions in rich media and suggested to play a role in maintaining pH homeostasis. Among LAB, an inducible ODC, structured as a dodecamer of about 1,000 kDa and requiring pyridoxal phosphate as cofactor, has been described in *Lactobacillus* sp. 30a (Momany et al. 1995; Vitali et al. 1999).

The origin of putrescine deserves a more detailed description because high concentrations of this diamine cannot depend only on the amount of free ornithine, since the concentration of this amino acid in both musts and wines is usually very low. Indeed, ornithine may also be produced from the catabolism of the amino acid arginine, one of the major amino acids found in grape juices and mostly metabolized by yeasts during alcoholic fermentation but generally still present in wines at significant amounts before the onset of MLF. This amino acid is catabolized by several strains of lactic acid bacteria, both lactobacilli and oenococci, via the arginine deiminase pathway (Liu and Pilone 1998). This metabolic route consists of three enzymes acting in series: arginine deiminase (ADI), ornithine transcarbamylase (OTC) and carbamate kinase (CK), as drawn in Fig. 10.2. As a result, bacterial cells catabolizing arginine excrete ornithine, ammonia and CO_2, besides small amounts of citrulline, in a molar ratio among the main products close to 1:2:1.

Fig. 10.2 Pathway of arginine breakdown by malolactic bacteria. (1) Arginine deiminase, (2) ornithine transcarbamylase and (3) carbamate kinase

In a paper by Mangani et al. (2005), some *O. oeni* strains demonstrated the capability to produce putrescine only from ornithine, but other strains were able to produce putrescine also from arginine, proving to possess the necessary enzyme system to degrade arginine to ornithine and then to decarboxylate this amino acid to putrescine, with the additional formation of ammonia and useful ATP. In the same paper, it was demonstrated that putrescine can be produced from arginine also by an association of strains possessing a complementary enzyme system: a coculture of one *O. oeni* strain, capable to metabolize arginine to ornithine but unable to decarboxylate ornithine to putrescine and another strain capable to produce putrescine from ornithine but unable to degrade arginine giving rise to putrescine in the presence of the sole arginine, as a practical consequence of an exchange of ornithine between the two strains. Consequently, the occurrence of a metabiotic association of this type might really contribute to the increase in concentration of putrescine in wines.

10.3.4 Production of Other Amines

Even if histamine, tyramine and putrescine have received major attention owing to their toxicity and abundance in wine, other amines can be produced by bacterial amino acid decarboxylases. However, information on the actual capability of wine LAB to produce agmatine, cadaverine and tryptamine is far too exhaustive. Most studies deal with chemical data on the presence of these amines during winemaking or in wines rather than with microbial populations responsible for amine formation.

Some *O. oeni* strains proved to be able to produce significant amounts of cadaverine, always coupled with a putrescine-producing activity (Guerrini et al. 2002). In the same study, it was observed that almost all *O. oeni* strains that produced cadaverine and putrescine were low producers of histamine and some low producers of histamine were unable to produce cadaverine or putrescine.

With regard to agmatine production by wine LAB, it can be stated that this polyamine could originate from decarboxylation of arginine, but the reaction has been ascertained only in *L. hilgardii* strain X_1B (Arena and Manca de Nadra 2001). This unique finding suggests that the strain might possess an anomalous pathway for catabolism of arginine, the amino acid being usually metabolized by wine LAB via the ADI pathway. Finally, no information is available on the LAB capability to produce tryptamine.

10.3.5 Physiological Role of Amine Production

If BA production is of concern to winemakers and to wine consumers, amino acid decarboxylation may play an important physiological role in microbial cells that might take advantage of this catabolic feature. In microorganisms, both biosynthetic and biodegradative amino acid decarboxylase enzymes have been described. The biosynthetic decarboxylases are constitutive, mainly associated with microbial growth, and it is found in considerably less quantities than that of the biodegradative form. The biodegradative decarboxylases are inducible by various environmental factors and are responsible for the BA accumulation in wine. The physiological role of BA synthesis by biodegradative decarboxylases seems to be the survival increase under acidic stress conditions thanks to the consumption of protons and the excretion of BA and CO_2. Indeed, detailed studies have demonstrated that BA production is enhanced when growth conditions become less favourable owing to the absence of fermentable substrates and the presence of ethanol at low pH values. These findings have suggested that amino acid decarboxylases might function as an additional mechanism for energy generation, as it happens with the decarboxylation of malic acid by the action of malolactic enzyme.

In the case of histidine decarboxylation, the energetic advantage for the strain might be due to an electrogenic exchange between histidine and histamine, as it was found in *Lactobacillus buchneri*: the amino acid enters the cell in the neutral form, while histamine is excreted with one positive charge, generating a proton motive force of sufficient value to drive ATP synthesis (Moolenar et al. 1993). This proton-consuming decarboxylation also generates a transmembrane pH gradient that enables the cells to protect themselves against the adverse effects of the acid environment (Fig. 10.3).

In the case of tyrosine decarboxylation as well as other amino acid decarboxylations, it has been envisaged an energetic advantage through a mechanism similar to that described above, but studies on this matter are still incomplete, and more detailed investigations are required to establish the physiological importance of these reactions. Nevertheless, the possible advantages of bacterial cells able to produce putrescine deserve some comments because of a possible involvement of arginine catabolism in putrescine production, with arginine acting as a remote precursor of the polyamine.

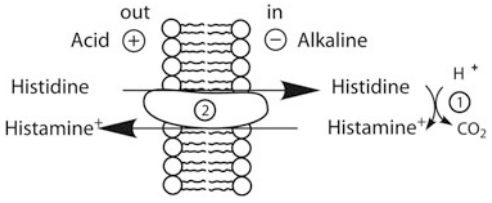

Fig. 10.3 Electrogenic exchange of histidine for charged histamine, produced by proton-consuming decarboxylation of histidine. (1) Histidine decarboxylase and (2) permease

In LAB strains able to catabolize arginine via the ADI pathway to ornithine, ammonia and CO_2 (Fig. 10.2), physiological advantages are due to a carbamate kinase-dependent phosphorylation at the substrate level, since the uptake of arginine is coupled with the excretion of ornithine through an electroneutral antiport system that is sustained by concentration gradients and does not require energy (Driessen et al. 1987; Poolman et al. 1987). On the other hand, in LAB strains able to decarboxylate ornithine to form putrescine, physiological advantages, as above reported, are due to an electrogenic substrate/product exchange. In addition, both metabolic routes cause an increase in cytoplasmic pH, protecting cells against acid damages. In LAB strains able to catabolize arginine via the ADI pathway and to decarboxylate ornithine to putrescine, as demonstrated for some *O. oeni* strains (Mangani et al. 2005), energetic advantages could take place only if ornithine produced by the ADI pathway is at first excreted, in order to satisfy the electroneutral exchange with arginine, and then reutilized, in order to sustain the electrogenic exchange with putrescine. However, experimental evidence of such mechanism is lacking, and it is to be proven that strains capable to catabolize arginine to putrescine take advantages over strains possessing either metabolic route.

10.4 Biogenic Amine Accumulation During Wine Fermentations

BA quantification in wine is still problematic due to their low concentration, the lack of chromophores of most biogenic amines, the complexity of the sample matrix and the presence of potentially interfering substances. Nowadays, HPLC is by far the most frequently used technique, due to its high resolution and sensitivity, especially when coupled with a fluorescence detector. As BA do not show satisfactory absorption in the visible and ultraviolet range nor do they show fluorescence, pre- or post-column chemical derivatization is considered a necessary analytical step for this detection technology. Currently, the International Organization of Vine and Wine (OIV) proposes two methods to determine BA in musts and wines by HPLC using fluorimetric (OIV-MA-AS315-18) and spectrophotometric detection methods (OIV-MA-AS315-26). Moreover, analytical methods for BA determination in fermented beverages including wines have been recently reviewed (Ordóñez et al. 2016).

Concerning the origin of BA in wines, a wide experimental evidence indicates MLF as the winemaking phase responsible for most BA production and accumulation in wines, at least for the most frequently found amines, namely, histamine, tyramine and putrescine (Soufleros et al. 1998; Moreno-Arribas et al. 2003; Lonvaud-Funel 2001; Guerrini et al. 2002; Marcobal et al. 2006; Lucas et al. 2008a).

However, in these studies, microbiological data have been only rarely included, so that it is difficult to ascertain possible relationships between the changes that occur in individual amine concentrations and those occurring in microbial populations during the winemaking process. Moreover, it has been suggested that amino acid decarboxylation may take place for a long time period independently of cell viability (Moreno-Arribas and Lonvaud-Funel 1999). Consequently, as stated by some researchers, no general rule for the time course of BA accumulation during winemaking is available yet. Nevertheless, by taking into consideration all the papers dealing with this matter as well as personal observations, some aspects appear elucidated.

During alcoholic fermentation, carried out by either indigenous or selected yeast strains, no remarkable increase in the BA concentration usually occurs, the levels of individual amines remaining at the values occurring in fresh grape must. With the onset of malolactic fermentation (MLF), after the development of the bacterial population to more than 10^5 CFU mL^{-1}, BA concentration begins to increase. The extent to which individual amines increase during MLF is unpredictable, each amount depending on both availability of precursor amino acid and catabolic properties of the participating bacterial strains. However, the BA-producing rate usually shows a remarkable increase towards the final phases of MLF, as a confirmation that BA production is enhanced as the growth conditions become less favourable owing to the progressive consumption of an important energy source such as malic acid. As soon as malic acid is exhausted, BA production process enters a delicate phase, determined by two main factors: (1) malolactic population might be still highly active and at high cell densities, and (2) catabolic activities of LAB population on some precursor amino acids might be delayed in comparison to MLF, as it has been demonstrated in the case of arginine breakdown by oenococci (Mangani et al. 2005). After completion of MLF, wines are generally sulfited in order to avoid growth or survival of undesirable bacteria, but it is known that, at usual concentrations, SO_2 does not immediately stop all the biochemical activities of the bacterial population that, otherwise, could survive for a long time after sulfitation, even if at a reduced cell density. The survival of the malolactic population and the availability of precursor amino acids might account for the increase in BA concentration often observed after completion of MLF or during wine ageing.

Other investigations, carried out to correlate the amount of BA in wines with factors suspected to play a significant role in affecting the level of BA accumulation, such as grape variety and degree of grape maturation, kind of soil and N fertilizer, content of BA precursors, addition of nutrients, pH, ethanol concentration, levels of sulphur dioxide, addition of lysozyme and oenological tannins, different maceration practices, duration of wine contact with yeast lees, type of vessel in which MLF takes place and other post-fermentative treatments (Bauza et al. 1995; Soufleros et al. 1998; Leitão et al. 2005; Herbert et al. 2005; Landete et al. 2005b; Pramateftaki et al. 2006; Alcaide-Hidalgo et al. 2007; Hernández-Orte et al. 2008; Cejudo-Bastante et al. 2010; Polo et al. 2011; López et al. 2012; Martínez-Pinilla et al. 2013; Smit et al. 2013), appear still too fragmentary, and

sometimes contradictory, to be used to draw a general picture. Nevertheless, general strategies to reduce the BA content in wine have been recently addressed as reported below.

10.5 Strategies to Minimize or Reduce BA in Wine

Based on the knowledge concerning the origin and factors involved in BA formation in wines, OIV published in 2011 a code of good vitivinicultural practices in order to minimize the presence of BA in vine-based products. In particular, the RESOLUTION OIV-CST 369-2011 reports the good practices that should be carried out in vineyard, during the grape harvest, and in cellar. A brief description of these good vitivinicultural practices is reported below.

Interventions in vineyard should consider all the recommendations contained in the OIV guide for sustainable vitiviniculture, with particular reference to issues involving fertilization, the ventilation of foliage and grape bunches and the phytochemical protection of the grape (Resolution CST 1/2008). All the preventive measures, designed to limit the formation of BA or their precursors in the agronomic phases, or the techniques favouring both conservation of the acidity of grapes and prevention of pH increase of the must should be applied in the vineyard. Similarly, viticultural practices (or the lack thereof) deteriorating the sanitary quality of grapes and/or increasing the nitrogen content of the must should be avoided.

Interventions recommended during the grape harvest include the elimination of bunches or parts of bunches that are damaged by fungi and the reduction to a minimum the delays in transport and maceration of the grape before its arrival at the cellar.

Finally, interventions recommended in cellar and concerning pre-fermentative, fermentation, and post-fermentative operations are listed in Table 10.3.

Another strategy to minimize or even reduce the content of BA in wine could be the selection of wine microorganisms able to degrade these components. In fact, considering that amino oxidases are responsible for the detoxification of dietary BA and enzymes with amino oxidases activity have been found in bacteria, recent studies focused on the screening of such activity in LAB isolated from wine (García-Ruiz et al. 2011; Alvarez et al. 2014). In particular, a recent study demonstrated the existence of strains belonging to different species (one *Lactobacillus casei*, one *Lactobacillus hilgardii*, one *Pediococcus parvulus*, one *Oenococcus oeni*, two *Lactobacillus plantarum* and three *Pediococcus pentosaceus*) able to degrade histamine, tyramine and putrescine in culture media and in wine (García-Ruiz et al. 2011). Although the BA-degrading ability of the selected LAB strains seemed to be suitable at wine pH, wine components such as ethanol and polyphenols or wine additives such as SO_2 might limit this capability. Moreover, during MLF, the BA-degrading ability was confirmed only for one *L. casei* strain. In another work, two strains of *L. plantarum*, isolated from wine and able to

Table 10.3 Interventions in cellar concerning pre-fermentative operations and treatments, fermentation operations and maturing and clarification operations as reported in RESOLUTION OIV-CST 369-2011

Pre-fermentative operations	Fermentation operations	Post-fermentative operations
– To ensure suitable hygiene practices are applied in the cellar – In case of spoiled berries, the maceration period must be minimized – In case of maceration, keep in mind that the maceration period is one of the important factors in the production of biogenic amines (enrichment in BA precursor amino acids, increase of the pH, activity of indigenous yeast and bacteria) – In must with a high pH (3.6–3.7), which favours the diversity and development of the bacteria population, it is recommended to lower the pH using suitable techniques (in accordance with the International Code of Oenological Practices of the OIV) and to avoid triggering spontaneous MLF before alcoholic fermentation (adding SO_2, lysozyme, etc.)	– The addition of ammoniacal nitrogen, inactivated yeasts, yeast cell walls or yeast autolysates should be restricted to minimal concentrations – Alcoholic fermentation should be carried out using *Saccharomyces* with a low predisposition for the formation of BA – MLF should be carried out by inoculation of LAB after alcoholic fermentation or by co-inoculation of LAB during alcoholic fermentation – During MLF it is recommended to inoculate the wine with LAB which have no or little decarboxylase activity and are able to prevent the proliferation of undesirable indigenous bacteria which could increase the levels of BA – After MLF, it is recommended to eliminate the microorganisms by adding SO_2 or by treatments described in the International Code of Oenological Practices – The preventive amounts of SO_2 must be increased even more if the wine has a high pH and is intended for maturing	• It is recommended to carry out microbiological analysis to determine the population of LAB with decarboxylase activity • Clarification can be performed in order to – Decrease the viable and viable but not cultivatable bacteria population, which uses amino acids in wine when the natural substrates such as sugar and malic acid have been consumed – Eliminate any protein compounds which have been transferred to the wine and which can act as substrates for the bacteria • Current clarification products have varying levels of effectiveness in reducing the contents of BA (es. bentonite) • When LAB-producing BA are present, the technique of maturing on lees is not recommended, since it can increase the content in BA precursor amino acids

degrade tyramine and putrescine, survived in a wine-like medium and, at the same time, degraded malic acid (Capozzi et al. 2012). Therefore, the use of LAB capable of degrading BA in wine seems to be a promising alternative. However, more studies are needed in order to prove their technological relevance during winemaking under real conditions (Alvarez et al. 2014).

Finally, a recent study investigated the possibility to absorb BA (histamine, putrescine, cadaverine and tyrosine) using a sodium form of the commercial zirconium phosphate (Na-ZrP) (Amghouz et al. 2014). In details, Na-ZrP was tested as an adsorbent for elimination of biogenic amines (histamine, putrescine, cadaverine and tyramine) from synthetic wine. The results showed that histamine is the

fastest adsorbed (individually and competitively), followed by cadaverine, putrescine and tyramine. Histamine and putrescine are the amines absorbed in higher amounts. In any case, further studies should be carried out to assess the possibility of using this tool with BA-containing wines.

10.6 Ethyl Carbamate Formation in Wine

Besides BA, another health concern in wine is the presence of ethyl carbamate (EC), also referred to as urethane. This compound has attracted the attention of many researchers because of its animal carcinogen potential and its possible origin from precursors produced by microbial catabolism of amino acids. Indeed, EC could originate in wine as a consequence of a spontaneous, non-enzymatic reaction between ethanol and a compound containing a carbamyl group, such as urea (produced from arginine breakdown by yeasts), citrulline and carbamyl phosphate (produced from arginine breakdown by LAB) (Jiao et al. 2014). This acid-catalysed alcoholysis of carbamyl compounds has been shown to be directly dependent on both concentration of reactants and temperature (Ough et al. 1988), the reaction occurring readily in the case of carbamyl phosphate and slowly in the cases of urea and citrulline at the normal wine storage temperatures. Consequently, EC levels are usually low or non-detectable in young wines and variable to different extent in aged or stored wines, depending on cellar or storage temperature. With reference to the reactants, urea is considered the major precursor for EC in wine (Ough et al. 1988; Jiao et al. 2014), and it is well known that it can be released by wine yeasts as metabolic intermediate from arginine breakdown. However, urea excretion by yeast cells is affected by a variety of environmental factors, including nitrogen availability, and is variable from strain to strain (Ough et al. 1991). In *S. cerevisiae*, the enzyme arginase, encoded by the gene CAR1, degrades arginine into ornithine and urea, which is further degraded into CO_2 and NH_4^+ by the bifunctional enzyme urea amidolyase, encoded by the DUR1,2 gene (Genbauffe and Cooper 1986). The expression of these two genes follows distinctive regulatory mechanisms, even if both genes are subject to nitrogen catabolite repression, a regulatory mechanism that causes the repression of genes encoding enzymes which degrade poor N-sources in the presence of good N-source. Since both arginine and urea are considered as secondary nitrogen sources, urea excretion should be the consequence of a lack or lower level of DUR1,2 expression in comparison to the expression of the CAR1 gene (Coulon et al. 2006). In any case, urea excretion is favoured by conditions of high availability of promptly assimilable nitrogen sources and mainly occurs during the first stages of wine fermentation.

As for the other reactants of microbial origin, citrulline has been suggested as the main EC precursor produced by LAB in wine (Azevedo et al. 2002; Jiao et al. 2014). Citrulline is an intermediate product of arginine breakdown by LAB through the ADI pathway and can be excreted by the bacterial cells to variable extent, depending on the strain (Granchi et al. 1998; Mira de Ordu et al. 2000). Carbamyl

phosphate, the other intermediate of arginine catabolism, is usually excreted to a much lower extent than citrulline and, although it is highly reactive to ethanolysis, does not seem to play a significant role in EC formation in wine.

10.7 Conclusions

The increasing attention given in recent years to BA and EC in wines seems not only due to a general demand for healthier foods and beverages but also dependent on the alcoholic nature of wine. Indeed, ethanol, besides being a reactant in EC formation, is known to be among the most active inhibitors of amine oxidases, so that wine consumers might really suffer the toxic effects of BA presence, even at low concentration.

The risks associated with the ingestion of BA-containing wines led some European countries to recommend maximum tolerable limits for histamine in wine: Germany, 2 mg L^{-1}; Holland, 3 mg L^{-1}; Finland and Belgium, 5 mg L^{-1}; France, 8 mg L^{-1}; and Switzerland and Austria, 10 mg L^{-1} (Busto et al. 1996; Lehtonen 1996). On the other hand, the perceived risk for EC led Canadian government to impose legal limit for this compound in imported wines (30 and 100 μg L^{-1} in table and fortified wines, respectively), and later, in 1988, the Food and Drug Administration in the USA brought to rule more stringent limits (15 and 60 μg L^{-1}, respectively, starting from the 1989 harvest).

In order to overcome any potential risk, different strategies have been tested or envisaged in the last 10 years, such as (1) to discourage spontaneous processes in favour of fermentations induced by selected yeast or malolactic strains with known biochemical properties; (2) to manage grape must pH, so that growth of BA-producing lactobacilli and pediococci is hampered; (3) to eliminate bacterial populations by means of lysozyme treatment suddenly after MLF completion, so that BA production is broken down; and (4) to add preparations of acidic urease of bacterial origin as well as to make use of metabolically engineered urea-degrading yeast strains in alcoholic fermentation of grape musts so that the presence of a major reactant in EC formation is eliminated.

However, in spite of these efforts, the challenge of BA and EC exclusions from wines is still to be won.

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Chapter 11
Usage and Formation of Sulphur Compounds

Doris Rauhut

11.1 Introduction

Sulphur is important for the growth of all microorganisms due to the formation of sulphur-containing amino acids. Among the wine-related microorganisms, extensive data has been accumulated for yeast of the species *Saccharomyces cerevisiae*. In recent years, also more and more research work is focused on the sulphur metabolism of non-*Saccharomyces* yeasts and lactic acid bacteria.

The goal of this review has been to assemble the literature concerning the usage and biosynthesis of sulphur amino acids and glutathione as well as on the formation of the high flavour-active volatile sulphur compounds during alcoholic and malolactic fermentation.

11.2 Sulphur Metabolism of Yeast

11.2.1 Sulphur Amino Acid Biosynthesis

Yeast has the ability to use various sulphur compounds in contrast to many other microorganisms due to the sulphur pathway in yeast, which allows it to use various organic and inorganic sulphur compounds as sole sulphur source.

The element sulphur can occur in a variety of stable compounds in which it can range from −2 in its most reduced form (sulphide) to +6 in its most oxidised form (sulphate). For all microorganisms, the biosynthesis of sulphur amino acids requires

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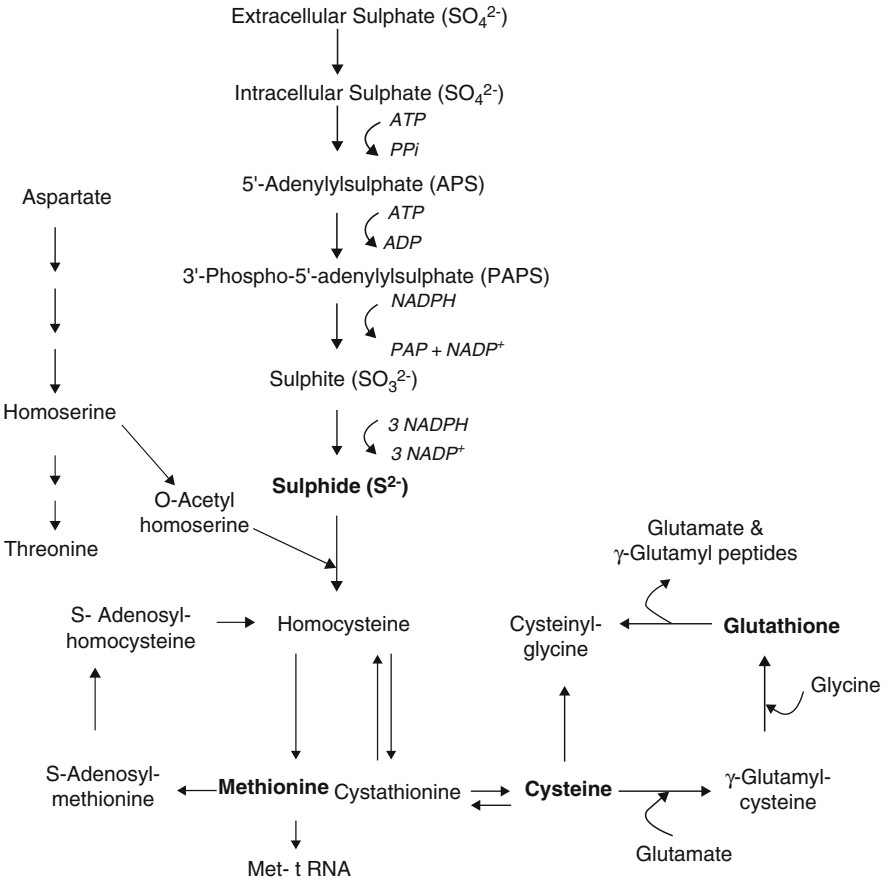

Fig. 11.1 Metabolism of sulphur amino acids in *Saccharomyces cerevisiae* [according to De Robichon-Szulmajster and Surdin-Kerjan (1971), Jones and Fink (1982), Henschke and Jiranek (1993), Rauhut (1993), Thomas and Surdin-Kerjan (2003), Wang et al. (2003), Linderholm et al. (2008)]

the ability to accumulate sulphur atoms from the growth medium and then the transformation of the transported intermediate compounds into the reduced form of the sulphur atom (S^{2-}). Basic research work to investigate sulphur metabolism in *Saccharomyces cerevisiae* was done by the research group of Surdin-Kerjan, which characterised more than 15 genes encoding enzymes of the sulphur amino acid pathway (Surdin-Kerjan 2003). Figure 11.1 gives a simplified overview on the metabolism of sulphur amino acids and glutathione in *Saccharomyces cerevisiae*.

The main sulphur source for yeasts during winemaking is sulphate, which occurs in grape must in a range of 160–400 mg L^{-1} or even more (Lemperle and Lay 1989), because the levels of methionine and cysteine are normally very low in grape musts (in most cases less than 10 mg L^{-1} methionine and cysteine) in comparison to other amino acids (Henschke and Jiranek 1991, 1993).

The first step of the sulphate reduction sequence (SRS) is the transport of extracellular sulphate from the environment into the cell through the enzyme sulphate permease. Cherest et al. (1997) have isolated and characterised two sulphate transporters. After that sulphate is activated by an adenylation and reduced by two successive reactions (requiring four molecules of NADPH + H^+ and two of ATP). The adenylation of sulphate lowers the electro-potential of sulphate that the subsequent reduction into sulphite and sulphide by means of NADPH + H^+ oxidation is possible. Activation of sulphate takes place by the transfer of the adenosyl-phosphoryl-moiety of ATP to sulphate and the formation of 5′-adenylylsulphate (APS). These reactions are catalysed through ATP sulphurylase. In a further step, APS is phosphorylated and 3′-phospho-5′-adenylylsulphate (PAPS) is formed through APS kinase. PAPS is then reduced through PAPS reductase to sulphite, which is reduced from sulphite reductase to form sulphide.

Sulphide is incorporated into amino acids through several more enzymatic steps.

The enzyme *O*-acetyl homoserine-*O*-acetyl serine sulphhydrylase is incorporating the sulphide, along with *O*-acetylhomoserine, into homocysteine. Cystathionine β-synthase converts homocysteine into cystathionine. γ-Cystathionase is then conducting the reaction to cysteine. Cysteine is only formed through the transsulphuration pathway. No direct synthesis of cysteine from sulphide occurs in yeast (Thomas and Surdin-Kerjan 1997). *O*-Acetylhomoserine is the amino acid precursor to form methionine with the reduced sulphur atom over homocysteine through the activity of *O*-acetylserine sulphhydrylase and homocysteinemethyl-transferase. Methionine is not only involved in protein synthesis. It is also an indispensable intermediate of the one carbon metabolism. *S*-Adenosylmethionine is the methyl donor in various transmethylation reactions of nucleic acids, proteins and lipids. It is involved in the biosynthesis of polyamines and is one of the substrates used in a number of reactions, including vitamin biosynthesis and nucleotide modifications (Surdin-Kerjan 2003).

It is expected that the equilibrium between methionine and *S*-adenosylmethionine plays a central role in the overall cellular homeostasis. The ratio of methionine and *S*-adenosylmethionine seems to be controlled through two recycling pathways that operate on the products of *S*-adenosylmethionine catabolism (Surdin-Kerjan 2003).

Kinzurik et al. (2017) noticed that *GLO1* gene is needed for full activity of *O*-acetyl homoserine sulphhydrylase encoded by *MET17*. These studies offer new possibilities for the study of other molecules in cell signalling and regulation of sulphur metabolism in yeasts.

Excellent reviews on metabolism of sulphur amino acids in *Saccharomyces cerevisiae* from Jones and Fink (1982), Hinnebush (1992) and Thomas and Surdin-Kerjan (1997) will offer more details also on the regulation of sulphur amino acids biosynthesis.

Further sulphur sources for yeasts during the winemaking process are sulphur dioxide, which is used due to its antimicrobial and antioxidative effect as well as its reaction with ethanal (acetaldehyde) to avoid an oxidative character and its inactivation of enzymes, and elemental sulphur from residues of the application of

wettable sulphur (fungicide) on grapes (Ribéreau-Gayon et al. 2000a, b; Romano and Suzzi 1993; Dittrich 1987; Wucherpfennig 1984; Wenzel and Dittrich 1978; Schütz and Kunkee 1977). An overview on sources of volatile sulphur compounds in wine is also given by Smith et al. (2015).

11.2.2 Sulphite Production

The production of sulphite by *Saccharomyces cerevisiae* is strain characteristic (Fig. 11.2) and is also affected by the grape must composition. Strains of *Saccharomyces cerevisiae* normally produce sulphite in the range of 10–30 mg L^{-1} (Eschenbruch 1974; Dott et al. 1976; Dittrich 1987). 'SO_2-forming yeasts' or 'yeasts with high-sulphite formation' can form sulphite in amounts exceeding 100 mg L^{-1} (Eschenbruch 1974). Suzzi et al. (1985) could demonstrate under comparable conditions that among 1700 strains of *Saccharomyces cerevisiae*, the majority (80%) produced less than 10 mg L^{-1} SO_2 and only four strains synthesised more than 30 mg L^{-1}. Dott and Trüper (1976, 1978), Dott et al. (1976, 1977), Eschenbruch (1972, 1974) and Würdig (1985) reported substantial variation in the activity of sulphate permease, ATP sulphurylase and sulphite reductase in the regulation of sulphur metabolism in high and low sulphite-producing yeast strains (reviewed by Rauhut 1993; Pretorius 2000).

An extreme formation of sulphite can be caused by defects in sulphate uptake and reduction, which is normally regulated by methionyl-t-RNA and *S*-

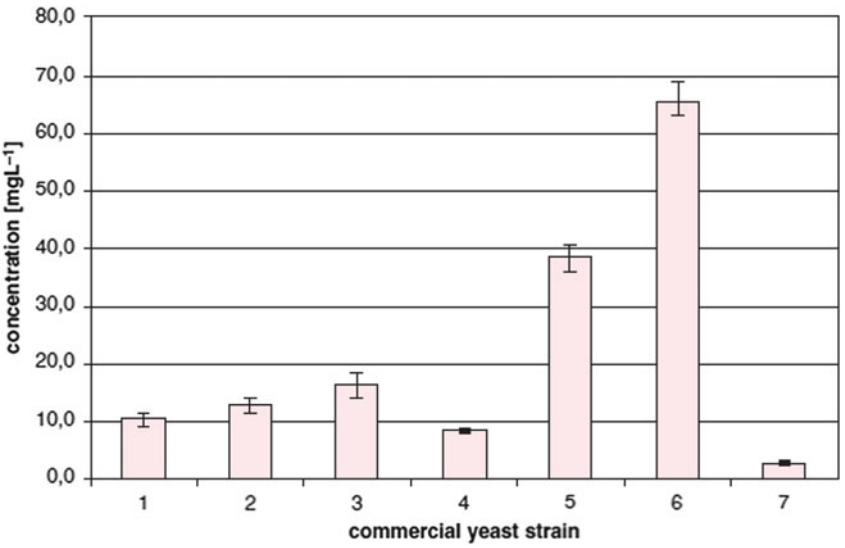

Fig. 11.2 Production of SO_2 by commercial yeast strains during alcoholic fermentation (Werner and Rauhut 2007, unpublished; Werner 2013)

adenosylmethionine (Henschke 1997). It could be demonstrated that in high sulphite-producing strains, sulphate permease is not repressed by methionine. In addition, ATP sulphurylase is not regulated by sulphur-containing intermediates in high and low sulphite-producing strains. Yeasts with low-sulphite production showed an increased biosynthesis of NADPH-dependent sulphite reductase during the exponential growth phase in comparison to yeasts with high-sulphite production. Sulphite production is very energy dependent, and the cellular metabolism of high SO_2-forming yeast strains is reduced, which explains a decreased production of biomass and a slow fermentation rate (Rauhut 1993; Pretorius 2000).

Higher levels of methionine and cysteine can diminish the levels of sulphite reductase (Rauhut 1993; Pretorius 2000). Sulphite production by wine yeasts is also influenced by nutrient composition of grape musts and in addition by the concentration of sulphate, must clarification, initial pH value, temperature and other environmental conditions (Minarik 1977; Larue et al. 1985; Bakalinsky 1996; Larsen et al. 2003; Fleet 2007).

An overview on the impact of sulphur dioxide on yeast cells and developed cellular and molecular mechanisms as strategies to reply to SO_2 exposure is given by Divol et al. (2012).

New tendencies and strategies in winemaking require starter cultures with low formation of sulphite and acetaldehyde obtained by selective breeding (without the use of genetic engineering) (Comitini et al. 2017).

11.2.3 Importance of Glutathione

One of the major antioxidants in living cells is the tripeptide, glutathione (L-γ-glutamyl-L-cysteinylglycine). It is formed through the reaction of cysteine with glutamate and glycine (Fig. 11.1). Glutathione prevents cellular destruction by maintaining certain thiols in their reduced stage due to its SH group of the cysteine molecule. Furthermore, it can react with heavy metals and other toxic compounds (Duncan and Derek 1996; Field and Thurman 1996; Penninckx 2002; du Toit et al. 2007). Glutathione is involved in the oxidative stress response through glutathione peroxidise and in detoxification processes. Vacuolar transport of metal derivatives of the tripeptide ensures resistance to metal stress (Penninckx 2000).

Glutathione was discovered in yeasts by Hopkins and Kendall in 1921 (Kocková-Kratochvílová 1990). It may account for 0.5–1% of the dry weight in the yeast *Saccharomyces cerevisiae*, represents more than 95% of the low-molecular-mass thiol pool and occurs in high concentrations up to 10 mM in yeast cells (Elskens et al. 1991; Mehdi and Penninckx 1997; Penninckx 2002). The sulphur-containing tripeptide can occur in reduced GSH form, the oxidised form GSSG and different mixed disulphides, e.g. GS-S-CoA and GS-S-Cys (Penninckx 2002).

Cheynier et al. (1989) showed that glutathione ranged from 14–102 mg L^{-1} in grape musts from different varieties. Dubourdieu and Lavigne-Cruège (2004) noticed that the glutathione levels in Sauvignon blanc musts are related to the

nitrogenous status of the vines. Musts with low levels of assimilable nitrogen had lower amounts of glutathione. Park et al. (2000a, b) found glutathione up to 1.3 mg L^{-1} in grape musts and up to 5.1 mg L^{-1} in wines. Du Toit et al. (2007) detected up to about 35 mg L^{-1} glutathione in wines. Final wine concentration of glutathione was correlated with both total nitrogen and assimilable amino acid concentration and an increase of glutathione towards the end of fermentation was observed.

Lavigne et al. (2007) investigated that the amount of glutathione after fermentation depends on the yeast strain. Glutathione levels in the wine can be increased through the choice of an adequate yeast strain and to store the wine on lees.

Glutathione may be involved to maintain mitochondrial and membrane integrity in *Saccharomyces* and non-*Saccharomyces* yeasts and can be mobilised during nitrogen and sulphur starvation and/or reproduction. About 50% of the glutathione was in the yeast cytoplasm and the remaining in the central vacuole during growth on nitrogen-sufficient medium. Glutathione stored in the yeast cell is used as an endogenous sulphur source in case of total sulphur deficiency. More than 90% of the cellular glutathione was transported to the central vacuole of the yeast, when *Saccharomyces cerevisiae* was subjected to nitrogen starvation (Mehdi and Penninckx 1997).

Glutathione in the must can be taken up by the yeast cell through two transport systems. Sulphur from glutathione is transferred to other metabolites along the sulphur metabolic pathway (Penninckx 2002). This explains that the supplementation of must with glutathione can lead to unpleasant volatile sulphur compounds under certain conditions, in particular at nitrogen deficiency (Rauhut et al. 2001; Rauhut 2003).

Glutathione can prevent oxidation of white musts due to trapping of *o*-quinones which limits the formation of browning pigments (Singelton 1987; Cheynier et al. 1986, 1989). Lavigne-Cruège and Dubourdieu (2002) found out that glutathione seems to play an important role in protecting volatile thiols that are responsible for the varietal flavour of bottled wines during their ageing. They demonstrated that the addition of 10 mg L^{-1} glutathione at bottling limits the yellowing of wine colour, protects varietal thiols and decreases the occurrence of volatile compounds which contribute to the atypical ageing of wines. Yeast strains with a higher formation and release of glutathione during fermentation and also during storage of wines on lees can contribute to the stabilisation of volatile thiols and can prevent atypical ageing of wines and act against browning to a certain extent (Dubourdieu and Lavigne-Cruège 2004). Thus, extensive research was conducted by different research groups during the last decade to study the impact of glutathione in the winemaking process and to develop strategies increasing its concentration and protecting its reduced form during the winemaking process. In particular, glutathione formation and metabolism for oenology were investigated to take advantage of the differences among yeast strains during the winemaking process and storage, in order to obtain strains better adapted to the frequent environmental stresses occurring before, during and after alcoholic fermentation (Penninckx 2002; Kritzinger et al. 2013a). Among inactive dry yeast (IDY) preparations, which have been widely

applied in winemaking to improve technological processes or sensory characteristics in recent years, special IDY preparations enriched with glutathione and probably other non-volatile thiols have been developed to protect flavour and colour of wines (Pozo-Bayón et al. 2009a, b, c; Rodríguez-Bencomo et al. 2016).

A comprehensive review about the role of glutathione in winemaking is given by Kritzinger et al. (2013b), but also limitations in existing knowledge about its reaction and effect on wine quality are pointed out. Additional information is available from Badea and Antoce (2015).

The International Organization of Vine and Wine (OIV) adopted a new oenological practice for the treatment of musts and wines using glutathione (Resolutions OIV-OENO 445-2015 and OIV-OENO 446-2015). The maximum allowed addition is 20 mg L^{-1} glutathione to must or wine. The intention of these applications is to lower oxidation in musts due to the properties of glutathione to trap specific quinones and to protect flavour compounds from oxidation (OIV 2015). It has to be pointed out that these new resolutions by the OIV do not lead to automatic authorisation of new oenological practices (e.g. any new application must be incorporated into European regulations which is in the case of glutathione still in process) (Oenoppia 2015). *Saccharomyces cerevisiae* strains with increased glutathione formation were evolved with the use of an evolution based strategy by Mezzetti et al. (2014).

11.2.4 Volatile Sulphur Compounds

11.2.4.1 Hydrogen Sulphide and Related Volatile Sulphur Compounds

Volatile sulphur compounds are essential aroma compounds for a huge amount of different foods and play a considerable role in the sensory characteristics of wine. This is related to their high volatility, reactivity and impact at very low concentrations. Sulphur compounds in wine can be classified as thiols (mercaptans), sulphides, thioesters and heterocyclic compounds. Some of the sulphur substances supply to the overall quality of wine, while others are the cause of strong objectionable flavours (rotten eggs, cooked cabbage, cauliflower, burnt rubber, cooked meat, etc.), even at extremely low concentrations [e.g. hydrogen sulphide (H_2S), methanethiol (MeSH), ethanethiol (EtSH)]. Because of their occurrence in very low concentrations (nanogram or microgram levels) and the high reactivity of thiols with metal residues (e.g. copper and silver) and their fast oxidation with traces of oxygen, it was very difficult to study the occurrence and formation of volatile sulphur compounds. After the development of soft analytical extraction methods and high sophisticated gas chromatographic systems in connection with high sensitive sulphur detectors, it was possible to get more information on their biosynthesis and presence in the last two decades. Sulphur aroma compounds were often separated in low and high volatile sulphur substances due to the broad range of different boiling points and the need of different analytical methods to

enrich the sulphur compounds from wines (Fedrizzi et al. 2007; Ferreira et al. 2007; Lopez et al. 2007; Mateau-Vivaracho et al. 2008; Rauhut et al. 2005, 2007; Schneider et al. 2006; Thibon et al. 2008a). In accordance with the new rules for the international nomenclature of chemical compounds (IUPAC), the prefix 'methyl-sulphanyl' should replace the prefix 'methylthio' and 'ethyl-sulphanyl' should replace the prefix 'ethylthio'; furthermore the prefix 'sulphanyl' must replace the prefix 'mercapto'. In the following, the more familiar chemical names of the sulphur compounds are mainly used to avoid confusion.

Volatile sulphur compounds are formed through several pathways involving enzymatic and/or non-enzymatic processes. Yeast fermentation biochemistry with sulphate-, sulphite-, sulphur-containing amino acids (methionine and cysteine) and oligopeptides (e.g. glutathione) plays a crucial role among the enzymatic processes.

Non-enzymatic processes involve chemical, photochemical and thermal reactions during winemaking and storage. Other factors for an increased development of unpleasant sulphur substances are an increase of wettable sulphur (used as fungicide) residues and other sulphur-containing pesticides in grape musts. Relevant overviews on the formation and occurrence of sulphur compounds in wine with a different focus are given from Maujean (2001), Rauhut (1993, 1996, 2003), Ribéreau-Gayon et al. (2000a, b), Swiegers et al. (2005), Vermeulen et al. (2005) and Smith et al. (2015).

In the following, a review is presented on the formation of hydrogen sulphide (H_2S) and related sulphur compounds through yeast sulphur and nitrogen metabolism during the winemaking process.

Sulphide is usually formed through the sulphate reduction sequence as indicated in Fig. 11.1 in response to a metabolic requirement, such as that induced by growth, for organic sulphur compounds like cysteine, methionine, *S*-adenosyl methionine and glutathione (Rankine 1963, 1964; Hallinan et al. 1999; Spiropoulos and Bisson 2000; Spiropoulos et al. 2000; Bell and Henschke 2005). Under certain conditions, mainly if insufficient or unsuitable nitrogen sources are available, a surplus of sulphide is released from the cell. It is converted spontaneously to H_2S as a consequence of the reductive conditions established in the anaerobic fermentation at low pH (Linderholm et al. 2008). The information on the threshold value for H_2S is in a range of 11–80 μg L^{-1}. The difference is depending on the used media for determination of the odour threshold value (Amoore and Hautala 1983; Wenzel et al. 1980). Concentrations exceeding these values cause an undesirable off-flavour that is reminiscent of rotten eggs. Lower levels in young wines contribute to the 'yeast' flavour or fermentation bouquet. Slight increased values take part in the so-called reductive off-odour in wines (Dittrich and Staudenmayer 1968; Monk 1986; Dittrich 1987).

One of the most influencing factors on H_2S production is the strain of *Saccharomyces cerevisiae*. Some strains produced amounts up to 1 mg L^{-1} H_2S (Acree et al. 1972; Eschenbruch 1974, 1978; Eschenbruch et al. 1978; Vos and Gray 1979; Giudici and Kunkee 1994; Jiranek et al. 1995a, b; Mendes-Ferreira et al. 2002). The variation among strains is genetically based but also influenced by environmental factors. Some yeast strains are constantly high or constantly low H_2S producers.

Most strains showed a strong influence on growth conditions and medium composition of sulphide formation. Genetic variation seen indicates a complex inheritance, meaning multiple genes are likely involved. Linderholm et al. (2008) identified several genes that have an impact on H_2S formation. Five genes (*MET17*, *CYS4*, *HOM2*, *HOM6 and SER33*) encode proteins directly involved in the biosynthesis of sulphur-containing amino acids, whereas other genes or their substrates and products may have key regulatory effects in the reduction of sulphate or play a more indirect role. Accumulation of acetaldehyde and the elongator histone complex are suggested as two cellular activities that have an impact on sulphide production during anaerobic fermentation.

H_2S is produced during the early to middle stages of fermentation and responds to nutrient composition and concentration (Vos and Gray 1979; Vos 1981; Monk 1986; Henschke and Jiranek 1991; Jiranek et al. 1995a, b). Ammonium salts like diammonium hydrogen phosphate (DAP) are widely used to compensate nitrogen deficiencies in grape musts and to control H_2S formation. Ammonium represses the *Met10* gene, which encodes the alpha-subunit of sulphite reductase (Hansen et al. 1994), but not in all cases its addition is effecting H_2S production due to other factors, e.g. methionine and other nitrogen sources that regulate amino acid transport into the yeast cell and sulphur metabolism (Spiropoulos et al. 2000; Spiropoulos and Bisson 2000).

H_2S formation can also occur in a second phase at the final stage of fermentation. Henschke (1996) observed limited evidence for a response to aeration and vitamin addition. Nutrient levels are very low at the end of fermentation; for that reason, it is proposed that a deficiency of vitamins or the degradation of sulphur reserves like glutathione is involved (Eschenbruch et al. 1978; Elskens et al. 1991; Hallinan et al. 1999). Insufficiencies in vitamins and micronutrients (pantothenate) and vitamin B_6 (pyridoxine) essential for the synthesis of sulphur-containing amino acids may also contribute to H_2S production (Jiranek et al. 1995a, b; Spiropoulos et al. 2000; Wainright 1970; Wang et al. 2003).

H_2S can be produced by the degradation of glutathione and the release of cysteine, which is then degraded by cysteine desulphhydrase, when nitrogen is limited (Tokuyama et al. 1973).

It is approximated that glutathione, which is accumulated in the yeast cell, can participate in up to 40% of the sulphide in cells with nitrogen starvation (Hallinan et al. 1999).

There are many other causes that increase H_2S production and the formation of other undesirable sulphur compounds, e.g. residues of elemental sulphur from wettable sulphur treatment of the vines and other sulphur-containing pesticides and their breakdown products, clarification level, concentration of SO_2, fermentation temperature, residues of copper ions and storage on lees (Bell and Henschke 2005; Henschke and Jiranek 1993; Maujean 2001; Rauhut 1993, 2003; Ribéreau-Gayon et al. 2000a, b).

An accelerated H_2S production leads to a higher formation of volatile sulphur compounds, especially if it is not carried out with the carbon dioxide produced

during fermentation and if increased amounts remain in the young wine (Rauhut 1996, 2003).

It has been suggested that ethanethiol is formed by the reaction of H_2S and ethanal (acetaldehyde) via a cyclic trithioethanal intermediate (Rankine 1963, 1968; Tanner 1969), but this reaction could not be demonstrated in model solutions and in wine (Bobet 1987; Rauhut and Kürbel 1994). On the contrary, it could be demonstrated that H_2S and ethanal is reacting to 1,1 ethanedithiol, which has a sulphury and rubbery flavour note, at wine-like conditions and in wine (Rauhut 1993, 1996). Further reaction products were identified. The same compounds were measured in yeast extract (Werkhoff et al. 1991). Therefore a contribution of these sulphur compounds to sulphur-related off-flavours can be expected (Rauhut 1993). It has also been suggested that ethanol and H_2S react to ethanethiol, but this could not be confirmed up to now (Rankine 1963, 1968; Rauhut 1993, 2003). A huge amount of all volatile sulphur substances is produced during the alcoholic fermentation. It could be demonstrated that yeast strains differ in their formation of sulphur compounds (Fig. 11.3; Rauhut and Kürbel 1994; Rauhut et al. 1995, 2000). An accelerated formation of H_2S leads to an increase of thioacetic acid esters. Matsui and Amaha (1981) supposed that high concentration of methanethiol and H_2S can hinder the growth of yeasts. It is therefore assumed that the formation of thioacetic acid esters is a detoxification process to transform sulphur substances with a free SH group, which can inhibit enzymes, to non-affecting compounds like the thioacetic acid esters. High H_2S formation in the early phase of fermentation leads to a high

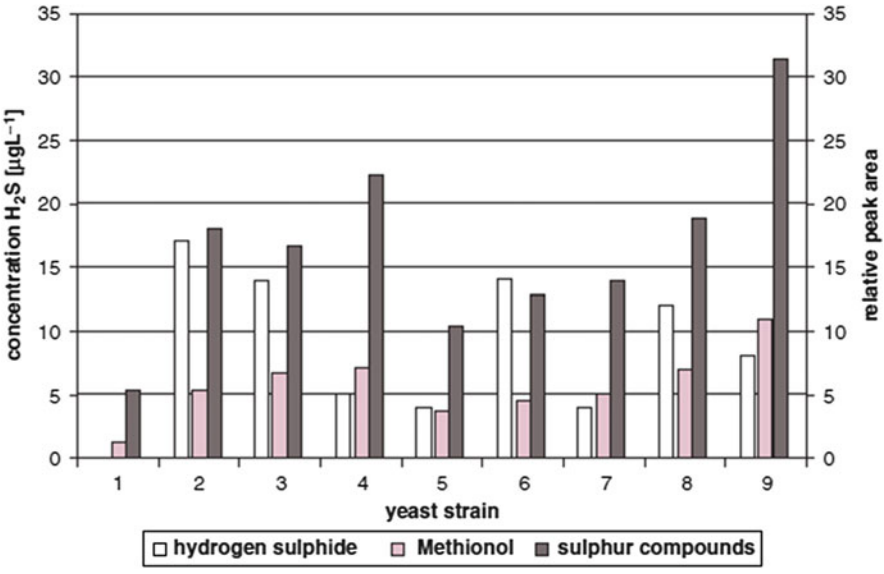

Fig. 11.3 Formation of hydrogen sulphide and the total sum of high volatile sulphur compounds and methionol in wine through nine different strains of *Saccharomyces cerevisiae* (according to Rauhut 1996, modified)

formation of thioacetic acid-*S*-methyl ester (MeSAc), and high formation of H_2S at the end of fermentation increased the formation of thioacetic acid-*S*-ethyl ester (EtSAc) (Rauhut 1996). Therefore, an increased formation of these two thioacetic acid esters can be used as an indicator for a high H_2S formation during the fermentation process. Thioacetic acid esters were probably produced through the reaction of the thiols and acetyl coenzyme A (Matsui and Amaha 1981, Walker and Simpson 1993). The addition of methanethiol and ethanethiol during fermentation leads to the corresponding thioacetic acid esters (Rauhut 1996). Furthermore, an increase of 3-(methylthio)-1-propanol (methionol), 3-(ethylthio)-1-propanol (ethionol) and other volatile sulphur compounds was observed. Increased levels of thioacetic acid ester can be seen as an indicator for a high H_2S production during fermentation. MeSAc can be detected in normal wine in a concentration up to about 20 μg L^{-1} (Leppänen et al. 1979, 1980). In off-flavour wines, more than 130 μg L^{-1} could be measured (Rauhut 1996).

Thioacetic acid esters can hydrolyse during wine storage like other acetic acid esters after fermentation due to the chemical equilibrium (Rapp 1989), which is influenced from pH value, storage temperature, etc. The hydrolysis of thioacetic acid esters leads to free thiols and acetic acid. The thiols have very low threshold values (<2 μg L^{-1}) in comparison to the thioacetic acid esters (>40 μg L^{-1}). A treatment of wine with copper sulphate has no influence on the concentration of the thioacetic esters, because copper ions mainly react with H_2S and thiols (Rauhut 1996, 2003). A reoccurrence of off-flavours in wines after a copper fining, bottling and storage is related to a release of unpleasant volatile compounds from non-volatile or volatile precursors like the thioacetic acid esters. In this content, the role of oxygen, the addition of copper ions, the occurrence of other transition metals, closure types and filling processes is discussed and investigated (Dimkou et al. 2011; Silva et al. 2011; Ugliano et al. 2011; Ugliano 2013; Viviers et al. 2013; Franco-Luesma and Ferreira 2016). There are several indications that other precursors exist, e.g. copper-thiol complexes, which are the source for the liberation of unpleasant sulphur compounds (mainly H_2S and methanethiol) during the storage of bottled wines (Franco-Luesma and Ferreira 2014; reviewed by Clark et al. 2015 and Waterhouse et al. 2016). Thiols can be oxidised to disulphides or trisulphides, which contribute to odours like ‘rubber’ or ‘garlic’. Disulphides or trisulphides cannot be removed by copper fining (Maujean 2001).

Kinzurik et al. (2015) noticed, as also indicated before from Rauhut (1996) and Rauhut et al. (1996), that notable variations in the time of production of volatile sulphur compounds during fermentation can be expected depending on yeast strains and composition of the media. Methanethiol production was detected early during anaerobic growth, whereas others like benzothiazole and thioacetic acid-*S*-ethyl ester were formed at a steady rate during the fermentation and diethyl disulphide was detected at the end of the fermentation process (Kinzurik et al. 2015).

The research studies of Kinzurik et al. (2016) provided more insight in the reaction of H_2S during fermentation. They could show with ^{34}S-labelled sulphate, which was added to the fermentations with a *MET-17*-deleted strain in a synthetic

grape juice medium, that ethanethiol, diethyl disulphide and thioacetic acid-*S*-ethyl ester were formed directly from H_2S.

Cordente et al. (2007, 2009) used classical biological non-GMO techniques to develop *Saccharomyces cerevisiae* strains that are not able to produce increased H_2S amounts in grape musts with low assimilable nitrogen levels.

Methionol is the main produced volatile sulphur compound through yeast metabolism. This sulphur-containing higher alcohol can give a raw potato or cauliflower aroma in concentrations exceeding 2000 µg L^{-1} (Meilgaard 1981). It was detected in wines up to concentrations of 6300 µg L^{-1} (Keck 1989). Methionol production is linked to sulphur and nitrogen metabolism of yeast. Figure 11.4 indicates the formation of the total sum of high volatile sulphur compounds and methionol in two different synthetic media (AAI is low in assimilable nitrogen, whereas AAII is high in assimilable nitrogen) through two *Saccharomyces cerevisiae* strains with a different ability to produce volatile sulphur compounds. It could be demonstrated that the addition of inorganic nitrogen in the form of 0.3 g L^{-1} diammonium hydrogen phosphate (DAP) can decrease the total sum of volatile sulphur compounds and methionol in both media.

In synthetic media with ammonium as only nitrogen source, methionol was produced as one of the main volatile sulphur compounds (Rauhut 1996). This indicates that methionol can be also synthesised like other higher alcohols from

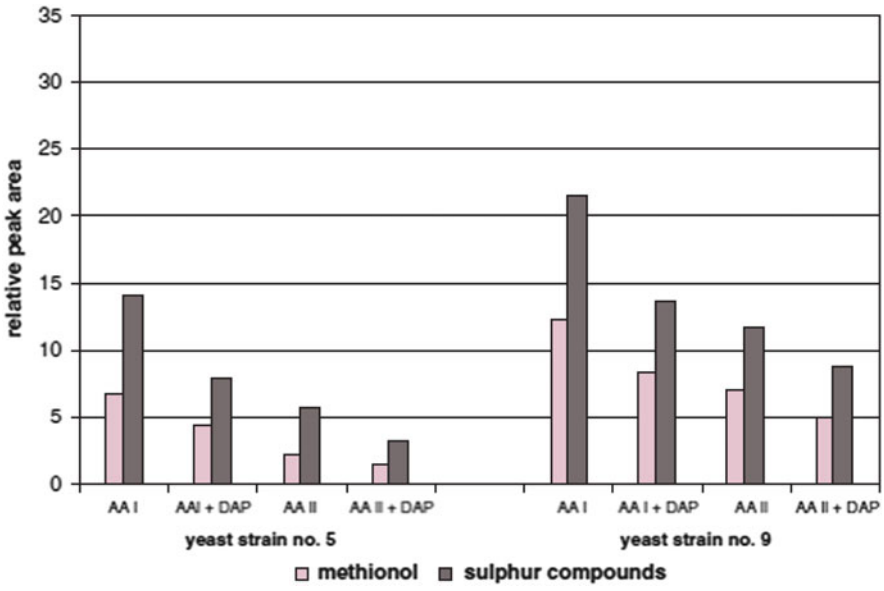

Fig. 11.4 Differences in the formation of the total sum of high volatile sulphur compounds and methionol in two different synthetic wine-like media [AAI (low in assimilable nitrogen) and AAII (high in assimilable nitrogen)] with and without addition of 0.3 g L^{-1} diammonium hydrogen phosphate (DAP) through two different strains of *Saccharomyces cerevisiae* (No. 5 and 9) (according to Rauhut 1996, modified)

an α-keto acid, 2-oxo-4-(methylthio) butyric acid, derived from sugars via glycolysis. The keto acid is then decarboxylised to 3-(methylthio)-propanal (methional), which is reduced to the alcohol. Methionol can be also produced from methionine through the Ehrlich pathway, which involves a transamination to the corresponding keto acid, decarboxylation to the aldehyde and enzymatic reduction to the alcohol. Methanethiol can be synthesised from methionine and the keto acid by demethiolase activity (Perpète et al. (2006). A supplementation of methionine before fermentation increased methionol, its corresponding acid and its acetic acid ester. These observations suggest that amounts of methionol in wine may be influenced by the levels of methionine as well as the assimilable nitrogen concentrations in the must (Rauhut 1996; Moreira et al. 2002; Bell and Henschke 2005; Ugliano and Henschke 2009).

The intermediate compound, methional, can only be detected in traces in wines after fermentation. Increased levels of methional are investigated in aged wines. There is also evidence that an accelerated level of methional and other sulphur compounds is involved in the so-called atypical ageing of wines (Rauhut 1996, 2003).

Further reaction products of methional like acetic acid-3-methylthiopropylester have an odour like 'mushroom' or 'garlic'. It has been also suggested that 4-methylthio-1-butanol and 2-mercapto-1-ethanol, which have both an unpleasant odour ('onion'/'garlic' and 'poultry'/'farmyard') can be probably formed by yeast in the same way like methionol via the amino acids homocysteine and cysteine, respectively (Moreira et al. 2002; Mestres et al. 2000; Swiegers et al. 2005).

Several studies demonstrated that increased additions of DAP can also increase the formation of H_2S and other volatile sulphur compounds and change their profiles (Rauhut 1996; Ugliano et al. 2009). These effects are strain dependent. It is evident that strains differ in the requirement for assimilable nitrogen as well as in certain regulation processes. In addition, it could be demonstrated by Wang et al. (2003) that also adequate levels of certain vitamins are required to avoid high H_2S formation at different levels of assimilable nitrogen. Therefore, complex nutrient supplements (combinations of DAP, thiamine, yeast cell hulls and/or inactive yeast preparations) are more and more recommended to support yeasts during alcoholic fermentation and to avoid off-flavours caused by volatile sulphur compounds.

Sulphur residues of more than 2.5 mg L^{-1} in the grape must from wettable sulphur applications in the vineyard can lead to an increased H_2S formation during the fermentation process (Thomas et al. 1993a, b, c; Rauhut 1996). The addition of elemental sulphur in amounts over 5 mg L^{-1} leads to an increased level of unpleasant sulphur compounds (EtSH, MeSAc, EtSAc, etc.) and extreme off-flavours (Fig. 11.5).

A similar effect could be observed with the supplementation of glutathione to must, which indicated that higher concentrations of this tripeptide can increase the development of unpleasant volatile sulphur compounds during fermentation at certain conditions (Rauhut et al. 2001, 2003). It is assumed that the addition of glutathione levels (>50 mg L^{-1}) to grape musts can lead to a higher H_2S formation, which can cause a further production of other undesired sulphur compounds like

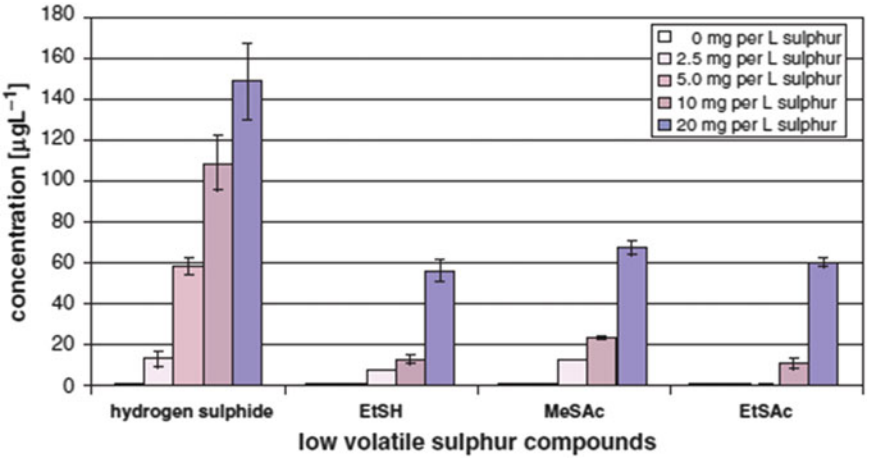

Fig. 11.5 Effect of different concentrations of elemental sulphur (added to must before fermentation) on the formation of hydrogen sulphide, ethanethiol (EtSH), thioacetic acid-*S*-methyl ester (MeSAc) and thioacetic acid-*S*-ethyl ester (EtSAc) during fermentation (Werner and Rauhut 2007, unpublished; Werner 2013)

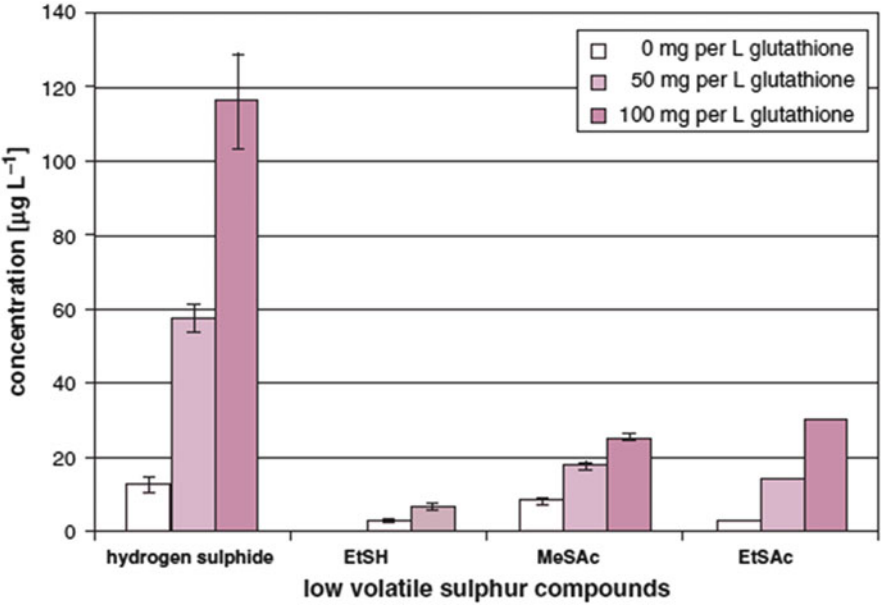

Fig. 11.6 Influence of different levels of glutathione addition to grape must on the formation of hydrogen sulphide, ethanethiol (EtSH), thioacetic acid-*S*-methyl ester (MeSAc) and thioacetic acid-*S*-ethyl ester (EtSAc) during fermentation (Werner and Rauhut 2007, unpublished)

ethanethiol (EtSH), MeSAc and EtSAc (Fig. 11.6) through yeast sulphur metabolism, in particular in the case of grape must nitrogen deficiency or depletion at the beginning of fermentation. This seems to be affected by the yeast strain, the nutrient composition and other ingredients of the grape must. On the other hand, the addition of glutathione is recommended to protect flavour compounds from oxidation (Sect. 11.2.3) (Dubourdieu and Lavigne-Cruège 2004; Ugliano et al. 2011, Tomašević et al. 2016). Research studies from Wegmann-Herr et al. (2016) with glutathione addition and the application of a glutathione-enriched inactive yeast product showed that pleasant aroma compounds (such as 3-mercaptohexanol) could be preserved by glutathione addition to must, but that at the same time the trapping of H_2S and other undesired thiols seemed to be inhibited due to the formation of glutathione phenol adducts. In particular, the authors noticed this effect in wines with a low phenolic content which can lead to sulphur off-flavours. These results, investigations by Burkert (2017) and own experiences point out that further research is required to study the difference in the application of glutathione-enriched inactive yeast preparations or addition of pure glutathione, the time of the addition as well as other oenological factors and conditions, such as the impact of the yeast strain, the nutrient composition of the must and the addition of nutrient supplements as well as the application and amounts of SO_2, ascorbic acid, etc. to strengthen the knowledge for a better understanding of the reaction processes and for adequate advice and consulting.

Several thiols were detected that seem to be synthesised in the presence of accelerated H_2S concentrations. Bernath (1997) detected increased levels of 2-methyl-3-furanthiol, a very powerful aroma compound with a very low threshold value. This thiol and its disulphide, bis(2-methyl-3-furyl)disulphide, contribute to the typical flavour of 'cooked meat'. It is supposed that these compounds are formed through the hydrolysis of thiamine (Belitz and Grosch 1992). Tominaga and Dubourdieu (2006) measured up to 100 ng L^{-1} of 2-methyl-3-furanthiol in different white and red wines. In off-flavour wines, concentrations of more than 300 ng L^{-1} were detected (Bernath 1997).

Furfurylthiol (2-furanmethanethiol) has a perception threshold of 0.4 ng L^{-1} (in water) and elicits odours like roasted coffee, meat, wheat bread and popcorn. It was found in Bordeaux red wines, white Petit Manseng and also in toasted barrel staves (Tominaga et al. 2000a). Blanchard et al. (2001) demonstrated that the addition of nitrogen can decrease the amount of furfurylthiol; therefore, it is assumed that its production is related to the formation of H_2S. Furfurylthiol was detected in wines in a concentration up to about 50 ng L^{-1} (Tominaga and Dubourdieu 2006).

Benzylthiol (benzenemethanethiol) contributes to the 'smoky' and 'flintstone' character of wines and has an odour threshold of about 0.3 ng L^{-1} (water-ethanol model solution) and was detected in wines in an amount up to about 15 ng L^{-1}. Furfurylthiol and Benzylthiol can increase during the ageing of wines, probably in the presence of H_2S (Tominaga et al. 2003).

A release of H_2S can also take place during the autolysis of yeasts (Suomalainen and Lehtonen 1979). It has been suggested that apart from glutathione, sulphur-

containing amino acids are degraded during autolysis, but the mechanism involved is unclear (Henschke and Jiranek 1993). Berry and Watson (1987) proposed that yeast with a low vitality such as in other sluggish or stuck fermentations can tend to autolyse faster. Furthermore, low alcohol-tolerant non-*Saccharomyces* yeasts in certain spontaneous fermentations lose viability through inhibition of the increasing levels of alcohol and presumably autolyse during the early to mid-phases of fermentation (Henschke and Jiranek 1991; Fleet and Heard 1993; Swiegers et al. 2005).

Lavigne-Cruège (1996) demonstrated that H_2S and methanethiol that are present at the end of fermentation decrease during barrel ageing. The dropping-off happens more rapidly in new barrels, probably due to a higher oxygen dissolution and the oxidising effect of new wood tannins (Ribéreau-Gayon et al. 2000a). Objectionable flavours can occur if an addition of sulphite (SO_2) is carried out after fermentation and if the wines are stored on lees. This is due to the activity of sulphite reductase, which can last up to about four weeks after fermentation. Sulphite reductase is producing H_2S from the added SO_2. It seems that the compacting of the lees under the pressure exerted at the bottom of high-capacity tanks is enhancing 'reductive' off-flavours in white wines after sulphite addition. Therefore, it is recommended to rack the wines after sulphite addition and to store the lees in barrels. This avoids the development of off-odours from gross lees. The separated lees should be reincorporated into the wine after approximately one month when the sulphite reductase activity is diminished, so that there is no longer a risk for the development of off-flavours. Yeasts have the opportunity to adsorb thiols due to their reaction with cell wall mannoproteins. A disulphide bond is formed between the cysteine of the cell wall mannoproteins and the thiols during aeration. More details on these enological practice can be taken from Lavigne-Cruège (1996) and Ribéreau-Gayon et al. (2000a).

Pixner et al. (2015) demonstrated that the addition of sulphite prior alcoholic fermentation increased reductive notes in Vernatsch wines.

Only a few research groups studied the contribution of non-*Saccharomyces* yeasts on the formation of volatile sulphur compounds (Romano et al. 1997, 2003). Trials in yeast-malt medium with mixed yeast cultures of *Hanseniaspora apiculata*, *Hanseniaspora guilliermondii* and *Saccharomyces cerevisiae* indicated that similar levels of 3-methylthiopropionic acid and acetic acid-3-(methylthio) propyl ester were produced like in the variant with pure inoculation of *Saccharomyces cerevisiae*. Non-*Saccharomyces* yeasts can also favour the formation of methionol production in fermented media (Moreira et al. 2005; Landaud et al. 2008).

There is a growing demand for yeast starter cultures obtained with conventional methods (non-GMO) with low formation of H_2S and if possible combined with other desired features like low formation of sulphite and acetaldehyde (Berlese-Noble et al. 2014).

Information on further volatile sulphur compounds that are determined in wines can be taken from Du Toit and Pretorius (2000), Dittrich and Großmann (2005), Keck (1989), Marchand et al. (2000), Pripis-Nicolau et al. (2000), Rauhut (1993,

1996), Ribéreau-Gayon et al. (2000a, b), Segurel et al. (2004) and Ugliano and Henschke (2009).

11.2.4.2 Occurrence and Formation of Thiols Involved in the Varietal Flavour of Wines

At the beginning of the 1980s, it was already proposed that certain volatile thiols contribute to the characteristic aromas of Chenin and Sauvignon wines (Augustyn et al. 1982; Marais 1994).

The first thiol identified in Sauvignon wines was 4-mercapto-4-methylpentan-2-one (4-MMP), whose odour threshold is very low (0.1–0.8 ng L^{-1} in water and model solution), and it elicits aromas like blackcurrant, box tree and broom (Darriet et al. 1991; Murat et al. 2001a). Characteristic nuances of the variety Sauvignon blanc were observed with concentrations of 4-MMP close to 40 ng L^{-1}. Increased concentrations of 4-MMP cause a 'catty' note in wines.

Apart from 4-MMP, further thiols, e.g. 3-mercaptohexanol (3-MH), 4-mercapto-4-methylpentan-2-ol (4-MMPOH) and acetic acid-3-mercaptohexyl ester (3-MHA), have been identified as major contributors to the varietal aroma of Sauvignon blanc wines (Darriet et al. 1995; Tominaga et al. 1995, 1998; Dubourdieu et al. 2001). Tropical fruit flavours such as passion fruit, grapefruit, citrus zest, lychee and guava in some of the Sauvignon wines are mainly related to 3-MH and its acetic acid ester. These compounds have a similar threshold value like 4-MMP.

4-MMP, 3-MH, 3-MHA and other related thiols have also been detected in wines made from other varieties like Petit and Grand Manseng, Arvine, Colombard, Chenin blanc, Alsace Muscat, Gewürztraminer, Riesling, Scheurebe, Bacchus, Cabernet Sauvignon, Merlot, etc. (Tominaga et al. 2000a, b; Murat et al. 2001a; Guth 1997a, b; Ribéreau-Gayon et al. 2000b). In addition, it could be shown that varietal thiols also play an important role in the typical flavour of wines from certain Greek autochthonous grape varieties (Kapaklis 2014).

The volatile thiols are almost not occurring in the grape must and develop during the fermentation process. Tominaga et al. (1998) identified odourless sulphur-cysteine conjugates as precursors for the high odour-active thiols. The release of the thiols was investigated by the use of a cell-free enzyme extract of the bacterium *Eubacterium* limosum that contain carbon-sulphur lyase enzymes. It could be shown that carbon-sulphur lyases can release 4-MMP from its precursor *S*-4-(4-methylpentan-2-one)-L-cysteine. Therefore, it was suggested that a yeast cysteine β-lyase releases a thiol, pyruvic acid and ammonium from the corresponding sulphur-cysteine conjugate as it is shown for 4-MMP in Fig. 11.7 (Tominaga et al. 1995, 2004; Masneuf 1996; Peyrot des Gachons et al. 2000, 2002a, b). This enzyme belongs to the carbon-sulphur lyases, which cleave a carbon-sulphur bond due to a β-elimination reaction (Dufour et al. 2013).

Peyrot des Gachons et al. (2002a, b) identified the precursor of 3-MH, *S*-3-(hexan-1-ol)-glutathione, in must of Sauvignon blanc for the first time. It is

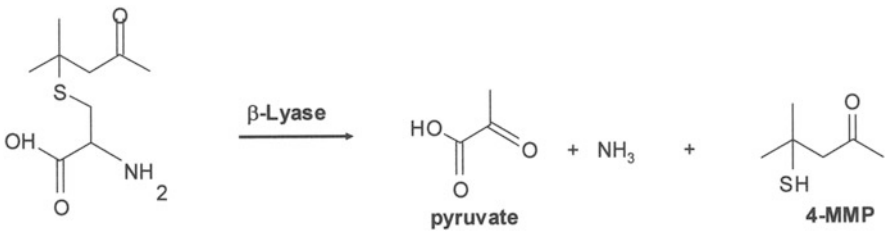

Fig. 11.7 Release of 4-MMP from the corresponding sulphur-cysteine conjugate (according to Peyrot des Gachons et al. 2000; Wüst 2003, modified)

assumed that a glutathione transferase is involved in the synthesis of the sulphur-glutathione conjugates, which are probably transported with the help of a glutathione-conjugate pump to the cell vacuole. The sulphur-cysteine conjugates are almost certainly formed through the activity of a γ-glutamyltranspeptidase and a carboxypeptidase (Wüst 2003).

Research work of Murat et al. (2001a) indicated that yeast strains vary in the ability to release 4-MMP, 3-MH and 4-MMPOH. It could be also demonstrated that commercial yeast strains differ in the release of the various volatile thiols. Strains of *Saccharomyces bayanus* and their hybrids created with *Saccharomyces cerevisiae* released even higher concentrations of the thiols. The activity of the enzymes involved in the release of the different thiols is strain dependent. A variation of the release of the enzymes and of the thiols can be achieved by the use of specific yeast strains. Dubourdieu et al. (2001, 2006) demonstrated in model fermentations that a synthesised precursor decreased in concentration, while the corresponding thiol increased, but only a small fraction (1.6% at day 6 of fermentation) of the cysteine-bound precursor was transferred to the thiol. The same effect was shown with Cabernet Sauvignon and Merlot musts (Murat et al. 2001a). Only 3.2% (average value) of the precursor was decomposed during the fermentation.

Therefore, the choice of the yeast strain is of considerable importance to enhance flavour complexity of wines and to use the huge potential of odourless sulphur-cysteine conjugate precursors to release the desired volatile thiols and to create specific wine styles (Swiegers et al. 2005). On the contrary, it is very crucial to avoid an overproduction of 'tropical' fruit flavours through specific yeast strains in wines from varieties that normally do not release this kind of aroma notes. Therefore, the adequate application of the yeast strains is very important to develop the 'typical' flavours of certain varieties and to activate the release of certain aromas for specific wine styles.

Howell et al. (2005) showed with a laboratory strain that four genes (*BNA3*, *CYS3*, *GLO1* and *IRC7*) are involved in the release of 4-MMP. This points out that the mechanism of release probably involves multiple genes (Swiegers et al. 2005, 2006). Subileau et al. (2008a) provided evidence that other transporters than the general amino acid transporter, *GAP1*, might be involved in the uptake of thiol precursors. Thibon et al. (2008b) showed by using a grape juice in which the cysteinylated precursor of 4-MMP was added that only the *IRC7* gene which

encodes for a cystathionine β-lyase is responsible for its release in *Saccharomyces cerevisiae*. Furthermore they could demonstrate that the related Ure2p/Gln3 proteins mainly control the release of volatile thiols by transcriptional regulation of the *IRC7* gene through nitrogen catabolic repression (NCR). Also Subileau et al. (2008a) and Winter et al. (2011a) demonstrated that NCR is effecting the thiol formation in some yeast strains, whereas Deed et al. (2011), Harsch and Gardner (2013) and Srisamattharakan (2011) noticed no influence in other strains. The genes involved in NCR seem to be strongly yeast strain dependent (Deed et al. 2011). Roncoroni et al. (2011) reported two alleles for the *IRC7* gene, a full-length version and a 38-bp deletion form, which is encoding for a less functional enzyme. These authors also showed that full-length *IRC7* is necessary and sufficient for 4-MMP release in grape juice. Thibon et al. (2008b) indicated that a deletion of full-length *IRC7* gene led only to a partially blocked release of 3-MH of the cysteinylated 3-MH precursor. Its participation in the release of 3-MH is not fully investigated (Roncoroni et al. 2011; Santiago and Gardner 2015). Belda et al. (2016a) indicated that most of *Saccharomyces cerevisiae* strains have the deleted allele of the *IRC7* gene.

Several research studies concluded that the majority of *Saccharomyces cerevisiae* strains are only able to release about 10% of the thiol precursors available in grapes and grape juices (Murat et al. 2001b; Coetzee and du Toit 2012; Belda et al. 2017a). Pinu et al. (2012) showed that the amount of 3-MH precursors does not correlate with the amount of thiols measured in the final wines.

Sulphur and nitrogen metabolism seem to be crucial in regulating the biosynthesis of 3-MA and 3-MHA during alcoholic fermentation of grape must (Harsch and Gardner 2013).

Swiegers et al. (2005) showed the link between ester and volatile thiol metabolism in yeast for the first time. It could be demonstrated that 3-MH is transformed to 3-MHA by the ester-forming alcohol acetyltransferase, encoded by the *ATF1* gene. Large differences in the ability of commercial strains were noticed to form 3-MHA. The ability to produce high levels of 3-MHA did not correspond with the ability to release 4-MMP.

Casu et al. (2016) revealed that increased levels of linoleic acid can lead to a remarkable decrease of 3-MHA. They proposed that linoleic acid lowered the acetylation by inhibiting an acetyltransferase. The effect of linoleic acid on 3-MH was strain specific. These results point out that the compositions of nutrients as well as other ingredients of the grape juice have to be much more considered in the research on the formation of varietal thiols and to get more insight in regulation processes of the yeast cell.

Swiegers et al. (2000) demonstrated the power of yeast in enhancing the aroma of wine due to the development of a prototype wine yeast able to release significantly more thiols than conventional yeast. The *Escherichia coli* tryptophanase gene, *tnaA*, was overexpressed in commercial wine yeast by the use of genetic modification technology. The transformant producing the bacterial cysteine β-lyase had more than a tenfold increase in 4-MMP concentration in comparison to the

commercial control strain. Lilly et al. (2006) indicated that the overexpression of *ATF1* in a wine yeast resulted in increased 3-MHA levels.

Schneider et al. (2006) demonstrated a new possible pathway leading to 3-MH and 4-MMP, starting from conjugated carbonyl compounds, alternative to the already known release from cysteinylated precursors. They described that 3-MH could be produced through the reaction of H_2S formed by yeasts during alcoholic fermentation by 1,4-addition with (E)-hexen-2-al to 3-mercaptohexenal-2 followed by a reduction step, whereas the formation of 4-MMP probably results from the reaction of H_2S with mesityl oxide. (E)-hexen-2-al is formed in damaged plant cells and occurs in grape juice, thus this could be an explanation for the ubiquitous occurrence of 3-MH in wines. Nevertheless, the presence of mesityl oxide or its hydrate in must should be analysed in a first step to investigate the contribution of the hypothesised alternative pathway to the total amount of 4-MMP in certain wines. The studies of Subileau et al. (2008b) revealed that the cysteinylated precursor of 3-MH and (E)-hexen-2-al are not the major precursors of 3-MH. In addition, they revealed that the glutathionylated precursor of 3-MH is transported via Opt1p into the yeast cell. Grant-Preece et al. (2010) demonstrated under model fermentation conditions that 3-MH can be formed from the glutathionylated precursor and the cysteine conjugate is also required in this process. The genes *DUG1*, *DUG2*, *DUG3*, *CPC*, *CPY* and *ECM38* seem to be involved in this pathway (Cordente et al. 2012, 2015; reviewed by Belda et al. 2017a). The release of 3-MH from its glutathionylated precursor could not directly be achieved with a known carbon-sulphur β-lyase. 3-MH seems to be more easily released from the cysteine precursor than from the glutathione precursor (Winter et al. 2011b).

Research studies of Roland et al. (2010a, b) indicated that certain reactions can also lead to other thiol precursors. A detailed review on the different biogenesis pathways for 4-MMP, 3-MH and 3-MHA during alcoholic fermentation was given by Roland et al. (2011). The authors also pointed out that the sulphur donor could be apart from H_2S also cysteine, glutathione or other molecules with a free SH group.

Harsch and Gardner (2013) demonstrated that supplying H_2S to grape juice led to the production of very high concentrations of 3-MH and that both (E)-hexen-2-al and (E)-hexen-2-ol can function as precursors. The authors proposed to investigate opportunities to increase the C6-precursors or to find legal ways to raise H_2S-formation during the early stage of fermentation to increase the potential for 3-MH release. It has to be taken into account that an increased production of H_2S can lead to off-flavours due to its low odour threshold value and its reaction with other substances forming unpleasant sulphur compounds.

The formation of labelled *S*-3-(hexanal)-glutathione was already shown by Capone and Jeffery (2011) with the addition of labelled (E)-hexen-2-al to whole grape berries before crushing. This was the evidence that the glutathionylated precursor is probably formed to a higher extent than expected during berry crushing. It is assumed that the aldehyde intermediate must be enzymatically reduced to achieve the glutathionylated 3-MH precursor by alcohol dehydrogenase or aldo-keto reductase. Furthermore it could be expected that dipeptide intermediates are involved in the breakdown process after cysteinylglycine conjugate of

3-MH was identified and quantified in Sauvignon blanc grape juice (Capone et al. 2011, Cordente et al. 2015). It is supposed that further reactions by peptidases can lead to the cysteinylated conjugate from which 3-MH is released (Parker et al. 2017). γ-Glutamyl transpeptidase seems to be required in the transformation of glutathione precursors to volatile thiols (Santiago and Gardner 2015).

Thibon et al. (2016) identified *S*-3-(hexanal)-glutathione and its bisulphite adduct in Sauvignon blanc grape juice and pointed out that these compounds could be considered as new direct 3-MH precursors. A transformation rate of these two precursors into 3-MH was estimated at 0.4%.

Finally, it has to be pointed out that Sarrazin et al. (2007) indicated a contribution of the identified and quantified thiols, 3-sulphanylpentan-1-ol, 3-sulphanylheptan-1-ol, 2-methyl-3-sulphanylbutan-1-ol and probably 2-methyl-3-sulphanylpentan-1-ol, to the overall aroma of sweet wines made from *Botrytis*-infected grapes.

A lot of factors influence the concentration of thiol precursors such as viticultural treatments (Peyrot des Gachons et al. 2005; Schüttler et al. 2011, 2013), grape ripening conditions, harvesting time and methods, condition of the grapes, oenological treatments, choice of yeasts and other issues (Parker et al. 2017). Nevertheless, the major precursor(s) to form 3-MH and/or its de novo synthesis and other varietal thiols are not fully understood and need further research.

On the basis of the above described studies, alternative strategies with conventional techniques are studied to optimise the release of thiols and the formation of 'tropical fruit' esters. For example, a large number of hybrid yeasts or natural selections of yeasts could be proved for their ability to release thiols or for the capacity of ester formation. Furthermore, co-inoculated fermentations of certain yeast strains have been applied and will be investigated more intensively in future as an appropriate enological tool to generate specific aroma characteristics in wines (Swiegers et al. 2006). It can be also expected that apart from other species of the *Saccharomyces* genus also species and strains of non-*Saccharomyces* yeasts have different abilities to release thiols (Wakabayashi 2004; Kagli et al. 2006; Sourabié et al. 2008), which provided the basis for an extremely promising area of research.

Anfang et al. (2009) studied the impact of co-fermentations with specific commercial strains of *Saccharomyces cerevisiae* and an isolate of *Pichia kluyveri*. Their results gave evidence that the co-fermentation could be a useful tool to increase 3-MHA formation. Zott et al. (2011) assessed 15 non-*Saccharomyces* strains from 7 species on 4-MMP and 3-MH release in model medium and Sauvignon blanc must after partial fermentation. They observed a low 4-MMP release in both media, but some of the *Metschnikowia pulcherrima*, *Torulaspora delbrueckii* and *Kluyveromyces thermotolerans* strains showed a high ability to liberate 3-MH. This indicates also a strain-dependent contribution for non-*Saccharomyces* yeasts as already revealed for *Saccharomyces* yeasts. An overview about non-*Saccharomyces* species to intensify the release of thiols due to carbon-sulphur lyase activity is given by Padilla et al. (2016).

Belda et al. (2016a, b) showed for the yeast species *Torulaspora delbrueckii*, *Kluyveromyces marxianus* and *Meyerozyma guilliermondii* a notable β-lyase

activity and a great intraspecific variability. Renault et al. (2016) could indicate that an industrial strain of *Torulaspora delbrueckii* is not able to release 4-MMP and showed a low activity to form 3-MHA. This strain led to a higher release of 3-MA but only from the glutathionylated precursor. Additionally, an increase of the cysteinylated precursor could be detected which forced the release of 3-MH in mixed cultures with *Saccharomyces cerevisiae*. In contrast, the research studies from Belda and coworkers showed in sequential fermentations that other strains of *Torulaspora delbrueckii* can lead to noticeable high amounts of 4-MMP and suggested that apart from the β-lyase activity other genes encoding oligopeptide and amino acid transporters differ due to intraspecific diversity (Belda et al. 2017a, b).

11.3 Sulphur Metabolism of Lactic Acid Bacteria

Lactic acid bacteria conduct malolactic fermentation, which usually occurs in wine a few days after alcoholic fermentation. Malolactic fermentation is forced in certain wines to degrade malic acid to lactic acid. This process softens the wine by decreasing its acidity and improves its organoleptic quality due to certain transformations. The lactic acid bacteria associated with grape must and wine mainly belong to the following genera: *Lactobacillus*, *Oenococcus* and *Pediococcus*. *Oenococcus oeni* mostly grows spontaneously in wine after the alcoholic fermentation or after commercial starters are added. There is a huge amount of literature available on the improvement of malolactic fermentation, on the formation of certain aroma compounds such as diacetyl and on health-related compounds such as biogenic amines (Bartowsky 2005; Henick-Kling 1993). Sulphur metabolism was only studied for lactic acid bacteria that are involved in cheese production for a great extent (Bonnarme et al. 2000; Dias and Weimer 1998; Sourabié et al. 2008).

Pripis-Nicolau et al. (2003, 2004) started to investigate the methionine catabolism of *Oenococcus oeni* and certain species of *Lactobacillus* at winemaking conditions for the first time. It could be indicated that in a laboratory media several lactic acid bacteria are able to metabolise methionine. The following sulphur compounds were detected: methanethiol, dimethyl disulphide, 3-(methylthio) propan-1-ol (methionol) and 3-(methylthio)propionic acid. Methionol and 3-(methylthio)propionic acid were formed in higher concentrations by *Oenococcus oeni* than by *Lactobacillus* species. It was observed that strains differ in their ability to produce the volatile metabolites. Figure 11.8 is giving an overview on the possible metabolites of methionine catabolism by *Oenococcus oeni* according to the research of Pripis-Nicolau et al. (2004). After malolactic fermentation in various red wines, only the level of 3-(methylthio)propionic acid increased in some cases significantly. It is assumed that this sulphur compound, which gives the impression of ‘chocolate’ and ‘roasted odours’ and a perception threshold of 50 μg L^{-1}, can probably contribute to the aromatic complexity often noticeable after malolactic fermentation. It is important to point out that no off-flavour

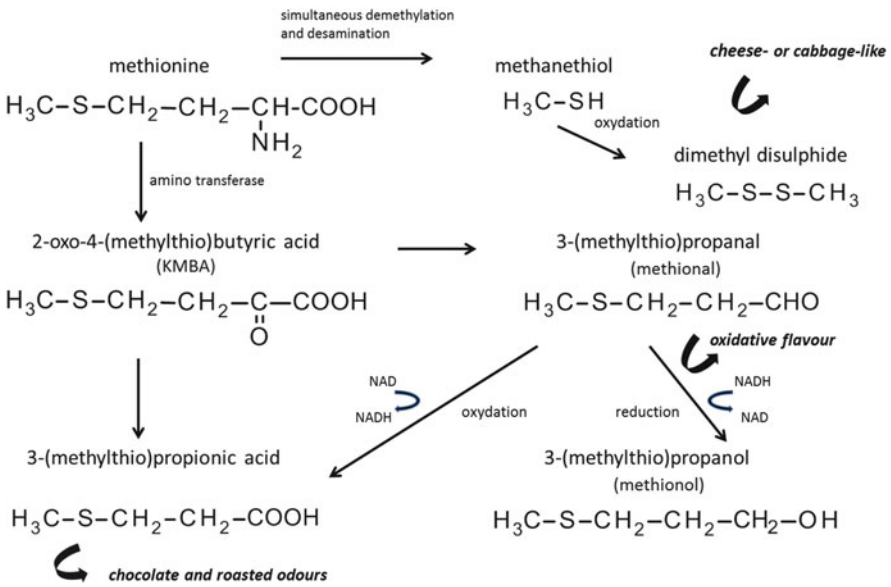

Fig. 11.8 Proposed metabolism of methionine by *Oenococcus oeni* in wine-like synthetic media (according to Pripis-Nicolau et al. 2003, 2004, modified)

compounds like methanethiol and dimethyl disulphide could be measured after malolactic fermentation conducted in wines.

Vallet et al. (2007, 2008) demonstrated that 2-oxo-4-(methylthio)butyric acid (KMBA) plays a central role in volatile sulphur compound synthesis.

The addition of low amounts of glutathione can have a positive effect on the growth of *Oenococcus oeni* in wine under certain conditions (Rauhut et al. 2004). The addition of methionine and glutathione seemed to accelerate the speed of malolactic fermentation a little bit at lower pH values. The catabolism of glutathione can lead to increased levels of H_2S in wine-like synthetic media, if the supplemented concentrations are far over the normal levels in wines. Furthermore, it could be shown that the catabolism of methionine to volatile sulphur compounds seems to depend on the pH value of the media (Rauhut et al. 2008). According to Pripis-Nicolau et al. (2004), no increase of volatile sulphur compounds that are related to off-flavours (e.g. H_2S, methanethiol, dimethyldisulphide, etc.) could be detected in wine-like model solutions and wine after addition of methionine and glutathione in amounts that can be usually expected in wines. As a result of the investigations, it is proposed that other factors such as the chemical or biochemical transformation of other volatile or non-volatile sulphur precursors in wine are the cause for 'reductive' sulphur off-flavours that can be sometimes noticed after malolactic fermentation and/or storage and ageing of wines.

11.4 Conclusions

Considerable research over more than three decades indicated that yeast sulphur metabolism influences to a high extent the wine flavour due to the occurrence of sulphur-related off-flavours that mainly occur due to deficiencies of assimilable nitrogen and other nutrients in grape musts. Research on an adequate and focused nutrient support of yeasts during fermentation has to be continued to fully understand the complex process of nutrient composition and formation of volatile sulphur compounds. This is of high interest especially with the regard to global climate change, which can decrease the nutrient composition in grapes as a result of increased stress conditions such as water deficiencies in certain years or can affect the development and incorporation of thiol precursors in the grapes or their formation during the winemaking process. More information is required about the demonstrated alternative pathways for varietal thiols and how they are regulated in the yeast cell.

Investigations of different research groups pointed out that yeast plays a great role in the characteristic of varietal aromas, in particular for wines from certain grape varieties like Sauvignon blanc, Muscat, Gewürztraminer, Scheurebe, etc.

Volatile sulphur aroma compounds are the best example to demonstrate that a comprehensive knowledge is necessary to avoid the formation of objectionable flavours and to optimise the release of specific thiols that offer wines specific desired 'tropical' fruit aromas. Any treatment or fining to get rid of developed 'reductive' sulphur off-flavours will also affect the varietal flavour triggered by volatile thiols. Ongoing research is necessary to process strategies to optimise the desired aromas through certain thiols and to avoid or to minimise the occurrence of malodorous sulphur compounds through yeast metabolism.

In addition, the interaction of yeasts from the same and/or from different species has to be studied to investigate their influence on the formation of volatile and non-volatile sulphur compounds in detail. The application of mixed yeast cultures and sequential fermentations to optimise the release and formation of varietal thiols seem to be interesting tools which need further research and experience.

In a further step, other microorganisms like lactic acid bacteria and fungi (e.g. *Botrytis cinerea*) should be integrated in these research investigations.

Appropriate wine yeast strains should be further on selected and scanned for a very low formation of sulphite, because a decrease of sulphites is generally required in food.

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Chapter 12
Polysaccharide Production by Grapes Must and Wine Microorganisms

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List of Abbreviations

EPS Exopolysaccharide
MLF Malolactic fermentation
MP Mannoproteins
PS Polysaccharide

12.1 Introduction

In this chapter, we describe the formation of polysaccharides (PS) by some of the microorganisms most frequently encountered in grapes, must and wine: *Botrytis cinerea*, *Saccharomyces cerevisiae*, non-*Saccharomyces*, *Oenococcus oeni* and other wine lactic acid bacteria. The structure of the polymer produced, the metabolic pathways identified, the putative or demonstrated benefits linked to capsular PS formation for the microorganism and the impact of the PS released on wine quality are described.

Several species of fungi, yeasts and bacteria develop on the grape berry during ripening and, afterwards, throughout the winemaking process. All contribute, via their own metabolic pathways, to the final chemical composition of the wine. Polysaccharides (PS) form part of the molecules produced by microbial metabolism which affect wine quality. They constitute the highest molecular weight component of wine and consist of repeating sugar units. These repeat units can be made of several different monosaccharides (heteropolysaccharides) or of the repetition of a

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single one (homopolysaccharides). The chain length, degree of branching and type of osidic bounds are also important characteristics of the molecule structure.

The PS content in must and wine varies throughout the winemaking process due to synthesis and degradation reactions. Only the more soluble grape PS are extracted in must (pectins and arabinogalactan). From picking until the end of alcoholic fermentation, pectins are gradually degraded into smaller PS, due to the action of grapes and microbial pectolytic enzymes (Pellerin and Cabanis 1998). The first microbial event that significantly modifies the wine's final PS composition is when the grapes are infected by *Botrytis* (Sect. 12.2): the pectins are hydrolysed and specific neutral polymers are formed (Dubourdieu 1982). In the next stage, during alcoholic fermentation and ageing on the lees, yeasts (*Saccharomyces* and non-*Saccharomyces*) release mannoproteins. These molecules constitute the second group of wine PS in quantitative terms, after those originating from grapes (Sect 12.3) (Ribéreau-Gayon et al. 2000). Pectolytic yeast species may also hydrolyse certain grape PS, thus providing substrates for the subsequent growth of other microbial species (Louw et al. 2006). Afterwards, as a result of the natural selection among bacteria occurring during alcoholic fermentation, *Oenococcus oeni* generally becomes dominant for the subsequent malolactic fermentation (MLF). During this stage, many changes occur in wine PS composition, indicating that, like *Botrytis* and yeast, *O. oeni* has the ability to produce and degrade PS (Dols-Lafargue et al. 2007). Though, most of the time, *O. oeni* PS have no evident impact on wine quality, some of them, which are also produced by other wine bacteria, have long been associated with the spoilage named "ropiness". Indeed, the bacterial PS structures and biosynthetic pathways are diverse and strain specific, and some associated genes are shared by several species (Sect. 12.4).

This chapter focuses on PS synthesis by microorganisms in grapes and wine, describing the structures of the polymers produced and, when identified, the biosynthetic pathways, with molecular aspects and regulation. Microbial PS are usually, at least partially, linked to the cells, thus forming a capsule, while the remainder is released into the surrounding medium (Sutherland 1993). The putative or demonstrated physiological benefits linked to capsular PS formation are discussed, and, finally, the impact of the released PS on wine quality is examined.

12.2 PS Produced by *Botrytis cinerea*

Botrytis cinerea is a deuteromycete (Hyphomycete) fungus. It is an important plant pathogen with an exceptionally broad host range. Its development on grapes may be dreaded (grey rot) or desired ("noble rot") (Ribéreau-Gayon et al. 2000).

In terms of PS, must extracted from rotten grapes no longer contains pectic PS, and its galactose and mannose concentrations are modified. Moreover, these musts contain exopolysaccharides (EPS), specifically produced by *B. cinerea*. When the fungus is cultivated on liquid medium, it is possible to separate two groups of soluble PS by alcoholic precipitation (Dubourdieu 1982):

- The more alcohol-soluble fraction consists of heteropolysaccharides.
- The less alcohol-soluble polymer is a glucan (glucose homopolysaccharide), known as cinerean. This is also the only polymer observed with certain strains of *B. cinerea* (Leal et al. 1976; Stahmann et al. 1992). Most of this extracellular polymer is attached to the hyphal cell wall, forming capsules (60%), while the rest (40%) is released as slime (Pielken et al. 1990).

12.2.1 Structure of the PS Produced

The heteropolysaccharide fraction has been less studied than the β-glucan. It consists of mannose, galactose, glucose and rhamnose (60/30/5/5), with molecular weights between 10 and 50 kDa (Dubourdieu 1982).

Cinerean has a linear backbone of β-1,3 linked glucosidic residues, with branched chains, consisting of a single β-1,6 linked glucosidic residue, attached to every second or third glucose molecule (Fig. 12.1) (Dubourdieu et al. 1981). This structure is common in cell wall polymer of yeast and filamentous fungi. The chains can be linked by low energy bonds. This increases the apparent molecular weight and leads to the trapping of a black pigment, melanin, by the glucan. The molecular weight of the glucan was estimated at 10^5–10^6 Da by size exclusion chromatography and 10^9–10^{10} by low-angle laser light scattering. Ultrasound treatment was used to separate the polymer from the melanin, resulting in glucan fibrils of 50–250 kDa (Dubourdieu et al. 1981; Dubourdieu 1982; Stahmann et al. 1995; Doss et al. 2003).

12.2.2 PS Production Kinetics

The two families of PS are produced during active growth on glucose in model medium: 300 mg l^{-1} cinerean and about 50 mg l^{-1} heteropolysaccharides (Dubourdieu 1982). In batch fermentation, a decrease in cinerean is observed after glucose exhaustion, leading to a striking decrease in viscosity. Indeed,

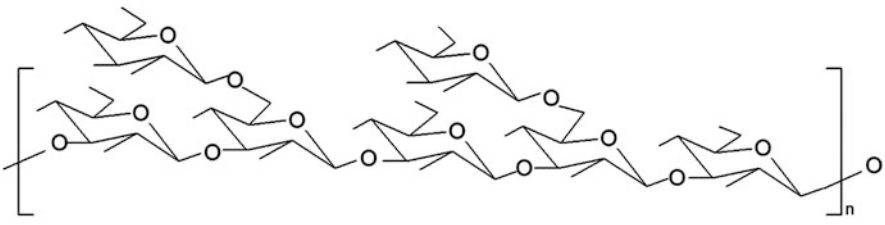

Fig. 12.1 Schematic representation of the repeating unit of *Botrytis cinerea* β-glucan (Dubourdieu et al. 1981)

B. cinerea produces several β-1,3 glucanases. Cinerean may be considered an external carbon reserve (Leal et al. 1976; Dubourdieu and Ribereau-Gayon 1980; Martinez et al. 1983; Stahmann et al. 1995). The PS content of wines produced from botrytised musts is up to 750 mg l^{-1} higher than in wines obtained from uncontaminated musts (Dubourdieu et al. 1978).

The genes and enzymes responsible for PS synthesis in *B. cinerea* have not been studied. Only Monschau et al. (1997) evidence the β-1,3 glucan synthase activity of membrane fraction of *B. cinerea* and suggest that the branching enzyme for the β-1,6 glycosidic bonds does not have the same location. Most studies have been done with other filamentous fungi but the biosynthetic pathway may be similar in *B. cinerea*. They suggest that the membrane-bound glucan synthase complex releases the polymer in the periplasmic space, where a remodelling occurs. In *Epicococcum niger*, Schmid et al. (2006) show that the synthesis of epiglucan (β-1,3 β-1,6 branched fungal glucan) occurs via the transfer of glucosyl residues (probably from UDP-glucose) to the non-reducing end of the growing chain. The side β-1,6 linked residues are incorporated gradually, as β-1,3 backbone glucan elongates. Furthermore, they suggest two PS formation mechanisms involving either (1) a single transmembrane glycosyltransferase, as proposed for *Streptococcus pneumoniae* and *Pediococcus parvulus* β-glucans (Sect. 12.4), or (2) a complex set of glycosyltransferases, as described for lactic acid bacteria EPS synthesis (Sect 12.4). Identification of single or multiple genes associated with β-glucan formation would clarify which mechanism is actually responsible.

12.2.3 Benefit for the Fungus

Like for other filamentous fungi, the *B. cinerea* glucan is essential for the cell wall rigidity. Most of the exocellular part of the β-glucan produced sticks to the cells, thus forming a thick capsule (Pielken et al. 1990). This capsule protects them from drought and assists in cell attachment on grapes (Dubourdieu 1982; Doss et al. 1995). Gil-ad et al. (2001) show that the presence of the glucan sheath strongly modifies the fungus morphology, protecting it from host responses, by slowing the diffusion of host secretions. In addition, the glucan sheath traps enzymes (peroxidase, laccase and catalase), which thus constitute an "arsenal" outside the cells (Doss 1999). Eventually, *Botrytis* PS undoubtedly play a key role in the biofilm established on the grape berry, containing yeasts, bacteria and other fungi.

12.2.4 Impact on Wine Quality

Cinerean is responsible for the high viscosity of musts produced from rotten grapes. After alcoholic fermentation, in the presence of ethanol, this glucan tends to form aggregates which block filters, making more difficult spontaneous clarification by

sedimentation and impairing wine filterability. Commercial glucanases are thus applied to such wines. They mainly display exo-β-1,3 glucanase and β-1,6 glucosidase activities, which finally hydrolyse the glucan to glucose (Villetaz et al. 1984; Dubourdieu et al. 1985; Humbert-Goffard et al. 2004). The direct pressing of rotten grapes without crushing them can also reduce the amount of glucan released into the must.

B. cinerea glucan affects yeast physiology and metabolism. Its addition to a fermenting medium slows down the alcoholic fermentation and stimulates the glyceropyruvic pathway, leading to increased excretion of glycerol and acetate (Ribéreau-Gayon et al. 1979; Dubourdieu 1982).

12.3 Yeast Mannoproteins

Mannoproteins (MP) constitute the outer part of the yeast cell wall polysaccharide layer. Some MP with enzyme activity (such as the external invertase) are immobilised in the structure of the MP matrix (Ballou 1976). During alcoholic fermentation and ageing on lees, some of these MP are released into the wine, where they interact with many other wine components.

12.3.1 Yeast Cell Wall Organisation and MP Structure

The most studied cell wall of *Saccharomyces cerevisiae* makes up 15–30% of the cell's dry weight, depending on growth conditions. It consists of separate, interconnected PS layers (Fig. 12.2). The outer layer is made of MP, connected to a matrix of amorphous β-1,3 glucan, while the inner layer consists of fibrous β-1,3 glucan, over a small quantity of chitin; β-1,3 glucan is the main component (85%) responsible for the mechanical properties of the cell wall. The β-1,6 glucan (15%) probably links the components of the inner and outer walls (Kollár et al. 1997; Klis et al. 2002).

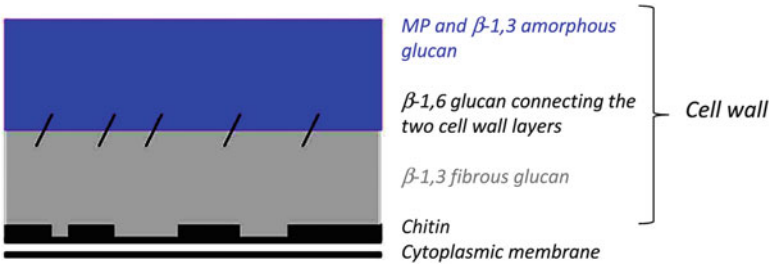

Fig. 12.2 *Saccharomyces cerevisiae* cell wall organisation

In the genus *Saccharomyces*, MP are made of mannose (about 90%), *N*-acetyl-glucosamine and mannosylphosphate (0.1–1%), in varying proportions, depending on the strain and growth phase (Ballou 1976, 1990; Jigami and Odani 1999; Klis et al. 2002). Their molecular weights vary from 20 to 450 kDa. A glycosylphosphatidylinositol anchor attaches the carboxylic group of the peptide chain of certain MP, which cross the cell wall, to the plasma membrane. Then, three forms of glycosylation have been described for *S. cerevisiae* MP, but they do not necessarily coexist in all of the MP (Fig. 12.3). The first form of glycosylation consists of mainly α-1,6-linked glucomannan chains, but their peptide point of attachment has not been clearly identified yet. The second form of glycosylation consists of small α-1,2- and α-1,3-linked mannooligosaccharide chains, which are sometimes phosphorylated. These small chains are attached to the peptide chain, via *O*-glycosidic bonds on serine or threonine residues. The last form of glycosylation is a *N*-linked PS attached to the peptide chain, via an asparagine residue. The core of this PS consists of a double unit of β-1,4-linked N-acetyl-glucosamine, to which a α-1,2-, α-1,3- and α-1,6-linked phosphorylated mannooligosaccharide is attached. A highly ramified outer chain (150–250 mannose units) is then attached to the core. This consists of a skeleton of α-1,6-linked mannosyl units, supporting short side chains of α-1,2- and α-1,3-linked mannosyl residues and phosphodiester-branched mannosyl residues (Ballou 1990; Jigami and Odani 1999).

The core of the PS fraction occurs in several yeast species, while the external PS chain is strain specific (Ballou 1976). The structure of the MP released into wine

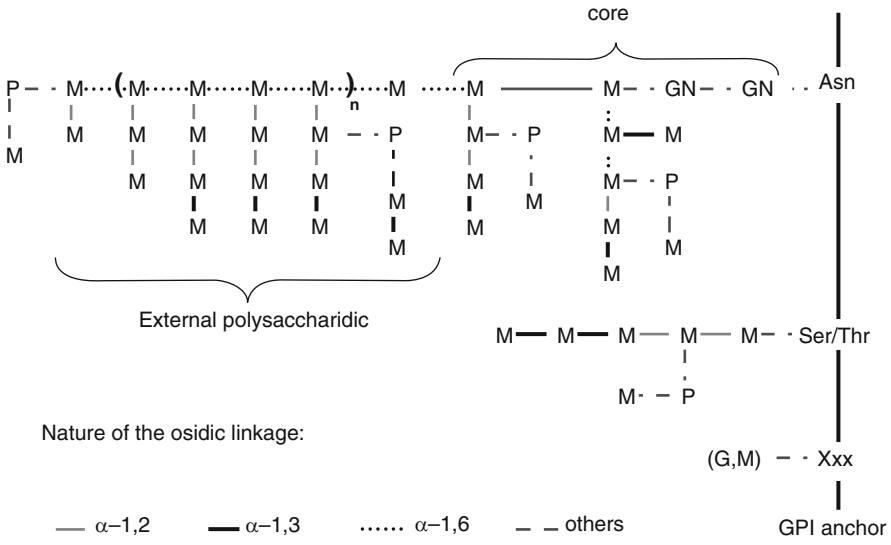

Fig. 12.3 Schematic representation of the O-linked oligosaccharide fraction and N-linked polysaccharide fraction of *S. cerevisiae* mannoproteins, MP ($n = 0$–10) (Adapted from Ballou 1990; Jigami and Odani 1999). *GNAc* N acetyl glucosamine, *M* mannose, *P* phosphate, *Asn* asparagine, *Ser* serine, *Thr* threonine

depends on the yeast strain, but is always similar to that of the yeast cell wall, with a molecular mass between 50 and 500 kDa (Villetaz et al. 1980; Llaubères 1987).

So far, non-*Saccharomyces* species MP have been less studied. However their structure is presumably similar to the one of *Saccharomyces*. These molecules are mainly mannoproteins with close composition to that of *S. cerevisiae*, excepted in *Schizosaccharomyces pombe*, whose MP contains also galactomannans (Giovani et al. 2012).

12.3.2 Physiology of MP Release

The cell wall construction is a dynamic, tightly regulated process, involving a large number of genes (Lussier et al. 1997; Smits et al. 1999; de Groot et al. 2001). The growing cells produce β-glucanases and other enzymes that partially degrade the β-1,3/β-1,6 glucan network, weakening the cell wall and facilitating cell division, budding or mating. No mannosidase or N-Ac-glucosaminidase is detected (Llaubères 1987; Klis et al. 2002; Gonzales-Ramos and Gonzales 2006). As a result, yeasts release PS, and especially MP, from the cell wall during active growth. In model medium, 100–250 mg l^{-1} MP are released, depending on the yeast strain, contact time, temperature and agitation of the yeast biomass. This phenomenon slows down when cells enter the stationary phase, as the walls become thicker and more resistant to β-glucanases, while the level of MP phosphorylation increases (Llaubères 1987; de Nobel et al. 1989, 1990; Shimoi et al. 1998; Jigami and Odani 1999).

The same phenomena occur during alcoholic fermentation in wine. *S. cerevisiae* MP are mainly released by active yeasts during the early stages of alcoholic fermentation but also by dying or dead cells (Giovani et al. 2010). According to Domizio et al. (2014), non-*Saccharomyces* PS are mainly released during growth. However, β-glucanases present in the cell wall maintain some residual activity a few months after cell death. As a result, ageing on the lees further raises the MP level by 150–200 mg l^{-1}, depending on the yeast strain, especially when lees are stirred and consist of fermented yeasts rather than additional dry yeasts (Llaubères 1987; Ribéreau-Gayon et al. 2000; Guilloux-Benatier and Chassagne 2003; Juega et al. 2015).

Given the positive effect of MP on wine (see Sect. 12.3.4), yeasts richer in MP are sought. Besides strain selection, genetic approaches such as recombinant genetics or random mutagenesis have been tried (Gonzalez-Ramos et al. 2008, 2010). Pérez-Través et al. (2015) obtained a *S. cerevisiae* strain with high producing MP ability and high fermentation performance. However another way to take advantage of yeast MP release is to use non-*Saccharomyces* yeasts. Namely, *Torulaspora delbrueckii* has been generally recognised as a high MP producing species (Giovani et al. 2012; Belda et al. 2014).

12.3.3 Benefit for the Wine Yeasts

MP in the outer cell wall layer play an important role in controlling the exchange of macromolecules (proteins, etc.) between the periplasmic space and the environment (de Nobel et al. 1989, 1990; Kapteyn et al. 1996). Several enzymes are thereby retained in the periplasmic space (Klis et al. 2002). Moreover, the external PS fraction of MP, which emanates from the cell surface, is involved in cell–cell recognition events.

MP are also involved in cell protection and survival in hostile environments, e.g. water retention and drought protection (Klis et al. 2002). Furthermore, various studies have shown that mannosylphosphorylation or modified MP patterns help the cells to overcome stress and contribute to yeast flotation during velum formation (Jigami and Odani 1999; Parascandola et al. 1997; Martinez et al. 1997; Alexandre et al. 1998, 2000). In an evolutionary engineered *S. cerevisiae* wine strain, genes linked to cell wall MP synthesis proved to be upregulated in response to low temperature, suggesting a direct involvement of MP in cold stress (López-Malo et al. 2015).

12.3.4 Impact on Wine Quality

Today, the use of yeast and yeast cell wall derivatives is accepted in winemaking, during or after fermentations, for fining or in replacement of lees for ageing. Most studies report that the presence of MP is beneficial to wine quality (Caridi 2006), although in specific cases, they may be responsible for a decrease in wine colour intensity or lower filterability (Vernhet et al. 1999; Morata et al. 2003; Rizzo et al. 2006).

In the pH range of wine, MP are negatively charged and establish interactions with other components, especially phenolic compounds (anthocyanins and tannins) and aromas, thus increasing colour stability, decreasing astringency and modulating aroma intensity and volatility (Lubbers et al. 1994; Vernhet et al. 1996; Escot et al. 2001; Riou et al. 2002; Caridi et al. 2004; Chalier et al. 2007; Juega et al. 2012; Mekoue Nguela et al. 2016; Gonzales-Royo et al. 2016). This property is used to stabilise wine via the legally authorised addition of purified MP (mannostab^{TM}) (Dubourdieu and Moine 1996). MP also inhibit the crystallisation of tartrate salts (Lubbers et al. 1993; Gerbaud et al. 1996) and prevent protein haze or adsorb molecules that would otherwise be implicated in oxidation reactions. This explains the stabilisation of white wines aged on lees (Waters et al. 1994; Escot et al. 2001; Charpentier et al. 2004; Dufrechou et al. 2015). Some MP have been shown to significantly adsorb ochratoxin A, a mycotoxin sometimes reported in grapes, must and wine (Caridi 2006). In addition, MP contribute to yeast flocculation as well as to yeasts and bacteria co-flocculation, during sparkling wine production (Suzzi et al. 1984; Peng et al. 2001; Fleet 2003; Pérez-Magariño et al. 2015). Some have

been reported to stimulate the growth of malolactic bacteria (Guilloux-Benatier et al. 1995; Guilloux-Benatier and Chassagne 2003). And last but not least, a keen interest for MP was recently observed for improving mouthfeel perception, aroma persistence and body or sweetness. This MP enrichment could be achieved through addition of purified molecules (Moine 2009; Pérez-Magariño et al. 2015) or by using selected MP producing strains or species. For example, the great impact on sensorial mouthfeel by *T. delbrueckii* is clear in all reports (Giovani et al. 2012; Belda et al. 2016; Domizio et al. 2014). This positive impact is also achievable by using *T. delbrueckii* lees in wine ageing (Belda et al. 2016).

12.4 Production of PS by Wine Lactic Bacteria

Many lactic bacterial species can be found in wines especially after alcoholic fermentation, when they drive malolactic fermentation, MLF (Chap. 1). Soluble PS concentrations increase or decrease during MLF, depending on the wine considered, suggesting that *Oenococcus oeni*, the bacterial species most often responsible for MLF, can both produce and degrade PS without altering the wine (Dols-Lafargue et al. 2007). However, in some cases, lactic acid bacteria cause "ropiness" or "oiliness", one of the four major types of bacterial spoilage in wine (Pasteur 1866). Spoiled wines display an oily, ropy texture, due to the liberation of a specific bacterial PS (Llaubères 1987).

However, recent studies show that it is not so easy to distinguish on one side harmless or beneficial bacterial EPS and, on the other side, those causing wine spoilage.

12.4.1 Structure and Location of Wine Bacterial PS

The first wine bacteria studied for their ability to produce EPS were chosen because they displayed visible and singular thickening or sticking properties (see examples Fig. 12.4a, b). They had been isolated from spoiled ropy wines, beer and cider. Such singular ropy strains belong to genera *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Lactobacillus* and *Oenococcus* (Luthi 1957; Van Oevelen and Verachtert 1979; Lonvaud-Funel and Joyeux 1988; Manca de Nadra and Strasser de Saad 1995; Duenas et al. 1995; Fernandez et al. 1995; Walling et al. 2005b; Werning et al. 2006; Ibarburu et al. 2007; Garai-Ibabe et al. 2010; Dimopoulou et al. 2014, 2016; Caggianiello et al. 2016). All ropy strains produce significant EPS amounts in model media, when compared to other strains of the same species.

The first and most studied ropy EPS is the high molecular weight (500–2000 kDa) β-glucan produced by *P. parvulus* 2.6 and IOEB_8801. These strains were first considered as *Pediococcus cerevisiae* (Lonvaud-Funel and Joyeux 1988), later classified as *Pediococcus damnosus* by DNA/DNA hybridisation

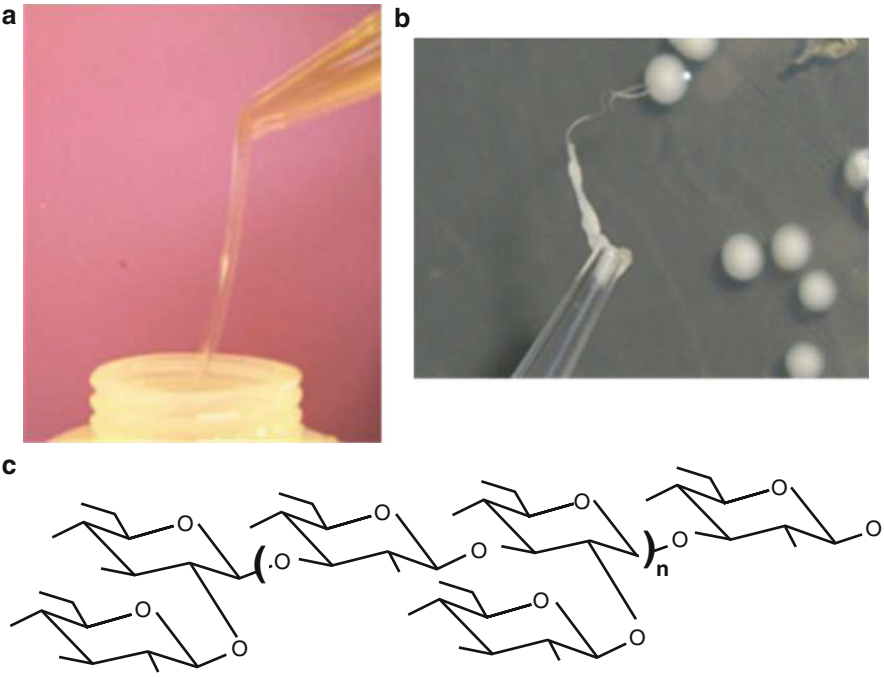

Fig. 12.4 (**a**) Ropiness induced in liquid model medium (MRS) by *P. parvulus* IOEB_8801. (**b**) Ropiness detection by picking colonies of *O. oeni* IOEB_0205 after growth on solid model medium (MRS) (**c**) Schematic representation of the chemical structure of *Pediococcus parvulus* β-glucan

(Lonvaud-Funel et al. 1993; Duenas et al. 1995, Walling et al. 2005a, b) and then, finally, as *Pediococcus parvulus* based on 16S RNA sequencing (Werning et al. 2006). The ropy β-glucan consists of a trisaccharide repeating unit with a β-1,3-linked glucosyl backbone branched with a single β-1,2-linked D-glucopyranosyl residue (Fig. 12.4c). Its structure is close to the one of capsular PS of *S. pneumoniae* type 37 (Adeyeye et al. 1988; Llaubères et al. 1990; Duenas-Chasco et al. 1997; Walling et al. 2005b). Transmission electron microscopy analyses show that the β-glucan forms a large but loosely attached layer around the cells (Fig. 12.5a). However, the β-glucan is probably not the only PS produced by *P. parvulus*, if one believes the dense halos still visible around the cells after β-glucan removal (Fernandez de Palencia et al. 2009; Coulon et al. 2012). Other less studied species, such as *Pediococcus damnosus*, *Lactobacillus diolivorans* and *Lactobacillus suebicus*, are described to produce this specific β-glucan (Walling et al. 2005b; Duenas-Chasco et al. 1998; Garai-Ibabe et al. 2010). However, PS other than this β-glucan may be responsible for the ropy character of *Lactobacillus collinoides* and *Lactobacillus hilgardii* strains (Walling et al. 2005b).

More recently, *O. oeni* was shown to produce EPS, independently of the ropy phenotype of the strain studied (Ibarburu et al. 2007; Dols-Lafargue et al. 2007,

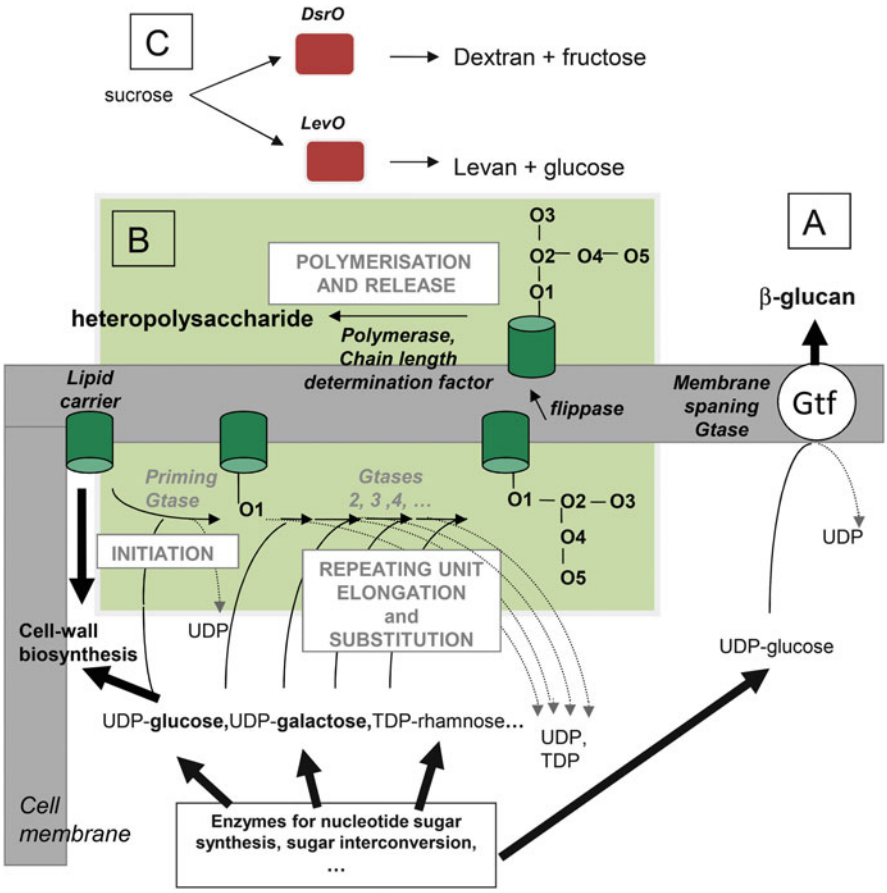

Fig. 12.5 Schematic representation of heteropolysaccharide biosynthesis by lactic acid bacteria. *O* osyl (e.g. glucosyl, rhamnosyl, galactosyl, etc.), *Gtase* glycosyltransferase [Adapted from Dimopoulou et al. (2012)]

2008; Ciezack et al. 2010; Dimopoulou et al. 2012, 2014). The EPS molecular weight distribution and chemical structure show that most strains produce a mixture of PS. With glucose as sole carbon source in the growth medium, the amounts of soluble PS recovered are low but significant. More than 75% of the studied strains produce heteropolysaccharides, made of glucose galactose and rhamnose, in varying proportions depending on the strain. These polymers are found in either a free or capsular form, but do not induce ropiness (Ibarburu et al. 2007; Dimopoulou et al. 2012, 2014). The capsule is dense but very thin, as shown in Fig. 12.5b. Some strains also produce the same β-1,3-β-1,2 glucan as *P. parvulus*, in either a free or a capsular form, and clearly display the ropy phenotype (Ibarburu et al. 2007; Dols-Lafargue et al. 2008; Dimopoulou et al. 2014). Moreover, with glucose and sucrose in growth medium, most *O. oeni* strains produce high amounts of soluble dextran

(>500 mg l^{-1}) and some strains also produce soluble levan (>1000 mg l^{-1}). Dextran is a glucose homopolymer with α-1,6-linked residues (95%) and some α-1,3-linked branched residues (5%), while levan is a β-2,6 fructan. None of these two polymers induce any obvious viscosity change in *O. oeni* growth media (Dimopoulou et al. 2012, 2014).

Furthermore, several *Leuconostoc mesenteroides* strains isolated from wine produce both dextrans and fructans in model media (Montersino et al. 2008).

12.4.2 Biosynthetic Pathways and Associated Genes

In *P. parvulus*, a single glucosyltransferase gene *(gtf)* is associated with β-glucan synthesis (Walling et al. 2005b; Werning et al. 2008). It codes a 567 amino-acid, 65 kDa protein (Gtf). The cloned *gtf* gene of *P. parvulus* expressed in *S. pneumoniae or L. lactis* produces a functional transmembrane Gtf which subsequently synthesises β-glucan (Werning et al. 2008; Dols-Lafargue et al. 2008). The role of Gtf in ropiness is thus clearly demonstrated. Gtf, like the glucosyltransferase of *S. pneumoniae* type 37, is a bifunctional transmembrane protein belonging to GT-2 family (www.cazy.org). It catalyses the synthesis of two distinct osidic bonds, as well as the export of the polymer (Fig. 12.6a) (Llull et al. 2001; Walling 2003; Werning et al. 2008).

In *O. oeni*, several complementary EPS biosynthetic pathways are active. Two have been characterised:

1. The glucan synthase pathway (Gtf), involved in ropy β-glucan synthesis from UDP-glucose (Fig. 12.6a) (Dols-Lafargue et al. 2008; Dimopoulou et al. 2014).
2. A Wzy-dependent synthetic pathway, resulting in production of heteropolysaccharides made of glucose, galactose and rhamnose from sugar nucleotides which originate in the central metabolic pathways (Fig. 12.6b). The repeating unit is assembled on a lipid carrier molecule, anchored in the cytoplasmic membrane. The first monomer is linked to the lipid carrier by the priming glycosyltransferase. Then, the following monomers are linked by other specific glycosyltransferases. Each glycosyltransferase uses the energy of the UDP-osyl bond to transfer the osyl to the growing repeating unit, forming in turn a specific osidic bond. After completion, the resulting repeating unit is assumed to be exported and polymerised on the outer face of the cell membrane. The lipid carrier is externalised by a flippase, and the repeating unit is added to the non-reducing end of the growing PS chain by a polymerase. A chain length determination factor may limit the extension of the molecule. This pathway is similar to that described in *Pneumococci* or in milk lactic bacteria (Dimopoulou et al. 2012, 2014).
3. The last pathway consists of homopolysaccharide synthesis from sucrose (α-glucan or β-fructan) thanks to glycoside hydrolases of the GH-70 (dextransucrase, DsrO) and GH-68 (levansucrase, LevO) families (Fig. 12.6c) (Dimopoulou et al. 2014).

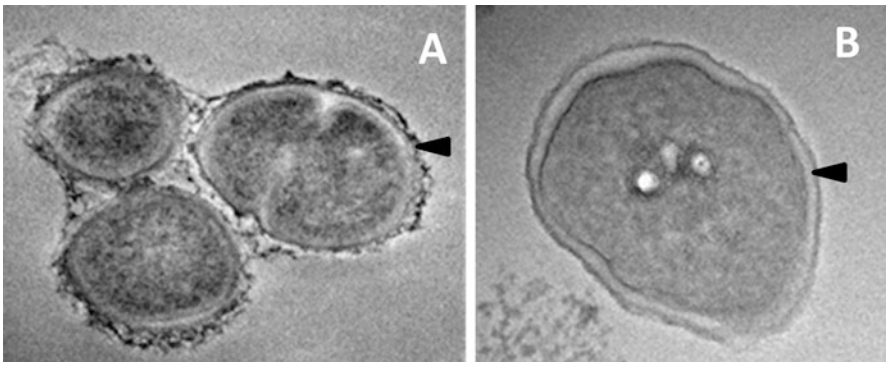

Fig. 12.6 Visualisation of PS capsules by transmission electron microscopy. (**a**) The β-glucan network around *P. parvulus* IOEB_8801 cells. (**b**) The thin PS layer around *O. oeni* IOEB_0607 cells (Adapted from Coulon et al. (2012) and Dimopoulou et al. (2014))

In *O. oeni*, all the genes dedicated to PS synthesis are located on the chromosome, and all strains studied display several *eps* genes. Most of them are inserted into two complex gene clusters named *eps1* and *eps2*. The composition of the *eps* gene clusters diverges from one strain to another and *eps2* is highly truncated or absent in specific strains. Other *eps* genes are spread over the chromosome: three glycoside hydrolase genes named *dsrO*, *dsrV* and *levO* and three glycosyltransferase genes named *gtf*, *it3* and *it4*. These last six genes are present or absent depending on the strain. Truncated genes or clusters are also found in some strains (Dimopoulou et al. 2014).

Analysis of sequences surrounding the *eps* genes and the *eps* gene distribution among distinct wine bacterial species and among distant strains in a same species (see Chap. 19 for *O. oeni*) brings some information on the mode of acquisition and mobility of the genes. More than 20% of the *Pediococcus* analysed by Garai-Ibabe et al. (2010), 20% of the *O. oeni* analysed by Dols-Lafargue et al. (2008) and 43% of *O. oeni* strains isolated from Champagne region (France) by Dimopoulou et al. (2016) display the *gtf* gene. In *O. oeni* strains originating from Champagne, *gtf* is located in a phage remnant (Dimopoulou et al. 2014, 2016). However, in a red wine *O. oeni* strain, the gene is inserted in a prophage of distinct origin, in another region of the chromosome. In red wine *Pediococcus*, the *gtf* gene is located on a 5.5 kb plasmid, on another 5.5 kb plasmid in *Lb. diolivorans* strains and on a 35 kb plasmid in cider *Pediococcus* (Gindreau et al. 2001; Werning et al. 2006). It displays over 98% identity from one bacterial species to another (Dols-Lafargue et al. 2008). The gene *gtf* is thus a mobile gene, via either phages or plasmids.

On the other hand, the *eps* gene clusters *eps1* and *eps2* of *O. oeni* display a mosaic structure. They are quite conserved in their 5′ end and more divergent in their 3′ end. Gene by gene, they have similarities with *eps* gene clusters found in bacteria isolated in very different ecological niches, and their mode of acquisition remains unclear (Dimopoulou et al. 2014).

12.4.3 Physiology of PS Release and Benefits for the Bacteria

All *O. oeni* strains studied so far have several genes dedicated to EPS metabolism. This suggests that these polymers are significant for the adaptation of *O. oeni* to its ecological niche and possibly contribute to the technological performance of malolactic starters. The same may apply to other wine lactic acid bacteria species.

In *O. oeni*, the exopolysaccharide production can be stimulated by changing the growth medium composition (Ciezack et al. 2010). Moreover, as previously stated, addition of sucrose to the growth medium may modify the biosynthetic pathway and final polymer structure (Dimopoulou et al. 2012). All the *Pediococcus* strains studied produce larger amounts of β-glucan when grown on glucose rather than other carbon sources, up to 140–200 mg l^{-1} β-glucan. Depending on the strain, β-glucan is also produced with fructose, maltose, galactose, xylose and arabinose as carbon source. It can be stimulated by adding malic acid or ethanol to the growth medium. β-glucan production is not directly linked to cell growth. However, an efficient preliminary growth phase is essential for subsequent "large-scale" EPS production. Agitation and aeration are detrimental (Llaubères 1987; Lonvaud-Funel and Joyeux 1988; Duenas et al. 2003; Walling et al. 2005a; Velasco et al. 2006, 2007).

Most of the EPS are not consumed by the bacteria that produce them and do not constitute external carbon sources (Walling et al. 2005a; Dols-Lafargue et al. 2008; Dimopoulou et al. 2012). Their biological role is probably to overcome stress commonly encountered in wine (Spano and Massa 2006; Dols-Lafargue et al. 2008; Dimopoulou et al. 2016; Caggianiello et al. 2016). Actually, the β-glucan capsule ensures resistance of ropy strains to SO_2, ethanol and low pH (Lonvaud-Funel and Joyeux 1988; Lonvaud-Funel et al. 1993; Walling et al. 2005a, b; Dols-Lafargue et al. 2008; Caggianiello et al. 2016) but also to lysozyme (Coulon et al. 2012). The β-glucan is supposed to enhance bacteria survival in the gut of insects or animals and hence contributes to dissemination of the bacteria present on fruits (Fernandez de Palencia et al. 2009; Stack et al. 2010; Deutsch et al. 2012). This polymer also modulates cell adhesion to biotic and abiotic surfaces (Dols-Lafargue et al. 2008; Fernandez de Palencia et al. 2009; Stack et al. 2010; Blättel et al. 2011). The heteropolysaccharidic capsule and the dextran released increase *O. oeni* resistance to cold shock, low pH or freeze-drying (Dimopoulou et al. 2016). Furthermore, *O. oeni* EPS may contribute to biofilm formation on grapes and winemaking equipment (Bastard et al. 2016). These biofilms are known to favour cell survival under extreme conditions, as well as genetic exchanges between species (Mah and O'Toole 2001). As a result, EPS production should contribute to the diversification of PS structures.

12.4.4 Impact on Wine Quality and Winemaking Practices

Ropiness due to beta-glucan production occurs all over the world in red and white wines, as well as beer and cider. The most frequently incriminated species is *P. parvulus*. Ropiness due to *O. oeni* in wine is not clearly reported, though it occurs in model growth media. High viscosity is sometimes reported during winemaking, in tanks or in barrels. At these stages, β-glucan is often produced by *Pediococcus* and 20 mg l^{-1} may be sufficient to spoil the wine (Lonvaud-Funel and Joyeux 1988). But afterwards, ropiness is easily decreased during the following winemaking steps like racking without any damage for the wine. The problem is when spoilage occurs later in bottles. Even if glucan has no impact on human health and has no specific taste, the wine's viscosity makes it impossible to market. Wine can be reconditioned after being agitated to reduce the viscosity and properly treated for its microbial stabilisation, especially for elimination of ropy bacteria (Ribéreau-Gayon et al. 2000). However, these are highly resistant to sulphur dioxide (Dols-Lafargue et al. 2008). It is the reason why bacterial detection and preventive treatment prior to the development of high population levels and the formation of ropiness are more appropriate. PCR-based methods were therefore developed to detect the presence of the *gtf* gene in wine microflora, as early as possible in the winemaking process (Gindreau et al. 2001; Delaherche et al. 2004; Walling et al. 2005b; Werning et al. 2006; Ibarburu et al. 2010). Then a well management of wine fermentations or ageing is generally sufficient to avoid the product alteration. Methods complementary to sulphuring like lysozyme treatment or beta-glucanases (Blättel et al. 2011; Coulon et al. 2012) have been proposed.

Conversely, the protective role of either cell-linked heteropolysaccharides or dextrans produced by *O. oeni* is demonstrated during freeze-drying or inoculation in wine and may be exploited in the future to produce even more resistant malolactic starters (Dimopoulou et al. 2016).

Furthermore, the soluble PS released by *gtf* negative *O. oeni* strains may interact with many wine molecules and contribute to the positive impact of MLF on wine quality. In addition, thanks to the EPS produced, *O. oeni* biofilm can develop on oak. This biofilm was shown to drive MLF more efficiently than free cells and it modulated the wood–wine transfer of volatile aromatic compounds during MLF and ageing by decreasing furfural, guaiacol and eugenol (Bastard et al. 2016).

12.5 Conclusion

All the microorganisms on grapes, in must and in wine produce exocellular PS. Acetic bacteria were not considered in this chapter, as their EPS-producing abilities have never been studied for wine strains. However, studies of strains of other origin suggest that the situation is as complex as that of lactic acid bacteria (Jansson et al. 1993; Geremia et al. 1999; Ua-Arak et al. 2016).

Whatever the species, some of the microbial PS remain attached to the cell, forming a capsule, which constitutes a protection to environmental constraints, especially in the final stages in winemaking. The remainder of the PS is released into the surrounding medium. Depending on the PS structure, on the species involved and on the winemaking stage, this may be neutral, beneficial or detrimental to wine quality and/or subsequent growth of other species (Lonvaud Funel 1999). Genetic exchanges between species are probably responsible for the present high diversity of microbial PS structure, particularly in bacteria. As a result, microbial PS remain important research topics in wine microbial ecology.

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Chapter 13
Microbial Enzymes: Relevance for Winemaking

Harald Claus

13.1 Introduction

Production of wine from grape juice is predominantly the result of complex enzymatic reactions. The primary bioconversion of grape sugar to ethanol and CO_2 by the yeast *Saccharomyces cerevisiae* is catalyzed by intracellular glycolytic enzymes. In spontaneous must fermentations, also strains of *Saccharomyces bayanus* or interspecies hybrids may dominate, probably because of better adaptation to specific environmental conditions (Christ et al. 2015). In addition, various enzymes released into the must influence the final composition, color, and sensory properties of wines. These enzymes originate from the grape itself, from epiphytic fungi like *Botrytis cinerea*, and from yeasts and bacteria associated with vineyards and wine cellars (Mojsov et al. 2015). Especially non-*Saccharomyces* yeasts, also called "wild" yeasts, belonging to the genera *Kloeckera*, *Candida*, *Debaryomyces*, *Rhodotorula*, *Pichia*, *Wickerhamomyces*, *Zygosaccharomyces*, *Hanseniaspora*, *Kluyveromyces*, and *Metschnikowia*, secrete different hydrolytic enzymes (esterases, lipases, glycosidases, glucanases, pectinases, amylases, proteases) which interact with grape compounds (Charoenchai et al. 1997; Fernández et al. 2000; Strauss et al. 2001; Bedrinana et al. 2012; Jolly et al. 2014; Belda et al. 2016a, b; Padilla et al. 2016). Apart from yeasts, lactic acid bacteria have an impact for vinification, i.e., the genera *Oenococcus*, *Leuconostoc*, *Pediococcus*, *Lactobacillus*, and *Weissella* (Wibowo et al. 1985; Fugelsang and Edwards 2007).

Because of its vitality under the extreme life conditions of wine (pH 3.0–4.0, alcohol concentration 10–15%), *Oenococcus oeni* is the primary species responsible for the malolactic fermentation. After completion of alcoholic fermentation, the cytosolic malolactic enzyme catalyzes the conversion of the dicarbonic acid

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Table 13.1 Oenological relevant enzymatic activities detected in lactic acid bacteria[a]

Enzyme	*Lactobacillus*	*Oenococcus*	*Pediococcus*
Protease	+	+	+
Cellulase	+	+	+
Xylanase	–	–	–
β-Glucanase	+	+	+
Lichenase	+	+	+
Glucosidase	+	+	+
Lipase	+	+	+
Esterase	+	+	+
Tannase	+	+	+
Phenoloxidase[b]	+	ND	+

[a]For references, see Matthews et al. (2004, 2006, 2007), Grimaldi et al. (2000, 2005a, b), Costello et al. (2013), and Perez-Martin et al. (2012, 2013)
[b]An intracellular laccase-like multicopper oxidase has been identified in *L. plantarum* J16 and *P. acidilactici* CECT 5930 (Callejón et al. 2014)
ND No data, but a gene encoding for a multicopper oxidase (Acc. ZB 00320225) is present in the genome of *Oenococcus oeni* PSU-1

L-malate to the monocarbonic acid L-lactate and CO_2. The result is a biological reduction of acidity, which is usually preferable in wines. On the opposite, different species of the genera *Lactobacillus* and *Pediococcus* are more active in the early stages of vinification often in connection with stuck fermentations and wine spoilage (Fugelsang and Edwards 2007; Sebastian et al. 2011).

A considerable number of publications emphasize the importance of enzymes from lactic acid bacteria for the winemaking process and its control (Matthews et al. 2004, 2006, 2007; Claus 2007; Mtshali et al. 2010). Immobilized malolactic enzyme was successfully tested to conduct malolactic fermentation outside the bacterial cell (Vaillant and Formisyn 1996). Proteases have been found in wine-associated strains of *Lactobacillus* and *Oenococcus* (Manca de Nadra et al. 1997, 1999; Farias and Manca de Nadra 2000). Davis et al. (1988) detected lipases and esterases in different wine-relevant lactic acid bacteria. Vaquero et al. (2004) demonstrated the existence of tannases in some *Lactobacillus plantarum* isolates. A group of enzymes of special interest are glycosidases, as they deliberate flavor compounds from the nonvolatile glycosidic bound state. Their occurrence has been reported for the wine-relevant genera *Oenococcus*, *Pediococcus*, and *Lactobacillus* (Boido et al. 2002; Barbagallo et al. 2004; D'Inecco et al. 2004; Grimaldi et al. 2000, 2005a, b). According to studies of Matthews et al. (2004, 2006, 2007), also esterases, lipases, tannases, cellulases, β-glucanases, and lichenases are produced by these genera (Table 13.1). All these enzymatic activities can have profound impacts on wine flavor and quality.

13.2 Enzymatic Transformations of Wine Ingredients

13.2.1 Proteases

Wine proteins originate from grapes and yeasts and may account for up to 2% of total nitrogen (Folio et al. 2008). They are sometimes unstable in the finished wine and precipitate to produce undesirable haze, which reduces the commercial value especially of white wines. More serious, some wine proteins, e.g., lipid transfer proteins, are suspected to possess allergenic potential (Wigand et al. 2009). In addition, proteinaceous wine fining agents (e.g., lysozyme, pectinase, ovalbumin, gelatin, casein) are hidden allergens and could present a risk for consumers (Deckwart et al. 2014; Liburdi et al. 2014; Veza et al. 2015; Rizzi et al. 2016).

Primarily pathogenesis-related proteins (β-glucanases, chitinases) and thaumatin-related proteins are responsible for haze formation. They are produced by the plants in response to microbial or fungal attack and abiotic stress factors. They deliver molecular masses between 13 and 30 kDa and isoelectric points between 4.1 and 5.8 (Selitrennikoff 2001). Protein removal is currently mainly achieved by bentonite addition, a process that unfortunately can be accompanied by losses of wine quantity and quality (van Sluyter et al. 2015). Bentonite acts essentially as a cation exchanger, and individual wine proteins adsorb onto the clay to different extents (Jaeckels et al. 2015). Proteins which are negatively charged at wine pH (ca. 3.5) and/or are high glycosylated as the laccase of *Botrytis cinerea* are hardly bound by bentonite (Claus and Filip 1988; Zivkovic et al. 2011). Thus, novel fining agents are under investigation to remove proteins from wine (Claus et al. 2014, van Sluyter et al. 2015).

Degradation of haze-forming proteins by enzymes is an attractive alternative to bentonite because it would minimize losses of wine volume and aroma. Appropriate proteases must be active under harsh winemaking conditions, i.e., low pH (~3.5), low temperature (~15 °C), and presence of ethanol (≥10% v/v), phenolic compounds, and sulfites. Another problem is the intrinsic stability of haze-forming proteins due to high numbers of disulfide bonds as present in lipid transfer proteins, chitinases, and thaumatin-related proteins (van Sluyter et al. 2015).

Papain, a cysteine protease from papaya, which is already used for beer brewing, has been studied for its capacity for wine protein reduction (Esti et al. 2013). However, in table white wines, the catalytic activity of papain is strongly diminished by free SO_2 and total phenol level (Benucci et al. 2015).

Bromelain, a cysteine protease extracted from the stem of pineapple plant, was tested for prevention of protein haze in white wine. Immobilized bromelain on chitosan beads, after 24h treatment in a laboratory-scale reactor, revealed a high capacity to reduce wine haze potential (approximately 70%), which was unaffected by wine composition (Benucci et al. 2014).

A thermotolerant fungal protease (aspergilloglutamic peptidase) has recently been approved for Australian winemaking (van Sluyter et al. 2015). The method of

Marangon et al. (2012) involves rapidly heating grape juice to 75 °C for 1 min to unfold wine proteins and make them susceptible to enzymatic degradation.

Other fungal proteases are currently being investigated that are active at winemaking temperatures and are specific against haze-forming grape proteins. The juice of grapes infected with *Botrytis cinerea* was found to have significantly lower concentrations of pathogenesis-related (PR) proteins than juice from healthy grapes (Girbau et al. 2004; Marchal et al. 1998, 2006). One particular protease from *B. cinerea*, BcAP8, has proven to be effective against grape chitinases during juice fermentation without the need for heating. When BcAP8 was added to juice prior to fermentation, the resulting wines produced significantly less heat-induced protein haze than wines made without BcAP8 (van Sluyter et al. 2013).

Acid proteases from yeasts may offer a microbial alternative or supplement to bentonite treatment to remove undesirable wine proteins (Rosi et al. 1987; Lagace and Bisson 1990; Theron and Divol 2014; Chasseriaud et al. 2015; Schlander et al. 2017). *Saccharomyces cerevisiae* is generally not famous as a producer of hydrolytic enzymes (Charoenchai et al. 1997; Strauss et al. 2001). None of 74 *Saccharomyces* isolates secreted an endopeptidase, but several strains exopeptidase activities in the investigation of Iranzo et al. (1998). However, an extracellular pepsin-like aspartic acid protease of 72 kDa has been characterized from a strain PIR1 (Younes et al. 2011, 2013). The enzyme was active during grape juice fermentations although it did not affect haze-inducing proteins, unless the wine was incubated for prolonged time at 38 °C. Nevertheless, the discovery of a secreted protease from a *S. cerevisiae* strain demonstrates that proteolytic activity can occur prior to autolysis of yeast cells and the release of vacuolar enzymes, which is a common source of yeast proteases in wine (Alexandre et al. 2001; van Sluyter et al. 2015).

Recently, Christ et al. (2015) described a triple hybrid strain *S. cerevisiae* × *S. kudriavzevii* × *S. bayanus* HL 78 being able to consume glucose and fructose with low levels of amino acids as the sole sources of nitrogen. Quantitative proteomics analysis revealed that the hybrid strain expressed two proteolytic enzymes at very high quantities compared to a commercial *Saccharomyces cerevisiae* wine strain Fermivin®: vacuolar proteinase Pep4 and carboxypeptidase Prc1 that may be the key to its unique properties (Szopinska et al. 2016).

Wine yeasts producing proteolytic exoenzymes are of high biotechnological interest for protein haze prevention because they could be directly added to the grape must as starter cultures without the need of enzyme production. Besides cost reductions, there are no administrative restrictions for their applications in must and wine, which has to be considered with enzyme preparations.

In contrast to *S. cerevisiae* itself, non-*Saccharomyces* wine yeasts are important sources of extracellular enzymes including proteases (Molnárová et al. 2014; Chasseriaud et al. 2015). In the study of Fernández et al. (2000), 53 from 141 isolates of "wild yeasts" hydrolyzed casein. The positive strains were identified as *Metschnikowia pulcherrima* and *Pichia membranifaciens*. In a similar study with 245 yeast isolates, 10 strains of *Candida stellata*, *C. pulcherrima*, and *Kloeckera*

apiculata and one strain of *Debaryomyces hansenii* showed proteolytic activity (Strauss et al. 2001). Oenological isolates of *Hanseniaspora* (Mateo et al. 2015; López et al. 2016), *Candida apicola* (Reid et al. 2012), *Metschnikowia pulcherrima* (Reid et al. 2012; Schlander et al. 2017), and *Wickerhamomyces anomalus* (Madrigal et al. 2013; Schlander et al. 2017) have been described which produce extracellular proteases with potential applications in biotechnological processes.

There are some studies which document the occurrence of proteolytic activities in wine lactic acid bacteria (Table 13.1). The presence of amino acids in the culture media is essential for the development of *O. oeni* due to its numerous auxotrophies. This bacterium is able to use small peptides and to hydrolyze wine proteins via extracellular enzyme activities. The released peptides enhanced the antioxidant and antihypertensive activities of a Cabernet Sauvignon wine as found in the study of Apud et al. (2013). Rollán et al. (1993, 1995, 1998) described two proteases I and II, which are produced by several strains of *O. oeni* during the early and final stages of growth, respectively. Proteases I displayed optimum activity at pH 4.0 and 30 °C and protease II at pH 5.5 and 40 °C. Both proteases were repressed by ammonium, tryptone, and casein hydrolysate, induced by nutrient starvation, and hydrolyzed protein and polypeptide extracts from red and white wines. A third extracellular protease EprA of *O. oeni* has been heterologous expressed in *Escherichia coli* (Folio et al. 2008). This protease differed from all lactic acid bacteria proteases identified so far. With a molecular mass of 21.3 kDa and a pI of 5.3, the enzyme showed maximum activity at pH 7.0 and 45 °C. These features appear not compatible with winemaking conditions and the question arises whether there is a nutritional benefit for the bacterium.

13.2.2 Glucanases

Polysaccharides in must and wine are directly derived from the grape berries (cellulose, hemicellulose, pectins) but also by the growth and autolysis of yeasts like *S. cerevisiae* (beta-glucans, chitin). A wide range of lactic acid bacteria is able to produce capsular or extracellular polysaccharides leading to viscous and "ropy" products (Caggianello et al. 2016). Especially strains of *Pediococcus* sp. (Llaubéres et al. 1990; Manca de Nadra and Strasser de Saad 1995; Velasco et al. 2007; Blättel et al. 2011) and the fungus *B. cinerea* produce glucan slimes (Dubourdieu et al. 1981) causing filtration problems during winemaking (Fig. 13.1). In addition, fermentation in presence of *Botrytis* glycans leads to yeast inhibition coupled with increased levels of acetic acid and glycerol (Fugelsang and Edwards 2007).

The yeast cell wall and the exopolysaccharides of *Pediococcus* and *B. cinerea* have substantial similarities in their biochemical compositions. Whereas the β-glucan of the exopolysaccharides possess a β-1,3-linked glucosyl backbone with branches made up of single 1,2-linked D-glucopyranosyl residues (*Pediococcus*) or 1,6-linked D-glucopyranosyl residues (*Botrytis*), the cell wall of yeasts mainly contains β-1,3 and β-1,6 but also variably linked β-glucans, too. Thus,

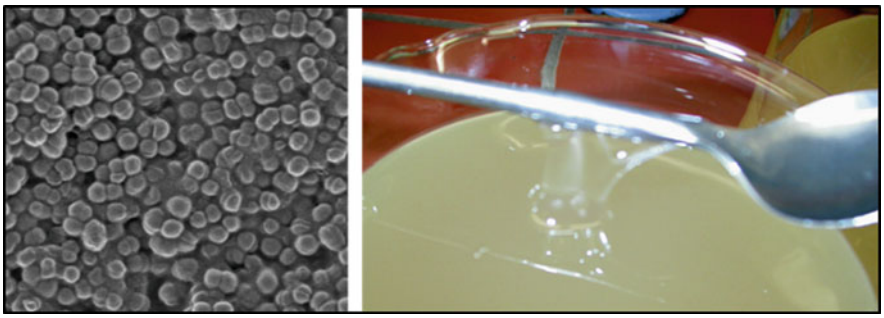

Fig. 13.1 *Pediococcus parvulus* strain B399 produces a polysaccharide slime (Blättel et al. 2011)

by means of suitable enzymes, not only polysaccharide slimes are degraded but also growth of spoilage yeasts could be inhibited (Enrique et al. 2010).

Two types of glucanases are important for winemaking: (1) exo–β1,3-glucanases catalyze the hydrolysis of β-glucan chains by sequentially cleaving glucose residues from the nonreducing end and releasing glucose as the sole hydrolysis product and (2) endo-β-1,3-glucanases catalyze intramolecular hydrolysis of β-glucans releasing oligosaccharides. Screening for such enzyme activities is usually performed with commercially available test substrates like lichenan (a linear β-1,3-1,4-glucan) or laminarin (a branched β-1,3 + β-1,6 glucan).

Fungal enzymes, e.g., from *Trichoderma reesei* or *T. harzianum*, have been found to dissolve *Botrytis* slime (Villetaz et al. 1984). Currently only glucanase preparations obtained from the species *T. harzianum* are approved for this purpose in German wines.

Blättel et al. (2011) examined an extracellular endo-β-1,3-glucanase from the Gram-negative bacterium *Delftia tsuruhatensis* strain MV01 with regard to its ability to hydrolyze both polymers, the β-1,3-glucan from *P. parvulus* and that from yeast cell walls. The 29-kDa glycolytic enzyme was purified to homogeneity. It exhibited an optimal activity at 50 °C and pH 4.0. The investigations indicated that this hydrolytic enzyme is still active under wine-relevant parameters such as elevated ethanol, sulfite, and phenol concentrations as well as at low pH values. Therefore, the characterized enzyme seems to be a useful tool to prevent slime production and undesirable yeast growth during vinification.

Also in wine-relevant lactic acid bacteria, such enzyme activities have been demonstrated (Table 14.1). A strain of *O. oeni* exhibited extracellular β-(1→3) glucanase activity (Guilloux-Benatier et al. 2000), which increased when cells were cultivated with cell wall compounds. In addition, the culture supernatant of the organism effectively lysed viable or dead cells of *S. cerevisiae*. This lytic activity appeared in the early stationary phase of bacterial growth. Yeast cells at the end of the log phase of growth were the most sensitive. The optimum temperature for lysis of viable yeast cells was 40 °C, which is very different from the temperatures observed under oenological conditions (15–20 °C).

A major source of polysaccharide-degrading exoenzymes are non-*Saccharomyces* wine yeasts. In a study of Strauss et al. (2001), 245 yeast isolates, representing 21 species belonging to the genera *Kloeckera*, *Candida*, *Debaryomyces*, *Rhodotorula*, *Pichia*, *Zygosaccharomyces*, *Hanseniaspora*, and *Kluyveromyces*, were positively screened for the production of extracellular pectinases, β-glucanases, lichenases, cellulases, xylanases, and amylases.

A multifunctional exo-β-1,3-glucanase (WaExg2) was purified from the culture supernatant of the yeast *Wickerhamomyces anomalus* AS1 (Schwentke et al. 2014). The encoding gene *WaEXG2* codes for a protein of 427 amino acids, beginning with a probable signal peptide (17 aa) for secretion. The mature protein has a molecular mass of 48 kDa with a calculated pI of 4.84. Glucose was detected as the sole hydrolysis product from laminarin. The glucanase activity of WaExg2 is of considerable interest to winemaking. As mentioned above, bacteria and fungi can spoil wine quality by producing slimes consisting of β-1,3-glucans similar to laminarin. Must and wine filtration in these circumstances can be difficult or impossible. Therefore, WaExg2 might be a tool to degrade such exopolysaccharides. The enzyme may also facilitate the release of beneficial cell wall components and cytoplasmatic proteins from (sparkling) wine yeasts (Zinnai et al. 2010; Torresi et al. 2014). In theory, the yeast *W. anomalus* could be directly added to grape must without the need of enzyme production.

The role of exo-β-1,3-glucanases from different *W. anomalus* strains as antagonistic "toxins" against fungi is a matter of debate. In case of strain K1, Gravesse et al. (2003) ruled out any involvement, whereas Friel et al. (2007) demonstrated by gene knockout experiments that glucanase PaExg2 is essential for the inhibition of the grape-infecting fungus *B. cinerea*. However, it should be considered that enzyme systems for fungal/yeast cell lysis are usually a mixture of several different enzymes, including one or more β-1,3- and β-1,6-glucanases, proteases, mannanases, or chitinases, which act synergetic (Salazar and Asenjo 2007).

13.2.3 Pectinases

Pectinases are applied in wine industry to facilitate juice extraction, viscosity reduction, and clarification, releasing more color and flavor compounds entrapped in the grape skins, thereby making a positive contribution to the quality of wine (van Rensburg and Pretorius 2000; Ugliano 2010). Commercial fungal pectinases used in food processing comprise mixtures of polygalacturonases, pectate lyase, and pectin methyl esterase. Ramirez et al. (2016) tested a pectinase from *Aspergillus niger* immobilized on a chitosan-coated chitin support for use in the juice and wine industries. The biocatalyst retained 100% of its original activity after nine cycles of reuse and delivered improved heat stability.

Wine strains of *S. cerevisiae* have no to weak natural pectinase activity, despite their genetic ability to secrete an endo-polygalacturonase (Eschstruth and Divol 2011). The *PGU1* gene, which encodes this enzyme, is present in most strains of

S. cerevisiae but is replaced by a partial transposon in strains lacking this activity (van Wyk and Divol 2010). In a study with 74 *Saccharomyces* isolates, it was found that 33% of the strains were capable of hydrolyzing galacturonic acid (Ubedo Iranzo et al. 1998). Because of these restrictions, so-called "wild" yeasts have been explored as enzyme sources (Fernández et al. 2000; Strauss et al. 2001).

In wine industries, cold fermentation (15–20 °C) is believed to increase the production and retention of volatile compounds, thereby improving the aromatic profile of wines. Therefore, cold-active pectinolytic enzymes are required both for extraction and clarification (Merín et al. 2011). Such cold-active pectinolytic enzymes were described in the psychrophilic yeast *Cystofilobasidium capitatum* SPY11 and psychrotolerant *Rhodotorula mucilaginosa* PT1 (Sahay et al. 2013). They exhibited 50–80% of their optimum activity under some major oenological conditions pH (3.5) and temperatures (6.0 and 12 °C).

Merín and Morato de Ambrosini (2015) investigated the influence of oenological factors on cold-active pectinases from 15 preselected indigenous yeasts belonging to *Aureobasidium pullulans*, *Filobasidium capsuligenum*, *Rhodotorula dairenensis*, *Cryptococcus saitoi*, and *Saccharomyces cerevisiae*. The study demonstrated that cold-active pectinases from some *A. pullulans* strains were able to remain active at glucose, ethanol, and SO_2 concentrations usually found in vinification and suggested their potential use as processing aids for low-temperature winemaking.

13.2.4 Glycosidases

The sensory profile of a wine is closely related to the composition of numerous volatile compounds. The monoterpenes make the most important contribution to the olfactory profile of wine due to their low odor threshold (Styger et al. 2011). Characteristic compounds include the acyclic terpene alcohols, linalool, geraniol, nerol, and citronellol, and the monocyclic terpineol. These and other aroma-active substances, such as C13-norisoprenoids, benzene derivates, aliphatic alcohols, and phenolics, are secondary plant metabolites and originate from the berry skin and, to a lesser extent, from the pulp (Terrier et al. 2010). However, up to 90% of these organoleptic active compounds do not exist in a free form. Most of these are aroma precursors conjugated to mono- or disaccharides, thereby forming water-soluble and odorless complexes (Sarry and Günata 2004; Styger et al. 2011; Ugliano and Henschke 2010; Hjelmeland and Ebeler 2015). Thus, enzymes that cleave sugar moieties from the precursors can have a major impact on the sensory profile of wine, as they release the volatile aroma compounds. The aglycone moiety in monoterpenyl glycosides is always linked to the β-D-glucopyranose unit. In diglycoside structures, the latter is further substituted with a second monosaccharide, which can be either β-D-glucopyranoside, α-L-rhamnopyranoside, α-L-arabinofuranoside, β-D-apiofuranoside, or β-D-xylopyranoside (Winterhalter and Skouroumounis 1997). Enzymatic cleavage of monosaccharide glycosides sugars requires at least a β-D-glucopyranosidase. In the case of disaccharide glycosides, an additional specific glycosidase is necessary which firstly cleaves the terminal sugar.

Besides a stepwise reaction, some endo-glucosidases are able to hydrolyze the glycosidic linkage to the aglycone, regardless of the number of sugar moieties (Hjelmeland and Ebeler 2015).

An important microbial source of such enzymatic activities are lactic acid bacteria (Table 13.1). Perez-Martin et al. (2012) screened 1464 isolates for glycosidase activities as biotechnological tools in oenology. The β-glucosidase activities occurred in both whole and sonicated cells but not in the supernatants of the cultures. Four *O. oeni* isolates retained their enzymatic activity under winemaking conditions. Similarly, an intracellular glucosidase and arabinosidase from *O. oeni* strains released high amounts of monoterpenes from natural substrates under optimal conditions (Michlmayer et al. 2012). The enzymes exhibited broad substrate specificities (release of both primary/tertiary terpene alcohols) and were still active in grape juice. Further, a sensory panel clearly preferred enzyme-treated Riesling wines compared to the controls.

Studies on the occurrence of glycosidases in *S. cerevisiae* strains are contradictory. The majority of *Saccharomyces* isolates do not show β-glucosidase activity on a natural substrate such as arbutin, and no gene in the genome of the haploid strain *S. cerevisiae* is known for coding a 1,4-β-glucosidase (EC 3.2.1.21). However, a putative β-glucosidase gene of a *S. cerevisiae* strain AL41, isolated on arbutin by Spagna et al. (2002), has been partially sequenced by Quatrini et al. (2006). None of 74 *Saccharomyces* isolates showed β-glucosidase activity in an investigation of Ubedo Iranzo et al. (1998). On the other hand, several *S. cerevisiae* strains showed varying ability to hydrolyze glycosides in a study by Zoecklein et al. (1997). However, these activities had no sensory impact, because the concentrations of the released compounds were below reported thresholds in wine. Another study examined the fate of Chardonnay glycosides in both a model matrix and fermenting wine (Chassagne et al. 2005). Sorption of glycosides to yeast cells was not a significant effect; however, the authors did not include an analysis of the released volatiles, so the fate of the hydrolyzed glycosides is not clear. Pérez et al. (2011) identified some β-glucosidase positive *S. cerevisiae* isolates from Uruguayan vineyards using an esculin glycerol agar. Intra- and extracellular β-glucosidase activities were detected in both *S. cerevisiae* strains investigated by Wang et al. (2015) using 4-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate. Mateo and DiStefano (1997) demonstrated the hydrolysis of grape glycosides by crude extracts of *Saccharomyces* strains. However, this reaction was suggested to be a side activity of the major exo-β-glucanase EXG1 of *S. cerevisiae* (Gil et al. 2005). In accordance, Schmidt et al. (2011) characterized EXG1 as the major hydrolytic enzyme involved in the cleavage of a wide range of *O*-glucosides of flavonoids and other natural compounds in living cells of *S. cerevisiae*. This underlines the assumption that only some *S. cerevisiae* strains possess a gene coding for an authentic functional β-glucosidase, whereas the majority do not. Delcroix et al. (1994) investigated the use of *Saccharomyces* strains with higher β-glucosidase activity to improve wine aroma but noted only few differences in the concentrations of terpenes and the sensory quality of the product.

Most research on β-glucosidases demonstrated intra- and extracellular activities in non-*Saccharomyces* yeasts. Selected species of *Candida*, *Hanseniaspora*, *Pichia*,

Metschnikowia, *Rhodotorula*, and *Trichosporon* with high β-glucosidase could potentially contribute to different aromas in wine (Rosi et al. 1994; Fernández et al. 2000; Ferreira et al. 2001; Rodriguez et al. 2004; Wang et al. 2015; Hu et al. 2016). A *Wickerhamomyces anomalus* strain AS1 hydrolyzed a number of synthetic and natural glycosides under oenological conditions (Sabel et al. 2014). Citronellol- and nerol-glucosides, among the most frequently occurring aroma precursors in wine, were cleaved. In contrast to a commercial β-glucosidase, whole cells of *W. anomalus* AS1 catalyzed deglycosylation of arbutin and salicin directly in a white and a red wine. Besides formation of intra- and extracellular glucoside hydrolases, strain AS1 exhibited arabinosidase and xylosidase activities which are also essential for the release of flavor compounds.

Attempts were undertaken for a technical application of yeast enzymes to improve the organoleptic quality of wine by enzymatic hydrolysis of glycosidically bound flavor compounds (van Rensburg and Pretorius 2000; Ugliano et al. 2006; Maicas and Mateo 2016). Although *Brettanomyces* has glucosidase activity (Daenen et al. 2008), the enzyme was not active against glycosides from grapes (Mansfield et al. 2002). On the other hand, wines treated with a β-glucosidase from *Debaryomyces hansenii* showed increased concentrations of terpenoids (Yanai and Sato 1999). An extracellular β-glucosidase from *Issatchenkia terricola* immobilized onto Eupergit C allowed enhanced aromatization of a white Muscat wine over a 16-h experiment (Gonzales-Pombo et al. 2011). The enzymatic treatment significantly increased the amount of monoterpenes and norisoprenoids. López et al. (2015) studied non-*Saccharomyces* yeast strains (*Pichia membranifaciens* Pm7, *Hanseniaspora vineae* Hv3, *H. uvarum* Hu8, and *W. anomalus* Wa1) showing high β-glucosidase and β-xylosidase activities to contribute to the production of quality wines. The use of these strains increased the levels of hotrienol, 2-phenylethanol, and 2,6-dimethyl-3,7-octadien-2,6-diol in wine. The moderated overall terpene increase (1.1- to 1.3-fold) inoculated with non-S*accharomyces* supports the potential of these strains to enhance wine aroma.

Polyphenols in red wine, such as resveratrol, have gained increasing public and scientific interest due to their presumed beneficial impact on human health (El Rayess 2014). A major section of the polyphenols in nature is conjugated with sugars or organic acids, rendering them more hydrophilic and less bioavailable for humans (Thilakarathna and Rupasinghe 2013). The level of glycosylated forms of resveratrol, known as piceid or polydatin, has been found in red wines as much as tenfold higher. As these modified forms are less bioactive, attempts were made with β-glucosidases from different fungal sources to increase the trans-resveratrol content in wines by hydrolyzing glycosylated precursors (El Rayess 2014). The multifunctional glucanase WaExg2 of *W. anomalus* AS1 released the polyphenolic aglycones from the model compounds arbutin, salicin, esculin, and polydatin (Schwentke et al. 2014).

WaExg2 proved active under typical wine-related conditions, such as low pH (3.5–4.0), high sugar concentrations (up to 20% w/v), high ethanol concentrations (10–15% v/v), presence of sulfites (up to 2 mM), and various cations. Therefore, the characterized enzyme might have multiple uses in winemaking to increase concentrations of sensory and health-promoting compounds by splitting glycosylated precursors or to reduce viscosity by hydrolysis of glycan slimes. Madrigal et al. (2013)

underlined that glucose- and ethanol-tolerant enzymes from oenological *Wickerhamomyces* isolates have great interest for the winemaking industry. Moreover, different strains of *W. anomalus* have been described which exert antagonistic activities against the wine spoilage yeast *Brettanomyces bruxellensis* and the phytopathogenic fungus *Botrytis cinerea* (Muccilli and Restuccia 2015).

13.2.5 Lipases

Lipids in wine originate from all parts of the grape berry; their composition (neutral lipids, glycolipids, phospholipids) and concentration are determined by factors like the variety, maturation, and climate (Gallander and Peng 1980; Izzo and Muratore 1993). In addition, autolysis of wine yeasts deliberates lipids, including tri-, di-, and monoacylglycerols and sterols (Pueyo et al. 2000). A significant number of biologically active lipids have been detected in Greek white wines and musts with antithrombotic and antiatherogenic properties in vitro (Fragopoulou et al. 2002). The authors found that the active lipids in must and wine have a glycerol backbone with some interesting differences. The lipids in must are phosphoglycolipids, while in wine it is a glycolipid. These findings suggest that the lipids of grape or yeast are subjected to chemical modification during fermentation and that the biologically active lipids come from the grape, since almost the same structure was found in must and wine.

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) from the yeast genera *Candida* and *Yarrowia* are one of the most important enzymes with industrial potential (Vakhlu and Kour 2006). In vinification they could be used, e.g., for better extraction of color compounds from red grape berries.

Several studies have provided information about lipase activities in genera that are of interest in winemaking, namely, *Lactobacillus*, *Pediococcus*, and *Leuconostoc*. Because lipases are located extracellularly or cell-associated, these bacteria have the ability to influence the wine lipid content when they are grown in grape juice or wine. By their action wine lipids are cleaved rendering different volatile compounds and fatty acids. Whereas the former (esters, ketones, aldehydes) may have a positive effect on wine flavor, the odors of fatty acids are usually not desirable.

13.2.6 Esterases

Esters (e.g., ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate) are qualitative one of the most important flavors in wine, where they contribute to a mostly desirable fruity taste (Fugelsang and Edwards 2007). Esters originate directly from the grapes (Rapp and Mandery 1986) as well as from the activity of yeasts during the alcoholic fermentation (Younis and Stewart 1998; Lambrechts and Pretorius 2000; Pfeiffer et al. 2007; Saerens et al. 2008). In the

course of malolactic fermentation (MLF), a concentration change of single esters has been observed, e.g., an increase of ethyl acetate (Maicas et al. 1999; Delaquis et al. 2000), isoamyl acetate (Maicas et al. 1999), and ethyl lactate (Delaquis et al. 2000). On the other hand, a decrease of different esters has been detected after completion of the malolactic fermentation (Zeemann et al. 1982).

Esterase activities (EC 3.1.1.1) have been found in different wine-relevant lactic acid bacteria, especially in *Oenococcus oeni* strains (Table 14.1). Their involvement in the synthesis and hydrolysis of wine esters has been studied by Matthews et al. (2007). Although all published esterase sequences lack a classical secretion signal sequence, there are reports of cell-associated esterases (Sumby et al. 2010). The enzymes generally deliver a broad pH activity range, with the majority of strains showing a maximum close to a pH of 6.0. Exceptions included an *O. oeni* strain that retained most activity down to a pH of 4.0. The majority of strains exhibited highest activity across the range 30–40 °C. Increasing ethanol concentration stimulated activity up to a maximum ethanol concentration of around 16%. Generally, strains were found to have greater activity toward short-chained esters (C2–C8) compared to long-chained esters (C10–C18). Even though the optimum physicochemical conditions for enzyme activity differed from those found in wine, these findings are of potential importance to oenology because significant activities remained under wine-like conditions.

Two purified intracellular esterases (EstA2 and EstB28) from *O. oeni* synthesized ethyl butanoate and ethyl hexanoate to varying degrees. Both purified esterases hydrolyzed ethyl butanoate, ethyl hexanoate, and ethyl octanoate. Strain-specific differences were observed, and strains with low esterase hydrolysis activity against artificial substrates had a higher level of total esters measured after MLF (Sumby et al. 2013).

An overexpressed arylesterase of *Lactobacillus plantarum* WCFS1 hydrolyzed most of the esters assayed including relevant wine aroma compounds (Esteban-Torres et al. 2014). Importantly, the enzyme exhibited activity at winemaking conditions, although optimal catalytic activity was observed at 40 °C and pH 5−7. From the compounds assayed (ethanol, sodium metabisulfite, malic acid, tartaric acid, lactic acid, and citric acid), only malic acid slightly inhibited arylesterase activity.

Ester synthesis and hydrolysis activities of several wine-associated yeast species have been described and a number of alcohol acetyltransferases and esterases have been purified. In a study with 74 *Saccharomyces* isolates, all strains delivered extracellular esterase activities (Ubedo Iranzo et al. 1998). Volatile esters are of particular interest as the presence of these compounds determines the fruity aroma of wine. Aroma-active esters are formed intracellularly by fermenting yeast cells, but since they are lipid-soluble, ethyl esters can diffuse through the membrane into the fermenting medium. The concentration of assimilable nitrogen is well known to have a major effect on fermentative aroma production by yeasts. An increase in initial nitrogen content is associated with the synthesis of acetate and ethyl esters (Rollero et al. 2016).

To date six distinct proteins of *Saccharomyces* species have been isolated and characterized as having ester synthesis or hydrolysis activity, with alcohol acetyltransferase having the greatest activity and being the most studied (Sumby et al. 2010).

13.2.7 Phenoloxidases

Phenoloxidases are copper-containing enzymes which use molecular oxygen to oxidize a broad range of aromatic compounds. The impact of fungal laccases on wine phenolics is described in Chap. 14. The occurrence of phenoloxidase activities in wine-related lactic acid bacteria was rather speculative for a long time (Matthews et al. 2004). For example, a gene encoding for a multicopper oxidase (Acc. ZB 00320225) is present in the genome of *Oenococcus oeni* PSU-1. More recently, Callejón et al. (2014) searched for enzymatic activities responsible for biogenic amine degradation in lactic acid bacteria strains isolated from wine. Fifty-three percent of the 76 cell extracts showed activity against a mixture of histamine, tyramine, and putrescine. Enzymes responsible for amine degradation were isolated and purified from *Lactobacillus plantarum* J16 and *Pediococcus acidilactici* CECT 5930 strains and were identified as intracellular laccase-like multicopper oxidases. When the laccase of *L. plantarum* J16 was overexpressed in *Escherichia coli*, it oxidized biogenic amines, mainly tyramine (Callejón et al. (2016).

However, in most known cases, degradation of biogenic amines is catalyzed by different classes of oxidases. According to the nature of the prosthetic group, they can be divided into FAD-dependent (EC 1.4.3.4) and copper-containing amine oxidases (CAOs, EC 1.4.3.6). The latter have been detected in several yeast species like *Debaryomyces hansenii* (Bäumlisberger et al. 2015). These enzymes belong to the class of type 2 or "non-blue" copper proteins and catalyze the deamination of primary amines, giving the respective aldehydes, with an equimolecular consumption of molecular oxygen and production of hydrogen peroxide and ammonia.

13.2.8 Tannases

Tannins are the most abundant class of soluble polyphenolics in grape berries found in the skins and seeds (Adams 2006). They vary in size from dimers and trimers up to oligomers with more than 30 subunits. The larger skin tannins contain epigallocatechin subunits, whereas in the smaller seed tannins, usually epicatechin gallate dominates. With the grapes and by leaching out from new oak barrels (Barrique), they enter must and wine. Especially in red wines, they are responsible for some bitterness and astringency. Tannases (tannin acylhydrolases, EC 3.1.1.20) cleave the ester and depsid bonds in hydrolyzable gallotannins, yielding gallic acid and D-glucose. They present an important group of biotechnologically relevant

enzymes, utilized for production of instant tee, beer, fruit juices, and wines (Yao et al. 2014).

The existence of such activities has been detected in wine bacteria (Vaquero et al. 2004; Table 14.1). The presence of an extracellular tannase in some *Lactobacillus plantarum* strains provides them an advantage for the degradation of complex tannins present in plant environments (Jimenez et al. 2014). The release of gallic acid may have a beneficial effect as it stimulates growth and malolactic fermentation of *O. oeni* (Vivas et al. 1997).

13.3 Mixed Starter Fermentations

Non-*Saccharomyces* yeasts are metabolically active during spontaneous and inoculated must fermentations, and by producing enzymes and by-products, they can contribute to the complexity of wine aroma (Jolly et al. 2014). Thus, use of *Saccharomyces* and "wild" yeasts as mixed starter cultures for inoculation of wine fermentations is of actual interest for quality enhancement and improving complexity of wines, e.g., by increasing the contents of terpenes, esters, or volatile thiols (Table 13.2). When using non-*Saccharomyces* yeasts in mixed starters, there are two general strategies of inoculation: co-inoculation (C) with *Saccharomyces* yeast at high cell concentrations or sequential inoculation (S) at high levels before *S. cerevisiae* is added to finish fermentation.

Comitini et al. (2011) combined each of four non-*Saccharomyces* yeasts (*Candida zemplinina*, *Lachancea thermotolerans*, *Torulaspora delbrueckii*, and *Metschnikowia pulcherrima*) with starter cultures of *S. cerevisiae* in mixed fermentation trials. The results indicated that the combinations were useful to increase polysaccharide, glycerol, and volatile compound production. Depending on yeast species and inoculum ration used, there was an increase or reduction of total acidity of the final wine.

The effect of mixed and sequential inoculations of *Wickerhamomyces anomalus* and a commercial *S. cerevisiae* strain in fermentations of non-sterilized red musts has been examined by Canas et al. (2014). The wines elaborated by sequential inoculation presented higher levels of acetates and ethyl esters, compounds that supply a fruity note, higher levels of lineal alcohols, which are responsible for herbaceous notes and lower concentrations of organic acids, which contribute to increase the aromatic quality, than wines produced by a *S. cerevisiae* monoculture. Both types of wines were comparable in levels of volatile acidity, glycerol, lactic acid, and succinic acid produced. Sensory analysis showed that red wines obtained by mixed fermentations were preferred by 71.5% of the tasters and were particularly appreciated for its floral and/or fruity notes.

Maturano et al. (2012) determined the ability of yeasts to produce extracellular enzymes of oenological relevance (β-glucosidases, pectinases, proteases, amylases, or xylanases) in pure and mixed *Saccharomyces*/non-*Saccharomyces* cultures during fermentation. Non-*Saccharomyces* species survived during 15–18 days

Table 13.2 Mixed starter fermentations (as reviewed by Padilla et al. 2016)

Yeast used in combination with *Saccharomyces cerevisiae*	Impact on wine aroma	Inoculation	Must
Candida zemplinina	3-Mecaptohexan-1-ol increase	C	Sauvignon Blanc
	Acetic acid decrease	C, S	Erbaluce dried grape must, Pinot Grigio
Debaryomyces pseudopolymorphus	Geraniol, nerol. and citronellol increase	C	Chardonnay
Debaryomyces vanriji	Geraniol increase	S	Muscat of Frontignan
Hanseniaspora guilliermondii	Acetate ester increase	C	Bobal, natural must
	Sulfur compound increase	C	Natural must
Hanseniaspora uvarum	Acetate ester increase	C	Synthetic must, Macabeo, natural must
Hanseniaspora vineae	Acetate and ethyl ester increase	C, S	Bobal, Chardonnay white, Tempranillo
Issatchenkia orientalis	Wine deacidification	C	Campell Early
Kazachstania gamospora	Acetate and ethyl ester increase	S	Ribolla
Lachancea thermotolerans	Wine acidification	C, S	Pasteurized natural must, sterile grape must
Metschnikowia pulcherrima	α-Terpineol increase	S	Muscat d'Alexandrie
	Acetic acid decrease	C	Pasteurized natural must
	Ethyl ester increase	C, S	Emir, Muscat d'Alexandrie
	Higher alcohol increase	C	Pasteurized natural must
Pichia fermentans	Acetic acid decrease	S	Sterile must
	Higher alcohol increase	C	Pasteurized natural must
Pichia kluyveri	3-Mecaptohexan-1-ol increase	C	Sauvignon Blanc
Schizosaccharomyces pombe	Wine deacidification	C, S	Airen, Garnacha
Torulaspora delbrueckii	α-Terpineol and linalool increase	S	Gewürztraminer
	Acetic acid decrease	C	Botrytis Semillon, pasteurized natural must

(continued)

Table 13.2 (continued)

Yeast used in combination with *Saccharomyces cerevisiae*	Impact on wine aroma	Inoculation	Must
	Acetate and ethyl ester increase	C, S	Sauvignon Blanc, Syrah, Tempranillo
	Higher alcohol increase	C, S	Chardonnay, Corvina, Corvinone, Rondinella, pasteurized natural must, Soave, Vino Santo
Wickerhamomyces anomalus	Acetate and ethyl ester increase	S	Mazuela
Wickerhamomyces saturnus	Acetate ester increase	C	Emir
Zygosaccharomyces bailii	Ethyl ester increase	C	Chardonnay

(*T. delbrueckii* BTd259) or until the end of the fermentation (*Hanseniaspora vineae* BHv438) after 36 days. Their secreted enzymes could be detected throughout the fermentation process. The results suggested that high concentrations of sugars did not affect enzymatic activities, but β-glucosidase and pectinase were adversely affected by an increase in ethanol. In a subsequent study, Maturano et al. (2015) assayed exoenzyme production in mixed cultures of *S. cerevisiae* BSc562/*Debaryomyces vanrijiae* BDv566 and *S. cerevisiae* BSc562/*Candida sake* BCs403. The first couple produced the highest concentrations of terpenes and higher alcohols which were associated with pectinase, amylase, and xylanase activities. For the second combination, high levels of β-glucosidase, proteolytic, and xylanolytic activities were correlated to concentrations of esters and fatty acids.

Mostert and Divol (2014) explored the exo-proteome of *Saccharomyces* and non-*Saccharomyces* yeasts (*Metschnikowia pulcherrima* and *Lachancea thermotolerans*) in pure and mixed cultures in a wine-like medium by a mass-spectroscopic LC-MS/MS approach. Apart from glucosidases and invertases, very few other enzymes of direct oenological interest could be identified.

Metschnikowia pulcherrima strain NS-EM-34 as a source of pectinolytic enzymes was analyzed by measuring its influence in filterability and turbidity and the increase on color, anthocyanin, and polyphenol content of wines fermented in combination with *S. cerevisiae* (Belda et al. 2016b). Improved results were obtained in the expected parameters in mixed fermentations, in comparison to wines fermented with *S. cerevisiae* alone or combined with other pectinolytic and non-pectinolytic yeasts (*Aureobasidium pullulans* and *Lachancea thermotolerans*, respectively), even working better than commercial enzymes preparations in most parameters. Additionally, strain NS-EM-34 was tested at a semi-industrial scale combined with three different *S. cerevisiae* strains, confirming its potential application for red wine improvement on the mentioned sensorial and technological properties.

13.4 Conclusions

Enzymatic activities of microorganisms play a key role for wine quality. They can influence color and taste as well as physical features like turbidity and ropiness of the final product. *Saccharomyces cerevisiae*, the principal wine yeast, is not a significant producer of hydrolytic extracellular enzymes (proteases, glycosidases, glucanases), unlike non-*Saccharomyces* yeasts and lactic acid bacteria. However, many strains of *S. cerevisiae* synthesize enhanced levels of aroma-active ester compounds by the action of cell-bound esterases and alcohol acetyltransferases. Volatile thiols are another important group of fragrances that contribute to the aroma profile of several grape varieties (Padilla et al. 2016). Thus, the expression of oenological relevant enzymes by wine microorganisms needs to be more deeply studied and managed to the benefit of wine production. By application of enzyme preparations or by mixed starter fermentations, wines with more individual sensory characters could be created.

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Chapter 14
Laccases of *Botrytis cinerea*

Harald Claus

14.1 Introduction

Due to their presumptive beneficial impact on human health, polyphenols in red wine, especially resveratrol, have gained increasing public and scientific interest (El Rayess 2014). They are believed to reduce the effects of cell-damaging free radicals in the body. In particular, these antioxidants slow down the removal of nitric oxide from the blood, lowering blood pressure, and therefore reducing the risk of heart problems and strokes. There is increasing evidence for similar health-promoting properties of white wine and champagne in spite of the fact that the total phenolic content is significantly lower than in red wines. The non-flavonoid phenolics tyrosol and hydroxytyrosol, present in white wine, seem to exert analogous effects as resveratrol (Zinnai et al. 2013).

Biotic and abiotic reactions alter the phenol composition of must and wine. *Saccharomyces cerevisiae* cells adsorb phenolic compounds or cause color loss by enzymatic hydrolysis involving a periplasmic anthocyanin-β-D-glucosidase (Caridi et al. 2004). Lactic acid bacteria such as *Lactobacillus plantarum* are able to metabolize cinnamic acids to 4-vinylphenol and 4-vinylguaiacol (Landete et al. 2007). The off-flavors ethylphenol and ethylguaiacol result from the enzymatic conversion of *p*-coumaric acid and ferulic acid by *Brettanomyces* strains (Dittrich and Großmann 2010). Glycosidases produced mainly by non-*Saccharomyces* yeasts split off sugar moieties from glycosylated polyphenols to yield the corresponding aglycons (Sabel et al. 2014; Schwentke et al. 2014).

Most of all, oxidative reactions, both spontaneously and enzymatically catalyzed (Du Toit et al. 2006; Li et al. 2008; Oliveira et al. 2011), have dramatic effects on the final phenol composition from the grape berry up to the bottled wine. Once the

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berry integrity has been destroyed, oxidative enzymes (phenoloxidases) and their phenolic substrates are exposed to air resulting in enzymatic browning (Macheix et al. 1991). The responsible copper containing enzymes tyrosinase and laccase use molecular oxygen for substrate oxidation (Mayer and Staples 2002; Claus and Decker 2006; Claus 2010). Tyrosinase (E.C. 1.14.18.1) hydroxylates monophenols to ortho-diphenols and oxidizes the latter to orthoquinone intermediates which polymerize to form brown pigments. Laccase (EC 1.10.3.2) has no monohydroxylase activity but oxidizes a wide spectrum of different polyphenols and other compounds by a radical mechanism. This enzyme is very stable under wine conditions and has serious impacts on the phenolic composition and quality of must and wine. Wine-relevant phenolic substrates of laccases are, e.g., caffeic acid, gallic acid, vanillic acid, ferulic acid, or resveratrol. Tyrosinase is produced by grape berries (Fronk et al. 2015), whereas laccases in must and wine originate from epiphytic fungi (Claus et al. 2014).

This chapter presents known and new facts on the interactions of *Botrytis* laccases with wine phenols.

14.2 Fungal Laccases

Laccase (EC 1.10.3.2) or laccase-like enzymes have been detected in all domains of life (Claus 2003). The best studied laccases are those of the lignin-degrading white-rot fungi (Baldrian 2006). The enzyme is a prominent member of the blue multi-copper oxidases which carry four copper ions in the polypeptide chain. The T1 copper has a trigonal coordination, with two histidines and a cysteine as conserved ligands. It is the site of substrate oxidation, and it has been widely argued that hydrophobic residues (Phe, Ile) in the near surroundings of the T1 copper strongly influence the oxidation potential of the enzyme which varies in the range between E^{o} +400 and +800 mV, depending on the individual laccase (Giardina et al. 2010; Jeon et al. 2012). The T2 and T3 copper atoms form a trinuclear cluster, where reduction of molecular oxygen to water takes place. The T2 copper is coordinated by two histidines and one water molecule and each of the two T3 copper atoms by three histidines.

A number of 3-D structures of laccases from basidiomycetes have been reported so far. On the other hand, crystal structures of ascomycetes have been so far resolved only for *Melanocarpus albomyces* and for *Botrytis aclada* (Kittl et al. 2012). The structures show that the protein monomer is normally organized in three sequentially arranged cupredoxin domains. Each of the three domains displays similar β-barrel type architecture, related to the small blue copper proteins such as azurin or plastocyanin. Disulfide bonds link domain one with domains two and three, while the trinuclear cluster bridges the first and third domains. The T1 copper located in domain three is the primary substrate electron acceptor site and is connected to the oxygen-reducing T2/T3 trinuclear cluster by a His-Cys-His bridge.

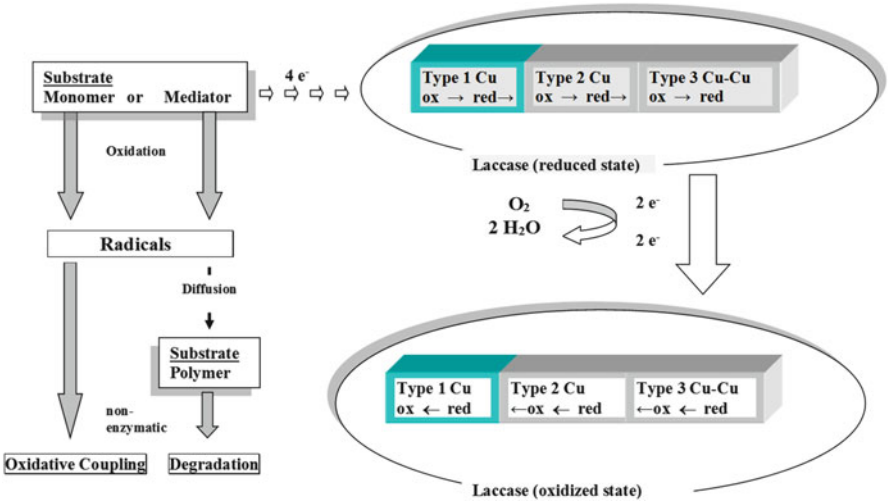

Fig. 14.1 Catalytic reactions of fungal laccases (Claus 2003)

The following simplified catalytic cycle has been proposed: the reducing substrate is bound in a cleft at the enzyme surface and is oxidized by the T1 copper site in domain three. Electrons donated by four equivalents of the reducing substrate are transferred via a strongly conserved His-Cys-His tripeptide and progressively lead to the reduction of all four Cu(II) ions in the polypeptide to the Cu(I) state. Reoxidation of the cuprous ions occurs at trinuclear T2/T3 cluster with the concomitant reduction of molecular oxygen, resulting in the formation of two water molecules. Reduction of oxygen by laccase occurs in two-electron steps involving a peroxide intermediate, bridging the trinuclear copper site. The free radicals generated from laccase oxidation are very reactive and undergo further nonenzymatic cross-linking reactions (Fig. 14.1).

Laccase is quite a unique enzyme in that it can oxidize small molecules that can in turn oxidize compounds that would not normally be a substrate of laccase. These small molecules are known as mediators and have increased the potential scope of laccase tremendously. The small size of these mediator compounds allows for the oxidation of bulky polymers such as lignin and humic acids, which cannot be oxidized by laccase under conventional conditions. Examples for synthetic mediators are TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy free radical), 1-hydroxybenzotriazole (HBT) and 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS). There are also natural lignin-derived compounds (vanillin, acetovanillone, methyl vanillate, acetosyringone, syringaldehyde, 2,4,6-trimethylphenol, *p*-coumaric acid, ferulic acid, sinapic acid) that can act as mediators (Strong and Claus 2011).

Laccases show relaxed specificity for their reducing substrates but a strong affinity for oxygen. Basically, any compound with characteristics similar to a

diphenol will be oxidized by laccase as long as its redox potential is not too high ($E^o < +1000$ mV). Classical substrates of fungal laccases include various lignin-derived phenols and aromatic amines. Currently, about 100 natural and artificial compounds are known to be oxidized by laccases (Reiss et al. 2013). Chromogenic compounds commonly used for detection and photometric measurement of laccase activities are 4-hydroxy-3,5-dimethoxy-benzaldehyde azine [syringaldazine], ABTS, or 2,6-dimethoxyphenol. Another method to quantify laccase activities is the polarographic determination of oxygen consumption in the course of substrate oxidation.

Often, several high-glycosylated laccase isoforms are produced by the fungi, as well as constitutively and/or after addition of inducers (copper, phenolic acids). Although usually active as monomeric proteins, some laccases consist of several subunits, forming hetero- or homodimers (Giardina et al. 2010).

Fungal laccases are rather resistant against detergents like SDS but, due to their reactive copper centers, are inhibited by halides, carbonates, and heavy metals. In addition to the general inhibitors of metal-containing oxidases like cyanide, sodium azide, or fluoride, hitherto no specific inhibitor has been described (Johannes and Majcherczyk 2000).

Due to the abundance of laccase and laccase-like enzymes, there are numerous and diverse natural functions for these oxidoreductases (Reiss et al. 2013). Fungal laccases probably play roles in spore pigmentation and morphogenesis, fungal plant-pathogen/host interactions, and stress defense. Furthermore they are involved in degradation of lignin and turnover of humic matter (Claus and Filip 1998; Baldrian 2006; Jeon et al. 2012). Bollag et al. (1988) showed that laccase of *Rhizoctonia praticola* reversed the inhibitory effects of a number of phenolic compounds on fungal growth. Laccases are also involved in antagonistic interactions between different fungal species in natural ecosystems (Sjaarda et al. 2015).

14.3 *Botrytis* Laccases

Botrytis cinerea (teleomorphic form: *Botryotinia fuckeliana*) has been identified as a pathogen of more than 235 plant species including grapes, lettuce, tomatoes, tobacco, and strawberries. Related species such as *Botrytis allii*, *Botrytis byssoides*, *Botrytis squamosa*, *Botrytis fabae*, and *Botrytis gladioli* are pathogens in onions, beans, and flowers. In viticulture, *B. cinerea* may cause both serious loss and enhancement of quality, depending not only on the stage of ripening in which berries are infected but also on weather conditions. Under dry and warm conditions, infections of ripe berries may raise the quality especially of white cultivars. In this case, berry ingredients are concentrated due to the perforation of the berry skin by the fungus. In addition, *B. cinerea* produces gluconic acid which confers a pronounced tastiness to the wine (Kassemeyer and Berkelmann-Löhnertz 2009). Consequently, late infections ("noble rot") of mature berries facilitate the production of dessert wines (Magyar and Soós 2016). On the other hand, berry infection at an

early stage of ripening and during long-lasting wetness reduces the quality, because of berry decay. This "gray mold" is caused by a cryptic complex of species. The core species *B. cinerea* has been genetically separated into distinct groups (N and S). Repeated fungicide applications have resulted in a dramatically increase of multiple resistances in *Botrytis* isolates (Rupp et al. 2017).

Phenol oxidizing enzymes produced by the phytopathogenic fungus help to detoxify plant phytoalexins, thereby increasing fungal virulence (Mayer and Staples 2002; Mayer 2006). Several glycosylated laccase isoenzymes with rather different molecular masses have been purified and biochemically characterized from different *B. cinerea* strains (Claus et al. 2014). Obviously when talking about *Botrytis* laccase, not necessarily the same enzyme is addressed. It might be argued, whether *Botrytis* species determinations in earlier studies could be verified by modern molecular approaches. Nevertheless, strain-specific variations, different culture conditions, and purification procedures deliver different laccase isoenzymes which may assemble to oligomers. The situation is even more complicated as the extracellular *Botrytis* laccase is partly entrapped in a glycan sheath (Gil-ad et al. 2001). It might be worth to mention that a laccase-like stilbene oxidase of only 32 kDa has been purified by Pezet (1998). This does not mean that "normal-sized" ≈60 kDa laccases, which were also present in the culture filtrate of this *B. cinerea* strain, did not oxidize stilbenes, but vice versa, the small enzyme did apparently not oxidize the typical laccase substrate syringaldazine.

A more homogenous picture appears when looking in the genome of *Botrytis cinerea* strains. Usually, two laccase genes are found with typical molecular sizes for fungal laccases around 60 kDa. Eight to eleven sequon structures (Asn-Xaa-Ser/Thr) indicate possible *N*-glycosylations sites. A signal peptide of 19–20 amino acids underlines that the gene products are probably secreted in the environment. The phylogenetic relatedness of the *Sclerotiniacae* (Amselem et al. 2011) is also manifested in the similarity of their laccase genes (Kittl et al. 2012).

For *B. cinerea* it has been reported that phenolic compounds, especially gallic acid or a product of gallic acid metabolism, can induce laccase production (Gigi et al. 1980; Viterbo et al. 1993b). Pectin acts as a second inducer of extracellular laccase production, in the presence of a phenolic substance as a first inducer. Pectin alone fails to induce enzyme formation (Marbach et al. 1985). Furthermore, terpenoids can stimulate laccase production (Cotoras et al. 2004). On the other hand, cucurbitacins have been found to inhibit laccase formation (Viterbo et al. 1993a, b).

14.4 *Botrytis* Laccase and Resveratrol

The hydroxystilbene resveratrol is one of the best analyzed compounds in wine, especially because of its beneficial influence on human health. The positive antioxidant, cardioprotective, neuroprotective, or anticancer effects have been discussed in numerous publications (e.g., by Soleas et al. 1997; Stevenson and Hurst 2007; Li et al. 2012; Stuart and Robb 2013).

The original well-investigated function of the molecule is that of a phytoalexin. The latter are secondary metabolites produced by plants in response to biotic and abiotic stress factors and have antifungal and antimicrobial activities. Resistance of *Vitis* species to fungal infection is generally correlated with their ability to produce stilbene phytoalexins. Exogenic application of trans-resveratrol to grape berries is an effective means of enhancing their resistance to fungal infection. In laboratory experiments, trans-resveratrol inhibits the downy mildew fungus *Plasmopara viticola* and a number of other fungal pathogens, including *Cladosporium cucumerinum*, *Oidium tuckeri*, *Pyricularia oryzae*, *Sphaeropsis sapinea*, and *B. cinerea*.

Trans-resveratrol is generated from *p*-coumaroyl-CoA and three molecules of malonyl-CoA in a reaction catalyzed by stilbene synthase. Trans-resveratrol is further converted into different derivatives:

- Piceid or polydatin (glycosylation)
- Pterostilbene (methoxylation)
- ε- and δ-viniferin (oxidative oligomerization)

It should be noted that some resveratrol derivatives are actually present in must and wine at higher levels than resveratrol itself. Piceid levels of red wines were found to be as much as tenfold higher. Pterostilbene and ε-viniferin are more potent inhibitors of fungal growth than trans-resveratrol, though they may not accumulate to levels required for physiological activity (Stuart and Robb 2013).

The mechanism of action of stilbenes against *B. cinerea* is not completely understood. It has been suggested that resveratrol inhibits the respiration of fungal cells, probably by acting as an uncoupling agent or by membrane lipid peroxidation. Pterostilbene is more active than the less hydrophobic resveratrol, due to its increased diffusion through the cytoplasmic membrane (Caruso et al. 2011). In vitro experiments suggest that phytopathogenicity of *B. cinerea* strains is associated with their ability to degrade stilbenes. In *B. cinerea* cultures, trans-resveratrol induces a specific laccase gene (*Bclcc2*), and trans-ε-viniferin has been identified as oxidation product of laccase activity (Pezet 1998; Schouten et al. 2002). Thus, *B. cinerea* would commit suicide, because the dimer has been found to be more toxic than the monomer (Schouten et al. 2002). The situation is puzzling, because the small stilbene oxidase has been postulated as the resveratrol-converting enzyme, whereas the gene product of *Bclcc2* is a normal-sized laccase. Other researchers have suggested that *B. cinerea* detoxifies trans-resveratrol by laccase activity (Pezet et al. 1991). The role of *B. cinerea* laccase in grape infection thus remains unresolved (Favaron et al. 2009). The situation is even more complicated as *B. cinerea* cultures convert trans-resveratrol not solely into ε-viniferin but also into a number of other dimers such as resveratrol trans-dehydrodimer, leachinol F, and pallidol (Cichewicz et al. 2000). Like resveratrol, trans-pterostilbene undergoes oxidative dimerization by the laccase activity of *B. cinerea* (Breuil et al. 1999).

Nevertheless, own results confirm the inhibiting effect of resveratrol on growth of *Botrytis* and the detoxificating reaction of laccase. After 5 days cultivation, growth of *B. cinerea* P16 on solid media was inhibited in presence of resveratrol

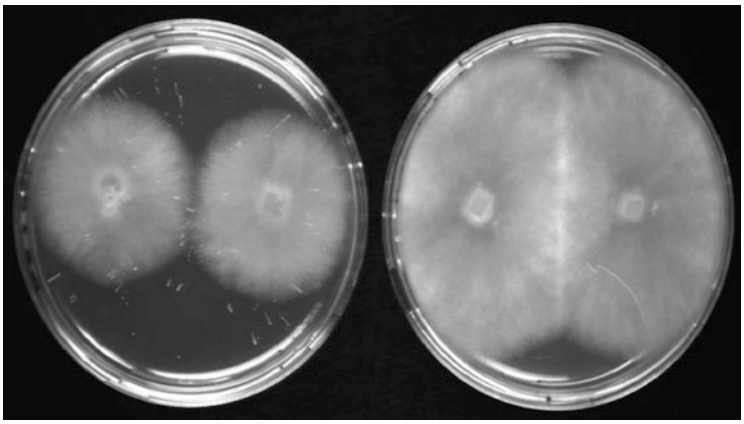

Fig. 14.2 Growth of *B. cinerea* strain P16 on Czapek-Dox agar. *Left* in presence of resveratrol (200 µg/ml), and *right* in presence of resveratrol oxidized by fungal laccase (Photograph by A. Sabel)

but not by laccase-oxidized resveratrol (Fig 14.2). No inhibition of this strain was observed in liquid media with added resveratrol. On agar plates the reversion of inhibition is properly restricted by the limited diffusion of laccase within the solid matrix. The glycosylated derivate polydatin had no impact on fungal growth.

Many studies have shown that certain phenolic compounds present in wine affect microbial growth and metabolism (ref. in Claus et al. 2014). In a survey with nine phenols (caffeic acid, gallic acid, resveratrol, polydatin, 3,4-dihydroxybenzoic acid, ferulic acid, sinapinic acid, syringaldehyde, and ethyl gallate), we found that resveratrol, ferulic acid, and syringaldehyde most significantly inhibited the growth of wine yeasts and lactic acid bacteria. Again, polydatin behaved neutral (Table 14.1). However, in presence of *Botrytis* laccase, the latter exerted an inhibition effect on some test organisms, and the impact of resveratrol usually was enhanced.

14.5 *Botrytis* Laccase and Wine Browning

The browning of wine, primarily due to the enzymatic and chemical oxidation of the phenolic compounds, represents one of the most feared processes which may arise during winemaking. The release of laccase into must from grapes infected with *B. cinerea* may cause a significant reduction of the content of phenolic compounds (Mayer 2006). As shown in Table 14.2, all major classes of wine phenols and even some terpenoids were oxidized by a laccase from the grape must isolate *B. cinerea* P16.

Laccase catalyzes the one-electron oxidation of a broad range of compounds including substituted phenolics, arylamines, and aromatic thiols to the

Table 14.1 Effect of resveratrol and polydatin on the growth of wine microorganisms as influenced by *B. cinerea* laccase

Species	Concentration	Relative growth (%)[a] in presence of			
		Resveratrol		Polydatin	
	(mg/ml)	Without laccase	With laccase	Without laccase	With laccase
Saccharomyces cerevisiae 16.1	0.25	88.69	0.00	103.25	17.02
	0.50	45.06	1.66	107.53	4.80
	1.00	0.00	2.30	106.64	3.65
Saccharomyces bayanus HL 77	0.25	16.90	1.22	95.78	1.68
	0.50	0.40	2.54	99.86	3.76
	1.00	0.00	0.00	106.90	0.96
Debaryomyces hansenii 525	0.25	71.94	33.66	102.78	122.37
	0.50	53.24	11.41	116.51	119.29
	1.00	5.04	0.30	123.24	108.95
Wickerhamomyces anomalus AS1	0.25	95.89	15.25	102.17	86.31
	0.50	61.84	14.16	106.65	49.68
	1.00	17.29	23.46	109.81	21.68
Lactobacillus hilgardii DSM 20176	0.25	11.37	8.94	97.08	104.04
	0.50	8.37	1.06	103.39	114.78
	1.00	0.00	0.00	99.79	115.36
Lactobacillus plantarum DSM 20174^T	0.25	4.41	8.22	90.34	100.45
	0.50	3.51	6.89	92.24	99.51
	1.00	0.00	0.00	97.66	88.62
Pediococcus parvulus DSM 20232^T	0.25	78.45	106.10	115.85	114.95
	0.50	26.21	1.89	113.33	117.59
	1.00	0.00	0.00	117.49	118.13
Oenococcus oeni DSM 20252	0.25	31.36	87.58	99.25	144.13
	0.50	4.27	37.68	77.05	134.90
	1.00	0.00	9.79	73.04	113.67

[a]The control without phenol additions was set as 100% growth (Sabel and Claus, unpublished)

corresponding radicals (Reiss et al. 2013). Polyphenolics, including the important classes of wine phenolics (phenolic acids, catechins, anthocyanins, tannins, and stilbenes), are converted into the corresponding quinones, which often react further to dark-colored polymers (Walker 1975; Fowler et al. 2011). The latter are generally insoluble in water and precipitate from must and wine. Furthermore, the oxidation of phenolic compounds may adversely affect the sensory and nutritional properties of wine and other foods (Oliveira et al. 2011; Zinnai et al. 2013). However, apart from resveratrol, currently only few studies focus on the fate of individual wine phenolics.

Due to its intrinsic stability and activity at low pH values, considerable laccase activities can persist in must (Zivkovic et al. 2011). The prevention of browning reactions by phenoloxidases has always been a challenge to food scientists (De Leonardis et al. 2010). In winemaking, sulfur dioxide is the generally used antioxidant to control wine browning. However, concerns about its ability to induce severe pseudoallergic reactions have created a great need for its reduction or replacement in recent years (Postolache et al. 2012). Bentonite is a common

Table 14.2 Activity of fungal laccases with phenolic and non-phenolic wine compounds[a]

Substance class	Compound (5 mM)	Relative activity (%)[a]	
		Laccase from *B. cinerea* P16	Laccase from *P. pinisitus*
Benzoic acids	Ethyl gallate	100	43
	Gallic acid	89	45
	Syringaldehyde	69	67
	3,4-dihydroxybenzoic acid	50	26
	2,5-dihydroxybenzoic acid	27	44
	Vanillic acid	12	15
	4-hydroxybenzoic acid	0	0
Cinnamic acids	Sinapinic acid	77	100
	Caffeic acid	69	78
	Ferulic acid	55	71
	Coumaric acid	0	7
Flavonoids	Quercetin	82	55
	Catechin	58	60
	Rutin	57	20
	Phloretin	17	12
	Naringin	11	0
	Phloridzin	10	5
	Naringenin	8	0
Stilbenes	Resveratrol	61	62
	Polydatin	57	80
Others	4-ethylguajacol	52	nd
	4-ethylphenol	16	nd
	Geraniol	7	nd
	Linalool	5	nd

nd not determined

[a]Measured by oxygen consumption: 100% correspond to 45 and 231 μmol O_2/min*l for *B. cinerea* and *Polyporus pinisitus* laccases, respectively

enological additive to remove undesirable grape wine proteins. In a former study (Zivkovic et al. 2011), we found bentonite treatment not effective for the removal of laccase from must. The probable reason is that laccases are acidic glycoproteins which are not efficiently bound to the negatively charged bentonite under wine conditions.

Unlike grape oxidases, which are inhibited by sulfites even at low concentrations, fungal laccases tend to be more resistant. Zivkovic et al. (2011) found an irreversible inhibition of a "natural" laccase activity in a white must by addition of as much as 200 mg/l sodium bisulfite. The most effective weapon to eliminate the laccase activity in the must is heat treatment. After 2 min at 75 °C, the enzyme was completely inhibited, whereas lower temperatures afford longer incubation times.

Due to the dramatic impact on wine quality, there is a strong demand to monitor *Botrytis* and its laccase activities in must and wine. Methods have been developed to quantify laccase activities by using chromogenic substrates such as syringaldazine (Grassin and Dubourdieu 1989; Cuadrado et al. 2005), use of *Botrytis*-specific antibodies (Sivertsen et al. 2005; Dewey et al. 2008), and detection of laccase genes by PCR methods (Hirschhäuser and Fröhlich 2007).

14.6 *Botrytis* Laccase, Wine Polyphenolics, and Human Health

14.6.1 Features of Polyphenols

Polyphenols (i.e., phenols with at least two hydroxyl groups linked to the aromatic ring) contribute to the red pigmentation, to the bitter and astringent components, and to the taste of grapes and wine (Eder and Wendelin 2002; Adams 2006; Landete 2012). The primary constituents of the phenolic wine compounds are flavonoids which share a basic C6–C3–C6 structure. They make up approximately 85% of the total phenols and enclose the anthocyanins, flavonols, flavanols, and condensed tannins. The non-flavonoid phenolic acids include the hydroxycinnamates (C6–C3), hydroxybenzoates (C6–C1), and stilbenes (C6–C2–C6). A major part of the polyphenols in nature is conjugated with sugars or organic acids (Landete 2012), rendering them more hydrophilic. Derivatives of cinnamic acid (including caffeic, *p*-coumaric, and ferulic acids) can form ester bonds with tartaric acid. The hydroxybenzoates, comprising *p*-hydroxybenzoic, protocatechuic, vanillic, gallic, and syringic acids are more prevalent in maturing wines. Hydrolysable tannins, polyphenols descending from gallic acid, are leached out from oak barrels (barrique). Especially in red wines, they are responsible for some bitterness and astringency (Moreno-Arribas and Polo 2010; El Rayess 2014).

The phenolic composition of wines is dependent on the grape variety and time of harvesting. Total polyphenol contents of red wines are in the range of 300–5000 mg/l and, thus, about ten times higher than that of white wines, which ranges between 60 and 200 mg/l (Eder and Wendelin 2002).

Due to their positive impact on human health, dietary polyphenols have got increasing scientific interest. Cardiovascular effects, tumor cell apoptosis, cytoprotective enzyme induction, antitumoral action, or activation of oxidative-stress-responsive transcription factors have been reported (Han et al. 2007; Stevenson and Hurst 2007; He et al. 2008; Hanhineva et al. 2010; Arranz et al. 2012; Landete 2012; Li and Förstermann 2012; Li et al. 2012; Stuart and Robb 2013).

In recent years, the moderate consumption of red wines has been shown to benefit human health. The effect has mainly been attributed to the antioxidant properties of polyphenols that are present in red wines, especially resveratrol (Espín and Wichers 2000; Eder and Wendelin 2002; Oak et al. 2005).

In view of this, it is necessary to analyze the possible interactions of phenoloxidases with these potentially beneficial components in wine. Espín and Wichers (2000) found that laccase did not modify the free-radical scavenging ability of resveratrol. However, according to Minussi et al. (2007), the treatment of a red must with laccase from *Trametes versicolor* mainly affected the phenolic compounds responsible for the antioxidant properties. They did observe that the treatment of white musts with the same laccase showed a greater reduction in total phenols than in total antioxidant potential, indicating the feasibility for the treatment of white wine musts.

The antioxidant capacity of substances is generally measured in vitro by radical scavenging assays (de Beer et al. 2002). These methods give hints but no proofs for antioxidant effects in vivo. Riebel et al. (2015) reported first results about the antioxidant capacity of red wines and individual polyphenolics on human cell lines before and after oxidation with tyrosinases and fungal laccases. In general, the common chemical 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (El Rayess 2014) and the biological tests using two different types of human cell cultures (monocytes and endothelial cells) delivered similar results. The oxidative capacities of all red wine samples (Merlot, Regent, Spätburgunder, St. Laurent) were drastically diminished after treatment with fungal laccases. The polyphenolics tested showed significant differences with respect to their antioxidant activity in all test systems. In most cases, their antioxidant capacities were reduced after enzymatic conversion by fungal laccases (Fig 14.3).

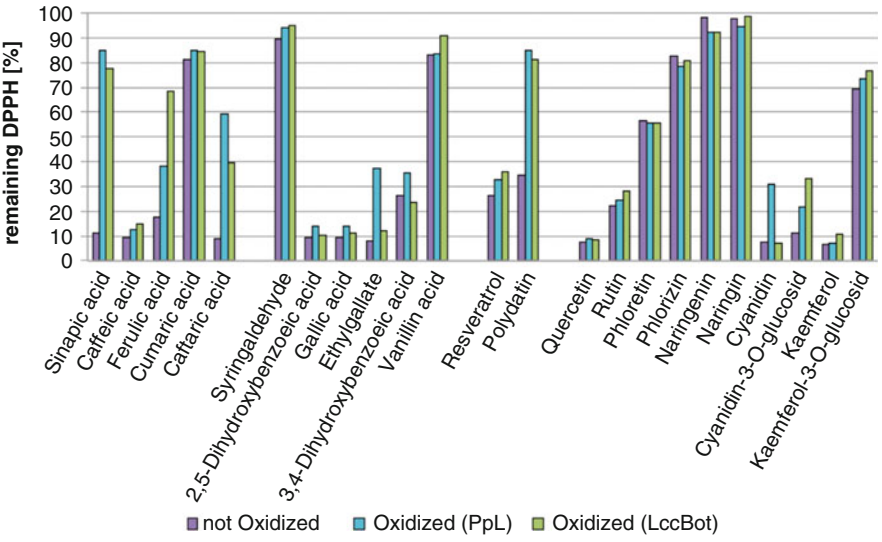

Fig. 14.3 Scavenger effect of phenolic wine compounds before and after oxidation by laccases from *Polyporus pinisitus* (PpL) and *B. cinerea* (LccBot), respectively. A low percentage of DPPH radicals indicates high antioxidant capacity

14.6.2 *Botrytis Laccase and Biogenic Amines*

The most significant and beneficial health properties of red wine consumption are related to compounds with high antioxidant capacity like polyphenols, including trans-resveratrol. However, red wines are also a source of biogenic amines that are detrimental to health (Preti et al. 2016). Biogenic amines in wine originate from the grape berries themselves or are produced during fermentation by undesirable activities of decarboxylase-positive microorganisms (Sebastian et al. 2011; Henríquez-Aedo et al. 2016). Although more abundant in foods like cheese, biogenic amines in wine have received much attention, because ethanol can increase the negative effects on human health by inhibiting the enzymes responsible for the detoxification of these compounds. Aromatic amines including the biogenic amines tyramine, phenylethylamine, tryptamine, or serotonin are another class of compounds, which are potential substrates for laccases. Table 14.3 demonstrates the effect of a *B. cinerea* laccase on selected biogenic amines (Sabel and Claus, unpublished). Significant degradation was observed especially at neutral pH values and in presence of small molecular mediator compounds like sinapinic acid and gallic acid that increase the oxidative potential of laccases.

14.7 Fungal Laccases in Wine Biotechnology

Although the uncontrolled oxidation of must and wine by *Botrytis* laccase is a nightmare for winemakers, attempts have been made for a directed laccase treatment of beverages.

Table 14.3 Oxidative degradation of selected biogenic amines by a laccase from *B. cinerea* P16 without and in presence of mediator compounds at two pH values

pH	Biogenic amine (0.5 mM)	Relative degradation of biogenic amines (%) by a *B. cinerea* laccase[a]				
		Without mediator	ABTS (1.0 mM)	HBT (1.0 mM)	Sinapinic acid (1.0 mM)	Gallic acid (1.0 mM)
3.5	Tyramine	24.24	100.00	23.53	60.98	14.56
	Histamine	0.00	17.95	3.09	37.34	10.04
	Putrescine	1.15	0.21	5.98	0.00	7.13
	Phenylethylamine	nd	nd	nd	nd	nd
7.4	Tyramine	51.32	100.00	57.30	100.00	24.86
	Histamine	25.86	74.11	11.57	100.00	47.16
	Putrescine	0.28	0.00	6.84	85.51	61.99
	Phenylethylamine	0.00	4.74	6.19	92.03	25.28

nd not determined; *ABTS* 2,2′-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid); *HBT* 1-hydroxybenzotriazole (Sabel and Claus, unpublished)

[a]Concentrations of biogenic amines were determined by HPLC after 48 h at 30 °C

Due to a complex sequence of events, where the polyphenols play an important role, oxidative reactions can occur in musts and wines causing flavor and color alterations. This phenomenon of oxidation is known as maderization. Different methods have been used in order to prevent these reactions in wines, such as the removal of phenolics with polyvinylpolypyrrolidone (PVPP) and the use of sulfur dioxide to block oxidizers, among others. An alternative is the use of phenoloxidases that selectively target specific polyphenols causing oxidative browning (Servili et al. 2000; Minussi et al. 2002, 2007). These polyphenolic substances are oxidized by the laccases and polymerized and then removed by clarification. Enzymatic treatment is advantageous for its specific action and as a "mild technology," with less drastic effects on the characteristics of the wine. Several publications in the literature report that laccase treatment promotes wine stabilization. The enzyme is consistent with some important requirements when used for the elimination of polyphenols from wines, such as activity and stability in acid media (pH 2.5–4.0).

A laccase of *T. versicolor* removed more than 90% of ferulic acid from a model solution and 34% of phenolic compounds from wines. A mutant laccase from *T. versicolor* (optimum pH 2.7) eliminated up to 70% catechin and 90% of anthocyanidins in a model solution in 3 h of treatment and removed 50% of total polyphenols from a black grape juice.

Maier et al. (1990) evaluated the polyphenol content, color, haze stability, and sensorial quality of Riesling wines prepared with and without enzymatic treatment of the must. The results showed that wines made by laccase treatment were the best, suggesting that a stable and high-quality wine can be made with little or no added SO_2. Since the use of laccase as a food additive is currently not allowed, this enzyme has been tested for wine production in an immobilized form, to ensure its elimination from the must and its reutilization (Brenna and Bianchi 1994). The results obtained suggest the potential of the immobilized enzyme for continuous enzymatic fruit juice clarification (Lettera et al. 2016).

Volatile ethylphenols are produced by *Brettanomyces*/*Dekkera* sp. yeasts and are associated with a serious taste defect in wine, known as "brett character." Effects of laccase from *T. versicolor* on 4-ethylguaiacol and 4-ethylphenol and their phenolic precursors (ferulic and *p*-coumaric acids) were investigated by Lustrato et al. (2015). Enzymatic treatment resulted in a significant reduction of all phenolic compounds from synthetic wines. After incubation at environmental temperature for 20 min, laccase decreased concentrations of 4-ethylguaiacol and 4-ethylphenol by 55.3 and 44.1%, respectively. These compounds are also oxidized by *Botrytis* laccase (Table 14.2).

Botrytized wines are specialities made of overripe grapes infected by *B. cinerea* with the form "noble rot" (Magyar and Soós 2016). Azzolini et al. (2013) gave an optimistic assessment of the possibility to modulate wine flavor of Italian sweet "passito" wines, through the artificially postharvest infection of selected *B. cinerea* strains, combined by the use of indigenous yeasts.

The oxidative power of laccase and tyrosinase has been used in biosensors for a rapid and reliable amperometric estimation of the total content of polyphenolic compounds in wines (Adamski et al. 2016). Another application of a fungal laccase

("suberase") is the treatment of cork stoppers. The enzymatic oxidation of the phenols avoids evolvement of the off-flavor tetra-chloroanisol (Sponholz 2000).

Many industrial wastewaters contain harmful phenolic compounds that are toxic and have high color content. The potential use of phenoloxidases (peroxidases, laccase, tyrosinases) to detoxify such effluents has been investigated already since the1980s (Strong and Claus 2011; Martínková et al. 2016).

14.8 Conclusions

Extracellular laccases of *B. cinerea* play an important role for its phytopathogenity and have a major impact on wine quality by altering wine phenolic composition and its health-promoting properties. Prevention of fungal infection would be the favorite instrument to avoid these unwanted enzymatic activities of gray mold, but unfortunately many isolates have become multiple resistant to common fungicides. Development of new chemical and/or biological strategies to combat *B. cinerea* infections is an urgent demand. Once released into must and wine, the fungal laccases persist due to their high intrinsic stability and can be only inactivated by high concentrations of sulfite and/or by heat treatment. Thus, a search for specific laccase inhibitors would be a worthwhile goal for winemaking. As *Botrytis* laccase is regularly present during production of dessert wines like "Trockenbeerenauslese," "Tokay," or "Passito" (Magyar and Soós 2016), one may wonder about the phenolic composition and nutritional value of such products.

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Chapter 15
Polyphenol Oxidases from Wine Grapes

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15.1 Introduction

Wine is produced from grapes which contain enzymes forming unfavorable dark polymers such as melanin, oxidizing proteins, and phenols which may influence their functions. Here we focus on the class of type-3 copper proteins presenting new insights on the structure, the occurrence during the ripening process and during wine making. Then, the reactivity of the enzymes on polyphenols from wine grapes are discussed and the possible consequences on health.

15.2 Type-3 Copper Proteins: Tyrosinase, Catecholoxidase, and Hemocyanins

Type-3 copper proteins comprise two different protein classes: oxygen-carrier proteins such as hemocyanins (Hcs) and the enzymes tyrosinase (TY) and catechol oxidase (CO) (van Holde et al. 2001). The latter ones are often summarized as polyphenol oxidases (PPOs). The active centers of type 3 copper proteins are similar with their two copper ions (CuA and CuB), which are each coordinated by three histidines. Oxygen is bound as peroxide in a "side-on" coordination (Decker et al. 2006; Rolff et al. 2011; Matoba et al. 2006; Solomon 2016).

Tyrosinases (EC 1.14.18.1) exert two different catalytic properties: the hydroxylation from monophenols (monophenolase or cresolase activity) and the oxidation in one step to *o*-quinones and the oxidation of diphenols to *o*-diquinones (diphenolase or catecholase activity). Catecholoxidases (EC 1.10.3.1) catalyze

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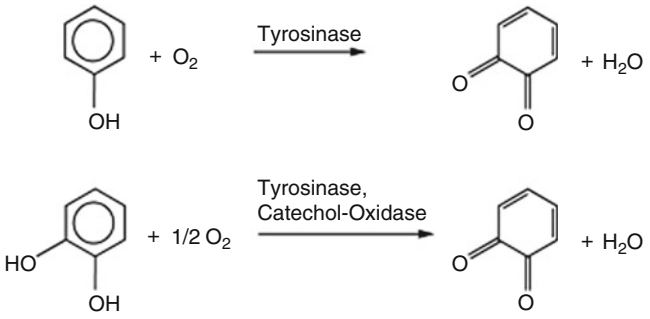

Fig. 15.1 Tyrosinase and catechol oxidase reaction. Tyrosinase catalyzes the conversion of mono- and diphenols to *o*-quinones. Catecholoxidase catalyzes only the reaction from *o*-diphenols to *o*-quinones (Decker et al. 2006)

only the latter step (Fig. 15.1). Quinones are highly reactive substances which spontaneously polymerize to melanins, which are polymeric substances with yellow to black color. For a long time, the molecular basis of the reaction was unclear, but recently a mechanistic explanation was given for the functional differences between tyrosinase and catechol oxidases based on crystal structures. Nevertheless, the exact catalytic mechanism of TY activity is still discussed. The crucial point is the deprotonation of monophenols to phenolate since only this form is able to bind to CuA (Goldfeder et al. 2014; Solem et al. 2016; Fujieda and Itoh 2016; Solomon 2016).

PPOs are ubiquitously found in almost all species, ranging from mammals to plants, fungi, and bacteria (Claus and Decker 2006) to produce highly reactive *o*-chinones which produce a black polymer without the use of other enzymes despite mammals (Sugumaran and Barek 2016). Many functions are discussed such as pigmentation, wound healing, radiation protection, and primary immune response (Cerenius and Söderhäll 2004; Kanteev et al. 2015; Solem et al. 2016; Coates and Decker 2017). In plants, they contribute to the defense system to pathogens and herbivores as well as oxidative processes (Mayer 2006; Boeckx et al. 2015). Pathogenic invaders are also attacked by *o*-quinones (Jiang et al. 2007; Boeckx et al. 2015).

Besides, PPOs gained importance through many applications in biotechnology such as bioremediation, dye production, biopolymer cross-linking, and melanoma therapy (Kanteev et al. 2015; Fairhead and Thöny-Meyer 2010, 2012; Zaidi et al. 2014; Buitrago et al. 2016).

In grapes and for wine production, PPOs from plant and fungi are of interest. PPOs naturally occur in grapes and in different fungi. Here we focus on plant PPOs especially tyrosinases in grape in this chapter. Laccase and their impact on wine making and health aspects are described in the chapter "Laccase" by Claus (cf. Chap. 14).

15.3 Tyrosinase and Catecholoxidase in Plants

PPOs are widely distributed in plant kingdom. They are responsible for the formation of mainly brown pigments, such as melanin and tannins. Thus, they cause the browning in fruits and vegetables of wounded tissue which is exposed to air, e.g., following bruising, cutting, or damage of the cell (Martinez and Whitaker 1995; Yoruk and Marshall 2003). The high number of phenolic substances in plants is accompanied by a high potential of browning. Especially the browning occurring during postharvest storage is of great concern for producers and the food industry. Not only the color but also flavor, texture, and nutritional value might be affected and have major impact on food quality (Yoruk and Marshall 2003). The oxidation may also affect the antioxidative capacity of phenols and their impact on health (Riebel et al. 2015, 2016; Oliveira et al. 2011).

PPOs can also be considered as pathogen-related proteins protecting the organisms against biotic and abiotic stress (Coupe et al. 1997). Richter et al. (2012) proposed that specific PPO isoenzymes should be included in a new family of pathogenesis-related (PR) proteins based on their results which indicate a strong contribution of a specific, single PPO isoform to disease resistance.

The general structure of plant PPO (about 55–70 kDa) is composed of the N-terminal catalytic domain containing CuA and CuB and the C-terminal domain with 15–20 kDa, which are connected through a short "linker unit" with about 2 kDa (Fig. 15.2). A transit peptide at the N-terminal directs the tyrosinase to the chloroplast and is then proteolytic removed. The activation of the latent PPO results from the cleavage of the C-terminal domain (Flurkey et al. 1995; Flurkey and Wichers 1997; Mayer 2006; Marusek et al. 2006; Kanteev et al. 2015; Leufken et al. 2015). However, the in vivo activator is not known yet.

For a long time, plant PPOs were considered as COs (Mayer 2006). But in recent years, monophenolase activity was described for several plant PPOs such as apple, kiwi, eggplant, walnut leaves, and grapes next to diphenolase activity (Yoruk and Marshall 2003). The first crystal structure of a plant tyrosinase was presented by Bijelic et al. (2015) from walnut leaves *Juglans regia*.

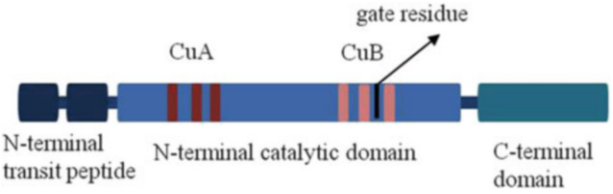

Fig. 15.2 Domain structure of a plant PPO. The N-terminal catalytic domain (about 40 kDa) follows a N-terminal transit peptide (about 100 amino acids) and a C-terminal domain (about 20 kDa) separated by a linker peptide (about 2 kDa). Each of the two copper ions (CuA and CuB) is fixed by three histidines (H_{A1}, H_{A2}, H_{A3} and H_{B1}, H_{B2}, H_{B3} shown as *red* and *orange stripes*) being provided by a 4-a-helix folding motif

Most plant PPOs are monomers, but in some plants, they are also multimeric, such as a tetramer in dandelion *Taraxacum officinale* (Dirks-Hofmeister et al. 2012). It should be noted that Pruidze et al. (1983) already reported about PPO from wine leaves. They found monomers (26–35 kDa) and dimeric forms with about 55–70 kDa. They showed that these PPOs have TY activity with higher affinity for monophenolic substrates than the dimers. During maturation/ripening, the dimeric fraction is predominant with an increase of the CO activity. No hydroxylation was observed anymore accompanied with an increase of phenol production.

15.4 Grape Polyphenol Oxidase

Decades ago, catecholoxidase activity was already reported for grapes and vine (Kidron et al. 1978). Lately, experiments revealed that PPO from grape *Vitis vinifera* cv Riesling can be considered as tyrosinase (Fronk et al. 2015), while for the closely related cultivar Grenache only a CO-activity could be shown (Virador et al. 2010) as it was the case in earlier reports for Muscat Bailey A Grape Juice (Okuda et al. 1999), DeChaunac Grapes (Lee et al. 1983), Koshu Grapes (Nakamura et al. 1983), and Ravat 51 and Niagara Grapes (Wissemann and Lee 1981). But for grape PPOs from Red Globe grape (García-García et al. 2013) and Victoria grapes (Rapeanu et al. 2006) both monophenolase and diphenolase activities have already been shown.

PPO of purified Sultana grapes consists only of a single protein with about 40 kDa as determined by SDS-PAGE (Dry and Robinson 1994). A complete cDNA encodes a 67 kDa protein consisting of a 10.6 kDa chloroplast transit peptide, a 40.5 kDa catalytic domain containing two copper-binding regions, and a 16.2 kDa C-terminal domain. High levels of gene expression were found in young developing berries, leaves, and roots, but there was little expression in mature tissues.

These results are supported by a recent structure of grape PPO by Virador et al. (2010). They presented the first crystal structure for wine PPO from *Vitis vinifera* cv. Grenache in its active form from biochemically purified grape. The molecular mass of the crystallized protein was determined to 38.4 kDa, while based on the cDNA a protein with 607 amino acids and a mass of about 67 kDa was calculated for the latent form. The mass difference can be explained by processing of the N- and C-terminal domains. First, proteolysis of the two transit peptides takes place after the protein is transported to the chloroplast. From the resulting 56.7 kDa protein, the C-terminal domain is cleaved at the "linker sequence" resulting in the active PPO with 38.4 kDa (Marusek et al. 2006; Virador et al. 2010). Based on a re-refinement of the crystal structure indicates the active form may also have a TY activity (Solem et al. 2016).

Southern analysis suggests a single gene for Grenache PPO (Virador et al. 2010). This is in accordance with an earlier publication by Dry and Robinson (1994). However, Sarry et al. (2004) found three major proteins corresponding to the PPO sequence (36, 20, 20 kDa) in the mesocarp of Gamay grapes suggesting multiple isoforms. A nonspecific cleavage of the C-terminus cannot be excluded for the different forms. For Riesling grapes, at least six spots with molecular masses of about 37 kDa and different IEPs could be detected (Riebel 2016).

In other plants, differential, tissue specific expression of PPO genes could also be detected. In potatoes, six genes coding for PPOs were found (Thygesen et al. 1995), and seven genes in tomatoes (Thipyapong et al. 1997; Mayer 2006). Kim et al. (2001) showed that two different genes were expressed at different stages of apple development. In dandelion, up to 11 isoforms of PPOs with different sequences were found (Dirks-Hofmeister et al. 2012, 2013). Thus, isoforms might result from the multiplicity of genes being differentially expressed in different plant compartments and at different stages of development (Mayer 2006).

15.5 Kinetic Characterization of Grape Tyrosinase

For PPO from Riesling grape berries, tyrosinase activity could be shown. Therefore, we refer to PPO from Riesling grapes as tyrosinase (Fronk et al. 2015).

Tyrosinase from Riesling grape berries shows activity over a broad temperature range and a substrate-dependent temperature optimum. For caffeic acid, the highest activity was in the range of 25–50 °C, but even at 5 °C 50% activity could be detected. However, for dopamine the highest activity was around 10–15 °C, but up to 35 °C activity declines only slightly and decreases at higher temperatures. For the monophenolic substrate *p*-coumaric acid activity at low temperature was lower compared to the diphenolic substrates. The optimum is located at 35 °C (Riebel 2016). An overview of kinetic parameters of tyrosinase from Riesling grapes is shown in Table 15.1.

The pH-optimum for Riesling tyrosinase was found between pH 5 and 5.6. At the physiological pH range between pH 2.8 and 4.0 in must, wine tyrosinase shows

Table 15.1 Kinetic parameters of tyrosinase from Riesling grapes

pH Optimum	**Buffer system [substrate affinity]**
Dopamine: 5–5.6	Citrate buffer > citrate-phosphate > sodium-acetate > acetate-phosphate
Temperature optimum	**Substrate specificity**
Dopamine: 10–15 °C	Catechol > dopamine > *p*-coumaric acid > *p*-tyrosol > tyramine
Caffeic acid: 40 °C	
p-Coumaric acid: 35 °C	

Measurements were performed at 25 °C and if not mentioned differently in citrate buffer pH 5.0

low or no activity. The enzyme, however, was active up to pH 7.8, where the activity reached 60% compared to the maximum (Fronk and Jaeckels 2016).

Next to pH the activity of Riesling tyrosinase was also dependent on the buffer system. At pH 5.0, the highest affinity was observed for citrate buffer and citrate-phosphate buffer, followed by sodium acetate and acetate-phosphate buffer (Fronk and Jaeckels 2016).

As already mentioned and as the name indicates tyrosinase from Riesling catalyze mono- and diphenolase reactions. For catechol, the highest activity was detected, but other diphenols such as dopamine, L-DOPA, and caffeic acid were catalyzed effectively as well. For monophenols, a lag phase of about an hour was observed. With the natural substrate *p*-coumaric acid tyrosinase showed a higher activity compared to other monophenols such as tyrosine, tyramine, and *p*-tyrosol (Fronk and Jaeckels 2016). An overview concerning kinetic characteristics for PPOs from different grape varieties is included in the paper by Fronk and Jaeckels (2016).

15.6 Activity of Riesling Polyphenol Oxidase During Grape Berry Development and Vinification

15.6.1 Activity of Riesling Tyrosinase During Grape Berry Development and Ripening

In 2007 the grapevine genome of Pinot noir was published (Jaillon et al. 2007). About 487 million base pairs code for 30.434 genes (Jaillon et al. 2007). The advances in genomics combined with those in mass spectrometry made it possible to describe the proteome of grape berries to an extent that was unreachable only few years ago. However, not all genes have already been annotated to particular proteins yet. In addition, not all genes are expressed in all stages of the developing and ripening grape (Newton et al. 2004). In Dornfelder grape berries more than 700 proteins could be identified. Most of them are involved in stress response and metabolic processes (Riebel 2016).

In compliance with previous studies, pathogen-related (PR) proteins such as thaumatin-like proteins (TLPs) and chitinases are the most abundant proteins in ripe grape berries (Pocock et al. 2000; Sarry et al. 2004). PPOs were detected in lower amounts. 2D gelelectrophoresis and subsequent mass spectrometry analysis as well as Western Blot analysis performed by Riebel (2016) revealed at least six different PPO spots in Dornfelder grapes and seven spots in Riesling grapes. They have a molecular mass of about 38 kDa but different isoelectric points (Fig. 15.3). As already mentioned above, Riesling PPO shows tyrosinase activity. However, since the isoforms were not purified separately and tested for tyrosinase activity, we refer to PPO in this case.

In Riesling grape berries, PPOs could be detected in green berries already at an early stage of berry development, and they are expressed throughout development

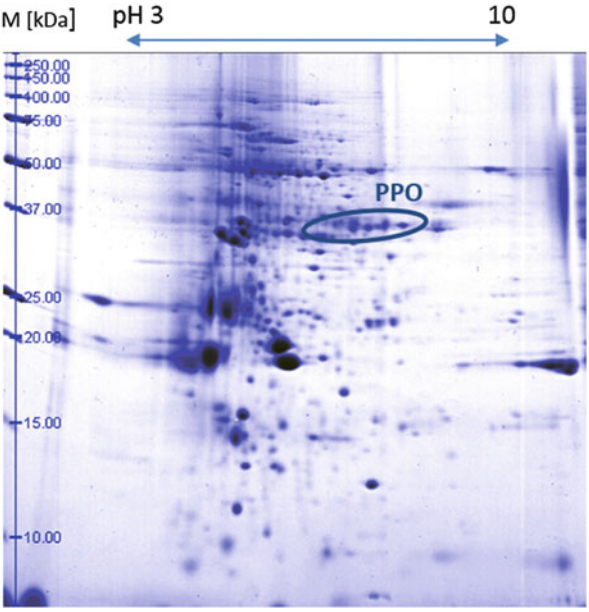

Fig. 15.3 Protein composition of mature Riesling grapes. Coomassie-stained 2D-SDS-PAGE (15%) after phenol precipitation of proteins

and ripening (Riebel 2016). This is in accordance with other studies showing that PPOs are active in grape berries from fruit set on (Lopez-Miranda et al. 2011; Martinez-Esteso et al. 2013). This result suggests an additional function for PPOs beside defense as discussed recently, such as an involvement in photosynthesis, amplification of wounding signals, and the phenylpropanoid pathway (Araji et al. 2014; Boeckx et al. 2015).

15.6.2 Activity of Riesling Tyrosinase During Vinification

An important question is up to which step of vinification tyrosinase shows activity. To answer this question, samples during the vinification of Riesling wine (winery Fleischer, Mainz, Germany) were taken: bevor and after fining, during fermentation, and after bottling. The samples were directly dialyzed and freeze-dried. Grapes were treated with a not further specified amount of potassium disulfate, due to attack with noble rot. To avoid any influence of laccase, measurements of tyrosinase activity were performed with *p*-tyrosol which is a substrate for tyrosinase, but not for laccase (the MBTH assay was used to increase the reaction signal in citrate buffer pH 5.0).

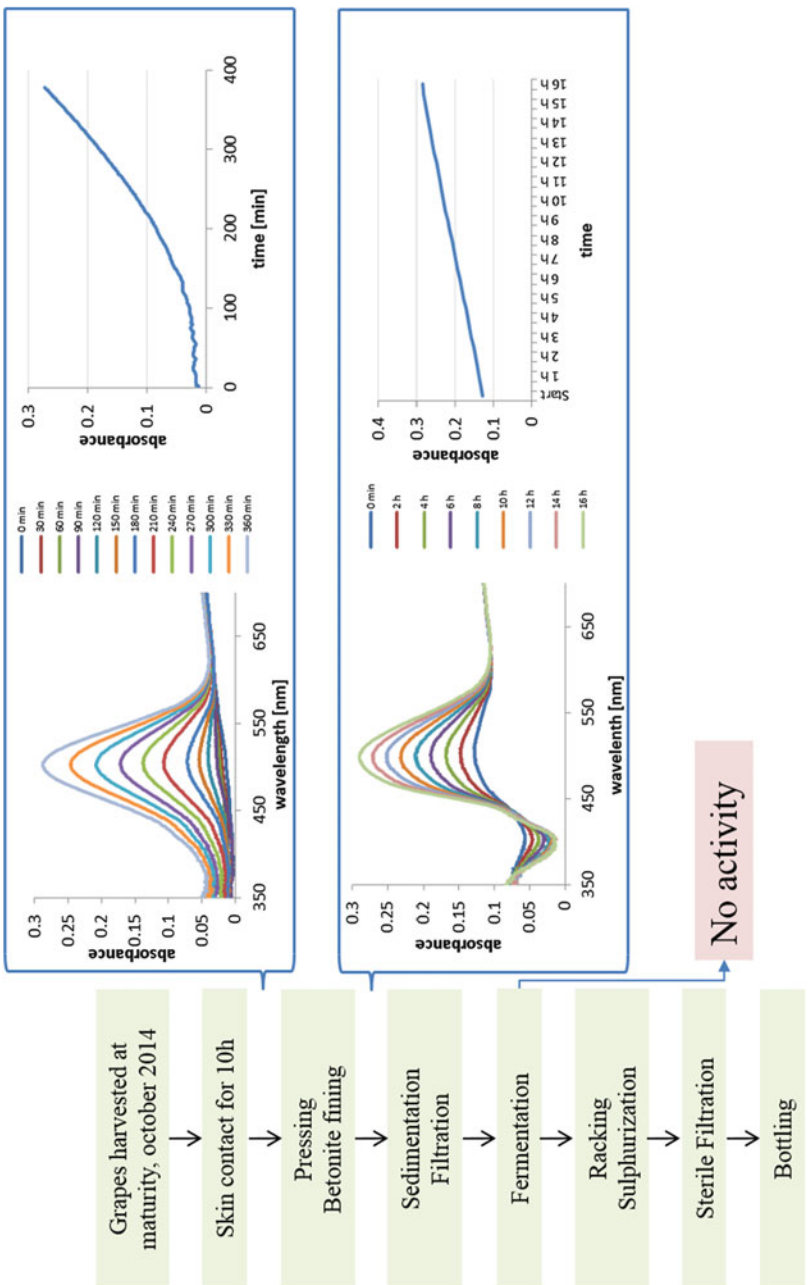

Fig. 15.4 Tyrosinase activity from Riesling grapes during vinification. No activity could be detected after fermentation

Tyrosinase is still active after treatment with disulfite and bentonite, even though activity after bentonite fining is very low (Fig. 15.4). During fermentation, tyrosinase activity is completely inhibited (Riebel 2016).

15.7 Influence of Sulfur and Bentonite on Tyrosinase Activity

As already mentioned in the previous section, disulfite and bentonite are added during vinification. To further quantify the influence of these substances on tyrosinase activity, measurements with purified tyrosinase from Riesling grapes were performed (Riebel 2016).

The permitted quantities of disulfite are 150 mg/L for red wines <5 g/L sugar, 200 mg/L for red wines >5 g/L sugar and for white und rosé wines <5 g/L sugar, and 250 mg/L for white and rosé wine with >5 g/L.

When tyrosinase was incubated with potassium disulfite before the measurement, already 10 mg/L inhibited its activity completely (MBTH assay with dopamine as substrate). Without incubation and with direct measurement after addition of disulfite, 10 mg/L decreased the tyrosinase activity to about 50% and then exponentially declined (Fig. 15.5). Temperature within a range of 10–30 °C did not notably influence tyrosinase activity (Riebel 2016).

Earlier investigations show that tyrosinase activity is completely inhibited applying sulfur dioxide concentrations of at least 50 mg/L (Wissemann and Lee 1980). In contrast, measurements by Riebel (2016) were performed without incubation time explaining the remaining activity of about 10% observed at 50 mg/L.

Bentonite is mainly added during the vinification process to remove proteins and prevent turbidity. Up to now, it is considered the most effective fining agent. But still, the fining with bentonite is also associated with negative aspects since in its function as cation exchanger it also removes important wine components such as phenols. Proteins are removed in different quantities (Jaeckels et al. 2016). Jaeckels et al. (2017) could show that varying adsorption behavior was even observed for different isoforms from TLPs ranging from no removal to 98% of reduction.

Usually the amount is determined with stability tests (especially heat and bento test).

Riebel (2016) applied bentonite concentrations up to 200 g/hL, which is usual praxis. Measurements with grape tyrosinase (58.9 U/mL) were performed after incubation for 1 h (MBTH assay with dopamine as substrate).

With a bentonite concentration of 10 g/hL, tyrosinase activity was decreased to 40%. 200 mg/hL bentonite resulted in a decrease of about 10% (Fig. 15.5). Earlier studies showed a reduction of polyphenol oxidase activity with bentonite up to 30% applying amounts of 20–100 g/hL depending on the commercial product used (Macheix et al. 1991).

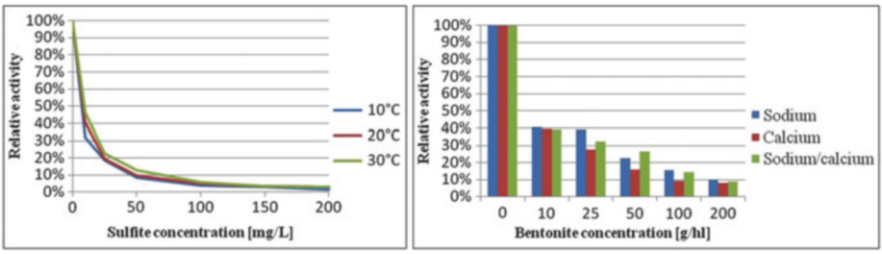

Fig. 15.5 Influence of sulfate and bentonite on relative tyrosinase activity. Measurements were performed with dopamine and the MBTH assay with detection of the activity at 505 nm (Riebel 2016)

15.8 Influence of Grape Tyrosinase on Wine Phenols and Their Antioxidant Capacity

Phenolic compounds are a chemically diverse group of secondary metabolites and occur in all fruits in different amounts. They are natural substrates for PPOs such as tyrosinase and laccase. Tyrosinases naturally occur in grape berries, and thus, the question arises whether their activity can change the antioxidant capacity of phenolic compounds. Besides, the oxidation might affect color and flavor. Especially in red wine, phenolic compounds are responsible for bitterness and astringency.

15.8.1 Oxidation of Phenolic Wine Compounds by Polyphenol Oxidases

In grapes, many different phenolic compounds are found which comprise flavonoids as well as non-flavonoids. Non-flavonoids in grapes and wine are hydroxycinnamic acids, hydroxybenzoic acids, and stilbenes. The flavonoids include four classes: catechins, flavonols, anthocyanins, and tannins. While the non-flavonoids are mainly located in the pulp of the grape, the flavonoids are mainly localized in the skins, seeds, and stems (Paixao et al. 2007).

The oxidation by tyrosinase and laccase of some of the most important wine phenols were investigated using absorption spectroscopy and oxygen consumption (Bauer 2015; Riebel 2016; Riebel et al. 2016). For most of the substrates, the conversion is visually detectable with a clear color change (Fig. 15.6).

The hydroxycinnamic acids caffeic acid and its tartrate ester caftaric acid were among the substrates with the highest conversion rate for tyrosinase, which was purified from Riesling grapes. The reaction with *p*-coumaric acid, a monophenolic hydroxycinnamic acid, was much slower and begins with a lag phase (Fig. 15.7). The methylated hydroxycinnamic acids ferula- und sinapic acid were not converted

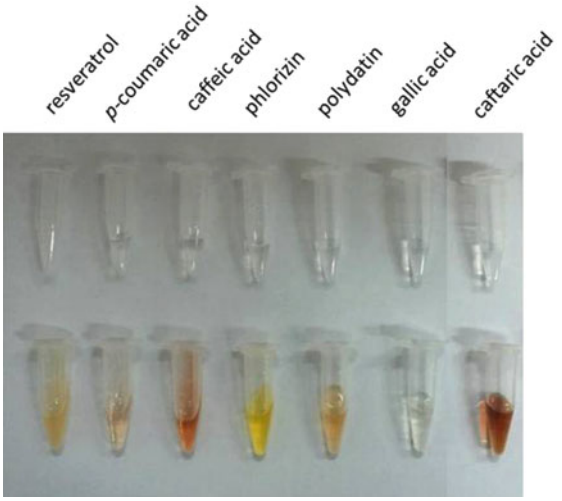

Fig. 15.6 Oxidation of phenolic compounds by tyrosinase from Riesling grapes (Riebel 2016). 5 mM of the substrate were incubated overnight with tyrosinase purified from Riesling grape berries (19.4 U/mL), in 0.1 M citrate buffer pH 5 at room temperature

by grape tyrosinase, but by *Agaricus bisporus* tyrosinase. Thus, the reasons might be the comparable low concentration of the purified grape tyrosinase rather than the inability of this enzyme to convert methylated hydroxycinnamic acids.

Overall, the conversion of hydroxybenzoic acids was slower and higher tyrosinase concentrations were required. The oxidation of the triphenolic gallic acid and 3,4-dihydroxy-benzoic acid (3,4-DHB) were the most effective within this group, while for ethylgallate and 4-hydroxybenzoic acid (4-HB), a reaction was detectably only after 24 h. The methylated vanillic acid was also a substrate for grape tyrosinase.

Resveratrol (3,5,4′-trihydroxystilben) and its 3-*O*-glycoside polydatin are both converted by grape tyrosinase after a lag phase, even though they show different absorption maxima indicating different oxidation products.

To quantify the conversion of phenolic substrates by tyrosinase, an oxygen consumption assay was performed. Due to the high amounts that are necessary, measurements were performed using tyrosinase from *Agaricus bisporus*. Phenolic compounds were converted in the following order:

caffeic acid > caftaric acid > ethyl gallate > resveratrol > 3,4 dihydroxybenzoic acid > polydatin > gallic acid > 2,5 dihydroxybenzoic acid > vanillic acid > sinapic acid > 4-hydroxybenzoic acid > *p*-coumaric acid > ferulic acid.

15.8.2 Antioxidative Activity of Phenolic Wine Compounds

Phenolic compounds in food are well known for their health-promoting effects. They are discussed to act as cardioprotectives, neuroprotectives, antifungals, antimicrobials, antidiabetics, anti-inflammatories, and antitumorals. The effect that is

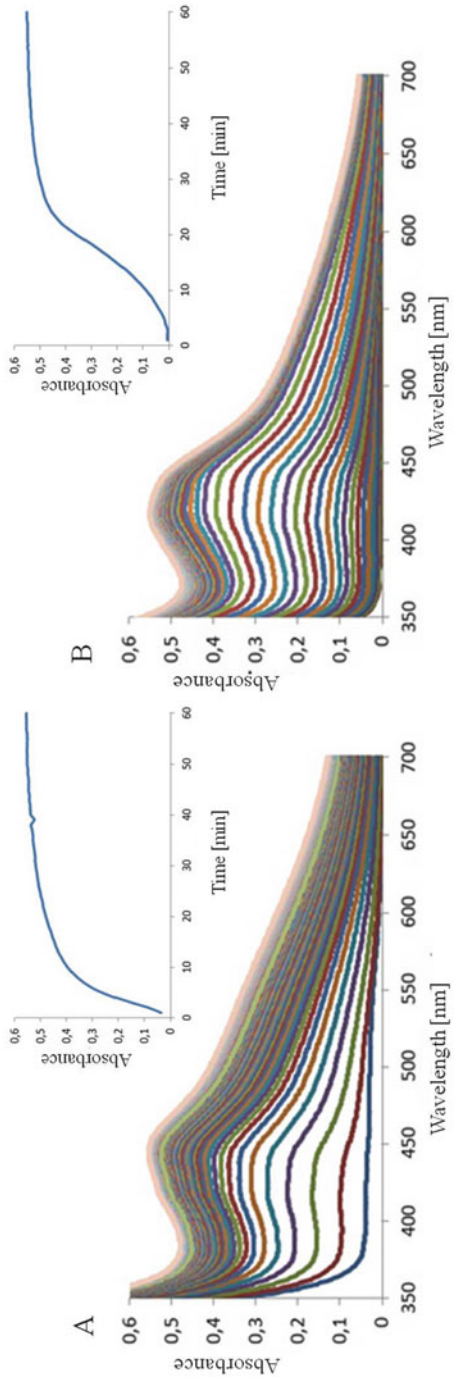

Fig. 15.7 Absorption spectrum of (**a**) caffeic acid and (**b**) *p*-coumaric acid. MBTH assay was performed in 0.1 M citrate buffer pH 5.0 at 25 °C (Bauer 2015)

most investigated and underlying some of the other effects is their role as antioxidants. They are especially known for quenching reactive oxygen (ROS) and nitrogen species (RNS). Thereby they can prevent mechanisms relevant for the development of cardiovascular disease. Functions in other degenerative diseases and cancer are discussed (Morton et al. 2000).

In this context, wine is one of the most discussed food products. In the beginning of the 1990s, the term of the "French paradox" arose describing the observation that mortality of coronary heart disease (CHD) is low in France despite a high intake of saturated fat. As reason a moderate consumption of red wine in French people was discussed (Renaud 1992). The French paradox is discussed controversy, but during the past 20 years, much research was conducted concerning the health-promoting effects of wine. Beneficial effects on cardiovascular diseases have been attributed to the actions of polyphenols with resveratrol being one of the most prominent representative (Frombaum et al. 2012).

To date, some studies investigated the antioxidative activity of phenolic wine compounds in detail (Villano et al. 2005; Paixao et al. 2007). Additionally, Villano et al. (2005) compared the antioxidative activity of polyphenolic metabolites with the polyphenolic compound itself. They showed that the metabolites tested had a similar antioxidative activity as the phenolic compounds and thus should be considered when analyzing the antioxidative activity in wine. Therefore, different metabolites that were found in the urine of rats fed with wine phenols and metabolites that were found after degradation by the colonic microflora were applied. However, not many studies have investigated the influence of oxidizing enzymes on the antioxidant capacity of phenolic wine compounds.

Riebel et al. (2015, 2016) performed measurements with human cell lines and could show that after tyrosinase oxidation the antioxidant capacity of many phenolic compounds was either not altered or even increased (Fig. 15.8).

During the last years, resveratrol has been discussed to be one of the major components in wine being associated with multiple cardiovascular and metabolic effects (Li et al. 2012; Xia et al. 2014). As expected, resveratrol showed strong scavenging activity in the DPPH assay and a high antioxidant capacity when tested in THP1 and EA.hy 926 cells. Interestingly, the oxidation of resveratrol by tyrosinase did beneficially affect antioxidative effects of resveratrol. Astudy by Espín and Wichers (2000) showed that laccase and tyrosinase did not change the antiradical capacity with respect to scavenger activity of resveratrol.

Phenolic acids in wine are also known for their antioxidative potential (Gülçin 2006). In the study by Riebel et al. (2015), the hydroxycinnamic acid caffeic acid as well as the hydroxybenzoic acid gallic acid showed strong scavenger and antioxidant activity. However, when comparing the phenolic compounds and their oxidation products, only slight differences in the DPPH assay and the cell line models could be observed, although both are substrates of tyrosinase. The monophenol *p*-coumaric acid showed low scavenging activity, and the antioxidant activity was also much lower compared to the diphenolic caffeic acid. After treatment with tyrosinase, both from mushroom and grape, the oxidation products displayed a strong scavenging effect, comparable to those of caffeic acid. This can be explained

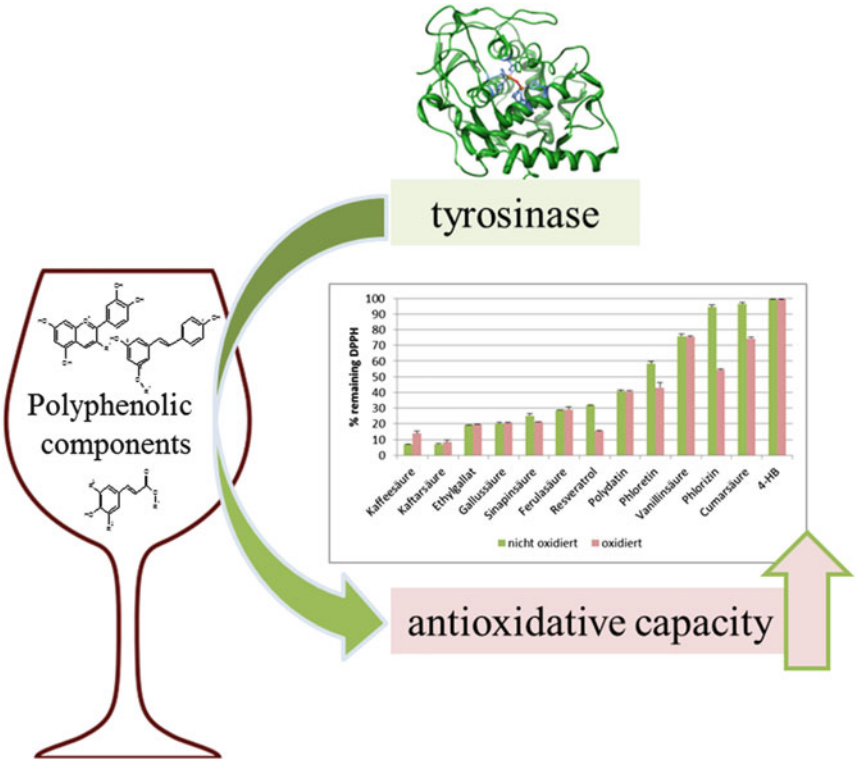

Fig. 15.8 Influence of tyrosinase oxidation on the antioxidative activity of phenolic compounds

by the monohydroxylation of *p*-coumaric acid in ortho-position, resulting in the same oxidation products as caffeic acid. In cell lines, the antioxidant capacity also increased after oxidation with tyrosinase, but was still lower compared to that of caffeic acid (Riebel et al. 2015, 2016).

The hydroxybenzoic acids gallic acid and its ethyl ester ethylgallate also showed high scavenging and antioxidant activities. Oxidation by tyrosinase revealed no change in the scavenging activity for these benzoic acids, although these phenols are tyrosinase substrates. Only for vanillic acid, a 4-hydroxy-3-methoxybenzoic acid, showing a comparably low scavenging and antioxidative effect, the antioxidative activity increased after treatment with tyrosinase whereas the scavenging activity was not influenced (Riebel et al. 2015, 2016).

In general, the antioxidative activity of the phenolic compounds depends on their chemical structure: The more aromatic OH-groups, the higher the antioxidative activity. This could be observed for hydroxycinnamic acids, benzoic acids, and flavonols. Another important aspect is the delocalization of electrons in the conjugated π-system, which stabilize the radical formed during oxidation and thus, leads to an increased antioxidative activity (Villano et al. 2005; Riebel et al. 2015, 2016).

Of course, next to the influence of oxidizing enzymes, many other factors during winemaking affect and can alter the phenolic composition of the end product such as time of maceration and fermentation, exposure to oxygen, pressing, maturation, fining, and bottle aging (Paixao et al. 2007).

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Part III
Stimulating and Inhibitory Growth Factors

Chapter 16
Stress Responses in Wine Yeast

Jürgen J. Heinisch and Rosaura Rodicio

16.1 Introduction

Saccharomyces cerevisiae is employed as pure starter cultures in industrial wine fermentations and usually within 2 days predominates in the must fermentations. Its physiology and genetics have been extensively studied, and it serves as a model organism for all eukaryotes. In this chapter, we will focus on the responses to different stresses the yeast encounters from its desiccation for distribution until the final stages of vinification. Stress has been defined as any environmental factor which impairs yeast growth (Ivorra et al. 1999). Although yeasts are thus continuously exposed to different stress factors in wine production, its growth kinetics in must fermentations basically follows that of laboratory batch cultures: inoculated yeast first encounters high sugar concentrations (osmotic stress) and after an adaptive lag phase start growing. Sugars are converted to carbon dioxide and alcohol-generating ethanol stress. Vigorous fermentation produces a rise in temperature, i.e. a heat shock, which drops rapidly afterwards with cells going into cold shock. The latter extremes are usually avoided in modern vinifications by a tight temperature control. Upon the rise in ethanol concentration, yeasts enter the stationary phase and respiratory functions, which were down-regulated by high sugar concentrations in the earlier phases, and become derepressed, creating oxidative stress. Nutrient supplies, such as carbon and nitrogen sources, also become

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limited causing nutrient stress. In addition, sulphite is routinely used for microbiological containment and, together with other must components like heavy metal ions, imposes additional stress on the yeast cells.

All these stresses may trigger specific and overlapping cellular responses (see Table 16.1 for abbreviations and reference to subchapters): (1) The HOG pathway governs the production of glycerol as a compatible solute. (2) The CWI pathway controls enforcement of the cell surface, i.e. cell wall and plasma membrane remodelling. (3) The HSR pathway increases the production of heat shock proteins and regulates folding or degradation of protein aggregates. (4) The OSR pathway triggers production of proteins needed to protect the cells against oxidative stress. (5) The GSR pathway overlaps with the other stress responses, with trehalose synthesis as a key feature. All these pathways have been covered by a number of excellent reviews, which will be cited in the corresponding sections. For more details on the underlying experimental evidence, we refer the reader to the works cited, therein.

Table 16.1 Stress factors frequently encountered by wine yeast and signalling pathways involved in the cellular response

Stress	Fermentation stage	Response[a]	Section (Figure)
High osmolarity	First and middle stages of must fermentation	HOG	Sect. 16.2.1 (Fig. 16.1)
Low osmolarity	On grape skins, after rain; during production of dried yeast	CWI	Sect. 16.2.2 (Fig. 16.1)
High temperature	Early and middle stages if not controlled, dried yeast production	HSR, CWI	Sect. 16.2.3 (Fig. 16.2)
Oxidative stress	During production of dried yeast, later stages of vinification	OSR	Sect. 16.2.5 (Fig. 16.4)
Ethanol	Middle and late stages	HSR, OSR, GSR, CWI	Sect. 16.3.1
Sugar limitation	Late stages	GSR, TOR	Sect. 16.2.4 (Fig. 16.2)
Nitrogen depletion	Late stages; middle stages in some "natural" fermentations (FAN[b])	TOR, GSR	Sect. 16.3.2
Acetic acid	Later stages or contaminating microflora	GSR, CWI, HOG, TOR	Sect. 16.3.3
Sulphite	Early stages	–	Sect. 16.3.4
Low temperature	Controlled white and rosé wine fermentations	CSR, OSR, CWI	Sect. 16.3.5

[a]*CWI* cell wall integrity pathway; *GSR* general stress response pathway; *HOG* high osmolarity glycerol pathway; *OSR* oxidative stress response; *CSR* cold stress response; *HSR* heat shock response; *TOR* target of rapamycin signalling. See text for detailed descriptions

[b]*FAN* free amino nitrogen, i.e. ammonium and free amino acids present in the must; limitations except for the early stage of fermentations may occur if not supplemented by ammonium salts

16.2 Stress Response Signalling Pathways

In the following sections, we will first describe basic components of the major stress response signalling pathways operating in *S. cerevisiae* as listed in Table 16.1 and how they may be integrated. The effect of stresses more specific to vinification will be discussed in subsequent sections.

16.2.1 The High Osmolarity Glycerol (HOG) Pathway

The HOG signalling pathway is used by yeast to cope with hyperosmotic stress (Hohmann 2015). Such conditions are encountered in the initial phase of must fermentation, exerted by the high sugar concentrations (>200 g L^{-1}; Chap. 8). To counteract osmotic stress and concomitant cell shrinking, glycerol is produced as a compatible solute and accumulates within the cell. To this end, glycolysis is activated, and enzymes diverting the flux at the level of the triosephosphates towards glycerol are produced (see Chap. 8). Signal perception and the proper cellular response are ensured by the HOG pathway, as one of four so-called *m*itogen-*a*ctivated *p*rotein *k*inase (MAPK) pathways operating in yeast. Such pathways are conserved throughout eukaryotes, and defects are a major cause of human cancer. Their central module is composed of three consecutive protein kinases, designated as MAPKKK, MAPKK and MAPK (Fig. 16.1). Besides stimulating glycerol production, the HOG pathway also triggers gene expression in response to heat shock (Varela et al. 1992).

The HOG pathway as depicted in Fig. 16.1 commences with two branches of signal perception and generation, mediated by the membrane-spanning sensors Sln1 and Sho1. While high external osmolarity activates the Sho1 signalling complex, which also requires either Msb2 or Hkr1, it inhibits Sln1 signalling. Sho1 then interacts with a number of intracellular mediators, among them the protein kinase Ste20, which phosphorylates the MAPKKK Ste11. Thus activated, Ste11 phosphorylates the MAPKK Pbs2, enabling it to phosphorylate the MAPK Hog1. High osmolarity also increases Hog1 phosphorylation through the Sln1-mediated signalling branch, which is reminiscent of bacterial two-component systems. Thus, Sln1 has an extracellular sensor domain and a cytosolic histidine kinase/receiver domain. Once activated, it transfers the phosphogroup to Ypd1, which delivers the phosphate group to Ssk1 as a response regulator. Phosphorylated Ssk1 cannot activate a redundant pair of MAPKKKs, namely, Ssk2/Ssk22, operating in this branch. Since high sugar concentrations inhibit the sensor and prevent the phosphogroup transfer, the MAPKKKs and the downstream kinase cascade are active under such conditions.

Dually phosphorylated Hog1 acts on various processes. In the cytosol, it increases glycolytic flux yielding triosephosphates as precursors for glycerol production, by acting on phosphofructo-2-kinase, which produces an allosteric

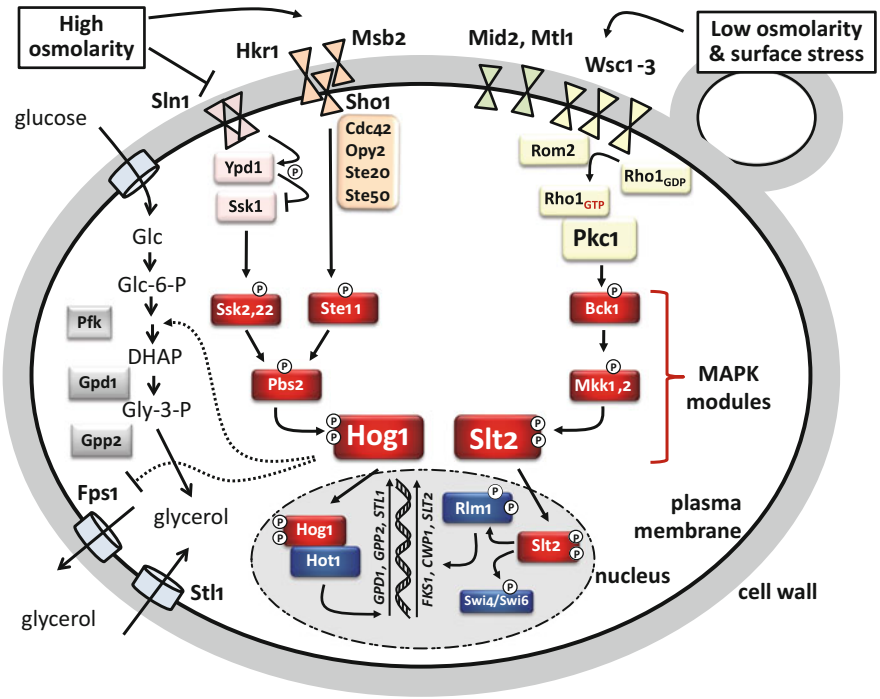

Fig. 16.1 High osmolarity glycerol (HOG, *left*) and cell wall integrity (CWI, *right*) pathway. *Arrows* indicate activation; *lines with bars* symbolize inhibition of the target proteins. Cytosolic targets regulated by Hog1 are connected by *dotted lines*. Phosphorylation of target proteins by ATP is symbolized by *circled "P"* attached to the protein. Direct transfer of phosphogroups in the two-component-like system of Sln1/Ypd1/Ssk1 is also indicated. Sensors are depicted as *hourglass-shaped symbols*, other proteins by *rectangular boxes*, with the central protein kinases shown in *red*, transcription factors in *blue* and metabolic enzymes in *grey*. *Other colours* refer to signal mediators. Membrane transporters are shown by *cylinders* with *arrows* indicating the direction of transport. Two branches of the HOG pathway mediated by Sho1 and Sln1, respectively, sense high medium osmolarity leading to activation of the MAPK Hog1. Hog1 activates target gene expression primarily by interacting with the transcription factor Hot1. Only some target genes are shown. Sensors of the CWI pathway activate the small GTPase Rho1 via interaction with Rom2. The sole yeast protein kinase C then mediates activation of the MAPK cascade culminating in the MAPK Slt2 (=Mpk1) which regulates the two transcription factors Rlm1 for cell wall synthesis, with some of the target genes shown, and Swi4/Swi6 (=SBF) for cell cycle control. *Pfk* heterooctameric yeast phosphofructo-1-kinase; *Gpd1* glycerol-3-phosphate dehydrogenase; *Gpp2* glycerol-3-phosphate phosphatase; *Fps1* glycerol channel; *Stl1* glycerol importer; *Glc* glucose; *Glc-6-P* glucose-6-phosphate; *DHAP* dihydroxyacetone phosphate; *Gly-3-P* glycerol-3-phosphate. See Chap. 8 for details on carbohydrate and glycerol metabolism

activator of the glycolytic phosphofructokinase. Hog1 also mediates degradation the glycerol channel Fps1, which is first closed and then endocytosed and degraded thus preventing glycerol export. The third regulation by Hog1 is exerted at the transcriptional level: Activated Hog1 enters the nucleus and induces target gene expression by interacting with various transcription factors, such as Hot1, Msn1,

Msn2, Msn4 and Sko1. Hog1 is also involved in transcription elongation of the target genes, which include those encoding enzymes of glycerol production (*GPD1*, *GPD2*) and import (*STL1*) as reviewed in Proft et al. (2006) and Saito and Posas (2012).

16.2.2 The Cell Wall Integrity (CWI) Pathway

The other extreme condition as compared to the hyperosmolarity at the beginning of fermentation is the low osmolarity encountered by wine yeast in washing steps in the preparation of dry yeast and in preparing the starter cultures. Such conditions would lead to a swelling and ultimately the disruption of the yeast cells, if not counteracted by the rigid yet flexible cell wall. The state of the latter and of the underlying plasma membrane is monitored by the CWI pathway depicted at the right in Fig. 16.1 (Levin 2011). Its central MAPK module consists of the MAPKKK Bck1, a dual pair of redundant MAPKKs, Mkk1 and Mkk2 and the MAPK Slt2 (=Mpk1). The activation of this module is triggered by phosphorylation of the MAPKKK by the sole yeast protein kinase C, which itself is activated by the small GTPase Rho1 in its GTP-bound state. The latter is acquired after activation by the GDP/GTP exchange factor Rom2, which is thought to interact with any of the five CWI sensors, Wsc1-Wsc3, Mid2 and Mtl1, upon cell surface stress. The signal perceived by these sensors is probably mechanical stress exerted either on the cell wall or the plasma membrane (Kock et al. 2015). The dually phosphorylated MAPK Slt2, like Hog1, operates both in the cytosol and in the nucleus. In the cytosol, it appears at the bud neck during cytokinesis, indicating a function in cell cycle regulation, and also at mitochondria under oxidative stress (Schmitz et al. 2015). Nuclear gene expression is controlled through activation of two transcription factors, Rlm1 and SBF, a dimer of Swi4 and Swi6. The former activates expression of genes whose products are involved in cell wall construction; the latter regulates cell cycle progression.

Interestingly, similar sensors and downstream-operating components have been described in other yeasts, e.g. in *Kluyveromyces lactis*, a close relative of the wine yeast *K. marxianus* (Rodicio and Heinisch 2013). In the light of increasing interest in non-*Saccharomyces* yeasts for improving wine quality, this indicates that CWI signalling is a common strategy to cope with cell surface and oxidative stress.

16.2.3 The Heat Shock Response (HSR) Pathway

Sudden temperature changes do not occur in modern wine production. Yet, throughout the history of winemaking and still today in smaller wineries, yeast metabolism during fermentation caused the temperature to rise well above 30 °C and to drop dramatically when fermentation ceased. *S. cerevisiae* and

non-*Saccharomyces* yeast dominating the first day of fermentation are also exposed to sudden temperature shifts while still on the grapes in the vineyard. Moreover, dry yeast used as starter cultures endure high temperatures in their preparation (Attfield 1997).

A major effect of higher temperatures on the cell is the increase in membrane fluidity and the unfolding and aggregation of cytosolic proteins. The HSR pathway triggers the production of heat shock proteins (Hsp's) which either help in refolding such proteins or target them to ubiquitin/proteasome-mediated degradation (Verghese et al. 2012). Thus, expression of >50 genes is increased after a shift to 38 °C, including 25 chaperones and co-chaperones of the HSP70/HSP90 protein family (Morano et al. 2012). Key players in this heat-induced signalling are depicted in Fig. 16.2. They include two types of transcription factors: the homotrimeric Hsf1 for specific heat shock responses recognizes heat shock response elements (HSE) in the promoter of its target genes. Some of these genes carry additional stress-responsive promoter elements (STRE) controlled by Msn2 or the redundant Msn4, the major transcription factors discussed in the general stress response, below (Sect. 16.2.4). Transcription activation by Hsf1 is inhibited by proteins of the Hsp70 family, of which there are 14 members in yeast. Upon heat shock, this inhibition is released, since the Hsp70 proteins are recruited to the Hsp104/Hsp40/Hsp70 complex required to regulate the levels of unfolded proteins. The complex either refolds such proteins, which tend to aggregate, to their functional conformation, or presents them for ubiquitinylation and degradation by proteasomes, to prevent cellular damage. The unfolded proteins are delivered to the trimeric complex by the intracellular heat shock sensors Hsp26 und Hsp42, which under heat stress form large oligomers to fulfil this function (Verghese et al. 2012).

Another important protein whose production is induced both by heat shock, and the GSR pathway discussed in the following section is Hsp12. In contrast to the Hsp family members described above, this protein is monomeric, associates with the plasma membrane, and does not function as a chaperone for other proteins but rather as a "lipid chaperone" required to maintain membrane integrity under various stress conditions. Besides revelation of its structure and its use as a cellular marker for industrial stress responses, e.g., desiccation stress, its exact physiological role is still unclear (Herbert et al. 2012).

16.2.4 The General Stress Response (GSR) Pathway

As already indicated by the overlapping functions of the Msn2/Msn4 transcription factors just described in the response to heat shock, many different stresses in yeast provoke a similar set of responses. The term "general stress response" (GSR; also called "environmental stress response," ESR) has been coined for this phenomenon. Exposure of yeast to sublethal doses of one stress condition, e.g. high temperature, not only confers protection to a later treatment with higher doses of the same stress

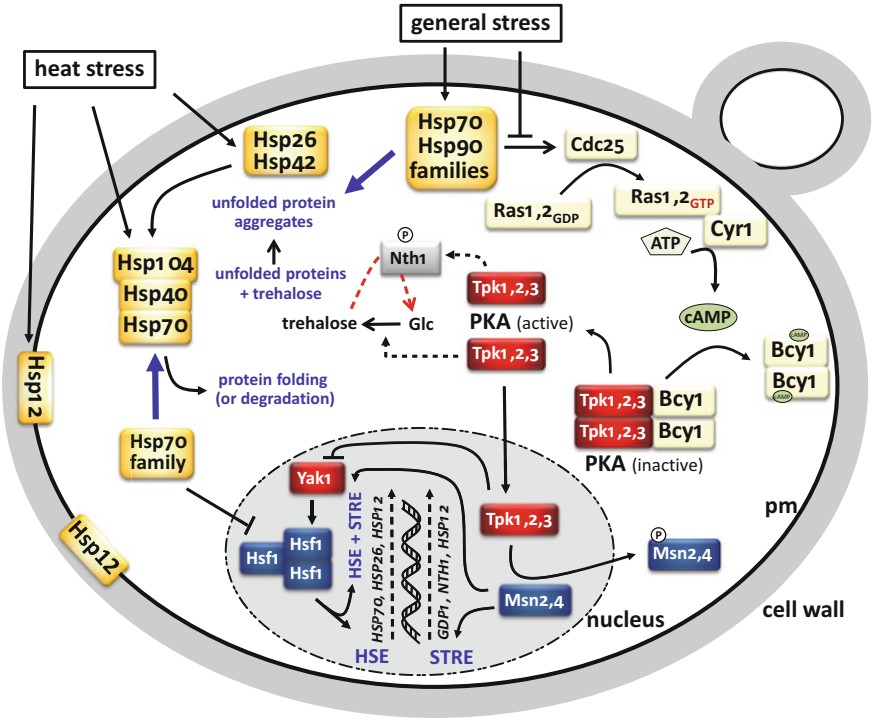

Fig. 16.2 Heat shock response (HSR, *left*) and general stress response (GSR, *right*). *Line* drawings for activation/inhibition and *colouring* of proteins are as in Fig. 16.1. Heat shock proteins are shown in *yellow boxes*. Members of the Hsp70 family inhibit the transcription factor Hsf1 under non-stress conditions. Heat shock leads to protein unfolding and titration of Hsp70 members, resulting in expression of Hsf1-regulated genes. In the general stress response, members of the Hsp70/Hsp90 family are also titrated by unfolded proteins, leading to inactivation of Cdc25 and finally of PKA, nuclear localization of the transcription factors Msn2 and Msn4 and thus expression of stress-responsive genes (see text for details). Only some of the target genes are shown. Trehalose metabolism is shown as one example for cytosolic targets of PKA, linking GSR to HSR (also see Fig. 16.3). *Glc* glucose; *cAMP* cyclic AMP; *pm* plasma membrane; *HSE* heat shock-responsive elements; *STRE* stress-responsive elements

but also against other stresses, such as high ethanol concentrations. This physiological effect is also known as "cross protection".

Control of gene expression in the GSR pathway depends on the presence of stress-responsive elements (STRE) in the promoters of target genes, recognized by Msn2 and Msn4. The latter co-activate gene expression in the heat shock response but are also targeted towards genes which are induced by osmotic and oxidative stress, nutrient limitation, presence of heavy metals and by DNA damage (Morano et al. 2012; Teixeira et al. 2011). Transcription activation confined to the nucleus is abolished by the export of Msn2/Msn4 into the cytosol. This translocation is

triggered by their phosphorylation by protein kinase A (PKA), whose regulation is depicted in the right half of Fig. 16.2. Under non-stress conditions, members of the Hsp70 and Hsp90 chaperone families stabilize the GDP/GTP exchange factor Cdc25, which converts Ras proteins, Ras1 or Ras2, into their active GTP-bound state. Active Ras then associates with and activates adenylate cyclase, which increases the levels of cAMP. The latter binds to the regulatory subunits, Bcy1, of the heterotetrameric, inactive PKA. These then dissociate from the catalytic subunits, which are constituted by one of the three redundant proteins Tpk1–Tpk3. The kinase subunits are inactive in the heterotetramer but become active when liberated from Bcy1. As one consequence, active PKA enters the nucleus and phosphorylates Msn2 and Msn4, causing their export into the cytosol. Target genes with STRE promoter elements are therefore not expressed. In parallel, PKA also phosphorylates the activating kinase Yak1 and thereby inhibits the heat shock transcription factor Hsf1.

Under various stress conditions, the Hsp70/Hsp90 proteins are required to maintain protein homeostasis, e.g. to prepare unfolded proteins for refolding or degradation (Fig. 16.2, *left side*). This leads to dissociation and degradation of Cdc25, resulting in a decrease in Ras-GTP and cAMP concentrations and reassociation of PKA into its inactive tetramer. Consequently, Msn2 and Msn4 in their dephosphorylated state remain in the nucleus and trigger expression of genes involved in the general stress response (Morano et al. 2012). Moreover, Msn2 and Msn4 also up-regulate the transcription of genes encoding heat shock proteins, oxidative stress detoxification enzymes and trehalose metabolism.

The disaccharide trehalose was viewed as a major stress protectant and has been implicated in conferring increased resistance to dehydration, freezing, heat and toxic compounds such as ethanol, oxygen radicals and heavy metals (Gancedo and Flores 2004). The metabolism of the reserve polysaccharide glycogen has also been implicated in increasing yeast cell viability in wine fermentations (Perez-Torrado et al. 2002). Since trehalose and glycogen both accumulate prior to entry into stationary phase and their functions are physiologically related, their metabolism is depicted in detail in Fig. 16.3 (Francois and Parrou 2001). A key role to provide the monomers for synthesis of both compounds is UDP-glucose. For glycogen synthesis, the first glucose molecules are covalently linked to the glycogenin enzymes Glg1 or Glg2. Glycogen synthase isozymes Gsy1 or Gsy2 then elongate the chains. They are allosterically activated by glucose-6-phosphate and inactivated by PKA-dependent phosphorylation. Branching activity to create alpha-1,6-linkages is provided by Glc3. For glycogen degradation, the glycogen phosphorylase Gph1 and the debranching enzyme Gdb1 liberate glucose-1-phosphate and glucose, respectively. Inversely to glycogen synthase, Gph1 is inhibited by glucose-6-phosphate and activated by PKA (Wilson et al. 2010).

Trehalose is synthesized by a multienzyme complex composed of two catalytic subunits, the trehalose-6-phosphate synthase Tps1 and the trehalose-6-phosphate phosphatase Tps2, and two regulatory subunits, Tps3 and Tsl1. Besides being synthesized by yeast, trehalose can also be imported from the medium *via* Agt1. Trehalose hydrolysis into two glucose molecules is catalyzed by either an acid

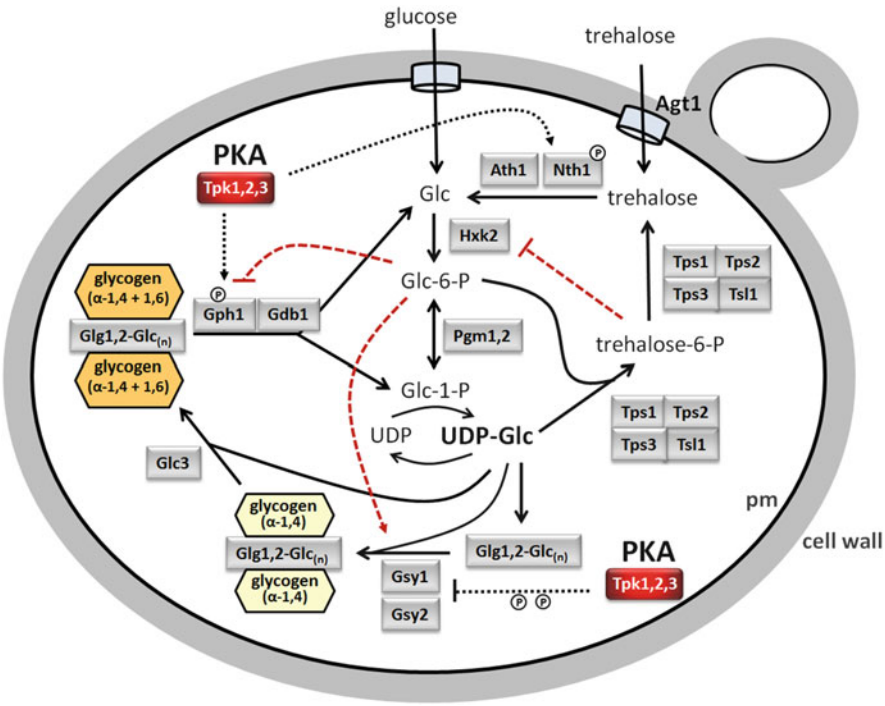

Fig. 16.3 Regulation of glycogen and trehalose metabolism. Protein colouring follows the rules explained in the legend of Fig. 16.1. *Hexagonal boxes* symbolize glycogen chains of different lengths and branching. *Black arrows* show interconversion of metabolites; *red dashed lines* indicate activation (*arrows*) or inhibition (*lines with bars*) of enzyme activities. A detailed description of reserve carbohydrate metabolism can be found in Francois and Parrou (2001)

isoform of trehalase, Ath1, or the neutral trehalase Nth1. Nth1 is phosphorylated and thus activated by PKA. Consistent with the kinetics of trehalose turnover in a laboratory strain, Nth1 activity is high in log-phase cells, decreases with the diauxic shift and remains low in stationary phase (Lillie and Pringle 1980). Similar kinetics for trehalose are observed during wine making, i.e. accumulation after nitrogen depletion and degradation in the initial growth phase (Novo et al. 2003).

Providing the connection to the heat shock response, trehalose is rapidly synthesized upon a sudden increase in temperature but is degraded soon after. This is consistent with the expression patterns of the genes encoding key enzymes of trehalose metabolism. Expression of all four genes for the biosynthetic subunits is induced under heat stress and repressed by the cAMP/PKA pathway, mediated by STRE sequences in their promoters (Fig. 16.2). Curiously, *NTH1* gene expression, which should increase trehalose degradation, is also induced. Nth1 is also activated post-translationally by PKA in response to external glucose availability (Alexandre et al. 2001). A model for the role of trehalose in stress protection may lend physiological significance to these findings (Fig. 16.2; Singer and Lindquist

1998): In a first reaction to stress, trehalose stabilizes protein structure and prevents aggregation of denatured proteins. In the next stage, heat shock proteins take over this function, and trehalose is degraded to avoid interference with this process. This also explains why trehalose confers resistance to a variety of stresses which affect protein folding, such as heat, cold and ethanol. In addition, trehalose stabilizes the plasma membrane upon exposure to these stresses. These conclusions may have to be revisited in the light of observations indicating that the enzyme Tps1, rather than trehalose itself, may mediate stress resistance (Petitjean et al. 2015).

16.2.5 The Oxidative Stress Response (OSR) Pathway

Yeast metabolism, especially respiration, produces reactive oxygen species (ROS) and radicals, which cause damage to proteins, lipids, carbohydrates and DNA and result in apoptotic cell death and ageing (Morano et al. 2012). In vinification, ROS are produced in the first stages of yeast preparation and propagation, as well as after prolonged exposure to ethanol in the presence of oxygen. ROS production is also triggered by heavy metals. In experimental laboratory setups, hydrogen peroxide, diamide or menadione are frequently used to apply oxidative stress.

The main components involved in OSR are shown in Fig. 16.4. Two types of transcription factors, Yap1 with five homologs in yeast, and Skn7 (=Pos9), regulate expression of genes encoding ROS detoxification enzymes. Their target genes are either regulated in concert or individually in response to different stresses. Yap1 function is regulated by its nuclear export sequence, which is only recognized by the nuclear exporter Crm1 if some key cysteine residues in its two cysteine-rich domains are reduced. In its oxidized state Yap1 enters the nucleus and activates transcription of target genes containing *y*east AP1-like *r*esponse *e*lements (YRE) in their promoters. Yap1 oxidation in the cytosol is catalyzed by the glutathione peroxidase-like enzyme Gpx3, which constitutes a sensor activated by hydrogen peroxide. Another sensor acting though Gpx3 in the same pathway is Ybp1.

The other transcription factor, Skn7, can also engage an oxidized conformation triggered by ROS and then binds to *o*xygen *s*tress *r*esponse *e*lements (OSRE) in the promoters of target genes (Fig. 16.4). This function is exerted by the non-phosphorylated, oxidized form of the transcription factor. On the other hand, Skn7 can also be regulated through the Sln1 branch of the HOG pathway under osmotic stress. In contrast to the inhibition of Ssk1 upon phosphotransfer from Ypd1 depicted in Fig. 16.1, the latter can also deliver its phosphate to the receiver domain of Skn7 and thereby trigger its binding to the promoters of other target genes. Rather than binding to OSRE, Skn7 then activates expression of genes involved in cell wall synthesis and cell cycle progression (Fassler and West 2011).

OSR pathway activation ultimately leads to production of at least 37 enzymes for detoxification, such as superoxide dismutase and catalase, as well as to the synthesis of protectants like glutathione, thioredoxin and glutaredoxin (see Morano et al. 2012, for lists of target genes). Oxidative stress also triggers a decrease in

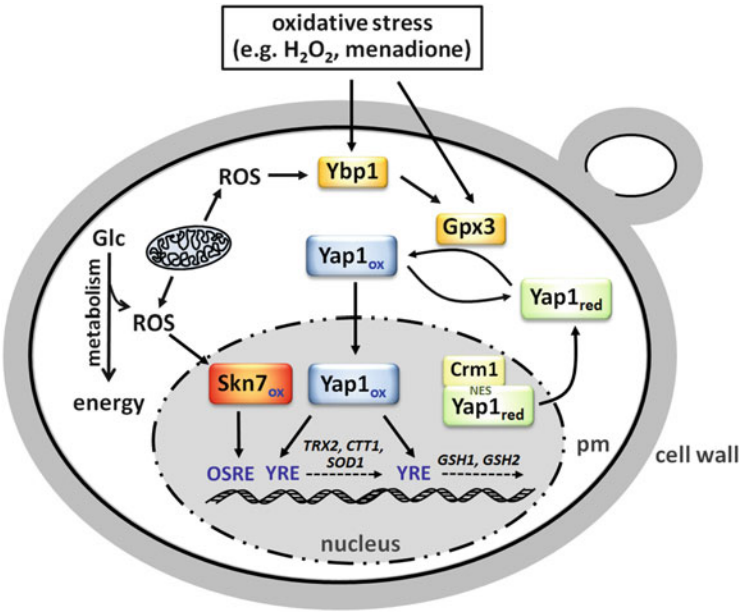

Fig. 16.4 Oxidative stress response (OSR). Reactive oxygen species (ROS) generated by respiratory metabolism or external agents trigger activation of two major transcription factors, Yap1 and Skn7. For Yap1 activation, the sensor proteins Ybp1 and Gpx3 may detect oxidative stress and mediate oxidation of the transcription factor. This favours a conformational change leading to its nuclear import and expression of target genes, some of which are depicted. In the absence of oxidative stress, Yap1 is reduced and exported into the cytosol mediated by Crm1. ROS can also lead to activation of Skn7 and co-activate expression of other target genes together with Yap1. *pm* plasma membrane; *Glc* glucose; *OSRE* oxidative stress-responsive element; *YRE* Yap1-responsive element; *NES* nuclear export sequence; *ox* oxidized; *red* reduced

glycolytic flux and an increase in the activity of the pentose phosphate pathway in order to provide the NADPH required in the detoxification reactions.

16.2.6 Crosstalk Between Different Stress Response Pathways

All stress responses share certain physiological hallmarks: They are transient, i.e. cells first increase production and/or activity of key proteins and then adapt their physiology, which permits them to decrease their concentrations. Stress also causes an initial slowdown in bulk protein synthesis attributed to the retention of bulk mRNAs in the nucleus, as well as a growth delay mediated by cell cycle control. These features are the result of coordination of different stress response pathways. Since literature on such crosstalk in *S. cerevisiae* is overwhelming, we

will only discuss a few examples, here. For a concise introduction on how different stresses may activate the same signal transduction pathways in yeast used in food industry, we recommend the review of Teixeira et al. (2011).

The response to heat shock provides a good example of how different pathways are addressed by the same external stress condition (Verghese et al. 2012). A sudden increase in temperature not only triggers the HSR pathway but also strongly affects CWI signalling to reinforce the strength of the yeast cell wall. Moreover, the general stress response is activated through the cAMP/PKA branch depicted in Fig. 16.2.

Likewise, the response to high and low medium osmolarity, mediated by the HOG and CWI pathways, also display an intimate crosstalk (Rodriguez-Pena et al. 2010). Thus, Ste11 regulates both Pbs2 and Mkk1/Mkk2 in the two pathways and Hog1 can activate the CWI transcription factor Rlm1 (Fig. 16.1).

As a final example, CWI signalling is also involved in the response of yeast cells to oxidative stress, with a crosstalk to the GSR and to TOR-mediated signalling, a pathway necessary for nutrient sensing as described below (Petkova et al. 2010).

16.3 Specific Physical and Chemical Stress Factors Encountered in Vinification

Yeast encounters a variety of vinification-specific stresses which may trigger either one or a combination of the response pathways described above but in addition elicit specific cellular responses. These will be discussed in the remaining part of this chapter.

16.3.1 Ethanol Stress

The most obvious stress encountered by yeast during must fermentations is a self-made one: ethanol concentrations rise well above 10% v/v upon conversion of the sugars. The alcohol affects plasma membrane integrity and permeability, especially for protons, and the activity of intracellular proteins, including glycolytic enzymes (Stanley et al. 2010). Many of the stress response pathways described above are activated in this process, in addition to specific physiological adjustments:

(1) Inhibition of cell growth and viability, with a general reduction in mRNA and protein synthesis. This is also accompanied by an initial delay in cell cycle progression, yielding larger cells.
(2) Induction of HSR is probably related to defective protein folding caused by ethanol, which also affects cell wall integrity.
(3) Induction of GSR, trehalose and glycogen accumulation.

(4) Alterations in vacuole morphology, i.e. a single huge vacuole is formed in ethanol-stressed cells.
(5) Inhibition of endocytosis and intracellular transport processes.

Two proteins, Ars1 and Rat8, accumulate in the nucleus after exposure to high ethanol concentrations and have been suggested to function in a specific ethanol response pathway (reviewed in Stanley et al. 2010). Since no recent literature is available on the subject, the exact structure of this pathway remains to be elucidated.

The investigation of several *S. cerevisiae* strains selected for increased ethanol tolerance, a trait desired in wine yeast, showed that it is not mediated by a single gene but rather involves a complex network of genetic interactions. For instance, ethanol tolerance has been associated with increased ATPase activity and higher levels of oleic acid and ergosterol in the plasma membrane (Aguilera et al. 2006). A relation to amino acid metabolism has also been suggested, first due to an increased ethanol tolerance after addition or intracellular accumulation of tryptophan (Stanley et al. 2010). Likewise, a protective effect of high arginine concentrations against ethanol was observed and attributed to effects on cell wall and plasma membrane integrity, underlining the necessity for a detailed knowledge of the stress response pathways discussed above (Cheng et al. 2016). In the latter work, ethanol tolerance was conferred by deletions in the arginase encoding gene *CAR1*, which has been previously employed to reduce the content of cancerogenous ethyl carbamate contributed by yeast metabolism in spirit production (Schehl et al. 2007).

At the genome level, a variety of transcriptome studies have yielded some conflicting results concerning ethanol effects (Stanley et al. 2010). Nevertheless, expression of genes encoding glycolytic and mitochondrial enzymes involved in energy production and heat shock proteins is consistently induced by ethanol stress. Likewise, transcription of some genes involved in trehalose synthesis, glycerol production, cell wall synthesis and detoxification enzymes for ROS is increased. The latter underlines the frequently observed relationship between ethanol and oxidative stress. On the other hand, genes necessary for cell growth and protein synthesis are generally down-regulated.

Other cellular responses strongly depend on the specific ethanol stress conditions applied prior to RNA preparations for transcriptome analyses. Several studies have been performed within 1 h after addition of ethanol, which yields hundreds of genes changing their expression level. Few studies have been performed under continued ethanol stress, i.e. for more than 3 h and up to 15 days, which is closer to what yeast encounters in wine making (Zuzuarregui et al. 2006; Marks et al. 2008). In these studies, it was found that:

(1) In contrast to sudden ethanol addition, accumulation of glycerol is not observed in long-term fermentations.
(2) Only Hsp104 and Hsp12 of a number of heat shock proteins primarily induced contribute to the response to longer ethanol exposures.
(3) Genes for mitochondrial metabolism show a permanently increased expression.

(4) Entry into stationary phase is caused by ethanol concentrations above 2% v/v, rather than by nitrogen limitation.
(5) Hints to a novel fermentation stress response (FSR) pathway obtained in these studies still need to be verified.

16.3.2 Nutrient Limitations

Sugars and the available nitrogen sources are the two major nutrients which determine the fate of must fermentations. Although not depleted until the very end of fermentation, sugar limitations trigger cAMP/PKA-dependent responses of the GSR pathway (Sect. 15.2.4). More frequently, nitrogen becomes limiting at concentrations lower than 140 mg L^{-1} and causes stuck fermentations. Stress responses provoked by nutrient limitations are mediated by TOR signalling (*T*arget *o*f *R*apamycin), involving the TORC1 and TORC2 complexes (Jacinto and Lorberg 2008). They control cell growth, i.e. the cell volume, rather than cell division and are connected to the other signalling pathways described above. Thus, targets of TOR have been identified in the cAMP/PKA pathway-dependent control of ribosome biogenesis and trehalose metabolism. Hog1 and Sch9, the latter involved in cAMP/PKA-independent glucose signalling (Chap. 8), cell cycle control through Rim15 and Pkc1-mediated CWI signalling are also intertwined with the TOR complexes. For example, the glucose-repressible protein kinase Rim15, known to establish stationary phase in laboratory strains, drives the efficient utilization of nitrogen sources and the production of glycerol in wine yeast (Kessi-Perez et al. 2016).

16.3.3 Acid Stress

Besides ethanol, yeast sugar and amino acid metabolism yield organic acids, of which acetic acid is the most prominent (Chap. 8). Together with the increased permeability of the plasma membrane to protons at higher ethanol concentrations, cytosolic acidification may cause protein unfolding and apoptotic cell death, which can be counteracted by the increase in vacuolar ATPase activity observed under such conditions. While vATPase also protects against ethanol-induced cell wall stress and ROS generation, the exact molecular mechanism is not clear, given that vATPase mutants do not display a higher cytosolic acidity as compared to wild-type cells (Charoenbhakdi et al. 2016). In transcriptome studies, low external pH induces the expression of genes encoding cell wall proteins, such as *CWP1*, enzymes involved in glycerol and trehalose synthesis, e.g. *GPD1*, *GPP2*, *TPS1* and *TSC1*, and in other stress responses, like *CTT1* or *HSP12* (Kapteyn et al. 2001). Regarding the underlying signalling pathway, Whi2 has recently been found to mediate acetic acid resistance upon its overproduction, correlating with an increased expression of

the encoding wild-type gene under acid stress (Chen et al. 2016). Although Whi2 has also been related to the GSR response probably interacting with Msn2, the detailed signalling pathway is not yet known.

As expected for a general stress affecting the plasma membrane, external low pH also activates both CWI and HOG1 signalling (de Lucena et al. 2015). Moreover, sphingolipid biosynthesis induced by the TORC2-Ypk1 pathway was recently found to contribute to the adaptation to acetic acid stress, also indicating that enforcement of the yeasts cell surface is an important way to cope with such adverse conditions (Guerreiro et al. 2016).

16.3.4 Sulphite Resistance

Sulphite is frequently added in vinification for microbiological containment, since the desired *S. cerevisiae* is one of the least sensitive microbes found in must. Sulphite exerts its toxic effects by reacting with various compounds, especially with carbonyl groups of cellular constituents (Divol et al. 2012). Accordingly, one of the cells' resistance strategies to cope with sulphite stress is the production of acetaldehyde as a scavenger. In addition, sulphite toxicity can further be reduced by diverting its flow towards the synthesis of the sulfur-containing amino acids methionine and cysteine. In fact, inhibition of methionine synthesis as a response to its external supply results in increased sensitivity towards sulphite. In contrast, deregulation of adenine metabolism leads to an increased tolerance (Aranda et al. 2006).

Resistance to sulphite has also been selected for by more than a hundred years of its use in wine making. Selective pressure thus applied resulted in a specific translocation between chromosomes 8 and 16, which placed a strong promoter in front of the *SSU1* gene. This gene encodes a pump located for sulphite excretion in the yeasts plasma membrane and is highly expressed at its new location by the positive transcription factor Fzf1, in contrast to its expression under its native promoter, which only allows a lower level expression (Perez-Ortin et al. 2002). An extensive study of the sulphite response in several wine yeast strains of *S. cerevisiae* attributed resistance to different mechanisms, with a general increase in basal transcription of many genes, rather than of a few specific ones (Nadai et al. 2016). Interestingly, again enforcement of the yeast cell wall and synthesis of membrane lipids were found among the primary adaptive responses.

16.3.5 Cold Stress

White wine fermentations at lower temperatures of 10–15 °C are of growing interest to improve aroma profiles. *S. cerevisiae* reacts to such conditions by a cold stress response (CSR), which counteracts the increased rigidity of the DNA

double strand, of RNA secondary structures impairing transcription and translation, the diminished fluidity of cell membranes interfering with transport and the denaturation of proteins due to reduced folding kinetics (Aguilera et al. 2007). The yeast reaction to low temperatures overlaps substantially with the OSR described in Sect. 16.2.5, since ROS generation and its counteraction by protective mechanisms are one of the hallmarks of the cold stress response. Moreover, the latter is related to an increase in sulfur assimilation, attributed to the fact that protective molecules such as glutaredoxin, glutathione and thioredoxin all contain sulfur and that methionine itself serves as a ROS scavenger (Garcia-Rios et al. 2016). The relation of CSR to oxidative stress response was also confirmed in transcriptome analyses in a wine fermentation at low temperature, in which transcriptional regulation by Rim15-Gis1 appeared to also affect nitrogen, sulfur and copper metabolism, as well as cell wall synthesis (Deed et al. 2015).

16.4 Emerging Issues

Since the last edition of this chapter in 2009, we have come a long way regarding our knowledge of the response of *S. cerevisiae* to different stress situations. Given the ever more affordable genome sequencing techniques, new discoveries lie ahead. The discovery that nitrogen limitations can be overcome by natural horizontal transfer of genes encoding oligopeptide transporters, which increases the spectrum of nitrogen sources available to *S. cerevisiae* and provides a selective advantage for the respective strains, is just one example of how new strategies to cope with certain stress situations act at the genome level (Marsit et al. 2016). Moreover, for polygenic traits like ethanol tolerance, quantitative trait analyses (QTL) are starting to produce results especially interesting for vinification yeast strains. The combination of genome-transcriptome-proteome analyses will also allow the study not only of *S. cerevisiae* itself, but also in its context of the "wine microbiome." Thus, interactions with the non-*Saccharomyces* yeast and the lactic acid bacterial flora have not yet drawn much attention, but will greatly aid in our understanding of what microbiological stresses may be encountered by yeast starter cultures and how they may either be avoided or exploited to improve wine quality (Ciani et al. 2016). One such interaction, the prevalence of *S. cerevisiae* in the course of fermentations, is a renewed matter of debate. While it is generally claimed that the production of and the high tolerance towards high ethanol concentrations causes its predominance, recent results indicate that *S. cerevisiae* may rather actively produce antimicrobial peptides to control the competitive microflora (Branco et al. 2016). If and how this constitutes a stress to the producing cell and if other non-*Saccharomyces* wine yeast produce similar compounds will be an exciting issue in future investigations.

Finally, what we learn from the studies on ethanol-tolerant wine yeast strains may well have applications to the growing interest in employing *S. cerevisiae* for the production of bioethanol and next-generation biofuels such as butanol

(Caspeta et al. 2015) and thus serve mankind far beyond the formidable task of producing high-quality wines.

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Chapter 17
Physical and Chemical Stress Factors in Lactic Acid Bacteria

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17.1 Introduction

The parameters influencing the malolactic fermentation (MLF) in wine are multiple. The winemaking process methods can be critical, but the main cause of delay appears to be the physical and chemical factors especially ethanol content, acidic pH, temperature, sulfites, or compounds present in the wine. Indeed, the cumulative effect of these multiple stresses increases the limitation for bacterial growth. However, several adaptative mechanisms at the genetical and physiological levels are implied for bacterial development and adaptation in wine. Some of them are discussed in this chapter.

Malolactic fermentation (MLF) takes place after alcoholic fermentation (AF) with a delay more or less long according to the (i) winemaking conditions, (ii) bacterial concentration, and (iii) physiochemical properties of the wine. This fermentation plays a key role in determining the final quality of most wines and contributing to the microbiological stabilization of the final wine. The consumption of residual sugars and L-malic acid makes it possible to inhibit any microbial activity (Nedovic et al. 2000). MLF is carried out by lactic acid bacteria (LAB) and most commonly by *Oenococcus oeni*. This bacterial specie is indigenous to wine, acidophilic, and is generally thought to be best suited to the harsh environment of wine (Sumby et al. 2014). The presence of ethanol, sulfites, and low pH results in this bacterium selection during AF (Britz and Tracey 1990). Moreover,

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O. oeni strains are known to have desirable flavor effects and produce different profiles of sensory compounds (Versari et al. 1999; Malherbe et al. 2012; Lasik 2013). *Lactobacilli* and more particularly *L. plantarum* can also carry out MLF and can produce desirable sensory attributes in red wines (Lerm et al. 2011; Bravo-Ferrada et al. 2013).

Consumption rate of the L-malic acid and the duration of MLF depend (i) on the malolactic activity of the lactic acid bacteria which is strain specific and (ii) the biomass concentration. This fermentation starts when LAB population reached about 10^6 UFC/mL, and its duration is more or less long, 5 days to 3 weeks according to the physical and chemical factors (Lonvaud-Funel 1995; Alexandre et al. 2004). Wine is a stressful environment for LAB, where their growth is particularly impaired. LAB and particularly *O. oeni* have to use several mechanisms to cope with these environmental stresses.

17.2 Parameters Influencing Malolactic Fermentation and Limiting the Growth of Lactic Acid Bacteria in Wine

A variety of factors affect the growth of LAB or their metabolic properties and consequently the timely completion of MLF. The four main parameters inducing stress and affecting MLF are ethanol (high concentrations, e.g., 12–16% v/v), low pH (<3.5), sulfites or sulfur dioxide (SO_2) concentrations (over 10 mg/L), and low temperature (Betteridge et al. 2015). Other factors such as enological practices and microbiological factors (yeast metabolites) and finally presence of chemicals inhibitors (e.g., pesticides residues and phenolic acids) can also affect bacterial growth and malolactic activity.

17.2.1 Enological Practices

Winemaking methods are the first factors that influence bacterial development and the progress of MLF. Indeed, several enological practices eliminate a part of nutriments (like nitrogen sources, e.g., amino acids) and suspended particles favorable to the growth of LAB. For example, the clarification process is unfavorable for the development of lactic acid bacteria. This process eliminates a part of the native microflore that is afterward responsible for spontaneous triggering off of MLF. Moreover, it reduces the quantity of nutriments necessary for the processing of AF and MLF.

At the end of AF, decanting (racking) also has an impact on MLF. This process eliminates yeast dregs which autolysis, thus depriving the bacteria of the growth factors (mannoproteins, vitamins), which can be liberated during this stage.

Addition of sulfite at the end of AF, in order to limit wine oxidation, is not advised. Due to its antimicrobial activity (Chang et al. 1997), sulfur dioxide (SO_2) keeps the LAB populations at very low levels and/or induces a decrease of bacterial population resulting a late starting of MLF (Andorra et al. 2008). The effect of SO_2 will be developed in the next part of the review.

Finally, usage of stainless steel tanks instead of wood barrels reduces natural developing of the wine during the winemaking process. However, recently a new method to control MLF in wine was developed using bacterial biofilm. *O. oeni* is able to form biofilms on winemaking material such as stainless steel and oak chips. This competitive mode of life in wine conferred to *O. oeni* an (i) increased tolerance to wine stress and (ii) functional performance with effective malolactic activities (Bastard et al. 2016).

17.2.2 Microbiological Factors

Winemaking is based on complex microbial collaboration. In musts and wines, *O. oeni* coexists with several other microorganisms (LAB and yeasts) and bacteriophages. Advances on molecular biology have provided new opportunities to study the evolution of the complex microbial communities along the winemaking (Borneman et al. 2012; Claisse and Lonvaud-Funel 2014; Campbell-Sills et al. 2015).

A potential cause of stuck or sluggish MLF is the fastidious nutritional requirements of malolactic bacteria whose growth typically depends on the availability of nutrients (Sumby et al. 2014). Firstly, wine is a poor environment from a nutritional point of view, and the nutritional composition of wine can vary. It depends on the grape variety, berry maturity, yeast strain, and winemaking conditions (Alexandre et al. 2004). Even though wine contains the necessary elements for growth, difficulties in development can appear. During AF and MLF, LAB in general and *O. oeni* in particular are in competition with other microorganisms and more specifically yeasts for the usage of the wine nutriments. Indeed, yeasts make the environment poorer in carbon and nitrogenous sources including amino acids, which is deleterious for LAB strains which require carbon and nitrogenous sources as well as remaining mineral elements for their growth. At the end of AF, residual sugars, essentially fructose, glucose, and pentose, are the principal sources of carbon and energy. Their concentration is variable according to the wine (10–0.1 g/L). This low concentration is nevertheless sufficient for bacterial growth (Henick-Kling 1995). It is the availability in nitrogenous sources (amino acids, peptides, and proteins) that is generally limiting (Alexandre et al. 2004; Remize et al. 2005). LAB and particularly *O. oeni* are auxotroph for a lot of amino acids (Garvie 1967). This deficiency phenomenon is compensated at the end of AF by the autolysis of the yeast, which allows the peptides, amino acids, and mannoproteins to be released in large quantities. This phenomenon stimulates the growth of LAB and malolactic activity (Alexandre et al. 2004; Comitini et al. 2005).Yeast/bacteria

interaction is complex, firstly opposing then synergic, and is still relatively unknown (Alexandre et al. 2004; Guilloux-Benatier et al. 1998). *O. oeni* is equally able to synthesize and to secrete (i) exoprotease which induces yeast lyses and allows for the liberating of the nitrogenous nutriments necessary for its growth (Guilloux-Benatier et al. 2000) and (ii) proteases and aminopeptidases that can hydrolyze macromolecules during autolysis (Farias and Manca de Nadra 2000; Manca de Nadra et al. 1997, 1999; Rollan et al. 1998). Protease activities seem to be dependent on stress conditions and nutritional deficiency; an increase is observed during stress conditions (Remize et al. 2005; Rollan et al. 1998).

Moreover, yeasts produce inhibiting factors against LAB during AF, such as ethanol, SO_2, medium-chain fatty acids, and antimicrobial peptides, which can affect MLF (Guilloux-Benatier et al. 1998; Alexandre et al. 2004; Comitini et al. 2005; Osborne et al. 2006). The effects of fatty acids are strongly dependent on the concentration and types of fatty acid present. For example, the presence of decanoic and dodecanoic acids in low concentration (respectively, 12.5 and 2.5 mg/L) stimulates the malolactic activity and growth, whereas high concentration of these fatty acids has an inverse effect (Capucho and San Romao 1994; Lonvaud-Funel et al. 1988). The toxic strength of fatty acids may be enhanced by the physicochemical conditions of the wine such as the pH or the presence of ethanol (Capucho and San Romao 1994), inhibiting, for example, the ATPase activity which is essential for the mechanism of MLF (Carrete et al. 2002). However, oleic acid has an important effect on the growth of *O. oeni*, since its assimilation acts as a survival or growth factor, according to the *O. oeni* strain used (Guerrini et al. 2002).

Some studies have investigated the production of antimicrobial peptides by strains of *S. cerevisiae*. However, the production of antimicrobial peptides seems to be strain dependent. For example, during AF in a synthetic grape juice, *S. cerevisiae* strain RUBY.ferm inhibits the growth of *O. oeni* and the MLF, while the yeast strain EC1118 do not despite the production of the same amount of total SO_2 (Osborne and Edwards 2007). This inhibiting molecule was revealed by SDS PAGE analysis as a peptide approximately 5.9 kDa. In a similar manner, Nehme et al. (2010) demonstrated that *S. cerevisiae* BDX is able to produce a peptidic fraction (MW between 5 and 10 kDa) responsible for the strong inhibition of *O. oeni* Vitilactic F during sequential fermentations (AF followed by MLF). In natural winemaking conditions, these yeast proteinaceous compounds are able to inhibit the L-malic consumption by directly targeting the malolactic enzyme activity (Rizk et al. 2016). For more information, see Chap. 19.

This phenomenon of competition also exists between the different species of LAB. During the winemaking process, antagonist effects between bacterial species including *Pediococcus*, *Lactobacillus*, *Leuconostoc*, and *Oenococcus* can be found (Lonvaud-Funel and Joyeux 1993). These effects are probably due to the liberation of components with antimicrobial properties such as bacteriocins (Yurdugül and Bozoglu 2002; Knoll et al. 2008). Indeed, some species of *Lactobacillus* and *Pediococcus* are able to produce bacteriocins (plantaricin and pediocin) that have been shown to successfully kill *O. oeni* cells (Nel et al. 2002). Moreover,

Rojo-Bezares et al. (2007) proposed to use reduced sulfur dioxide concentration in combination with bacteriocin in order to control bacterial population during winemaking. Although the use of bacteriocins to control LAB in wine has great potential, its use has not yet been approved in winemaking (Bartowsky 2009).

As for during the fermentation of dairy products, phage represent a threat for the MLF process since *O. oeni* strains can be infected by phage (Henick-Kling et al. 1986; Kot et al. 2014). Even if the role of phage has often been neglected, they could be successfully isolated from wines with sluggish or stuck MLF (Davis et al. 1985; Henick-Kling et al. 1986; Jaomanjaka et al. 2013). However, the sensitivity of *O. oeni* strains to phage is very variable and is now well studied for the production of malolactic starters (Jaomanjaka et al. 2013).

17.2.3 Physical and Chemical Factors

Wine is a complex environment, and its physical and chemical characteristics vary according to numerous conditions: vine variety, climatic conditions, and winemaking conditions. These physicochemical properties do not correspond to the optimum conditions for the growth of LAB and are, thus, stressful. These multiple stresses therefore have a major impact on the progress of MLF.

Ethanol Content Ethanol, produced by yeast during AF, is considered as being one of the main factors, which inhibits the growth of LAB in wine. The final ethanol content is very variable according to the wine (10–16% v/v). The tolerance of LAB species to ethanol is strain dependent (Henick-Kling 1995). *O. oeni* can tolerate content attaining 14% (v/v), and that small quantities of ethanol (3–7% v/v) can stimulate their growth (Alegria et al. 2004; Britz and Tracey 1990). Resistance to ethanol also diminishes when environmental pH is low and when temperature increases. However, with concentrate higher than 8% (v/v), ethanol is responsible for the inhibiting, or even bacterial death, of *O. oeni* strains (Capucho and San Romao 1994; Teixeira et al. 2002). Ethanol affects the latency phase and growth rate of LAB in wine. Indeed, ethanol presence induces an increase in the *O. oeni* membrane fluidity (Da Silveira et al. 2002, 2003; Chu-Ky et al. 2005; Maitre et al. 2014). The membrane then becomes permeable to many solutions and produces a loss of intracellular material such as cofactors (NAD^+/NADH and AMP) and ions (Da Silveira et al. 2002). The composition of cell membrane is equally dependent on ethanol presence (Teixeira et al. 2002). Cells of *O. oeni* modify the composition in fatty acids of its membrane during culture in the presence of ethanol: (i) the proportion of cyclic fatty acids increases (Teixeira et al. 2002; Grandvalet et al. 2008), and (ii) the membrane protein/phospholipid ratio increases in order to limit the effect of ethanol on lipids (Da Silveira et al. 2003). Exposure to ethanol can equally induce a dissipation of the membrane electrochemical gradient (Da Silveira et al. 2002, 2004). An influx of protons can then occur which will affect the cell processes dependent on the pH gradient such as ATP synthesis, transportation of

amino acids, and L-malate. Ethanol also has an impact on enzymatic activities. The malolactic activity is notably modified according to the ethanol concentrate. When the concentrate in ethanol is higher than 12% (v/v), the malolactic activity can be inhibited (Capucho and San Romao 1994). Nevertheless, this inhibiting phenomenon is less pronounced when ethanol is produced in a progressive manner. In these cases, LAB can adapt itself progressively to the ethanol presence, triggering the "adaptive response" (Weber and de Bont 1996; van de Guchte et al. 2002). Indeed, to counteract the effect of the ethanol, the cells use several mechanisms, such as the recruitment of stress protein interacting with the membrane (Maitre et al. 2012). Ethanol toxicity is generally attributed to the insertion of this molecule into the hydrophobic part of the membrane lipid double layer (Weber and de Bont 1996). There is then destabilization of the membrane structure which afterward affects several cell processes such as DNA replication, enzymatic activities, metabolites transport, and peptidoglycan synthesis (Jones 1989; Weber and de Bont 1996). Membrane permeability is modified and it no longer plays its barrier role. Membrane polarity is increased and can favor the passage of other polar molecules through the membrane. Recently, omics approaches such as transcriptomic coupled with proteomic allowed to study the global ethanol stress response in *O. oeni* (Olguin et al. 2015), which is mainly focused on the control of envelope composition (membrane, cell wall, and EPS) but also concerns general stress proteins such as chaperones and proteases (Costantini et al. 2015).

Ethanol has therefore an important impact on the physiology of the cells because its presence generates important modifications that are the basis of the adaptation of the cells to this stress. Due to these effects on the cell, this component influences MLF development, by notably modifying the latency time between AF and the beginning of MLF necessary for the adaptation of the cells.

Acidity Low pH (<3.5) is another factor which has a high influence on the development of LAB in wine. Most of LAB species are neutrophilic, with an optimum pH growth close to neutrality (Hutkins and Nannen 1993). Some genus such as *Lactobacillus* and *Oenococcus* show more acidophilic behavior. However, during winemaking, the average pH is low (pH between 3.0 and 3.8); the bacterial growth rate is therefore longer, increasing the latency phase between AF and MLF. When pH values are lower than 3.0, bacterial growth is very difficult or impossible according to the other physical and chemical factors (Lonvaud-Funel 1995).

Acidity creates major damages at cell level, with especially protein denaturation (Molina-Gutierrez et al. 2002). In fact, low pH modifies the survival of bacteria and can induce a slowdown and/or a stop of their growth. Metabolic changes linked to acid stress are therefore numerous and complex. The main effect of wine pH is to generate a decrease of the intracellular pH (pH_{int}), which is a critical factor for controlling cell processes such as enzymatic activity including malolactic activity, ATP and ARN synthesis, and protein and DNA replication (Britz and Tracey 1990; Belguendouz et al. 1997; Molina-Gutierrez et al. 2002; Carrete et al. 2002). Once the lower limit of pH_{int} is reached, cell damages are major, and enzymatic activities can no longer take place and induce bacterial death (Hutkins and Nannen 1993).

In addition to modifying the pH_{int}, extracellular pH (pH_{ex}) plays an important role in sugar metabolism (Henick-Kling 1995). An optimal pH level exists for sugar assimilation. This pH level corresponds to the lower pH from which sugar is used. For example, at pH 3.0, glucose is practically no longer metabolized, while L-malic acid is transformed into L-lactic acid and carbon dioxide. Moreover, transport of L-malic acid is regulated according to the pH_{ex}. The diffusion of L-malic acid in its non-dissociated form increases at low pH. There is thus a modification in metabolism according to the wine pH.

Acidity can also impair proteins by modifying their ionic interactions thus causing aggregation and denaturation (van de Guchte et al. 2002; Cotter and Hill 2003). DNA structure damage, notably to its topology, was also observed (Drlica 1992). Intracellular acidification is the origin of a DNA rolling decrease and generates DNA depurination and depyrimidination (van de Guchte et al. 2002). These modifications result in affecting the expression of several genes and induce a DNA repair system (Hartke et al. 1996; van de Guchte et al. 2002).

Finally, cytoplasmic membrane is the first target of acidity. Acidity generates large modifications in the composition and fluidity of this membrane. At low pH, the barrier role of the membrane is altered, permeases no longer function correctly, molecules can be freely distributed throughout the cell, and exchanges with the extracellular environment are then disrupted.

In conclusion, pH is an essential factor in wine; it influences the survival and the development of lactic acid bacteria by intervening at different levels: modification of the growth rate and pH_{int} and total membrane change.

Temperature Temperature has an important role in the final quality of wine (Reguant et al. 2005b). It modifies directly the growth of all microorganisms (yeasts and bacteria). Indeed, all bacteria show growth optimum temperatures. The majority of LAB being mesophilic (van de Guchte et al. 2002), its optimum growth is between 25 and 30 °C in laboratory culture. In wine the optimum temperature of growth is different to that obtained in a laboratory. The ideal temperature for the growth of *O. oeni* in wine and consumption of L-malic acid is between 20 and 25 °C, according to the strain tested (Britz and Tracey 1990). This value is modified according to the physical and chemical parameters and notably ethanol content. Indeed, the optimum growth temperature is decreased in wine containing high content of ethanol.

The average temperature at which MLF is carried out in the cellars is between 18 and 22 °C. These conditions are therefore favorable for the growth of *O. oeni*. However, in certain cases the temperature is <18 °C, bacterial growth is then slower, enzymatic activities are slowed down, and MLF starts late. At 15 °C, MLF is very slow and growth is almost impossible (Britz and Tracey 1990).

Low temperature has an impact on the molecules and notably on transcription, ARNm translation, and DNA replication (van de Guchte et al. 2002). In fact, cold temperatures induce the forming of secondary structures in the ARNm which slow down translation (Sanders et al. 1999). Temperature changes can equally induce

negative DNA rolling and modify the topoisomerase activities and DNA gyrase (Drlica 1992; Abee and Wouters 1999).

Finally, as for ethanol and pH, temperature has an important effect on membrane fluidity. However, few studies were carried out in order to measure the impact of low temperatures on *O. oeni* physiology. Chu-Ky et al. (2005) have demonstrated that cold shocks (14 and 8 °C) strongly rigidified cytoplasmic membrane but did not affect cell survival. To maintain optimal fluidity, *O. oeni* cells regulate the lipid composition in their cell membranes, and this ability represents a stress-tolerance mechanism (Tourdot-Marechal et al. 2000). These authors suggested that the cold-induced rigid membrane prevented an increase in permeability induced by ethanol in wine, diminishing passive proton influx and loss of intracellular materials. More recently, Zhang et al. (2013) have demonstrated that unsaturated fatty acid/saturated fatty acid (UFA/SFA) ratio was not modified after a cold shock (4 and 15 °C), but the cold shock induced a slight increase in the proportion of cyclopropane fatty acids (CFA). However, CFA contribution to membrane properties is not yet completely understood, especially concerning the modifications of membrane fluidity in response to environmental stress.

Temperature has an important impact on MLF. However, this parameter is much easier to control compared to pH and/or ethanol content.

Sulfites or Sulfur Dioxide Sulfur dioxide (SO_2) is another factor which plays an essential role in the growth of *O. oeni* and can cause a sluggish or stuck MLF (Reguant et al. 2005a). This component, found in wine with variable concentrate, has two origins: (i) an exogenous origin and (ii) an endogenous origin.

The exogenous SO_2 originates from sulfuring during winemaking. This method consists of adding sulfur anhydrous in salt (sodium or potassium metabisulfite) to the must. These compounds release SO_2, which reacts with water to form sulfites. Sulfur dioxide is mainly used for its antioxidant effect and antimicrobial activity. It can be added to the grape must while it is being put into the vat, so as to limit proliferation of lactic acid bacteria and thus avoiding an interruption of AF (Chang et al. 1997). It also permits better extraction of the anthocyanes.

Endogenous SO_2 originates from yeast metabolism. During AF, yeasts synthesize and naturally release molecular SO_2 in wine. The quantity varies according to the yeast strain and winemaking conditions (Alexandre et al. 2004; Comitini et al. 2005). Larsen et al. (2003) have demonstrated that different strains of *S. cerevisiae* produced amount of total SO_2 ranging from 15 to 75 mg/L during FA.

In wine, the SO_2 present is in equilibrium in a free and combined form. Three liberated forms of sulfur dioxide are present: (i) molecular (SO_2), (ii) bisulfite (HSO_3^-), and (iii) sulfite (SO_3^{2-}). The predominant form varies according to the environmental pH (Fig. 17.1). In wine pH, the bisulfite form predominates in equilibrium with the hydrated form (H_2SO_3), which constitutes the active form of the free SO_2. This form is responsible for the antioxidant activity and antimicrobial effect. Only the molecular form of SO_2 can freely diffuse through the membrane of bacteria. Then, SO_2 reacts with cell constituents, interferes with the protein disulfurous bridges, and associates itself with coenzymes and vitamins (Chang

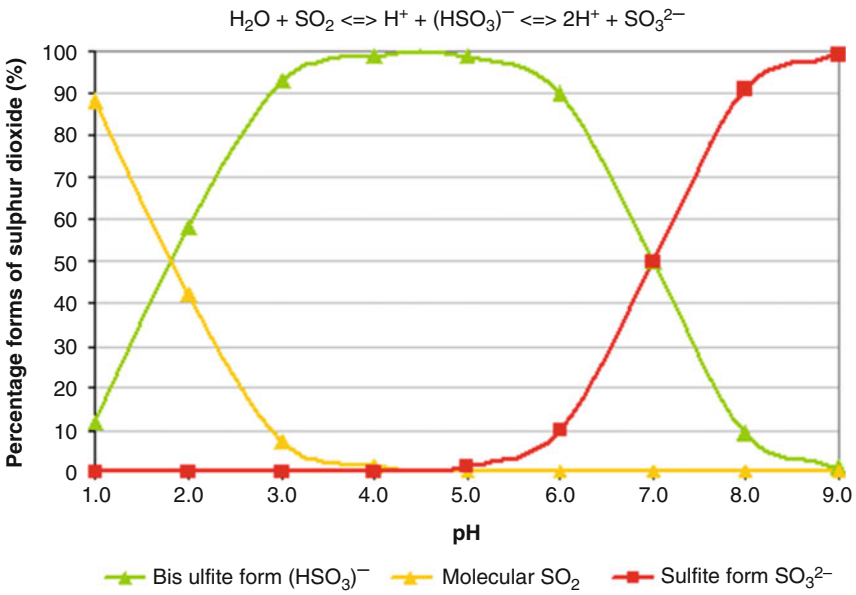

Fig. 17.1 Equilibrium reactions between free sulfur dioxide and proportion of the three types for different pH values. The equilibrium equations between the different forms are indicated above the graph

et al. 1997). These reactions can result in bacterial death. SO_2 also has an impact on the different enzymatic activities. The malolactic activity of cells is sensitive to the SO_2 concentration (Henick-Kling et al. 1989; Henick-Kling 1995). Carrete et al. (2002) showed that SO_2 also has a major inhibiting effect on the ATPase activity, this activity being reduced by more than 50% for concentrations in total SO_2 of 40 mg/L. Reduction of this activity induces major loss of viability in the cells. In fact, the ATPases, by their implication in the mechanism of MLF and the maintaining of pH_{int}, are implied in the survival of *O. oeni*.

Apart from these free forms, SO_2 can be found in combined forms (also called bound sulfites or bound SO_2). Combined SO_2 results from the combination of the bisulfite form with other molecules such as sugars, phenolic compounds (such as anthocyanes), and carbonyl or keto compounds (such as aldehyde, α-ketoglutaric acid, and pyruvic acid). The physicochemical properties of wine and the composition of wine, notably in organic acids and in aldehydes, regulate the balance between the free form and the combined form (Osborne and Edwards 2007). For example, the lower the pH in wine, the more SO_2 presents a larger antimicrobial effect. Moreover, temperature and ethanol concentration modulate the antimicrobial effect of SO_2 while modifying the proportion of the liberated and combined forms (Britz and Tracey 1990). Antimicrobial activity of combined forms is firstly considered as weak or nonexistent in this form and is often reported as being as a consequence of the release of SO_2 following degradation of SO_2-bound

acetaldehyde by *O. oeni* or *Lactobacillus* spp. strains (Osborne et al. 2000, 2006; Jackowetz and Mira de Orduña 2012). However, there is still some debate regarding the inhibitory action of bound SO_2. Many researchers have suggested that bound SO_2 may be more antimicrobial than previously believed, particularly toward LAB (Wells and Osborne 2011).

Tolerance of wine LAB to SO_2 and bound SO_2 is very variable as well as to the type of bound SO_2 (Britz and Tracey 1990; Wells and Osborne 2012). Concentrates of 1–10 mg/L of free SO_2 and 50–100 mg/L of combined SO_2 (100–150 mg/L of total SO_2) are able to inhibit the growth of LAB (Reguant et al. 2005a). *O. oeni* is able to develop tolerance to SO_2 (Guzzo et al. 1998). More recently, Wells and Osborne (2012) have studied the impact of acetaldehyde- and pyruvic acid-bound SO_2 on strains of *P. parvulus*, *P. damnosus*, *L. hilgardii*, and *O. oeni*. These tests were conducted at wine pH into media containing various concentrations of acetaldehyde or pyruvic acid and an equimolar concentration of SO_2. In these experimental conditions, acetaldehyde-bound SO_2 and pyruvic acid-bound SO_2 were inhibitory to wine LAB growth at concentrations as low as 5 mg/L of bound-SO_2, and growth of *L. higardii* was completely inhibited in media containing acetaldehyde-bound SO_2. Furthermore, SO_2 has therefore a selective effect on the native microflore of LAB. This parameter allows the selection of *O. oeni*, as for pH and ethanol content.

The effect of SO_2 on MLF has not received much attention in the literature. Two studies concern the impact of the production of SO_2 and SO_2-binding compounds by different strains of *S. cerevisiae* on MLF (Larsen et al. 2003; Wells and Osborne 2011). Both have demonstrated that combined form of SO_2 rather than free SO_2 was mainly responsible for MLF. Thus, the impact of combined form of SO_2 on MLF may need to be considered when choosing a *S. cerevisiae* strain for conducting AF (Sumby et al. 2014).

Finally, Larsen et al. (2003) have suggested that the SO_2 production by yeast did not always account for the inhibition of *O. oeni*, and the presence of other inhibitory mechanisms is probable.

17.2.4 Other Factors Specific to Wine

As mentioned above, wine is a complex environment, and several other factors, including phenolic compounds and pesticides, also have an influence on the growth of *O. oeni* and on the malolactic activity.

Phenolic Compounds Interaction between wine phenolic compounds (e.g., phenolic acid, anthocyanins, and tannins) and LAB can be considered two-way: LAB can degrade polyphenols, and, on the other hand, bacteria growth and metabolism can be affected by wine phenolics or even by phenolic metabolites produced by other microorganisms (Garcia-Ruiz et al. 2009).

Some polyphenols and anthocyanins inhibit LAB development (Figueiredo et al. 2008). However, the effect of these molecules on the malolactic activity is still quite unknown (Reguant et al. 2000; Vivas et al. 1997). Phenolic acids influence can be either positive or negative in terms of growth stimulation, depending (i) on the bacterial species (*O. oeni* or *L. hilgardii*), (ii) the specific phenolic acid used, and (iii) its concentration (Campos et al. 2003). Most of the studies refer to *O. oeni*. However, several studies have reported the effect of phenolic compounds against LAB wine. Reguant et al. (2000) have showed that phenolic compounds depending on their type and concentration affected the growth of *O. oeni*, and their role is very complex. Studies focusing on *O. oeni* and *P. pentosaceus* strains indicate that these species appear to be more sensitive to phenolic acid inactivation than *L. hilgardii* (Campos et al. 2003, 2009a, b; Garcia-Ruiz et al. 2009). Lot of articles report on the impact of polyphenols on wine lactic acid bacteria, but it is clear that the results still remain confusing because the system is complicated both in term of chemical composition and of diversity of strains (Chasseriaud et al. 2015). Certain of these components such as gallic acid helps to stimulate growth (Rozes et al. 2003) and increase the malolactic activity, whereas others have an inhibiting effect (Vivas et al. 1997; Figueiredo et al. 2008). Requant et al. have demonstrated that the consumption of L-malic acid was inhibited in cases where growth was affected (Reguant et al. 2000). For all these molecules, the mechanisms involved in the inhibitory effects remain unclear. It is known that phenolic compounds can damage the bacterial cell membrane, causing leakage of intracellular constituents (such as proteins, nucleic acids, and inorganic ions), an increase of potassium efflux, and an increase of proton influx (McDonnell and Russell 1999; Campos et al. 2009a, b). Phenolic acids may diffuse through the cytoplasmic membrane and induce an increase of the cell membrane permeability in LAB from wine. But, little work has been done concerning the effect of phenolic compounds on the cell membrane, and the effect of these compounds cannot be explained by the simple adsorption to cell walls (Campos et al. 2003). Garcia-Ruiz et al. (2011) have confirmed the damage of the cell membrane integrity by scanning electron microscopy. In this study, the incubation of *O. oeni* cells with ethyl gallate and ferulic acid induced a breakdown of the cell membrane and the release of the cytoplasm material. Finally, as described for the other factors, low pH may facilitate the diffusion of phenolic acids through the cytoplasmic membrane, and other antimicrobial agents may have synergistic effects with these compounds on the bacterial membrane (Campos et al. 2009a, b).

However, the role of these molecules remains secondary in comparison with that of the physical and chemical parameters of the wine. The influence of the physical and chemical factors (pH, ethanol content, temperature, and sulfite presence) is crucial for the realization of MLF.

Pesticides Other factor, often neglected but highly significant for inhibiting MLF, is the presence in must of pesticide residues, substances protecting plants against undesirable microorganisms and diseases (Lasik 2013). Chemical treatments against fungi, such as mildew and *Botrytis*, not only affect yeast but also LAB in

wine and may delay MLF. However, the effect of pesticides on MLF is variable and depending on the molecule considered (Vidal et al. 2001; Ruediger et al. 2005; Cabras et al. 1994). For example, the effect of seven fungicides and three insecticides on red wine fermentation was investigated (Ruediger et al. 2005), and results indicated a variable effect of these pesticides on MLF according to the molecule studied with a major inhibitory effect of dicofol and a minor effect of chlorpyrifos and fenarimol. In another study, Cabras et al. (1994) studied the influence of six fungicides (azoxystrobin, cyprodinil, fludioxonil, mapanyperim, pyrimethanil, and tetraconazole) on two LAB species (*O. oeni* and *Lactobacillus plantarum*), showing in the same time a different effect according to the specie and the pesticide considered. Moreover, the examination of the effect of two commonly used pesticides, copper and dichlofluanid, on several strains of *O. oeni* and on MLF in simulated wine, showed that inhibition of the MLF resulted in a decrease in cell number (Vidal et al. 2001). The different studies on pesticide effect on LAB suggest an effect strongly dependent on the malolactic bacteria strain, variety of fermented grapes, vinification technique used, as well as the type of the used pesticide (Lasik 2013).

17.2.5 Common Molecular Responses to Physical and Chemical Stress

The parameters presented previously induce physiological and metabolic changes and injuries at the cellular level. LAB have to adapt their physiology to environmental changes to void the death. Metabolic changes are numerous and complex. The response to the different stress can be considered as bimodal with a physiological response that implicates enzymatic systems and an induced response that necessitates changes of genome expression (van de Guchte et al. 2002). Even if many studies on wine stress response focused on *O. oeni*, this bacterium is not the only bacterium able to grow in wine and to develop the MLF. Indeed, *Lactobacillus* spp. have been shown to survive in winemaking conditions and to possess many favorable biological properties that would make them suitable candidate for MLF starter culture mechanisms (Kleerebezem and Hugenholtz 2003; Spano and Massa 2006; Du Toit et al. 2011; Testa et al. 2014; Bravo-Ferrada et al. 2013). Several mechanisms have been associated with a stress response and adaptation. These mechanisms concern (i) the regulation of the intracellular pH, (ii) modification of the membrane composition (adjusting membrane fluidity), and (iii) synthesis of stress proteins.

Regulation of the Intracellular pH In order to maintain its pH_{int}, *O. oeni* uses different enzymatic mechanisms including H^+-F_0F_1-ATPases (Tourdot-Marechal et al. 1999; Fortier et al. 2003), malolactic enzyme (Augagneur et al. 2007), and decarboxylation of certain amino acid (Bonnin-Jusserand et al. 2011; Romano et al. 2012). Indeed, H^+-F_0F_1-ATPases coupled to decarboxylases have a key role in pH_{int}

homeostasis and proton-motive force. Entry of mono-anionic L-malate, which is decarboxylated in L-lactate, consumes a proton H^+ (Salema et al. 1996; Labarre et al. 1996). This modification of charge generates a proton-motive force used by H^+-F_0F_1-ATPases. The importance of this mechanism was underlined by Carrete et al. (2002). These authors have showed that sulfites inhibit the H^+-F_0F_1-ATPase activity, limiting the ability of bacteria to maintain a physiological pH_{int}. Other decarboxylases involved in amino acid metabolism allow regulating the pH_{int} by liberating biogenic amines such as putrescine, tyramine, or histamine (Bonnin-Jusserand et al. 2011; Romano et al. 2012). Membrane exchange between amino acid and biogenic amine conducts to the establishment of a H^+ gradient and generation of the proton-motive force.

Modification of the Membrane Composition One major adaptation following stress involves mechanisms that counteract the modification in membrane fluidity. Indeed, such fluidization or rigidification impairs the functionality of membrane-bound proteins. Different mechanisms are used by bacteria to maintain the membrane integrity such as de novo synthesis of fatty acids (Lu and Rock 2006), modification of their saturation and desaturation (Denich et al. 2003), or cyclisation of fatty acids (Cronan 2002). Several studies have demonstrated that *O. oeni* membrane fluidity is impaired following stresses such as presence of ethanol or temperature increase (fluidization) or temperature decrease (rigidification) (Chu-Ky et al. 2005). *O. oeni* is able to modify its membrane phospholipid content in sublethal conditions (Teixeira et al. 2002; Grandvalet et al. 2008). Indeed, during growth in presence of acid and ethanol or in cells in stationary phase, the oleic acid content is reduced in favor of cyclofatty acids (Grandvalet et al. 2008). This is also true for *Lactobacillus plantarum*. In response to ethanol, this bacterium synthesizes large amounts of saturated fatty acids, resulting in a decrease in the relative proportion of unsaturated fatty acids, which helps to counteract the deleterious effects of fluidizing agent such as ethanol on membrane fluidity (Bokhorst-van de Veen et al. 2011; Bravo-Ferrada et al. 2014).

Synthesis of Stress Proteins Among the numerous proteins induced during stress, stress proteins and notably proteins which are induced by thermal shock (named HSP for "heat shock protein") play an important role. These HSP are classified in different families according to their molecular mass and activity (Parsell and Lindquist 1993). During different stresses, these proteins have several functions: (i) degradation of damaged proteins (proteins not able to return to its initial state) and (ii) refolding into active conformation known as molecular chaperone activity (Fig. 17.2).

A wide variety of stress (ethanol, temperature, acid, osmotic, nutritional deficiency, oxidative) induces protein synthesis of the HSP. Certain of these proteins, such as the molecular chaperones GroES and DnaK (Kilstrup et al. 1997; Koch et al. 1998) and proteins of the Clp family (caseinolytic protein), constitute a common response to the different stress (Abee and Wouters 1999; Sanders et al. 1999). The universal molecular chaperones GroES and DnaK have a crucial role since these proteins limit the aggregation of the damaged proteins and allow for

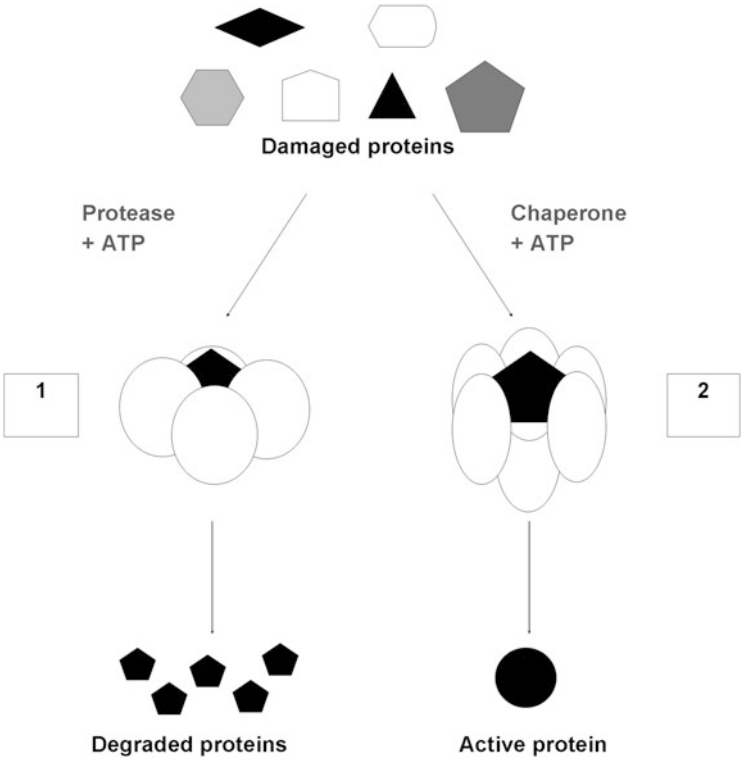

Fig. 17.2 Heat shock protein (HSP) simplified action mode according to Abee and Wouters (1999). The proteins damaged by the different stress are recognized by the protease (1) either by the chaperone (2) resulting in degradation or the restoration of an active protein

folding into active conformation. The Clp family is constituted of ATPases (ClpX, ClpC, . . .) and protease (ClpP) (Gottesman et al. 1997). The ClpP protease associates itself with a Clp ATPase subunit in order to damage those denatured in an irreversible manner during the different stress and enable recycling of monomers as amino acids. Overexpression of these proteins is induced in *O. oeni* cells following different stress such as temperature increase (Beltramo et al. 2004).

Besides intervention of these HSP, other stress proteins more specific to other stress take place in the adaptation and the resistance of bacteria to stress. This is notably the case of acid shock protein (ASP), cold shock protein (CSP), and general stress protein (GSP) that, respectively, intervene in the adaptation of the lactic acid bacteria to acid stress, low temperature, and nutritional deficiency (Hartke et al. 1996; Sanders et al. 1999; van de Guchte et al. 2002). The roles of these proteins are different and depend on stress. CSPs, induced by low-temperature stress, play a role in the stability of the ARNm and in translation effectiveness. These proteins are implied in the adaptive response of the lactic acid bacteria to a drop in temperature

(van de Guchte et al. 2002). ASPs intervene in the repairing and deterioration of damaged proteins during acid stress (Abee and Wouters 1999).

The variety and amount of stress proteins induced are variable according to the stress applied and the LAB studied. It is really of interest to note that different ethanol condition (8 or 12%) can control different stress response pathways by activating, refolding, or degrading chaperones, respectively (Costantini et al. 2015). With *O. oeni*, stress proteins were detected after exposure to different kinds of stress (Guzzo et al. 1997). In fact, ethanol presence in high concentration induces the stress protein synthesis with *O. oeni* (Garbay and Lonvaud-Funel 1996) and notably small HSP such as the Lo18 protein (Guzzo et al. 1994, 1997). A study based on the monitoring of several stress gene expression in *O. oeni* after direct inoculation in wine revealed an increase of mRNA rate for stress genes as *hsp18* (encoding Lo18 protein), *groEL*, *clpP*, *clpX*, and others. These results showed clearly that *O. oeni* can develop an adaptative response in wine (Beltramo et al. 2006). The most-studied protein involved in *O. oeni* adaptation is the small HSP named Lo18. Several studies pinpoint out its role more precisely. The small HSP Lo18 can act as a lipochaperone on phospholipids (increases the molecular order of phospholipids and regulates the membrane fluidity) and as a molecular chaperone on damaged proteins by preventing aggregation of proteins during stress conditions (Coucheney et al. 2005a, b). Since this small HSP is an essential actor of the stress response in *O. oeni*, Maitre et al. (2014) proposed a model of Lo18 involvement to ethanol tolerance in *O. oeni* (Fig. 17.3). Membrane fluidity abruptly increases in response to the ethanol (Chu-Ky et al. 2005), and the envelope stress signal leads to an increase in *hsp18* gene expression. Oligomers of Lo18 dissociate to dimers, which can interact with the phospholipid membrane, where they participate in membrane stabilization. The distribution of the protein between the membrane and the cytoplasm (Jobin et al. 1997; Coucheney et al. 2005a, b) suggests that Lo18 can participate both in membrane stabilization and in the protection of cytoplasmic proteins from aggregation. This adaptation process includes a modification of the membrane phospholipid content, which helps to counteract the fluidizing effect of ethanol. Lo18 affinity decreases for the ethanol-adapted membrane, which may allow the release of this sHsp to the cytoplasm, where it exerts its chaperone activity on ethanol-aggregated proteins. Since this small HSP is involved in the survival of *O. oeni* in stressful environment, the corresponding gene could be a marker used in the selection of new strains as starters for MLF (Coucheney et al. 2005b; Bordas et al. 2013).

The overproduction of sHsp was equally studied in *L. plantarum* (Spano et al. 2005; Spano and Massa 2006). One of them, the Hsp18.55, was studied more in detail, and it was suggested that it was involved in the maintenance of the membrane fluidity and the physicochemical surface properties of *L. plantarum* WCFS1 (Capozzi et al. 2011).

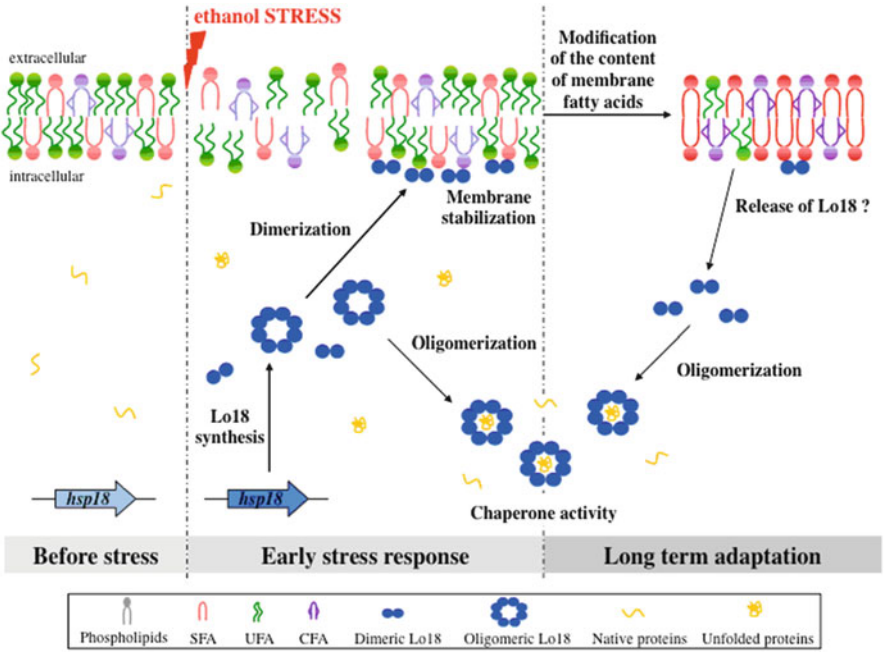

Fig. 17.3 Model of Lo18 activities in response to ethanol stress in *O. oeni* cells. To maintain membrane integrity, *O. oeni* produces the sHsp Lo18 that has the dual role of both membrane stabilization and protection of proteins from aggregation. Dissociation of Lo18 oligomers may be crucial for these activities since dimers are preferentially found at the membrane surface. As soon as the fatty acid content of the bacterial membrane is modified, Lo18 is release from the membrane, making it available for oligomerization and chaperone activity for cytoplasmic proteins. *SFA* saturated fatty acids; *UFA* unsaturated fatty acids (Maitre et al. 2014)

17.3 Conclusions

In conclusion, we can note that wine is an extremely complex environment. Several factors have an effect on the survival and the development of bacteria and notably of *O. oeni*. Furthermore, all these factors can interact according to synergic or opposing effects (Britz and Tracey 1990; Versari et al. 1999): pH and SO_2 show opposing effects (Nielsen et al. 1996); low temperature influences the tolerance of bacteria to ethanol. The modification of one of these parameters can have serious consequences on the starting and development of MLF. It is also necessary to take account of the fact that the different *O. oeni* strains highly differ by their capacities in tolerating different stress and their developing in wine (Britz and Tracey 1990). In wine, *O. oeni* survives and develops in hostile conditions which form a "multistress" environment for this bacterium. In order to survive and then to develop, this bacterium has therefore adapt itself during the winemaking process. Multiple adaptation mechanisms are implied for bacterial development in wine.

The intervention of all these technological, microbiological, physical, and chemical factors leaves spontaneous MLF uncertain and difficult to foretell and control.

In order to master this step, several alternatives are currently at the disposal of winemakers and enologists, notably strain selection and strain improvement. The use of adapted (pre-acclimated) strains to wine stresses was largely developed during the last decade.

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Chapter 18
Influence of Phenolic Compounds and Tannins on Wine-Related Microorganisms

Helmut Dietrich and Martin S. Pour Nikfardjam

18.1 Introduction

Polyphenols represent an important family of compounds found in grapes and wine. To date, more than 9000 flavonoids have been described and structurally elucidated in plant kingdom. Research on polyphenols mainly started in the early 1930s after the discovery of vitamin P and the "ascorbic acid cofactor" by Rusznyak and Szent-Györgyi (1936). Research on these compounds was intensified especially after the development of more sophisticated analytical methods. The general interest in polyphenols was also intensified after the publication of several epidemiological studies in the early 1990s, which suggested that the negative correlation between coronary diseases and a diet rich in saturated fats in France is mainly due to the relatively high consumption rate of red wine in this country. The polyphenols in red wine have been made responsible for this effect.

To date many polyphenols have been identified in grapevines, grapes, and grape products. They play an important role in the sensorial characteristics of the wine, as they not only contribute to color but also to tasting sensations, such as bitterness, astringency, and velvety, and hitherto, although to a lesser extent, to the volatile aromatics of a wine.

With regard to the interactions between the polyphenols from grapes and microorganisms, mainly antimicrobial effects have been studied due to the

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aforementioned belief that polyphenol-rich diets may have an impact on human health. Some researchers have also studied the direct interactions between yeasts and polyphenols.

The following chapters give an overview over the classification, biosynthesis, typical contents of polyphenols in grapes and grape products, and their antimicrobial effects in vitro and in vivo.

18.2 Classification of the Polyphenols

Generally, polyphenols can be divided in two large groups: flavonoids and non-flavonoids. In the first large group, more than 8000 substances have been identified to date. Thus, a further classification is necessary, which in this case is limited to the flavonoids occurring in grapes and grape-related products.

The flavonoids of grapes and wines are being subdivided into three groups:

(1) Anthocyanins (anthocyanidin glucosides): malvidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, and their corresponding acylated forms
(2) Flavonols and flavonol glycosides: kaempferol, quercetin, and myricetin
(3) Flavan-3-ols: also often referred to as catechins (catechin, epicatechin, epigallocatechin) and procyanidins or tannins (procyanidins B_1–B_4, T_1–T_6).

The non-flavonoids are also being subdivided into three groups:

(1) Hydroxybenzoic acids: gallic acid, ellagic acid, and vanillic acid
(2) Hydroxycinnamic acids: *p*-coumaric acid, caffeic acid, and ferulic acid
(3) Stilbenes: resveratrol, resveratrol glucosides (piceids; see Fig. 18.1), and resveratrol polymers (viniferins).

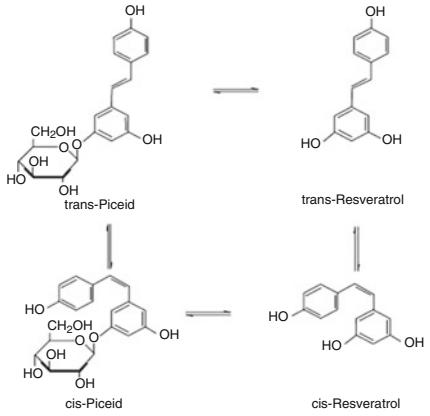

Fig. 18.1 Resveratrol and its glucosides

18.3 Polyphenol Biosynthesis

The precursors of the polyphenols emanate from the carbohydrate metabolism. The biosynthesis of the polyphenols has been investigated in detail (Harborne 1988; Forkmann 1993; Heldt 1996; Winkel-Shirley 2001; Macheix et al. 2005; Jeong et al. 2006; Davies and Schwinn 2006; Moreno-Arribas and Polo 2008; Yonekura-Sakakibara and Saito 2014) and can be divided into three partitions:

- *Shikimic acid partition*: synthesis of the amino acids phenylalanine, tyrosine, and tryptophan
- *Phenylpropanoid partition*: synthesis of the hydroxycinnamic acids and the precursors of the flavonoids and lignins
- *Flavonoid partition*: flavonoid synthesis.

18.3.1 Shikimic Acid Partition

The schematic biosynthesis of the polyphenols is shown in Fig. 18.2. Shikimic acid is formed from phosphoenolpyruvate and erythrose-4-phosphate. The enzyme phenylalanine-ammonium-lyase (PAL) catalyzes the formation of *trans*-cinnamic acid from phenylalanine. The ammonia released during the conversion of both amino acids is probably bound to glutamine synthetase and brought back into the cycle (Heldt 1996; Wen et al. 2005; Chen et al. 2006).

18.3.2 Phenylpropanoid Partition

The biosynthesis of the phenylpropanes emanates from *trans*-cinnamic acid and, thus, is a subsequent reaction of the shikimic acid partition. The enzyme cinnamic acid-4-hydroxylase (4CH, a P450 monooxygenase) hydroxylizes the benzene structure in *para*-position, which leads to *p*-coumaric acid. Further hydroxylation and methylation lead to the hydroxycinnamic acids caffeic acid, ferulic acid, and sinapic acid. *S*-Adenosylmethionine acts as the methyl donor. Elimination of a C_2 fragment leads to the formation of the benzoic acids, such as salicylic acid (Moreno-Arribas and Polo 2008). 4-Coumaroyl-CoA is produced from *p*-coumaric acid by 4-coumarate/CoA ligase (4CL).

18.3.3 Flavonoid Partition

The flavonoid biosynthesis starts from the chalcone. The latter is formed from three molecules malonyl-CoA and one molecule *p*-coumaroyl-CoA. The enzyme

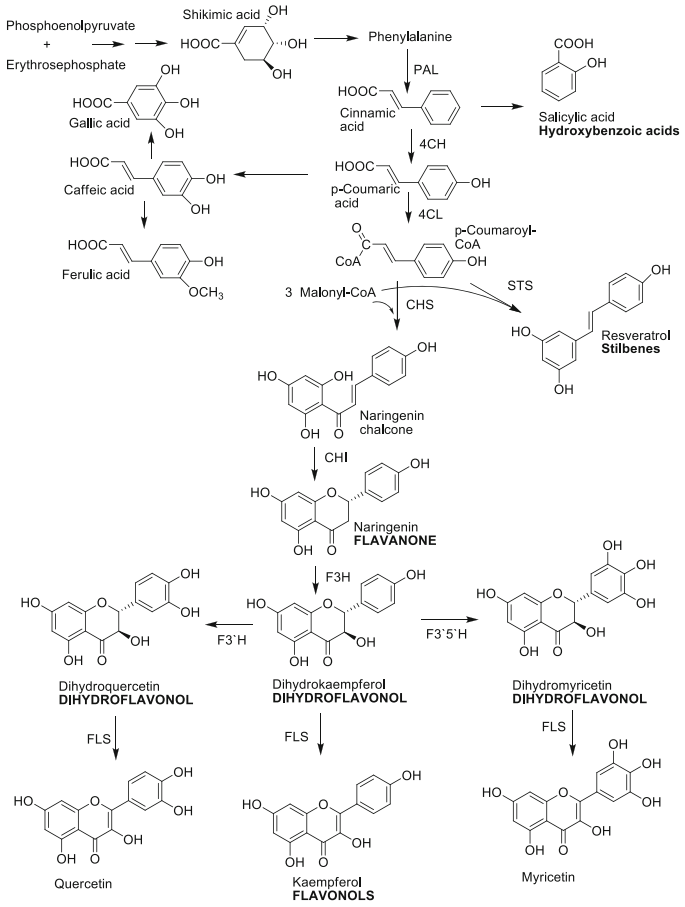

Fig. 18.2 Principle of polyphenol biosynthesis

chalcone synthase (CHS) catalyzes the reaction, which initially leads to the formation of tetrahydroxy chalcone and concurrent elimination of CO_2. This pathway is also called malonate pathway.

Some plants, such as grapevine, peanut, pine, and mulberry, also possess a stilbene synthase (STS), which reacts with three molecules malonyl-CoA and one molecule *p*-coumaroyl-CoA. Yet, the C9′ atom of the phenylpropanoid is eliminated as CO_2. The formed structure is called resveratrol and belongs to the subclass of the stilbenes. Its effect as an agent against fungal attacks classifies it as a typical phytoalexin (Pezet et al. 2003; Püssa et al. 2006; Ali et al. 2011). Recent research has shown that the biosynthesis of stilbenes competes with the synthesis of flavonoids (chalcones) as the expression of STS genes increases when chalcone synthase genes are reduced (Li et al. 2014).

The C_6–C_3–C_6 skeleton of tetrahydroxy chalcone is the structural basis for all flavonoids. After ring closure, catalyzed through the chalcone isomerase (CHI),

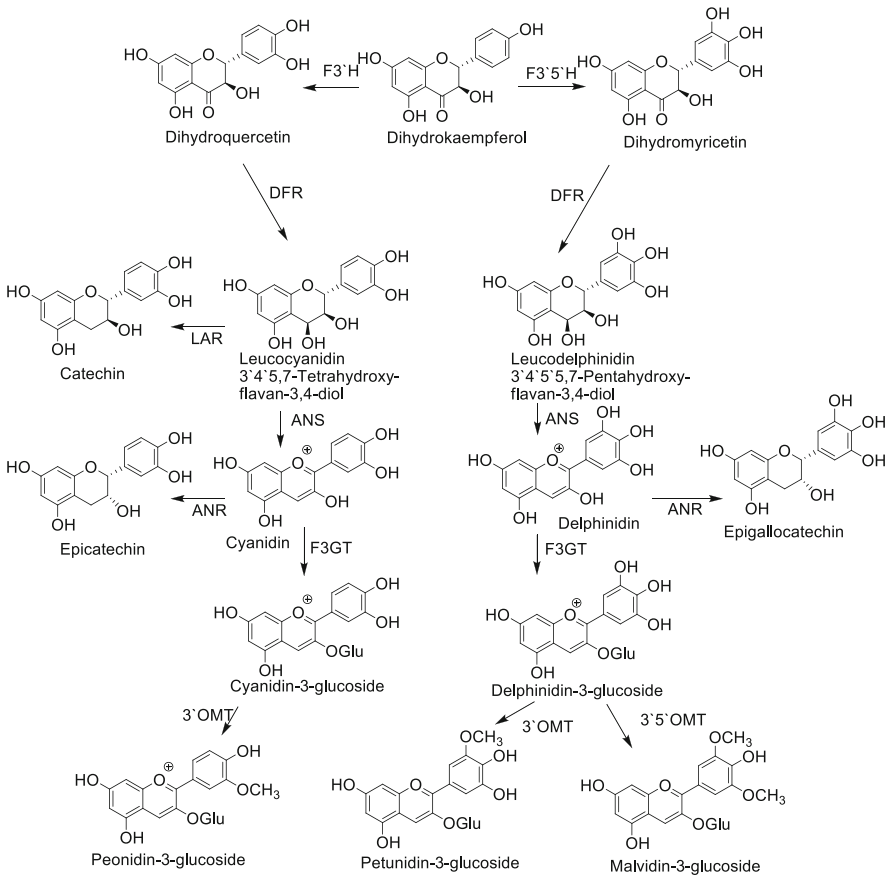

Fig. 18.2 (continued)

naringenin is being formed. The latter is transformed to dihydrokaempferol by the enzyme flavanone 3-hydroxylase (F3H). The enzyme flavonol synthase (FLS) catalyzes the formation of a double bond between the C_2 and the C_3, which leads to the formation of kaempferol (a flavonol).

Other dihydroflavonols can be formed from dihydrokaempferol by the activities of flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′5′-hydroxylase (F3′5′H), leading to dihydroquercetin and dihydromyricetin, respectively (Jeong et al. 2006). Both enzymes belong to the cytochrome P450 family, and it is presumed that the ratio of F3′H and F3′5′H controls the anthocyanin composition of grape skins. Recent research has shown that F3′H is expressed from *VviF3′H* gene. The transcript level of *VviF3′H* was higher at early developmental stages and gradually decreased during véraison and then increased in the mature phase (Sun et al. 2015). The resulting dihydroflavonols are then transformed to quercetin and myricetin by means of flavonol synthase (FLS) and subsequently glycosylated.

Dihydroflavonol can also be converted to a flavane-3,4-diol through a catalytic reduction by means of dihydroflavonol reductase (DFR). The crystal structure of *Vitis vinifera* DFR, heterologously expressed in *Escherichia coli*, was determined recently by Petit et al. (2007).

Flavan-3,4-diols (also called leucoanthocyanidins) are regarded as transient precursors of the anthocyanins, the flavan-3-ols, and the proanthocyanidins (PA), with the latter being polymerized flavan-3-ols. The complete synthesis of this compound is still not yet completely revealed. The interaction of several enzymes is probable, and these compounds might even be converted from anthocyanidins by anthocyanidin reductase (Harborne 1988; Forkmann 1993; Li et al. 2014).

The glucosides of cyanidin, delphinidin, peonidin, petunidin, and malvidin are the characteristic pigments of red grapes and wines. Cyanidin and delphinidin are formed from leucocyanidin and leucodelphinidin, respectively, by anthocyanin synthases (ANS) or, more precisely, leucoanthocyanin dioxygenases (LDOX). These aglycons are glucosylated by means of flavonoid 3-glucosyltransferases (F3GT) using UDP-glucose. The enzymes are therefore UDP-glucose: flavonoid 3-*O*-glucosyltransferases (UF3GT, Jeong et al. 2006). Further modifications, like methylations, occur under the enzymatic control of 3′*O*- and 3′5′*O*-methyl transferases 3′OMT, 3′5′OMT, leading to peonidins, petunidins, and malvidins. Numerous enzymes catalyzing flavonoid modifications by hydroxylation, methylation, glycosylation, acylation, and some other reactions have been described (Forkmann 1993; Davies and Schwinn 2006; Hugueney et al. 2009; Figueiredo-González et al. 2012). Pelargonidins are not found in grape wine, but pelargonidin-3-*O*-glucoside was found in trace amounts in the berry skins of Cabernet Sauvignon and Pinot noir (He et al. 2010). It can be assumed that the reaction from dihydrokaempferol to leucopelargonidin is not possible due to high activities of F3′H and F3′5′H providing a strong biosynthetic drive away from the pelargonidin precursors. Another possible explanation is that the grape DFR does not accept dihydrokaempferol as a substrate, and therefore leucopelargonidin cannot be formed. This situation is met in the case of *Petunia*, which does not produce pelargonidin (Macheix et al. 2005).

Flavan-3-ols may be formed by two biosynthetic routes, from either leucoanthocyanidins or anthocyanidins (Davies and Schwinn 2006; Li et al. 2014). The reaction type leading to monomeric flavan-3-ols is catalyzed by the NADPH-dependent leucoanthocyanidin reductase (LAR) and is presumably initiated by the separation of the hydroxyl group at the C_4 atom, which leads to a carbocation, which is then reduced to the flavan-3-ol. Thus, the monomeric catechin is formed from leucocyanidin, whereas gallocatechin is formed from leucodelphinidin (Davies and Schwinn 2006; Jeong et al. 2006; Adams 2006). In contrast, epicatechin and epigallocatechin are formed from the anthocyanidins cyanidin and delphinidin (presumably the pseudobase forms), respectively. The corresponding enzyme anthocyanidin reductase (ANR) was first described by Xie et al. (2003).

Bogs et al. (2005) found that the ANR of grape is encoded by a single gene and LAR is encoded by two closely related genes. The ANR gene is expressed in the skin and seeds until the onset of ripening, and the two LAR genes show a different

pattern of expression in the skin and seeds. The expression of ANR and LAR genes is consistent with the accumulation of proanthocyanidins (condensed tannins) in the berry. The recent findings on tannin synthesis support the idea of a close relationship between anthocyanins and proanthocyanidins. Epicatechin is the predominant extension unit in grape hypodermis cells and seeds, suggesting that cyanidin plays a role as an intermediate in tannin biosynthesis in grape berries. In grape skins, where also epigallocatechin is found as a tannin subunit, cyanidin and delphinidin would be important intermediates in proanthocyanidin biosynthesis (Adams 2006). The polymerization is still a matter of debate (Zhao et al. 2010; Zhao and Dixon 2010). Beside the monomeric flavan-3-ols, derivatives of leucoanthocyanidins seem to play a role, like carbocation products and quinone methide. Recent investigations strongly support an active role of ANS (LDOX), ANR, and LAR genes in proanthocyanidin biosynthesis, which is regulated on the transcription level (Huang et al. 2012; Liu et al. 2013; Zhu et al. 2013). It is still not known whether the polymerization of proanthocyanidin occurs spontaneously in all tissues or is enzyme catalyzed in some or all cases (Davies and Schwinn 2006).

18.4 Typical Contents of Phenols in Grapes and Grape Products

Polyphenols in grapes and in the respective products vary with season, climatic conditions, soil structure and composition, viticultural practices, and winemaking technology. Light intensity and light quality received under field conditions have been shown to modulate anthocyanin and flavonoid biosynthesis remarkably (González et al. 2015; Friedel et al. 2016). Also the impact of various winemaking procedures on polyphenolic composition has been studied thoroughly (Garrido and Borges 2013).

Many researchers have studied the polyphenolic composition of grape berries. For example, Gómez-Alonso et al. (2007) have published the phenolic compounds in Spanish grape skins and wines of the red grape variety “Cencibel.” Table 18.1 summarizes the results.

Trans-coutaric acid is the main hydroxycinnamic acid derivative in the skins. It is well known from literature that this compound acts as a vehicle for the anthocyanins.

Relatively few data are available on the flavonol composition of grapes and their distribution within the grape berry. Tables 18.2 and 18.3 show the flavonol and anthocyanin contents of grape skins and wines of the Cencibel variety.

Newer research has shown that catechin, epicatechin, gallic acid, and resveratrol are the most abundant phenolics in grape skins of the cv. Ghara Shani in West Azerbaijan (Farhadi et al. 2016). In cv. Ghara Shira also rutin was identified as a major compound.

Table 18.1 Molar (%) hydroxycinnamic acid composition of grape skins and respective wines from the Spanish grape variety "Cencibel" (Gómez-Alonso et al. 2007)

Hydroxycinnamic acid/ester	Grape skins (molar%) ($n = 10$)	Wines (molar%) ($n = 10$)
Grape reaction product (GRP)	n.d.	15.65 ± 5.02
cis-Caftaric acid	6.75 ± 2.81	0.64 ± 0.10
trans-Caftaric acid	28.26 ± 2.73	34.28 ± 7.56
Caffeic acid	n.d.	6.25 ± 4.29
cis-Coutaric acid	13.03 ± 1.91	5.91 ± 0.54
trans-Coutaric acid	48.88 ± 4.32	31.17 ± 2.04
p-Coumaric acid	n.d.	2.73 ± 2.46
cis-Fertaric acid	0.81 ± 0.65	0.26 ± 0.20
trans-Fertaric acid	2.27 ± 1.11	2.50 ± 0.59
Ferulic acid	n.d.	0.35 ± 0.34

Table 18.2 Molar (%) flavonol composition of grape skins and respective wines from the Spanish grape variety "Cencibel" (Gómez-Alonso et al. 2007)

Flavonol	Grape skins (molar%) ($n = 10$)	Wines (molar%) ($n = 10$)
Myricetin-3-glucoside	37.37 ± 5.10	38.21 ± 4.69
Quercetin-3-rutinoside	3.35 ± 0.81	n.d.
Quercetin-3-galactoside	4.22 ± 0.76	3.26 ± 0.51
Quercetin-3-glucoside	22.62 ± 3.68	25.37 ± 1.62
Quercetin-3-glucuronide	14.02 ± 1.66	20.52 ± 5.74
Kaempferol-3-rutinoside	2.74 ± 0.58	1.34 ± 0.11
Kaempferol-3-glucoside	4.84 ± 2.66	1.98 ± 1.27
Isorhamnetin-3-glucoside	7.87 ± 2.42	7.25 ± 1.43
Myricetin	1.84 ± 0.38	0.91 ± 0.36
Quercetin	0.53 ± 0.18	0.92 ± 0.35
Kaempferol	0.41 ± 0.25	0.14 ± 0.08
Isorhamnetin	0.18 ± 0.09	0.11 ± 0.06

Table 18.3 Molar (%) anthocyanin composition of grape skins and respective wines from the Spanish grape variety "Cencibel" (Gómez-Alonso et al. 2007)

Anthocyanin	Grape skins (molar%) ($n = 10$)	Wines (molar%) ($n = 10$)
Delphinidin-3-glucoside	15.75 ± 1.84	10.87 ± 1.29
Cyanidin-3-glucoside	2.99 ± 0.69	0.48 ± 0.17
Petunidin-3-glucoside	11.97 ± 0.80	12.41 ± 0.54
Peonidin-3-glucoside	5.77 ± 1.18	4.07 ± 2.06
Malvidin-3-glucoside	37.37 ± 1.85	55.10 ± 3.10

Grape pomace, i.e., skins and seeds, of cv. Malbec has recently been analyzed on polyphenolic content. Antoniolli et al. (2015) have identified catechin, epicatechin, syringic acid, quercetin, and gallic acid as the main constituents of grape pomace.

Table 18.4 Polyphenolic composition of grape juices from the "Thompson seedless" variety (Spanos and Wrolstad 1990)

Polyphenol (mg/L)	Concentration
Caftaric acid	n.d.–14.0
Coutaric acid	n.d.–2.2
Grape reaction product (GRP)	2.4–8.6
Caffeic acid	n.d.–8.6
p-Coumaric acid	0.2–3.3
Ferulic acid	n.d.–0.5
Gallic acid	n.d.–1.6
(−)-Epicatechin	n.d.–2.2
(+)-Catechin	n.d.–7.4
Procyanidin B_1	n.d.–15.4
Procyanidin B_2	n.d.–1.8
Procyanidin B_3	n.d.–4.7
Procyanidin B_4	n.d.–3.5
Procyanidins (Trimers–Tetramers)	n.d.–2.3
Quercetin-glycosides	n.d.–7.5

Downey et al. (2003) have studied the biosynthesis of flavonols in Shiraz and Chardonnay berries. The main flavonols were quercetin-3-glucoside and kaempferol-3-glucoside. Their results show that flavonol biosynthesis in Shiraz starts about 7 weeks before véraison and reaches a maximum of 6 weeks after véraison followed by a decline toward harvest date. In Chardonnay biosynthesis starts about 9 weeks before véraison reaching a peak about 2 weeks after véraison followed by a decline toward harvest date.

Masa and Vilanova (2008) have studied the flavonol composition of Albarín blanco grapes in three consecutive years. According to their results, 30% of the flavonols is made up of dihydroquercetin glycosides, 26.8% is quercetin glycosides, and 10.3% is kaempferol glycosides.

Spanos and Wrolstad (1990) published some data on the polyphenolic composition of white grape juices of the "Thompson seedless" variety. According to their results, only very low amounts of polyphenols can be found in these grape juices (Table 18.4).

Some more detailed data was published by Pour Nikfardjam et al. (2000) on the polyphenolic content of commercial and varietal red grape juices from own production (Table 18.5).

As can be seen from Table 18.5, the varietal juices contain much more polyphenols than the commercial samples. This might be due to several reasons: (1) the varietal juices were analyzed shortly after production; (2) other red varieties than in the commercial samples were used; and (3) commercial red grape juices may contain also white varieties.

Fuleki and Ricardo-da-Silva (2003) measured the concentrations of catechin and procyanidins in grape juices of North America. According to their results, especially high amounts of procyanidins were found in Seyval (13 mg/L) and Niagara

Table 18.5 Polyphenolic composition of commercial and varietal grape juices from own production (Pour Nikfardjam et al. 2000)

Red grape juice	Commercial juices ($n = 27$)			Varietal juices ($n = 7$)		
Phenol (mg/L)	Mean	Min.	Max.	Mean	Min.	Max.
Hydroxycinnamic acids						
Caftaric acid	22.1	7.7	30.3	28.3	19.4	41.0
Coutaric acid	25.1	13.7	67.8	49.8	22.9	105.4
Fertaric acid	1.8	n.d.	5.8	7.5	n.d.	29.9
Ferulic acid	1.4	n.d.	10.0	4.0	n.d.	9.4
Grape reaction product (GRP)	7.9	4.6	13.7	21.1	12.3	30.7
Caffeic acid	4.8	1.6	7.9	9.7	n.d.	22.3
p-Cumaroylglucosyltartrate (*p*-CGT)	1.1	n.d.	n.d.	n.d.	n.d.	n.d.
p-Coumaric acid	0.7	n.d.	n.d.	2.6	n.d.	10.3
Sum	64.9	30.3	120.0	122.9	65.5	226.2
Hydroxybenzoic acids						
3-OH-Benzoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-OH-Benzoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ellagic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gallic acid	9.9	1.9	23.4	4.3	1.6	9.6
Protocatechuic acid	10.3	n.d.	24.2	3.2	0.0	8.7
Sinapic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Syringic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tyrosol	9.2	2.3	17.9	11.7	4.8	20.2
Sum	29.4	6.5	55.9	19.2	10.5	29.8
Flavonols						
Avicularin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hyperoside	7.4	n.d.	20.2	4.2	n.d.	8.5
Isoquercitrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Rutin	15.5	n.d.	42.6	38.2	24.0	47.6
Sum	22.9	n.d.	62.0	42.4	32.5	55.7
Flavan-3-ols						
Catechin	13.8	n.d.	7.6	2.4	0.0	5.4
Epicatechin	8.8	0.4	15.7	32.4	7.9	68.5
Sum	22.6	1.5	23.3	34.8	11.8	68.5
Proanthocyanidins						
Procyanidin B_2	14.9	0.3	19.1	12.9	0.6	39.9
Anthocyanins						
Delphinidin-3-glucoside	2.9	n.d.	4.3	33.8	10.3	98.4
Cyanidin-3-glucoside	1.6	n.d.	3.6	21.2	2.4	48.2
Petunidin-3-glucoside	2.8	n.d.	4.4	33.6	12.3	92.3
Peonidin-3-glucoside	2.4	n.d.	4.5	113.2	16.2	355.4
Malvidin-3-glucoside	8.3	n.d.	17.5	213.9	45.0	527.0
Peonidin-3-acetylglucoside	1.3	n.d.	2.2	22.3	n.d.	51.8
Malvidin-3-acetylglucoside	1.5	n.d.	4.2	46.7	n.d.	154.2

(continued)

Table 18.5 (continued)

Red grape juice	Commercial juices ($n = 27$)			Varietal juices ($n = 7$)		
Peonidin-3-coumaroylglucoside	1.2	n.d.	2.0	12.1	n.d.	42.9
Malvidin-3-coumaroylglucoside	1.4	n.d.	3.2	55.2	n.d.	195.8
Sum	23.5	n.d.	41.9	552.2	137.4	1550.4
Stilbenes						
cis-Piceid	1.6	n.d.	6.0	1.8	n.d.	7.3
cis-Resveratrol	0.0	n.d.	0.5	n.d.	n.d.	n.d.
trans-Piceid	1.0	0.5	3.2	1.2	n.d.	4.8
trans-Resveratrol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sum	2.6	0.5	9.2	3.0	n.d.	12.1
Sum (HPLC)	115.7	43.0	176.1	664.5	202.8	1729.3

(4.4 mg/L), catechin and epicatechin in Elvira (8 and 12 mg/L, respectively), and Chardonnay (4.1 and 4.8 mg/L, respectively). In red grape varieties, Vincent (32 mg/L) had the highest contents on procyanidins, and Vincent, Maréchal Foch, and Baco noir were highest in catechin (18, 30, and 6 mg/L, respectively).

Moreno-Montoro et al. (2015) have recently published a study on polyphenols in white and red grape juices. According to their results, red grape juice contains significantly higher contents on polyphenols and antioxidative capacity. Yet, their results show much lower polyphenol contents than the aforementioned papers (Table 18.6). The impact of various treatments shows strong influence on the final polyphenolic content of grape juice. Especially thermal treatment of red grapes has been shown to enhance extraction of anthocyanins from grapes significantly (Lambri et al. 2015).

Table 18.7 summarizes the polyphenolic composition of white wines (Vrhovsek et al. 1997; Pena-Neira et al. 2000; Carando et al. 1999; Teissedre et al. 1996).

Pour Nikfardjam et al. (2007) reported on the polyphenolic content of German white wines (Table 18.8). According to their results, tyrosol is the most abundant polyphenol in German white wines of the Bacchus, Müller-Thurgau, Riesling, and Silvaner varieties. In Red Traminer, *p*-coumaric acid was the main polyphenol, and in Rieslaner 3-hydroxybenzoic acid was dominant. White wine polyphenolic composition has also been used for the differentiation among varieties (Ritter et al. 1994). Other authors could not support these findings (Pour Nikfardjam et al. 2007). More sophisticated methods have successfully used for this purpose, such as MALDI/ToF-MS, yet these techniques do not rely on polyphenols only but on the whole chemical composition (Rešetar et al. 2016). Other authors have applied HPLC-MS/MS techniques to identify 61 monomeric phenolic compounds in Apulian Italian wines. This detailed information could be used for discrimination among varieties (Barnaba et al. 2016).

Table 18.9 shows the composition of colorless polyphenols of red wines (Pour Nikfardjam et al. 2006a; Makris et al. 2006; Woraratphoka et al. 2007).

Table 18.6 Polyphenolic composition of Spanish white and red grape juice (Moreno-Montoro et al. 2015)

Polyphenol (mg/L)	White grape juice ($n = 11$)	Red grape juice ($n = 9$)
Gallic acid	1.14 ± 0.68	2.01 ± 0.71
m-Hydroxybenzoic acid	2.66 ± 2.83	0.73 ± 0.31
Syringic acid	0.14 ± 0.02	0.34 ± 0.28
Vanillic acid	0.41 ± 0.12	0.54 ± 0.32
Caffeic acid	0.61 ± 0.71	0.65 ± 0.06
p-Coumaric acid	1.20 ± 0.87	0.62 ± 0.21
Ferulic acid	0.31 ± 0.13	0.25 ± 0.09
Catechin	0.57 ± 0.34	1.64 ± 0.62
Epicatechin	0.11 ± 0.07	1.93 ± 0.99

Table 18.7 Polyphenolic composition of white wines (Vrhovsek et al. 1997; Pena-Neira et al. 2000; Carando et al. 1999; Teissedre et al. 1996)

Polyphenol (mg/L)	Riesling	Riesling	White wine	White wine
Origin	Germany	Germany	Canada	Various countries
Caftaric acid	30.2 (12.1–75.6)	n.d.	n.d.	25.1 (14.2–32.6)
Grp	1.3 (0.2–6.0)	n.d.	n.d.	12.5 (4.0–32.2)
p-CGT	0.5 (0.1–1.2)	n.d.	n.d.	n.d.
Coutaric acid	2.5 (0.9–5.1)	n.d.	n.d.	4.4 (1.2–6.9)
Fertaric acid	2.3 (1.1–3.4)	n.d.	n.d.	2.9 (1.8–3.4)
Caffeic acid	2.3 (0.5–4.8)	n.d.	(1.51–5.20)	1.9 (0.8–3.3)
p-Coumaric acid	1.7 (0.9–2.8)	2.0	(1.57–3.21)	1.8 (1.0–2.8)
Ferulic acid	1.7 (0.9–4.5)	n.d.	(tr-4.42)	0.6 (0.4–0.9)
Protocatechuic acid	n.d.	n.d.	n.d.	0.06
Tyrosol	25.2 (6.3–53.8)	n.d.	n.d.	1.7
Gallic acid	n.d.	n.d.	2.8	0.19
Ellagic acid	n.d.	n.d.	n.d.	0.09
Sinapic acid	n.d.	n.d.	n.d.	0.1
(+)-Catechin	0.9 (tr-2.5)	3.7	(3.80–4.20)	9.8/34.9
Procyanidin B_2	4.9 (tr-9.5)	n.d.	n.d.	n.d.
(−)-Epicatechin	6.1 (1.0–11.8)	1.2	(1.70–3.80)	5.3/21.2
(−)-Epicatechin-3-*O*-gallate	12.3 (0.4–47.2)	n.d.	n.d.	n.d.

The data already show that the origin of the wines has a distinctive effect on its polyphenolic composition. Besides climatic conditions, other factors, such as winemaking style and technology, also play a very important role. Especially, fining agents are known to reduce the polyphenol content in wines (Tschiersch et al. 2008).

Wang and Huang (2004) studied the flavonol content of wines by means of HPLC and capillary electrophoresis. Their results show that on a quantity basis, the most important flavonol is quercetin followed by kaempferol, myricetin, and baicalein (5,6,7-trihydroxyflavone).

Table 18.8 Polyphenolic composition (mg/L) of German white wines (Pour Nikfardjam et al. 2007)

Cultivar	Bacchus	Müller-Thurgau	Rieslaner	Riesling	Silvaner	Traminer
Number of samples	$n = 14$	$n = 75$	$n = 7$	$n = 17$	$n = 68$	$n = 10$
Vintage	1997	1989–1998	1992–1996	1996–1998	1993–1998	1998
Gallic acid	0.8 (n.q.–2.9)	1.3 (n.q.–10.1)	0.6 (n.q.–1.7)	n.q.	1.6 (n.q.–14.8)	1.4 (n.q.–2.8)
Protocatechuic acid	4.5 (1.7–11.0)	2.1 (n.q.–7.7)	2.4 (0.7–3.7)	n.q.	3.5 (n.q.–11.0)	3.0 (1.8–4.4)
Tyrosol	13.9 (9.9–22.0)	19.9 (n.q.–39.2)	16.1 (10.8–19.6)	18.0 (n.q.–42.2)	17.4 (3.5–26.7)	16.0 (7.5–24.1)
3-Hydroxybenzoic acid	n.d.	0.2 (n.q.–12.0)	23.1 (n.q.–36.8)	n.q.	0.9 (n.q.–38.3)	n.d.
Caftaric acid	1.4 (n.q.–3.6)	4.0 (n.q.–19.9)	19.9 (17.2–28.4)	13.7 (0.5–25.9)	8.3 (n.q.–40.2)	2.8 (n.q.–21.1)
Catechin	n.d.	0.3 (n.q.–7.0)	6.0 (n.q.–10.9)	n.q.	1.1 (n.q.–26.3)	n.d.
GRP	2.3 (n.q.–12.4)	3.7 (n.q.–18.6)	8.1 (6.6–12.8)	1.7 (n.q.–5.1)	4.1 (n.q.–18.0)	4.0 (1.5–8.4)
Procyanidin B_2	n.d.	1.7 (n.q.–39.5)	8.1 (n.q.–16.3)	n.q.	0.3 (n.q.–6.7)	n.d.
p-CGT	8.2 (n.q.–16.7)	4.3 (n.q.–23.0)	2.1 (n.q.–3.9)	0.5 (n.q.–1.7)	10.6 (n.q.–61.4)	7.5 (n.q.–24.2)
Syringic acid	n.d.	n.d.	0.6 (n.q.–2.1)	n.q.	n.d.	n.d.
Caffeic acid	1.9 (n.q.–10.0)	0.7 (n.q.–2.5)	2.4 (1.1–2.9)	1.6 (n.q.–3.9)	2.0 (n.q.–13.5)	8.4 (2.1–25.9)
Epicatechin	n.d.	0.3 (n.q.–12.3)	3.3 (n.q.–5.7)	0.9 (n.q.–6.4)	0.3 (n.q.–10.3)	n.d.
Coutaric acid	2.5 (n.q.–6.3)	2.0 (n.q.–8.4)	0.6 (n.q.–2.9)	1.7 (0.7–3.0)	2.7 (n.q.–6.9)	1.3 (n.q.–7.5)
Sinapic acid	n.d.	0.1 (n.q.–3.0)	1.0 (n.q.–5.1)	n.q.	0.2 (n.q.–4.7)	n.d.
Fertaric acid	1.0 (n.q.–2.5)	1.3 (n.q.–2.7)	3.6 (2.5–4.8)	1.1 (n.q.–2.6)	1.6 (n.q.–3.3)	2.6 (n.q.–5.8)
p-Coumaric acid	12.4 (6.2–18.8)	4.4 (n.q.–15.4)	4.5 (2.2–5.7)	1.3 (n.q.–3.7)	8.2 (0.8–33.3)	21.9 (8.5–47.2)
Ferulic acid	1.1 (n.q.–3.5)	0.3 (n.q.–1.7)	3.2 (1.0–4.6)	0.7 (n.q.–4.7)	0.6 (n.q.–5.5)	1.3 (n.q.–3.6)
Ellagic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin-3-galactoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin-3-rutinoside	n.d.	0.1 (n.q.–3.5)	n.d.	n.d.	n.d.	n.d.
Quercetin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
trans-Resveratrol	n.d.	0.2 (n.q.–1.5)	n.d.	n.q.	0.1 (n.q.–1.7)	n.d.
Quercetin	n.d.	n.d.	1.4 (n.q.–4.1)	n.q.	n.d.	n.d.
Sum (HPLC)	50 (37–65)	49 (24–97)	111 (55–140)	48 (14–87)	64 (22–124)	70 (29–137)

Table 18.9 Colorless polyphenolic composition of red wines from various countries (Pour Nikfardjam et al. 2006a; Makris et al. 2006; Woraratphoka et al. 2007)

Polyphenol (mg/L)	Cabernet Sauvignon ($n = 10$)	Cabernet Sauvignon ($n = 7$)	Merlot ($n = 10$)	Merlot ($n = 5$)	Pinot noir ($n = 4$)	Shiraz ($n = 3$)	Barbera ($n = 3$)
Origin of wines	Hungary	Greece	Hungary	Greece	Hungary	Thailand	Thailand
Gallic acid	57.8	n.d.	65.9	n.d.	45.2	16.9	19.5
Tyrosol	89.1	n.d.	81.2	n.d.	117.0	n.d.	n.d.
Caftaric acid	53.5	22.8	51.6	24.3	55.5	n.d.	n.d.
Catechin	81.8	40.5	89.1	42.4	103.0	6.4	4.4
Grp	1.1	n.d.	2.5	n.d.	n.d.	n.d.	n.d.
Procyanidin B_2	43.7	14.1	47.5	5.3	33.9	n.d.	n.d.
Caffeic acid	23.1	3.1	18.5	1.4	28.9	10.7	1.3
Epicatechin	102.8	19.4	126.0	18.6	64.6	3.5	2.1
p-Coumaric acid	6.6	0.9	10.2	1.8	8.9	19.4	2.9
Fertaric acid	3.4	n.d.	3.0	n.d.	8.3	n.d.	n.d.
Rutin	13.1	30.9	16.9	20.7	9.7	1.8	n.d.
Ferulic acid	n.d.	n.d.	2.6	n.d.	n.d.	n.d.	n.d.
trans-Resveratrol	2.8	n.d.	3.9	n.d.	3.2	1.9	0.4
Quercetin	5.6	13.7	11.2	29.9	7.5	2.1	0.6

Amico et al. (2004) analyzed the flavonol content of Nerello mascalese grape pomace using HPLC-MS-ESI. According to their results, quercetin-glucoside was the main flavonol in grape pomace (170 mg/kg) followed by quercetin-glucuronide (130 mg/kg), isorhamnetin-glucoside (63.8 mg/kg), myricetin-glucoside (21.3 mg/kg), and quercetin (15.3 mg/kg).

Table 18.10 shows the anthocyanin composition of the wines already mentioned in Table 18.9 (Pour Nikfardjam et al. 2006a; Makris et al. 2006; Woraratphoka et al. 2007). Unfortunately, the wines from Thailand were not analyzed on their anthocyanin content.

The anthocyanin content and its composition are mainly dependent on the variety. Some authors have studied the feasibility of using the anthocyanin composition for the determination of the cultivar. It has been shown that the anthocyanin composition remains relatively constant irrespective of the winemaking technology used (Eder et al. 1994; Holbach et al. 1997; Kennedy 2008; Gil-Muñoz et al. 2010; Kontoudakis et al. 2011; Figueiredo-González et al. 2012; Garrido and Borges 2013).

18.5 Resveratrol

The phytoalexin resveratrol is one of the best analyzed compounds in wine. A plethora of papers has been published on resveratrol, its analysis, and its impact on the antioxidative and anticancer effect of moderate wine consumption.

Table 18.11 summarizes the *trans*-resveratrol and resveratrol derivative contents of various red varieties and origins.

A strong dependence of resveratrol on vintage and variety has been emerged from these studies. Vintage is important because of the climatic influence on *Botrytis cinerea* growth. Several studies have shown that *Botrytis*-free grapes contain less resveratrol than those with a slight infection. In the event of a very strong infection, the resveratrol concentration is drastically reduced. This is due to the activity of a laccase-like enzyme, a stilbene oxidase, which is produced by the fungus as a means against the antifungal effect of resveratrol. The enzyme catalyzes the oxidation of resveratrol to various products. One of those is the resveratrol *trans*-dehydrodimer, which was first mentioned by Breuil et al. (1998).

Figure 18.3 shows the dependence of the *trans*-resveratrol content of Hungarian red wines from the "Villány" region on vintage. Especially, the wines from the 2002 vintage had very high resveratrol contents.

Besides vintage, variety has been identified as a very important factor for resveratrol levels. Here a clear trend to varieties of the Pinot family and other varieties high in phenolics can be observed (Figs. 18.4 and 18.5). Very high levels on *trans*-resveratrol could be found in the varieties Kékfrankos (synonym: Blaufränkisch, Lemberger), Merlot, Pinot noir, and Zweigelt.

In white wines, much lower concentrations of resveratrol can be found (Goldberg et al. 1999). One reason for this difference is the lower biosynthesis rate. One

Table 18.10 Anthocyanin composition of red wines from various countries (Pour Nikfardjam et al. 2006a; Makris et al. 2006; Woraratphoka et al. 2007)

Anthocyanin (mg/L)	Cabernet Sauvignon ($n = 10$)	Cabernet Sauvignon ($n = 7$)	Merlot ($n = 10$)	Merlot ($n = 5$)	Pinot noir ($n = 4$)	Shiraz ($n = 3$)	Barbera ($n = 3$)
Origin of wines	Hungary	Greece	Hungary	Greece	Hungary	Thailand	Thailand
Delphinidin-3-glucoside	92.8	14.1	53.7	17.3	43.0	n.q.	n.q.
Cyanidin-3-glucoside	n.d.	0.6	n.d.	1.4	n.d.	n.q.	n.q.
Petunidin-3-glucoside	74.2	22.1	49.6	31.8	39.9	n.q.	n.q.
Peonidin-3-glucoside	43.5	16.2	38.2	18.8	48.1	n.q.	n.q.
Malvidin-3-glucoside	565.0	391.5	276.0	311.5	316.0	n.q.	n.q.

Table 18.11 Resveratrol derivatives (mg/L) in red wines from various countries

Origin	*trans*-Piceid	*cis*-Piceid	*trans*-Resveratrol	*cis*-Resveratrol	Literature
Worldwide	0.5–11.3	n.q.	0.4–10.6	0.4–7.5	Burns et al. (2000)
Hungary	3.8–16.4	n.q.	0.1–14.3	n.q.	Márk et al. (2005)
Canada	0.02–0.98	0.02–0.68	0.71–2.5	0.27–0.88	Soleas et al. (1997)
Austria	1.7–3.6	4.2–5.7	1.3–2.4	0.9–1.6	Vrhovsek et al. (1997)
Spain	0.9–4.0	0.3–1.9	0.6–8.0	0.1–2.5	Lamuela-Raventos et al. (1995)
Spain	–	–	0.06–36.13	n.d.–0.24	Feijóo et al. (2008)
Portugal	n.d.–50.8	n.d.–17.9	n.d.–5.7	n.d.–9.5	Ribeiro de Lima et al. (1999)

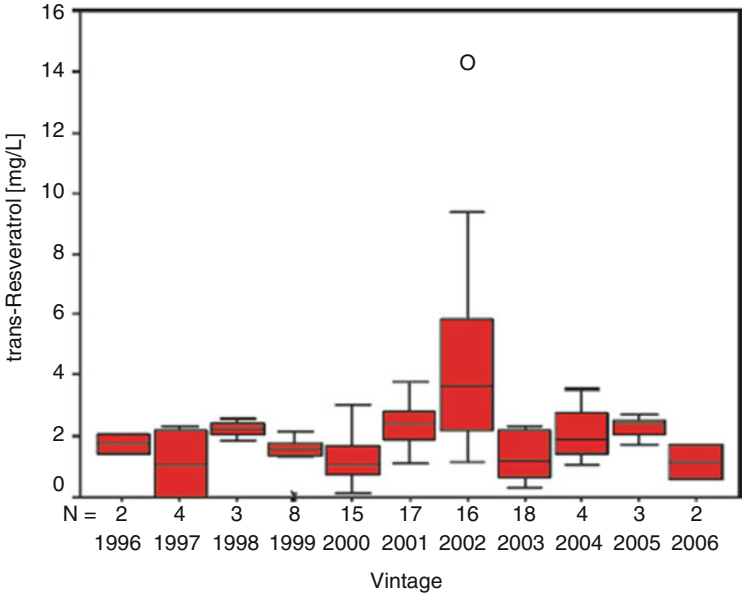

Fig. 18.3 Trans-resveratrol content (mg/L) of Hungarian red wines from the "Villány" region according to vintage (Pour Nikfardjam et al. 2006a)

of the precursors of *trans*-resveratrol is the *p*-coumaroyl-CoA. This substance is also a precursor of the anthocyanins. It is thus comprehensible that in red grapes more *p*-coumaroyl-CoA is being formed, since it is needed for the biosynthesis of the anthocyanins (Conn et al. 2003). This also explains the occurrence of resveratrol in the grape skins. Firstly, in the skins, it can act as a defensive agent against fungal attacks. Secondly, it is self-evident to place the biosynthesis at the location where the substance is needed. Research has shown a direct negative correlation between resveratrol formation and anthocyanin content in grape skin cells. This has been attributed to the competitive action of the two enzymes chalcone synthase and STS.

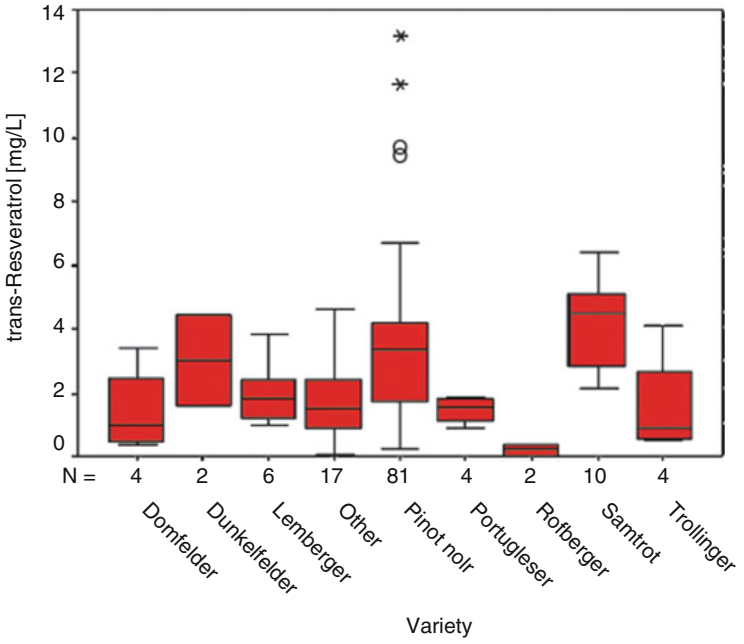

Fig. 18.4 Trans-resveratrol content (mg/L) of German red wines (Pour Nikfardjam 2001)

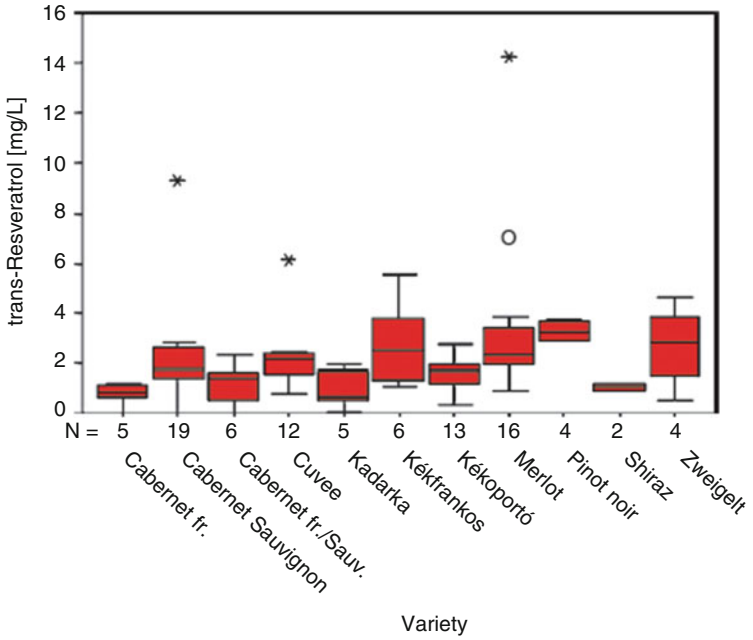

Fig. 18.5 Trans-resveratrol content (mg/L) of Hungarian red wines (Pour Nikfardjam et al. 2006a)

The decrease of ability of grapes to synthesize resveratrol in response to UV irradiation following the onset of véraison could be a consequence of the concomitant rise of anthocyanin accumulation in these fruits (Jeandet et al. 1995). Despite this switch in biosynthesis pathways from resveratrol to anthocyanins at véraison, recent studies have shown that postharvest UV-C irradiation of the grapes can be used to increase resveratrol and piceatannol concentration in the finished wine up to 26 times and 3.2 times, respectively (Guerrero et al. 2010).

Another reason for the much lower resveratrol levels in white wines is the different winemaking procedures. Mash fermentation or mash heating is generally not applied to white grapes, thus not leading to an opportunity to sufficiently extract the resveratrol from the grape skins (Vrhovsek et al. 1995).

Still there are white wines, which, due to their different winemaking style, show resveratrol levels, which are comparable to those of red wines. These wines are, for instance, the wines from the Tokaj region in Hungary. Especially, those Tokaj wines with a high amount of botrytized berries (Tokaji Aszú) show high resveratrol levels. Obviously, the concentrating effect of the berry shrinking caused by *B. cinerea* and the extraction of these berries in the high alcoholic base wine lead to these high resveratrol levels (Pour Nikfardjam et al. 2006b; Magyar 2011).

Another typical product of the Tokaj region is the so-called Forditás (twist). Here the already extracted Aszú berries are extracted again with the aid of a high alcoholic base wine. The result is a wine rich in extract and polyphenols. Folin values for these wines can reach 1700 mg/L and an antioxidative capacity (TEAC value) of 10.8 mmol/L. These are values which otherwise are only reached by red wines (Pour Nikfardjam et al. 2006b).

Resveratrol derivatives can also be found in grape juices. Because of the missing fermentation step, most of the derivatives are present in their glucoside form (piceid). Partly, resveratrol levels are reached that are comparable or even higher than in wines. Generally, the *cis*-isomers are more common in grape juices (see Fig. 18.1). Table 18.12 shows an overview over the resveratrol levels in commercially available German grape juices and variety-pure grape juices (Pour Nikfardjam et al. 2000).

Table 18.12 Resveratrols in commercial and variety-pure grape juices (Pour Nikfardjam et al. 2000)

Grape juice	*trans*-Piceid (mg/L)	*cis*-Piceid (mg/L)	*trans*-Resveratrol (mg/L)	*cis*-Resveratrol (mg/L)
Red (commercial)	0.53–7.34	0.08–5.66	n.q.–1.09	n.d.–0.23
Red (variety-pure)	n.d.–4.8	n.d.–8.7	n.d.–0.5	n.d.–0.5
White (commercial)	n.d.–0.48	n.d.–0.34	n.d.–0.19	n.q.
White (variety-pure)	n.d.–0.2	n.d.–1.0	n.d.	n.d.

The high resveratrol levels of some juices are partly due to the variety influence. Hitherto, the effect of the production technique has to be taken into account. Especially during grape juice production, the length of the mash enzymation has a decisive influence on the resveratrol levels. Modern techniques, such as ultrasonication, have been successfully applied to increase resveratrol concentration in grape juice (Hasan et al. 2014).

18.6 Antimicrobial Effects of Polyphenols in Grapes

Grapes can suffer from several stress factors, which can be divided into biotic and abiotic stress: herbivory, fungal and viral pathogens, wounding, high light and UV radiation, ozone, extreme temperature, drought, salinity, nutrient deficiency and imbalances, and application of herbicides and fungicides. Secondary plant metabolites, like the polyphenols of grapes, are part of a generalized stress response. Leshem and Kuiper (1996) introduced the term *general adaption syndrome*. Plants use a broad repertoire of defense strategies against phytopathogenic fungi and other biotic stressors. These are among other things based on the following:

1. Presence of several polyphenol subclasses (see above) with high chemical reactivity (antioxidant and radical scavenging properties)
2. Cross-linking of microbial enzymes
3. Inhibition of microbial cellulases and pectinases
4. Chelation of metal ions necessary of enzyme activity
5. Formation of a physical barrier
6. Accumulation of phytoalexins after contact with fungal elicitors
7. Formation of flavonoid oxidation products (FOP).

The early activation of the phenylpropanoid metabolism is a strict prerequisite in the expression of disease resistance in plants and is, to a certain extent, dedicated to the reinforcement and chemical modification of plant cell walls (Matern and Grimmig 1993). Cinnamic acids can rapidly be incorporated into the polysaccharide fraction within 24 h following fungal elicitation. The acylation confers increased mechanical rigidity and renders the polysaccharides insensitive to hydrolytic enzyme activities by pathogenic fungi. Elicitation is also known to cause an oxidative outburst by reactive oxygen species (ROS) as a very rapid response. As ROS are toxic to cell membranes and other plant structures, the cells need a kind of self-protection. Hydroxycinnamic acids and esters are known as efficient radical scavengers and can fulfill this important task. Also other elicitor-inducible substances with antifungal properties were described, like the formation of aromatic aldehydes (vanillin, 4-hydroxybenzaldehyde,) produced from cinnamic esters (Matern and Grimmig 1993).

18.6.1 Resveratrols

The grapevine produces a multitude of chemical agents against fungal and bacterial attacks. Phytoalexins are defined as low molecular weight substances, which are synthesized de novo by the plant cell after fungal infection as part of a hypersensitive reaction. This concept excludes those substances that are present in the plant before any infection occurs. The most prominent agent is resveratrol, a stilbene derivative (Fig. 18.1), which occurs as *cis*- and *trans*-form. For steric reasons, the *trans*-form is the favored structure. In grapes the stilbene monomers occur as glucosides; they are termed piceids or polydatins. A number of derivatives are known in the meanwhile, present in different tissues, like in root tissue. Typical substances are the amurensins, viniferins, astringin, piceatannol, pterostilben, cyphostemmin, pallidols, and gnetins (Bavaresco et al. 2002; Pezet et al. 2003; Baderschneider and Winterhalter 2000; Huang et al. 2000; Ducrot et al. 1998; Carando et al. 1999; Kong et al. 2011).

The biosynthesis of resveratrol is different within the *Vitis* species. There is a significant correlation between their sensitivity against fungal diseases and the biosynthesis of resveratrol. In *V. amurensis* and *V. labrusca*, several authors could prove a significantly higher concentration of resveratrol and its derivatives compared with *V. vinifera*. The biosynthesis is carried out primarily before the véraison. Young, unripe grape berries are highly resistant against *Botrytis*. After véraison the anthocyanins are formed, whereas the biosynthesis of resveratrols declines (Jeandet et al. 1995).

The fungus *B. cinerea* is able to produce a stilbene oxidase, to be protected against the toxic resveratrol (Pezet 1998). This enzyme oxidizes resveratrol to higher molecular weight substances. Such a degradation product is resveratrol-*trans*-dehydrodimer (Breuil et al. 1998).

18.6.2 Flavonoids

Resveratrol is a quite efficient protective molecule for the grapevine, but not enough to be on the safe side. In most grapes, resveratrol comprises only 1% or less of the total concentration of polyphenols. Therefore, a broad arsenal of other polyphenolics is found in grapes, like flavonol glycosides and flavan-3-ols, including proanthocyanidins. Flavonol glycosides, like quercetins, myricetins, and kaempferols, are only localized in the berry skin, acting mainly as protective agents against radiation and UV light, whereas flavan-3-ols are situated in the berry skins and the grape seeds. Flavonols and flavanols possess remarkable antimicrobial properties (Harborne 1988; Treutter 2006).

In spite of the fact that anthocyanins are mainly discussed in relation to their function as attractants or to their protective function against UV radiation and light, they seem also to support plants against microbes. The structures of nearly

540 different anthocyanins have been elucidated, and more than half of these have been reported after 1992 (Anderson and Jordheim 2006). Several dozens of anthocyanin-based structures are found in grapes and red wines.

18.6.3 Formation of Flavonoid Oxidation Products (FOP)

The biological effects of flavonoids are linked to their antioxidative properties and their potential cytotoxicity. They act as scavengers of free radicals, like ROS, and prevent their formation by metal chelation. The behavior of oxidized flavonoids (quinones and related oxidation products) is different and has attracted much attention in plant physiology. Quinones are strong antibiotics, possess tanning properties, and are able to alkylate proteins (Pourcel et al. 2006). For example, kaempferol and quercetin polymers, produced by polyphenol oxidases (PPO), have a stronger ROS scavenger effect than the corresponding monomers.

Three enzymes are responsible for flavonoid oxidation: laccase (EC 1.10.3.2), catecholoxidase (EC 1.10.3.1), and peroxidase (EC 1.11.1.7). Laccases are *o*-diphenol- and *p*-diphenol dioxygen oxidoreductases, belonging to the group of blue copper oxidases. Those multi-copper glycoproteins consist of four histidine-rich copper-binding domains. Catechol oxidases are less glycosylated and possess two copper-binding domains. Peroxidases (POD) are hemoproteins, which oxidize phenols with concomitant reduction of hydrogen peroxide to water. PODs are able to form ROS, like superoxide anion radicals and hydroxyl radicals. Not all plants possess these three groups of enzymes.

Most PPOs are found in a latent form and must be activated. Catechol oxidases are localized in plastids, whereas PODs can have different locations. In healthy, non-senescent cells, the enzymes and substrates are distributed in different subcellular compartments. This is a kind of self-protection. Anthocyanins, flavonol glycosides, flavan-3-ols, and proanthocyanidins are sequestered in vacuoles. Oxidation can occur only after senescence or plant stress (wounding, elicitation by phytopathogenic microorganisms).

It is assumed that the physiological role of flavonoid oxidation is protection during seed and plant development and the defense against pathogen attacks. Flavonoids lead to browning of seed hulls and reinforce the barrier against water permeation. There is a positive correlation between oxidation of procyanidins and their cross-link in the cell wall. The autoxidation of quercetins leads to activated oxygen and subsequently to the formation of hydrogen peroxide. This is the substrate for POD, which enhances the autoxidation and induces the formation of 3,4-dihydroxybenzoic acid, a well-known antifungal agent. Quinones are toxic against pathogens. Wounding increases the formation of polyphenols and corresponding oxidizing enzymes, and the oxidized tannins can react by covalent binding with pectinases, cellulases, and laccases of fungi, leading to their inhibition (Pourcel et al. 2006).

18.6.4 Salicylic Acid: Systemic Acquired Resistance

Salicylic acid (2-hydroxybenzoic acid; see Fig. 18.2) is formed through elimination of a C_2 fragment from the phenylpropanoids. Following fungal infects or UV irradiation, higher salicylic acid concentrations can be found in plants. Presumably, the influences from outside induce the biosynthesis of defensive and protective agents (Heldt 1996). This is exploited during the induction of the so-called systemic acquired resistance (Durner et al. 1997), where salicylic acid has a role as regulatory molecule (Métraux et al. 2008). Grapes are sprayed with an aqueous salicylic acid solution. This induces the biosynthesis of phytoalexins and protects the plant and its fruits (Ryals et al. 1996; Qin et al. 2015). There is big interest especially in ecologically orientated wineries to use salicylic acid against downy mildew. Several studies have confirmed a dosage-dependent fungistatic and fungicidic effect of salicylic acid (Amborabé et al. 2002). Furthermore, salicylic acid is an important mediator with hormonelike character in plants. Kreava et al. (1998) have shown that injection of an aqueous salicylic acid solution into grape berries retards ripening of these berries. Obviously, the plant possesses the potential to delay ripening in certain cases.

To date only little is known on the incorporation of salicylic acid after wine ingestion in humans. Yet, this seems to be of reasonable importance, given the fact that high consumption of salicylic acid can lead to hypersensibilization (Haeberle 1987). In contrast to this negative impact, also positive effects have been reported, such as antithrombotic and blood-diluting effects (Muller and Fugelsang 1994).

One study has analyzed the impact of an exogenous salicylic dosage on the final salicylic acid levels in wines. No elevated salicylic acid levels were found. Generally, the concentrations on this compound were very low. In white musts and wines, a mean of 0.01 mg/L was found. In red musts and wines, the mean was slightly higher with 0.16 mg/L. In total none of the wines had salicylic levels above 0.43 mg/L (Pour Nikfardjam et al. 1999).

18.7 Antimicrobial Effects of Polyphenols in General

Flavonoids in food and their health effects have been studied thoroughly (Rice-Evans and Packer 1998; Yao et al. 2004; Hoensch and Oertel 2015). Especially, berry fruits are rich sources of bioactive compounds, such as flavonoids, phenolics, and organic acids, which have antimicrobial activities against human pathogens. Among different berries and berry phenolics, cranberry, cloudberry, raspberry, strawberry, and bilberry especially possess clear antimicrobial effects against, for example, *Salmonella* and *Staphylococcus*. Complex phenolic polymers, like ellagitannins, are strong antimicrobial and antibacterial agents present in cloudberry, blackberry, strawberry, pomegranate, and raspberry (Quideau 2009; Yoshida et al. 2009). Several mechanisms of action in the growth inhibition of bacteria are

Table 18.13 Examples for antimicrobial effects of different polyphenols

Product	Polyphenols	Effects and target	Literature
Artichoke	Chlorogenic acid isomers, combined with flavones	Antimicrobial	Zhu et al. (2004)
Finnish berry fruits	Flavonoids, including anthocyanins, hydroxycinnamic acid derivatives	Antimicrobial	Heinonen (2007)
Finnish berry fruits	Flavonoids, phenol carbonic acids	Antimicrobial against pathogenic colon bacteria, mostly gram-negative, *Salmonella*, *Staphylococcus*	Puuponen-Pimiä et al. (2001, 2005)
Native olive oil	Hydroxytyrosol derivatives tyrosol, oleuropein, decarboxymethyl- ligstrosid aglycon, pinoresorcinol	Antibacterial, *Helicobacter pylori* (partially resistant against antibiotics)	Romero et al. (2007)
Cranberry	Proanthocyanidins	Inhibition of adherence of uropathogenic P-fimbriated *Escherichia. coli*	Foo et al. (2000)
Red wine, Cranberry	Flavonoids, anthocyanins	Anticariogenic, growth inhibition of *Streptococcus mutans*	Thimothie et al. (2007)

involved, such as destabilization of the cytoplasmic membrane, permeabilization of the plasma membrane, inhibition of extracellular microbial enzymes, direct actions on microbial metabolism, and deprivation of the substrates required for microbial growth. Antimicrobial activity of berries may also be related to antiadherence of bacteria to epithelial cells, which is a prerequisite for colonization and infection of many pathogens. Antimicrobial berry compounds may have important applications in the future as natural antimicrobial agents for food industry as well as for medicine (Puuponen-Pimiä et al. 2005; Yang and Kortesniemi 2015).

Every plant has developed its own strategy to fight against fungal, bacterial, and viral diseases (Hammerschmidt and Hollosy 2008). This is demonstrated with a few examples from different plant sources (Table 18.13). It can be expected that the phenolics of grapes and wines possess similar effects, based on their broad range of these substances and their similar structures.

18.8 Polyphenols Formation During Winemaking

Some polyphenols are formed when the berries are crushed and fermented. One of these phenols is tyrosol. It is formed through deamination from the amino acid tyrosine. Further hydroxylation leads to hydroxytyrosol. The latter was first analyzed in olive oil of extra vergine quality. Later it was shown that it is ubiquitous in all parts of the olive tree. In 1982, it was discovered in fermented products. Tommaso et al. (1998) analyzed several white wines from Italy and found a mean concentration of 1.8 mg/L. The final content on hydroxytyrosol depends on several

factors, such as variety, fermentation technology, and yeast strain (Romboli et al. 2015). Due to its antioxidative properties, hydroxytyrosol has been tested as an alternative to SO_2. Raposo et al. (2016a, b) have shown that wines treated with hydroxytyrosol show better color and flavor properties at bottling but show weaker quality and less varietal character after 6 months storage compared to wines treated with SO_2.

The grape reaction product (GRP, 2-*S*-glutathionyl-caftaric acid) and the GRP2 (2,5-*S*-diglutathionyl-caftaric acid) are also only formed after crushing and under oxidative conditions (Du Toit and Oberholster 2014). Caftaric acid is oxidized by tyrosinase to the respective quinone, which then reacts with glutathione (GSH) to GRP. Thus, the GRP can be regarded as an indicator for an enzymatically driven oxidation. Especially, in grape material with a high amount of infected berries, the laccase from *B. cinerea* can further oxidize GRP and lead, after incorporation of another GSH molecule, to the GRP2. Tyrosinase cannot oxidize GRP or GRP2. The GSH level of the must and wine is of utmost importance for further browning reactions. As long as enough GSH is present, the quinone formed from caftaric acid reacts with GSH and browning does not occur (Hosry et al. 2009).

A further large group of phenols which is only formed during winemaking are degradation products of the anthocyanins and flavan-3-ols. They can react with several wine compounds and form new substances with interesting characteristics. Mainly three compounds or compound groups take part in the reactions:

1. Flavan-3-ols (catechins)
   It is well known that anthocyanins react with flavan-3-ols to form higher molecular compounds. Partly acetaldehyde, which is formed during fermentation, acts as a bridge between the anthocyanin and the flavan-3-ol.
2. Ellagitannins
   Ellagitannins are extracted from the wood and, thus, mainly occur in wines with maturation in barrique. Ellagic acid is part of the lignin structure of the wood and is hydrolyzed from the wood by enzymatic and chemical processes. It preferably reacts with flavan-3-ols forming new structures.
3. Various wine compounds with low molecular mass
   The anthocyanins can react with various wine compounds. This leads to the formation of the so-called pyranoanthocyanins. Molecules, like pyruvate, vinylphenol, or acetone, react with an anthocyanin molecule, forming a new pyran ring. These pyranoanthocyanins have been found in fermenting and aged wines. Other wine compounds can react with the newly formed pyran, leading to highly complex structures, which have been identified in port wines and partly account for their blue color tonality (Mateus et al. 2003; Oliviera et al. 2013).

For more details, see Ribéreau-Gayon et al. (2000), Monagas et al. (2005), Cheynier (2006), and Garrido and Borges (2013).

18.9 Interactions of Polyphenols with Wine Yeasts (*Saccharomyces cerevisiae*) and Other Microorganisms Before/During/After Fermentation

The levels of anthocyanins and proanthocyanidins recovered in red wines at the end of fermentation represent 20–40% of their amounts in the corresponding grapes (Cheynier 2006). Principally, the amount of polyphenols is decreased by mashing and pressing, and a large quantity of these secondary grape metabolites remain in the pomace (Hang 1998; Kammerer et al. 2005; Drosou et al. 2015). The biggest loss is found for white wines, which contain only minor concentrations of flavonoids, stilbenes, and hydroxycinnamic acid derivatives. Also, yeasts can decrease a part of these substances by adsorption. It was shown that the *Saccharomyces cerevisiae* strain 9CV could decrease especially *p*-coumaric acid derivatives up to 29%. In the case of anthocyanins, the 3-*O*-glucosides and the *p*-coumaroylated anthocyanins were depleted by 52.6% and 36.6%, respectively (Morata et al. 2005).

Some research has also shown that the yeast strain has a clear impact on the final polyphenolic composition of the wines. Caridi et al. (2004) have analyzed the effect of two *S. cerevisiae* strains (Sc2659 and Sc1483). According to their results, Sc2659 led to deeper color, higher polyphenol content, and higher antioxidative capacity.

Saccharomyces yeasts are sufficiently tolerant of the presence of polyphenols. Therefore, it is no problem to ferment grape musts to wines or fruit juices to fruit wines. Nevertheless, a certain inhibitory effect on fermentation, but also on the inhibition of microbial spoilage and deterioration, can be found for several microorganisms, depending on the concentration and composition of the polyphenols. A remarkable inhibition of fermentation is known for several benzoic acid derivatives. Free benzoic acid is found in different *Vaccinium* fruits, for example, 0.6–1.3 g/L in lingonberries, which are difficult to ferment. 0.2–0.3 g/L prevent the growth of yeasts (Visti et al. 2003). Inhibitory effects on the fermentation are also found for the gallic acid and ellagic acid derivatives.

Under certain circumstances, the undesired fermentation of fruit juices can be delayed for days and weeks. We found that pasteurized and bottled apple juices made from polyphenol-rich cider apples are stable for a long period after opening the bottles. On the contrary, if we investigated apple juices made from dessert (table) apples with low polyphenol concentrations, the fermentation started within a few days after opening of the bottles (unpublished). It seems that polyphenolics inhibit foreign microorganisms.

The antimicrobial effect of apple, grape, orange, and red beet phenolics was shown for *Alicyclobacillus acidoterrestris*, a thermophilic spoilage bacterium (Wieland et al. 2002). Cloudy juices contained a significantly higher amount of polyphenolics and inhibited the growth of *Alicyclobacillus*, whereas the corresponding clear products, where a part of polyphenolics was removed by clarification, were sensitive against spoilage. The best growth was found in apple juices made from clear apple juice concentrates, due to their low polyphenol

concentration. The authors recommend gentle fruit processing and the preservation of these protective substances in the juices. Similar results for *Alicyclobacillus* were found by Brodbeck et al. (2004) for iced tea beverages. Besides the factors temperature (>25 °C), pH (<5.8, optimum <4.6), oxygen (>0.1%, strictly aerobic), ascorbic acid, redox potential, and storage time, growth is dependent on the presence or absence of polyphenolics. Freshly prepared tea contains antimicrobial flavan-3-ols, whereas their content in soluble tea extracts is diminished by 90%. Therefore, the inhibiting effect on microorganisms gets lost. The addition of polyphenolics to iced teas is recommended by the authors to ameliorate stability.

After fermentation, yeast cell walls are protected by wine polyphenols toward the action of hydrolytic enzymes. Salmon et al. (2003) studied the effect of wine polyphenols on the shape of wine yeast after fermentation. Their results show that yeast cells in the absence of wine polyphenols rapidly reach a flat shape after the end of alcoholic fermentation. With wine polyphenols, they keep a spherical and almost intact shape, which might be an indicator for a decelerated yeast cell autolysis evoked through the aforementioned antioxidative protection against hydrolytic enzymes through the wine polyphenols.

Polyphenols interact also with the bacteria of the malolactic fermentation. Some phenolics can influence the growth of bacteria in a positive or a negative way. 50–150 mg/L caffeic acid supported the growth and the degradation of malic acid in Merlot wine, whereas ferulic acid was inhibitory; coumaric acid had even a more negative effect (Krieger-Weber 2007). In the same study, the tannins from grape seeds inhibited the growth of malolactic bacteria.

18.9.1 Increasing the Live Expectancy of Saccharomyces with Resveratrol

Studies, which have been conducted at the Harvard Medical School in the USA, have revealed that resveratrol increases the live expectancy of *S. cerevisiae*. The activity of sirtuin 2 (Sir2) is increased. Sirtuins are enzymes (NAD-dependent histone deacetylases), which are important for DNA regeneration. The calorie consumption is mediated by Sir2 (Horwitz et al. 2003). In some organisms, this "calorie restriction" has led to an increased live expectancy (Barger et al. 2003). This could be shown for rats, mice, and *Drosophila melanogaster* (Conti et al. 2006; Rauser et al. 2004). In the studies of Horwitz et al. (2003), the life span of *S. cerevisiae* was extended by 70%. The impact of calorie restriction on human health has been intensively reviewed by Ravussin et al. (2016).

Studies on other organisms, such as mice, have revealed that resveratrol promotes longevity and improves glucose homeostasis by stimulating the Sir1-mediated deacetylation of the transcriptional coactivator PGC-1α (Koo and Montminy 2006; Olesen et al. 2013).

18.10 Conclusions

The biosynthesis of polyphenols is an energy-costly process for the grape but necessary for the survival of the fruit. The antimicrobial properties play a major role in this context. Grapes and wines are characterized by a very complex composition of these secondary plant metabolites. This complexity is also due to the fact that the original polyphenols are intensely transformed during the processing into must and wine. A further factor is the subsequent chemical transformation of anthocyanins and colorless polyphenols during wine aging. Presently, it is essentially nothing known on inhibiting or supporting effects of these products on yeasts and other microorganisms.

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Part IV
Molecular Biology and Regulation

Chapter 19
Genomic Evolution and Adaptation to Wine of *Oenococcus oeni*

Hugo Campbell-Sills, Marc Lorentzen, and Patrick M. Lucas

19.1 Introduction

O. oeni is the main driver of malolactic fermentation. It grows slower than other lactic acid bacteria (LAB) but flourishes late in wine fermentation because it has better tolerance to high acidity and alcohol content. The first sequenced *O. oeni* genome in 2005 revealed several adaptations to the wine environment, such as loss of metabolism and biosynthetic pathways of many vitamins, cofactors, sugars, and amino acids, which are compensated by the richness of grape juice and autolysis of yeast. *O. oeni* also lacks several DNA maintenance genes and mutates faster than other related LAB species.

In recent years, the number of available genomes has increased rapidly and has enabled the creation of detailed phylogenomic trees and comparisons of genetic repertoires. These tools have given important clues about the evolution of the species and the adaptation and specialization of strains in specific environments. Two main genetic groups have been identified to date, as well as a third putative group and several subgroups. Regions, grape variety, and year of isolation seem to have little effect on the grouping, but strains isolated from wine and cider—two products with different conditions for fermentation—appear to group differently, as do certain styles of wine, suggesting that the adaptation is due rather to the wine environment, and not to geographical constraints.

Oenococcus oeni is the lactic acid bacteria (LAB) species most frequently detected in wine. It was first described by Garvie in 1967 and named *Leuconostoc oenos* on the basis of phenotypic similarities with other species of this genus, while it was also distinguished by its ability to develop in an acidic medium (Garvie

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1967). Molecular methods later revealed that it is indeed distantly related to the *Leuconostoc* genus, and in 1995 it was reclassified in a newly created genus, *Oenococcus* (Dicks et al. 1995). *O. oeni* was the only known species of its genus until 2006, when *Oenococcus kitaharae* was discovered in composting distilled shochu residues in Japan (Endo and Okada 2006). More recently, a third species named *Oenococcus alcoholitolerans* was isolated from cachaça and alcohol fermentation vats in Brazil (Badotti et al. 2014). Although all three species are adapted to alcohol-rich environments, up to date only *O. oeni* has been reported in wine. The species is also associated with cider but rarely found in other niches. It is a minor species in the environment and hardly detectable in the vineyard at the surface of grape berries. In contrast, thanks to its tolerance to the grape must acidity and the ethanol produced by yeasts during alcoholic fermentation (AF), it develops faster than other LAB in wine and often becomes the only detectable species after AF (Fleet et al. 1984).

O. oeni is responsible for the malolactic fermentation (MLF)—also called the secondary fermentation—which is commonly performed after AF in red wines and in some varieties of white wines. The main goal of MLF is to reduce the sourness caused by malic acid that is still present in wine following AF. During MLF *O. oeni* converts L-malate into L-lactate and carbon dioxide, which reduces the acid taste of wine, slightly increases the pH, and provides a softer mouth feel (Henick-Kling 1993). *O. oeni also* transforms a variety of sugars, organic acids, amino acids, and aroma precursors, among other metabolites, into esters, alcohols, thioesters, thiols, and other products that contribute to flavor modifications and increase the microbiological stability of wine by removing potential substrates (Bartowsky 2005). The good progress of MLF and the changes it brings to the wine are greatly dependent on *O. oeni* strains. Indigenous strains may delay the start or cause sluggish MLF and sometimes produce undesirable aromas or compounds such as biogenic amines (Lonvaud-Funel 1995, 1999).

Winemakers who want to control MLF often inoculate industrial malolactic starters in wine. These are *O. oeni* strains selected for their ability to survive in wine, to perform MLF rapidly, and to enhance positive attributes of wine (Lonvaud-Funel 2001; Torriani et al. 2011). Understanding the diversity and genetic attributes of the species is necessary to select the best-adapted strains to produce particular wines. This is why *O. oeni* was one of the first LAB species whose genome was fully sequenced (Mills et al. 2005) and also one of the LAB species with the most genomes reported to date (Borneman et al. 2010, 2012a; Campbell-Sills et al. 2015; Capozzi et al. 2014; Jara and Romero 2015; Lamontanara et al. 2014; Mendoza et al. 2015; Sternes and Borneman 2016). Consequently, this review describes the general properties of the *O. oeni* genome in relation to the oenological role of bacteria, as well as the new insights on the evolution and adaptation of strains that come from the comparison of nearly 200 genomes.

19.2 The *O. oeni* Genome

19.2.1 General Features

The genome of *O. oeni* strain PSU-1 was the first to be released in 2005 (Mills et al. 2005). Although nearly 200 additional genomes are now available, strain PSU-1 is still the reference because it is the single fully assembled sequence reported to date. *O. oeni* has a compact genome of approximately 1.8 Mb, ranging from 1.7 to 2.0 Mb depending on strains (Table 19.1) (Sternes and Borneman 2016). It is rather small compared to other LAB and results presumably from specialization to the narrow ecological niches of wine and cider (Gibbons and Rinker 2015; Makarova et al. 2006). Compared with the nearest *Leuconostoc* genome, *O. oeni* has lost genes involved in sugar uptake and metabolism and biosynthetic pathways for cofactors such as glutathione, riboflavin or thiamine, and many amino acids (Makarova et al. 2006). Its auxotrophies are compensated by the abundance of sugars and amino acids in grape juice and wine and also by the capacity of bacteria to import the essential vitamins, amino acids, and nutrients released by yeast cells during their autolysis that takes place at the end of AF. Only two copies of the rRNA operon are present in the *O. oeni* genome, whereas LAB genomes contain very rarely less than four copies, and up to nine have been observed (Klappenbach et al. 2001; Stoddard et al. 2015). It has been suggested that the rRNA copy number is positively correlated with rapid growth that requires high translational activity or fluctuating growth conditions that require the capacity to respond rapidly (Klappenbach et al. 2000). This is consistent with the behavior of *O. oeni* which is a slow-growing species that is unable to outcompete other LAB species in the natural environment but that takes advantage of its adaptation to acidity and ethanol to slowly become the prevailing species in wine. The number of tRNA genes—40 to 45 depending on strains—which correlates with the number of rRNA operons, is also among the lowest in LAB, but they represent all 20 amino acids and are redundant for most of them (Mills et al. 2005).

19.2.2 Hypermutability

O. oeni was early considered a fast-evolving species on the basis of 16S rRNA analysis (Yang and Woese 1989). Its genome has revealed a possible explanation: one of its most striking features is that it lacks the *mutS* and *mutL* genes involved in the DNA mismatch repair system (Mills et al. 2005). This system is highly conserved among all living organisms and greatly contributes to maintaining genome stability through the correction of mismatched base pairs. Its absence is most likely a characteristic of the genus *Oenococcus*, since it is also absent from the sister species *O. kitaharae* and *O. alcoholitolerans* (Borneman et al. 2012b; Campbell-Sills et al. 2015). It correlates with the high mutation rate and

Table 19.1 General features of *Oenococcus* genomes

Species/Strains	Origin	Sequences		Size (Mbp)	Genes	rRNA operons	tRNAs	CRISPR	Malate utilization	Citrate utilization
		Draft	Complete							
O. alcoholitolerans	Bioethanol plant, cachaça	1	0	1.143	1619	1	18	+	+	–
O. kitaharae	Shochu residues	3	1	1.786–1.840	1820–1883	2	43–44	+	–	–
O. oeni	Wine, cider	197	1	1.727–2.013	1621–1974	2	40–45	–	+	+
O. oeni/group A	Wine	160	1	1.727–2.013	1862–2200	2	40–45	–	+	+
O. oeni/group B	Wine, cider	25	0	1.753–1.873	1918–2044	2	40–45	–	+	+
O. oeni/group C (putative)	Wine, cider	12	0	1.789–1.912	1842–1987	2	42–43	–	+	+

compositional bias of spontaneous mutations in both *O. oeni* and *O. kitaharae* (Marcobal et al. 2008). It is possible that hypermutability has allowed the fast adaptation of *O. oeni*, *O. kitaharae*, and *O. alcoholitolerans* to their respective and restricted environments: wine/cider, shochu distillate residues, and cachaça/bioethanol plants, respectively. In agreement with this hypothesis, an analysis of single nucleotide polymorphisms (SNP) among 50 *O. oeni* strains has revealed specific mutations for each subgroup of strains, with as much as 1085 unique SNPs in strains isolated from champagne, with more than half corresponding to missense mutations that can affect the functionality of the encoded proteins and possibly contribute to adaptation to this specific type of wine (Campbell-Sills et al. 2015).

19.2.3 Plasmids and Phages

LAB are frequently endowed with plasmids carrying functions that improve their adaptation to specific environments through the use of substrates or the resistance to stressors and phages (Mills et al. 2006). In contrast, less than ten different plasmids were reported to date in *O. oeni*, which is very low compared to the number of genomes available. Most of the strains do not carry any plasmid, and, even when one has been detected, it is generally a small cryptic plasmid encoding only the machinery required for its own replication and propagation. Nevertheless, a group of closely related plasmids of around 20 kb has been detected among commercial malolactic starters and indigenous strains performing MLF (Favier et al. 2012). Their genetic repertoire revealed two candidate genes which are possibly linked to the fitness of the strains: a putative sulfite exporter and an oxidoreductase of the old yellow family. More plasmids encoding adaptive functions are expected in environmental strains, but they might be lost during cultivation in laboratory conditions. For example, the capacity to produce the biogenic amine histamine is supposedly linked to a large unstable plasmid that is often present in wild strains but lost in laboratory isolates (Lucas et al. 2008).

Several prophage sequences have been identified in *O. oeni* genomes, and most of the strains—including industrial ones—have been confirmed as lysogenic (Borneman et al. 2012a, b; Jaomanjaka et al. 2013). Up to date, the temperate bacteriophages that have been characterized in *O. oeni* can be classed into four groups on the basis of their integrases, from int_A to int_D (Jaomanjaka et al. 2013). Their contribution to genomic changes in *O. oeni* has been poorly investigated until now, but it is likely that oenophages might benefit to the fitness of their host strains by interrupting or silencing genes, introducing new factors or promoting genomic rearrangements as described in other LAB. Bacteriophages are also active in wine and possibly responsible for delayed or sluggish MLF (Henick-Kling et al. 1986; Jaomanjaka et al. 2013). CRISPR are part of the prokaryotes' defense mechanisms against phagic DNA. Up to date, no CRISPR-associated proteins have ever been detected in *O. oeni* strains, although a type II CRISPR RNA-guided endonuclease Cas9 protein can be found by BLAST in *O. kitaharae*. Some small contigs of the

only *O. alcoholitolerans* sequenced strain match for the same protein (Campbell-Sills, personal data), although the sequences are truncated by contig gaps and no integral predicted protein has been identified. It is still unknown whether *O. oeni* lost these genes at some point of its divergence from the *Oenococcus* branch or if *O. kitaharae* and *O. alcoholitolerans* acquired them independently.

19.3 Intraspecific Genomic Variability

19.3.1 Phylogenomic Structure of O. oeni

The population structure of *O. oeni* was first investigated using molecular methods such as multilocus sequence typing. Despite the high genetic diversity and recombination rate observed among strains, it was possible to discern groups of clonal descents—particularly two main groups designated A and B—and several subgroups that sometimes contained strains isolated from the same region, such as Chile or South Africa, or the same product, such as cider or champagne (de Las Rivas et al. 2004; Bilhere et al. 2009; Bridier et al. 2010). Phylogenomics now makes it possible to reconstruct the evolutionary history of microorganisms using whole genome data and methods such as SNP concatenation, average nucleotide identity, or genomic signatures (Delsuc et al. 2005). Recent analyses performed using data sets of 50 and 191 *O. oeni* genomes have confirmed the previously reported distribution of strains in the main groups A and B and also in a putative third group C (Fig. 19.1) (Campbell-Sills et al. 2015; Sternes and Borneman 2016). The region, grape variety, and year of isolation do not appear as the main factors delimiting the groups of strains. In contrast, strains from cider and wine group apart, as do strains isolated from certain types of wines. Group B strains form subgroups associated with either cider or wine, while group A strains are found only in wine. In fact group A strains are much more frequently detected in wine during MLF than those of group B (Campbell-Sills et al. 2015). In addition, group B strains are genetically more diverse than group A strains, and the putative group C seems to belong to a more ancestral branch. This distribution suggests that group A strains were domesticated to wine, and strains from several subgroups of A were further domesticated to specific types of wines such as champagne and Burgundy wines (Campbell-Sills et al. 2015).

19.3.2 Core- and Pan-Genomes

In genomics, it is often more informative to study the genomic features of a group of strains as a whole, instead of the genes of each strain individually. Two concepts that are commonly used in comparative genomics are the pan-genome, which refers

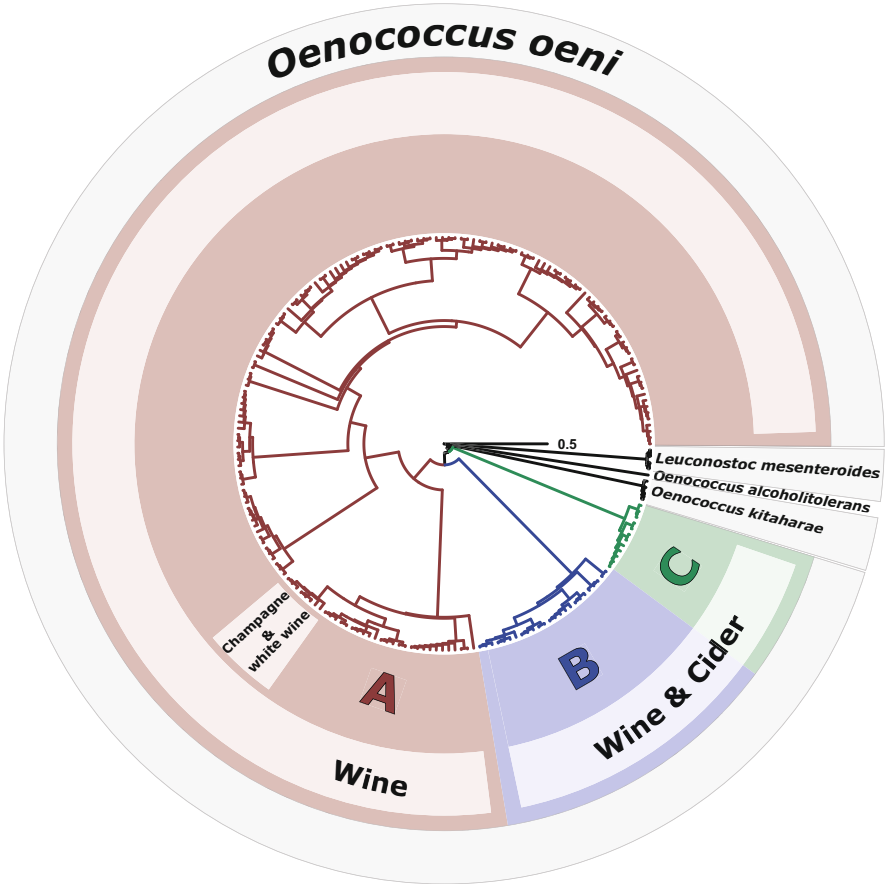

Fig. 19.1 Phylogenomic tree of publicly available genomes of *Oenococcus* genus and strains of the closest species *Leuconostoc mesenteroides ssp. mesenteroides* and *L. mesenteroides ssp. cremoris*. The genomic distances have been calculated by average nucleotide identity, and the branches lengths have been rescaled by Grafen's method for better display. The genetic groups of *O. oeni* **a**, **b**, and **c** are indicated in *red*, *blue*, and *green*, respectively. The products in which the strains are found are indicated by the *light boxes*

to the whole set of genes that can be present in a group of organisms, and the core-genome, which is the portion of these genes that are shared by every single individual of the group. If the individuals of the group are not identical, the core-genome size will always be smaller than the pan-genome, and when adding individuals to the group, the core-genome size will tend to decrease as the pan-genome size will tend to increase. The first analysis of the pan-genome of three *O. oeni* strains revealed a core-genome of 1216 and a pan-genome of 2360 ORFs (Bartowsky and Borneman 2011). Further analyses including 14 strains revealed core- and pan-genome sizes of 1165 and 2846 ORFs, respectively (Borneman et al. 2012a, b), while an analysis of 50 strains revealed core- and

pan-genome sizes of 1368 and 3235 ORFs (Campbell-Sills et al. 2015). The inconsistency of the core-genome size increasing with a higher number of strains can be explained by the differences in the methods that were used to estimate the core- and pan-genomes. Moreover, a bias has been observed in the composition of the pan-genomes of strains from group A and B: although strains from group B are less numerous in the examined samples, their core-genome is smaller of that of group A, but their pan-genome is comparable in size. This is consistent with the higher genetic diversity of group B strains and might also be a sign of different degrees of domestication (Campbell-Sills et al. 2015).

19.3.3 Industrially Relevant Genes

The availability of numerous genomic sequences is an opportunity to unravel genes of industrial relevance which might explain the different aptitudes of strains to survive in wine, to perform MLF, and to modify the sensory properties of wine more or less favorably.

Up to date, the malolactic pathway, which comprises the *mleR*, *mleA*, and *mleP* genes, has been detected in all genomes, suggesting that the capacity to perform MLF is ubiquitous among *O. oeni* strains. The sister species *O. kitaharae* carries the *mleA* gene, but it is unable to perform MLF due to a nonsense mutation in the coding sequence (Endo and Okada 2006; Borneman et al. 2012a, b). A genomic analysis of *O. alcoholitolerans* suggests that it is likely capable of performing MLF, since the coding sequence of the operon genes does not carry any nonsense SNP (Badotti et al. 2014).

As for malate, it seems that all *O. oeni* strains sequenced to date can consume citrate as they all contain the citrate operon (*citI*, *citM*, *maeP*, *citC*, *citD*, *citE*, *citF*, *citX*, *citG*). The consumption of citrate is well known from winemakers as it produces diacetyl and its derivatives butanediol and acetoin that confer a buttery attribute to wine. This aroma is generally well appreciated when low amounts of diacetyl occur in wine, but it becomes unpleasant when diacetyl is in excess. Surprisingly all the strains carry the same citrate operon, but they differ in the amounts of diacetyl that they release during MLF. Their differences may come from the regulation of gene expression levels or their capacity to co-metabolize citrate and sugars (Ramos and Santos 1996). *O. kitaharae* shows again an opposite behavior as it lacks the whole citrate operon (Borneman et al. 2012b).

A survey on 191 *O. oeni* strains identified 390 flexible genomic islands, some of which contain industrially relevant genes. The most relevant four include genes implied in amino acid biosynthesis, phosphotransferase systems, sugar utilization, and natural competence (Sternes and Borneman 2016). Independent studies on the auxotrophies for amino acids in *O. oeni* strains, as well as sugar utilization profiles, confirm these findings and might explain the different performances of *O. oeni* strains to achieve MLF (Remize et al. 2006; Hocine et al. 2010). Moreover, strains from certain genetic groups or subgroups have exclusive genetic features, some of

which belong to phage elements (Campbell-Sills et al. 2015). It has also been observed that strains from group A have different truncated versions of the competence gene *com*EA, which remains intact on strains of groups B and C (Sternes and Borneman 2016). Natural competence genes confer bacteria the capacity to assimilate foreign DNA into their own cytoplasm, which can act as a horizontal gene transfer mechanism to increase fitness. The fact that these genes are truncated in group A strains might be one of the reasons why these strains are genetically less diverse, while at the same time they are more domesticated to wine than the rest.

19.4 Biogeography

Numerous studies based on molecular methods were performed during the past 30 years to evaluate the diversity of *O. oeni* strains in regional wines. They revealed that there is a huge diversity in each region, with up to ten different strains simultaneously in the same vat during MLF. However, some strains persist in the cellar during several consecutive vintages (Kelly et al. 1993; Reguant and Bordons 2003; Larisika et al. 2008; Gonzalez-Arenzana et al. 2015). Until recently it was unclear whether strains were specific to a region or not. This is an important issue for determining whether they contribute to the unique properties of regional wines or if they can be considered as a microbial component of the terroir. Recent surveys have shown that strains present in a region may belong to different genetic groups (A and B, as described above) and that they ferment the local wines more or less efficiently (Bordas et al. 2013; Gonzalez-Arenzana et al. 2014; Garofalo et al. 2015). A comparison of nearly 3000 *O. oeni* isolates from different vineyards has confirmed that there is a considerable strain diversity in the regions and that each region holds a unique set of several hundreds of strains, which is in agreement with previous studies suggesting that vineyards represent different microbial terroirs (Bokulich et al. 2014; Knight et al. 2015). However, the strains present in a region belong to different genetic groups, some of which are also detected in distant locations, indicating that they are not genetically exclusive to any particular region (El Khoury et al. 2017). In contrast, there are clear cases of adaptation to different products (cider, wine) or different types of wines (white wines from champagne) (Campbell-Sills et al. 2015; El Khoury et al. 2017).

19.5 Conclusions

The number of available *O. oeni* genomes has increased rapidly in recent years with the advance of next-generation sequencing. The evolution of the species is being unraveled by comparative genomics and population structure analysis, which provides the basis for a segregation of *O. oeni* into two major groups, as well as a third putative group and several subgroups. The pan-genome, meanwhile, of the

sequenced strains provides detailed information on the metabolic pathways and paves the way for computational and "omics" approaches to model the organism and to relate the metabolism of *O. oeni* strains to specific wine flavors and fermentation strategies for the industry. Genomic intraspecies comparison will provide more clues for the evolution and adaptation for each group to a specific environment. With the move toward more comprehensive data sets, like the community shotgun approach, the "omics" disciplines are expected to further delve into the interactions between *O. oeni* and its environment in the winery.

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Chapter 20
The Genomes of Acetic Acid Bacteria

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Abbreviations

EDP	Entner–Doudoroff pathway
PPP	Pentose phosphate pathway
PQQ	Pyrroloquinoline quinone
TCA cycle	Tricarboxylic acid cycle

20.1 Introduction

Acetic acid bacteria are strictly aerobic, acidophilic organisms that are known for their rapid incomplete oxidation of alcohols, polyols, or sugars and their derivatives. They are the elicitors of various wine faults, mainly the formation of vinegar taste due to direct oxidation of ethanol to acetic acid with acetaldehyde as an intermediate when oxygen is available. Complete genome sequences provided rich information on the physiology and biochemistry of acetic acid bacteria and reflected their adaptation to nutrient-rich habitats. The stereo- and regioselective direct oxidations are performed by membrane-bound pyrroloquinoline quinone (PQQ) or flavine-dependent dehydrogenases with their active sites facing toward the periplasm. The membrane-bound dehydrogenases feed the electrons derived from the oxidations directly into a short electron transport chain, conserving energy by forming a proton motive force. Besides the membrane-bound dehydrogenases, the organisms have an additional set of dehydrogenases located in the cytoplasm. They may function mainly in carbon assimilation. The central metabolism seems to be specialized in providing building blocks for biosynthesis. Glycolysis is incomplete due to a missing phosphofructokinase, but a pentose phosphate cycle is functional. In the genus *Gluconobacter*, the tricarboxylic acid (TCA) cycle is not closed, because succinate thiokinase and succinate dehydrogenase are missing. Also, there is no glyoxylic acid cycle and gluconeogenetic phosphoenolpyruvate

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formation from acetate. Therefore, it is not possible to overoxidize acetate. Organisms like *Acetobacter* have a complete TCA and a glyoxylic acid cycle, allowing them to overoxidize acetate in a second growth phase. For the activation of acetate in some strains, a succinyl-coenzyme A (CoA):acetate CoA-transferase (AarC) is present, which replaces succinyl-CoA synthetase in the TCA.

Acetic acid bacteria (AAB) classified in the family *Acetobacteraceae* are a diverse group of Gram-negative, rod-shaped, acidotolerant to acidophilic organisms with a strictly aerobic type of metabolism. The group has been taxonomically revised many times and currently comprises 18 genera (Trcek et al. 2015; Yamada and Yukphan 2008). Overview on the literature is hindered by many taxonomic changes resulting in some strains renamed several times. This can pose problems when, for example, enological literature sticks to traditional names; while in the literature of microbiology, more recent names are used for the same organism. For example, the traditional *Acetobacter xylinum* was first reclassified as *Gluconacetobacter xylinus* and is recently renamed *Komagataeibacter xylinus*.

Acetic acid bacteria are fastidious organisms that thrive in complex, nutrient-rich environments such as on the surfaces and in the gastrointestinal tracts of honey bees or other insects, on the surface of fruits, in nectar, plant saps, fruit juices, cider, must, wine, sake, beer, or other sweet or alcoholic beverages (Mamlouk and Gullo 2013). Some genera such as *Gluconobacter* can grow in highly concentrated sugar solutions of up to 30% (w/v) glucose and others at pH values as low as 2.5, tolerating high acetate concentrations. Acetate is toxic, because it uncouples the proton motive force especially at low pH values. While most bacteria cease to grow at acetate concentrations of $<0.5\%$, acetic acid bacteria that are typically associated with vinegar production such as *Acetobacter pasteurianus*, *Gluconacetobacter europaeus* (now *Komagataeibacter europaeus*) (Vegas et al. 2010), and others can endure acetate concentrations of up to 18% (Trcek et al. 2000, 2015). Using sophisticated process engineering, it is even possible to produce alcohol vinegar with an acidity of up to and exceeding 20% acetic acid by submerged fermentation with acetic acid bacteria. The reasons for this high resistance are not yet completely clear but seem to be attributed to the presence of transporters indicated from the genome sequence that are homologs of multidrug resistance transporters that pump acetate out of the cell (Trcek et al. 2015). Proton motive force-dependent efflux systems for acetic acid have also been physiologically characterized (Matsushita et al. 2005). Since some high acid-tolerant strains seem to lack genes for such transporters, it might not be the whole story, but nevertheless it suggests that an active energy metabolism is required for the maintenance of acetic acid resistance.

The most prominent physiologic feature of acetic acid bacteria is that they derive their energy from incomplete oxidations of a multitude of substrates, mainly alcohols, sugars, and other polyols. The stereo- and regioselective oxidations occur in single steps while retaining the carbon skeleton of the oxidized compounds. They are carried out by a multitude of membrane-bound dehydrogenases with often broad substrate spectra, located in the cytoplasmic membrane. The active sites of such dehydrogenases are facing toward the periplasm. The most important of the oxidations catalyzed by these enzymes with respect to enology are the

oxidation of ethanol to acetate and the oxidation of glucose to gluconic acid. As most of their oxidation products are acids and as acetic acid bacteria rapidly convert large amounts of substrate, they quickly acidify their environment during growth. This inhibits growth of many other bacteria and gives acetic acid bacteria a selective advantage as they can tolerate low pH values.

Another aspect of this group of organisms important in oenology is their tendency to form biofilms by secretion of various, sometimes complex, exopolysaccharides (Ali et al. 2011; Serrato et al. 2013). A remarkable example of these biofilms is the "mother of vinegar." This is a massive gelatinous or leathery biofilm layer that develops on the surface of nutrient-rich, unstirred alcoholic solutions such as wine during prolonged aerobic incubation. This biofilm transforms the wine to vinegar by oxidizing the ethanol to acetic acid. The matrix of the biofilm consists of cellulose fibrillae that are synthesized by *G. xylinus* and other strains (Castro et al. 2013; Valera et al. 2015). Cellulose biosynthesis in several acetic acid bacteria seems to be astonishing complex, being directed by up to three distinct cellulose biosynthesis operons. Bacterial cellulose is of high purity and is biotechnologically produced. It is used in many applications, for example, as dressing material or for care of large area burn wounds or as an additive for foods and drinks. For example, the dessert "nata de coco" is produced mainly from bacterial cellulose. Besides cellulose fibrillae, a number of other exopolysaccharides are formed by acetic acid bacteria, such as levans, a neutral polyfructan containing D-fructofuranosyl residues linked predominantly with β-(2,6) linkages as a main chain, or the acidic water-soluble polysaccharide acetan, the structure of which is similar to xanthan, consisting of glucose, mannose, glucuronic acid, and rhamnose. Acetan production also seems to play an important role in the cellulose biosynthesis (Ishida et al. 2002). Some strains of *Gluconobacter* show viscous growth in beer, resulting in the formation of "ropy beer" due to the formation of dextrans. These are α-D-glucans that have consecutive α(1,6)-linked glucose residues in the main chains and a variable amount of α(1,4), α(1,3), and α(1,2) branch linkage (De Muynck et al. 2007). A major function of cellulose and probably other exopolysaccharides for acetic acid bacteria seems to be that those obligate aerobic organisms are kept on the surface of liquids or stick to places with contact to air and therefore ensure sufficient oxygen supply.

20.2 Acetic Acid Bacteria in Oenology

The production of vinegar from wine was documented as early as 4000 B.C. (Deppenmeier et al. 2002). The English word "vinegar" derives from the old French "vinaigre," meaning "sour wine." A diluted sour wine with modest acetic acid concentration has been used at all times as a drink in Mediterranean countries with their warm climate. The sensory threshold of acetic acid in wine is around 700 mg/L, while concentrations larger than 1.2–1.3 g/L are generally regarded as unpleasant (Dittrich and Großmann 2011). But things are complex, as a certain

level of acetic acid is required in some wines to develop a more complex, "desirable" taste. Acetic acid bacteria are also important for the development of a characteristic aroma in several other foodstuffs such as Kombucha. The characteristic sweet and sour flavor results from ethanol, acetate, and gluconic acid produced by yeasts, acetic acid bacteria, and lactic acid bacteria (Chen and Liu 2000; Greenwalt et al. 2000).

The formation of stronger vinegar taste is seen as a major and irreversible wine fault, and acetic acid bacteria are the major source for this spoilage. The acetic acid is either formed in the must or later in the wine, if oxygen is available (Bartowsky and Henschke 2008).

Acetic acid bacteria can be found ubiquitous on the surface of grapes. Therefore, they are inoculated in high numbers in the must where they can proliferate. They are responsible for rapid acidification during the initial stages of fermentation (Drysdale and Fleet 1988). Under prolonged sticky weather in autumn, the acetic acid bacteria can even form acetic acid at the grape by infecting the sap through tiny injuries. These injuries can be caused by insects or by *Botrytis* infections. As the fungus breaks through the skin of the grape, tiny droplets of sap can leak from the berry providing substrate for the bacteria (Barbe et al. 2001).

If acetic acid bacteria develop in the must, increased amounts of acetic acid, gluconic acid, keto-gluconic acids, as well as dihydroxyacetone are formed, although the latter will usually be reduced again during fermentation by yeasts (Dittrich and Großmann 2011). The produced carbonyl compounds account for an increased SO_2-binding capacity of the must (Barbe et al. 2001).

As the must contains high sugar concentrations and comparatively little ethanol, these are especially favorable conditions for members of the "suboxydans" group of acetic acid bacteria. In contrast to must, wine contains only small amounts of sugar and high concentrations of ethanol. As wine, especially red wine, is not always sterile filtered prior to bottling, it often has a small resident bacterial population of acetic acid bacteria, which might proliferate under conducive conditions. This results in the prevalence of acetic acid bacteria of the "peroxydans" group, whenever the wine is stored with exposure to air (Joyeux et al. 1984; Bartowsky and Henschke 2008).

Their obligate oxygen dependence alleviates the control of acetic acid bacteria. Bottled red wines, sealed with natural cork closures, and stored in a vertical upright position may develop spoilage by acetic acid bacteria. This spoilage is evident as a distinct deposit of bacterial biofilm in the neck of the bottle at the interface of the wine and the headspace of air and is accompanied with vinegar, sherry, bruised apple, nutty, and solvent like off-aromas, depending on the degree of spoilage (Bartowsky and Henschke 2008).

As the preparation of red wine as compared to white wines usually requires more intense mechanical processing of the mash, more oxygen is available to the acetic acid bacteria. This manifests itself in higher concentrations of acetic acid in many red wines. The concentration of acetic acid in wines is commonly measured in the form of volatile acidity. The accepted critical value of volatile acidity is 1.6 g/L for red wines as opposed to 1.2 g/L for white wines (Dittrich and Großmann 2011).

Ethyl acetate is another important wine fault that has been attributed to acetic acid bacteria, although wine spoilage yeasts or lactic acid bacteria seem to be the more important elicitors (Rojas et al. 2003). The transcription of an esterase that seems to be responsible for ethyl acetate formation in *A. pasteurianus* is induced by ethanol (Kashima et al. 1999). Ethyl acetate does not contribute to the volatile acidity and is formed by esterification of acetate with ethanol. Wines with a high level of acetic acid are therefore more likely to suffer from ethyl acetate formation. While at low concentrations, ethyl acetate contributes to the richness and sweetness of the wine, above a sensory threshold of 150–200 mg/L, it produces a "nail polish remover" aroma. In vinegars the esters weaken the strong smell of acetic acid (Kashima et al. 1999). Several strains of *Acetobacter* and *Gluconobacter*, particularly strains of *A. pasteurianus*, can oxidize lactate to acetoin. Acetoin has a characteristic "butter-like" aroma and flavor, occurring in spoiled wine (Mamlouk and Gullo 2013).

Besides causing off-flavors in wine and other alcoholic beverages such as beer, acetic acid bacteria are generally regarded as harmless and are not pathogenic to humans or animals (Gupta et al. 2001). They have only sporadically been described to induce bacterial rot of apples, pears, or other fruits. Those infections are accompanied by different shades of browning. Sometimes they also cause the spoilage of canned pineapples. After the heating in the canning process, the diseased tissue turns pink to brown due to the presence of 2,5-diketogluconic acid (Cho et al. 1980). Recently occasional infections of humans by strains of *Asaia bogorensis* and *Granulibacter bethesdensis* were described, both of which belong to the *Acetobacteraceae*, but those organisms are not known to be relevant for oenology (Greenberg et al. 2007; Kawai et al. 2015). Generally, acetic acid bacteria are regarded as generally recognized as safe (GRAS) organisms and are therefore very attractive for the industry.

20.3 The Lifestyle of Acetic Acid Bacteria

Traditionally, acetic acid bacteria have been taxonomically divided into the "suboxydans" and the "peroxydans" group. These two groups are physiologically defined with respect to their further utilization of the acetate formed from the oxidation of ethanol: the members of the "suboxydans" group, represented by the genus *Gluconobacter*, show strong ketogenesis from polyols and prefer habitats rich in sugar (De Ley and Swings 1984; Gupta et al. 2001). They are not capable of complete acetate oxidation to CO_2 also called overoxidation. While *Gluconobacter* is able to oxidize the glucose in the must with a high yield of gluconic acid, members of this genus are not very active in forming acetic acid or ethyl acetate.

In contrast to the "suboxydans" group, members of the "peroxydans" group prefer alcohol-enriched niches and are capable of slowly oxidizing acetate or lactate completely to CO_2 after depletion of the primary carbon sources (De Ley et al. 1984). Whereas *Gluconobacter* can be found during the early stages of cider

manufacture, *Acetobacter aceti* and *A. pasteurianus* as members of the "peroxydans" group are usually isolated in later stages (Passmore and Carr 1975). Despite their ability for overoxidation of acetate, mainly organisms of the "peroxydans" group seem to be responsible for vinegar formation. The capability for overoxidation requires a complete TCA cycle as well as a glyoxylic acid shunt.

Traditionally all acetic acid bacteria of the "peroxydans" group have been attributed to the genus *Acetobacter*. But later on, the genus has been split into the genera *Acetobacter* and *Gluconacetobacter*; many of the latter have recently been placed in the genus *Komagataeibacter*. Numerous new genera have also been added to this group over time (Yamada and Yukphan 2008; Trcek et al. 2015).

While incomplete oxidation is a common phenomenon in many microorganisms, acetic acid bacteria are highly specialized in this kind of metabolism. Using their membrane-bound dehydrogenases located in the cell membrane with their active site facing toward the periplasm, they do not degrade sugars to acetic acid but instead preserve the carbon skeleton of their substrates and perform only a few stereo- and regioselective oxidation steps. This architecture has the advantage that the substrates and products need not to be transported across the cell membrane as they can enter or leave the periplasmic space by diffusion. Such enzymes typically have a very broad substrate spectrum converting up to 30 different tested substrates. Substrates converted by homologous enzymes in different strains can be very different. *Gluconobacter oxydans* 621H, for example, codes for at least ten different experimentally verified membrane-bound dehydrogenases (Peters et al. 2013b; Mientus et al. 2017). Therefore, over the years, a multitude of such dehydrogenase activities located in the cytoplasmic membrane of acetic acid bacteria have been described in the literature. The purified enzymes are either flavoproteins containing covalently bound FAD or quinoproteins containing pyrroloquinoline quinone (PQQ) as cofactor (Matsushita et al. 1994; Adachi et al. 2003). Although a large number of membrane-bound dehydrogenases are characteristic for acetic acid bacteria, these enzymes are not confined to this group of organisms. Several organisms such as *Sphingomonas wittichii* also contain a large number of those membrane-bound dehydrogenases, though not much is known about the substrate specificity of such enzymes (Zeiser et al. 2014). Even *Escherichia coli* contains a PQQ-dependent glucose dehydrogenase in its membrane (Toyama et al. 2004). In contrast to acetic acid bacteria, *E. coli* is only able to synthesize the apoenzyme and needs to take up the PQQ from the environment to form an active enzyme (Neijssel 1987).

Acetic acid bacteria carry out the oxidation of ethanol to acetate in two consecutive steps using the membrane-bound PQQ-dependent ethanol dehydrogenase and acetaldehyde dehydrogenase. This ethanol oxidation occurs in all acetic acid bacteria except in the genus *Asaia*. Besides ethanol, a large number of alcohols, sugars, and other polyols are oxidized to their corresponding acids or ketones by typical acetic acid bacteria in one or very few steps by the action of various membrane-bound dehydrogenases. For example, glycerol is oxidized to dihydroxyacetone by the polyol dehydrogenase, or D-sorbitol is oxidized to L-sorbose by a

D-sorbitol dehydrogenase (Adachi et al. 2003; Deppenmeier et al. 2002). The occurrence of different dehydrogenases is species or even strain specific, and their classification based on the literature is difficult. In most studies the enzymes have been purified and characterized only according to their activities, while the corresponding genes were not cloned and sequenced. As subunit composition and molecular weights are often similar, the number of different enzymes that are responsible for the observed activities is not known except in some recent studies for *G. oxydans* 621H. For example, the alcohol and aldehyde dehydrogenases of acetic acid bacteria oxidize many straight and branched-chain alcohols and aldehydes to their corresponding carboxylic acids (Sievers and Swings 2005), or the polyol dehydrogenase is able to oxidize glycerol, D-arabitol, D-sorbitol, and many other sugar alcohols (Peters et al. 2013b; Mientus et al. 2017).

Numerous of the oxidations carried out by the membrane-dependent enzymes can be described empirically according to the Bertrand–Hudson rule, stating that polyols with *cis*-arranged secondary hydroxyl groups in D-configuration to the adjacent primary alcohol group (D-*erythro* configuration) are oxidized to the corresponding ketoses.

As compared to complete oxidations, these incomplete oxidations extract only few electrons per mole of substrate. Because substrates are predominantly converted by the membrane-bound dehydrogenases, acetic acid bacteria have to convert large amounts of substrates to produce little biomass. When *G. oxydans* is growing on glucose, more than 90% of the glucose is oxidized by membrane-bound dehydrogenases and remains extracellular and $<10\%$ is metabolized in the cytoplasm (Krajewski et al. 2010). On the one hand, this is an adaptation to an environment with high substrate concentrations, where speed not efficiency is crucial; on the other hand, this fact makes these organisms interesting for industrial applications, because acetic acid bacteria couple rapid stereo- and regioselective oxidation reactions of many substrates to the reduction of oxygen, and only little substrate is lost for biomass formation (Deppenmeier et al. 2002). From this perspective, they can be seen as living oxidative catalysts. This is the reason for the employment of acetic acid bacteria in many biotechnological processes such as the production of vitamin C, gluconic acid, dihydroxyacetone, miglitol, and many others (Macauley et al. 2001).

In addition to the set of membrane-bound dehydrogenases catalyzing irreversible oxidations, a second set of dehydrogenases, using NAD(P) as the cosubstrate, is located in the cytoplasm. These soluble enzymes convert similar or even the same substrates as their membrane-bound counterparts in reversible reactions. For example, a soluble, NADP-dependent alcohol dehydrogenase, a glucose dehydrogenase and a gluconat dehydrogenase have been isolated and characterized (Deppenmeier et al. 2002). The physiological role of this second set of dehydrogenases in metabolism is not completely clear. It is assumed that the soluble, NAD(P)-dependent enzymes contribute only little to the overall oxidation of the substrates but are instead required to channel a multitude of compounds in the central metabolism in order to obtain building blocks for biosynthesis. Accordingly, a membrane-bound quinoprotein glucose dehydrogenase has been shown to be

30 times more active than a NADP-dependent soluble glucose dehydrogenase (Pronk et al. 1989).

20.4 Characteristics of Acetic Acid Bacteria Genomes

Currently 12 closed genome sequences of acetic acid bacteria can be found in the databases. For an overview on their characteristics, see Table 20.1. Beside the *G. oxydans* strains 621H, DSM3504, and H24 (Prust et al. 2005; Ge et al. 2013; Kostner et al. 2015), these include *Gluconacetobacter diazotrophicus* Pal5 and the *Gluconactobacter xylinus* strains E25 and NBRC3288 (Bertalan et al. 2009; Kubiak et al. 2014; Ogino et al. 2011). Also, the genome sequences of the *A. pasteurianus* strains 386B, NBRC 3283, CICC20001, and CGMCC 1.41 have been published (Illeghems et al. 2013; Azuma et al. 2009; Wang et al. 2015). Furthermore, the complete genome sequences of the opportunistic pathogenic isolates *G. bethesdensis* CGDNIH1 and *A. bogorensis* NBRC 16594 were determined (Kawai et al. 2015; Greenberg et al. 2007). The first published genome of an acetic acid bacterium was *G. oxydans* 621H, a strain used in biotechnological production, due to its strong incomplete oxidation. It allowed for the first time to reconstruct the unique metabolism of this ketogenic acetic acid bacterium that is adapted to growth in concentrated sugar solutions.

The complete genome of *G. oxidans* 621H has a size of 2.9 Mb and consists of a circular chromosome of 2.7 Mb coding for 2601 predicted protein-encoding open reading frames, 55 tRNA genes and four copies of rRNA operons (Prust et al. 2005). 89.9% of the DNA codes for proteins or stable RNAs.

20.4.1 Transposable Elements

A prominent genetic feature is the high number of insertion sequences and transposase genes found in the genome: a total of 82 insertion sequences and 98 transposase genes together with two genome regions potentially representing inserted prophages could be recognized in the genome of *G. oxydans* 621H. According to the classification of Mahillon and Chandler (1998), ten copies of the insertion sequences can be attributed to the family IS12528 and eight copies to IS1032. Although several of the insertion sequences seem to be defective, the functional copies may be responsible for the marked genetic and, as a consequence, physiological instability that has also been observed in several other strains of acetic acid bacteria. The genome of *A. pasteurianus* NBRC 3283, a strain isolated from the surface of a vinegar fermentation, comprises a 2.9-Mb chromosome and six plasmids and contains more than 280 transposons and five genes with hypermutable tandem repeats in the genome. This amounts to approximately 9% of the total genes in the genome. Seventy-five of these insertion sequences belong to

Table 20.1 Comparison of complete *Acetobacteraceae* genomes

Organism	Size of chromosome (Mb)	GC (%)	Number of plasmids	Open reading frames	Number of transposases	rRNA operons	Reference
Gluconobacter oxydans 621H	2.7	60.8	4	2668	104	4	Prust et al. (2005)
Gluconobacter oxydans H24	3.6	56.2	1	3732	80	5	Ge et al. (2013)
Gluconacetobacter diazotrophicus Pal5	3,9	66.3	2	3938	129	4	Bertalan et al. (2009)
Gluconacetobacter xylinus E25	3.4	62.6	5	3156	81	5	Kubiak et al. (2014)
Gluconacetobacter xylinus NBRC 3288	3.1	60.6	7	3195	204	5	Ogino et al. (2011)
Acetobacter pasteurianus 386B	2.8	52.9	7	2875	50	5	Illeghems et al. (2013)
Acetobacter pasteurianus NBRC 3283	2.9	53.1	6	3050	268	5	Azuma et al. (2009)
Acetobacter pasteurianus CICC 20001	2.8	52.9	10	3623	5		Wang et al. (2015)
Acetobacter pasteurianus CGMCC 1.41	2.9	52.9	7	3250	4		Wang et al. (2015)
Granulibacter bethesdensis CGDNIH1	2.7	59.1	0	2437	15	3	Greenberg et al. (2007)
Asaia bogorensis NBRC 16594	3.1	59.8	0	2758	14	5	Kawai et al. (2015)

the IS1380 type, an insertion sequence also abundant in many other *A. pasteurianus* strains and could be connected to the genetic instability of these acetic acid bacteria (Azuma et al. 2009). The endophytic *Ga. diazotrophicus* Pal5, present in large numbers in the intercellular space of sugarcane roots, stem, and leaves, fixes molecular nitrogen inside sugarcane plants, without causing apparent disease. Remarkable characteristics of this bacterium are the acid tolerance and the ability to fix nitrogen in the presence of ammonium in media with high sugar concentration (Bertalan et al. 2009). The genome of *Ga. diazotrophicus* contains 245 transposable elements, which represent 6% of the total open reading frames of this organism (Bertalan et al. 2009; Skraban and Trcek 2017).

On the other hand, *A. pasteurianus* 386B contains only 50 transposases and lacks transposons of the IS1380 type (Illeghems et al. 2013), and *A. pasteurianus* CICC 20001 and *A. pasteurianus* CGMCC 1.41 contain even less transposase genes than strain *A. pasteurianus* 386B, implying a higher genetic stability of these strains (Wang et al. 2015). *Acetobacter pasteurianus* 386B originates from a spontaneous cocoa bean heap fermentation carried out in Ghana and has been characterized as an ethanol-oxidizing, lactic acid-oxidizing, and acetic acid-producing strain. Furthermore, *A. pasteurianus* 386B is a thermotolerant strain with high resistance to ethanol and acetic acid. The strains CICC 20001 and CGMCC 1.41, which were isolated from a vinegar factory in Dandong by the Shanghai Institute of Brewing and are still widely used to brew vinegar by solid-state and liquid-state fermentation, are displaying high stabilities in acetic acid production (Wang et al. 2015)

20.4.2 Plasmids

The occurrence of numerous plasmids is another characteristic property of the genomes of acetic acid bacteria. It was already reported in several studies before genome sequences became available. Some of the plasmids contain identified dehydrogenase- or antibiotic-resistant genes, but most of them are cryptic plasmids with no obvious function (Trcek et al. 2000; Krahulec et al. 2003). The genome of *G. oxydans* 621H contains five cryptic plasmids of 163.1, 26.6, 14.6, 13.2, and 2.7 kb size. The genome sequence did not help much in deciphering their role since nearly 70% of the open reading frames located on them, code for hypothetical proteins of currently unknown function, based on sequence homologies. Among other things open reading frames with suggested functions code for putative proteins of plasmid replication, a DNA helicase II (*umuD*), a restriction and modification system, a heavy metal resistance system, a conjugation system, as well as for a C_4-dicarboxylate transporter and two alcohol dehydrogenases of unknown substrate specificity (Prust et al. 2005). The five plasmids show no homology to known plasmids of other *G. oxydans* strains, for instance, pAG5 from *G. oxydans* IFO 3171 (Tonouchi et al. 2003) and pGO128 from *G. oxydans* DSM 3504 (Sievers M., direct submission to GenBank, Gen ID:NC_003374) (Skraban and Trcek 2017). However, the smallest plasmid pGOX5 has substantial similarities to the plasmid

pJK2-1 from *G. europaeus* DSM 13109 (Trcek et al. 2000). While the chromosomes of sequenced *A. pasteurianus* strains have a high homology, the plasmids from several strains have only few sequences in common. For example, the comparison of the two largest plasmids of *A. pasteurianus* NBRC 3283, pAPA01-020 with 174 open reading frames and pAPA01-011 with 178 open reading frames with the largest plasmid of *A. pasteurianus* 386B, APA386B_1P with 220 open reading frames, reveals that they share only 44 and 16 homolog open reading frames, respectively. Furthermore, the plasmid APA386B_1P contains 165 unique genes that are not present on the two largest plasmids of *A. pasteurianus* NBRC 3283 (Illeghems et al. 2013; Skraban and Trcek 2017). Similarly, *A. pasteurianus* CICC 20001 contains a large plasmid of 474 kb, which shows almost no homology to plasmids from other *A. pasteurianus* strains (Wang et al. 2015). It contains clustered, regularly interspaced, short palindromic repeat (CRISPR) elements, which function to prevent phage infections (Caliando and Voigt 2015). In contrast *A. pasteurianus* CGMCC 1.41 and *A. pasteurianus* CICC 20001 have one and two putative CRISPR elements on the chromosome, respectively. In addition, *A. pasteurianus* CICC 20001 contains one putative and two confirmed CRISPR elements on the large plasmid (Wang et al. 2015).

20.4.3 Phages

In *G. oxydans* 621H two putative prophages were identified on the chromosome (Prust et al. 2005). In the genome of *A. pasteurianus* 386B, the chromosomal synteny with the chromosome of *A. pasteurianus* NBRC 3283 is also interrupted by an inserted prophage. This prophage has a size of approximately 28.8 kb and contains 61 genes. Several of the prophage genes found in *A. pasteurianus* 386B have homologs in *A. pasteurianus* NBRC 3283, *G. diazotrophicus* Pal5, and *G. oxydans* 621H (Illeghems et al. 2013). The prophage region in *A. pasteurianus* 386B includes an integrase that has a homolog in *G. oxydans* 621H and *G. bethesdensis* CGDNIH2, a phage terminase and a λ family phage portal protein. However, the region lacks virulence-associated genes as well as genes coding for a head maturation protease and a tail tape measure protein, which suggests that the prophage is defective (Canchaya et al. 2003; Illeghems et al. 2013).

20.5 The Membrane-Bound and the Soluble Dehydrogenases

Using bioinformatic tools, more than 75 genes coding for potential oxidoreductases, mainly with unknown substrate specificity, were identified in the genome of *G. oxydans* (Prust et al. 2005). Ten of them are predicted to be located in the

cytoplasmic membrane (Peters et al. 2013b). This exemplarily illustrates the large oxidative potential just of this organism. The presence of a rich set of membrane-bound dehydrogenases that transfer electrons directly in the respiratory chain and a second set of soluble dehydrogenases in the cytoplasm that transfer their electrons to NAD(P) is the property that in general characterizes the physiology of acetic acid bacteria. At least in *G. oxydans*, the membrane-bound dehydrogenases compete with NADH dehydrogenase for channeling electrons into the electron transport chain. Synthesis of cell mass is dependent on assimilating and oxidizing substrates taken up into the cell. Membrane-bound dehydrogenases just channel electrons into the organism. In result this means that strains with more active membrane-bound dehydrogenases incompletely oxidize more substrate but have lower growth yields and vice versa (Kostner et al. 2015).

20.5.1 Membrane-Bound Dehydrogenases

Recently, the set of substrates converted by membrane-dependent dehydrogenases of *G. oxydans* 621H was comprehensively described using deletion mutants and expression of a single enzyme at a time in *G. oxydans* (Peters et al. 2013b; Mientus et al. 2017). Only two of the enzymes convert only one substrate out of the 55 substrates tested; the inositol dehydrogenase oxidized only *myo*-inositol and the gluconate-2-dehydrogenase only gluconate. The remaining dehydrogenases showed a broader substrate spectrum. All tested aldehydes (acetaldehyde, butyraldehyde, valeraldehyde, and formaldehyde) were oxidized by the aldehyde dehydrogenase. These substrates were also oxidized by the alcohol dehydrogenase together with glyceraldehyde, D-threose, ethanol, isopropanol, 3-hexanol, 1,3-butandiol, 1,2-pentandiol, 1,2-hexandiol, L-fucose, cellobiose and further 16 substrates. Altogether, the membrane-bound alcohol dehydrogenase oxidized 30 of 55 substrates tested. The glucose dehydrogenase oxidized D-xylose, L-arabinose, D-glucose, D-galactose, D-allose, D-altrose, D-mannose, turanose, and like the alcohol dehydrogenase L-fucose and cellobiose. The second most substrates were oxidized by the polyol dehydrogenase. The enzyme exhibited activity for 25 of the 55 substrates tested, e.g., glycerol, *meso*-erythritol, D-arabitol, D-mannitol, D-sorbitol, ribitol, D-ribose, 2,3-butanediol, 2,4-pentandiol, 2-hexanol, and gluconate. The substrates glyceraldehyde, D-threose, D-erythrose, isopropanol, 3-hexanol, 1,3-butandiol, 1,2-pentandiol, 1,2-hexandiol, and L-erythrulose were oxidized by the polyol dehydrogenase as well as the alcohol dehydrogenase. The membrane-bound sorbitol dehydrogenase in this particular strain is inactivated by an amber stop mutation in one subunit. The sorbitol dehydrogenase from *G. oxydans* DSM3504 tested instead, converted only mannitol and sorbitol. From the substrates tested, no substrate could be identified for two of the membrane-bound dehydrogenases, the PQQ-dependent dehydrogenase 3, and the PQQ-containing dehydrogenase 4, but nevertheless the vast oxidative potential of a single strain of acetic acid bacteria was demonstrated. Given that homologous membrane-bound dehydrogenases from different species

and even strains have different substrate spectra, acetic acid bacteria seem to be able to oxidize a huge number of alcohols, aldehydes, polyols, as well as sugars and their derivatives. Given the oxidative activity for structurally different alcohols and aldehydes, it can be expected that acetic acid bacteria have an influence on the aroma of vinegar and possibly of wine, far beyond acetic acid production.

The PQQ-dependent alcohol dehydrogenase complex is exclusively known from acetic acid bacteria (Reid and Fewson 1994; Toyama et al. 2004) and is composed of three subunits in *G. oxydans* as well as in *A. pasteurianus* (Kondo and Horinouchi 1997). The larger subunit is the catalytically active quinohemoprotein that binds the PQQ cofactor, contains a heme *c* and requires Ca^{2+}. The second largest subunit, coded adjacent to the large subunit, is the triheme cytochrome *c* that transfers the electrons from the quinoprotein to the ubiquinone pool in the membrane. Therefore, it connects ethanol oxidation to the electron transport chain as it is characteristic for the membrane-bound dehydrogenases. The smallest subunit, encoded in a distant genome location, is of uncertain function. It has been suggested, that it might be a kind of chaperone that helps to keep the complex in correct conformation (Kondo et al. 1995). The subunits of the purified enzyme complexes have molecular masses of 83, 51, and 15 kDa in *G. oxydans*, as deduced from the genome sequence (Prust 2004). The comparison of the number of paralogs of membrane-bound alcohol dehydrogenases in the efficient acetic acid producers *A. pasteurianus* CICC20001, *A. pasteurianus* CGMCC 1.41, *A. pasteurianus* 386B, *A. pasteurianus* NBRC 3283, *Ga. oboediens* 174Bp2, and *Ga. europaeus* 5P3 revealed that the genomes of these strains contain 1, 1, 2, 2, 6, and 7 paralogs, respectively. Therefore, the highly acetic acid-resistant strains *Ga. oboediens* 174Bp2 and *Ga. europaeus* 5P3, which tolerate up to 8 and 18% of acetic acid, contain considerably more genes coding for the membrane-bound alcohol dehydrogenases than the more sensitive *A. pasteurianus* strains (Wang et al. 2015; Skraban and Trcek 2017). This might reflect an adaptation of those strains to environments rich in ethanol, though the substrates converted by the isoenzymes are not yet known.

Acetic acid bacteria form acetic acid from ethanol in two consecutive steps. The acetaldehyde resulting from the alcohol dehydogenase complex is oxidized to acetate by the aldehyde dehydrogenase. The aldehyde dehydrogenase complex of *G. oxydans* 621H is composed of three subunits of 83, 52, and 17 kDa size. Although the enzyme systems that carry out the formation of acetic acid in vinegar fermentations are present in *Gluconobacter*, they are more active in members of the genera *Acetobacter* and *Gluconacetobacter*. These genera seem to play a much more important role in vinegar formation than *Gluconobacter* (Gullo and Giudici 2008; Trcek et al. 2000).

The major polyol dehydrogenase is a quinoprotein that is composed of a large 79.6 kDa SldA subunit and a small 13.7 kDa SldB subunit. The SldA subunit contains the active site with the PQQ-binding domain. SldB seems to be involved in processing or stabilizing SldA (Shinjoh et al. 2002b; Hoshino et al. 2003).

The quinoprotein glucose dehydrogenase consists of a single subunit of 87 kDa and has an amino acid sequence that is 56% identical to the well-studied enzyme from *E. coli* (Prust 2004; Yamada et al. 2003).

A second group of membrane-bound dehydrogenases contains flavin in the form of covalently bound FAD as cofactor. Examples are the gluconate-2-dehydrogenase (Elfari et al. 2005) and the sorbitol dehydrogenase, an enzyme with FAD and heme *c* as prosthetic groups coded by the *sldSLC* genes. It is composed of three subunits of 61.5, 52, and 22 kDa size (Adachi et al. 2003). For *Gluconobacter* two types of membrane-bound sorbitol-oxidizing dehydrogenases were described (Toyama et al. 2005). Beside the sorbitol dehydrogenase, the major polyol dehydrogenase also oxidizes sorbitol (see above) and has therefore been described in the literature as a PQQ-containing sorbitol dehydrogenase (Shinjoh et al. 2002b). This is an example that in older studies, activities were described as distinct enzymes but not associated to genes. Due to the wide substrate spectrum of some of the membrane-bound dehydrogenases as mentioned above, this led to the description of many enzymes that were actually identical.

An interesting feature of the genome of the pathogenic acetic acid bacterium *G. bethesdensis* is the presence of genes coding for a putative methanol dehydrogenase complex predicted to be located in the periplasm, which are absent from *G. oxydans* and most other acetic acid bacteria (Greenberg et al. 2007). Moreover, *Gr. bethesdensis* is capable of growing on methanol as the sole carbon source (Greenberg et al. 2006).

20.5.2 Soluble Dehydrogenases

Besides the membrane-bound dehydrogenases, there is an alternative set of soluble NAD(P)-dependent enzymes in the cytoplasm for metabolizing sugars, sugar derivatives, polyols, and alcohols in reversible reactions (Adachi et al. 2001; Prust et al. 2005). There are several soluble enzymes that catalyze oxidations of polyols or reduction of ketosugars. For example, two cytosolic NADPH-dependent carbonyl reductases from *G. oxydans* 621H were studied in detail (Schweiger et al. 2010). Both enzymes are dimers with native molecular masses of 66.1 and 74.5 kDa, respectively. The enzymes have broad substrate specificities and reduce α-ketocarbonyls at the keto moiety most proximal to the terminus of the alkyl chain to produce α-hydroxy carbonyls. The smaller protein display highest activities with 2,3-diones, α-ketoaldehydes, α-keto esters, and 2,5-diketogluconate. The larger enzyme was less active with these substrates but displayed a broader substrate spectrum reducing a variety of alpha-diketones and aldehydes (Schweiger et al. 2010).

Other soluble enzymes can oxidize ethanol, acetaldehyde, or glucose. Two cytosolic NADPH-dependent aldehyde reductases were characterized recently in more detail (Schweiger and Deppenmeier 2010). The purified proteins exhibit subunit masses of 26.4 and 36.7 kDa. Both proteins form homo-octamers exhibiting

native masses of 210 and 280 kDa, respectively. They both efficiently catalyze the reduction of medium- and long-chain aliphatic aldehydes. The best activity for the smaller enzyme is with chain lengths of C6–C10. In contrast, the larger enzyme has a limited substrate spectrum and only reduces octanal, nonanal, and decanal. Both enzymes are unable to oxidize primary alcohols (Schweiger and Deppenmeier 2010).

Therefore, like their membrane-bound counterparts, at least some of those enzymes seem to act on a broad substrate spectrum (Schweiger et al. 2007). The resulting products are phosphorylated and can be channeled into the central metabolism. For example, a NADPH-dependent sorbose reductase converts L-sorbose to D-sorbitol (Shinjoh et al. 2002a; Shibata et al. 2000; Soemphol et al. 2007). D-sorbitol can then be oxidized by a sorbitol dehydrogenase to D-fructose (Parmentier et al. 2003) that in turn could be channeled into the pentose phosphate pathway. The physiological role of the multitude of soluble enzymes is not completely clear. They might be responsible for assimilating a wide variety of sugars, alcohols, or their oxidation products and channel them into the central metabolism, mainly the pentose phosphate cycle, in order to allow further oxidation and for the supply of biosynthetic precursors (Deppenmeier and Ehrenreich 2009). In one case the role of two NAD- and NADP-dependent cytoplasmic mannitol dehydrogenases for the osmotolerance of *G. oxydans* has been shown by biochemical and mutant studies. The organism produces and accumulates mannitol as compatible solute under osmotic stress conditions (Zahid and Deppenmeier 2016; Zahid et al. 2015).

20.6 Structure of the Respiratory Chain

All acetic acid bacteria, with the exception of *Acidomonas methanolica* when grown on methanol, are oxidase negative (Sievers and Swings 2005).

This means that a cytochrome *c* oxidase should be missing. Biochemical investigations identified ubiquinol oxidases of the cytochrome *o* type in *Gluconobacter*. A second, cyanide-insensitive alternative ubiquinol oxidase was also detected. In summary, the biochemical studies predicted a respiratory chain in *Gluconobacter* that consists of cytochrome *c* as part of many membrane-bound dehydrogenases, ubiquinone, and ubiquinol oxidase. In contrast *Acetobacter* and *Gluconacetobacter* have terminal ubiquinol oxidases of the cytochrome a_1, cytochrome *d*, or cytochrome *o* type (Matsushita et al. 1994). *A. aceti* expresses cytochrome a_1 in shaking cultures and cytochrome *d* in static cultures (Matsushita et al. 1994).

Gluconobacter, *Gluconacetobacter*, as well as *Acidomonas*, *Asaia*, and *Kozakia* mainly contain ubiquinones of the Q-10 type, whereas *Acetobacter* uses ubiquinones of the Q-9 type (Yamada et al. 1997). The terminal oxidases are of the cytochrome a_1, cytochrome *d*, or the cytochrome *o* type (Matsushita et al. 1994). Figure 20.1 gives an overview on the components of the respiratory chain of acetic acid bacteria.

The data from the genome sequence allow deriving a detailed picture of the components of the respiratory chain in *G. oxydans* 621H. The numerous membrane-bound dehydrogenases transfer electrons to ubiquinol. The electrons are transferred via the cytochrome *c*-containing subunits or domains, if present in the respective enzyme. The organism contains a non-proton-translocating NADH:ubiquinone oxidoreductase that allows feeding of electrons from NADH into the ubiquinol pool. In contrast to a proton-translocating NADH:ubiquinone oxidoreductase (complex I), this enzyme generates no proton gradient during its redox reaction. Two operons were identified that code for ubiquinol oxidases, one of a bo_3 type, the other of a *bd* type. The bo_3-type enzyme has been studied biochemically and shown to generate a proton gradient (Matsushita et al. 1987). The *bd*-type ubiquinol oxidase represents the observed cyanide-insensitive oxidoreductase that is produced at low pH values (Matsushita et al. 1989). Deletion of the genes for cytochrome *bd* in *G. oxydans* 621H had no obvious influence on growth, whereas the lack of the genes for cytochrome bo_3 severely reduced the growth rate and the cell yield (Richhardt et al. 2013b).

There is also an enigmatic cytochrome bc_1 complex (ubiquinol:cytochrome *c* oxidoreductase) and a cytochrome c_{552} encoded in the genome of *G. oxydans*. Their function is not yet clear, because a cytochrome *c* oxidase is missing that would be needed to reoxidize this enzyme. The putative cytochrome bc_1 complex lacks histidine residues serving as Cu_B and heme a_3 ligands, making it unlikely that this complex functions as expected. However, a deletion mutant of the cytochrome bc_1 complex shows a 13% diminished growth rate on mannitol (Hanke et al. 2012). Genes for cytochrome bc_1 complexes without the presence of a cytochrome *c* oxidase were also detected in *A. aceti* NBRC14818, *A. pasteurianus* NBRC3283 (Sakurai et al. 2012; Azuma et al. 2009), and *A. pasteurianus* 386B (Illeghems et al. 2013) as well in the α-*Proteobacterium Zymomonas mobilis* (Balodite et al. 2014). Therefore, this situation is not unique to *Gluconobacter* but seems to be widespread in acetic acid bacteria and other α-*Proteobacteria*. However, a good reasoning is not yet known. One idea is a transfer of the electrons to a cytochrome *c* peroxidase, reducing hydrogen peroxide (Bringer and Bott 2016).

There are no genes found in the genome of *G. oxydans* and other acetic acid bacteria that could be involved in any anaerobic respiration. This is in good agreement to the obligate aerobic growth physiology of these organisms. The proton motive force that is generated by the respiratory chain (Fig. 20.1) is used by an F_1F_0-type ATPase to generate ATP. The short electron transport chain seems to have a rather limited ability to conserve energy by proton translocation during its redox reactions. But it must be considered that many oxidation products are acids. Therefore, protons are formed at the outer side of the cell membrane, while proton consumption by oxygen reduction occurs at the inner side, contributing to building up a proton motive force. Overall, the respiratory system of *Gluconobacter* and other acetic acid bacteria seems to be more adapted to fast oxidation of large amounts of substrate than to efficient energy conservation.

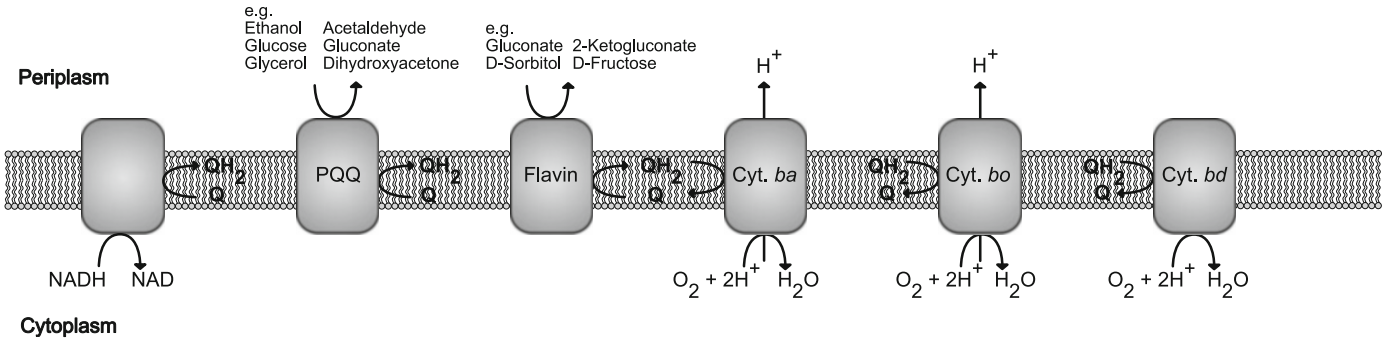

Fig. 20.1 Overview on electron transport in acetic acid bacteria. Various PQQ- and flavin-dependent dehydrogenases with their active site facing toward the periplasm are present in the cell membrane or periplasm. Often products are acidic, thereby generating protons at the periplasmic side. They transfer the electrons from substrate oxidation to quinone. A type I NADH dehydrogenase that is unable to translocate protons is also present to feed electrons into the quinone pool-pumping protons during its redox cycle. The quinone is reoxidized either by cytochrome *ba*, present in representatives of the "peroxydans" genera, as well as by cytochrome *bd* or cytochrome *bo*-containing quinone oxidases consuming protons at the cytoplasmic side. The cytochrome *ba*- and *bo*-containing enzymes additionally transfer protons

20.7 The Central Metabolism

When common heterotrophic bacteria like *E. coli* grow, they transport their substrate in the cell and use it as electron and carbon source. During progressing oxidation of the substrate in central metabolism, electrons are transferred in the form of NADH to the respiratory chain. At the same time, intermediates of central metabolism are used as precursors for biosynthetic pathways. In acetic acid bacteria in contrast, there is a tendency to separate electron source and carbon source. The largest amount of the substrate is oxidized by the membrane-bound dehydrogenases without transporting them in the cell, and the electrons of those oxidations are directly channeled into the respiratory chain. Only a small part, in *G. oxydans* $<10\%$ (Krajewski et al. 2010), of the substrates are transported into the cell, and the main purpose of the central metabolism is the formation of biosynthetic building blocks. For substrate oxidation, the central metabolism is of less importance. This seems to be an adaptation to habitats with high substrate concentrations. Fast acidification gives the acid-tolerant acetic acid bacteria a distinct growth advantage. Therefore, speed of substrate conversion is more important than efficiency of substrate utilization. Organisms of the "suboxydans" group, the genus *Gluconobacter*, show this pattern most pronounced. On the other hand, in vinegar, organisms of the "peroxydans" group, for instance *Acetobacter* species, exhibit a biphasic growth curve. The first phase corresponds to ethanol oxidation with acetate production by membrane-bound dehydrogenases, and the second growth phase corresponds to an overoxidation of the acetate. The acetate is taken up and oxidized via the tricarboxylic acid (TCA) cycle. Other acids such as lactate, pyruvate, malate, succinate, citrate, and fumarate are similarly metabolized (Mamlouk and Gullo 2013).

The mentioned growth patterns are reflected in organization of the central metabolism of acetic acid bacteria as shown in Fig. 20.2. The central metabolism of *G. oxydans* is mainly concerned with providing the building blocks for biosynthesis. Because of the absence of the phosphofructokinase, there is no glycolysis, but an Entner–Doudoroff pathway (EDP) and a pentose phosphate pathway (PPP) are present. While the significance of the PPP for biosynthesis is clear, the advantage of the EDP might be that this pathway converts gluconate in only two reaction steps into the important biosynthetic precursor pyruvate. Ultimately, the importance of the EDP for *G. oxydans* is not yet clear, because a mutant in this pathway showed no growth defect, at least in the presence of yeast extract (Richhardt et al. 2013a).

Pyruvate is converted via the pyruvate dehydrogenase to acetyl-CoA, which can be fed into the incomplete TCA cycle. *G. oxydans* and other acetic acid bacteria contain a pyruvate decarboxylase that degrades pyruvate to acetaldehyde, which in turn results in acetate formation by the organism using a soluble acetaldehyde dehydrogenase (Peters et al. 2013a).

The TCA is incomplete due to the absence of the succinyl-CoA synthetase and the succinate dehydrogenase. This is a situation very uncommon in aerobic

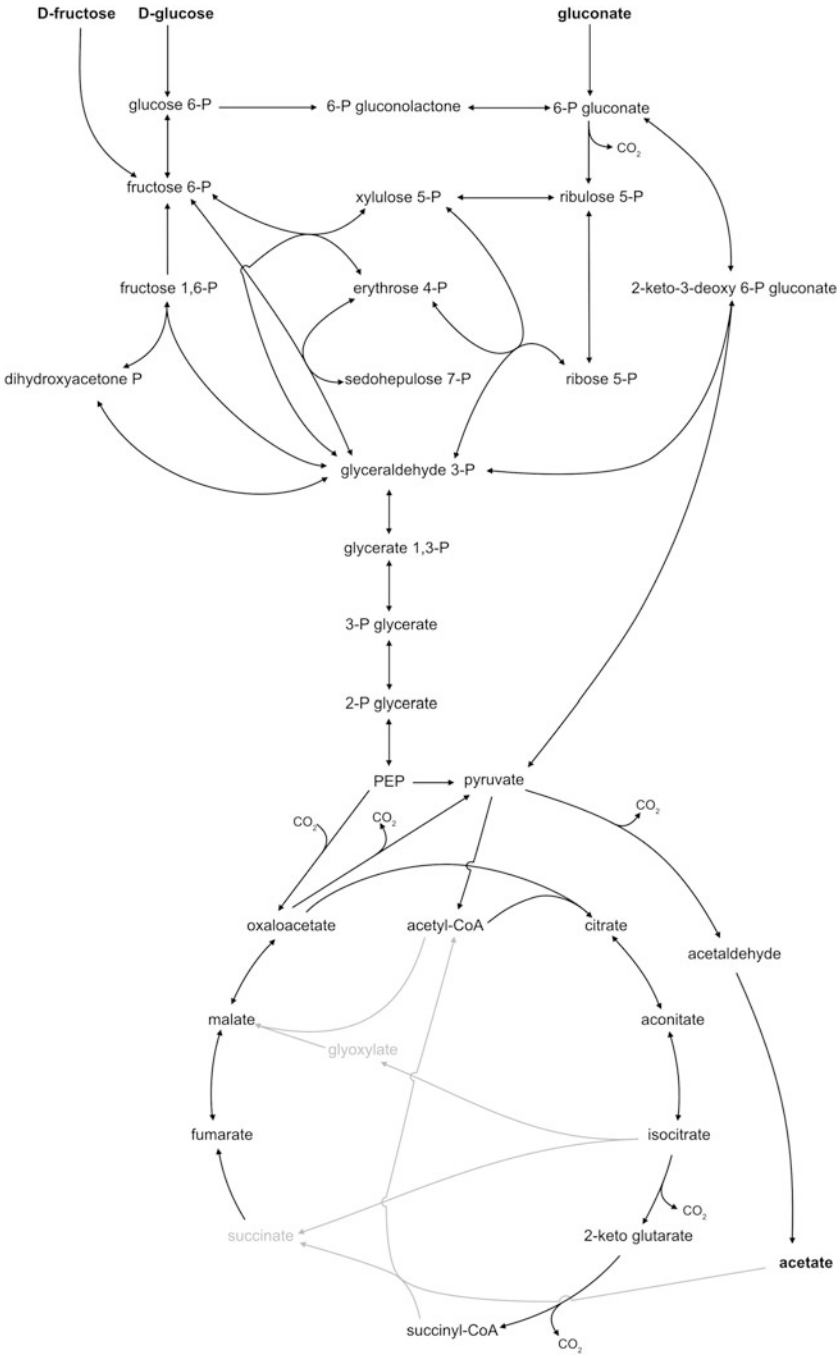

Fig. 20.2 Overview on the central metabolism of acetic acid bacteria. An oxidative pentose phosphate pathway is present as well as an Entner–Doudoroff pathway. There is no glycolysis due to a missing phosphofructokinase. Also, a gluconeogenesis from pyruvate is not possible because no PEP-forming enzyme has been identified. The TCA cycle is not complete in organisms of the

organisms. Furthermore, no genes for a glyoxylic acid shunt are present in the genome of *G. oxydans* 621H.

As the TCA cycle is not closed, a putative PEP carboxylase supplies oxaloacetate as an acceptor for acetyl-CoA (Prust 2004). The lack of a complete TCA cycle has profound consequences for the biology of *G. oxydans*, because a complete oxidation of substrates degraded via acetyl-CoA is not possible (Fig. 20.2). Therefore, no overoxidation of acetate is possible (Deppenmeier and Ehrenreich 2009).

In this context, it is worth mentioning that the *G. oxydans* 621H genome sequence reveals the presence of a membrane-bound transhydrogenase. This enzyme couples the equilibrium between the NADH and NADPH pools to the proton motive force. In *E. coli*, the membrane-bound transhydrogenase functions in the direction of generating NADPH from NADH (Sauer et al. 2004). Accordingly, its physiological function might be to supply *Gluconobacter* with the NADPH needed to reduce assimilated products of direct oxidation in order to channel them into the biosynthetic metabolism.

The gluconeogenetic abilities of the metabolism are limited. While gluconeogenetic hexose or pentose formation is possible from trioses, there is no phosphoenolpyruvate synthase, pyruvate orthophosphate dikinase, or any other phosphoenolpyruvate-synthesizing enzyme. This means that *G. oxydans* cannot produce hexoses from pyruvate or acetate.

Opposed to its restricted central metabolism, *G. oxydans* 621H is able to synthesize all amino acids, nucleotides, and many vitamins de novo (Prust 2004). Nevertheless, all *Gluconobacter* strains do require growth factors. Pantothenic acid, niacin, thiamin, and *p*-aminobenzoic acid are required by 96%, 40%, 8%, and 4% of the strains, respectively (Gosselé et al. 1980). Nitrogen is assimilated at the level of ammonium that is taken up by specific transporters and incorporated into the metabolism via the glutamine synthetase and the glutamate synthase reactions, while a NAD(P)-specific glutamate dehydrogenase is missing (Prust et al. 2005).

According to the *G. oxydans* 621H genome sequence, sulfur is taken up as sulfate by a permease and reduced via a phosphoadenosine-phosphosulfate synthase (PAPS) system. The serine-*O*-acetyltransferase and cysteine synthase reactions then incorporate the sulfur in the amino acid cysteine (Deppenmeier and Ehrenreich 2009).

An important cofactor synthesized by *G. oxydans* is PQQ, as it is essential for the synthesis of the quinoproteins. All but one of the essential genes has been identified

Fig. 20.2 (continued) "suboxydans" group (drawn in *black*) and can therefore only be used for providing biosynthetic precursors, because the thiokinase and the succinate dehydrogenase are absent. Characteristic for acetic acid bacteria is acetate formation via a pyruvate decarboxylase. Additionally, organisms of the "peroxydans" possess a glyoxylic acid shunt and a closed TCA cycle (drawn in *gray*). Often, a succinyl-CoA:acetate CoA-transferase (AarC), which replaces succinyl-CoA synthetase, is present for the activation of acetate. Those reactions seem to be responsible for acetate overoxidation. Beside the shown reactions, a multitude of soluble dehydrogenases are present that can channel many substrates as carbon sources into various points of the central metabolism

in the genome of *G. oxydans*. The missing *pqqF* gene that has low sequence similarity between species was later identified by transposon mutagenesis (Hölscher and Görisch 2006). Beside *G. oxydans* 621H, two other *G. oxydans* strains have been sequenced. *G. oxydans* H24, a strain used in biotechnological L-sorbose production (Ge et al. 2013) and *G. oxydans* DSM3504, a strain with a significant higher growth yield than *G. oxydans* 621H (Kostner et al. 2015). The discussed features of central metabolism, such as incomplete glycolysis and TCA, are conserved among all three strains.

In *A. pasteurianus* 386B (Illeghems et al. 2013), *A. pasteurianus* NBRC 8283 and the *A. pasteurianus* strains CICC20001 and CGMCC 1.41 (Wang et al. 2015) belonging to the "peroxydans" group, the central metabolism also reflects the growth physiology discussed for this group (Fig. 20.2). Analogous to *G. oxydans* there is no glycolysis due to a missing phosphofructokinase, but a functional PPP is present, providing access to biosynthetic building blocks. Also, in analogy to *G. oxydans*, a pyruvate decarboxylase is present, at least in *A. pasteurianus* 386B, presumably also responsible for a certain amount of acetate formation from other substrates than ethanol. In all, this might reflect similarity to *G. oxydans*, where the central metabolism seems mainly concerned with providing biosynthetic building blocks, while the main part of the energy is formed by electrons channeled in the respiratory chain by the membrane-bound dehydrogenases. The main difference between *Gluconobacter* and the organisms of the "peroxydans" group is the ability for overoxidation by the latter. This is reflected in the central metabolism by the presence of a functional TCA allowing these organisms to completely oxidize acetyl-CoA. *A. pasteurianus* NBRC 8283 as well as *A. aceti* contain a succinyl-CoA:acetate CoA-transferase (AarC), which replaces succinyl-CoA synthetase. Overoxidation of acetate requires activation to acetyl-CoA. The unique modification of the TCA transfers CoA from succinyl-CoA to acetate saves energy for the activation of acetate to acetyl-CoA and might also help in the detoxification of acetate (Mullins et al. 2008). In *A. aceti* an active glyoxylic acid shunt has been shown, specifically induced during overoxidation, allowing effective degradation of acetate to CO_2 (Sakurai et al. 2012, 2013).

20.8 Conclusion and Outlook

Acetic acid bacteria are truly fascinating organisms with an unusual physiology and biochemistry. While the consequences of their metabolism are mostly detrimental in oenology, namely, the formation of an excess of acids from sugars in the must or from ethanol in wine during storage, their metabolism in the right dosage also gives a balanced aroma to the wine. The future will probably demonstrate profound influences on aroma components. Although acetic acid bacteria have been studied since Pasteur's times, their physiology and biochemistry still hold many unanswered questions. The genome sequence of *G. oxydans* 621H allowed a detailed look on the metabolic pathways and enzymology of a representative of the

"suboxydans" group of acetic acid bacteria, providing a solid fundament for further studying physiology and molecular biology, demonstrating the enormous value of genome sequencing for microbial research. This data was complemented by more publicly available genome sequences of other acetic acid bacteria, especially from representatives of the "peroxydans" group, opening the field for future research.

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Chapter 21
Plasmids from Wine-Related Lactic Acid Bacteria

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21.1 Introduction

This chapter presents a review of the most important plasmids isolated to date from wine-related lactic acid bacteria (LAB). The chapter is organised in four main parts dealing, respectively, with plasmids from four genera of LAB found on grapes and in must and wine (*Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*). Some of these genera, notably *Lactobacillus*, include a large number of plasmid-carrying strains that have been isolated from non-wine-related sources, but this chapter focuses on plasmids of strains isolated from wine-related sources. When information on the genetics (nucleotide sequence, replication mechanism, use as cloning vectors and/or transformation procedures) of these plasmids, or their effects on phenotype, has been reported in the literature, this information is summarised here. Finally, in this chapter it is concluded that future work on wine-related-LAB plasmids will require new vectors and transformation systems, notably for *Oenococcus oeni*.

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Lactic acid bacteria are important in winemaking for several reasons. First, they are responsible for malolactic fermentation (MLF) that provokes deacidification of wine by transformation of malic acid into lactic acid. Second, they can spoil wine as a consequence of metabolism of various substrates, leading to so-called diseases of wine. Third, some strains can produce undesirable molecules that can be considered as toxic products, such as the biogenic amines and the precursors of ethyl carbamate. Finally, wine-related LAB have been reported to be a source of enzymes of interest in winemaking. For reviews of LAB in winemaking, see Gasson and de Vos (1994), Lonvaud-Funel (1999, 2015), Matthews et al. (2004) and Swiegers et al. (2005).

In view of the above, the genetic manipulation of wine-related LAB to improve their beneficial effects and to minimise their negative effects is one of the main objectives of research on winemaking LAB (Pretorius and Høj 2005; Sumby et al. 2014).

A large number of LAB isolated from different sources contain one or more plasmids: some of these are cryptic and have been used to develop cloning vectors (Shareck et al. 2004; Cui et al. 2015; Landete 2016), while others have been studied for the traits they encode, including bacteriocin production, sugar catabolism, heat stress response, antibiotic resistance, bacteriophage resistance, metal ion resistance and polysaccharide biosynthesis (Salminen and von Wright 1998; Gasson and Shearman 2003; Landete 2016). Parallel to these findings on plasmids from LAB, winemaking researchers have investigated the presence of plasmids in LAB from grapes, must and wine, their putative roles and their possible use as cloning vectors enabling manipulation of wine-related LAB.

The LAB associated with grapes, must and wine belong to the genera *Lactobacillus* (*Lb.*), *Leuconostoc* (*L.*), *Oenococcus* (*O.*), *Pediococcus* (*P.*) and *Weissella* (*W.*). The present chapter aims to review findings on plasmids from LAB strains of these genera which have been isolated from wine-related sources.

21.2 *Lactobacillus*

Lactobacillus is a diverse genus of homo- and heterofermentative LAB whose species are widespread in a variety of natural habitats including the gastrointestinal tract of man and animals, wine and other alcoholic beverages and fermented vegetables. Many species of the genus *Lactobacillus* are important in the industrial preparation of fermented milk, meat and vegetable products like wine (Du Toit et al. 2011; Sun et al. 2016). Mesas et al. (2011) reported *Lb. plantarum* as the main contributor of the MLF to Ribeira Sacra wines which have high alcohol content.

Plasmids are present in most, but not all, *Lactobacillus* species. According to Wang and Lee (1997), at least 38% of the species of *Lactobacillus* contain plasmids that vary widely in size (from 1.2 to more than 150 kb), in number (from 1 to more than 10 types of plasmids in a single strain) and in mode of replication [generally theta replication for large plasmids and rolling-circle (RC) replication for small

plasmids]. Although most of the plasmids of *Lactobacillus* remain cryptic (Shareck et al. 2004; Cui et al. 2015), some functions have been found to be plasmid-linked. According to Wang and Lee (1997), such functions can be grouped into four classes: (1) hydrolysis of proteins; (2) metabolism of carbohydrates, amino acid and citrate; (3) production of bacteriocins, exopolysaccharides and pigments; and (4) resistance to antibiotics, heavy metals and phages.

A large number of vectors based on native plasmids of *Lactobacillus* strains have been developed to transform and/or to conjugate species of *Lactobacillus* and other gram-positive bacteria, and a number of vectors derived from plasmids of non-lactobacilli strains can replicate in *Lactobacillus* species (Wang and Lee 1997; Shareck et al. 2004; Alegre et al. 2009; Rodríguez et al. 2015). Nevertheless, very few such plasmids have been reported from strains of *Lactobacillus* species isolated from wine-related sources.

De las Rivas et al. (2004) described the complete nucleotide sequence of pPB1, a small cryptic plasmid (2899 bp) isolated from *Lb. plantarum* BIFI-38 (a wine-related strain). This plasmid replicates via an RC mechanism and is composed of two modules, a replication module that shows 94.5% identity to an analogous region of the *L. lactis* plasmid pCI411 (Coffey et al. 1994) and a mobilisation module that shows 97.5% identity to *Lb. plantarum* plasmid pLB4 (Bates and Gilbert 1989). These findings suggest that pPB1 originated by modular exchange of large DNA fragments between two plasmids. Evolution based on accumulation of modular units is well established in RC plasmids (Francia et al. 2004).

Lactobacillus plantarum IWBT B 188, a strain isolated from South African wine, contains several plasmids. Strain-specific primers based on the sequence of one of these plasmids resulted useful to follow this strain by qRT-PCR method when it was used as a starter culture during the MLF in Grauburgunder wine (Cho et al. 2011).

In some cases, functions undesirable for winemaking have been associated with the presence of plasmids in *Lactobacillus*. For example, Lucas et al. (2005) reported a strain of *Lb. hilgardii* isolated from wine that contains several plasmids, one of which (pHDC, 80 kb) encodes histamine production and is probably also harboured by some histamine-producing strains of *Tetragenococcus muriaticus* and *O. oeni*. Later, Suzuki et al. (2005, 2006) reported three plasmids involved in resistance to hop compounds of three LAB isolated from spoiled beers. These plasmids are pRH45 (15,136 bp) from *Lb. brevis*, pRH20690 (13,022 bp) from *Lb. lindneri* and pRH478 (14,567 bp) from *P. damnosus*. The very similar characteristics and high percentage of nucleotide sequence identity between these three plasmids suggest a common origin. In addition, a *P. pentosaceus* plasmid (10.1 kb) named pRS5 (Alegre et al. 2009) shows a region with high nucleotide sequence identity (Fig. 21.1) with the plasmids reported by Suzuki's group (Suzuki et al. 2005, 2006). This suggests a close relationship between some plasmids of LAB present in beer and wine. Recently, genome sequencing of the virulent beer-spoilage *Lb. brevis* BSO 464 revealed the presence of eight plasmids (from 15 to 85 kb); some of them carry the genes *hor*A, *hor*C and *hit*A involved in hop tolerance. The loss of three out

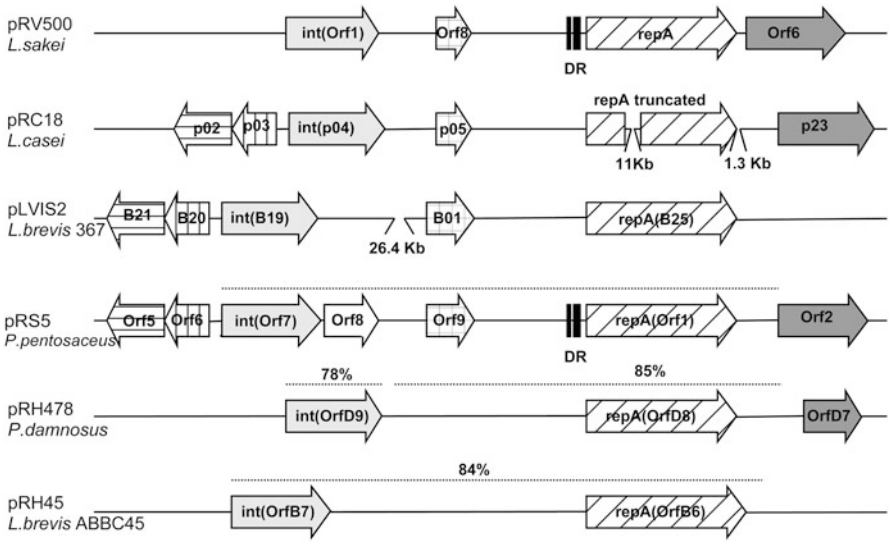

Fig. 21.1 Sequence relationships between pRS5 and other plasmids from LAB. *Black boxes* represent stretches of direct repeats (DR). *orf* genes putatively coding for integrases and replication proteins are indicated by *light-grey arrows* and *arrows* with *slanted lines*, respectively. ORFs sharing amino acid similarity with ORFs 5, 6 and 9 of pRS5 are represented by *arrows shaded* with *horizontal lines*, *vertical lines* and *squares*, respectively. ORFs showing similarity with ORF 2 are indicated by *dark-grey arrows*. pRC18 is represented in the reverse direction relative to its entry in the database. A *horizontal dotted line above* the *sequence lines* indicates large DNA fragments sharing high nucleotide identity among pRS5, pRH478 and pRH45. Reprinted from Alegre et al. (2009) with permission from Elsevier

of the eight plasmids affects hop tolerance and growth of *Lb. brevis* BSO 464 in beer (Bergsveinson et al. 2015).

21.3 *Leuconostoc*

The genus *Leuconostoc* comprises a diverse group of heterofermentative LAB of considerable industrial importance, traditionally used in various food fermentations. Few species of *Leuconostoc* have been documented to be related with wine: *L. oenos*, now reclassified as *O. oeni* (Dicks et al. 1995), and *L. mesenteroides* are the most frequent. In general—and with the exception of *O. oeni*, which has been always isolated from wine-related sources—the species of the genus *Leuconostoc* have been isolated from other sources, including fermented vegetable, milk and meat products (Dellaglio et al. 1995). The species of *Leuconostoc* isolated from musts usually disappear from their own wines during the MLF with the exception of *O. oeni* (Mesas et al. 2011). There have been few studies reporting the presence of

plasmids in species of *Leuconostoc*: those concerning *O. oeni* will be discussed in Sect. 21.4 and the rest here.

O'Sullivan and Daly (1982) were the first to report the presence of plasmids in *Leuconostoc*. They found at least one plasmid, ranging in size from 3.8 to 61 kb, in every one of the ten strains analysed. Later, Orberg and Sandine (1984) documented plasmids from 1.5 to 115 kb in *L. lactis*, in *L. paramesenteroides* now reclassified as *W. paramesenteroides* (Collins et al. 1993) and in several subspecies of *L. mesenteroides*, while Dellaglio et al. (1984) reported the presence of plasmids from 1.8 to 5.3 kb in 15 strains of *W. paramesenteroides*. In 1988 Cavin et al. reported a mutant derived from strain *L. mesenteroides* ssp. *mesenteroides* 19D unable to metabolise citrate and lacking a plasmid of 22.2 kb. In 2011, Mesas et al. reported the presence of plasmids in strains of *L. citreum*, *L. fructosum*, *L. pseudomesenteroides* and *W. paramesenteroides* isolated from musts. Finally, Eom et al. (2010, 2012) reported the presence of two types of plasmids—both RC and theta replicated plasmids—in *L. citreum* isolated from kimchi.

Although some *Leuconostoc* plasmids have been reported to be associated with metabolic functions such as lactose utilisation and citrate permease activity (O'Sullivan and Daly 1982; David et al. 1992), and bacteriocin production and immunity (Hastings et al. 1991), most of the plasmids of *Leuconostoc* remain cryptic.

Four cryptic plasmids replicated by the RC mechanism have been characterised in *Leuconostoc*, pCI411, pFR18, pIH01 and pCB18. pCI411 from *L. lactis* (2926 bp) has a replication origin of the pE194 type and can be introduced into *Leuconostoc*, *Lactococcus*, *Streptococcus*, *Lactobacillus* and *Bacillus* (Coffey et al. 1994). pFR18 (1828 bp) from *L. mesenteroides* ssp. *mesenteroides* has similar characteristics of the pT181 family of plasmids (Biet et al. 1999), and its derivatives are able to transform *Lb. sakei* and several species of *Leuconostoc*. pIH01, a small plasmid (1822 bp) from *L. citreum*, has been characterised by Park et al. (2005) as a new member of the pT181 family. A pIH01 derivative carrying the erythromycin resistance gene (*ermC*) from pE194 has been able to transform *Leuconostoc* strains, *Lb. plantarum* and *Lactococcus lactis*. Finally, pLeuCM, a derivative of pCB18 (3.37 kb) from *L. citreum*, was able to replicate in *L. citreum*, *L. mesenteroides*, *L. lactis*, *Lb. plantarum*, *Lb. reuteri*, *Streptococcus thermophilus*, *Weissella confusa* and *O. oeni* (Eom et al. 2010).

Three cryptic plasmids replicated by the theta mechanism have been characterised in *Leuconostoc* (pTXL1, pCB42 and pRS6). pTXL1 (2665 bp) from *L. mesenteroides* ssp. *mesenteroides*, the first small theta-replicating plasmid described in *Leuconostoc*, was noted by Biet et al. (2002). Derivatives of this plasmid replicate in several LAB. pCB42 (4.3 kb) from *L. citreum* was used to construct derivatives able to replicate in *L. citreum*, *L. mesenteroides*, *L. lactis*, *Lb. plantarum*, *Lb. reuteri*, *S. thermophilus*, *W. confusa* and *O. oeni* (Eom et al. 2012). pRS6 (5.3 kb) from *L. citreum* QV93 (Mesas et al. 2011), a strain isolated from wine, is currently under study by our group. The replication region of pRS6 shares a high sequence identity with the replication region of pCB42. Derivatives of pRS6

replicate in several species of *Pediococcus* as well as in *Lb. plantarum* and *Enterococcus faecalis* (unpublished results).

21.4 *Oenococcus oeni*

This LAB is a heterofermentative coccus which is usually present on grapes, in must and in wine and is the major agent of the MLF (van Vuuren and Dicks 1993; Lonvaud-Funel 1995; Versari et al. 1999; Bartowsky and Borneman 2011; Mesas et al. 2011). According to Lonvaud-Funel (1999), *O. oeni* could be the sole LAB that is genuinely beneficial in winemaking. Due to the important role of *O. oeni* in the process of winemaking, the presence of plasmids in this LAB and their putative roles have been investigated for the last 30 years; there have also been various attempts to develop cloning vectors and transformation protocols for *O. oeni* based on these plasmids.

21.4.1 Plasmids

The earliest reported attempt to find plasmids in *O. oeni* appears to have been that of Sgorbati et al. (1985), who examined extra chromosomal DNA in 52 strains belonging to the heterolactic bacterial species most commonly found in alcoholic beverages (wine, cider, beer, etc.). More than 50% of the strains, belonging to *O. oeni* species and *Lactobacillus* genus, carried plasmids. Two years later, the same group (Sgorbati et al. 1987) analysed 35 new strains of *O. oeni*, finding plasmids ranging from 5 to 59 kb in 11 of these strains. One of these plasmids, pBL34 from strain *O. oeni* Lco 34, was associated with resistance to the pesticides aldrin, bromophos-methyl and heptachlor.

Using new methods of DNA extraction, Janse et al. (1987) isolated 11 plasmids, ranging from 2.4 to 4.6 kb, from 8 of a total of 42 strains of *O. oeni*. Five of these strains contained only one plasmid, and the other three contained two plasmids each. According to these authors, the relatively small size of these plasmids, their low frequency of appearance and their low copy number together suggest that *O. oeni* carries little genetic information in these extrachromosomal elements.

In 1988 Cavin et al. investigated the presence of extrachromosomal DNA in 22 strains of *Leuconostoc*, 8 of them of *O. oeni*. They found that only two of the *O. oeni* strains carried plasmids: strain 8413 from the Institute of Oenology of Bordeaux which carried a plasmid of 5.3 kb later called pLo13 (Fremaux et al. 1993) and strain ATCC 23279 which carried a plasmid of 6.2 kb. These authors concluded that the frequency of appearance of plasmids is low in *O. oeni* by comparison with other *Leuconostoc*.

Table 21.1 Plasmids of *Oenococcus oeni* with complete nucleotide sequence in databases

Plasmid	Size (pb)	Source	Accession number	Characteristics[a]	Reference
pLo13[b]	3948	French wine, IOB[c]	M95954	RC, cryptic	Fremaux et al. (1993)
p4028[b]	4410	Spanish wine, CECT[d]	Z29976	Theta, ATPase	Zúñiga et al. (1996a)
pOg32[b]	2544	Portuguese wine	X86402	RC, cryptic	Brito et al. (1996)
pRS1	2523	Spanish wine	AJ006467	RC, cryptic	Alegre et al. (1999)
pRS2	2544	Spanish wine	AJ310613	RC, cryptic	Mesas et al. (2001)
pRS3	3948	Spanish wine	AJ310614	RC, cryptic	Mesas et al. (2001)
pOM1	3926	Japan	AB208028	Unknown	Unpublished
pOENI-1	18,332	French wine, IOB		Theta	Favier et al. (2012)
pOENI-1v2	21,926	French wine, IOB		Theta	Favier et al. (2012)

[a]*RC* replication by rolling-circle mechanism; *Theta* replication by theta mechanism; *cryptic* unknown function; *ATPase* DNA-dependent ATPase
[b]From strains previously designated as *L. oenos*
[c]*IOB* Institute of Oenology of Bordeaux
[d]*CECT* Spanish Type Culture Collection

In an attempt to differentiate strains of *O. oeni* from New Zealand isolates, Kelly et al. (1993) found three new strains harbouring unreported plasmids of 4, 22 and 33 kb, with unknown metabolic roles.

The first plasmid of *O. oeni* to be analysed at the molecular level (Table 21.1) was pLo13 (3948 bp) present in strains 8413 and HB156, both from French wines. Sequence analysis indicated that pLo13 replicates by the RC mechanism. Later, Prévost et al. (1995) reported the isolation and partial characterisation of 6 cryptic plasmids present in 6 of 15 strains of *O. oeni* isolated from wines and ciders. Every one of the six strains harboured a single plasmid, with three different plasmids altogether, pUBLO1 (3.9 kb), pUBLO5 (4.3 kb) and pUBLO6 (26 kb); pUBLO1 is the previously reported pLo13.

Zúñiga et al. (1996a) reported the nucleotide sequence of p4028, a plasmid of 4410 bp present in *O. oeni* CECT 4028 isolated from wine. Sequence analysis revealed five ORFs grouped in two clusters separated by a short non-coding sequence. No relationships between pLo13 and p4028 were found.

The nucleotide sequence of pOg32, a cryptic plasmid of 2544 bp stably maintained in several strains of *O. oeni* isolated from Portuguese wines, was reported by Brito et al. (1996), who described some similarities between pOg32 and pLo13, such as the presence of three major ORFs and the use of an RC replication mechanism.

Following the change of denomination of *L. oenos* to *O. oeni*, studies on wine-related heterofermentative cocci have tended to centre specifically on *O. oeni* rather than on the genus *Leuconostoc*. Zavaleta et al. (1997) studied the genetic diversity of different strains of *O. oeni* finding that 26% of the strains have extrachromosomal DNA, a higher percentage than the 16 and 8% found by Janse et al. (1987) and Fremaux et al. (1993), respectively. Brito and Paveia (1999) used a large-scale isolation technique to screen 30 strains of *O. oeni* for extrachromosomal DNA, finding large plasmids (ca. 40 kb) in 18 strains and small plasmids (2.5–4.5 kb) in 6 strains. Some of the small plasmids corresponded with, or were similar to, other plasmids already reported, such as pOg32, pLo13 and p4028. This study suggested that large plasmids might be frequent in *O. oeni*, but were difficult to detect due to their low copy number and problems of isolating them.

In 1999 Alegre et al. published the nucleotide sequence of pRS1 (2523 bp), a cryptic plasmid of a strain of *O. oeni* isolated from Spanish wines. This plasmid shows high homology with pOg32, and like pLo13 and pOg32, it contains three ORFs coding for a replication initiation protein (Rep), a plasmid recombination enzyme (Pre) and an unknown protein. These features prompted the authors to postulate the existence of a family of small cryptic plasmids in *O. oeni* that is widespread among strains isolated in diverse countries. Mesas et al. (2001) reported the nucleotide sequences of two other RC plasmids stably maintained in a single strain of *O. oeni*, its natural host. One of these plasmids, pRS2 (2544 bp), shows high homology with pOg32 and pRS1, while the other, pRS3 (3948 bp), is practically identical to pLo13. This finding prompted the authors to suggest that the family of small RC plasmids of *O. oeni* could be split into two subfamilies that could coexist in a single strain of *O. oeni*. Attempts to identify possible roles of these plasmids using cured strains (Mesas et al. 2004) were unsuccessful. Walling et al. (2005) detected strains of *O. oeni* containing a putative *dps* gene by using the same primers that correlate this gene with plasmid pF8801 of *P. damnosus*. This finding raised the possibility of searching for this gene among small plasmids of *O. oeni* without known functions.

Recently, the large theta-replicating plasmids from *O. oeni* have newly attracted the interest of researchers due to their technological appeal, for instance, plasmids pOENI-1 and pOENI-1v2 (18.3 kb and 21.9 kb, respectively) (Table 21.1; Favier et al. 2012), which exhibit high identity in their nucleotide sequences. The ORF 15 of both plasmids encodes replication protein A (RepA). It shares high sequence identity with the replication region of pRS7, a large plasmid of *O. oeni* more recently found that has been used to develop the cloning vector for LAB pRS7Rep (Fig. 21.2; Rodríguez et al. 2015).

A second member of the genus *Oenococcus* isolated from composting distilled shochu residue is *Oenococcus kitaharae*. Sequencing of the genome of the type strain of *O. kitaharae* DSM 17330 reveals the presence of an 8.3 kb plasmid (Borneman et al. 2012).

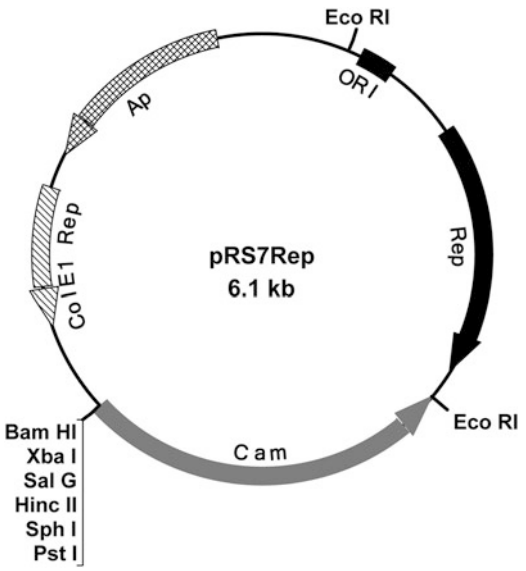

Fig. 21.2 Genetic map of the cloning vector for LAB pRS7Rep. The components of this vector are as follows: (i) the *Eco*RI fragment of the PCR-amplified replication region of pRS7 containing the origin of replication (ORI, *dark rectangle*) and the gene encoding the replication initiator protein (Rep, *dark arrow*); (ii) *Eco*RI-linearised pEM64, which contains the gene for chloramphenicol resistance (Cam, *grey arrow*), and pIJ2925 (*thin line*), which includes the replication region of pUC18 (ColE1 Rep, *diagonal-striped arrow*) and the gene for ampicillin resistance (Ap, *diagonal-cross-striped arrow*). Only relevant restriction sites are shown. Reprinted from Rodríguez et al. (2015) with permission from Elsevier

21.4.2 Development of Cloning Vectors and Transformation Systems for O. oeni

Despite increasing knowledge of the genetics of *O. oeni*, no useful procedures for its genetic manipulation have been developed. Dicks (1994) reported the transformation by electroporation of *O. oeni* using a protocol in which competent cells were prepared in the presence of lysozyme; however, it has proved difficult to reproduce these results, and no new reports based on this protocol have been reported. Several other groups of researchers, including our own group, have dedicated a lot of effort to developing cloning vectors based on small cryptic plasmids of *O. oeni*, and trying to develop an efficient transformation system for *O. oeni*, but so far without success. In our laboratory, we have tried to electrotransform *O. oeni* using well-established electroporation protocols developed for other LAB (see Kim et al. 1992; Berthier et al. 1996; Caldwell et al. 1996; Alegre et al. 2004; Rodríguez et al. 2007) with gram-positive vectors like pCU1 (Augustin et al. 1992), pBT2 (Brückner 1997), pRS4C1 (Alegre et al. 2004), derivatives of pRS5 (Alegre et al. 2009), derivatives of pRS6 and pRS7Rep as well as derivative plasmids of pRS1, pRS2 and pRS3 (Table 21.1). Again, we had not any success. Eom et al. (2010, 2012) reported the

transformation of pBC18 and pBC42 by electroporation in *O. oeni* KCTC3200 at a very low rate (1.7×10^1 and 1×10^1 transformants/μg of DNA, respectively). However, no new reports based on this protocol have been reported.

The introduction and expression of plasmids in *O. oeni* using conjugation appears to give better results than those obtained by electroporation. Zúñiga et al. (1996b, 2003) were able to develop reproducible methods to transform *O. oeni* by conjugation using conjugative transposons and plasmids. They transferred transposons Tn916 and Tn925 from *Enterococcus faecalis*, and plasmids pIP501 and pVA797 from *Lactococcus lactis*, into *O. oeni* by conjugation. However, these conjugative plasmids showed structural instability. Later, Beltramo et al. (2004) developed pGID052, a new plasmid that can be successfully transferred by mobilisation from *Lactococcus lactis* to *O. oeni*. This low-copy-number plasmid seems to be structurally and segregationally stable. To date, the sole realistic candidate vectors for future genetic manipulation of *O. oeni* seem to be pGID052, pBC18, pBC42 and perhaps pRS7Rep.

Later an electroporation protocol using ethanol as a membrane-fluidizing agent and plasmid pGID052 as vector succeeded in the introduction of foreign DNA into *O. oeni* BAA-1163. However, this result has not yet led to an increase in published accounts of molecular transformations of this bacterium, possibly due to the low copy numbers of pGID052 (Assad-García et al. 2008).

Recently, a comparative analysis of the *O. oeni* pan-genome revealed intraspecific genetic variations in the DNA uptake machinery of this microbe (Sternes and Borneman 2016). These authors suggest that a careful selection of strains, which may be more amenable to transformation, provides a sensible avenue for researchers to explore.

21.5 *Pediococcus*

Pediococci are homofermentative LAB that are commonly found in nature in fermenting plant materials (Giacomini et al. 2000), as well as in beer, cider and wine (Fernández et al. 1995; Gindreau et al. 2001; Mesas et al. 2011). Several pediococcal strains are important in the fermentation of vegetables, soy milk and meat and in flavour development of Cheddar cheese (Fleming and McFeeters 1981; Smith and Palumbo 1983; Thomas et al. 1985). In addition to their ability to produce fermented foods, there are pediococcal strains that produce bacteriocins active against gram-positive pathogenic and food-spoilage bacteria (Daeschel and Klaenhammer 1985; González and Kunka 1987; Hoover et al. 1988; Motlagh et al. 1994). Many pediococcal strains harbour plasmids that encode a variety of traits, while others, being cryptic, have been used to develop cloning vectors (Shareck et al. 2004; Alegre et al. 2005, 2009; Cui et al. 2015). To date, genetic studies on pediococcal plasmids have yielded considerable knowledge; however, only few studies on plasmids from wine-related strains of *Pediococcus* have been reported.

21.5.1 Plasmids

One of the first indications of the presence of plasmids in *Pediococcus* was reported by González and Kunka (1983), who detected plasmids from 7.1 to 46 kb in strains of *P. pentosaceus* and *P. acidilactici*, though no information was provided about their metabolic function. Later, curing studies with novobiocin indicated that production of a bacteriocin-like substance by *P. cerevisiae* FBB63 might be linked to a 16 kb plasmid (Graham and McKay 1985). Daeschel and Klaenhammer (1985) reported that the production of a bacteriocin named pediocin A was associated with a plasmid present in two strains of *P. pentosaceus* isolated from cucumber fermentations. Both bacteriocin immunity and bacteriocin production were encoded by the plasmid. González and Kunka (1986) found that the abilities to ferment raffinose, melibiose and sucrose by three strains of *P. pentosaceus* were encoded by plasmids. The same two authors also reported in 1987 the association of sucrose fermentation and production of pediocin PA-1 with plasmids pSRQ10 and pSRQ11, respectively, in *P. acidilactici* and speculated on the natural role of such plasmid-linked properties that may give to the strains containing these plasmids a selective advantage in nature.

Other authors (Hoover et al. 1988; Halami et al. 2000) have reported linkage of pediococcal sugar utilisation, and bacteriocin production and immunity, to plasmids, but there have been no published reports relating such functions with plasmids of strains of pediococci isolated from wine. However, because some strains of *Pediococcus* isolated from wine seem to produce bacteriocins (Strasser and Manca 1993), it seems likely that some functions of wine-related pediococci may be plasmid-linked.

Some LAB can induce viscosity in wine, beer and cider by production of exopolysaccharides (EPS); these LAB are designated ropy strains. In a study of the presence of plasmids in a ropy strain of *Pediococcus* isolated from Basque Country ciders, Fernández et al. (1995) found six plasmids, one of them related with the ropy character and resistance to oleandomycin. These results are in line with those of Lonvaud-Funel et al. (1993), who observed that non-ropy derivatives of ropy strains of *Pediococcus* had lost some of their plasmids. Plasmid pF8801 was detected in ropy strains of *P. damnosus* (Lonvaud-Funel et al. 1993) and partially sequenced in order to develop primers specific for the detection by PCR amplification of other ropy strains. The complete nucleotide sequence of this plasmid (Walling et al. 2005) revealed genes for maintenance (*rep*) and transfer (*mob*) and a putative glucosyltransferase gene named *dps*.

In addition to pF8801, only a few plasmids of *Pediococcus* have been completely sequenced (Table 21.2). The first reported complete nucleotide sequences of plasmids of *Pediococcus* were those of pMSB 74 from *P. acidilactici* (Motlagh et al. 1994) and of pUCL287 from *P. halophilus* (Benachour et al. 1997), later reclassified as *Tetragenococcus halophila*. Because plasmid pUCL287 was shown to be a theta-replicating plasmid unrelated with previously well-characterised theta-type replicons, a new family of theta-type replicons

Table 21.2 Plasmids of *Pediococcus* with complete nucleotide sequence in databases

Plasmid	Size (bp)	Species	Source	Accession number	Characteristics[a]	Reference
pSMB 74	8877	*P. acidilactici*	–	U02482	Theta, pediocin AcH	Motlagh et al. (1994)
pUCL287	8738	*T. halophila*[b]	–	X75607	Theta, cryptic	Benachour et al. (1997)
pMD136	19,515	*P. pentosaceus*	Cucumber	AF033858	Theta, pediocin A	Giacomini et al. (2000)
pRS4	3550	*P. pentosaceus*	Spanish wine	AJ68953	RC, cryptic	Alegre et al. (2005)
pF8801	5558	*P. damnosus*	French wine	AF196967	(RC), *dps*	Walling et al. (2005)
pRH478	14,567	*P. damnosus*	Japanese beer	AB218963	(Theta), *hor* A	Suzuki et al. (2006)
pRS5	10,153	*P. pentosaceus*	Spanish wine	FM163399	Theta	Alegre et al. (2009)
pPCEL1 to pPCEL8	From 1.8 to 36 kb	*P. claussenii*	Beer	CP003138 to CP003145	Several	Pittet et al. (2012)

[a]*Theta* replication by theta mechanism; *RC* replication by rolling-circle mechanism; (RC or Theta) putative replication mechanism; *cryptic* unknown function; *Pediocin AcH* contains *pap* gene cluster of the Pediocin AcH; *Pediocin A* contains regulatory genes involved in pediocin A activity; *dps* glucan synthase gene; *hor* A hop resistance gene

[b]Previously called *P. halophilus*

represented by pUCL287 was set up. Plasmid pMD136 of *P. pentosaceus* (Giacomini et al. 2000) is another completely sequenced pediococcal plasmid that has been reported to use theta replication; however, while pMD136 showed homology with the theta-type replicons of *Lactococcus lactis*, pSMB 74 was suggested to be a member of the pUCL287 replicon family.

The nucleotide sequence of cryptic plasmid pRS4 from a strain of *P. pentosaceus* isolated from wine was reported by our group (Alegre et al. 2005). This was the first RC-replicating plasmid of a *Pediococcus* strain that was completely sequenced and used as a cloning vector for LAB. A second plasmid named pRS5, which replicates via the theta mechanism and isolated from the same strain, was also sequenced and used as a cloning vector for LAB (see Sect. 21.2).

Pediococcus claussenii is a common beer-spoilage organism. *P. claussenii* ATCC BAA-344T contained eight plasmids (pPECL-1 to pPECL-8) that encode a variety of traits, including drug resistance, conjugation protein, the toxin-antitoxin (TA) system and bacteriocin, among others (Pittet et al. 2012). From the point of view of beer spoilage, several genes are interesting like the hop resistance gene *hor*A that codes an ATP-binding cassette multidrug transporter found on pPECL-8. The overall G + C content of the genome is 36.8%, whereas that of the plasmids ranges from 34.9 to 42.5%. The results show that there is a horizontal gene transfer in this bacterium.

21.5.2 Plasmid Transfers into Pediococcus

A number of reports show that some broad-host-range plasmids can be transferred by conjugation and/or electroporation into *Pediococcus* species (González and Kunka 1983; Kim et al. 1992; Benachour et al. 1996; Caldwell et al. 1996; Rodríguez et al. 2007). However, only pRS4 and pRS5 have been isolated from a wine-related strain of *Pediococcus*. In addition, pRS4 derivatives seem to be a useful vector for food-related LAB, since they replicate in species of *Lactobacillus* and *Pediococcus* but not in *Enterococcus faecalis*, while pRS5 derivatives seem to be more stable vectors (Alegre et al. 2004, 2009; Mesas et al. 2006).

21.6 Conclusion

Plasmids are frequently present in LAB species, but only a few plasmids have been reported from wine-related LAB strains. Some genera like *Lactobacillus* and *Pediococcus* seem to be plasmid-rich, while *Leuconostoc* and *Oenococcus* are not. In general, large plasmids replicate via the theta mechanism and contain genes coding for different traits, while small plasmids usually replicate by the RC mechanism and are cryptic. The stable maintenance of such small cryptic RC plasmids in their hosts, under an apparent absence of selective pressure, can be

explained—as in the case of the RC plasmids of *Bacillus* (Guglielmetti et al. 2006)—by considering that they promote recombination and consequently enhance the ecological adaptability of their hosts.

The presence of plasmids in *O. oeni*, the most important LAB in winemaking, has attracted the attention of several groups of researchers. As a consequence of these studies, it can be concluded that small plasmids are less frequent in *O. oeni* than in other LAB. A few small cryptic plasmids are widespread among strains of this species (Fremaux et al. 1993; Brito et al. 1996; Zúñiga et al. 1996a; Alegre et al. 1999; Mesas et al. 2001), and there have been some attempts to use these plasmids as tools for genetic manipulation of LAB, but without successful results to date. The low degree of conservation of *rep* and *pre* sequences in the small RC plasmids of *O. oeni* can be attributed to the fast rate of evolution of *O. oeni* compared with other gram-positive bacteria (Yang and Woese 1989; Brito et al. 1996; Makarova and Koonin 2007). Large plasmids, sometimes with known roles, are also more frequently present in *O. oeni*, than would be expected a priori (Sgorbati et al. 1987; Brito and Paveia 1999; Favier et al. 2012; Rodríguez et al. 2015), suggesting that a lot of genetic information from *O. oeni* may be contained in large plasmids.

Future work on wine-related-LAB plasmids will require new vectors and transformation systems, notably for *O. oeni*. To date, the ability to reproducibly transform it for research purposes remains a considerable challenge. Nevertheless, the availability of LAB genome sequences (Makarova and Koonin 2007) together with the development of other genetic tools (Sumby et al. 2014; Sternes and Borneman 2016) will undoubtedly facilitate the development of strains of LAB with improved winemaking properties.

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Part V
Modern Methods

Chapter 22
Molecular Methods for Identification of Wine Microorganisms and Yeast Development

Jürgen Fröhlich, Helmut König, and Harald Claus

22.1 Introduction

A prerequisite for the biochemical and physiological investigation of microorganisms is the isolation and management of pure cultures. Nevertheless, most of the environmental microorganisms are graded as "yet not cultivable" because the nutritional requirements are unknown or they could not be isolated due to the fact that fast-growing strains overgrow other microorganisms of a microbiota. In addition to plating techniques, isolation without cultivation and analysis of microbes could be performed by micromanipulation techniques or the application of optical tweezers followed by the utilization of PCR-based technologies.

Many different phenotypic and genotypic methods are presently used for microbial identification and classification. Several of these methods are suitable for the simultaneous detection on species level, like the analysis of the rRNA genes. Strain-related fingerprint techniques like RFLP-PFGE, nSAPD-, or RAPD-PCR as well as total genome sequencing are dependent on the purity of cultures or genomic DNAs and therefore not suitable for simultaneous detection. For the identification of uncultivable microbes of certain microbiota, different micromanipulation methods for isolation of single cells can be employed. The usage of micromanipulation

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techniques along with PCR is thereby not limited to the identification of single cells. The method can also be applied for the development of new yeast strains by using protoplast fusion technique when different yeast cells have to be placed together side by side. In this context, the technique is still by far the most reliable method for the selection of a pure strain. Large-scale fermentations for active dry yeast productions are sensitive for infections and syntrophic cultures, which were generated by protoplast fusions. These mixed populations are hardly separable by common plating techniques and can cause problems during the propagation of competing cells leading to loss of biomass. The only absolute criterion of purity for a microbial culture is that it has been derived from the progeny of a single cell. Failure to apply this criterion may lead to much effort in proving the purity of a culture. All strains upon which research is to be based should therefore be rigorously purified before starting to investigate the properties of individual organisms (Johnstone 1969). Ecologically oriented wine microbiologists are especially faced with the problem of how to obtain a pure culture of certain microbial strains from their densely populated natural habitats. The used methods comprise thereby a range from simple devices up to very complex machines. Most approaches to identify and enumerate microbes in wine use enrichment techniques (Fugelsang and Edwards 2007). Such indirect methods do not enumerate the original cell number in the sample, but their progeny, as enriched in a specific medium. Fugelsang and Edwards (2007) described both general and selective growth media for plating yeasts and bacteria from wine. Unfortunately, plating and enrichment procedures are time consuming as colonies for some wine-related microbes take up to a week or more to appear on a Petri dish. Additionally, once colonies appear on a plate, the identification of the microbes requires further testing. Moreover, sublethally injured or viable but nonculturable cells, common in wine, may fail to grow on plates but are metabolically active. As a rule, culture-based techniques typically underestimate the size and diversity of a population (Kell et al. 1998; Millet and Lonvaud-Funel 2000). For monitoring the succession of a microbiota, cultivation-free molecular biological approaches were applied which give a more realistic view of a population. These spatiotemporal "snapshots" are often presented in the form of gel-electrophoretic pattern of PCR amplicons or pictures of fluorescence in situ hybridization (FISH) which allow a simultaneous visualization of the main role-players within a population on species level (Amann et al. 1995; Mills et al. 2002; Hirschhäuser et al. 2005; Röder et al. 2007a, b). Unfortunately, there are no cultivable-free techniques available that could represent and monitor populations on strain level. Up to now, all methods that fit the strain level are culture dependent. With focus on a single cell, micromanipulation techniques are alternative methods to traditional cultivation approaches and a useful tool when complex habitats are investigated without cultivation (Fröhlich 2002; Fröhlich and König 1998, 1999a, b, 2000; Fröhlich et al. 2002). After the isolation of a single cell, different methods for identification on species or strain level can be applied, which are described below. The combination of micromanipulation techniques with methods like protoplast fusion, (n)SAPD-PCR, or mutagenesis to get

new yeast strains has substantially broadened their application spectrum (Fröhlich et al. 2016).

22.2 Micromanipulation Techniques

22.2.1 Historical Perspective

Since the beginning of the twentieth century, several attempts have been made to improve the management of single prokaryotic and eukaryotic cells by using micromanipulator techniques. Thereby, a suspension of an adjusted concentration of microorganisms was aspirated into a simple capillary tube, so that a single cell was transferred statistically in a defined volume (Harbeck and Rothenberg 1995).

Moreover, a survey of the chief methods devised for single organism cultures was presented by Johnstone (1969, 1973). These include the block cut method for the selection of an isolated organism on a lightly inoculated nutrient gel; formation of droplets with micropipettes, which are searched for those containing single organisms; and isolation by carrying the selected organisms across the sterile gel surface with a microneedle. Because of technical problems and disadvantages, these methods were not adopted for routine isolation.

Other attempts to improve the management of single microbial cells by using micromanipulator techniques have been described in the literature. Either microneedles or microcapillaries were used for the separation of single bacterial cells (Skerman 1968; Bakoss 1970; Johnstone 1973; Thomsen et al. 2004). The techniques firstly suggested more than 40 years ago were based on the state of the art at that time. They were faced with several technical disadvantages, which hampered routine usage of the isolation techniques for a broad spectrum of prokaryotes in a microbiological laboratory. The magnification was limited, and a transfer of single cells was hardly possible (Skerman 1968). It was designed for use with low power objectives (e.g., 10×) with a working distance of 7 mm or more. The instrument consists of a lens collar and magnetic tool carrier. The lens collar was clamped onto the objective, and it contained two steel slides which permitted the magnet tool carrier to slide along freely. Knobs or microloops were the most useful tools for the isolation of cells from colonies on solid agar plates. By several operations, cells were floated across the surface of solid media by lateral movement of the Petri dish, and they were well separated from the original population. Attempts to lift single organisms in a loop for transfer were rarely successful. So far, this method has been applied for the isolation of large filamentous bacteria (Bradford et al. 1996) and cyanobacteria (Bowyer and Skerman 1968). Bakoss (1970) cloned single cells of leptospires with a micropipette connected to a syringe via a thin polyethylene tube, which was fastened to the holding clip of a micromanipulator. He used a syringe as a simple pneumatic system. The disadvantage of this micromanipulator technique was that it was laborious. A mechanical

micromanipulator with a microneedle was also used (Sherman 1973) to separate the four spores from a cluster in a yeast ascus. This technique was also applied for this purpose in our institute, and it is also suitable for the separation of larger bacterial cells (>3 μm) and yeasts by moving them onto an agar surface. Coccoid bacteria from the "corn cob" of human dental plaques were successfully isolated by Mouton et al. (1977) with microneedles designed to be a double-angulated microhook as described by Johnstone (1973). Single selected spores of *Bacillus cereus* adhering to the glass point of capillary tubes were selectively removed from Petri dishes (Hamilton 1978). Micromanipulation was also successfully applied for the isolation of *Pedomicrobium* cultures from water samples (Sly and Arunpairojana 1987). Luttermann et al. (1998) described a micromanipulation method for transferring micro-objects such as bacteria from agar plates with micro-capillary tubes. An angulated capillary tube (angle of 90°) is positioned between the condenser and the objective. The agar plate with the selected bacteria is moved below the opening of the capillary tube with the microscope stage. The aspirated bacterium is placed on the surface of a solid medium or in liquid media in microtiter plates.

22.2.2 Modern Equipment

Since the 1960s, the technical equipment of micromanipulators has been greatly improved. A long-distance objective (Zeiss, Oberkochen, Germany) with a magnification of a hundredfold is now available. This allows manipulation at a magnification of a thousandfold and more with an inverse microscope. The capillary tools can be positioned quickly and precisely. The available pneumatic or hydraulic systems are very accurate pressure devices.

For the isolation of microbial cells, a commercial micromanipulator (Eppendorf, model 5171) equipped with a pressure device (Eppendorf model 5246 plus or CellTram Oil) and mounted onto an inverse phase contrast microscope (Axiovert 25; objective CP "Achromat" 100×/1.25 Oil Ph2; Zeiss) is used (Bactotip method) (Fröhlich 2002; Fröhlich et al. 1998a, b, 2002; Fröhlich and König 1999a, b, 2000; Prüss et al. 1999; Fig. 22.1). The magnification is adjusted from 400× to 1,000×. The micromanipulator is used according to manufacturer's instructions (micromanipulator 5171: Operating Manual; CellTram Oil: Operating Manual; Transjector 5246: Operating Manual; Eppendorf, Hamburg, Germany). The diameter of the opening of the capillary tip can be adjusted to the size of the bacterial cell of interest. For the isolation of bacteria, a sterile capillary tube ("Bactotip"; Fig. 22.2a, b) is used, which preferably possesses a beveled tip (angle 45°) usually with an opening of about 5–10 μm at the anterior end. The sterile Bactotips are produced by Eppendorf (Hamburg) or can be manufactured with a capillary puller (Saur, Reutlingen, Germany) and a microgrinder (Saur, Reutlingen) using capillary tubes type GB 100 TF-8P (Science Products GmbH, Hofheim, Germany). The posterior end of the Bactotip is sealed with a droplet of sterile oil. If desired, the inner surface of the tip can be siliconized with dichlorodimethylsilane (Fluka Chemie AG, Buchs,

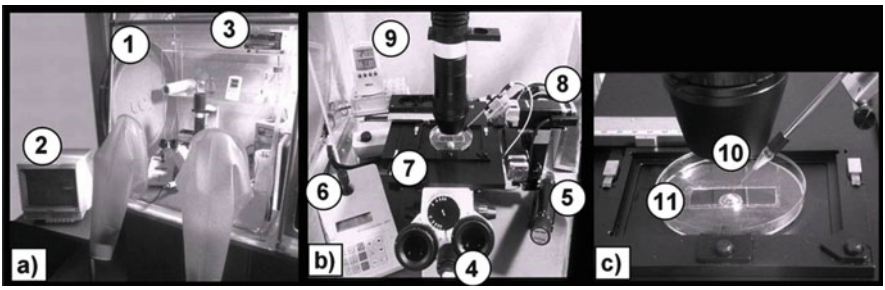

Fig. 22.1 Working station for the manipulation of single cells with a COY chamber (**a**) for the aerobic and anaerobic isolation. The isolation of single cells is performed using an inverse microscope and a micromanipulator device (**b**). The spread cells are aspirated by the application of a Bactotip (**c**). Technical specifications: COY chamber (*1*), monitor (*2*), O_2/H_2 electrode (*3*), camera (*4*), CellTram Oil (*5*), joystick (*6*), inverse microscope (*7*), micromanipulator (*8*), thermometer/hygrometer (*9*), Bactotip (*10*), cover slip with spread bacteria (*11*)

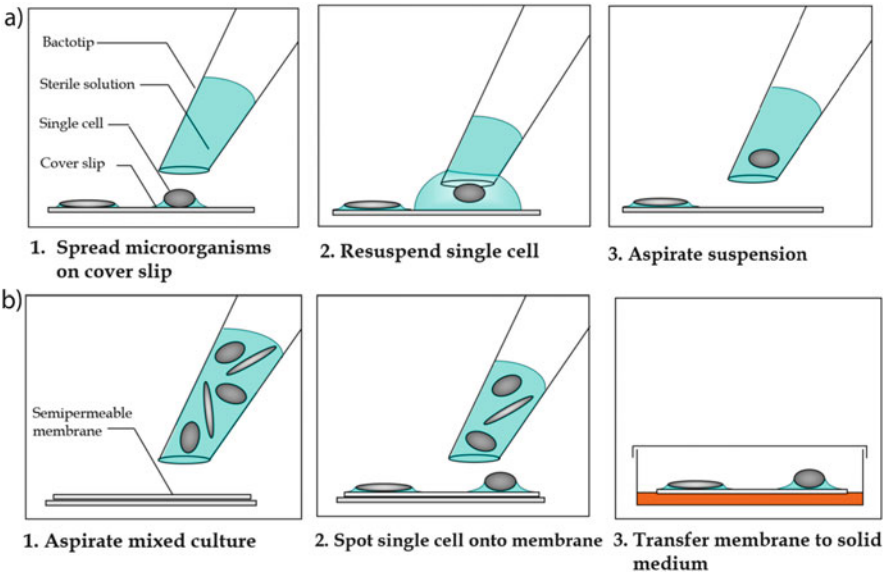

Fig. 22.2 Bactotip (**a**) and membrane (**b**) methods for the isolation of single cells

Switzerland). This is advisable if the bacteria tend to adhere to glass surfaces. Our experiments show that desiccation and oxygen stress (Krämer 1997) for the isolation of anaerobic and aerobic microorganisms can be avoided by using a glove box with a N_2/H_2 (95:5; v/v) atmosphere (COY chamber, Toepfer Lab Systems, Göppingen, Germany). The relative humidity in the chamber is adjusted from 95 to 100%. The microscope bulb is replaced by an optical fiber device (Schott, Mainz, Germany) which reduces the IR radiation. The microscope is equipped with a CCD camera (Type AVTBC12CE, Zeiss) and a monitor (Type PM 95 B, Zeiss).

22.3 Isolation Techniques

22.3.1 Bactotip Method

This technique (cf. Fig. 22.2a) is used when single cells are cultured in liquid media or genes are going to be amplified by single-cell PCR. Cultures or complex mixtures of prokaryotic or eukaryotic strains are diluted in 1–10 mL phosphate-buffered saline (1× PBS). An aliquot of the suspension (10 µL) is spread as a thin film on a sterile microscopic cover slip (24 × 60 mm). A small volume of buffer or medium (ca. 0.1–0.2 µL) is aspirated into the capillary tube. When the opening of the Bactotip is brought close to the surface of a distinct microorganism, a droplet flows out of the tip and moistens the selected cell. The cell is suspended in the droplet after detaching from the glass surface and aspirated into the Bactotip. About ten single microbial cells are successively removed from the microscopic slide within 30 min by aspirating them together with the droplet into the Bactotip. The withdrawn single cells can be transferred in Eppendorf reaction tubes or Hungate tubes (anaerobes) containing 0.3 mL of the corresponding liquid medium. The tubes are incubated at, e.g., 37 °C for 10–72 h (Fröhlich and König 1999a, 2000; cf. Prescott et al. 2002).

22.3.2 Membrane Method

In contrast to the Bactotip method, an appropriate dilution (ca. 0.1 µL) of a mixed culture is sucked into the capillary tube (cf. Fig. 22.2b; Fröhlich et al. 2002). The tip is brought close to the surface of a semipermeable membrane (dialysis hose; Roth) and single cells are spotted under visual control on the membrane in a distance of 5–10 mm to each other. Subsequently, the membrane will be removed with sterile tweezers and transferred onto a solid medium. Nutrients diffuse through the membrane and enable individual cells to grow up to colonies. In contrast to the Bactotip method, more cells can be isolated in a little while. The use of a dialysis membrane has the advantage of a very smooth surface compared with the application of agar layers, so that very small microorganisms can be separated without limitation of the visual control.

22.3.3 Efficiency of the Cloning Procedure

The efficiency rate of the cultivation from freshly grown laboratory cultures was between 30% (*Escherichia coli*) and 70% (*Staphylococcus aureus*) (Fröhlich and König 1999a), and the isolation of lactic acid bacteria (LAB) could be performed with similar rate. The *Oenococcus oeni* strains B70 and B139^T could be isolated

with an efficiency between 63 and 67%. Similar results could be obtained with the species *Lactobacillus brevis* (66%) and *Pediococcus damnosus* (70%). The fastidious anaerobe *Bifidobacterium bifidum* could be micromanipulated anaerobically in the anaerobic chamber with a yield of 30% (Fröhlich et al. 2002). Thereby, the selection of the isolation method did not have any influence. The single cells grew up to a visible density or a visible colony in 10–72 h. A single cell could also be transferred onto solid media in Petri dishes as proved with *B. cereus*. The colonies become visible after incubation overnight at 37 °C. Furthermore, single cells (e.g., *B. cereus*) were directly grown in the Bactotip.

Spreading of the bacterial suspension onto a microscopic slide after an appropriate dilution of the original culture was a prerequisite for the rapid isolation of the single cells, while the isolation of a single bacterial cell directly out of a droplet containing a suspension of a mixed microbial population was not successful. The cells should be transferred to the culture medium within 30 min after spreading. The application of the Bactotip method allows the transfer of single prokaryotic cells to different culture vessels such as Eppendorf reaction tubes and Hungate tubes, onto the surface of solid media in Petri dishes or to subject the isolates to single-cell PCR. Cells were also directly grown in the Bactotip. The advantage of the Bactotip method compared with conventional isolation methods can be seen in the ability to pick out a single prokaryotic cell under direct visual control and to grow pure cultures of distinct aerobic and anaerobic cells directly out of a mixed natural or laboratory population in a relatively short time.

22.3.4 Use of Micromanipulation Techniques and nSAPD-PCR for Protoplast Fusion and Strain Development

Standard GMO techniques as well as modern tools for gene manipulation, like CRISPR/Cas9, showed that genes of *Saccharomyces* species can be easily genetically manipulated (Pretorius and van der Westhuizen 1991; Pretorius 2000; Ryan et al. 2016). Nevertheless, the international world on wine and in particular the EU regarded GMO as hazardous, and the use of them is more or less restricted in the majority of wine-producing countries. Only the application of two GMO wine yeasts (*S.c.* strain MLO1, malolactic fermentation, and *S.c.* strain ECMo01, degradation of urea) is in agreement with the regulations of Canada and the USA and therefore accepted in the winemaking process (cf. Louie 2005; Pretorius et al. 2012). On the other hand, natural crossbreeding and protoplast fusion as well as non-GM technique, like random mutagenesis by chemical mutagens or UV light, are very useful in generating new yeast strains. Traditionally, improvements in an industrial strain, e.g., alcohol tolerance, aroma profile, and adaptation to changing fermentation conditions, have involved different techniques followed by selection of strains having the new characteristics.

Using the example of strain ML01, the necessity of GMO techniques seems to be questionable in view of the publication about the fusion of fission yeast *Schizosaccharomyces pombe* and wine yeast *Saccharomyces cerevisiae* (Carrau et al. 1990). The generated hybrids were described as good fermenting yeasts and able to degrade malic acid. Unfortunately, such interesting non-GMO hybrids never became commercial products, despite the increased robustness compared to SO_2-sensitive MLF-bacteria (*O. oeni*). In order to identify reasons for this, hybrids with the *Ss. pombe* and *S. cerevisiae* were produced. The investigation of these hybrids which could be clearly identified alone on the basis of their unique mixed cell morphology by microscopy showed interesting behavior (Fröhlich et al. 2017, unpublished data). A few isolates picked from single colonies of a Petri dish exhibited the fermenting power of the wine yeast combined with the capability for MLF of *Ss. pombe*. Unfortunately, these apparently pure cultures revealed as nonstoichiometric blends of hybrids and parental strains tested by selective medium for *Ss. pombe*. Using micromanipulation techniques for isolation of single cells, the hybrids showed good fermenting ability but were not able to ferment malic acid indicating that failure of plating techniques for isolation can be attributed to the formation of syntrophic cultures. Therefore, the method is however also associated with risks and has its limits particular when metabolic pathways of cell organelles and membrane proteins are involved.

Focusing on protoplast fusion methods, generally two techniques can be applied. The first technique uses mixed cultures consisting of numerous cells in a vial, and fusion occurs therefore in a "black box" system without visual control (Kavanagh and Whittaker 1996). In order to mitigate this drawback, flow cytometry could be applied for the difficult separation of fusants and also exclusion of undesired phenotypes (Urano et al. 1994). At first glance, the second method seemed to be more labor-intensive, but it provides the advantage of visual control by fluorescence microscopy. In addition, the protocols for fusions are comparable, but the micromanipulator- and microscope-assisted method is independent on complex screening and isolation procedures (Kavanagh and Whittaker 1996; Fröhlich and König 2000; Katsuragi 2001). Here, the selection of the fittest parental strains can be executed before placing the fusion partners by using viability dyes under microscopic view (cf. inlet Fig. 22.3, Katsuragi 2001).

Assuming that selected high-performance hybrids become successful commercial products, it is absolutely conclusive that scientists are interested in the investigation of genomic alterations of such strains. Therefore, the genomes of five different commercial wine yeast strains AWRI 696, QA23, VIN7, VIN13, and VL3 were sequenced. Especially the analysis of VIN7 revealed that the yeast is an allotriploid hybrid fusion product possessing genes of *S. cerevisiae* and *Saccharomyces kudriavzevii* (Borneman et al. 2012). Other commercial yeasts (Oenoferm®/Erboferm™ X-treme and Oenoferm®/Erboferm™ X-thiol) derived by protoplast fusion using micromanipulation techniques are crossbreedings of *S. cerevisiae* subsp. *cerevisiae* and *S. cerevisiae* subsp. *bayanus*. Comparing DNA pattern analysis of hybrid and parental strains by using (n)SAPD-PCR (Primer C*NOT*G) assesses the success of fusion (cf. Fig. 22.3, Fröhlich and Pfannebecker 2007). A

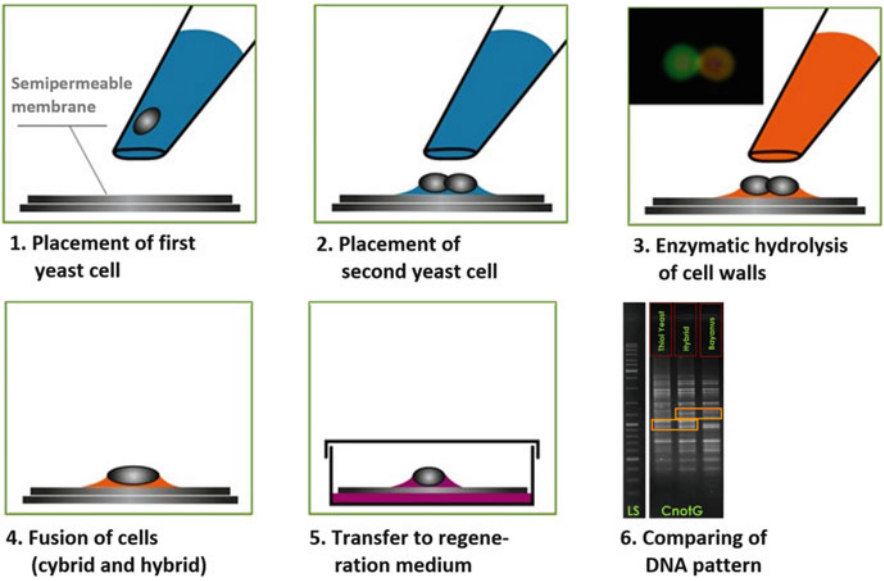

Fig. 22.3 Course of protoplast fusion by using micromanipulator mounted to an inverse fluorescence microscope and combined application of viability dyes and nSAPD-PCR in order to confirm the success of the fusion. *Orange boxes* indicate identical DNA pattern of hybrid and parental strains

general overview and application procedures of protoplast fusion are outlined by Kavanagh and Whittaker (1996), and the combination with micromanipulation technique is described by Katsuragi (2001).

22.3.5 Development of a Less Alcohol-Producing Yeast Using Mutagenesis, nSAPD-PCR, and Micromanipulation

As a result of the climate change, rising sugar content in grape must and the concomitant increase in alcohol levels in wine are some of the main challenges affecting winemaking nowadays. Among the several alternative solutions currently applied, the use of special wine yeasts which were isolated after different selective pressures shows promising results to relieve this problem. Attempts to produce such yeasts in particular comprise intentional genetic modification without application of GMO techniques and processes based on selective cultivation. Besides GM technologies, further methods can be applied to manipulate yeasts to produce less alcohol and increased amount of glycerol by using their molecular response to osmotic stress. When exposed to highly osmolar conditions, yeast cells show enhanced uptake of glycerol from the juice and fast accumulation of glycerol to

mitigate the dehydration. In addition, increased retention of glycerol by the plasma membrane is induced (Nevoigt and Stahl 1997). Glycerol was mainly synthesized during the early phase of fermentation because of the osmotic stress but released in the later stage due to cellular lysis and higher membrane permeability (Orlić et al. 2010).

Gene modifications can be introduced by mutagenic substances or UV light as well as different selection pressures are suited to adapt yeasts (Fröhlich et al. 2016). The object can be achieved by using combined different mutagenesis techniques, whereby the first and the second mutagen differ from each other and are selected from the following groups: nucleotide-alkylating agent, nucleotide-deamination agent, and UV radiation. A first selection step is performed after the first round of mutagenesis, and a second selection step is performed after the second mutagenesis, whereby the mutants resulting from the respective preceding mutagenesis are exposed to selection factors like hypertonic medium or alcohol dehydrogenase inhibitor (cf. Fig. 22.4). Finally, these strains were selected by a pipette robot, and the best of them are subjected to RNA microarray tests showing that genes of the high osmolarity glycerol (HOG) pathway were mainly affected by the mutagenesis (Fröhlich 2017, unpublished data).

Other procedures like adaptive evolution can also lead to a slight reduction of ethanol yield and improvement of glycerol formation (Tilloy et al. 2014).

With a view to the yeast metabolism, it could be assumed that an increase in glycerol formation requires an equimolar amount of cytoplasmic NADH, when cells are osmotically stressed. In order to maintain the redox balance, the reduction of acetaldehyde to ethanol decreased partially, and on the one hand, an increased oxidation to acetate takes place (Blomberg and Adler 1989; Scanes et al. 1998). Although the mitochondria are no longer functional under anaerobic conditions, the enzymes of the tricarboxylic acids cycle are present in the cytoplasm. Pyruvate carboxylase catalyzes the carboxylation of pyruvate into oxaloacetate. The citric acid cycle cannot be completed since the succinate dehydrogenase activity requires the presence of FAD, a strictly respiratory coenzyme. The chain of reactions is therefore interrupted at succinate, which accumulates. In contrast to higher amounts of succinic acid which leads to salty and bitter taste, esters contribute positively to the wine aroma. In compensation of these by-products, improved fermentation performance in terms of shortening fermentation period, effective uptake of ammonium leads to clear reduction of acetaldehyde, acetic acid, and fusel alcohols (Csutorás et al. 2014).

The staggered mutagenesis and selection procedures play the key role in the modification of the yeast genetics. Surprisingly, the subsequent mutagenesis cannot successfully carry out in random order. It rather indicates that a very particular sequence of mutagenic treatments leads to best results in combination with a certain selection pressure (cf. Fig 22.4). The following mutagens have been applied: ethyl methane sulfonate (produces alkylated DNA), nitrite (produces deaminated nucleotides), and UV light (produces thymidine dimers, etc.). It could be elicited beforehand in a separate test that mutagens, like ethidium bromide (EB), which causes frame shift alterations in DNA, are not suited for the production of glycerol-

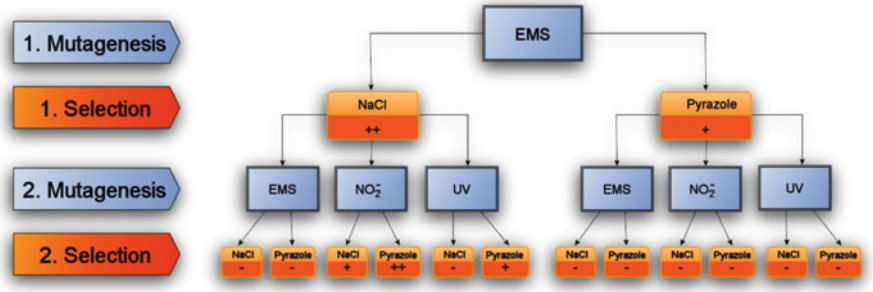

Fig. 22.4 Mutagenesis and selection strategy for successful yeast gene modification

producing yeast mutants. Mainly, these mutants were impaired in the respiratory chain and the mitochondrial DNA, which is only slowly repairable. Furthermore, it was demonstrated that EMS along with high amounts of sodium chloride produces useful mutants for the second mutagenesis. At this stage, the application of pyrazole (ADH inhibitor) seems to be less effective for selection. It is also not advisable to apply EMS repeatedly. Best mutants were obtained after the application of EMS and the selection under high osmophilic conditions followed by nitrite treatment and the use of pyrazole for the second selection pressure (Fröhlich et al. 2016, cf. Fig. 22.4). After mutagenesis and selection, strains were isolated by micromanipulation to get pure cultures. For the identification of these pure cultures, (n) SAPD-PCR was applied (Fröhlich et al. 2016).

Finally, the production of glycerol instead of ethanol provides further advantages by balancing the alcoholic strength and astringency. Glycerol conferred smoothness on the palate and gave the impression of full-bodied wines. The overall flavor intensity was positively influenced by glycerol. For the bitter taste, glycerol was reported to suppress astringency and roughness perception of a wine (Jones et al. 2008). Aroma enhancement of glycerol is subject of controversial debate, and no overall aroma impacts have been claimed for the wine. Nevertheless, the volatility of two fruity aromas (3-methyl butyl acetate and ethyl hexanoate) was supported by glycerol in concentration <15 g/L (Lubbers et al. 2001).

22.4 Laser Micromanipulation Systems

22.4.1 Optical Tweezers

Ashkin et al. (1987) described the use of infrared laser beams (1064 nm) for trapping and manipulation of biological specimens such as the single cells of *E. coli* and *S. cerevisiae*. This method was improved and successfully applied for the isolation of hyperthermophilic bacteria and archaea (Huber 1999; Huber et al. 1995).

A neodymium laser is focused by a microscope objective. The movement of the microscope stage is computer controlled. A rectangular glass capillary with a predetermined breaking point is used as separation chamber (inside dimensions 0.1 × 1 mm, length 10 cm), which is filled with fresh medium (90%) and the mixed microbial population (10%). A single selected cell is fixed with the laser beam and is separated from the mixed culture by moving the microscope stage. The capillary is broken at the predetermined breaking point, and the single cell is transferred to the culture medium. The culture efficiency after an incubation time of up to 5 days was 20–100%. The isolation of dead cells could be prevented by application of fluorescent dyes staining viable cells, for example, with bis-(1,3-dibutylbarbituric acid) trimethine oxonol (Beck and Huber 1997). Photodamage can be reduced to background level under anaerobic conditions (Neumann et al. 1999). This method is a promising tool for the isolation of microorganisms, which cannot be obtained in pure culture by conventional methods. A survey about further insight in the method including advantages and disadvantages is described by Zhang and Kuo-Kang (2008).

22.4.2 Laser Microdissection

Schütze et al. (1998) described a laser pressure catapulting method (LPC), which uses a laser (Robot MicroBeam) for the microdissection and transfer of single cells. This method has been successfully applied for the isolation of single cells from human tissues. The specimens are spread on a sheath of a 1.35-µm thin polyethylene membrane. With the high photonic energy of a focused nitrogen laser, a selected single cell is precisely circumscribed, and the selected cell together with a small surrounding strip of the polyethylene membrane is cut out. The round polyethylene slip with the selected cell still adheres to the polyethylene membrane. The laser is then focused below the microdissected target cell, and the microdissected sample is catapulted into the oil-dampened cap of a common Eppendorf reaction tube positioned above the sample with a laser shot of increased energy. The cells are subjected to single-cell nested PCR. In principle, this method can be applied to cells of any size, but an application for the isolation of viable prokaryotes has not been published so far. A summery on single cell analysis of bacterial isolates by laser microdissection was given by Yanagihara et al. (2011).

22.5 Molecular Biological Techniques

22.5.1 Resolution of Molecular Biological Methods

The identification and classification of microorganisms are of great importance in microbial ecology. Many different molecular biological methods are presently

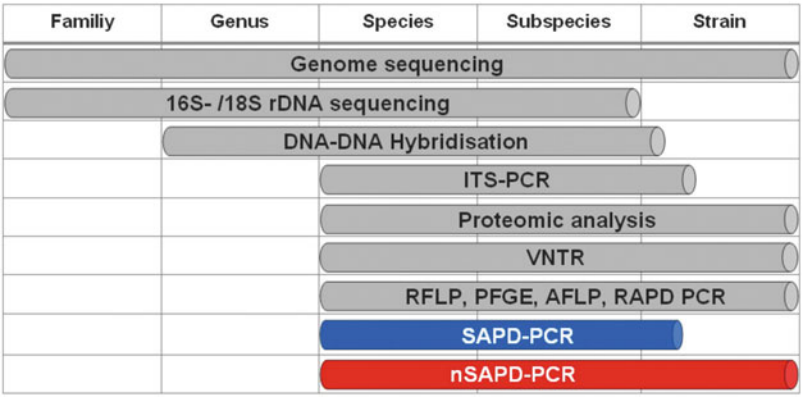

Fig. 22.5 Taxonomic resolution and discrimination performance of diverse techniques for identification of microorganisms and assessing their taxonomic relationships. However, sequencing of genomic DNA (gDNA) or SSU rDNA fits also these phylogenetic levels

being applied for microbial identification and classification. Each of these methods permits a certain level of phylogenetic classification from higher levels to the genus-, species-, subspecies-, and strain-specific level (Fig. 22.5). Moreover, each method has its advantages and disadvantages with regard to convenience of application, reproducibility, equipment, and level of resolution. Despite of a greater genome in size, the comparison of eukaryotic and prokaryotic microorganisms reveals that classical phylogenetic marker like small subunit (SSU) rDNA is less sensitive in resolution for eukaryotic cells. For yeast, only the species level could be reached (Fig. 22.5) or for fungi like *Botrytis* sp. ITS analysis or SSU rDNA matches only the genus level or phylogenetic levels below (Hirschhäuser and Fröhlich 2007). Bacterial species are considered to be groups of strains that are characterized by a certain degree of phenotypic consistency, by a significant degree of DNA hybridization (>70%) and over 97% of SSU ribosomal RNA (rRNA) gene sequence identity (Stackebrandt et al. 2002). Although 16S rRNA gene sequences and DNA–DNA hybridization continue to be considered as molecular criteria for species delineation, it is anticipated that much additional taxonomic information and therefore a more adapted natural species concept can be extracted from complete genome sequences. On the base of comparison of whole-genome sequencing, modern phylogenetic marker beyond the unsatisfactory rRNA approach was compiled by Coenye et al. (2005).

22.5.2 Molecular Biological Techniques

For identification on species level, the rRNA approach could be applied. After isolation of a clone from an axenic culture or manipulated cell, the rDNA could be

amplified by a standard PCR technique following methods like cloning and sequencing. Also, rDNA-derived techniques like FISH or T/DGGE could be used.

DNA-based typing methods, in which a DNA banding pattern is generated, include the separation of macrorestriction fragments by pulsed-field gel electrophoresis (PFGE) and various PCR-based methods like rep-PCR, BOX-PCR, nSAPD-PCR, RAPD-PCR, and AFLP fingerprinting (van Belkum et al. 2001; Tenover et al. 1995; Gurtler and Mayall 2001; Fröhlich and Pfannebecker 2007; Pfannebecker and Fröhlich 2008). The banding patterns obtained by these methods can be altered in various ways. Chromosomal rearrangements including large insertions, deletions, or mobile genetic elements like transposons or retrotransposons can have an enormous effect on banding patterns generated by restriction enzyme analysis-based methods like PFGE, as well as on patterns obtained with several PCR-based methods. The loss or gain of restriction sites or primer-binding sites can also result in modified patterns. Methylation of the restriction sites also hampers the cleavage by restriction enzymes. Subsequently, a part of the methods is described more precisely. Further assembled methods for acetic acid bacteria and yeasts could be consulted in further sections of this text books.

22.5.2.1 Decontamination and DNA Enrichment Techniques for Low Contents of Genomic DNA (gDNA)

In the past, PCR of single cells was difficult to perform because the reaction often failed or amplification techniques like nested PCR are very sensitive to contaminated DNA. Nowadays, standard PCR approaches can be decontaminated using psoralen (Jinno et al. 1990) or genomic DNA of a single cell can be amplified in an isothermal PCR approach (Notomi et al. 2000; Hayashi et al. 2007). Moreover, after application of FISH, single cells with a bright fluorescent signal were isolated using a micromanipulator and the genome of the single isolated cells served as a template for multiple displacement amplification (MDA) using the Phi29 DNA polymerase (Kvist et al. 2007).

22.5.2.2 Amplified Ribosomal rDNA Restriction Analysis (ARDRA)

On the surfaces of grapes, in musts and wines, LAB are found in a great variety. The occurrence of wine-spoiling LAB and yeasts during fermentation highlights the close relationship between the wine microbiota and the quality of the wine. For these reasons, the analytical technique ARDRA allows a fast and reliable identification of wine microorganisms. The analyses launch with the amplification, directly from colony, of 16S/18S rDNA and later digestion with one of the following restriction enzymes *Bfa*I, *Mse*I, and *Alu*I. A sequential use of the three enzymes is proposed to simplify LAB wine identification, first *Mse*I, then *Bfa*I, and finally *Alu*I digestion. The discriminated isolates belonged to the species *Lb. brevis*, *L. collinoides*, *L. coryniformis*, *L. hilgardii*, *L. mali*, *L. paracasei*, *Leuconostoc*

mesenteroides, *O. oeni*, *Pediococcus parvulus*, and *P. pentosaceus* (Rodas et al. 2003).

22.5.2.3 Nested Specifically Amplified Polymorphic DNA-PCR (nSAPD-PCR)

The nSAPD-PCR was developed as a versatile method for identification and discrimination of strains and genotypes from various organisms from bacteria to humans and was also successfully applied to distinguish between strains of LAB like *O. oeni*, *P. parvulus*, *Lb. hilgardii*, and *L. mesenteroides*; yeasts like *S. cerevisiae*, *Dekkera bruxellensis*, and *Candida* sp.; and fungi like *Botrytis cinerea*, *Sclerotinia minor*, *Sclerotinia sclerotiorum*, and *Monilinia fructigena* (Fröhlich and Pfannebecker 2007, 2012).

The method is based on the principle of the RAPD-PCR. Unlike the RAPD-PCR, the nSAPD-PCR uses specific primers including the *Not*I recognition site and additional nucleotides. The whole primer set consists of 20 primers (first PCR, 4 primers; nested PCR, 16 primers). In contrast to the RAPD-PCR, the nSAPD-PCR primers are not restricted to a small group of species. The nSAPD-PCR is a method which improves the strain discrimination power of RAPD-PCR in combination with a high reproducibility. This could be achieved by using a prolonged ramp in the first PCR (SAPD-PCR) which supports the annealing step. The nested PCR without a ramp and the usage of an enhancer solution improves the specificity. After electrophoretic separation, reliable DNA fingerprints were generated for cluster or descent analysis. As a rule, the first PCR discriminates in dependence from the investigated species on the species or subspecies level, while the nested SAPD-PCR is able to resolve strains (Fig. 22.5).

Since the introduction of this new PCR technique in 2007 (cf. Fig. 22.6), several applications were performed as described in the following. Pfannebecker et al. (2016) used this method for the identification of natural isolates of food spoiling osmophilic species of the genera *Zygosaccharomyces*, *Torulaspora*, *Schizosaccharomyces*, *Candida*, and *Wickerhamomyces*. Fermentation restart after a stuck fermentation can occur by natural microbiota when adapted strains have a selective advantage and form the majority within a population after a number of generations. Christ et al. (2015) studied the restart of stuck fermentations of spontaneously fermented wines and used the (n)SAPD-PCR for discrimination of species and strains.

Lactic acid bacteria can cause spoiling of wine by the formation of biogenic amines when occurring in must and wine. For studying these lactic acid bacteria, SAPD-PCR was used for their rapid identification (Sebastian et al. 2011). Comparing approaches of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Maldi-TOF) and (n)SAPD-PCR were performed for the identification of wine yeasts (Blättel et al. 2013) and oenococci (Petri et al. 2015).

For the first time, also non-foodborne microbes from biogas plants were investigated by SAPD-PCR. Stantscheff et al. (2014) compared different methods like

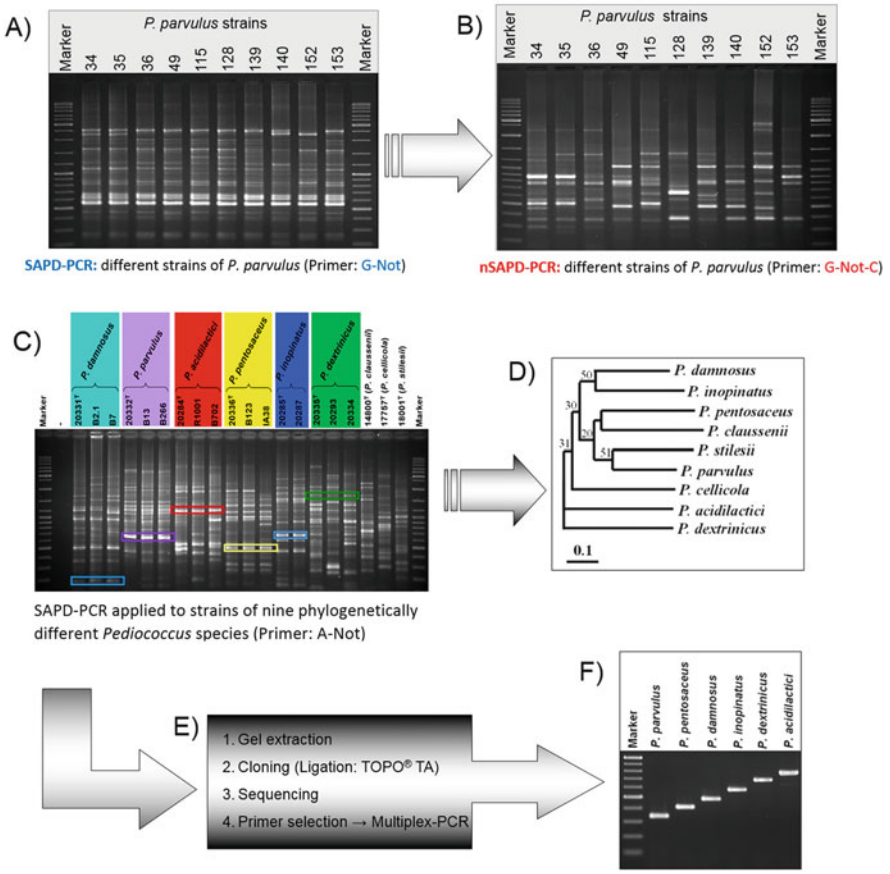

Fig. 22.6 (**a**) Application of SAPD-PCR to *Pediococcus parvulus* strains indicating the species level. (**b**) DNAs of the SAPD-PCR were used as templates for the nSAPD. All *P. parvulus* strains were clearly discriminated by nSAPD-PCR on the strain level. (**c**, **d**) After gel-electrophoretic separation, reliable DNA fingerprints of different strains of several pediococci species were generated for cluster or descent analysis. (**e**, **f**) Following gel extraction, appropriate bands were ligated (TOPO® TA) and transformed in *E. coli*. Positive clones were selected and sequenced. After primer selection and screening for selectivity, a set of primers could be combined to a whole set for multiplex PCR. DNA electrophoresis and gel staining are used for visualization PCR products

denaturating gradient gel electrophoresis of 16S rRNA gene amplificates, Maldi-TOF, as well as SAPD-PCR for the affiliation of *Methanobacterium* isolates and Reuß et al. (2015) identified methanotrophic bacteria from the termite gut.

22.5.2.4 Sequence Characterized Amplified Region PCR (SCAR-PCR) and Multiplex PCR

A further method which could be applied directly after (n)SAPD-PCR is the sequence characterized amplified region PCR. A species- or strain-specific band of a pattern was cut off the gel, reamplified by PCR, and subsequently cloned (Nakano et al. 2004). Finally, the insert was sequenced and specific primers flanking the amplified segment were generated (Fröhlich and Pfannebecker 2007). Several applications followed which combined the (n)SAPD-PCR with SCAR-PCR to generate specific primers for multiplex PCR. Lactic acid bacteria are often associated with wine spoilage and can form biogenic amines as well as ropiness, volatile acidity, butter aroma, mousy taste, and further off-flavors. Specific DNA bands of lactic acid bacteria amplified by (n)SAPD-PCR were used for the development of multiplex PCR primers. Since, the method provides a rapid and simultaneous detection for 13 different lactic acid bacteria species and supports a better monitoring of the vinification process to improve wine quality (cf. Fig. 22.6; Petri et al. 2013). Miranda-Castilleja et al. (2016) used this multiplex PCR for the investigation of the diversity of wine-associated LAB in Mexico and particularly in the wine region of Queretaro. Four wineries were studied, and five different species (*O. oeni*, *P. parvulus*, *L. plantarum*, *L. hilgardii*, and *L. brevis*) were monitored. Also non-wine related isolated were analyzed by (n)SAPD-PCR. Stantscheff et al. 2014 used DNA bands of (n)SAPD-PCR and derived a PCR-based primer system for the detection of *Methanobacterium formicicum*-related isolates and the reference strains.

22.5.2.5 Fluorescence In Situ Hybridization (FISH)

A rapid method for the identification and enumeration of LAB and yeasts from wine is the fluorescence in situ hybridization (Amann et al. 1995). This technique uses fluorescently labeled oligonucleotide probes targeting the rRNA of a species. Probes were used to identify species in different wines, making it evident that direct identification and quantification from natural samples without culturing are also possible. The results show that FISH is a promising technique for the rapid identification of LAB and yeasts, allowing positive identification within a few hours for common wine species *O. oeni*, *P. damnosus*, *P. parvulus*, *P. pentosaceus*, *Lactobacillus plantarum*, *Lb. casei/paracasei*, *Lb. brevis*, *Lb. hilgardii*, and most *Leuconostoc* species among others (Blasco et al. 2003; Hirschhäuser et al. 2005; Röder et al. 2007a, b). Xufre et al. (2006) developed 26S rRNA gene probes targeting the D1–D2 region for identification of numerous wine-related yeast including *S. cerevisiae*, *Candida stellata*, *Hanseniaspora uvarum*, *H. guilliermondii*, *Kluyveromyces thermotolerans*, *K. marxianus*, *Torulaspora delbrueckii*, *Pichia membranaefaciens*, and *Pi. anomala*. Moreover, Röder et al. (2007a, b) demonstrated that there are further target sides downstream the common

used D1–D2 regions of the large subunit (LSU) rRNA of yeasts. Though, low-signal intensity due to poor probe hybridization efficiency is one of the major drawbacks of rRNA-targeted in situ hybridization. To overcome problems with a poor signal-background ratio or confusion with autofluorescent wine constituents, several attempts were performed to increase the yield in fluorescence. Stender et al. (2001) used peptide nucleic acid probes to identify the spoilage yeast *D. bruxellensis* because PNA–rRNA hybrids are very stabile under stringent hybridization conditions. The usage of unlabeled helper probes (Fuchs et al. 2000) or side probes, a set of partially complementary fluorescently labeled probes, also supports the microscopic analysis (Hirschhäuser et al. 2005; Röder et al. 2007a, b). It could also be shown that the nature of the labeled fluorescent dye was also involved in hybridization. Differences in quantum yield could be measured if dyes like carbocyanine 3 (CY3), carboxyfluorescein (FAM), or carboxytetramethylrhodamine (TAMRA) were used. In comparison to FAM and TAMRA, CY3 is the most used label in FISH, since it has a high absorption coefficient and a high quantum yield, shows little bleaching, and is pH insensitive (Fuchs et al. 2001). On the other hand, FAM shows a fluorophor-dependent quenching at various target sites by electronic interaction with guanosine. This quenching phenomenon led to the development of so-called smart probes. They, like molecular beacon, are self-complementary at the 5′ and 3′ endings but instead of a fluorescence resonance energy transfer (FRET) dye system, the overlapping fluorochrome is quenched via charge transfer by the cumulative guanosine nucleotides. If the probe is involved in hybridization with the requested DNA target, the dye nucleotide interaction will be finished and finally emission occurs. Another approach for improving FISH is the use of fluorescent DNA oligonucleotides modified to contain locked nucleic acid (LNA) residues. This increases the thermal stability of hybrids formed with RNA. The LNA-based probes detect specific RNAs in fixed yeast cells with an efficiency far better than conventional DNA oligonucleotide probes of the same sequence (Thomsen et al. 2005; Kubota et al. 2006). Further practical applications demonstrated the successful use of FISH techniques for the monitoring of *S. cerevisiae* and *Hanseniaspora guilliermondii* during fermentations (Andorra et al. 2011). Moreover, the combination of live/dead staining and FISH could be applied for the simultaneous assessment of the identity and viability of *S. cerevisiae* and *H. guilliermondii* (Branco et al. 2012).

22.5.2.6 PCR Temperature/Denaturing Gradient Gel Electrophoresis (PCR-T/DGGE)

PCR–DGGE and PCR–TGGE of rRNA gene fractions were applied to differentiate individual wine yeast isolates (Manzano et al. 2004, 2005) or to monitor the succession of the yeast microbiota during fermentation (Mills et al. 2002). The study of Mills and coworkers revealed that PCR–DGGE signals for several non-*Saccharomyces* yeast populations could persist into the fermentation and long after these yeasts could be identified on culture media. Furthermore, these

methods were used to investigate the LAB microbiota on grape surfaces that were enriched by means of different media. Spano et al. (2007) used the PCR–DGGE for monitoring *Lb. plantarum* and *O. oeni* in red wine. They reported that the PCR–DGGE method, based on the *rpoB* gene as molecular marker, is a reproducible and suitable tool to monitor spoilage microorganisms during wine fermentation. PCR–DGGE was also used to examine the bacteria that developed in enrichment cultures from grapes. Species of the genera *Lactobacillus*, *Enterococcus*, *Lactococcus*, and *Weissella* were detected in enrichments by plating and PCR–DGGE (Bae et al. 2006). The complexity and diversity of the wine microbial consortium on grape berries, in must during fermentation and in wine during aging, were investigated by Renouf et al. (2007). On grapes, 52 different yeast species and 40 bacteria could be identified. The diversity was dramatically reduced during winemaking then during aging. Nevertheless, the routine usage of these techniques for identification purposes is technically problematic because each visible band of a gel must be previously identified by control strains. Also, it was observed that different DNA amplicons could be "felted" during PCR amplification and band comigration while gel electrophoresis occurs (Gafan and Spratt 2005). This aspect could be a reason for lower sensitivity of DGGE when compared to PCR. The sensitivity of these methods was investigated for different wine-spoiling microorganisms (*Lb. plantarum*, *Pediococcus pentosaceus*, *Acetobacter pasteurianus*, *D. bruxellensis*) showing that PCR detection limits were more sensitive (10–100 cfu/mL) compared to DGGE detection limits (10–10^4 cfu/mL). Inoculation tests also revealed that DGGE detection limits were higher for mixed populations when compared to single strains (Bester et al. 2010). Finally, a summary about studies exploiting the DGGE technique to investigate the microbial ecology of various food products was provided by Cocolin et al. (2013).

22.5.2.7 Real-Time PCR/Quantitative PCR (qPCR)

Real-time PCR is a PCR-based method using fluorescently labeled probes and DNA-intercalating fluorescence stain. The method permits a quantification of DNA during the amplification. For indirect cell counting standardization with housekeeping genes or reference samples with known DNA content were used. A few grapes- and wine-related microbes could be identified and monitored so far. Hierro et al. (2007) applied the real-time PCR for the rapid quantification of *Saccharomyces* sp. and *Hanseniaspora* sp. during fermentation. They designed specific primers for the region spanning the internal transcribed spacer 2 (ITS2), and the 5.8S rRNA gene or universal yeast primers were designed from the variable D1/D2 domains of the 26S rRNA gene (Hierro et al. 2006; Phister et al. 2007). The qPCR assay for enumeration of *Hanseniaspora* sp. in must and wine can detect 10 cells/mL. The approach is linear over four orders of magnitude and is not influenced by high concentrations of contaminating *S. cerevisiae* DNA. Also, *D. bruxellensis*-infected wines were investigated by using the same gene region. The assay was linear over a range of cell concentrations (6 log units) and could

detect as little as 1 cell/mL in wine (Phister and Mills 2003). Rawsthorne and Plister (2006) analyzed *Zygosaccharomyces bailii*, a major food and beverage spoilage organism in grape juice and wine. They could detect 22 cells/mL from grape juice. The assay was equally efficient in wine, detecting 6 cells/mL, and provides a rapid and accurate method to establish the levels of the total *Z. bailii* population which consists of both viable and nonviable cells. The correlation was high between qPCR and total cell count as determined by fluorescent microscopy. For the detection of spoiling pediococci, also a real-time PCR approach could be established. The detection limit in wine was 40 cells/mL for ropy *P. damnosus* (Delaherche et al. 2004). Ultee et al. (2013) investigated stuck Riesling fermentations by qPCR. During the stuck period another microbiota has established and restarted the fermentation later on. Here, the main fermenting organism was *Saccharomyces uvarum*.

22.5.2.8 Pulsed-Field Gel Electrophoresis (PFGE) for Strain-Specific Differentiation

Placing DNA samples in a solid matrix (most commonly agarose or polyacrylamide) and forcing the molecules to migrate through the gel under a static electric field is the basis of conventional gel electrophoresis. The separation of molecules of different sizes predominantly depends on the sieving properties of the gel matrix. In conventional agarose, all DNA molecules larger than 20 kb will show essentially the same mobility in a static electric field and, thus, will not be separated from each other.

PFGE avoids molecular sieving (and its limitations) by using a completely different separation mechanism that maintains size-dependent electrophoretic mobilities of large DNA molecules. In the absence of external forces, DNA molecules exist in a relaxed form. Under the influence of an electric field, the DNA samples elongate, align with the field, and migrate toward the anode by a process termed "reptation." After the removal of an electric field, the elongated DNA molecules relax back to their original state. When a second electric field is applied in a different angle to the first field, the DNA must change conformation and reorient before it can migrate in the direction of the second field. The time required for this reorientation has been found to be very sensitive to the length of the molecule. Smaller molecules that rapidly reorient migrate most of the time along the electric field. Larger DNA molecules take more time to realign after the field is switched than smaller ones, because of the physical barrier of the agarose matrix. Hence, molecules of increasing size must spend a large portion of each switching cycle reorienting before they can migrate through the gel. As long as the alternating fields are equal with respect to length and voltage, the DNA will migrate in straight path down the gel that reflects the sum of the many zigzag steps actually taken.

PFGE allows separation of large DNA molecules (up to 12 Mb) due to molecular reorientation produced by periodic changes in the electric field (Herschleb et al.

2007). The change frequency of electric fields is referred as switch interval, switch of time, or time pulse. Duration of alternative electric fields establishes the DNA dimension ranges that are possible to resolve in PFGE. Pulses can go from some seconds to resolve molecules with some kb to more than 1 h for molecules larger than 5 Mb.

The opposing electric fields create two distinct directions for the DNA molecules as they travel through the gel. The reorientation angle or rotor angle is the difference between these two paths. For most purposes, a fixed angle of 120° is sufficient; however, adjusting the reorientation angle can improve separation of small and large DNA molecules in a mixture.

Besides pulse time and reorientation angle, PFGE resolution is remarkably sensitive to changes in all electrophoretic parameters such as agarose concentration and quality (Kirkpatrick et al. 1993), buffer composition, and temperature. Selection of the gradient voltage is also very important since any change in this parameter drastically changes the dimension of resolved molecules.

Modifications of the original method introduced by Schwartz and Cantor (1984) are field inversion gel electrophoresis (FIGE), contour-clamped homogeneous electric fields (CHEF), or transversal alternating field electrophoresis (TAFE). Rotating field electrophoresis (RFE) is an improvement of the original method, allowing continuous variations of all relevant PFGE parameters, as well as two-dimensional separations and conventional electrophoresis. Straight-line separations of DNA fragments in the 0.1–6000 kb range are accomplished by varying pulsed time and orientation of the electrodes (Ziegler et al. 1987).

Conventional methods of DNA extraction use forces that lead to breakage of DNA molecules, reducing their dimensions. With adequate protocols, it is possible to obtain DNA molecules of 500 kb in solution. For larger molecules, it is necessary to protect them from both mechanical shearing and nucleolytic degradation during the entire isolation process. Individual cells are embedded in agarose, which protects the DNA against breakage while allowing the free flow of solutions necessary for lysis and digestions (Herschleb et al. 2007).

The development of methods to prepare and analyze large DNA molecules contributed to the development of PFGE as a powerful instrument in molecular biology. It is used to evaluate genome dimensions, to construct physical genome maps, to clone large DNA inserts, to separate large plasmids, and to analyze genomes of eukaryotic cells.

PFGE analysis of large genomic DNA fragments obtained by digestion with rare cutting enzymes (macrorestriction analysis) and of whole chromosomes (karyotyping) is a technique with high reproducibility and discriminative power, which are widely used for epidemiological studies with clinical bacteria (Ribot et al. 2001; Andrei and Zervos 2006) and yeasts (Bellis et al. 1987; Jang et al. 2005; Lukácsi et al. 2006).

The strain specificity of macrorestriction profiles obtained by PFGE makes this technique also useful to track biotechnological-relevant bacteria in natural environments (Claus et al. 1992, 1995) and food (Huys et al. 2006). For vinology, it might become a powerful tool for monitoring the fate of starter cultures and analyzing microbial wine communities (Oliveira et al. 2008). Moreover,

ecologically aspects and new genetics resources from so far unknown wild Chinese *Saccharomyces* species and strains could be investigated by electrophoretic karyotyping (Wang et al. 2012).

Tables 22.1 and 22.2 comprise PFGE approaches to differentiate wine-relevant bacteria and yeasts on the strain level.

Table 22.1 Use of macrorestriction profiles to characterize wine-relevant lactic acid bacteria

Species	References
Oenococcus oeni	Lamoureux et al. (1993)
	Daniel et al. (1993)
	Kelly et al. (1993)
	Tenreiro et al. (1994)
	Zapparoli et al. (2000)
	Sato et al. (2001)
	Guerrini et al. (2003)
	Malacrinò et al. (2003)
	Lechiancole et al. (2006)
	Larisika et al. (2008)
Leuconostoc sp.	Tenreiro et al. (1994)
Pediococcus sp.	Luchansky et al. (1992)
	Barros et al. (2001)
	Simpson et al. (2002, 2006)
Lactobacillus sp.	Zapparoli et al. (1998)
	Rodas et al. (2005)

Table 22.2 Use of karyotyping to characterize wine-relevant fungi

Species	References
Saccharomyces sp.	Giudici et al. (1998)
	Vaughan-Martini et al. (1993)
	Versavaud et al. (1995)
	Nadal et al. (1996)
	Guijo et al. (1997)
	Petersen et al. (1999)
	Naumov et al. (2001)
	Mitterdorfer et al. (2002)
	Naumova et al. (2003)
	Špirek et al. (2003)
	Martinez et al. (2004)
	Cocolin et al. (2004)
	Glover et al. (2005)
	Puverenti et al. (2005)
	Valero et al. (2005, 2007)
	Divol et al. (2006)
	Ribeiro et al. (2006)
	Le Jeune et al. (2007)
	Oliveira et al. (2008)
	Wang et al. (2012)
Botrytis cinerea	Vallejo et al. (1996)

Disadvantages of electrophoretic karyotyping include being time-consuming, laborious, and relatively expensive. The duration of the PFGE procedure (4 days from sample preparation to result) might be regarded as the main drawback of the method, especially when there is a demand of rapid reactions toward spoiling microorganisms to control vinification processes. In future, this hindrance might be overcome by new technical developments which could shorten PFGE runs (Birren and Lai 1994; Wagner and Lai 1994) and short protocols for sample preparation (Ribot et al. 2001; Simpson et al. 2006). Nevertheless, even newer technologies placing to the foreground, electrophoretic karyotyping shows comparatively greater resolution than other techniques, like RAPD-PCR, microsatellites, or other markers for *S. cerevisiae* (Oliveira et al. 2008).

22.6 Conclusions

Driven by the climate change, the demand in microbial test methods will increase due to the correlating rise of spoiling microbiota on grapes. By application of these methods, wine risks incurred are therefore recognizable already at an early stage of fermentation. Cultivation-dependent techniques, for instance, selection on specified media or for uncultivable microbes isolation of single cells by micromanipulation or other methods like microbial detection by FTIR, can be used. Yet to determine complex microbial communities, cultivation-independent methods are particularly suitable. Mentionable in this respect are the fluorescence in situ hybridization (FISH) as well as a multitude of modern PCR techniques (real time, multiplex, etc.). The nSAPD-PCR (nested specifically amplified polymorphic DNA-PCR) was introduced as a new central PCR technique, which not only allows to analyze any organisms on a species and strain level but is also suitable to develop special DNA probes, respectively, PCR primer, which enable to identify individual starter cultures or spoiling organisms without cultivation from the wine sample (SCAR-PCR—sequence characterized amplified region PCR). For a lot of wine-spoiling bacteria, already a multiplex PCR variant was developed which is based on this new technology and simultaneously detects several microorganisms.

Modern techniques for separation of microbial cells followed by DNA amplification and application of molecular biological tools permit even the investigation of a single cell without cultivation. Many of the technical problems regarding the separation of a single cell have been solved. For cultivation, the remaining problems to be solved are now less of technical nature than choosing a suitable medium composition for growing cells of, e.g., unknown systematic affiliation.

As aforementioned different diagnostic techniques support the identification and characterization on variable phylogenetic levels for culturable or nonculturable microorganisms. Techniques like FISH, DGGE, qPCR, fingerprint approaches, and others are suitable for simultaneous identification or cell counting or monitoring of wine-related microorganisms. Nevertheless, the technical progress in the development of genetic tools and devices cannot hide the fact that there are

unsolved problems in investigation of cell-to-cell communication, cofermentation, and genetic drift in focus of environmental changes particularly the effects of climate change and changes of the gene regulation and physiology of wine yeast as well as spoiling microbes.

In the coming years, the climate change will give us the opportunity to grow up new vine varieties and to modify wine profiles because of reduced acidity and increased sugar contents of juice, leading to wines which are too heavy on alcohol. Besides technical solutions, the production of active dry yeasts which produce less alcohol has only just begun, and first new strains are developed from science all the way to product and market maturity.

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Chapter 23
Maintenance of Wine-Associated Microorganisms

Helmut König and Beate Berkelmann-Löhnertz

23.1 Introduction

A great variety of microorganisms growing on grapes, in must, or in wine have been isolated, which also have an influence on wine quality. They belong to acid-tolerant microorganisms such as lactic acid bacteria, acetic acid bacteria, and yeasts. On grapes also molds can be found (Table 23.1). The most important species for conversion of must into wine are the yeast *Saccharomyces cerevisiae* and the lactic acid bacterium *Oenococcus oeni*, which perform alcoholic and malolactic fermentation, respectively. Both species are used as starter cultures. A variety of techniques and media are available for the enrichment, culture, and preservation of these microorganisms (Kirsop and Doyle 1991; Atlas and Parks 1993). For selected species culture and preservation procedures are described (Tables 23.1, 23.2 and 23.3).

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Table 23.1 Recommended media for cultivation of fungi from grapes

Group	Genus	Culture media	Species (examples)
Ascomycota	1. *Acremonium* Link	DRBC	*A*. spp.
	2. *Arthrinium* Kunze	DRBC	*A*. spp.
	3. *Aspergillus* Fr.: Fr.	CZ (CBS), MEA, AFPA, DRBC	*A. aculeatus* Iizuka; *A. alliaceus* Thom & Church; *A. auricomus* Saito; *A. candidus* Link; *A. carbonarius* Bainier; *A. carneus* Blochwitz; *A. clavatus* Desm.; *A. flavipes* Thom & Church; *A. flavus* Link; *A. fumigatus* Fresenius; *A. japonicus* Saito; *A. niger* aggregate; *A. ochraceus* K. Wilh.; *A. ostianus* Wehmer; *A. parasiticus*; *A. terreus* Thom; *A. terreus* var. africanus Raper & Fennell; *A. ustus* Thom & Church; *A. versicolor* Tirab; *A. wentii* Wehmer
	4. *Aureobasidium* Viala & G. Boyer	DRBC	*A*. spp.
	5. *Beauveria*	DRBC	*B. bassiana* Vuill.
	6. *Botrytis* P. Micheli	OA, DRBC	*B. cinerea* (Sclerotinia fuckeliana, causal agent of gray mold)
	7. *Chaetomium* Kunze	DRBC	*C*. spp.
	8. *Chrysonilia* Arx	DRBC	*C*. spp.
	9. *Curvularia* Boedijn	DRBC	*C*. spp.
	10. *Dendryphiella*	DRBC	*D*. spp.
	11. *Drechslera* S. Ito	DRBC	*D*. spp.
	12. *Emericella* Berk.	DRBC	*E*. spp.
	13. *Epicoccum* Link	DRBC	*E. nigrum*
	14. *Eurotium* Link: Fr.	DRBC	*E. amstelodami* L. Mangin; *E. chevalieri* L. Mangin
	15. *Fusarium* Link	DRBC	*F*. spp.
	16. *Geotrichum* Link	DRBC	*G*. spp.
	17.*Gliocladium* Corda	DRBC	G. spp.
	18. *Guignardia*	OA (Guignardia)	*Guignardia bidwellii* (causal agent of black rot)
	19. *Histoplasma* Darling	DRBC	*H*. spp.
	20. Neurospora	DRBC	*N. tetrasperma* Shear & Dodge
	21. *Nigrospora* Zimm.	DRBC	*N*. spp.

(continued)

Table 23.1 (continued)

Group	Genus	Culture media	Species (examples)
	22. *Oidium*	Obligate biotrophic! Cultivation only on potted vines	*O. tuckeri* (Erysiphe necator, causal agent of powdery mildew)
	23. *Penicillium* Link	CZ (CBS), MEA, DRBC	*P. aurantiogriseum* Dierckx; *P. bilaiae* Chalabuda; *P. brevicompactum* Dierckx; *P. canescens* Sopp; *P. chrysogenum* Thom; *P. citrinum* Thom; *P. corylophilum* Dierckx; *P. crustosum* Thom; *P. echinulatum* Fassatiova; *P. expansum* Thom; *P. fellutanum* Biourge; *P. funiculosum* Thom; *P. glabrum/spinulosum*; *P. griseofulvum* Dierckx; *P. implicatum* Biourge; *P. janczewskii* K.M. Zalessky; *P. miczynskii* Zaleski; *P. minioluteum* Dierckx; *P. novae-zeelandiae* J.F.M. Beyma; *P. olsonii* Bainier & Sartory; *P. oxalicum* Currie & Thom; *P. pinophilum* Hedgcock; *P. purpurogenum* Stoll; *P. raistrickii* G. Sm.; *P. restrictum* J.C. Gilman & E.V. Abbott; *P. roqueforti* Thom; *P. rugulosum* Thom; *P. sclerotiorum* van Beyma; *P. simplicissimum* Thom; *P. solitum* Westling; *P. thomii* Maire; *P. variabile* Sopp; *P. verruculosum* Peyronel; *P. waksmanii* Zaleski
	24. *Periconia* Tode ex Fr.	DRBC	*P.* spp.
	25. *Pestalotiopsis* Steyaert	DRBC	*P.* spp.
	26. *Phoma* Sacc.	DRBC	*P.* spp.
	27. *Pithomyces*	DRBC	*P. chartarum* Ellis
	28. *Scytalidium* Pesante	DRBC	*S.* spp.
	29. *Trichothecium*	MEA	*T. roseum* Link
	30. *Truncatella* Steyaert	DRBC	*T.* spp.

(continued)

Table 23.1 (continued)

Group	Genus	Culture media	Species (examples)
	31. *Ulocladium* Preuss	DRBC	*U*. spp.
Deuteromycotina	32. *Alternaria* Nees: Fr.	MEA, PCA (CBS), DRBC	*A. alternata*
	33. *Cladosporium* Link	MEA, DRBC	*C. herbarum*
	34. *Monilia*	OA	*M. fructigena*, *M. fructicola*
	35. *Paecilomyces* Bainier	MEA, OA, DRBC	*P. variotii*
	36. *Stemphylium* Wallr.	DRBC	*S*. spp.
	37. *Trichoderma* Pers.	OA, MEA	*T*. spp.
Oomycota	38. *Plasmopara*	Obligate biotrophic! Cultivation only on potted vines	*P. viticola* ("*Peronospora*" causal agent of downy mildew)
Zygomycota	39. *Cunninghamella* Matr.	DRBC	*C*. spp.
	40. *Mucor* P. Micheli: Fr	MEA 4% (CBS), DRBC	*M. mucedo*, *M. hiemalis*, *M. piriformis*
	41. *Rhizopus* Ehrenb.	MEA	*R. stolonifer*
	42. *Syncephalastrum*	DRBC	*S. racemosum* J. Schröt.

AFPA Aspergillus flavus/A. parasiticus selective medium; *CZ (CBS)* Czapek agar (formula used at CBS); *MEA* malt extract agar; *MEA 4% (CBS)* mout extract agar 4% (formula used at CBS); *OA* oatmeal agar; *PCA (CBS)* potato carrot agar (formula used at CBS); *PDA* potato dextrose agar. *CBS* Centraalbureau voor Schimmelcultures, The Netherlands. *DRBC* Dichloran Rose-Bengal Chloramphenicol Agar (Oxoid) (cf. Serra et al. 2005)

Table 23.2 Recommended media for cultivation of bacteria from grapes, must, and wine

Group	Genus	Culture media	Species (examples)
Gram-positive bacteria			
Lactic acid bacteria	1. *Lactobacillus*	MRS, SL, FYP	*Lb. brevis*, *Lb. buchneri*, *Lb. casei*, *Lb. curvatus*, *Lb. delbrueckii*, *Lb. diolivorans*, *Lb. fermentum*, *Lb. florum*, *Lb. fructivorans*, *Lb. hilgardii*, *Lb. jensenii*, *Lb. kunkeei*, *Lb. mali*, *Lb. nagelii*, *Lb. oeni*, *Lb. paracasei*, *Lb. plantarum*, *Lb. vini*
	2. *Leuconostoc*	MRS	*Lc. mesenteroides*
	3. *Oenococcus*	MRS, TJ	*O. oeni*
	4. *Pediococcus*	MRS	*P. damnosus*, *P. parvulus*, *P. pentosaceus*; *P. inopinatus*
	5. *Weissella*	MRS	*W. paramesenteroides*
Proteobacteria			
Acetic acid bacteria	1. *Acetobacter*	360, 989	*A. aceti*, *A. cerevisiae*, *A. malorum*, *A. oeni*, *A. orleanensis*, *A. pasteurianus*, *A. syzygii*, *A. tropicalis*
	2. *Ameyamaea*	GY	*Am. chiangmaiensis*
	3. *Asaia*	105, GEA	*As. lannaensis*, *A. siamensis*
	4. *Gluconacetobacter*	360	*Ga. liquefaciens*
	5. *Gluconobacter*	105, 360, 626, GY, GYG	*G. albidus*, *G. cerinus*, *G. frateurii*, *G. japonicus*, *G. oxydans*, *G. thailandicus*
	6. *Komagataeibacter*	GY, GYG	*Km. europaeus*, *Km. hansenii*, *Km. intermedius*, *Km. saccharovorans*
	7. *Kozakia*	105	*Ko. baliensis*

Table 23.3 Recommended media for cultivation of yeasts from grapes, must, and wine

Genus	Culture media[a]	Species[b] (examples)
1. *Brettanomyces* (A)	GPYA + $CaCO_3$, 25 °C	*B. anomala*; *B. bruxellensis* (teleomorphic form: Dekkera)
2. *Candida* (A)	GPYA, 25 °C	*C. agrestis*, anamorphic (synonym of Saturnispora zaruensis); *C. albicans*; *C. apicola*; *C. boidinii*; *C. cantarellii*; *C. catenulata*; *C. colliculosa* (synonym of Torulaspora delbrueckii); *C. diversa*; *C. famata* (synonym of *Debaryomyces hansenii*); *C. glabrata*; *C. incommunis*; *C. inconspicua*; *C. intermedia*; *C. montana*; *C. norvegica*; *C. parapsilosis*; *C. pelliculosa* (synonym of *Pichia anomala*); *C. pulcherrima* (synonym of *Metschnikowia pulcherrima*); *C. rugosa*; *C. sake*; *C. solani*; *C. stellata*; *C. tenuis*; *C. tropicalis*; *C. vanderwaltii*; *C. veronae* (synonym of *Pichia mexicana*); *Candida valida* (synonym of *Pichia membranifaciens*); *C. versatilis*; *C. vinaria*; *C. vini* (synonym of *Kregervanrija fluxuum*); *C. zeylanoides*
3. *Citeromyces* (A)	GPYA, 25 °C	*C. matritensis*
4. *Cryptococcus* (B)	PDA, 25 °C	*C. albidus*; *C. humicola*; *C. laurentii*; *C. luteolus*
5. *Debaryomyces* (A)	GPYA, 25 °C	*D. carsonii*; *D. etchellsii*; *D. hansenii*; *D. polymorphus*
6. *Dekkera* (A) (anamorphic form: *Brettanomyces*)	GPYA + $CaCO_3$, 25 °C	*D. anomala*; *D. bruxellensis*
7. *Dipodascus* (A)	GPYA, 25 °C	*D. ingens* (synonym of *Magnusiomyces ingens*)
8. *Endomyces* (A)	GPYA, 25 °C	*E. fibuligera* (synonym of *Saccharomycopsis fibuligera*)
9. *Endomycopsella* (A)	GPYA, 25 °C	*E. vini* (synonym of *Saccharomycopsis vini*)
10. *Filobasidiella* (B)	PDA, 25 °C	*F. neoformans*
11. *Filobasidium* (B)	PDA, 25 °C	*F. capsuligenum*
12. *Geotrichum* (A)	EMSA, 24 °C	*G. fermentans*
13. *Guehomyces* (B)	PDA, 25 °C	*G. pullulans*
14. *Hanseniaspora* (A)	GPYA, 25 °C	*H. guilliermondii*; *H. occidentalis*; *H. osmophila*; *H. uvarum*; *H. valbyensis*; *H. vineae*
15. *Hasegawaea* (A)	GPYA; 25 °C	*H. japonica* (synonym of *Schizosaccharomyces japonicus*)
16. *Hyphopichia* (A)	MYA, 25 °C	*H. burtonii* (synonym of *Pichia burtonii*)
17. *Issatchenkia* (A)	GPYA, 25 °C	*I. terricola*, *I. orientalis* (*C. krusei*)

(continued)

Table 23.3 (continued)

Genus	Culture media[a]	Species[b] (examples)
18. *Kazachstania* (A)	GPYA, 25 °C	*K. exigua*; *K. transvaalensis*; *K. unispora*
19. *Kloeckera* (A)	GPYA, 25 °C	*K. apiculata* (synonym of *Hanseniaspora uvarum*); *K. corticis*
20. *Kluyveromyces* (A)	GPYA, 25 °C	*K. marxianus*; *K. thermotolerans* (synonym of *Lachancea thermotolerans*)
21. *Kregervanrija* (A)	GPYA, 25 °C	*K. fluxuum*
22. *Lachancea* (A)	GPYA, 25 °C	*L. kluyveri*; *L. thermotolerans*
23. *Leucosporidium* (B)	PDA, 20 °C	*L. scottii*
24. *Lipomyces* (A)	GPYA, 25 °C	*L. starkeyi*
25. *Lodderomyces* (A)	GPYA, 25 °C	*L. elongisporus*
26. *Magnusiomyces* (A)	GPYA, 25 °C	*M. ingens*
27. *Metschnikowia* (A)	GPYA, 25 °C	*M. pulcherrima*; *M. reukaufii*
28. *Nadsonia* (A)	GPYA, 25 °C	*N. fulvescens*
29. *Octosporomyces* (A)	GPYA, 30 °C	*O. octosporus* (synonym of *Schizosaccharomyces octosporus*)
30. *Pachytichospora* (A)	GPYA, 25 °C	*P. transvaalensis* (synonym of *Kazachstania transvaalensis*)
31. *Pichia* (A)	GPYA, 25 °C	*P. anomala* (asexual form: *Candida pelliculosa*); *P. burtonii*; *P. canadensis*; *P. carsonii* (synonym of *Debaryomyces carsonii*); *P. etchellsii* (synonym of *Debaryomyces etchellsii*); *P. farinosa*; *P. fermentans*; *P. guilliermondii*; *P. jadinii*; *P. membranifaciens*; *P. silvicola*; *P. subpelliculosa*
32. *Rhodotorula* (B)	PDA, 25 °C	*R. acuta* (synonym of *Sterigmatomyces elviae*); *R. aurantiaca*; *R. bogoriensis*; *R. glutinis*; *R. minuta*; *R. mucilaginosa*
33. *Saccharomyces* (A)	GPYA, 25 °C	*S. cerevisiae*; *S. bayanus*; *S. exiguus* (synonym of *Kazachstania exigua*); *S. kluyveri* (synonym of *Lachancea kluyveri*); *S. unisporus* (synonym of *Kazachstania unispora*) Triplehybrid: *S. cerevisiae* × *S. kudriavzevii* × *S. bayanus*
34. *Saccharomycodes* (A)	GPYA, 25 °C	*S. ludwigii*
35. *Saccharomycopsis* (A)	GPYA, 25 °C	*S. fibuligera*; *S. vini*
36. *Saturnispora* (A)	GPYA, 25 °C	*S. zaruensis*

(continued)

Table 23.3 (continued)

Genus	Culture media[a]	Species[b] (examples)
37. *Schizosaccharomyces* (A)	GPYA; 25 °C	*S. pombe*; *S. japonicus*; *S. versatilis*; *S. octosporus*
38. *Sporidiobolus* (B)	PDA, 25 °C	*S. pararoseus*; *S. salmonicolor*
39. *Sporobolomyces* (B)	PDA, 25 °C	*S. roseus*
40. *Sterigmatomyces* (B)	PDA, 25 °C	*S. elviae*
41. *Torulaspora* (A)	GPYA, 25 °C	*T. delbrueckii*; *T. globosa*
42. *Trichosporon* (B)	PDA, 25 °C	*T. beigelii* (synonym of *Trichosporon cutaneum*); *T. pullulans* (synonym of *Guehomyces pullulans*); *T. cutaneum*
43. *Torulopsis* (A)	GPYA, 25 °C	*T. versatilis* (synonym of *Candida versatilis*)
44. *Wickerhamiella* (A)	GPYA, 25 °C	*W. domercqiae*
45. *Williopsis* (A)	GPYA, 25 °C	*W. californica*; *W. saturnus*
46. *Yarrowia* (A)	GPYA, 25 °C	*Y. lipolytica*
47. *Zygoascus* (A)	GPYA, 25 °C	*Z. hellenicus*
48. *Zygosaccharomyces* (A)	GPYA, 25 °C	*Z. bailii*; *Z. bisporus*; *Z. florentinus* (synonym of *Zygotorulaspora florentinus*); *Z. rouxii*
49. *Zygotorulaspora* (A)	GPYA, 25 °C	*Z. florentinus*

(A) Ascomycota; *(B)* Basidiomycota
[a]cf. also Barnett et al. (1990), Robert et al. (2008)
[b]Index fungorum (2008), Robert et al. (2008)

23.2 Bacteria

23.2.1 Genera of Acetic Acid Bacteria

Acetic acid bacteria (AAB) are acid and ethanol-tolerant aerobic bacteria, which oxidize ethanol to acetic acid. Species of the genus *Acetobacter* can also completely oxidize acetate to CO_2 in the presence of oxygen. Therefore, AAB are common wine spoilage microorganisms, because higher concentrations of acetic acid (>1 g L^{-1}) cause an off-flavor in wine (volatile acidity). Species of the seven genera *Acetobacter*, *Ameyamaea*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, *Komagataeibacter*, and *Kozakia* have been found on grapes, in must, and in wine (Table 23.2). They are grown at 25–30 °C.

Culture media for acetic acid bacteria	
Medium 626 (2017) (5% sorbitol medium) (DSMZ 2017)	
D-Sorbitol	50.0 g
Yeast extract	10.0 g
Peptone	10.0 g
Agar	15.0 g
Distilled water	1000.0 mL
Adjust medium for final pH 6.0 with HCl	
Medium 105 (2017) (Gluconobacter oxydans medium) (DSMZ 2017)	
Glucose	100.0 g
Yeast extract	10.0 g
$CaCO_3$	20.0 g
Agar	15 g
Distilled water	1000.0 mL
Adjust pH to 6.8	
Medium 989 (2017) (acetic acid bacterium medium) (DSMZ 2017)	
Bacto Peptone	5.0 g
Yeast extract	5.0 g
Glucose	5.0 g
$MgSO_4 \times 7\ H_2O$	1.0 g
Agar	15 g
Distilled water	1000.0 mL
pH 6.6–7.0	
Medium 360 (2017) (YPM medium) (DSMZ 2017)	
Yeast extract	5.0 g
Peptone	3.0 g
Mannitol	25.0 g
Agar	12 g
Distilled water	1000.0 mL
pH not adjusted	
GEA medium (glucose/ethanol/acetic/acid medium) (Malimas et al. 2008)	
D-Glucose	15 g
Ethanol	5 mL
Acetic acid	3 mL
Peptone	8 g
Yeast extract	5 g
Distilled water	1000.0 mL
pH 3.5	
GYC Medium (Du Toit and Lambrechts 2002)	
Glucose	50 g
Yeast extract	10 g
$CaCO_3$	30 g
Agar	20 g
Distilled water	1000.0 mL

(continued)

Culture media for acetic acid bacteria	
GY Medium (Mateo et al. 2014)	
Yeast extract (Oxoid)	10 g
Glucose (Oxoid)	50 g
Agar (Oxoid)	20 g
Distilled water	1000.0 mL

Preservation Agar cultures of the bacteria can be kept at 4 °C for 1 or 2 months. Lyophilized cells can be kept alive for 10 years. Methods are described by Kirsop and Doyle (1991).

23.2.2 Genus Lactobacillus

Species of the genus *Lactobacillus* have complex nutritional requirements for amino acids, peptides, nucleic derivatives, vitamins, salts, fatty acids or fatty acid esters, and fermentable carbohydrates. Nutritional requirements are generally characteristic for species or strains. Ten compounds were essential for all wine LAB tested, the carbon and phosphate source, manganese, as well as several amino acids (proline, arginine, and the branched amino acids valine, leucine, and isoleucine) and vitamins (nicotinic acid and pantothenic acids). Nucleotides were not essential for any of the bacteria studied (Terrade and Mira de Orduña 2009). Pantothenic acid and nicotinic acid are required by most species and thiamine by heterofermentative species. Some may require folic acid, riboflavin, pyridoxal phosphate, *p*-aminobenzoic acid, biotin, and B12. Nutritional requirements are the result of minor defects in the chromosome, and they are met when the media contain fermentable carbohydrates, peptone, meat, and yeast extract. Supplementation with tomato juice, manganese, acetate, and oleic esters are even essential for some species (Kandler and Weiss 1986). These compounds are included in the MRS medium (de Man et al. 1960). In general, specific amino acids and monosaccharides were related to a stimulating effect, whereas fatty acid composition and likely some volatile compounds seemed to show an inhibitory effect on the growth of the lactic acid bacteria (Andújar-Ortiz et al. 2010). Yeast mannoproteins in concentrations up to 200 mg L^{-1} activated the growth of 23–48% of the studied LAB strains when ethanol was present in the culture broth (Diez et al. 2010). Ten compounds were essential for all wine LAB tested, the carbon and phosphate source, manganese, as well as several amino acids (proline, arginine, and the branched amino acids valine, leucine, and isoleucine) and vitamins (nicotinic acid and pantothenic acids). Nucleotides were not essential for any of the bacteria studied (Terrade and Mira de Orduña 2009).

Culture media for lactic acid bacteria	
MRS medium (de Man et al. 1960)	
Casein peptone	10.0 g
Meat extract	10.0 g
Yeast extract	5.0 g
Glucose	20.0 g
K_2HPO_4	5.0 g
Diammonium citrate	2.0 g
Sodium acetate	5.0 g
$MgSO_4 \times 7\ H_2O$	0.2 g
Tween 80	1.0 g
Agar	15.0 g
Distilled water	1000.0 mL
pH	6.2–6.4
Sterilization	121 °C, 15 min
SL medium (Rogosa et al. 1951)	
Casein peptone	10.0 g
Yeast extract	5.0 g
Glucose	20.0 g
$K_2\ HPO_4$	6.0 g
Diammonium citrate	2.0 g
Sodium acetate $\times$ 2 H_2O	25.0 g
$MgSO_4 \times 7\ H_2O$	0.5 g
$MnSO_4 \times 4\ H_2O$	0.2 g
$Fe_2SO_4 \times 7\ H_2O$	0.04 g
Tween 80	1.0 g
Agar	15.0 g
Distilled water	1000.0 mL
Sterilization	121 °C, 15 min
FYP broth (Endo et al. 2010)	
D-Fructose	10 g
Yeast extract	10 g
Polypeptone	5 g
Sodium acetate	2 g
Tween 80	0.5 g
$MgSO_4 \times 7\ H_2O$	0.2 g
$MnSO_4 \times 4\ H_2O$	0.01 g
$FeSO_4 \times 7\ H_2O$	0.01 g
NaCl	0.01 g
Cycloheximide	0.05 g
Sodium azide	0.05 g
pH	6.8
Distilled water	1000.0 mL
Sterilization	121 °C, 15 min

Lactobacilli grow best in slightly acidic media with an initial pH between 4.5 and 6.5. Growth ceases below 3.5. Most species are aerotolerant but grow better under microaerophilic or anaerobic conditions. 5% CO_2 stimulates growth. Surface growth on solid media is enhanced by anaerobiosis of reduced oxygen pressure and 5–10% CO_2. The growth temperature ranges from 2 up to 53 °C. Optimal growth is usually at mesophilic temperatures between 30 and 40 °C. The optimal pH usually is 5.5–6.2. Growth is often inhibited above pH 7.0. Most strains exhibit proteolytic activity.

Nonselective MRS medium (de Man et al. 1960) can be applied, when lactobacilli form a predominant flora. A more selective medium is the acetate medium (SL medium) (Rogosa et al. 1951), required when lactobacilli are part of a complex microbial flora. Growth of yeasts may be prevented by the addition of cycloheximide (100 mg L^{-1}). Manganese is required as cofactor for enzyme activity (e.g., lactate dehydrogenase, malolactic enzyme, RNA polymerase, xylose isomerase, NADH oxidase, superoxide dismutase) (Caspritz and Radler 1983; Archibald 1986; De Angelis and Gobbetti 1999; Jakubovics and Jenkinson 2001).

Agar is dissolved separately by steaming in 500 mL distilled water. All other ingredients are dissolved without heating in 500 mL water. The pH is adjusted to 5.4 with glacial acetic accmelted agar and boiled for 5 min. *Preservation*. For short-term preservation, cultures are preferably inoculated into MRS medium stabs after colonies become visible. The cultures are stored at 4–7 °C for 1 month or at −20 °C for several months. For long-term preservation, the cells of the late growth phase are collected by centrifugation, resuspended in skim milk or horse serum containing 7.5% glucose, and lyophilized. Ampules are sealed under vacuum and stored at 5–8 °C. Strains can be kept for 10–20 years at −76 °C or in liquid nitrogen over 30 years. Freezing in glass capillary tubes is convenient (Kirsop and Doyle 1991).

Some special growth requirements of selected species (Kandler and Weiss 1986):

Lactobacillus brevis Calcium pantothenate, niacin, thiamine, and folic acid are required for growth, while riboflavin, pyridoxal, and vitamin B_{12} are not.

Lactobacillus buchneri As described for *L. brevis*.

Lactobacillus casei Riboflavin, folic acid, calcium pantothenate, and niacin are required for growth. Pyridoxal or pyridoxamine is essential or stimulatory. Thiamine, vitamin $B_{12,}$ and thymidine are not needed.

Lactobacillus curvatus Some strains grow at 2–4 °C.

Lactobacillus delbrueckii Pantothenic acid and niacin are required for growth. Some strains require riboflavin, folic acid, vitamin B_{12}, and thymidine. However, thiamine, pyridoxine, biotin, and *p*-aminobenzoic acid are not required.

Lactobacillus diolivorans Fermentative growth on 1.2-propanediol producing 1-propanol and propionic acid.

Lactobacillus fermentum Calcium pantothenate, niacin, and thiamine are required for growth, while riboflavin, pyridoxal, and folic acid are not. Stimulants are tomato and orange juice, extracts of green beans, beetroots, bulb, cabbage, and spinach.

Lactobacillus florum Only glucose and fructose are fermented out of 49 tested carbohydrates. Fructose is fermented faster than glucose (fructophilic). Cells grow in the presence of 300 g fructose or 5 g NaCl L^{-1}.

Lactobacillus fructivorans Enhanced growth takes place in the absence of O_2. Fructose is reduced to mannitol. Acidophilic, pH optimum 5.0–5.5, and no growth happens at an initial pH above 6.0. Mevalonic acid, tomato juice, and/or ethanol are required for growth.

Lactobacillus hilgardii Optimal growth occurs at pH 4.5–5.5. Growth takes place in the presence of 15–18% ethanol.

Lactobacillus kunkeei Citrate and malate are utilized in the presence of glucose. Mannitol is produced from fructose.

Lactobacillus paracasei Growth at 10 and 40 °C, some strains at 5 and 45 °C.

Lactobacillus plantarum Calcium pantothenate and niacin are required for growth. Thiamine, pyridoxal, pyridoxamine, folic acid, vitamin B_{12}, riboflavin, thymidine, and deoxyribosides are not required.

Lactobacillus oeni No growth at pH 3.3 or 10% ethanol.

Lactobacillus vini Citric and malic acids are utilized.

23.2.3 Genus Leuconostoc

Leuconostoc can be isolated on media containing thallous acetate and crystal violet (Cavett et al. 1965). Rich media with complex growth factors and amino acids are required (Dellaglio et al. 1995). Optimal growth occurs at pH 6–7. Growth is stimulated by addition of 0.05% cysteine. Growth on agar plates is stimulated in the presence of a gas mixture of 19.8% CO_2, 11.4% H_2, and nitrogen.

Glucose is used by all species, but fructose is preferred except for *L. mesenteroides* subs. *cremoris*. All species require nicotinic acid, thiamine, biotin, and pantothenic acid, while cobalamine and *p*-aminobenzoic acid are not required. Growth occurs between 5 and 30 °C with an optimum between 20 and 30 °C (Garvie 1986a).

Leuconostoc mesenteroides* subsp. *mesenteroides
Growth occurs between 10 and 37 °C with an optimum between 20 and 30 °C. *Leuconostoc mesenteroides* requires up to eight amino acids, some strains more than eight amino acids. Glutamic acid and valine are required by all.

L. mesenteroides subsp. *mesenteroides* requires only glutamic acid and valine. None require alanine.

Culture Media MRS medium (see Sect. 23.2.2).

Preservation Stock cultures can be prepared from all species in the late growth phase by lyophilization in horse serum containing 7.5% glucose. Once dried, cultures can be kept under vacuum at 10 °C.

Lc. mesenteroides* subsp. *cremoris
Optimal growth is observed between 18 and 25 °C. Sucrose and fructose are not fermented. A large number of amino acids and vitamins are required for growth.

Lc. mesenteroides* subsp. *dextranicus
Growth temperature is comparable to that of *Lc. mesenteroides* subsp. *mesenteroides*, but more amino acids and vitamins are required for growth.

23.2.4 Genus Oenococcus

Until 2006, the genus *Oenococcus* contained only one specie, *O. oeni* (formerly *Leuconostoc oenos*; Dicks et al. 1995), which was isolated from must. A second species, *O. kitaharae* (Endo and Okada 2006), was isolated from a composting distilled shochu residue.

O. oeni is more acid and ethanol tolerant than other lactic acid bacteria. It can grow at pH 3.0 and 10 vol% ethanol (van Vuuren and Dicks 1993; Versari et al. 1999). *O. oenos* can be enriched from must and wine on tomato juice agar with an initial pH below 3.5 and 6 vol% ethanol containing cycloheximide to prevent growth of yeast (Kunkee 1967) for 8 days.

This more defined medium can replace the tomato juice medium for culturing some *Oenococcus* strains (Theobald et al. 2005, 2007b, 2008).

Culture media for Oenococci	
Tomato juice medium (TJ)	
Basal medium	
Peptone from meat	5.0 g
Yeast extract	5.0 g
Tryptone from casein	2.0 g
Glucose	5.0 g
Fructose	5.0 g
Citric acid	3.0 g
Tween 80	1.0 g
Magnesium sulfate × 7 H_2O	0.5 g
Distilled water	1000.0 mL
pH 6.0	

(continued)

Culture media for Oenococci	
75% basal medium +25% tomato juice (Garvie and Mabbitt 1967) (pH 6.0) (v/v)	
20 min 121 °C	
MAC medium (Theobald et al. 2008)	
Peptone from meat	5.0 g
Tryptone from casein	20.0 g
Glucose	5.0 g
Fructose	5.0 g
Arabinose	1.5 g
Citric acid	3.0 g
Tween 80	1.0 g
Manganese sulfate × H_2O	1.9 g
Magnesium sulfate × 7 H_2O	0.5 g
Cysteine	1.5 g
Vitamin solution	10.0 mL
Amino acid solution	100.0 mL
Distilled water	1000.0 mL
pH 6.0	

Amino Acid Solution DL-Alanine (2 g L^{-1}), L-arginine × HCl (2 g L^{-1}), L-aspartic acid (3 g L^{-1}), L-glutamate (3 g L^{-1}), glycine (2 g L^{-1}), L-histidine × HCl (2 g L^{-1}), L-leucine (2 g L^{-1}), L-lysine × HCl (2 g L^{-1}), L-proline (2 g L^{-1}), DL-aminobutyric acid (1 g L^{-1}), L-asparagine (1 g L^{-1}), L-cysteine (1 g L^{-1}), L-isoleucine (1 g L^{-1}), L-methionine (1 g L^{-1}), L-phenylalanine (1 g L^{-1}), L-serine (1 g L^{-1}), L-threonine (1 g L^{-1}), L-tryptophan (1 g L^{-1}), L-tyrosine (1 g L^{-1}), and L-valin (1 g L^{-1}). The amino acids are dissolved in 1 L of distilled water by heating and stored at −18 °C.

Vitamin Solution Pyridoxal hydrochloride (100 mg L^{-1}), nicotinic acid (100 mg L^{-1}), calcium D-(+) pantothenic acid (100 mg L^{-1}), riboflavin (100 mg L^{-1}), thiamine (50 mg L^{-1}), folic acid (20 mg L^{-1}), *p*-aminobenzoic acid (10 mg L^{-1}), cyanocobalamine (1 mg L^{-1}), D(+) biotin (1 mg L^{-1}), and myo-inosit (1 mg L^{-1}).

Riboflavin is dissolved in distilled water by heating folic acid in 50 mL distilled water by adding some drops 1 M NaOH, biotin in a solution of 0.2 g KH_2PO_4, and 0.2 g K_2HPO_4 in 10 mL. The other vitamins are dissolved in 600 mL distilled water. The solution is combined and filled up to 1 L.

Oenococcus oeni Guanine, adenine, xanthine, uracil, riboflavin, folic acid, nicotinic acid, and thiamine are essential. Stimulants are pyridoxal, biotin, yeast preparations, juice, manganese, cysteine, arabinose, and epigallocatechin gallate (Theobald et al. 2005, 2007a, b). A natural source of arabinose is araban and arabinogalactan. Arabans occur as side groups of pectins and in soft fruits (e.g., grapes) and sugar beets.

Inosite, cobalamine, and 4-aminobenzoic acid are not necessary. *Oenococcus* strains need up to 16 amino acids. Glutamic acid and valine are not required, but all strains need α-aminobutyric acid. Asparagine and lysine (Weiler and Radler 1972)

and carotinoids (β-carotin) do not stimulate growth. Polyphenols such as epigallocatechin-3-gallate can act as stimulators (400–500 mg L^{-1}) or inhibitors (>543 mg L^{-1}) on growth (Theobald et al. 2007a). In the case of some strains, tomato juice can be completely replaced by 34 mM manganese (Theobald et al. 2005).

Oenococcus oeni can be used as starter culture for decreasing the concentration of malic acid, which reduces the acidity of wine.

Preservation Stock cultures can be kept on tomato juice agar stabs for 1 month at room temperature. They can be stored for several years in glass capillary tubes at −76 °C in fresh tomato juice medium supplemented with glycerol (10%) (Kirsop and Doyle 1991).

23.2.5 Genus Pediococcus

All species need nicotinic acid, pantothenic acid, and biotin, while none requires thiamine, *p*-aminobenzoic acid, and cobalamine. In the case of *P. damnosus* and *P. parvulus*, 5 days may be required for growth. *P. pentosaceus* grows more rapidly. It grows well aerobically on agar plates. Growth can be improved in an atmosphere of H_2 and 10% CO_2. All species grow at 30 °C but optimum temperatures range from 25 to 40 °C. Acti-dione can be used to suppress yeast growth (Garvie 1986b).

Pediococci grow best in rich media. Most strains need amino acids such as alanine, aspartic acid, glutamic acid, arginine, histidine, isoleucine, phenylalanine, proline, threonine, tyrosine, valine, tryptophan, cysteine, glycine, and leucine, and some strains need lysine, methionine, and serine (Simpson and Tachuchi 1995). Many strains are stimulated by peptides.

No single medium or incubation condition can be used for isolation and growth of all species. Pediococci can be isolated in the presence of lactobacilli by using MRS medium in which glucose has been replaced by 1% mannose, cellobiose, or salicin (Back 1978). Yeast and gram-negative bacteria can be inhibited by the addition of cycloheximide, crystal violet, 2-phenylethanol, sorbic acid, and acetic acid (thallous acetate). Many gram-positive species—except strains from *Pediococcus*, *Leuconostoc*, and some lactobacilli—are inhibited by vancomycin (Simpson et al. 1988).

Culture Media In general the MRS medium (de Man et al. 1960; see Sect. 23.2.2), YPG medium (Garvie 1978), and TGE medium (Biswas et al. 1991) are sufficient.

Pediococcus damnosus Cells grow within 2–3 days at 22 °C. Addition of cysteine improves growth. On agar surfaces, colonies grow better under anaerobic conditions. The final pH is 4.0. The optimal pH is 5.5. Cells grow at pH 4.2, but not at pH 8.5. The maximum pH for growth is 6.5–7.0. The pH of the MRS medium is adjusted to 5.2. Growths occur in the range of 8–30 °C. Hop bitter acid can be used for the isolation of *Pediococcus damnosus*.

Pediococcus parvulus Cultures are improved by addition of cysteine. Some strains require asparagine but no folinic acid. Upper pH limit is between 7.0 and 7.5; the pH optimum is at about 6.5. Optimum growth temperature is 30 °C; maximum temperature is between 37 and 39 °C. Colonies are obtained at 30 °C within 48 h. Strains grow in the presence of 8% NaCl. They are unable to use pentoses.

Pediococcus pentosaceus Colonies are visible after 24 h at 30 °C. Folinic acid is required by some strains. Growth is obtained at pH 4.5 and 8.0. The final pH in MRS broth is 4.0; the optimum is between 6.0 and 6.5. The optimal temperature is between 28 and 32 °C; the maximum temperature is 39–45 °C. Growth occurs in the presence of 10% NaCl.

Pediococcus inopinatus Colony growth can take 5 days. The optimal temperature for growth is between 30 and 32 °C with a maximum of 40 °C. Cells tolerate up to 8% NaCl. Growth occurs between pH 4.5 and 7.5. Pentoses or lactose are not fermented and arginine not hydrolyzed. Starch is no substrate. Slime can be produced.

Preservation Pediococci can be kept on agar slopes at 4 °C for about 3 months. They can be preserved by lyophilization (Garvie 1986b). Storage is improved by the addition of calcium carbonate (1%) to the culture medium. Cells from the late exponential phase should be suspended in horse serum containing 7.5% glucose. Cells can also be stored in growth medium and glycerol (1:1) (Weiss 1991). They can be lyophilized in the presence of horse serum and 7.5% glucose. Cells of the late growth phase should be used. Dried cultures will survive at 10 °C under vacuum. Cultures can be preserved for 3–4 months in skim milk supplied with glucose (1.0%), yeast extract (0.3%), and calcium carbonate (1.0%).

23.2.6 Genus Weissella

Together with some lactobacilli, *Leuconostoc paramesenteroides* has been assigned to the new genus *Weissella* due to RNA-sequence analysis.

Weissella paramesenteroides Temperature for optimal growth is 30 °C. Some strains require lower temperatures between 18 and 24 °C and reduced conditions. Amino acid requirements are complex.

Culture media: MRS medium (see Sect. 23.2.2).

Preservation Agar cultures in MRS medium can be kept at 4 °C for 2 weeks. See also Sect. 23.2.3.

23.3 Yeast

More than 100 yeast species have been isolated from grapes, must, and wine (Table 23.1). The most important for wine making is *S. cerevisiae*, which converts glucose and fructose to ethanol and CO_2. Wine-related yeasts can be grown on one of four solid media.

Preservation Yeast can be kept on agar slopes at 4 °C for several months. They can be stored in growth medium supplemented with glycerol (10%) at −76 °C or liquid nitrogen for several years. The maintenance of yeast (subculturing, drying, and freezing in liquid nitrogen) was described by Kirsop (1991). *Saccharomyces* hybrids have the potential to offer new possibilities for must fermentation. Christ et al. (2015) showed that the triple hybrid *S. cerevisiae* × S. *kudriavzevii* × S. *bayanus* needs no free ammonia as nitrogen source. In contrast to *S. cerevisiae*, the triple hybrid can use amino acids and peptides as sole nitrogen source.

23.4 Fungi

Different fungi can grow on grapes. They can be grown in the following culture media (Table 23.2). Ingredients are dissolved in 1 L distilled water and sterilized by autoclaving at 121 °C for 15 min, unless stated otherwise.

Addition of 1 mL trace elements solution per liter is recommended for avoiding atypical colony growth and color.

Culture media for yeasts	
Emmons' modified Sabouraud's agar (EMSA) (ATTC 2008)	
Sabouraud dextrose broth (BD 238220)	30.0 g
Agar	20.0 g
pH	6.8–7.0
GPYA (glucose-peptone-yeast extract agar) (CBS 2008)	
Glucose	40.0 g
Peptone	5.0 g
Yeast extract	5.0 g
Agar	15.0 g
Distilled water	1000 mL
Sterilize 15 min at 110 °C (0.5 atm)	
MYA (malt-yeast agar) (CBS 2008)	
Yeast extract	3.0 g
Malt extract	3.0 g
Glucose	10.0 g
Bacto Peptone	5.0 g
Agar	15.0 g
Distilled water	1000 mL

(continued)

Culture media for yeasts	
PDA (potato dextrose agar) (CBS 2008)	
Potato extract	0.23 L
Dextrose	20.0 g
Agar	15.0 g
Distilled water	0.77 L
pH = 6.6	

Culture media for fungi	
Aspergillus flavus/A. parasiticus selective medium (AFPA; Oxoid)	
Peptone	10.0 g
Yeast extract	20.0 g
Ferric ammonium citrate	0.5 g
Dichloran	0.002 g
Chloramphenicol	0.1 g
Agar	15.0 g
Distilled water	1000 mL
Final pH ± 6.3	
Note: dichloran and chloramphenicol can be added before sterilization	
Czapek agar (CZ; CBS)	
Saccharose	30.0 g
$NaNO_3$	3.0 g
K_2HPO_4	1.0 g
KCl	0.5 g
$MgSO_4 \times 7\ H_2O$	0.5 g
$FeSO_4 \times 7\ H_2O$	0.01 g
Agar	15.0 g
Distilled water	1000 mL
pH = 6.0–6.5	
Malt extract agar (MEA; Difco, Bacto)	
Malt extract, Difco	30.0
Bacto Agar	15.0
Distilled water	1000 mL
Final pH 5.5 ± 0.2 at 25 °C	

Trace elements solution: 1 g $ZnSO_4 \times 7\ H_2O$ and 0.5 g $CuSO_4 \times 5\ H_2O$ in 100 mL water.

Malt Extract Agar 4% (2%) (MEA 4% (2%); Formulae Used at CBS) Add water to malt extract from the brewery until it contains 10% sugar (measurement with aerometer). Mix 400 mL (200 mL) of this solution with 15 g agar and 600 (800) mL water. Malt agar may also conveniently be prepared with malt syrup (10–40 g L^{-1}) or malt powder (10–20 g L^{-1}).

Oatmeal Agar (OA; Formula Used at CBS) Boil 30 g oat flakes in 1 L water and simmer gently for 2 h. Filter through cloth and fill up to 1 L. Add 15 g agar to 1 L

and sterilize by autoclaving at 121 °C for 15 min. When using powdered oatmeal, filtering is superfluous. Lupin stems may be placed in slants with oatmeal agar. This is also commercially available.

Oatmeal Agar (Guignardia) Add 20 g oat flakes (Bio Hafer Gold "Holo" of *Neuform*) to 500 mL distilled water and stir gently. After 15 min add 10 g agar and sterilize by autoclaving at 121 °C for 15 min. For 500 mL medium, use a 1 L bottle for autoclaving (Attention! Use a 1 L bottle for 500 mL medium because it foams over easily). Final pH: 6.5.

Potato Carrot Agar (PCA; Formula Used at CBS) 40 g carrots and 40 g potatoes are separately washed, peeled, chopped, boiled in 1 L for 5 min, and filtered off. It is then sterilized for 60 min at 1 atm overpressure (121 °C). 250 mL potato extract, 250 mL carrot extract, 500 mL distilled water, and 15 g agar are taken and sterilized at 121 °C for 15 min.

Potato Dextrose Agar (PDA) 300 g scrubbed and diced potatoes are added to 900 mL water and boiled for 1 h. This is then passed through a fine sieve and sterilized for 60 min at 121 °C (1 atm overpressure), 230 mL potato extract, 15 g agar, and 20 g dextrose are then mixed, filled up to 1 L, and boiled until dissolved. This is also commercially available.

Preservation The maintenance of filamentous fungi (subculturing, drying, and freezing in liquid nitrogen) is described by Smith (1991).

23.5 Conclusions

In Tables 23.1, 23.2 and 23.3, most of the microbial species (bacteria, yeast, fungi), which have been isolated from grapes, must, and wine are compiled. Relatively few media are required to grow most of them. Since conventional isolation procedures need several days, they are not very suitable for diagnostic purposes. For isolation and rapid detection of certain species or strains, micromanipulation and molecular methods (fluorescently labeled probes, PCR procedures) have been developed (Fröhlich 2002; Fröhlich and König 1998, 1999, 2000, 2004; Hirschhäuser and Fröhlich 2007; Röder et al. 2007a, b; Pfannebecker and Fröhlich 2008). It is expected that molecular detection methods will be helpful tools to learn more about the diversity and identity of microbial strains on grapes, in must, and wine that have not yet been cultured.

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Chapter 24
Functional Genomics in Wine Yeast: DNA Arrays and Next Generation Sequencing

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24.1 Introduction

The transformation of a grape juice into wine results from the biochemical activity of many microorganisms, particularly yeast. *Saccharomyces cerevisiae* wine yeast strains are able to completely ferment sugar-rich natural musts under conditions that other strains are unable to. Additionally, they are particularly well adapted to the harsh conditions of fermentation, characterised by high sugar content, high alcohol content, low pH, the presence of sulphites, copper, limiting amounts of nitrogen, anaerobiosis and other environmental stresses. For those reasons, *S. cerevisiae* is still referred as the wine yeast par excellence.

Over the last years, winemaking industry have benefit tremendously from the established interest of the scientific community in *S. cerevisiae* fundamental research, being a model organism for studies in cell biology, biochemistry and in molecular biology for many years. The sequence of the reference laboratory strain S288c entire genome was accomplished before any other eukaryote in 1997 (Goffeau et al. 1996, 1997), and since then about 420 laboratory, industrial and wild strains have been extensively annotated (Borneman and Pretorius 2015; Borneman et al. 2016; Gallone et al. 2016). Given the considerable genetic

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information available, the leading role of this eukaryotic model has been evident in the development of powerful tools for analysis. For instance, global gene expression studies by means of microarray analysis were first performed using *S. cerevisiae* (Schena et al. 1995; Wodicka et al. 1997; DeRisi et al. 1997; Hauser et al. 1998) and proved to be instrumental in the unravelling of the complexity of gene expression regulation under several conditions. Moreover, it has been continuously being improved because this yeast is the working horse for the development of different technical improvements (Hughes et al. 2001; García-Martínez et al. 2004; David et al. 2006). Logically, these DNA array studies were first done in laboratory strains of *S. cerevisiae* growing in laboratory conditions. These strains do not exhibit the same properties as industrial strains, and the growth conditions are significantly different; therefore, their responses may be quite different. However, rapidly this genome-wide approach received a strong interest in the subsequent years to address the question of the adaptation of industrial wine yeasts to the actual winemaking conditions. This review presents a synopsis of DNA array and next-generation sequencing (NGS) technologies and focus mainly in their use in studying wine yeast gene expression profiles, recapitulating the major findings about *S. cerevisiae* biology that have emerged from its application and how they contributed to the improvement of industrial winemaking process. Although the use of microarrays to generate gene expression data has become widespread, thanks to the advent of NGS, RNA-seq has recently become an attractive alternative method in the studies of transcriptomes, promising several advantages compared with microarrays.

24.2 Short Overview of the DNA Array Technology

By definition "array" means "to place in proper or desired order". A DNA array (also commonly known as gene or genome chip, DNA chip or gene array) is a collection of DNA spots, commonly representing single genes arrayed on a solid surface (glass, plastic, silicon chip or nylon) by the covalent attachment to chemically suitable matrices or simply by electrostatic binding. The immobilised DNA segments are known as probes, and many thousands can be placed in known locations on a single DNA microarray (see Fig. 24.1 for a schematic representation of DNA chip technology).

DNA arrays can be fabricated using a variety of technologies, including printing with fine-pointed pins onto either glass slides or nylon membranes, photolithography using pre-made masks, ink-jet printing or electrochemistry on microelectrode arrays. By regarding the printing surface and the technology used for fabrication and processing, different kinds of DNA arrays can be distinguished:

Spotted Microarrays The probes are cDNA or small fragments of PCR products that correspond to mRNAs and are spotted onto a glass surface. This type of array is typically hybridised with cDNA from two samples to be compared and is labelled

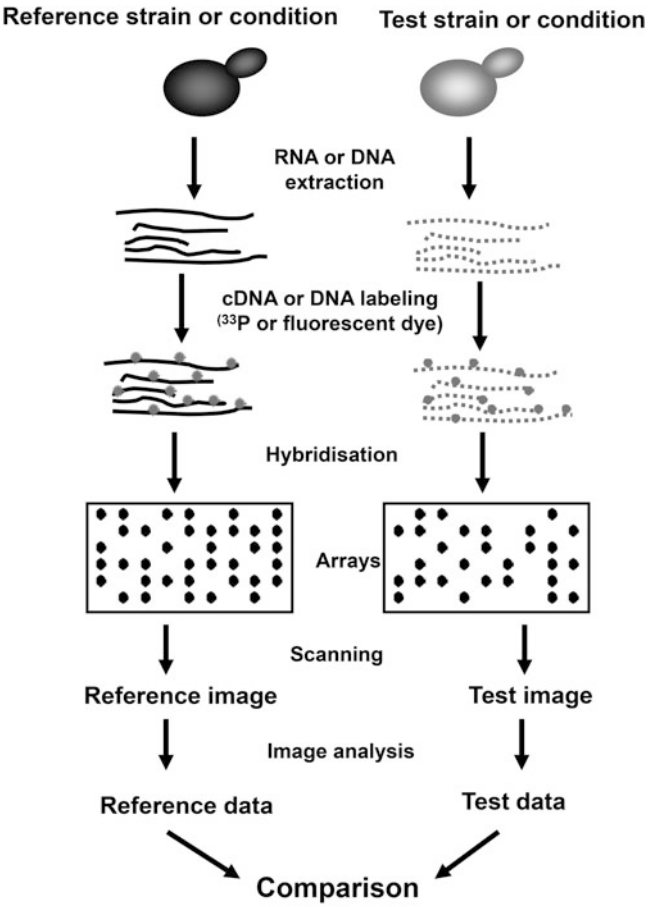

Fig. 24.1 Schematic representation of the different steps in the DNA array processing and analysis. Note that in the hybridisation step in macroarrays and in some kinds of oligonucleotide arrays, two independent hybridisations are performed whereas in most glass microarrays, both test- and reference-labelled samples, are hybridised simultaneously on the same slide

with two different fluorophores. The two labelled cDNA samples are mixed and hybridised to a single microarray that is then scanned to visualise the two fluorophores after excitation with a laser beam of a defined wavelength. Relative intensities of each fluorophore signal may then be used in ratio-based analysis to identify upregulated and downregulated genes. Absolute levels of gene expression cannot be determined in the two-colour array, but relative differences in the expression among different spots (=genes) can be estimated.

Spotted Macroarrays Equivalent to the previous one but in which the probes are immobilised onto a positively charged nylon membrane. mRNA is radioactively labelled (usually ^{33}P). Each condition (e.g. wild type and mutant) is hybridised

independently with a stripping step between them, which allows the use of the same arrays for different sample replicates.

Oligonucleotide Microarrays The probes are designed to match parts of the sequence of known or predicted mRNAs. There are commercially available designs that cover complete genomes from different companies. These microarrays can provide estimations of the absolute value of gene expression. Oligonucleotide arrays can be either produced by piezoelectric deposition with full-length oligonucleotides or by *in situ* synthesis. Long oligonucleotide arrays are composed of 50–60 mers and are produced by ink-jet printing on a silica substrate. Short oligonucleotide arrays are composed of 25–30 mer and are produced by photolithographic synthesis on a silica substrate or piezoelectric deposition on an acrylamide matrix.

Genotyping Microarrays They are spotted macro- or microarrays than can be used to identify genetic variation in individuals and across populations. In this array, the labelled genomic DNAs from the strain to be tested along with the reference strain S288c are competitively hybridised to a spotted array containing probes of each gene of the later. The comparison of the signal intensities of both strains is then associated with the enlargement or deletion of genes in the tested strain relative to the reference. Short oligonucleotide arrays can be used to identify the single nucleotide polymorphisms (SNPs) that are thought to be responsible for genetic variation.

Tiling Arrays They are a kind of microarray that includes overlapping oligonucleotides designed to blanket the entire genome each 5–20 nucleotides without any previous knowledge of the coding regions. They can be used either for genotyping or expression studies.

24.3 Impact of DNA Array Technology on Yeast Gene Expression Research

The availability of the *S. cerevisiae* genome sequence has led to the discovery of many gene sequences but not their function. Since then, many functional analysis projects have been dedicated to the investigation of the molecular biology of this yeast, making use of omic tools developed based on genome knowledge. The first of these high-throughput techniques, DNA arrays, provided one entry point for functional genomics, changing the paradigm of gene expression analysis that has been limited to small number of genes (Lockhart et al. 1996). Global expression analyses have helped to elucidate their role in both cellular physiology and the way in which their mechanism works. The first studies compared expression patterns of one third of the yeast genome in different metabolic states (Lashkari et al. 1997). The advances in the array-based techniques allowed the expression of approximately 6000 genes of the yeast *S. cerevisiae* grown under a few different conditions to be

monitored on a single chip (DeRisi et al. 1997; Wodicka et al. 1997), using probes and primers obtained or designed from the laboratory strain S288c sequence. While Wodicka et al. (1997) compared gene expression in yeast cells grown on rich and minimal media, other pioneering comprehensive studies characterised the genes that were differentially expressed during the shift from fermentation to respiration (DeRisi et al. 1997; ter Linde et al. 1999; Kuhn et al. 2001), during sporulation (Chu et al. 1998), during the cell cycle (Cho et al. 1998; Spellman et al. 1998) or in response to conditions or treatments of interest, such as chemical or environmental agents (Jelinsky and Samson 1999; Jelinsky et al. 2000). In a landmark experiment that studied yeast response to 13 varied environmental conditions (Gasch et al. 2000), the authors found that while some genes altered its expression in a particular condition, a large set of genes showed a similar response to almost all the conditions studied, being generally termed environmental stress response (ESR) genes. The authors actually found that while some genes alterations were specialised for specific stresses, a large set of genes (the ESR ones) showed a similar response to almost all the conditions studied. This ESR share features with the previously recognised general response to stress, comprising a set of ≈50 genes induced by a variety of stresses through the stress response element (STRE) promoter sequence and recognised by the transcription factors Msn2p and Msn4p (see Estruch 2000 for a review). The majority of 900 ESR genes are repressed in response to acute stresses and are involved in growth-related processes including ribosomal protein genes, along with the large set of genes involved in RNA metabolism and protein synthesis. On the other hand, approximately 300 genes are induced in the ESR and involved in a wide variety of processes, including carbohydrate metabolism, detoxification of reactive oxygen species, cellular redox reactions, cell wall modification, protein folding and degradation, DNA damage repair, fatty acid metabolism, metabolite transport, vacuolar and mitochondrial functions, autophagy and intracellular signalling (Gasch et al. 2000). Later, it has been revealed that the ESR is not only a transcriptional response, but it also encompasses a post-transcriptional (mRNA stability) response that contributes to the fine adjustment of the induction and repression peaks (Canadell et al. 2015). More recently, it has been shown that there are differences in environmental stress response among yeast species with the more pronounced differences mostly found in the induced genes, whereas the repressed ones are highly conserved (Brion et al. 2016).

Other major work in this field discerned the function of regulatory proteins, such as transcription factors or subunits of transcription complexes, and either studied the consequences of overexpression or examined mutants (DeRisi et al. 2000; Holstege et al. 1998; Kobor et al. 1999; Myers et al. 1999; López and Baker 2000; Lee et al. 2000; Sudarsanam et al. 2000; Carmel-Harel et al. 2001).

For yeast biologists, the main achievement of the early gene expression studies was the discovery of genetic regulatory mechanisms, providing data to link genes and pathways to phenotypes in such a way that components of any metabolic and regulatory pathway could be determined. The wealth of data provided by the microarrays allowed the formulation of hypotheses that could be tested with other more traditional experiments. On the other hand, genome-wide expression

experiments on yeast validated the wide application of the technology and led to the development of a variety of other genome-scale technologies, which allowed mapping the binding sites of transcription factors in vivo by chromatin precipitation followed by DNA microarray (ChIP-chip) (Horak and Snyder 2002), analysis of screens of pooled mutants (Giaever et al. 2002; Pierce et al. 2007), quantification and detection of distinct spliced isoforms (Clarck et al. 2002) or genome-wide assessment of transcription rates (García-Martínez et al. 2004).

In the pursuit of a more comprehensive understanding of yeast physiology and metabolism, along the last years, numerous large-scale functional genomics studies have been performed, and *S. cerevisiae* response to different perturbations has been investigated. Presently, there are 1371 and 398 gene expression experiments hosted by public gene expression databases such as Gene Expression Omnibus (Edgar et al. 2002; Barrett et al. 2013) and ArrayExpress (Brazma et al. 2003; Kolesnikov et al. 2015), respectively. Restricting the search for wine yeast, we found fewer transcriptomic studies in both databases, 60 and 9 experiments, respectively. The development of the Saccharomyces Genome Database (SGD) (Cherry 2015) was essential for collecting, organising, storing and accessing the data from yeast large-scale studies. Also the curating of the data derived primarily from focused studies to generate machine-readable Gene Ontology (GO) annotations for yeast genes (Ashburner et al. 2000) turned possible for the yeast scientific community to address the roles of previously uncharacterised genes and to map novel functional connections between seemingly unrelated processes (Boone 2014).

24.4 Impact of RNA Sequencing on Yeast Gene Expression Research

As denoted above, transcriptome analysis by DNA arrays has played a central role in yeast functional genomics unravelling the complexity of gene expression regulation. Nevertheless, it is acknowledged that this methodology suffers from several caveats. For instance, for the construction of DNA microarrays, it is mandatory to have prior knowledge about genome sequence of the organism being studied. Up to 37,000 SNPs can be found when comparing laboratory strains (Schacherer et al. 2007), and the problem can become even more complicated for non-laboratory yeast strains. Indeed, in the sequence comparison of a wine strain AWRI1631 to S288c, an SNP frequency of 1 per 150 base pairs or roughly 7 SNPs per kilobase was found (Borneman et al. 2008). Also, microarrays often cannot readily distinguish closely related sequences due to cross-hybridisation jeopardising specificity and the quantification of RNAs expressed at a low level. On the other hand, saturation of spot signals puts an upper limit on the amount of expression that can be reliably quantified. To address these last two limitations, real-time qPCR is commonly used to validate microarray-generated data (Chuaqui et al. 2002; Brazma et al. 2001). Finally, there are several different microarray platforms

commercially available and other DNA arrays produced in-*house* using completely different probe sets which turn difficult the comparison of the data generated. Indeed, most of cross-platform comparisons are done by analysing each platform data set independently using the most appropriate normalisation method and statistical tests for each, and only afterwards the lists of significantly differentiated genes are compared.

In this line, next-generation RNA sequencing (RNA-seq) has recently become an attractive method in the studies of transcriptomes. Briefly, total or fractionated RNA is converted to a library of cDNA fragments with attached adaptors which are then sequenced. These reads are aligned to a reference genome or transcriptome set and can be counted to determine differential gene expression (Nagalakshmi et al. 2010) (see Fig. 24.2 for a schematic representation of RNA-seq technology). An RNA-seq protocol, covering yeast RNA extraction, cDNA synthesis, cDNA fragmentation and Illumina cDNA library generation with some brief remarks on bioinformatics analysis, is presented by Waern et al. (2011). This technique provides several advantages compared with microarrays. For once, RNA-seq does not depend on prior knowledge of sequence as RNA-seq labelled cDNA in parallel and multiple times. Also, cross-hybridisation and range of detection are not a concern since there is no hybridisation step involved, and due to the digital nature of RNA-seq, there is an unlimited dynamic range of detection (reviewed in Wang et al. 2009). While surpassing the mentioned microarray disadvantages, in order for RNA-seq technology to reach its full potential, a number of experimental and computational challenges need to be addressed, including the handling of read mapping uncertainty, sequencing non-uniformity, estimation of potentially novel isoform (alternatively spliced transcript) expression levels and efficient storage and alignment of RNA-seq reads (Li et al. 2010).

Again, *S. cerevisiae* was one of the first species in which transcriptome reconstruction RNA-seq was evaluated (Nagalakshmi et al. 2008). In that study, the authors revealed the transcriptional landscape of the yeast being able to detect novel sequences through de novo assembly of sequences that did not match with the reference genome. RNA-seq yielded a comprehensive view of both the transcriptional structure and the expression levels of transcripts showing that nearly 75% of the non-repetitive sequence of the yeast genome is transcribed (Nagalakshmi et al. 2008; Wang et al. 2009). Besides *S. cerevisiae*, RNA-seq has already been applied to other yeast species including *Candida albicans* (Bruno et al. 2010), *Candida parapsilosis* (Guida et al. 2011), *Candida glabrata* (Linde et al. 2015), *Schizosaccharomyces pombe* (Bitton et al. 2015), *Cryptococcus neoformans* (Toh-E et al. 2015), *Pichia anomala* (Fletcher et al. 2015), *Pichia pastoris* (Valli et al. 2016), *Brettanomyces bruxellensis* (Capozzi et al. 2016) and *Kluyveromyces marxianus* (Schabort et al. 2016). On the other hand, only a few studies have been carried out with this technology in biofuels (McIlwain et al. 2016), Chinese rice wine (Li et al. 2014) and baker's (Aslankoohi et al. 2013) and wine (Treu et al. 2014b; Nadai et al. 2015, 2016, see below) industrial *S. cerevisiae* yeasts.

RNA-seq has proven to be extremely powerful and continues to advance raising the question about the future of microarrays technology in gene expression studies.

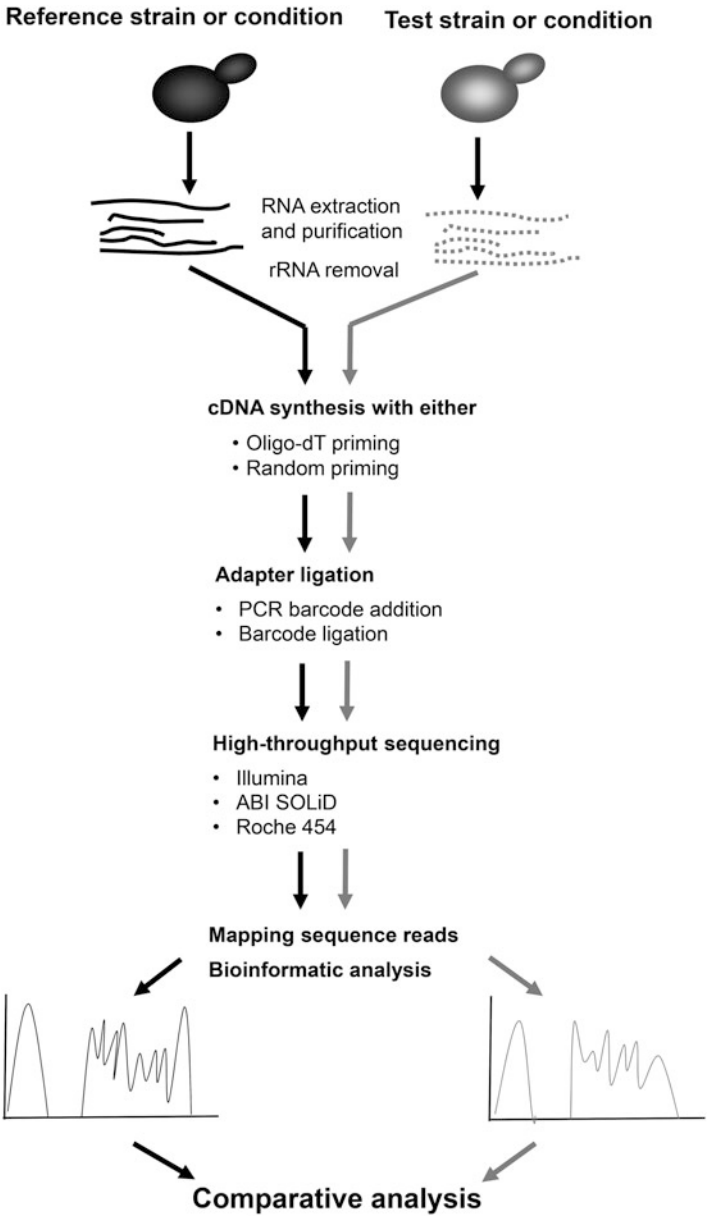

Fig. 24.2 Schematic representation of the different steps in the NGS processing and analysis

Recently, Nookaew et al.(2012) presented the first comprehensive comparison of both methods for analysis of transcriptome data of *S. cerevisiae* using the laboratory strain CEN.PK113-7D grown under two different metabolic conditions: respiro-fermentative (batch) or fully respiratory (chemostat) metabolism. Their results

underlined the importance of accurately mapping the reference genome to estimate gene expression level and to identify differentially expressed genes. Nevertheless, the authors found high consistency between microarray and RNA-seq platforms. More recently, a single extraction of mRNA from *S. cerevisiae* was quantified by both microarrays and RNA-seq in parallel (Robinson et al. 2015). In this study, they multiplexed each lane of RNA-seq profiling so that it exactly mirrored the eight-array per chip design of the microarray platform that was used. The authors concluded that microarrays, while more consistent in their estimates across technical replicates, may show systematic biases at low intensities that confound differential expression detection suggesting that low-expressed genes of special interest should be monitored cross-platform. Taken together, both studies encourage the continual use of microarray as a versatile tool for differential gene expression analysis. In some way, RNA-seq technology will certainly contribute to the improvement of microarrays; actually, as new sequences are discovered, they could be incorporated in the *S. cerevisiae* arrays increasing their coverage, keeping microarrays relevant. In addition, this technology can boost the development of arrays for other biotechnological important yeast species which has been limited by the lack of sequence information available.

24.5 Transcriptional Response of *Saccharomyces cerevisiae* to Oenological Relevant Stresses

The transformation of grape juice into wine is accomplished by the activity of several microorganisms, mainly yeasts that are responsible for conducting alcoholic fermentation. During winemaking, yeast strains come across acidic pH (2.9–3.6), hyperosmotic stress due to the high sugar concentration in musts (up to 260 g/L), low nitrogen content and the presence of inhibitors such as sulphite, occasionally low temperature and, later, anaerobiosis, nitrogen starvation and high ethanol concentration (up to 15% v/v) (reviewed in Attfield 1997; Pizarro et al. 2007), being selected based on their ability to adapt to this harsh environment. Although there is a great variety of wine-related yeast species harboured in the skin of grapes, *S. cerevisiae* is still referred as the "wine yeast" mostly due to its stress resilience and unequalled fermentative ability, being able to adjust and completely ferment sugar-rich natural musts under conditions that other strains are unable to (Camarasa et al. 2011). The impressive adaptation of these wine strains to the oenological environment is related to variation in gene expression, as a consequence of genetic differences, either on coding or non-coding regions (Salinas et al. 2016) with regard to other *S. cerevisiae* strains of different origins (Cavalieri et al. 2000; Fay et al. 2004; Wang et al. 2007; Carreto et al. 2008), and in some cases correlates with the niche from which the strains have been isolated (Warringer et al. 2011). Recently, whole genome sequencing performed on 196+19 wine strains of *S. cerevisiae*, including commercial and natural isolates, indicated that these strains contain

relatively little genetic variation compared to the global pool of *S. cerevisiae* diversity (Borneman et al. 2016; Gallone et al. 2016, see below).

Unlike the genome sequence, the transcriptome is very dynamic with genes being high or lowly expressed according with the external stimulus. DNA microarrays have been extensively used to study yeast molecular responses to stress situations. The already cited study by Gasch et al. (2000) on a laboratory strain was used to elucidate how *S.* cerevisiae yeast cells respond when exposed to 13 different environmental stresses including osmotic shock amino acid starvation, nitrogen depletion, progression into stationary phase and oxidative stress which are relevant in the winemaking context.

Also the molecular responses of *S. cerevisiae* exposed to various wine-relevant stresses, including osmotic shock (Kaeberlein et al. 2002; Jiménez-Martí et al. 2011), ethanol (Alexandre et al. 2001; Fujita et al. 2004; Hirasawa et al. 2007; Lewis et al. 2010), sulphite (Park and Hwang 2008), nutrient limitation (Boer et al. 2003; Pizarro et al. 2008), acclimatisation to low temperature (Leng Tai et al. 2007) and CO_2 pressure (Aguilera et al. 2005), have been also addressed. Logically, these DNA array studies were mostly done in laboratory strains of *S. cerevisiae* growing in laboratory conditions. The inclusion of wine yeasts in some of these studies lead to the uncovering of some transcriptomic and genomic differences between wine and non-wine yeast strains. For instance, in the T73 wine yeast strain in relation to oxidative metabolism, *YHB1*, a gene encoding a flavohaemoglobin, whose expression is elevated in aerobic conditions in laboratory strains (Liu et al. 2000), is only slightly expressed in wine yeast. A small deletion found in its promoter is thought to be the reason (Hauser et al. 2001). This event may reflect the physiological features of the wine strain, which has been evolving for billions of generations under the almost anaerobic conditions of wine fermentation. Also, genes involved in sulphur (*SUL1-2*) and ammonia (*MEP2*) transport (Cavalieri et al. 2000) or that involved in sulphite resistance (*SSU1*) were found to be highly expressed in wine yeast strains (Hauser et al. 2001). The overexpression of these genes might be a developed detoxification strategy giving the continuous contact of these strains with copper sulphate and sulphur dioxide, used in controlling mould growth on grapes or in preservation during the winemaking process, respectively. In this line, Pérez-Ortín et al. (2002a) investigated in great detail the possible mechanisms for the expression regulation of the *SSU1* gene of the T73 wine yeast strain. A rearrangement of the promoter of *SSU1* was detected and led to an upregulation in its expression. We concluded that human involvement and the traditional vinification protocols led to a selection of wine yeasts which resist these agents. Also, Aa et al. (2006) analysed both the population genetic variation and population structure of *S. cerevisiae* by sequencing the coding region of *SSU1* and three other loci (*CDC19, PDH1, FZF1*) in 27 strains from very different locations in Italy and Pennsylvania, collected from oak forests and vineyards. The phylogenetic reconstruction showed the existence of differences between oak strains and wine strains, indicating that differences within *S. cerevisiae* populations are more likely due to ecological factors than to geographic factors. Recently, NGS and quantitative trait loci (QTL) mapping have discovered a different reciprocal chromosome translocation involving *SSU1*

promoter that increases sulphite resistance in other wine yeast strains (Zimmer et al. 2014). The high sequence polymorphism found in the *SSU1* gene suggests the existence of a diversifying selection on its protein product, thus supporting our previous proposal of a strong selection for this gene during the historical use of sulphur-based fungicides in winemaking. Additionally, it is known that wine strains diverge on their susceptibility to sulphite (Barbosa et al. 2014). Recently, Nadai et al. (2016) using RNA-seq to study strain-dependent SO_2 resistance have confirmed the main role of Ssu1p transporter in SO_2 tolerance and its importance in discriminating resistant from sensitive strains. Also, the *CUP1* gene, which is related to copper resistance (Karin et al. 1984; Winge et al. 1985), was found to be less expressed in YPD in the T73 wine strain than in the S288c background (Hauser et al. 2001). This could be due to a small deletion in the *CUP1* locus region (Pérez-Ortín et al. 2002b) or a higher number of copies of *CUP1* among wine strains compared with other isolates (Almeida et al. 2015). Recently, a promoter variant of *CUP1* with increased expression variability was identified in the wine yeast strain EC1118 conferring improved resistance to environmental stress conditions (Liu et al. 2015).

Also, growth temperature was found to lead to differential transcriptional responses among laboratory (CEN.PK113-7D) and wine (EC1118) strains of *S. cerevisiae*, centred on genes involved in sugar uptake and nitrogen metabolism (Pizarro et al. 2008). The levels of expression of both the low-affinity transporter *HXT1* gene and the high-affinity transporter *HXT6* and *HXT7* genes were higher in the wine yeast than in the laboratory strain. On the other hand, the authors showed that the levels of expression of high-affinity nitrogen transporters and amino acid biosynthetic genes were higher in the laboratory strain, whereas in the wine yeast, there was increased transcription of anabolic and catabolic genes involved in nitrogen metabolism, suggesting that the laboratory yeast is more starved for nitrogen than the wine yeast.

Differences in the genome-wide expression profile between laboratory (W303 diploid) and wine strains have also been found when exposed to osmotic stress caused by high sugar concentrations (Jiménez-Martí et al. 2011). The authors associated the improved adaptability of the ICV16 wine yeast, as seen by the higher percentage of viable cells and increased ability to grow in 20% of glucose, with the higher expression of genes related with amino acid and nucleotide metabolism (particularly biosynthesis), glycolysis, alcohol and ergosterol metabolism and DNA replication. In this sense Pizarro et al. (2008) observed higher expression of genes associated with the cellular response to nitrogen starvation in the laboratory strain used in their study (CEN PK113-70) when compared with the wine counterpart EC1118. In a comparative genome hybridisation on array (aCGH) study (see later), Carreto et al. (2008) reported that among the genes depleted in five commercial wine *S. cerevisiae* strains, relative to the reference strain S288c, were four copies of tandemly repeated cell-wall asparaginase genes (*ASP3-1*, *ASP3-2*, *ASP3-3* and *ASP3-4*), which are induced in response to nitrogen starvation. Taken together, these studies reinforce the suggestion that nitrogen metabolism is differentially regulated among these strains.

In sum, these studies have shown that although wine and laboratory strains are genetically highly related, the genetic basis of their distinct technological properties under fermentation conditions is still largely unknown. Recently RNA sequencing performed on the four vineyard strains, as well as on the industrial wine yeast strain EC1118 and on the laboratory strain S288c, revealed that *cis* and, more significantly, *trans* variations have a markedly different effect on transcriptional variability among strains with the latter being the major determinant of the fermentation characters that differentiated the strains examined (Treu et al. 2014a). Nevertheless, the data acquired in the studies using laboratory strains allowed a better understanding of the molecular mechanisms underlying yeast stress response and paved the way for the identification of gene targets or gene expression patterns that allow industrial yeast strains to adapt to each particular condition. The use of standard laboratory conditions enabled the comparison of specific metabolic and physiological features of natural isolates or commercial wine yeast strains in relation to the laboratory strains. However, those experiments in which the cells are transiently exposed to a single stress at a time do not efficiently reproduce the natural environment for wine yeast considering the dynamic succession of stresses occurring along the winemaking process.

24.6 Expression Responses of Wine Yeasts to Stress Situations During Vinification

Until the development of DNA microarray analysis, some traditional gene expression studies including only a small number of genes were conducted with wine *S. cerevisiae* yeasts. The first gene expression study in wine yeasts was conducted on a haploid strain, V5 (a non-usual wine strain) by Northern blot analysis of 19 genes which had been previously described as being expressed in laboratory growth conditions or on molasses during the stationary phase and/or under nitrogen starvation. Nine genes, including members of the *HSP* family, showed a transition-phase induction profile (Riou et al. 1997). A more comprehensive study was conducted on the same haploid wine strain and on a reference strain FY69 (S288c background) by the same group. In this case, 99 genes from chromosome III were studied by Northern blot analysis (Rachidi et al. 2000). A particular wine strain, T73, isolated from Alicante wines (Querol et al. 1992), has been selected in our laboratory for the expression studies of particular sets of genes. A molecular study using Northern blot was conducted on it (Puig and Pérez-Ortín 2000a, b). The expression patterns of glycolytic genes, and of nine other genes that were characterised by DeRisi et al. (1997) as showing a peak of induction at the diauxic shift, were studied. The T73 strain (and other commercial wine yeast strains) has also been useful to demonstrate the relevance of the expression of genes involved in the response to osmotic stress (mainly *GPD1*, encoding the glycerol-3-phosphate dehydrogenase gene) during the first hours of vinification (Pérez-Torrado et al. 2002;

Zuzuárregui et al. 2005). Gene expression analysis have also been carried out along benchtop trials of industrial wine yeast propagation in order to identify stress responses that might be relevant for the performance of active dry yeasts. After testing the expression profiles of a selected set of stress gene markers, the induction of the stress responsive gene *TRX2* during the batch stage of industrial growth suggests that an oxidative stress response can occur at the transition from fermentative to respiratory metabolism (Pérez-Torrado et al. 2005).

Partial transcriptomic analysis with commercial wine yeast strains, which differ in their fermentative behaviour, has also helped to understand these differences and to obtain clues to understand the best adaptation of several strains. Our research groups have carried out several analyses in this sense. A first study limited to two commercial strains and several well-characterised stress-responsive genes (HSP family and others) showed that *HSP12* could serve as a molecular marker for stress resistance in wine yeasts (Ivorra et al. 1999). Later on, analyses of this kind with 14 oenological strains demonstrated that it is possible to establish a correlation between stress resistance and fermentative behaviour (Zuzuárregui and del Olmo 2004a). Besides, although each strain shows a unique pattern of gene expression (Carrasco et al. 2001), higher (and in some cases maintained) mRNA levels of many stress genes tested were found in the strains with severe fermentative problems (Zuzuárregui and del Olmo 2004b), which suggest the requirement of and accurate stress response during vinification.

24.7 Genome-Wide Expression Studies in Wine Yeast

As previously mentioned, the natural environment of *S. cerevisiae* has shaped the evolution of this organism's metabolism to allow it to exploit the harsh winemaking environment. From its inoculation into grape juice until the end of the fermentation process, *S. cerevisiae* is exposed to stress situations that are reflected in the yeast's gene expression pattern.

Inoculation of grape musts with active dry yeast is a common practice in wine industry. Little is known about the transcriptional changes occurring during the biomass propagation step used in the industrial production of dry yeast, but a transcriptomic and proteomic analysis carried out by Gómez-Pastor et al. (2010) revealed that the most critical step is the metabolic transition from respiration to fermentation-based growth. Its use requires a previous rehydration process in which yeast cells restore their cellular functions. Some studies have analysed the genomic response in commercial wine yeast strains to rehydration and adaptation to osmotic stress at the beginning of vinification. In the first study, rehydration was carried out in a complete glucose medium to identify events related to re-establishment of fermentation (Rossignol et al. 2006). The authors reported substantial transcriptional changes. The expression profile observed in the dried yeasts was characteristic of cells grown under respiratory conditions and exposed to nitrogen and carbon starvation and considerable stress during rehydration. Furthermore, many genes

involved in biosynthetic pathways (transcription or protein synthesis) were coordinately induced, while those subject to glucose repression were downregulated. While expression of general stress-response genes was repressed during rehydration, despite the high sugar levels, that of acid-stress genes was induced, probably in response to the accumulation of organic acids. In the second study, rehydration was carried out in water to separate this process from adaptation to osmotic pressure (Novo et al. 2007). The results of the study showed that rehydration for an additional hour (following an initial period of 30 min) did not induce any relevant changes in global gene expression. The incubation of rehydrated cells in a medium containing fermentable carbon sources activates genes involved in the fermentation pathway, the nonoxidative branch of the pentose phosphate pathway, ribosomal biogenesis and protein synthesis. Also addition of the rehydration nutrient mix downregulated the expression of genes involved in the biosynthesis of different amino acids and vitamin/cofactor transport, consistent with its composition in these nutrients (Winter et al. 2011). Previously, Erasmus et al. (2003) analysed yeast response to high sugar concentrations by inoculating rehydrated wine yeast in Riesling grape juice containing equimolar amounts of glucose and fructose to a final concentration of 40% (wt/vol) and comparing global gene expression with that observed in yeasts inoculated in the same must containing 22% sugar. Although the sugar concentration used is not generally found in winemaking conditions, some of the results coincided with those reported by Rossignol et al. (2003), with sugar stress resulting in the apparent upregulation of glycolytic and pentose phosphate pathway genes and structural genes involved in the formation of acetic acid from acetaldehyde and succinic acid from glutamate and the downregulation of genes involved in the de novo biosynthesis of purines, pyrimidines, histidine and lysine. The authors also reported considerable changes in the expression levels of stress-response genes. These changes affected, among others, genes involved in the production of the compatible osmolyte glycerol (*GPD1*) and genes encoding the heat shock proteins *HSP104/12/26/30/42/78/82* and *SSA3/4*. In agreement, Jiménez-Martí et al. (2011) by means of gene expression analyses with several wine yeast strains found that the higher expression of genes involved in both biosynthetic processes and glycerol biosynthesis was directly associated with the improved ability of yeasts to growth in grape juice.

Large-scale transcriptome monitoring during alcoholic fermentation under conditions mimicking an oenological environment was first reported by Rossignol et al. (2003) that analysed samples taken at different time points during fermentation of a synthetic must. The authors found genes involved in C-compound metabolism, mitochondrial respiration/oxidative phosphorylation, stress responsive genes and a large number of genes with no biological process associated [130 genes from various subtelomeric families of unknown function (*PAU, AAD, COS*)] to be induced during wine fermentation. On the other hand, genes primarily involved in cell growth, protein biosynthesis and ribosomal processing functions were repressed in response to stress associated with alcoholic fermentation progression. A common description of gene expression during fermentation of synthetic or natural grape juices has consistently been described, although with differences in

gene expression patterns between strains (Rossouw et al. 2008). The greatest effect on gene expression is produced upon entry into the stationary phase, probably explained by a cell proliferation arrest in response to nitrogen depletion, a process regulated by the TOR pathway (Rossignol et al. 2003). The changes in gene expression seen in this phase, however, appear to differ from those observed under laboratory conditions (Gasch et al. 2000). In a latter comprehensive study of the dynamics of the yeast transcriptome during wine fermentation, Marks et al. (2008) discovered 223 genes that were dramatically induced along the process. They called this the "fermentation stress response" (FSR). The most interesting point was that the FSR was found to overlap only partially with the ESR (Gasch et al. 2000). Interestingly, 62% of the FSR genes were novel, suggesting that the stress conditions in wine fermentation were rather different from those observed in laboratory conditions. Also of interest was the fact that respiratory and gluconeogenesis genes were expressed even in high glucose concentrations and that ethanol accumulation was the main reason for entry into the stationary phase.

The amount of available nitrogen is considered to be one of the main limiting factors for yeast growth in musts (reviewed in Mendes-Ferreira et al. 2011). Studies performed with wine yeasts have generally found high expression levels for genes linked to amino acid and purine biosynthesis (Backhus et al. 2001; Cavalieri et al. 2000; Hauser et al. 2001), which are indicative of high growth rates. Activation of the methionine biosynthesis pathway and alterations in sulphate and nitrogen assimilation are known markers for metabolic phenotype as they are connected with cell-cycle progression (Patton et al. 2000). The effect of nitrogen availability on the growth of wine yeasts has been analysed in recent studies. One of these compared global gene expression profiles in synthetic media containing high and low concentrations of arginine (a source of nitrogen) (Backhus et al. 2001), whereas the other compared expression profiles in a Riesling must with normal concentrations of nitrogen and another to which diammonium phosphate (DAP) was added during the late fermentation phase, when yeast growth is no longer active (Marks et al. 2003). In the first study, it was found that nitrogen limitation induced genes that would normally be repressed by the high concentrations of glucose in the must. This suggests that, in the growth conditions that characterise the fermentation of must containing high concentrations of sugars and nitrogen, the use of glucose might be diverted, at least partly, to a respiratory metabolism (Backhus et al. 2001). This effect would be similar to what is known as the Pasteur effect, which is the inhibition of fermentation in the presence of oxygen. Although this effect has been reported to be irrelevant for yeast in laboratory growth conditions (Lagunas 1986), it might occur in the fermentation of musts with low levels of nitrogen, and, accordingly, cause sluggish or stuck fermentations. Curiously, they also found a slight increase in the expression level of genes encoding ribosomal proteins and those involved in ribosome biogenesis after nitrogen has been depleted. A more comprehensive and realistic study of transcriptional response in *S. cerevisiae* to different nitrogen concentrations during alcoholic fermentation was performed by Mendes-Ferreira et al. (2007a, b). The authors compared 11 samples from different time points of a series of control vinifications, nitrogen-limiting fermentations and

fermentations to which DAP was added. They found alterations in approximately 70% of the yeast transcriptome in at least one of the fermentation stages and also showed a clear association between these changes and nitrogen concentrations. In agreement with earlier findings published by Backhus et al. (2001), their results indicated that early response to nitrogen limitation involved the induction of genes associated with respiratory metabolism and a subsequent general decrease in the levels of genes associated with catabolism. More recently, our group (Barbosa et al. 2015a) performed a genome-wide study of the transcriptional response of three wine yeast strains with distinctive nitrogen requirements and fermentative profiles, under two contrasting nitrogen levels. This comparative transcriptomic analysis revealed common and strain-specific responses to nitrogen availability. In particular, domains of yeast metabolism related to nitrogen and sulphur (including amino acid metabolism and catabolism of nitrogen compounds) were heavily impacted at early fermentation stages by both differences in composition of fermentation medium and most importantly by the yeast strain. These differences were, at some extent, attenuate in latter fermentation stages, suggesting that the yeast strains may in fact alter the expression of a similar set of genes to cope with the stresses imposed during fermentation, but their adaptation to both nitrogen environments takes place in a different manner, in line with the specific fermentative and metabolic behaviour of each strain (Barbosa et al. 2014). Similar conclusions have been reached by Treu et al. (2014b) while using RNA-seq to analyse the expression profile of four vineyard strains of *S. cerevisiae* having different fermentation performances and compared with those obtained for the industrial wine strain EC1118 and for the laboratory strain S288c. Accordingly, the analysis of the genes involved in fermentation stress response revealed a lower expression in strains characterised by low fermentation efficiency, particularly in the first fermentation phase evidencing the high variability of transcriptional profiles among different wine yeast strains and their connection with complex phenotypic traits, such as the fermentation efficiency and the nitrogen sources utilisation. In search for the genetic basis of such variability on yeast nitrogen requirement, Brice et al. (2014) using a QTL approach identified four polymorphic genes (*GCN1*, *MDS3*, *ARG81* and *BIO3*) associated with differences in fermentative activity in a medium in which nitrogen was limiting.

The most common strategy used by winemakers to avoid premature fermentation arrest and to avoid the risk of sulphur off-flavours production is the addition of nitrogen compounds, such as DAP. A study by Marks et al. (2003) found that the addition of DAP affected the expression of 350 genes. The 185 genes that were found to be downregulated encoded small-molecule transporters and nitrogen catabolic enzymes, including enzymes involved in the synthesis of urea, which is a precursor of ethyl carbamate. The other 165 genes affected were all upregulated. These included genes involved in the biosynthesis of amino acids, purines and ribosomal proteins (suggesting a more active metabolism despite an absence of cell proliferation) and assimilation of inorganic sulphate (necessary for the elimination of hydrogen sulphide). The results of the study by Marks et al. (2003) provided a possible explanation for why the addition of DAP reduces the production of ethyl

carbamate and hydrogen sulphide, two undesirable components in wines. Similar results were later obtained by Mendes-Ferreira et al. (2007a) who found that the main transcriptional effect of adding DAP to a nitrogen depleted medium was an upregulation in genes involved in glycolysis, thiamine metabolism and energy pathways. A study performed by Jiménez-Martí and del Olmo (2008) showed that the effect of nitrogen refeeding depended on the source of nitrogen used, as they detected differences in gene expression reprogramming depending on whether ammonia or amino acids were added. The addition of ammonia resulted in higher levels of genes involved in amino acid biosynthesis, whereas that of amino acids directly prepared cells for protein biosynthesis.

Genome-wide expression analysis has emerged as a powerful tool for identification of genes that behave in a similar trend in a particular condition. The identification of genes that specifically respond to a specific stimulus (molecular biomarkers or signature genes) could be important for refining or complementing the existing diagnostic procedures. The genome-wide analysis performed on the yeast strain PYCC4072, growing in nitrogen-replete and nitrogen-depleted conditions, led to the identification of a set of 36 genes as promising candidates for prediction of problematic fermentations due to low nitrogen (Mendes-Ferreira et al. 2007b). A list of 46 potential nitrogen-dependent genes under winemaking conditions were also uncovered by Barbosa et al. (2015a), with a special emphasis to *CAR1*, *ATF1*, *DUR1,2 and PUT1*, which displayed the higher upregulation and to the ORF with unknown function, *YML057C-A,* which was the most downregulated gene under limitation of nitrogen. The fact that in that study we have used three contrasting yeast strains in gene expression analysis prompts this biomarkers identification more reliable, accurate and reproducible. Ethanol stress is another major pressure that *S. cerevisiae* has to deal with during vinification. Ethanol tolerance is still not fully understood, but it is known to partly depend on alterations in the plasma membrane (Alexandre et al. 1994). Global gene expression studies have provided a better understanding of the molecular basis underlying yeast response and resistance to ethanol stress (Alexandre et al. 2001; Fujita et al. 2004; Hirasawa et al. 2007; Lewis et al. 2010) under laboratory conditions. Using microarray analysis to identify target genes and analyse ethanol sensitivity in knockout strains, Hirasawa et al. (2007) found that the biosynthesis of tryptophan can confer ethanol tolerance. In our laboratory, we have studied the yeast response to sudden ethanol addition. A laboratory strain stops growing when ethanol is added to 7.5%. Growth is reassumed after several hours. At that time, a specific increase in the level of mRNAs of genes encoding cell wall components, hexose transporters and enzymes for carbohydrate metabolism is seen (Antúnez and Pérez-Ortín, unpublished data). Despite the data available from the global analysis of ethanol response in yeast laboratory strains, there are no published papers in which this topic is considered in wine yeasts. Usually wine strains are much more ethanol resistant than laboratory ones. Particularly, the flor yeasts involved in the biological ageing of sherry wines should cope with ethanol concentrations above 15% (Aranda et al. 2002). In this line, Lewis et al. (2010) have shown extensive natural variation in the response to acute ethanol stress among yeast strains while studying the

transcriptional response of a lab strain S288c, vineyard isolate M22 and oak-soil strain YPS163 exposed to ethanol. While targets of the "general stress" transcription factor Msn2p, the oxidative stress factor Yap1p and the proteasome regulator Rpn4p were all affected coordinately across the strains, thousands of gene expression differences in response to ethanol have been found.

The global transcriptomic studies conducted with wine yeast strains during alcoholic fermentation (Backhus et al. 2001; Rossignol et al. 2003; Marks et al. 2008), although not specifically devoted to ethanol stress, provided some insights into the topic, particularly the stress caused by progressive ethanol production. For instance, Backhus et al. (2001) and Rossignol et al. (2003) found changes in the levels of the expression of genes involved in biosynthesis of fatty acids, phospholipids and ergosterol during vinification. Genes encoding enzymes involved in the synthesis of fatty acids, phospholipids and ergosterol are highly expressed (Backhus et al. 2001) in *S. cerevisiae* yeasts but decrease towards the stationary phase. The results of the fermentation monitoring study conducted by Rossignol et al. (2003) indicated that anaerobic stress is a characteristic of wine fermentation and that the absence of ergosterol synthesis, one of the main growth-limiting factors for yeasts in musts with low oxygen and high ethanol levels, is due to the continuous decrease in the expression levels of genes involved in ergosterol biosynthesis. In agreement, while studying gene expression changes in *S. cerevisiae* at the late stage of very high gravity (VHG) fermentation, Zhang et al. (2012) found *ERG7*, *ERG20*, *ERG1 and ERG8* being highly repressed. On the other hand, only 5% of short-term ethanol stress genes (Alexandre et al. 2001) were found among the FSR genes which are considered to mediate long-term adaptation to the increasing ethanol levels, suggesting that ethanol activates unidentified ethanol signal transduction pathway which regulates FSR response (Marks et al. 2008).

Fermentation temperature is also an important factor in winemaking. For instance, white and rose wines fermentations are usually conducted at lower temperatures (12–17 °C) than red wines (22–28 °C) in order to reduce the volatility of aromatic compounds improving the sensory quality of wine. Global gene response of the wine strain QA23 has been analysed in fermentations carried out at 13 and 25 °C (Beltrán et al. 2006). The authors observed that the lower temperature induced cold stress response genes at the initial stage of fermentation and increased levels of genes involved in cell cycle, growth control and maintenance in the middle and late stages of fermentation. Furthermore, several genes involved in mitochondrial short-chain fatty acid synthesis were found to be overexpressed at 13 °C compared to 25 °C. These transcriptional changes were correlated with higher cell viability, improved ethanol tolerance and increased production of short-chain fatty acids and associated esters. Similar conclusions were obtained in a more recent study conducted in similar conditions (12.5 and 25 °C) but using different strains and grape juice varieties (Deed et al. 2015). Additionally, this comparative study found, along with changes in the cell wall and stress response, genes linked to three key nutrients to be strongly influenced by low temperature fermentation: nitrogen, sulphur and iron/copper. In agreement, García-Ríos et al. (2014) using an integrative approach, combining genomics, proteomics

and transcriptomics, reported that the upregulation of genes of the sulphur assimilation pathway and glutathione biosynthesis has a crucial role in the yeasts adaptation at low temperature. Only 137 genes out of 787 (17%) identified by Deed et al. (2015) were in common with those expressed in the cold wine fermentation performed by Beltran et al. (2006) confirming the strain specificity of the cold stress response between *S. cerevisiae* strains (García-Ríos et al. 2014), as seen for other stress conditions (Treu et al. 2014b; Barbosa et al. 2015a).

Under industrial conditions wine is obtained by a microbial consortia possessing various metabolic activities. Even in inoculated fermentations, there is a substantial yeast and bacterial biodiversity observed on grapes and musts that can persist during the fermentation process. The understanding of the microbial interactions that may occur during winemaking and how they affect the composition and quality of wines obtained are far from being known. A limited number of recent studies, involving lactic acid bacteria, have indicated that genome-wide transcriptome analysis can provide a better insight into the nature and molecular basis of microbial interactions (bacteria-bacteria) in mixed cultures of industrial organisms (Sieuwerts et al. 2010; Maligoy et al. 2008; Hervé-Jimenez et al. 2008). More recently, *S. cerevisiae* genome-wide transcriptional profiling in mixed culture has also been conducted to assess yeast-bacteria interaction, using *Lactobacillus delbrueckii* subsp. *bulgaricus*, which co-occur in kefir fermentations (Mendes et al. 2013) and cocultivated with the wine malolactic bacterium *Oenococcus oeni* (Rossouw et al. 2012). In this last work, the transcriptome of a commercial yeast strain in single and in co-inoculated fermentations with *O. oeni* was evaluated. This analysis showed that a significant number of genes were differentially expressed in *S. cerevisiae* under these two conditions. While genes involved in stress response, sulphur metabolic pathway, lipid biosynthesis and nutrient uptake were overexpressed in the co-inoculated fermentations, genes encoding for sterol biosynthesis and metabolism of phosphorus, proline and glycine were downregulated.

A first attempt to study yeast-yeast interaction in mixed culture wine fermentation using transcriptome-based approach has been carried out by our group using DNA arrays (Barbosa et al. 2015b). In that study, transcriptome profiling on mixed-culture fermentations was performed at three different time points, in mid-exponential growth phase (24h), in early stationary phase (48h), and in late stationary growth phase (96h), and compared to single *S. cerevisiae*-culture fermentations. We have detected a large set of genes that were differentially expressed that were associated to the presence of *Hanseniaspora guilliermondii* during fermentation confirming the importance of such a global approach for the study of yeast-yeast interactions during fermentation. The observed changes in the expression level of genes associated with vitamins biosynthesis and amino acid uptake and biosynthesis confirmed the nutritional interactions revealed or at least suggested by growth-based methodologies including competition for vitamins (Bataillon et al. 1996; Medina et al. 2012) and for nitrogen available (Fleet and Heard 1993; Medina et al. 2012) on grape must. The transcriptomic analysis carried out in our study were only performed in *S. cerevisiae* since microarray analysis is limited to organisms with sequenced genomes. The global response to mixed-culture growth in

H. guilliermondii remains to be established. Furthermore, it remains to be understood if these adjustments are specific to this strain or more generally linked to the presence of any competing yeast. In recent years, however, the development of high-throughput sequencing techniques such as RNA-seq has been successfully used to characterise the transcriptome of other wine non-*Saccharomyces* strains. Accordingly, RNA-seq approach was recently used to identify genes differently expressed after exposure to SO_2 in *Brettanomyces bruxellensis*, considered to be the main spoilage yeast in red wines (Capozzi et al. 2016). Global transcriptional analysis revealed that entrance and recovery of viable but non-culturable SO_2-induced state are associated with yeast sulphite toxicity and the consequent oxidative stress response.

The application of DNA array technology to wine strains has extended the landscape of expression studies. The studies on wine yeast using DNA array analysis have used various approaches in relation to growth conditions as well as the experimental design of the assay. Thus, whereas some experiments simulate the vinification conditions, by growing the wine yeast strain on a chemically defined synthetic must in an attempt to increase reproducibility and study particular stresses (Backhus et al. 2001; Rossignol et al. 2003; Zuzuárregui et al. 2006; Mendes-Ferreira et al. 2007a; Jiménez-Martí and del Olmo 2008; Rossouw et al. 2008; Jiménez-Martí et al. 2011; Carreto et al. 2011; Brice et al. 2014; García-Ríos et al. 2014; Orellana et al. 2014; Barbosa et al. 2015a), others used natural grape juices in their studies which are far more complex and variable (Marks et al. 2003, 2008; Erasmus et al. 2003; Beltrán et al. 2006; Deed et al. 2015; Barbosa et al. 2015b). In addition, we find a great heterogeneity among these studies in terms of the volume of fermentation used in the experiments. Thus, the studies conducted by F. Bauer group on the applicability of those experiments performed in synthetic medium to study conditions experienced in industrial fermentations and on the prospective extrapolation of the results obtained in small-scale laboratory fermentations to large-scale industrial environments were very pertinent (Rossouw and Bauer 2009; Rossouw et al. 2012). To answer the first topic, the transcriptomes of two phenotypically diverging commercial strains in two simulated wine must or real grape must (Colombard) at three stages of wine fermentation were analysed (Rossouw and Bauer 2009). The authors showed that gene regulation throughout fermentation, either on synthetic or real grape musts, did not differ significantly concluding that synthetic musts are indeed a valid model of real grape must fermentations. Later, Rossouw et al. (2012) used the same comparative transcriptomic approach assessing the response of an industrial wine yeast strain in parallel fermentations of a natural grape juice in small-scale laboratory (80 mL) and large-scale industrial conditions (110 L). Again, the authors found that yeast gene expression profiles in both conditions followed the same trend, concluding that small-scale fermentations in synthetic must are valid experimental models for investigation of microbial biology in real commercial fermentation processes. Most importantly, both studies validate the usefulness of all transcriptomic studies performed in *S. cerevisiae* towards the understanding of industrially relevant

aspects of winemaking that could be used by winemakers to improve the fermentation process and the quality of wines obtained.

24.8 Structural Genomics Studies in Wine Yeast Strains

Since the developing of NGS technologies, the whole genome sequencing of wine yeast strains has been applied to an increasing number of natural and commercial isolates (reviewed in Borneman and Pretorius 2015). These kinds of analyses have allowed to discover the similarities and differences of wine yeast genomes and to develop hypothesis on the origin and evolution of those strains (revised in Pérez-Ortín and García Martínez 2011). Several studies have recently investigated the diversity of *S. cerevisiae* species by sequencing the genomes of hundreds of different strains, providing a first glimpse of the complex evolution of this species (Almeida et al. 2015; Liti et al. 2009; Schacherer et al. 2009; Gallone et al. 2016). Indeed, from the comparative genomic analysis of up to 196 wine strains of *S. cerevisiae*, Borneman et al. (2016) concluded that all appear to represent a highly inbred population containing relatively little genetic variation compared to the global pool of *S. cerevisiae* diversity. This conclusion has been reinforced by another study that showed that wine yeast group is phenotypically distinct from wild strains and stems from a limited set of ancestral strains that have been adapting to winery environments. In spite of this, wine yeasts group in just one clade much more homogenous than beer yeasts which have stronger hallmarks of domestication (Gallone et al. 2016).

In spite of the recent application of NGS to wine yeast, DNA arrays are still used because of their simplicity and relative low price, for a variety of genomic research applications: systematic characterisation of genes discovered by sequencing projects, identification of new transcripts, detection of aneuploidies or partial chromosome deletions, chromosomal rearrangements and identification of interesting QTLs, among others.

Allelic variations can be detected in any strain by analysing the patterns obtained by hybridising genotyping arrays with total genomic DNA (Winzeler et al. 1998). Array hybridisation is strictly dependent on the precise sequence of the target; therefore, changes in the genes may produce differences in signal intensity or even no signal at all. Point variations (SNPs) are, however, difficult to detect with long probe arrays. The use of oligonucleotide arrays is the only way to analyse allelic differences in detail. For instance, Primig et al. (2000) have shown that SK1 strain has more genetic variation as polymorphisms and deletions (34%) when comparing the S288c standard background with the W303 background (5%).

With full-length ORF PCR-product arrays, it is possible to make array aCGH and monitor chromosome aneuploidy or chromosomal segment duplications (Hughes et al. 2000). aCGH is a simple but powerful technique that allows gross-comparisons of genomes, using a reference strain. It allows to test differences in gene copy number, ploidy and gross-chromosomal rearrangements that are, in part,

responsible for different developmental, morphological and physiological characteristics of the industrial yeast strains, as already indicated. The first aCGH study was performed by Hauser et al. (2001) who found important differences between laboratory and wine strains when both expression and genomic hybridisation values for transposon (Ty) ORFs were analysed. The low expression of these ORF in the wine yeast strain seems to be due to the fact that the laboratory strain (S288c genetic background) has more copies of transposable elements (Ty1–Ty4) than the wine yeast strain. This factor, also shared by other industrial yeast strains such as brewer's yeast strains (Codón et al. 1998), agrees with the suggestion that a negative selection for transposon accumulation might exist in the wild for the Ty elements. Ty elements recently expanded in laboratory strains because they lack of the strongly competitive wine or beer fermentation environment (Jordan and McDonald 1999; Codón et al. 1998). Another difference found in that study was the different number of subtelomeric genes in the T73 wine strain. In fact, it has been found that subtelomeric regions are the most variable region in the *S. cerevisiae* (and specially in wine yeast strains) genome including not only different copy number of subtelomeric gene families but also wine strain-specific loci (reviewed in Borneman and Pretorius 2015). For instance, *FSY1* gene, encoding a H+/fructose symporter, was first identified as a member of the large multigenic strain-specific locus present in the EC1118 group of *S. cerevisiae* wine strains (Novo et al. 2009). The presence of this gene is thought to support active transport of fructose into the cell, a phenotypic trait that is lacking (perhaps lost during laboratory evolution) from most *S. cerevisiae* strains and is predictable to provide a selective advantage during wine fermentation.

Using aCGH technique, Infante et al. (2003) found that two prominent variants of *S. cerevisiae flor* yeast strains differ from one another in the DNA copy number of 116 genomic regions that comprise 38% of the open reading frames (ORFs). They also found that the majority of them correspond to a widespread amplification of genomic fragments. By analysing the different situations found, the authors suggest that the amplifications have been produced by gross chromosomal rearrangements (GCRs) mediated by identified hotspots (transposon LTRs, tRNAs, subtelomeric repeated sequences, etc.), helped by bursts of double-strand breaks (DSBs) mainly produced by both acetaldehyde and ethanol. One of the unique properties of *flor* yeast is the production and release of high amounts of acetaldehyde as a consequence of ethanol assimilation. Since some of the genes among those involved in these copy number variations have functions related to the specific phenotypes that are characteristic of *flor* yeast strains, one possible suggestion is that this mechanism is responsible for the adaptive evolution of these yeasts. Actually, two changes in *FLO11* (a large deletion in the promoter and another one in the coding region) differentiate *flor* yeast strains from other non-floating strains (Fidalgo et al. 2006). Recently, the aCGH profiles of six flor strains from Spain, Hungary, France and Italy were compared (Legras et al. 2014). This analysis revealed differences in the subtelomeric regions but disagree with the previous study (Infante et al. 2003) arguing that copy number variations in

subtelomeric regions are not enough to explain the flor yeast adaptation to its environment.

The aCGH technique also allowed Dunn et al. (2005) to analyse four commonly used commercial wine yeast strains. They assayed three independent isolates from each strain and compared them with laboratory strain S288c. All four wine strains displayed common differences with regard to laboratory strain S288c. Some may be specific to commercial wine yeasts. Slight differences inter- or intra-strain were observed, indicating that they are closely related and quite genetically stable. Among the variations, there are genes that code for transporter proteins (similarly to the case of *FSY1* gene described before). Moreover, genes exist that are involved in drug resistance (or detoxification). The authors not only propose a "commercial wine strain signature", comprising the genes whose copy number is altered in all the wine yeast isolates examined in relation to the S288c strain, but also suggest that the differences in the fermentation and organoleptic properties of the different strains may arise from a small number of genetic changes.

Finally, aCGH has been also used by the A. Querol group to analyse ploidy and genome identity in *S. cerevisiae* × *S. kudriavzevii* hybrids from beer and wine (Peris et al. 2012). They found that all hybrids share a common set of depleted *S. cerevisiae* genes, which also are depleted or absent in the wine strains previously studied, and the presence of a common set of *S. kudriavzevii* genes, related with their capability to grow at low temperatures. They also found chromosomal rearrangement events in the hybrid genomes, which differentiate two groups of wine strain originated by different rare-mating events.

24.9 Conclusions

DNA array technology has been widely used on wine yeast research. DNA arrays are currently much more feasible and straightforward and are providing more clues towards an understanding of the biotechnology process. They have been particularly important in the disclosure of why some yeast strains are able to perform winemaking whereas others are not, why some of them are more resistant to particular stresses, and how the evolution has modelled the genome of this organism. To date, transcriptomic studies undertaken in the vinification context have only been carried out with *S. cerevisiae* strains. NGS techniques such as RNA-seq have recently started, allowing the characterisation of the transcriptome of other wine non-*Saccharomyces* strains. This technology will provide important genomic and transcriptomic data on these yeasts that is expected to revolutionise the manner in which global regulatory responses and development of the yeast-yeast interactions throughout alcoholic fermentation will be analysed. This knowledge will be of great importance in the improvement of current winemaking technologies and the accompanying yeast strains.

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Chapter 25
Application of Yeast and Bacteria as Starter Cultures

Sibylle Krieger-Weber

25.1 Introduction

Although wine yeasts have been known for a long time, the production of wine has remained more of an art than a science until 40 years ago. The production and use of active dry yeasts (ADY) began in the United States in the mid-1960s and expanded worldwide thereafter (Degré 1993). In inoculated fermentations, selected strains of *Saccharomyces cerevisiae* are usually added to achieve a population of about 10^5–10^6 cells ml^{-1} in the must to ensure a quicker start for fermentation, to outcompete and dominate indigenous yeast strains, and to provide a wine with distinctive characteristics.

The history of controlled malolactic fermentation (MLF) is even shorter. Despite the early discovery of Müller-Thurgau in 1891 of lactic acid bacteria (LAB) contributing to the acid reduction in wine, by degrading malic acid to lactic acid and CO_2, commercial starter cultures were only introduced to the markets in the beginning of the 1980s. Most commonly *Oenococcus oeni* (ex *Leuconostoc oenos*) starter cultures are used, but there are also some preparations with lactobacilli reported to give good results (Prahl 1989). Malolactic (ML) starter cultures for easy direct inoculation were only made available in the early 1990s.

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25.2 Application of Yeast Starter Cultures

In spontaneous alcoholic fermentations, there is an early and rapid succession of yeast species such as *Hanseniaspora*, *Kloeckera*, *Candida stellata*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, or *Pichia*, which commonly grow in must (Henschke 1997), but subsequently die, while generally *S. cerevisiae* dominate and lead and complete alcoholic fermentation (Fleet and Heard 1993). The critical point of a spontaneous alcoholic fermentation is around 4% vol. alcohol (Dittrich and Grossmann 2005), when the non-*Saccharomyces* yeast die off and strains of *S. cerevisiae* become dominant. However, dominance of *S. cerevisiae* does not guarantee a successful alcoholic fermentation; it also depends on genetic disposition of the dominant strain. There has been much discussion over the years concerning the relative merits of spontaneous alcoholic fermentation versus induced alcoholic fermentations. A definitive resolution of this issue is unlikely, as it partially depends on stylistic preferences, grape variety, grape juice composition, and vintage.

25.2.1 Selection of the Yeast Strains for Winemaking

Today worldwide more than 200 different yeast strains are commercially available. These yeast strain were selected or rejected for specific properties (Table 25.1), which can be divided in two groups, desirable and undesirable features (Degré 1993), and also technological and qualitative properties as described by Dittrich and Grossmann (2005).

25.2.2 Wine Yeast Characterization

Oxygen and Nitrogen Requirements As already reported in Chaps. 3 and 8, yeast can generally synthesize all amino acids and nitrogenous bases required for their growth from ammonium ions, although yeast growth is accelerated when ready-made building blocks, amino acids, are available in the growth medium. Nitrogen content of must can often be a limiting factor (Amerine et al. 1980); a relationship had been found between the initial concentrations and the maximum fermentation rate (Bely et al. 1991). A value less than approximately 150 mg l^{-1} assimilable nitrogen (YAN) in the must is associated with greater chance of fermentation problems (Henschke and Jiranek 1993). Additions of nitrogen during the stationary phase can be effective, but some authors have demonstrated this effect being strain specific (Jiranek et al. 1991). Julien et al. (2000) proposed a method to quantify nitrogen and oxygen yeast requirements and to study these requirements depending on the yeast strain. Nitrogen requirements were determined during the stationary

Table 25.1 Selection criteria for yeast strains for commercial use

Desirable	Undesirable
Qualitative properties	*Qualitative properties*
Production of positive fruit aromas and esters	Production of sulfur dioxide
Production of β-glucosidase	Production of hydrogen sulfide
Production of glycerol	Production of S-deriving compounds
Production of manno-proteins	Production of volatile acidity and ethyl acetate
	Production of SO_2-binding compounds (acetaldehyde, pyruvate, etc.)
For special applications	Formation of ethyl carbamate precursors
Degradation of malic acid	Production of polyphenol oxidase
Formation of lactic acid	Production of biogenic amines
Formation of isoamyl acetate	
Fast autolysis	
Technological properties	*Technological properties*
Complete fermentation of sugar	Foaming properties
High tolerance to alcohol	Biofilm formation
Resistance to sulfur dioxide	Activity during fermentation
Minimal lag phase on rehydration	
Fermentation at low temperatures	
Tolerance to high temperatures	
Fermentation under pressure	
Activity during fermentation	
Killer phenomena	
For special applications	
Agglomerization properties	
Sedimentation properties	

phase of yeast fermentation. To quantify the effectiveness of nitrogen addition during this phase, constant rate fermentations were performed. Very important differences were observed: some strains needed twice the nitrogen compared to other strains to maintain the same fermentation rate. Based on these results, selected yeast strains have been classified as low, medium, or high nitrogen demanding. Oxygen is another important factor for yeast metabolism during winemaking since it is required for the synthesis of sterols and fatty acids. Sablayrolles et al. (1996) demonstrated the advantage of combined additions of oxygen and nitrogen to prevent sluggish or stuck alcoholic fermentations. Again different selected yeast strains vary in their oxygen requirement (Julien et al. 2001).

Temperature and Alcohol Tolerance Temperature strongly influences yeast growth. *Saccharomyces cerevisiae* can grow over a range of 0–45 °C, and the optimum temperature for alcoholic fermentation ranges between 20 and 30 °C (Henick-Kling 1988). Again it is important to look at selected yeasts for their temperature tolerances, which is normally done in a minimal synthetic medium at

10, 12, 15, 20, and 30 °C, to select the best yeast strain for specific vinification conditions.

Alcohol tolerance is tested in the same medium. Most selected yeast strains will tolerate up to 14% vol., but for fermentation of high maturity red grape juices, the use of yeast with higher alcohol tolerances, 16% vol. and above, is highly recommended.

25.2.3 Choice of the Active Dry Yeast Starter Cultures

In the 1970s and 1980s, when the first active dry yeast starter cultures were used for winemaking, yeast had been selected mainly for their technological advantages; nowadays their sensory properties and contribution to overall wine quality are just as important. Thus, the more recently selected strains have more than a simple role of fermenting sugar into ethanol. Although there is a high probability that inoculated *S. cerevisiae* will dominate fermentation (Schütz and Gafner 1993), seeding will not necessarily guarantee 100% dominance of the strain or its exclusive contribution to fermentation. Significant factors that affect this outcome will be the population of the indigenous yeasts already in the juice, choice of yeast strain, and its adaptation to specific wine environment. It is crucial to choose a suitable yeast strain, which can grow and express its metabolic activity under given conditions, e.g., a yeast strain with good tolerance to low temperatures for cool white wine fermentations, or a yeast strain, which can tolerate higher temperatures in high pH, high alcohol red wine vinifications. Since it is not always easy to select the right yeast strain for specific wine conditions and sensory contribution, Lallemand has put a "yeast chart" (Table 25.2) to help winemakers conquer the challenge of choosing the right yeast for every fermentation.

The plurality and variation of selected active dry yeast preparations available for winemaking can be even more confusing because producers provide different information on their yeast strains. To help the winemaker with the best choice, the Research Station of Geisenheim has developed a unique data sheet to record most important yeast strain characteristics of selected yeast strains available in the German market. This data was collected in a database and can be accessed in electronic form on the Web page of the research station or under www.hefefinder.de. The system proposes the most suitable yeast strain for a particular wine environment and wine style based on details given in a questionnaire by any person and also proposes a ranking within several yeast strains.

25.2.4 Utilization of Active Dry Yeast Starter Cultures

Beside the traditional method of inoculating freshly prepared juice with an amount of actively fermenting juice, two types of yeast starter cultures are used: liquid

Table 25.2 Quick yeast chart

Criteria for selecting a yeast strain	Rating
Suited for white wine vinification	1–4
Suited for rosé wine vinification	1–4
Suited for red wine vinification	1–4
Suited for restart stuck fermentations	1–4
Sensory effect	Neutral–esters–EVC[a]
Temperature range (°C)	Range does not indicate "optimum temperature range"
Fermentation speed	Slow–moderate–fast
Competitive factor	Sensitive–neutral-active
Alcohol tolerance	Max. alcohol levels tolerated
Relative nitrogen needs[b]	Low–medium–high
H_2S production (60 ppm N)	Low–medium–high
H_2S production (170 ppm N)	Low–medium–high

Highest rating (compatibility) = 4, lowest rating = 1
[a]*EVC* enhances varietal character
[b]"Relative nitrogen requirement" refers to how much nitrogen one strain requires relative to the other strains on the chart under nitrogen-limiting conditions

starter cultures and active dry yeast preparations. Most yeast starter cultures are pure and consist of only one strain of *S. cerevisiae*. Liquid yeast preparations have a limited market because of their short shelf life. Liquid starter cultures are prepared by the winery or by a commercial supplier (e.g., local wine laboratories or institutes). The main difference between these cultures and active dry yeast cultures is that they are not subjected to drying, and thus have a high population of viable cells, but only for a short time frame. Main applications are for specific juices like fermentation of dry berry selections or ice wines and even in the preparation of sparkling wines.

Active dry yeast starter cultures are grown over different propagation steps with adequate supply of oxygen and nutrients to produce yeast that contain optimal amounts of protein, ergosterol, unsaturated fatty acids, and reserve materials (Monk 1986). The yeast is then dried to conserve it during transport and storage. In addition to the strain, the trehalose content in the cell is one of the most important factors that affects the resistance of yeast to drying and subsequent rehydration. Therefore, there is a strong incentive for yeast producers to stimulate formation of trehalose during production in order to increase resistance of yeast cells to the stresses of dehydration and rehydration (Degré 1993).

Active Dry Yeast Rehydration Commercial preparations of dry yeast normally contain <8% residual moisture, in most cases even less (6%). Thus, active dry yeast must be rehydrated for revitalization. Rehydration of active dry yeast is very critical, because if it is not done properly, it can cause leakage of large amounts of cellular components and subsequent loss of viability and vitality (Henick-Kling 1988). Although yeast rehydration is a straightforward operation and several scientific and technical papers have been published on correct techniques for

obtaining healthy membranes and subsequent optimum technological performance, manufacturer's instructions vary.

Degré (1993) proposed a general procedure for vinification:

- Sprinkle 500 g of dry yeast into 5 l of warm water (35–40 °C).
- Stir the suspension after 5 min to resuspend all cells.
- Leave yeast cells not more the 30 min in this suspension to avoid use-up of their reserve material.
- Add yeast to 20–25 hl must to be fermented, which would correspond to a dosage 25–30 g hl^{-1} (ca. 2–4 × 10^6 cfu ml^{-1}).

Some yeast producers recommend manufacturer precise "clean, chlorine-free water and 15–30 min holding time before stirring." Other producers prefer to carry out "the soaking of the yeast" in a mixture of juice and water at 35–40 °C, because with the addition of juice the yeast cells, which will start budding according to theory during rehydration, will have a source of nutrition and can also adapt to the juice/must environment. This procedure may have advantages if the rehydration exceeds the recommended 30 min. Radler et al. (1985) had, in his most complete studies on wine yeast rehydration, obtained maximum values for cell viability and fermentation activity when rehydration temperatures ranged between 38 and 45 °C. Within a time frame of 2 h for rehydration, no change in these activities was observed, but the composition of the rehydration medium had an important influence. It was found that a mixture of grape juice and water, solutions containing sugars, and vitamins or salts did have an impact on the metabolic activity of rehydrated yeast. Best activity was achieved when rehydrating in 1% KCl solution. Rehydration in more than 50% juice is not recommended, because of high osmotic pressure, low pH, and sometimes high SO_2 levels or fungicide levels.

Active Dry Yeast Rehydration Using a Yeast Rehydration Nutrient Studies of Fornairon-Bonnefond et al. (2002) have shown positive impact of specific sterols during the rehydration phase on the structure of the plasma membrane resulting in better fermentation capacity, particularly under difficult wine conditions. Since the membranes are stressed from the drying and rehydration processes, the yeast needs to mobilize lipid reserves for repair as shown by Beker et al. (1984). More recently, Soubeyrand (2005) showed that yeast can also incorporate extracellular lipids, including sterols, which is again interesting as these molecules can play an important role in yeast cell vitality in the final stages of alcoholic fermentation (Luparia et al. 2004). In grape musts sterols are present in the form of phytosterols, but their nature differs from the sterols synthesized by the yeast during growth. Because of the differences in the chemical structure, these phytosterols were not sufficient to guarantee yeast integrity during the whole alcoholic fermentation (Luparia et al. 2004). Soubeyrand (2005) has studied the possibility of incorporating specific yeast sterols during rehydration by addition of specific inactive yeast preparations naturally rich in sterols to the rehydration medium. The influence of rehydrating ADY in the presence of micronutrient and/or sterol and unsaturated fatty acid enriched inactivated yeast suspension on yeast viability (Kontkanen et al. 2004) which was

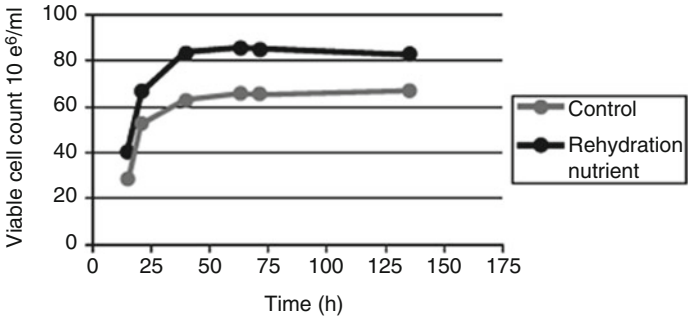

Fig. 25.1 Effect of rehydrating ADY in a micronutrient and sterol-enriched inactivated yeast suspension on the cell viability at the end of alcoholic fermentation (potential alcohol 14% vol., fermentation temperature 28 °C). *Gray line* control (rehydration without addition): 28×10^6 cfu ml^{-1} (42%). *Dark line* rehydration in presence of a specific rehydration nutrient: 42×10^6 cfu ml^{-1} (50%)

remarkable. Higher maximum yeast cell density (Fig. 25.1) and shorter overall fermentation lengths (Fig. 25.2) were observed when using these types of rehydration nutrients especially under high sugar concentrations. The impact obtained on the later performance of the yeast is excellent. The recommended procedure for yeast rehydration using a rehydration nutrient is shown in Table 25.3.

Utilization of Multiple Strain: S. cerevisiae *Starter Cultures* Spontaneous alcoholic fermentations are generally driven by more than one yeast strain. New molecular biological methods allowed detecting different yeast populations in wild fermentation depending on the phase of the fermentation. The succession of different yeast strains could lead to more complexity in the aroma profile of the final wine in both a positive and a negative sense. Besides the recommendation of achieving complexity in a more controlled manner by preparing separate ferments with different selected yeasts and then blending the ferments mixed cultures of *S. cerevisiae* had been developed, in imitation of the variability of a spontaneous alcoholic fermentation. The yeast strains are produced as single cultures, and the final mix is done by blending the dried pure cultures. Still the market for mixed *S. cerevisiae* starter cultures is small also due to the mixed success of these inoculations. Due to the variability of the juice/wine matrix, wine conditions may favor one or the other strain and allow dominance by one strain as otherwise it can induce a negative interaction between strains, which will have an impact on the sensory profile of the final wine.

Inoculation of Grape Must with: Saccharomyces and Non-Saccharomyces Strains In some cases, wine produced with pure yeast monocultures lack flavor complexity that may originate from good indigenous fermentations. But wild fermentations require more vigilance and are a gamble, as explained earlier in this chapter, leading to off-flavors or stuck fermentations with high amounts of residual sugar because of the dominance mainly of undesirable non-*Saccharomyces*

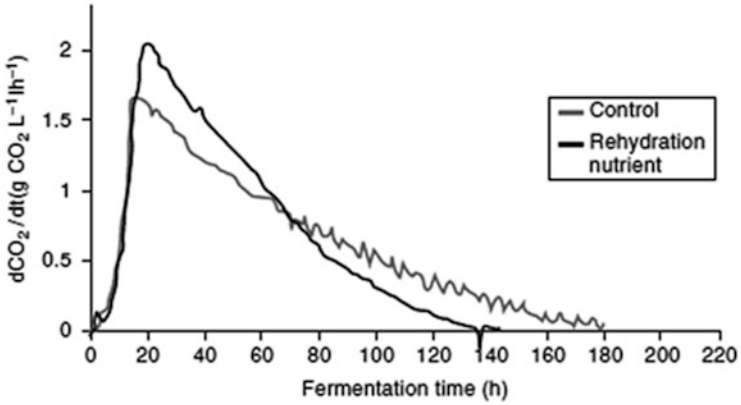

Fig. 25.2 Variation of CO_2 production by *Saccharomyces cerevisiae* strain EC1118 in a Chasan must (240 g l^{-1} sugar and 266 mg l^{-1} FAN) rehydrating ADY in a micronutrient and sterol-enriched inactivated yeast suspension compared a standard rehydration in water only. *Gray line* control (rehydration without addition); *dark line* rehydration in presence of a specific rehydration nutrient. Fermentations carried out in 1.1 l fermenters under isothermal conditions (28 °C) with gentle stirring. The CO_2 production rate was calculated automatically from the weight loss of the fermenter expressed as a function of fermentation time

Table 25.3 Instructions for optimal yeast rehydration using a rehydration nutrient for the inoculation of 100 hl must

Step	Action
1	Suspend 3 kg (30 g hl^{-1}) of yeast rehydration nutrient in 20 times of its weight in clean water (43 °C)
2	Once the temperature of yeast rehydration nutrient solution has dropped to 40 °C, add 2.5 kg active dried yeast (25 g hl^{-1}). Stir gently to break up any clumps. Let suspension stand for 15–30 min, and then stir gentle again
3	Over a period of 5 min, slowly combine an equal amount of must to be fermented with the yeast suspension. This will help the yeast to adapt to the cooler temperatures in the must and will avoid cold shock caused by a rapid temperature drop exceeding 10 °C. This attemperation step may need repeating for very low temperature must. Each attemperation step should last about 5 min
4	Add the yeast suspension to the bottom of the fermentation tanks as you begin filling the vessels with must

strains. Only recently wine research has discovered these "exotic" yeast strains, and more knowledge is available on their real impact on the sensory profiles of wines (Ciani 1997). Some of these strains like *Pichia fermentans*, *C. stellata*, or *T. delbrueckii* have been studied for their interesting organoleptic contributions (Clemente-Jimenez et al. 2005; Ciani and Ferraro 1996; Moreno et al. 1991). Although some of these strains could improve the wine bouquet, most of them are not able to complete alcoholic fermentation. For this reason incorporation of a *S. cerevisiae* strain with non-*Saccharomyces* strains was studied to overcome these shortcomings. The first blended commercial *S. cerevisiae*/non-*Saccharomyces*

starter cultures was introduced at the beginning of the twenty-first century with mixed success due to unpredictable interactions between the yeast populations induced by the wine matrix favoring the dominance of one strain over the other. A recent study by Languet et al. (2006) showed good success by reproducing the natural succession of yeast population with sequential inoculation of a non-*Saccharomyces* strain first followed by a good fermenting *S. cerevisiae* strain during a later stage of alcoholic fermentation. These sequential inoculations have not only shown better results in terms of intensity but also in terms of sensory complexity.

Utilization of Yeast Starter Cultures in the Production of Sparkling Wine For the production of sparkling wine or champagne-style wines, both liquid yeast starter cultures and dried yeast starter cultures are used for secondary in-bottle fermentation. The liquid cultures have to be built up under sterile conditions not only to increase the volume of the inoculums but also to adapt to difficult conditions in the sparkling base wines. It is also obligatory to acclimatize active dried yeast cultures prior to inoculation for the secondary fermentation, since the direct addition of rehydrated yeast suspension to a medium containing higher levels of alcohol can damage the yeast cell. Again variations of protocols exist; one widely used is described below:

- Rehydration of the ADY according to the yeast producer's instructions, preferably in the presence of a rehydrating nutrient.
- Addition of the yeast suspension to a part of the sparkling base wine (3–10% of the total volume) supplemented with grape juice (up to 50 g l^{-1}) or sugar (between 50 and 100 g l^{-1}) and ammonium phosphate (0.5–2 g l^{-1}). A variation of the traditional method of starter culture preparation is the use of a mixture of equal parts of base wine, water, and tirage liqueur (Wilkinson 1986).
- Acclimatization for 12–20 h (the yeast starts to produce alcohol) at 20–25 °C. The suspension must be occasionally stirred for oxygenation to stimulate yeast growth. If conditions are very difficult or the base wine temperatures are very low, acclimatization can be also done at constant lower temperatures.
- Again, it is crucial to avoid temperature differences of more than 5 °C when transferring the acclimatized yeast solution to the final wine volume.

Utilization of Yeast Starter Cultures to Restart Stuck Fermentations Dr. Paul Monk used to say: "The best solution for a stuck fermentation is prevention." Problems occur because of highly clarified must, low temperature fermentations, and high temperatures especially in the presence of alcohol, lack of nitrogen, micronutrients, sterols, high concentrations of sugar or alcohol, negative interactions with other wine microbes, and spray residues. Various factors can have a negative impact on yeast vitality (Dittrich 1977), and as many circumstances can cause stuck alcoholic fermentations as numerous protocols studied and proposed to cure stuck fermentations (Graf and Bannister 1996; Leske and Henschke 1996; Bisson and Butzke 2000; Fischer 2000). All protocols recommend racking off the

old yeast and using an alcohol-tolerant and vigorous fermenting yeast strain, to restart the stuck fermentation. Most protocols also recommend the addition of SO_2 (30 mg l^{-1}) and/or lysozyme to avoid growth of spoilage bacteria or wild yeast. If potential inhibitory substances are expected to be in the wine, addition of yeast hulls at 25 g hl^{-1} is recommended (Lafon-Lafourcade et al. 1984). After yeast hulls have settled (ca. 48 h), the wine has to be racked or filtered. The preparation of the rescue yeast varies between different yeast producers and research groups. Grossmann has listed in Sect. 7.6 of their wine microbiology book (2005), four different recommendations published in wine literature.

Lallemand recommends:

1. Rehydration of rescue yeast (50 g hl^{-1}) in a rehydration nutrient: Calculation of the appropriate amount of yeast rehydration nutrient at 1.25 times the weight of the yeast to be used. Suspension of the rehydration nutrient in 20 times of its weight of 43 °C clean water, gentle mixing, allowing the solution to cool to 40 °C. The rescue yeast is sprinkled on the suspension and stirred gently to mix and avoid clumping. The suspension stands for 15–30 min.
2. In the meantime, in another container, preparation of starter mixture with 2.5% of volume of stuck wine and 2.5% of volume of water and a complete yeast nutrient (50 g hl^{-1} wine and water mix). Sugar levels are adjusted to 5 Brix (50 g l^{-1}) with juice, concentrate, or sugar. The temperature of the mixture has to be adjusted to 25–30 °C.
3. The rehydrated rescue yeast suspension has to be slowly added to this wine/water/sugar mix. The temperature should be maintained at 15–30 °C. The sugar levels are monitored, and when the sugar level has dropped by half (approximately 2.5 Brix—ca. 25 g l^{-1} sugar), stuck wine is added to the starter in batches of 20% of the total volume of stuck wine (total of five additions to the starter). Temperature should be maintained between 20 and 25 °C. A very critical point is to avoid sugar completion before the addition of the next batch. Only at the last batch of added stuck wine should the sugar be allowed to completely deplete.

More recently Gafner's group (Sütterlin et al. 2004) proposed the use of *Zygosaccharomyces bailii* to rebalance the glucose-fructose ratio back to values above 0.1 because of its fructophilic character. Best results were achieved when the *Z. bailii* strain was inoculated together with a strong fermenting *S. cerevisiae* strain since the *Z. bailii* was losing viability after the correction of the glucose-fructose ratio, and the *Saccharomyces* strain took over and fermented the wine to dryness. The first *Z. bailii* starter culture to restart stuck alcoholic fermentations was introduced to the German market in 2007.

Another innovative approach is the use of immobilized *S. cerevisiae* yeast strains selected for its strong fermenting properties and its high tolerance to alcohol. One typical technique of immobilization is encapsulation which involves coating microorganisms in a rigid alginate matrix (natural polysaccharide extracted from seaweed). The encapsulation allows substrates and metabolites to diffuse easily throughout the gel matrix without releasing yeast cells into must or wine. They have

the advantage of being easily introduced and removed from the media after transformation of sugar into alcohol.

25.3 Application of Bacterial Starter Cultures

For a long time, spontaneous acid reduction observed in wine was related to precipitation of tartaric acid only, though in 1891, Müller-Thurgau had already postulated that acid reduction could be due to bacterial activity. In 1913 Müller-Thurgau and Osterwalder, with their epoch-making investigation into LAB in wine, explained bacterial degradation of malic acid to lactic acid and CO_2 according to the formula:

$$C_4H_6O_5 = C_2H_6O_3 + CO_2$$

They called this phenomenon biological deacidification or MLF, and *Bacterium gracile* was described as the responsible agent. Since these early findings, research on LAB has progressed. The name, *B. gracile*, which was used frequently in the past to recognize the organism which caused the MLF, was revised. Findings by Radler (1963) showed that LAB of grape must and wine belong to the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and more recently to *Oenococcus* (Dicks et al. 1995). Different LAB enter into grape juice and wine from the grape berry surface, stems, leaves, soil, and winery equipment. However, due to the highly selective environment of different juices and wines, only few types of LAB are able to grow in wine (Wibowo et al. 1985). Studies from several countries indicate that *O. oeni* is the predominant species conducting MLF in wine, even though the LAB composition of grape must at the beginning of the alcoholic fermentation is dominated by *Lactobacillus* strains described in more detail in Chap. 1 of this edition by König and Fröhlich.

Historically, MLF was described as a phenomenon that is capricious and not completely understood, but is of great importance to the final product. In the not-too-distant past, winemakers were content to let nature take its course and to merely wait for the MLF to occur spontaneously (Morenzoni 2005). This practice was responsible for typical MLF comments such as “It doesn’t go when I want it to” and “I don’t like what it does to the wine.” Recent research into MLF has helped us to understand better this process of biological wine deacidification and the limiting factors in wine impacting on the performance of LAB responsible for the biotransformation. “When we encounter a wine that has undergone a spontaneous MLF, it means that a lactic acid bacterium has overcome these hardships and has taken up residence in the wine. However, it does not mean that these bacteria will give us a malolactic fermentation that we can predict, nor will it give us one that has the positive organoleptic and sensory profiles that we want. It only means that a lactic acid bacterium is present in the wine, and that it, not the winemaker, has the ultimate control upon the quality of the finished product” (Morenzoni 2005).

25.3.1 Selection and Characterization of Lactic Acid Bacteria for Winemaking Starter Culture Preparations

As explained previously, relying on indigenous bacterial microflora to complete timely a desirable MLF can be precarious, even in low pH musts and wines. Even when desirable malolactic acid bacteria are established in a winery, the onset of the MLF may take several months and may occur in some barrels and tanks but not in others. For this reason, induction of the MLF by the use of selected bacterial starter cultures is becoming the preferred option. *Oenococcus oeni* is the MLF organism of choice, but not all strains of this bacterium are good candidates for use as starters. Selecting strains of *O. oeni*, which are best in terms of performance and most interesting in flavor production, is a multifaceted and challenging task (Bou and Powell 2005). It is imperative to isolate and domesticate only natural malolactic bacteria (MLB) strains. Wines which employ natural selective pressures of low pH, low cellar temperature, high alcohol, and high SO_2 are used to supply isolates of malolactic bacteria. The physiology and genetic profiles of new and interesting strains are determined in the laboratory and in pilot vinifications. One of the first selection criteria for a selected bacteria isolate is its ability to withstand rigors of stress induction during the production process (Bou and Powell 2005) and the freeze-drying step. Besides high resistance to limiting wine conditions as pH, alcohol, SO_2, and temperature, the bacteria are also selected for desirable metabolic activities and absence of undesirable features (Table 25.4).

25.3.2 Malolactic Starter Culture Preparations

Control of MLF, which is an integral part of the winemaking process, was often ignored until ML starter cultures became available. Liquid ML cultures were available and used for decades until the early 1980s when frozen and freeze-dried malolactic bacteria starter cultures were developed. The 1990s saw the development of direct inoculation freeze-dried ML starter cultures, and their use has virtually revolutionized the control and predictability of MLF in wine (Specht 2005). Table 25.5 summarizes the parameters which are applicable to different types of ML starters used in winemaking.

Most starter cultures available for winemaking benefit from storage under refrigerated and/or frozen conditions in their original, unopened package; the container should not be opened until just before use. In addition, the freeze-dried bacteria should avoid contact with oxygen, excess moisture, and high temperature as these conditions are detrimental to survival of the bacteria. In order to obtain the maximum effect from ML bacteria starter cultures, always follow bacteria producers' recommendations for handling and storage.

Table 25.4 Selection criteria for malolactic bacteria strains for winemaking

Desirable	Undesirable
Technological properties	*Technological properties*
Resistance to stress during production	Formation of excessive exopolysaccharides
Resistance to freezing and freeze-drying	Host of prophages
Resistance to low pH	Too high tolerance to SO_2
High tolerance to alcohol	Too fast degradation of malic acid (for red wine application and color stabilization)
High tolerance to SO_2	
Good performance at low temperatures	
Short lag phase	
Fast degradation of malic acid	
Good tolerance of oxygen	
Tolerance of pesticides	
Elevated resistance to lysozyme	
Production of bacteriocins	
Qualitative properties	*Qualitative properties*
β-glucosidase activity	Production of biogenic amines
Esterase activity	Production of ethyl carbamate
Production of positive fruit aromas	Production of S-deriving compounds
Reducing vegetative notes	Production of volatile acidity
Rounding the mouthfeel	Production of ethyl lactate
Lowering astringency	Production of mousy taint
Lowering bitterness	Production of volatile phenols
Increasing complexity	Production of geranium off-flavors
Lowering overall SO_2 (degradation of acetaldehyde and keto compounds)	Production of (excessive) amounts of diacetyl
	Fast degradation of citric acid
Production of acetaldehyde (red wine color stabilization)	Production/degradation of acetaldehyde
	Degradation of citric acid
Production of moderate amounts of diacetyl	
Production of butanediol	
Low affinity to glucose	

The listing below gives an overview of the most common instructions for ML starter culture preparations:

Frozen ML Starter Cultures

1. Thaw in room temperature water and not in the refrigerator. Mix 3 l water, 3 l grape juice, and 30 g yeast extract. Adjust pH to 4.0 with calcium carbonate or other permitted buffer, and mix thoroughly. Add 170 g of thawed culture, seal carboy, and mix thoroughly. Hold at 18–24 °C for 48 h before inoculation.
2. Directly add the frozen pellets to the wine.

Table 25.5 Properties of ML starter cultures (adapted from Specht 2005)

Property	Frozen	Type of malolactic bacterial culture			
		Liquid suspension	Direct inoculation (mbr)	Quick buildup culture (one step)	Traditional freeze-dried (standard)
Storage tempert. and shelf life	Up to 120 days at −26 °C or up to 1 year at −29 °C in a non-defrosting freezer	Up to 2 days at room temperature or up to 2 weeks at 4 °C	Up to 18 months at 4 °C or up to 30 months at −18 °C	Up to 18 months at 4 °C or up to 30 months at −18 °C	Up to 18 months at 4 °C or up to 30 months at −18 °C
Open container	Once thawed, *use immediately*; do not refreeze	*Use immediately*	*Use immediately*	*Use immediately*	*Use immediately*
Time for starter preparation	48 h before inoculation	Tenfold expansion in 3–7 days	0–15 min	18–24 h	3–14 days
Nutritional supplements	30 g yeast extract to activation media	~1 g yeast extract per liter growth medium	Proprietary MLB nutrients recommended under more challenging MLF conditions	Proprietary activator. MLB nutrients recommended under more challenging MLF conditions	Proprietary MLB nutrients recommended under more challenging MLF conditions
Usage rates	Red wine ~1 g hl^{-1} White wine ~3–8.5 g hl^{-1}	2–5% inoculation volume or when using finished wine to prepare the starter, then 5–10% inoculation volume	~1 g hl^{-1}	~0.5 g hl^{-1}	~1 g hl^{-1}

Liquid ML Starter Culture Suspensions
Use clean settled juice without added SO_2. If possible heat the juice to 60 °C. Adjust sugar level to 180 g l^{-1} with water [if juice is not available, substitute with a mix of 50% finished wine (<10 ppm free SO_2 and low total SO_2), 25% water, and 25% apple juice]. Adjust pH 3.5–3.6 with calcium carbonate. If inoculating wine at pH < 3.2, adjust pH again to 3.4 as an intermediate step.

Add culture and maintain temperature at 22–26 °C.

Monitor to 100% malic acid degradation, and then expand again as a 10% inoculum at each buildup stage or inoculate.

If finished wine is used to prepare the starter, then expand culture by doubling starter volume with wine until it is 5–10% of the amount to be inoculated.

Direct Inoculation Starter Cultures (MBR®)
A special preparation is *not required* but may be suspended in clean chlorine-free water at 20 °C for a maximum of 15 min to help in handling.

Quick Buildup Starter Cultures (1-STEP® Kit)
Rehydration phase: Mix and dissolve content of the activator mix in 100 l of potable water at 18 and 25 °C. Add content of the bacteria sachet and dissolve carefully by gentle stirring. Wait for 20 min.

Acclimatization phase: Mix the bacteria/activator solution with 100 l of wine, pH > 3.5, temperature between 20 and 25 °C. Wait between 18 and 24 h.

Transfer the activated culture to 1000 hl of wine.

Traditional Freeze-Dried Standard Starter Cultures
Rehydrate in 50:50 water/wine mix. Wine should be pH > 3.3 and total SO_2 < 30 mg l^{-1}.

Monitor malic acid drop, and when ~2/3 is converted to lactic acid, expand as a 5% inoculum into wine. Make sure pH > 3.3 and alcohol <12.5%.

Monitor malic acid drop, and when ~2/3 is converted to lactic acid, expand as a 4% inoculum into wine.

25.3.3 Choice of the Appropriate Malolactic Starter Cultures

There are two basic considerations when selecting a malolactic starter culture:

1. Security—the culture's compatibility with the wine environment
2. Sensory—the desired contribution of different ML strains

For successful induction of malolactic fermentations, it is critical that the most appropriate preparation of malolactic bacteria is selected for the prevailing wine conditions (Table 25.6).

Since the four main limiting factors (alcohol, pH, temperature, and SO_2) have a cumulative stress effect on cultures, Lallemand has developed a table, which allows

Table 25.6 General characterization of wine conditions for MLF

Wine conditions for MLF	Alcohol (% v/v)	pH	Free SO_2 measurable (mg l^{-1})	Total SO_2 (mg l^{-1})	Temperature (°C)	Problems associated with alcoholic fermentation	Recommended method of MBR® inoculation
Favorable	<13	>3.4	<8	<30	18–22	None	Direct (MBR)—*no acclimatization*
Difficult	13–15	3.1–3.4	8–12	30–40	14–18	Yeast stress	Direct (MBR) or with acclimatization method
Harsh	15–17	2.9–3.1	12–20	40–60	10–14	Sluggish/stuck	Direct (MBR) culture with acclimatization procedure
Extreme	>17	<2.9	>20	>60–80	<10		MLF unlikely
Stuck/Partial MLF							MBR acclimatization methods for inoculation of wines with stuck MLF (see Sect. 2.4)

Table 25.7 Scorecard for determining the ease of malolactic fermentation

	1 point each	2 points each	8 points each	10 points each	Score
Alcohol (% vol)	<13	13–15	15–17	>17	=
pH	>3.4	3.1–3.4	2.9–3.1	<2.9	=
Free SO_2 (mg l^{-1})	<8	8–12	12–15	>15	=
Total SO_2 (mg l^{-1})	<30	30–40	40–60	>60	=
Temperature (°C)	18–22	14–18 or 18–24	10–14 or 24–29	<10 or >29	=
Yeast's nutritional needs	Low	Medium	High	Very high	=
Ease of Alcoholic Fermentation	No problems	Transient yeast stress	Sluggish/ stuck AF	Prolonged yeast contact	=
Initial level of malic acid (g l^{-1})	2–4	4–5 or 1–2	5–7 or 0.5–1	>7 or <0.5	=
Maximum AF rate (maximum loss of brix/day)	<2	2–4	4–6	>6	=

Note: Other, currently less well-known factors that are not considered in this scorecard may include the level of dissolved oxygen, polyphenolic content, lees compacting, pesticide residues, etc.

scoring cumulative "points" of the impact of different wine parameters (Table 25.7).

The resulting "TOTAL" corresponds to the level of difficulty of a wine to start MLF:

- <13 points = favorable
- 13–22 points = not so favorable
- 23–40 points = difficult
- >40 points = extreme

Depending on the *O. oeni* strain, direct inoculation starter cultures will tolerate in general:

- Alcohol tolerance <15% vol
- PH tolerance >3.1
- Total SO_2 tolerance <60 ppm
- Temperature tolerance >12 °C

In addition to the wine conditions described in Tables 25.6 and 25.7, other conditions for alertness when planning selection, preparation, and inoculation for MLF include:

- Wines which have struggled to complete alcoholic fermentations are more likely to be deficient in nutrients required to support bacteria during the MLF.
- Nutrient limitation is considered to be one of the major causes of incomplete malolactic fermentations.
- Lower the wine pH below 3.5, higher the bacterial nutrient demand to perform the MLF.

- Bacterial ability to grow and conduct MLF will gradually decrease as wine temperatures fall. Depending upon the wine alcohol content, higher wine temperatures can also be inhibitory to the development and activity of ML bacteria. A general guideline to avoid inhibitory effects is:

Total score for the ease of MLF:
Total

1. Wine alcohol content (% v/v) temperature for MLF should not fluctuate:

   Less than 14.5% 28 °C
   Greater than 14.5% 23 °C

2. Wine volatile acidity above 0.4 g l^{-1} (as tartaric acid) is likely to inhibit malolactic bacteria.
3. Wines stored for more than 3 months on yeast lees are best racked clean before attempts to conduct MLF.

Various acclimatization procedures exist to help overcome very limiting wine conditions or in presence of inhibitory compounds. Protocol described in Sect. 25.3.4 below has been developed to aid inoculation of MBR bacteria preparations into wines, to cure stuck malolactic fermentations.

25.3.4 Restarting Stuck Malolactic Fermentations

Winemakers are aware that *O. oeni* bacteria, responsible for MLF, are successful only if they adapt to the harsh environment of a fermenting must or finished wine. Direct-addition MLB strains from serious producers that have been selected both for their positive sensory contributions and their ability to perform under the difficult situations are described above. During production, MLB cells undergo a biophysical conditioning that induces the formation of a protective protein. In this physiological state, the cells are harvested and then freeze-dried. As a result, they are able to develop a natural resistance to wine conditions and can therefore be added directly to wine without a significant loss of viability.

Sometimes, a stuck MLF can be completed simply by adding a freshly rehydrated direct-addition malolactic bacteria culture. At other times a more extensive adaptation of the MLB is needed to achieve completion. This adaptation can be critical in reducing the effect of an unfavorable wine matrix on the bacteria, favoring successful completion of the MLF. Lallemand Australia has worked in conjunction with their MLF R&D team to develop a MLB acclimatization strategy for finishing wines with stuck malolactic fermentations.

Adaptation Protocol for Handling Stuck Malolactic Fermentations (Specht 2005)

Stage 1

- Pretreat wine and adjust temperature.
- Prepare the wine with the stuck MLF by removing any lees and potential inhibitory toxins and inhibiting spoilage organisms. A small amount of SO_2 and/or lysozyme (or filtration) may be necessary to control undesirable *Lactobacillus* or *Pediococcus* bacteria.
- Lysozyme is very effective at inhibiting spoilage LAB, especially when the wine is above pH 3.5. If using lysozyme, be sure that no residual activity remains in the treated wine before inoculation with malolactic bacteria.
- In a wine with a stuck MLF suspected of containing substances toxic to malolactic bacteria, a pretreatment with inactive yeast residues (yeast hulls) at 6.25–12.5 g hl^{-1} is recommended. Prepare the yeast hull suspension in water or wine, and then add it to the stuck wine while mixing.
- Finally, adjust the temperature of the MLF-stuck wine to 18–22 °C (65–72 °F).

Stage 2

Acclimatize the bacteria culture in three steps:
Step 1: Prepare medium.
Step 2: Rehydrate culture.
Step 3: Add rehydrated culture to medium to acclimatize.
Note: The volumes below are based on restarting 10,000 l of stuck-MLF wine.

Step 1: Preparation of the Acclimatization Medium

Combine:

- 10 l of grape juice (free of SO_2).
- 10 l of water (free of chlorine).
- 20 l of stuck-MLF wine.
- After adding all ingredients, adjust the pH to between 3.6 and 4.0.
- Adjust temperature to 25–30 °C.

Step 2: Rehydration of ML Bacteria Starter Culture

1. Adjust the temperature of 5 l of tap water (free of chlorine) to 22–25 °C. Suspend 1 kg of ML rehydration nutrient into the 5 l of tap water.
2. Rehydrate 100 g of direct inoculation malolactic bacteria in the 5 l of tap water/nutrient suspension.

   Allow bacterial suspension to stand for 15 min.

Step 3: Acclimatization of Malolactic Bacteria

- Mix acclimatization medium (from Step 1) with the rehydrated malolactic bacteria (from Step 2).

- Allow malolactic bacteria to acclimate at 22–25 °C for at least 2 h and not more than 4 h.
- After this first acclimatization step, double the volume of acclimatization culture with the stuck wine (e.g., 50 l culture and 50 l wine). If on-site malic acid analysis is not available to monitor MLF progress, it can be assumed that the inoculation culture will be ready in 4–6 h. CO_2 evolution should be evident and/or a slight lactic smell detected. If rapid malic acid analysis is available, 50–70% of the malic acid should be converted before proceeding to Stage 3.

Stage 3

- Add ML nutrient and then acclimatized culture from Stage 2 to the stuck wine.
- Add the nutrient (20 g hl^{-1}) to wine prior to inoculation. The aim is to overcome any nutrient shortages and minimize the risk of residual nutrients in the wine.
- With gentle stirring to avoid excessive aeration, transfer the active acclimatized malolactic culture to 10,000 l of stuck-MLF wine.
- Regular analysis for malic acid (every 2 weeks) and volatile acidity (weekly) is recommended.

25.3.5 Contribution of the Malolactic Starter Culture to the Sensory Quality of Wine

Reduction of wine acidity and modification of wine flavor due to this secondary bacterial fermentation are often considered to benefit wine quality. The advantage of induction of MLF by inoculation with selected strains of LAB is twofold. First there is a better control over the time and speed of malic acid conversion and, second, a positive influence on wine flavor and quality. Research in recent years has revealed the positive contribution of specific bacteria starters and conditions, including the rate and timing of inoculation for MLF, to the sensory profile of white, red, and rosé wines. The metabolic activity of malolactic bacteria (MLB), as well as the kinetics of MLF, will influence the sensory profile of the wine in relation to different winemaking techniques, physical and chemical composition of the wine (pH, alcohol, temperature, citric acid level, SO_2, and aeration), and presence of lees (Lallemand Winemaking Update 01/2007).

MLF Reveals Varietal Aromas Of all the lactic bacteria active in wine, *O. oeni* is the one most often responsible for MLF. It reduces acidity and modifies the sensory profile of the wine, which has beneficial effect on its quality. For example, the intensity of the floral, fruit, spice, and honey notes is associated with the increase of volatile compounds linked to glycosides and released during MLF. A study done by Ugliano and Moio (2006) validates the role of *O. oeni* in the evolution of the varietal's volatile compounds. Their work shows that the concentration of total

glycosides drops significantly during MLF. The hydrolysis of glycosylated aromatic precursors and, consequently, corresponding aromas from the grapes are revealed. The importance of this phenomenon depends both on the bacteria used for MLF and the composition of the wine. In other words, the expression of these varietal aromas, whose importance is considerable to the overall aroma of the wine, depends not only on the potential of the grape varietal but on the type of malolactic culture as well. This confirms previous observations on the glucosidase activity of MLB. For example, during MLF the glucosidase activity of *O. oeni* releases volatile compounds linked to the aromatic precursors of the grape, including 3-hydroxydamascone, alpha-terpineol, vanillin, methyl vanillate, 4-hydroxybenzoate, and tyrosol, from extracts of Chardonnay (Bartowsky and Henschke 2004), as well as linalool, alpha-terpineol, nerol, and geraniol extracts of Muscat (Ugliano et al. 2003). These studies suggest that the glycosidic activity of *O. oeni* and subsequent release of the aroma moieties during MLF have the potential to increase the sensory characteristics of the wine.

Diacetyl Management: The Influence of MLB Inoculation Rate and Timing of Addition on the Aroma Profile Diacetyl is one of the main aromatic compounds produced during MLF and is responsible for the butter and hazelnut notes typical of MLF. Its impact is very important on the profile of the wine, and depending on the desired wine style, it is either sought after or very undesirable. Indeed, various studies by Martineau and Henick-Kling (1995) and Bartowsky and Henschke (2004) have shown that the production of diacetyl by different *O. oeni* starters could result in completely different aromatic profiles. Each bacteria starter's potential for producing diacetyl is a criterion to consider when choosing a malolactic culture. Beyond diacetyl, the type of starter chosen can also modify other aroma families.

A high level of inoculation with the malolactic cultures not only accelerates the start and speed of the MLF but also results in a low level of diacetyl. In general, it is recommended the wine is inoculated at a population level above 10^6 CFU ml^{-1} to reach the critical bacteria population to ensure the rapid initiation of MLF and the regular degradation of the malic acid. Krieger (2005a, b) studied the diacetyl level in a Pinot noir wine where MLF was initiated with different inoculation rates for the malolactic cultures. A low inoculation rate of 2×10^4 CFU ml^{-1} had a prolonged lag phase (14 days) and produced 3.9 mg l^{-1} of diacetyl, whereas a rate of 4×10^6 CFU ml^{-1} immediately initiates the degradation of malic acid and produced 0.8 mg l^{-1} of diacetyl. Inoculation at a rate $>2 \times 10^6$ CFU ml^{-1} resulted in wines under the diacetyl perception threshold of at nearly 1.5 mg l^{-1} for white and rosé wines.

The timing of inoculation can be just as crucial on the final wines sensory properties. Riesling wines were made using different timing for inoculation with malolactic cultures and carried out in collaboration with DLR Neustadt and Trier (Krieger 2006). These experiments have demonstrated that co-inoculation—the simultaneous inoculation of yeast and bacteria—does not influence the alcoholic fermentation or increase volatile acidity; but it does reduce the overall MLF

duration. The co-inoculated Riesling wines did not have buttery or milky aromas associated with MLF, but did have a high intensity of varietal fruit aromas. The diacetyl produced under such reducing conditions during the alcoholic fermentation was immediately transformed into butanediol, which has no odor at this concentration. The same wines inoculated for MLF after alcoholic fermentation had more typical MLF sensory character with dominant notes of butter hazelnut, while the fruit was diminished. The control wines with no MLF were more acidic, green, and vegetative.

The Sensory Impact of Post-MLF Winemaking Techniques The choice between aging on lees and filtering after MLF influences the sensory profile of the wine. The yeast lees can degrade the diacetyl, and bâtonnage can reduce or even eliminate the buttery aroma. The production of diacetyl increases, while the wine is in contact with oxygen. Oxygen encourages the oxidation of acetolactate into diacetyl. Nielsen and Richelieu (1999) showed that the accumulation of diacetyl in a semi-aerobic environment could be six times higher than in a completely anaerobic environment. Moreover, the reduction of diacetyl into acetoin and butanediol depends on the redox potential of the wine. A low redox potential is associated with a low level of diacetyl.

25.4 Innovations and New Applications for Enological Products

The wine industry has changed a lot over the past two decades not only because of the global warming, which impacts on the grape composition, but also because of the challenges coming from new regulation from regional, European, and international authorities (OIV, EU, WHO, Codex Alimentarius, under others), regulation concerning organic, biodynamic wine production, demand for sustainability, environmental protection, and last but not least consumer demands. This was the base for the development of a range of new enological products to serve the needs and help the wine industry to produce safe and good quality wines, wines with less SO_2 or lower alcohol, good acidity balance, and desired aromatic profile.

25.4.1 Low SO_2 Approach

Sulfur dioxide (SO_2) is an important food additive. In Europe it is indicated on labels as E220 (sulfur dioxide) or as E224 (potassium metabisulfite). Sulfur dioxide is also the key additive for the preservation of wines. Wines have to be labeled: "contains sulfites." In certain conditions, SO_2 has health negative impacts. It destroys part of B1 vitamin and may induce allergenic reactions that affect blood circulation and respiratory system and generate headache. The US FDA estimates

that 1% of the US population show an increased degree of sensitivity to sulfites. The World Health Organization has determined a daily admissible dosage of 0.7 mg^{-1} kg^{-1} day^{-1}. Therefore, the European Union has determined legal levels of total SO_2 for red wines as 160 mg l^{-1}; for white/rosé wines 210 mg l^{-1}, and sweet wines 250 mg l^{-1}. Minimally processed foods and wines with little or no sulfites have been increasingly popular with consumers. To meet the new regulations and the increasing consumer demand, research has worked on different solutions.

Molecular Bases for Low Sulfite and/or Sulfide Production by Wine Yeasts Enological yeasts contribute greatly to the final aromatic balance of wines through the production of volatile compounds of interest; nevertheless, they can also be responsible for the production of negative off-flavors, such as sulfur compounds. Sulfite and sulfide are two of those compounds whose production has to be controlled. The sulfate assimilation pathway has been widely studied; however, little is known about the molecular basis responsible for the differences in sulfite and/or sulfide production between yeast strains. In order to address the genetic determinism of such properties, we implemented a QTL mapping approach along with phenotypic and transcriptomic characterization of a couple of wine yeast strains highly differing in their sulfite production. This global study resulted in the identification of two new allelic variants of the MET2 and SKP2 genes. Functional validations demonstrated their involvement in the control of the production of sulfite, sulfide, and acetaldehyde. The combination of both alleles of the low sulfite producer strain is responsible for a strong control of the entire sulfur assimilation pathway, and we show that it is strong enough to control the production levels of sulfur compounds in other wine yeast. Transfer of those alleles in a high sulfite producer strain has already been performed through backcrossing cycles using marker-assisted selection and allowed us to construct an optimized strain of low SO_2, low H_2S, and low acetaldehyde producer.

Role of Carbonyl Compounds and MLF In addition to the partial reutilization of acetaldehyde by yeast in the second half of the alcoholic fermentation, acetaldehyde is also degraded by LAB. During MLF, acetaldehyde is typically degraded simultaneously with malic acid or a little later. If a complete degradation of acetaldehyde is desired, wines should not be stabilized until 5 days after malic acid depletion. Malolactic fermentation also leads to the substantial reduction of pyruvic acid and partial reduction of α-ketoglutaric acid. Hence, MLF can make a significant contribution toward achieving lower bound and total SO_2 levels. Co-inoculation techniques, inoculating selected wine LAB 24 h after yeast, can lead to a further reduction of SO_2-binding compounds.

25.4.2 Low Alcohol Approach

Over the past two decades, the level of ethanol in wine has increased in most wine-producing regions, raising a number of issues related to consumer health,

prevention policies, the effectiveness of the fermentation, and wine sensorial quality. While different approaches have been proposed at all stages of the winemaking process to reduce this trend, microbial strategies have the advantage of being inexpensive and easy to implement. Many research efforts during the last two decades have therefore focused on developing novel yeast strains that produce less ethanol during wine fermentation.

Reducing Alcohol Levels in Wines Through Adaptive Evolution of Wine Yeast Because the diversity between *S. cerevisiae* isolates for ethanol yield is very low, the development of strains producing less ethanol requires a marked modification of yeast metabolism. This is a challenging task that requires considering a number of important constraints, such as maintaining carbon, redox, and energetic balances and preserving wine quality and yeast performance.

Metabolic engineering approaches have been first used to generate strains producing less ethanol. Among various strategies, rerouting carbons toward glycerol has emerged as the best option to reduce ethanol yield. As a proof of concept, wine yeast strains overproducing glycerol and 2,3-butanediol with a lower ethanol yield and without accumulation of unwanted by-products have been successfully constructed by metabolic engineering strategies. These strains have the potential to decrease alcohol levels in wines by up to 3% (vol/vol).

Evolutionary engineering is a GM-free strategy that has proven which has proven its effectiveness to reshape the yeast metabolism. The concept of adaptive evolution is that microorganisms tend to evolve their intrinsic characteristics to adapt to new conditions. During this process of evolution, random genetic mutations occur, and if a selection pressure is applied, strains having one or beneficial mutations in the selective medium will dominate in the culture medium and can thus be selected. This approach is based on the extended cultivation of a strain in controlled selective medium to select for natural genetic variants having beneficial mutations under the conditions used. Since the emergence of mutations is a rare event, several hundred generations are usually necessary before observing an evolution, which can last several months.

Recent adaptive evolution work has been used for developing wine yeast strains with reduced ethanol yield. Different strategies to redirect carbons toward other by-products other than ethanol were used to reprogram the metabolism of a commercial *S. cerevisiae* wine yeast strain and resulted in the selection of an evolved strain producing less ethanol. These strategies and the characteristics of the wines produced at lab scale and pilot scale and in 2016 on commercial scale by the selected evolved strains were awarded with a special innovation price at Intervitis Interfructa, as the jury recognized the growing concern regarding the increased levels of alcohol in wine as well as decreased acidity. Overall, this work shows that adaptive evolution is a valuable alternative to rational modification for reducing ethanol yield in wine yeasts.

25.4.3 Acidity Management Approach

In enology, one of the consequences of global warming is the steady decline in acidity in musts and wines of many hot climate regions, but increasingly also cooler climates are concerned depending on the vintage year. Higher temperatures over a longer time period generate early crops with low values of total acidity and malic acid and high sugar concentration. Musts with low acidity and high pH show unbalanced organoleptic properties and are very susceptible to microbial spoilage. The most commonly used solution to this phenomenon is the chemical acidification with organic acids such as malic, tartaric, and lactic acid. Another alternative is physical acidification by methods such as cation exchange resins or electrodialysis treatments. These strategies have disadvantages and are subject to strict legislation. However, the biological acidification is not legislated, and it is also applicable to organic wines.

Lactobacillus plantarum In collaboration with the University of Valencia and the group of Isabel Pardo and Sergi Ferrer, a program on the selection of *Lactobacillus* strains was started, to find strains with a high potential for biological acidification to be applied as starters for winemaking. Different selection criteria were used such as ability to grow in must, carry out MLF, must acidification, synthesis of lactic acid from sugars, resistance to lysozyme and sulfur dioxide, and biogenic amines' forming inability. Two strains have been selected and characterized which are suitable as starter cultures for biological acidification in low acidity wines. The use of these strains ensures the microbiological stability by lowering pH and providing a faster vinification by parallel early induction of MLF; a prompt stabilization of wines can be made just after the end of the alcoholic fermentation.

25.4.4 Microbial Safety Approach

The wine pH is one of the most important factors which limits LAB growth and MLF in wine (Radler 1966) and determines the type of LAB which will be present. Ideally for table wines, the pH should be between 3.1 and 3.6 (Amerine et al. 1980), but due to global warming, wine pH increased over the last years in almost all wine regions. Wine pH affects the metabolism of sugars (Peynaud and Domercq 1970) and also has a selective effect on the species (Mayer and Vetsch 1973). In wines of pH below 3.5, strains of *O. oeni* will generally dominate, and in wines of pH above 3.5, various strains of *Lactobacillus* and *Pediococcus* will dominate. Within these species we find a good potential of strains that negatively influence the final product and may cause a range of undesirable changes to wine sensory properties, such as masking varietal fruit characters, altering wine color, and producing undesirable metabolites, e.g., biogenic amines (Davis et al. 1985). The biogenic amines are of importance due to their potential toxic effects in sensitive humans. Ingestion can cause various symptoms. Histamine, e.g., can cause headaches, low blood pressure,

heart palpitations, and in extreme cases anaphylactic shock, while putrescine and cadaverine not only enhance histamine toxicity but can also contribute putrefaction and rotten meat aromas to wine (Palacios 2006). These toxic effects are amplified by the presence of alcohol, SO_2, and other amines. Others of these bacteria can cause problems in the wine both during and after completion of the MLF due to the production of high amounts of acetic acid, exopolysaccharides, and compounds associated with mousy taint.

Co-inoculation Approach Findings show the production of biogenic amines being curtailed with co-inoculation strategies versus sequential inoculation strategies. It is also stated that co-inoculation often results in the inhibition of the production of 4-ethylphenol and 4-ethylguaiacol, both of which can cause a myriad of sensory and organoleptic problems (Gerbaux et al. 2009). Early inoculation with a high amount of selected *O. oeni* strains will not only allow a dominance over the indigenous bacteria flora, but due to the fact that bacteria cells are added very early in the winemaking process, the MLF will complete promptly after AF, which will then allow the product to be microbiologically stabilized at an earlier date.

Co-inoculation Approach with Lactobacillus plantarum Lactic acid bacteria are present in wine at all stages of winemaking. Wine pH is most selective, and at pH below 3.5, generally only strains of *O. oeni* can survive and express malolactic activity. Wine pH has been increasing gradually for the last several years. Red wines with pHs over 3.5–3.6 are more and more frequent. At those pH levels, we can observe very fast growth of various indigenous microorganisms, some of which are spoilage bacteria that can cause loss of wine quality. Among these species, *Lactobacillus plantarum* strains have shown most interesting results for their capacity to induce MLF under high pH conditions, their facultative heterofermentative properties that avoid acetic acid production from hexose sugars, and their more complex enzymatic profile compared to *O. oeni*, which could play an important role in the modification of wine aroma.

Dating back to 2005, a new selection of *L. plantarum* at the Università Cattolica del Sacro Cuore in Italy resulted in a very effective *L. plantarum* culture, adapted to high pH wines. The new starter culture, called ML Prime™, is a pure *Lactobacillus*, and due to its facultative heterofermentative metabolism, it is best suited for co-inoculation (inoculation 24 h after the wine yeast) without any risk of volatile acidity production during MLF. Because of its specific optimized production process, ML Prime™ expresses very high malolactic enzymatic activity on musts, resulting in a fast MLF during alcoholic fermentation. This way wines can be stabilized early and protected from further contamination and, thus, retain their sensory integrity. The trials, where implantation controls had been applied, showed *L. plantarum* ML Prime™ achieving 100% implantation in the must during co-inoculation, and consequently the indigenous contaminating LAB were under control and under the detection threshold of 2×10^3 cfu ml^{-1}. This is in line with 2014 OIV regulation (OIV-Oeno-264-2014) on good vitivinicultural practices for controlling *Brettanomyces* which specifies “co-inoculation of selected yeast and

selected wine bacteria may avoid *Brettanomyces* spoilage." Another regulation proposes co-inoculation strategy to limit biogenic amine formation.

25.5 Conclusion

More than 200 strains of active dry wine yeast are available worldwide, offering the wine industry a significant biological diversity. The number of commercially available active dry malolactic starter cultures is still rather limited, but has increased more recently. While active dry yeast starter cultures mostly belong to *S. cerevisiae*, starter cultures for the induction of the MLF mainly consist of *O. oeni*. Both yeast and bacteria strains had been selected for their tolerances to limiting wine conditions and their sensory and enological properties to meet creative and security needs of the modern wine industry. It is crucial to know wine parameters and properties of the selected starter cultures to select the right yeast strain, the right bacteria strain, and the correct nutrition strategy to match the grapes, fermentation conditions, and stylistic goals.

Challenges coming from climate changes; new regulation from regional, European, and international authorities (OIV, EU, WHO, Codex Alimentarius, under others); regulation concerning organic, biodynamic wine production; demands for sustainability; environmental protection; and last but not least consumer demands have stimulated the development of a range of new enological products to serve the needs and help the wine industry to produce safe and good quality wines and wines with less SO_2 or lower alcohol and good acidity balance, e.g., the application of evolved yeast strains or yeast strains other than *S. cerevisiae* or LAB starter cultures other than *O. oeni*.

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Chapter 26
Application of Microbial Enzymes During Winemaking

Eric Hüfner and German Haßelbeck

26.1 Introduction

Making wine is not possible without enzymes. The joint action of yeast enzymes together with those originating from the grape is responsible for the biotransformation processes occurring from juice to wine. However, winemaking poses adverse conditions to grape enzymes, which therefore display only poor activity (Ducasse et al. 2011). This results in long reaction times and limited conversion, both of which are not acceptable in industrial winemaking. Microbial enzymes, especially from fungal origin, display better tolerance to the prevailing physicochemical conditions and are therefore commonly used to facilitate controllable processes, improve overall quality, and produce distinguished wines.

26.1.1 History of Enzyme Use in Winemaking

The first use of enzymes in European winemaking started in the late 1960s, introducing the fruit juice pectinase preparation Pectinex (Swiss Ferment AG, subsidiary of Novo Industri A/S) in red wine mash treatment. The use of this pectinase led to a better free run juice, more intense red color, and an accelerated maturation of the resulting red wine. Within a few years, special wine enzymes came on the market, e.g., Ultrazym 40 (Swiss Ferment AG), Vinibon

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(C. H. Boehringer Sohn GmbH & Co. KG), Rapidase (Société Rapidase), and Trenolin (Erbslöh Geisenheim Getränketechnologie GmbH), preferred in red winemaking and also in white wine processing for better pressing performance. The benefit of an earlier aging in red wine production turned out to be a disadvantage in white wine processing. Cinnamoyl esterases, undesired side activities in most of the pectinase preparations for winemaking in the 1970s, were detected by wine researchers, leading to the development of naturally pure (PV 8, Miles Kali Chemie) or especially purified pectinase preparations (Panzym Super DF, C. H. Boehringer Sohn GmbH & Co. KG). While the use of enzymes in winemaking first was limited on mash treatment, it soon was applied to grape juice and young wine also. The growing knowledge about viticulture and vinification, the composition of grapes and grape juice, and the production of enzymes fostered the design of special pectinase preparations by boosting valuable side activities, e.g., aroma-enhancing glucosidases (Rapidase AR 2000, Gist-Brocades N.V.), and the introduction of new enzyme activities in wine processing, e.g., glucanase for the degradation of botrytis glucan (Glucanex, Novo Nordisk A/S), urease for the prevention of urethane formation (Nagapsin, Nagase Biochemicals Ltd.), and laccase for the improvement of cork stoppers (Suberase, Novo Nordisk A/S). Today enzyme producers, enzyme formulators, and enzyme traders from all over the world offer numbers of special wine enzymes in winemaking worldwide.

26.1.2 Regulation of Enzyme Use in Winemaking

The use of enzymes in winemaking in most of the wine-growing countries actually is regulated by the International Organisation of Vine and Wine (OIV), which is a multinational semigovernmental organization ruling all matters of vine and wine in specific resolutions. Except for the USA, Canada, China, and some smaller Asian countries, actually 46 member states are organized in OIV. In most of the member states, OIV regulations immediately are adopted by the states as national law in winemaking. In all European wine-growing countries, OIV regulations come into force by the EU administration after a delay of 6 months, with the option of national restrictions. The USA, Canada, and China have national regulations in winemaking. Bilateral agreements between USA and EU (USA-EU Wine Trading Agreement) and Canada and EU (CETA) provide for mutual acceptance of the national regulations in force. The actual enzyme regulations by OIV define general aspects of enzymes in winemaking (OIV/OENO 14/2003), the permitted enzyme activities (OIV/OENO 27/2004b and others), its use on enological practices (OIV/OENO 11/2004a and others), and the analytical methods of enzyme activity measurements (OIV/OENO 9/2008 and others).

26.1.3 Development and Production of Food Enzymes

In the beginning of industrial fungal pectinase production, the surface fermentation process (solid state fermentation) was the standard production technique, e.g., Pectinol by Röhm & Haas AG (Darmstadt, Deutschland) for fruit juice processing. Also all early produced wine enzymes resulted from surface fermentation, already improved in pectinase activity. In the 1970s pectinase production in submerged fermentation process reduced the costs of pectinase production drastically, increasing the number of pectinase producers. In submerged fermentation improvement of activity, the enhancement of special pectinase activities and enzyme purification was more efficient than in surface fermentation. Especially genetic engineering as modern tool of enzyme development is generally combined with submerged fermentation processes. While in fruit juice processing use of GMO and self-cloning pectinases is already standard, wine still is processed using conventionally produced pectinases from surface and submerged fermentation. Today special enzymes in winemaking mostly are produced and marketed by enzyme formulators, e.g., Oenobrands (France), AEB (Italy), and Erbslöh Geisenheim AG (Germany).

26.2 Application of Enzymes in Winemaking

The application of enzymes in winemaking may occur at several stages during processing and serve a variety of purposes. The aim can be technological benefits like lowering viscosity and increase of yield, or impact on the sensorial quality or stability of the final product wine. The main targets or substrates for enological enzymes are macromolecular colloids, in particular polysaccharides, present in juice and wine. These carbohydrates are either originating from the grape berry (pectin) or are produced by microorganisms (glucan) and introduced into the wine during processing. The exceptional water-holding capacity and ability to form gel networks lead to problems with clarification and filtration. Also, precipitation events can lead to haze formation. The most prominent colloid in terms of quantity and technological effect is pectin (Voragen et al. 2009). Pectin is referring to the most heterogeneous group of polysaccharides in nature (Caffall and Mohnen 2009). Consequentially, suitable enzymes for winemaking are carbohydrate-active enzymes that degrade polysaccharide structures (see Table 26.1). The most frequent sources are plant-pathogenic molds, where the enzymes are part of virulence mechanisms like invasion and tissue disintegration (Kubicek et al. 2014; De Vriess and Visser 2001). In recent years, great effort has been done for classification regarding mode of action, substrate specificity, and overall characterization. The carbohydrate-active enzymes (CAZy) database has evolved into a benchmark reference for this vast enzyme category (Cantarel et al. 2009; http://www.cazy.org/). Most enological enzymes can be assigned to two groups, glycoside hydrolases (EC 3.2.1.-) and polysaccharide lyases (EC 4.2.2.-). Hydrolases cleave

Table 26.1 Typical enzyme constituents of commercial enological enzyme products

	IUBMB no.	CAZy family	Substrate	Product
Pectinases				
Pectin lyase	4.2.2.10	PL1	Pectin/polygalacturonan	Homogalacturonan oligomers
Pectate lyase	4.2.2.2	PL1	Pectate	Homogalacturonan oligomers
Polygalacturonase	3.2.1.15	GH28	Pectin/polygalacturonan	Homogalacturonan oligomers
Exo-polygalacturonase	3.2.1.67	GH28	Pectin/polygalacturonan	Galacturonic acid
Pectin methylesterase	3.1.1.11	CE8		
Rhamnogalacturonan hydrolase, endo-, or exo-acting	3.2.1.171/ 3.2.1.174	GH28	RG-I	RG-I oligomers, rhamnose, galacturonic acid
Rhamnogalacturonan lyase, endo- or exo-acting	4.2.2.23/4.2.2.24	PL4	RG-I	RG-I oligomers, rhamnose, galacturonic acid
Rhamnogalacturonan acetylesterase	3.1.1.86	CE12	RG-I	Deacetylated RG-I
Endo-xylogalacturonan hydrolase	3.2.1.-	GH28	Xylogalacturonan	Xylogalacturonan oligomers
Arabinogalactan endo-β-1,4-galactanase	3.2.1.89	GH53	AG-II	Galactooligomers
Arabinan endo-1,5-α-L-arabinanase	3.2.1.99	GH43	Araban	Araban oligomers
Galactan endo-1,6-β-galactosidase	3.2.1.164	GH5	AG-II	Galactooligomers
Galactan endo-β-1,3-galactanase	3.2.1.181	GH16	AG-II	Galactooligomers
Cellulases and hemicellulases				
Cellulase/β-1,4-glucanase	3.2.1.4	GH5	Cellulose	Cellooligomers
Cellulose 1,4-β-cellobiosidase	3.2.1.176	GH48	Cellulose	Cellobiose
β-D-glucosidase	3.2.1.21	GH1	β-D-glucosides	D-glucose
α-L-rhamnosidase	3.2.1.40	GH78	α-L-rhamnosides	L-rhamnose
Endo-1,4-β-xylanase	3.2.1.8	GH5 and others	Xylan	Xylan oligomers
Xyloglucan-β-1,4-glucanase, endo- and exo-acting	3.2.1.151/ 3.2.1.155	GH12	Xyloglucan	Xyloglucan oligomers, xylose, glucose

Abbreviations: *RG-I* rhamnogalacturonan I; *AG-II* arabinogalactan II

glycosidic bonds by addition of water; prominent examples are polygalacturonases acting on homopolygalacturonan (Bussink et al. 1992). Lyases like pectin lyases degrade polymers by a beta-elimination mechanism that results in the formation of 4,5-unsaturated oligosaccharides (Yadav et al. 2009). Both groups feature endo- as well as exo-acting enzymes that may use either soluble polysaccharides as substrates or are active on unsoluble fractions like protopectinases (unsoluble pectin) or avicelase (crystalline cellulose). The majority of enological enzymes are produced by classically developed strains; the main enzyme activity pectinase is generally accompanied by numerous side activities like hemicellulases and cellulases. They act as accessory activities in the application and often make the difference between a standard product and a superior wine enzyme.

26.2.1 Mash Treatment

After the crushing of the grape berries, the resulting mash may be treated in several different ways, according to the grape variety and the intended type of wine. The use of enzymes can serve several purposes. On the one hand, the juice yield can be strongly increased; on the other hand, the extraction of value-adding constituents is significantly facilitated.

Yield Improvement The yield of grape juice is mainly determined by the mechanical disruption of grape cells during crushing. It can be significantly increased by the destruction of the water-holding capacity of colloids, which results in a decrease of viscosity and, at the same time, to a gain of free run juice. Free run juice is the initial juice fraction obtained from crushed fruit prior to the application of any mechanical pressure. Here, commercial enzyme products can be used to a great effect (Ough and Berg 1974). Depending on the type of wine (white, rosé, red), juice yield improvement is the main aim of microbial pectinase application. In white wine processing, free run juice is strongly depending on the colloid content of the grape mash, mostly influenced by the grape variety and the temperature conditions while mashing (Ough and Crowell 1979). In red wine processing, juice yield is depending on the process of color extraction, either by mash fermentation or thermovinification. Especially in thermovinification, the application of microbial pectinases is needed to substitute grape pectinases that have been inactivated by the heat treatment.

In more specialized processes, where the aim is to minimize the extraction of grape components (blanc de noir) or to maximize the extraction (orange wine), the application of pectinases is used to reduce process time. The demand for quick-acting pectinase reaction in cold climate vinifications led to the development of cold-active enzymes (Adapa et al. 2014).

Extraction Wine quality depends on the optimum and, often maximum possible, extraction of value-adding substances of the grape cells. The so-called maceration enzymes were introduced to facilitate polyphenol extraction in red winemaking but

are nowadays also frequently used in white and rosé winemaking. The main application purpose is the effective disintegration of the grape cell wall, which constitutes the major diffusion barrier for aroma precursors, pigments, tannins, and other constituents. Many commercial enzyme preparations are characterized by a cocktail of different enzymes acting on the structural macromolecules of the cell wall, combined to achieve degradation of the diverse insoluble polysaccharides. Commercial products have been intensively examined throughout the last years (Romero-Cascales et al. 2008). Two active principles can be differentiated that are analogies of natural processes of tissue disintegration. Firstly, macerating protopectinases cleave the pectin in the middle lamella, thus leading to a loosening of the tight cell–cell junction and thus softening of the tissue (Sakai et al. 2000). This is virtually an analogy to fruit-ripening processes (Paniagua et al. 2014). Secondly, pectinases together with cellulases and hemicellulases like xylanase, xyloglucanase, and galactanase interact in the decomposition of the primary cell wall, mimicking the pathogenetic processes characteristic for microbial infection of the grape (De Vriess and Visser 2001). This cell wall perforation is effective for increased release of anthocyanins from vacuoles and cell wall-bound tannins (Amrani Joutei et al. 2003). The successful application of maceration enzymes in red winemaking has been well documented (Río Segade et al. 2015; Parley et al. 2001; Puértolas et al. 2009; Busse-Valverde et al. 2011; Bautista-Ortín et al. 2005; Ducasse et al. 2010; Gump and Haight 1995). Beneficial effects have been reported concerning higher polyphenol content, color intensity, and a more stable color. Color-stabilizing copigmentation reactions of anthocyanins, tannins, and catechins are evidently promoted by enzyme treatment. The application of cold-active pectinases supports the recirculation of the mash in the beginning of mash fermentation at low temperatures. This is a growing demand due to late harvesting of the grapes in order to maximize “aroma ripeness.” Furthermore, extraction enzymes can have a distinct impact on sensory characteristics like improved mouthfeel, astringency, and structure (Canal-Llaubères and Pouns 2002) and can lead to wines easier to clarify and filter, and with a higher pressing yield. However, there are several studies stating no significant effect of maceration enzymes on wine quality. Obviously, factors like state of maturity and grape variety can influence the efficacy. For example, the extraction of overripe grapes is per se very good and cannot be further improved by enzymes (Ortega-Regules et al. 2008). Thermovinification is a physical method for extraction improvement. The heat treatment of the mash has as a consequence the complete inactivation of the grape enzyme system and at the same time an increased leaching of colloids into the juice. As a consequence, the addition of exogenous microbial enzymes is necessary for compensation and to ensure processibility.

26.2.2 Juice Treatment

Clarification The must obtained after pressing is naturally turbid, due to a content of insoluble solids, originating predominantly from the berry flesh (pulp). This particulate matter is composed mainly of fruit tissue cells and remnants thereof, cell walls and cell organelles, and clusters of polysaccharides like cellulose with proteins (Vernhet et al. 2016). Soluble pectin that is released from the middle lamella of grape cell junctions is responsible for keeping the solids in suspension. It functions as "protective colloid," a model first introduced by Yamasaki et al. in 1964 for apple juice, and still the accepted view today. The negatively charged pectin polymers build a coating that encloses positively charged insoluble particle clusters and thus keeping them in suspension (Yamasaki et al. 1964). This "metastable" situation is often referred to as the "cloud" in fruit and vegetable processing.

A clear must is the prerequisite of high-quality wines (Ribereau-Gayon et al. 1975). Musts with high turbidity are prone to develop off-flavors during fermentation like a herbaceous or sulfurous aroma and a higher content of yeast metabolites like isoamyl alcohol or acetic acid/acetaldehyde in the wine (Armada and Falqué 2007; Delfini and Costa 1993; Crowell and Guymon 1963). Therefore, must clarification is an important processing step in winemaking, especially for white and rosé wines.

On the other hand, too excessive clarification may lead to fermentation problems (Wucherpfennig and Bretthauer 1970). Insufficient supply of nitrogen sources and unsaturated fatty acids and sterols can negatively influence yeast growth and metabolism and thus cause hampered and even stuck fermentation (Valdés et al. 2011; Malherbe et al. 2007). Furthermore, the lack of solid particles leaves few adherence sites for yeast and lactic acid bacteria, which have a negative impact on gas exchange and growth.

Since many decades, fining agents are used to achieve effective sedimentation of the particulate matter. Examples are bentonite, activated carbon, gelatine, casein, and polyvinylpyrrolidone, often used in combination for synergistic effects. These processing aids are nevertheless not efficient or quick enough, when the colloid amount, foremost pectin, is high. To overcome this problem, industrial pectinases have been used since many years with great success.

All enzymes of the pectinase complex exert a distinct effect in the process of cloud destabilization, leading in combination to the degradation of the negatively charged pectin coat. The result is electrostatic aggregation and the flocculation of the cloud and subsequent clarification of the must. The common characteristic is the action on soluble pectin, particularly homogalacturonan, as substrate.

Pectin/pectate lyase (PL), polygalacturonase (PG), and pectin methylesterase (PME) are involved, as well as accessory enzymes as hemicellulases like xylanase (Table 26.1). Pectin lyase acts on methyl-esterified homogalacturonan stretches, leading to strand breakage. The related pectate lyase recognizes only unmethylated homogalacturonan generated by PME. Both groups have in common to cause a rapid decrease in viscosity, due to a predominantly endo-cleaving action.

Polygalacturonases show a diverse specificity for high or low methylation and also endo and exo activity. Besides the pectinases degrading "smooth" homogalacturonan, several enzymes acting on the branched "hairy" pectin regions contribute to clarification efficiency. Recent research has focussed on the pectin side branches and their effect on technological and sensory parameters. RG-I and AG-II are the most prominent pectin constituents that call for special enzyme activities to achieve degradation. The enzyme systems from fungi, foremost molds, have great potential in this regard (Benoit et al. 2012; van den Brink and de Vries 2011). Several commercial enzymes are commercially available that show activity against pectin side chains, but the quest for more efficient solutions is still ongoing.

As opposed to sedimentation, flotation is an important alternative clarification method. By using pectinases with high portion of pectin methyl esterase, rapid viscosity reduction and simultaneous demethylation of the homogalacturonan are achieved. The resulting negatively charged pectin (pectate) acts as reaction partners with oppositely charged flocculants, causing rapid agglomeration and clarification.

Protein Stabilization Protein is one of the main polymeric constituents found in wines, mainly originating from the grape itself and only to a lesser extent from yeast or other microorganisms. Its concentration may vary from a few milligrams to more than a gram per liter. The protein content is in part prone to instability and may lead to "protein casse," the formation of turbidity or deposit. This protein haze is a major wine quality fault predominantly for white wines, comparable in adverse impact to tartrate crystallization (van Sluyter et al. 2015; Tattersall et al. 2001; Ferreira et al. 2002). The precipitation is induced by shifting conditions, such as in pH, storage temperature, or compositions of phenolic compounds (Siebert et al. 1996; Tattersall et al. 2001). Among polyphenolic substances, proanthocyanidins seem to possess the most inducing power (Koch and Sajak 1959). It may occur during the blending of wines with different chemical properties or after bottling during storage. Haze-forming proteins vary in concentration and composition in ripe grapes and grape juice with cultivar, vintage, disease pressure, and even harvest conditions. Generally, proteins with defensive and pathogenesis-related (PR) functions for the grape berry appear to be susceptible to precipitation. PR proteins share a globular structure and are of low molecular size: chitinases (28–32 kDa), thaumatin-like proteins (20–25 kDa), and lipid transfer proteins (9–12 kDa) (Waters et al. 1996, 1998; Marangon et al. 2011, 2014; Gazzola et al. 2012).

The traditional solution for protein stabilization is fining with bentonite, which is used since the 1930s. Bentonite is a type of clay with a very high water-binding capacity and a negative charge that allow it to function as cation exchanger. Positively charged proteins are adsorbed and can be removed from wine or juice by precipitation and subsequent decanting or filtration. Due to high efficiency and reliability and low costs, bentonite fining is the standard treatment to obtain protein-stable wines throughout the world. Nevertheless, there are some drawbacks of this method. For example, the sediment causes a considerable loss of volume that may be up to 10%, and the loss of aroma compounds as well as color is frequently

reported (Miller et al. 1985; Rankine 1987; Tattersall et al. 2001). The use of exogenous proteolytic enzymes for protein degradation and thus stabilization has been a subject of evaluation since many years (Heatherbell et al. 1984). However, PR proteins display a striking resistance to enzymatic action under normal winemaking conditions (Heatherbell et al. 1984; Waters et al. 1992, 1995). This stability is due to their exceptionally compact conformation, which is enabled by a high number of intramolecular disulfide bonds (Hamel et al. 1997; Marangon et al. 2014). Other proposed mechanisms like phenolic association or glycosylation are not responsible for the resistance to proteases (Waters et al. 1995).

Recently, a new proteolytic treatment has been developed by the Australian Wine Research Institute (AWRI) that seems to accomplish the aim of wine protein stabilization. The use of an acid protease derived from *Aspergillus niger* rich in aspergillopepsin I (EC 3.4.23.18) and aspergillopepsin II (or aspergilloglutamic peptidase, EC 3.4.23.19) in combination with flash pasteurization (75 °C) of the must was demonstrated to remove haze-forming proteins and result in stable wines (Marangon et al. 2012). The heat treatment proved to be essential for protein degradation, because no significant proteolysis was observed in unheated control wines. It was therefore concluded that thermal pretreatment of the grape proteins renders them susceptible to protease attack partial unfolding in the denaturation process. While being a promising option, the requirement of a heat treatment will limit the applicability of proteases to winemakers with the necessary equipment, which is nowadays still an exception. Moreover, heat treatment in white wine processing is often avoided due to the risk of negative sensory effects. However, Marangon and coworkers found no detrimental sensory impact and also reported decent energy input with flash pasteurization treatment. In this regard, instant recooling of the must can have a protective effect. Presently, the use of protease in winemaking is in the application process for OIV approval. In the meantime, the search for proteases that display good activity against native PR proteins is ongoing. Enzymes from *Botrytis cinerea* and yeast have been identified, but no commercial products are available yet (Van Sluyter et al. 2013; Lagace and Bisson 1990).

Aroma Enhancement The organoleptic quality of wine is determined by a multitude of substances that are either already present in the grape (aroma) or develop during fermentation and storage (bouquet). Besides organic acids like tartaric, malic and citric acid, and phenolics, volatile compounds like monoterpenes, thiols, alcohols, and esters are significantly influencing the aroma profile. Monoterpenes are the most important determinants of varietal white wine aroma. The quantity and composition are highly variable among the different grape cultivars from, for example, highly aromatic varieties like muscatel or Traminer to more restrained types from the Pinot family. Dominant substances are, e.g., linalool, nerol, geraniol, and α-terpineol, as well as a large number of closely related derivatives. The predominant part of the monoterpenes is present in the grape in a sugar-bound form as glycosides (Maicas and Mateo 2005; Black et al. 2015), which are nonvolatile and thus nonaromatic. The sugar moieties are predominantly diglycosides that

contain D-glucose and several other monosaccharides like L-arabinose, L-rhamnose, D-apiose, or D-xylose connected by α- or ß-linkages, but also monoglucosylated terpenes may be present.

The glycosidic conjugates are essentially the aroma reservoir of the wine. Usually the glycosides outnumber the free terpene aglycones by several magnitudes (Günata et al. 1985, 1990; Arévalo-Villena et al. 2006; Flamini et al. 2014). For this reason, winemakers strive to exploit this aroma pool by using enological practices. The application of microbial β-glycosidases has been established as the most simple and effective method for aroma increase during the past years (Günata 2002). Glycosidase enzymes of fungal origin are the common products on the market, either as main activity or as side activity accompanying pectinases produced by *A. niger*. Enzymes of fungal origin show significantly higher activity under winemaking conditions than other glycosidases from *Vitis vinifera* and *Saccharomyces cerevisiae*. For efficient aglycone release, enzymes must work sequentially in a two-step hydrolysis reaction (see Fig. 26.1). Firstly, β-apiosidase, α-rhamnosidase, α-arabinosidase, or β-xylosidase removes the terminal sugar, and then β-glucosidase cleaves the glucose from the aglycone. The first fungal enzyme displaying this set of enzymes used in enology for aroma increase was Rapidase AR2000 from Gist-Brocades/DSM. Obviously, the quality of the enological enzyme preparation strongly depends on the composition and ratio of these different functionally related enzymes. Furthermore, a one-step hydrolysis of diglycosides has been demonstrated for plant and fungal enzymes (Ogawa et al. 1997; Günata et al. 1998, Šimčíková et al. 2015) (Fig. 26.1). The efficacy of

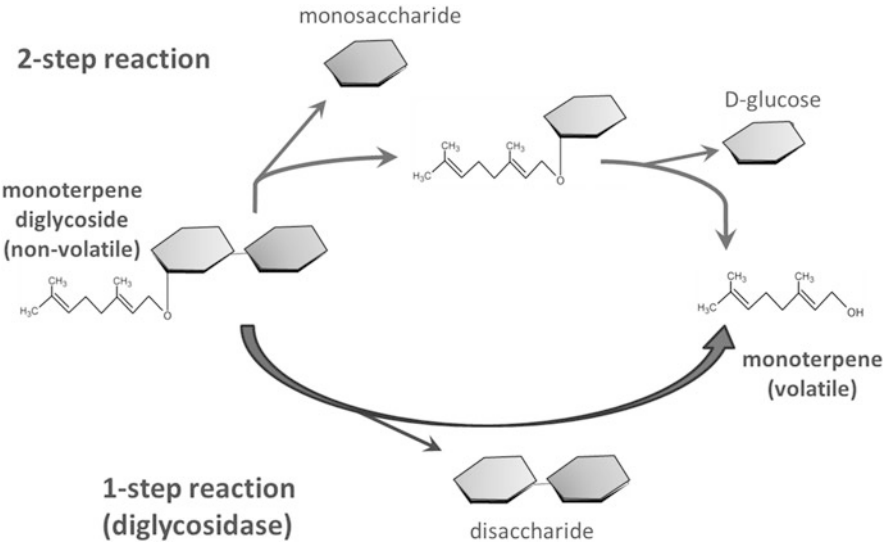


Fig. 26.1 Representation of the glycosidase action on monoterpene diglycosides. The sequential action of two enzymes is depicted opposed to the diglycosidase reaction

glycosidase application in winemaking is well documented for many grape varieties (Maicas and Mateo 2005; Black et al. 2015). Besides monoterpenes, also other aroma substances like C-13 norisoprenoids, such as 3-oxo-α-ionol and β-damascenone, benzene derivatives, or aliphatic alcohols, have been demonstrated (Günata 2002).

Several features of aroma enzymes have been subject of debate in the recent past. Firstly, glycolytic enzymes are typically prone to feedback inhibition by glucose to a significant degree. This has limited the use of aroma enzymes in winemaking to dry wines, where the enzyme dosage is applied at the end of fermentation when the sugar has to a large extent been consumed by the yeast. However, there is actually a need for glycosidases that are more sugar tolerant and thus suitable for the production of sweet wines. For example, Trenolin® Bouquet ^{PLUS} (Erbslöh) has been developed to overcome this drawback. Secondly, glycosidases, especially produced by *A. niger*, are usually accompanied by cinnamoyl esterases. This group of enzymes poses a risk due to the potential development of phenolic off-flavors (see Sect. 26.3.1). Thirdly, many glycosidases can also use anthocyanins as substrates, leading to the loss of color in red or rosé wines (see Sect. 26.3.2). Obviously, there is considerable potential to improve existing enological aroma enzymes regarding efficiency and avoidance of unwanted side effects.

26.2.3 Wine Treatment

Improvement of Filterability Pectic polymers or remnants thereof are potent disturbing factors in juice and wine filtration leading to filter blocking and membrane fouling (Vernhet et al. 1999; Vernhet and Moutounet 2002). The application of the aforementioned array of pectinase enzymes for clarification/settling will be sufficient to allow economical filtration in the majority of cases. Nevertheless, depending on the grape material and winemaking process, other colloids of microbial origin may be present. Bacterial glucans from lactic acid bacteria, for example, *Pediococcus damnosus* causing ropy wines, only play a minor role with regard to filtration problems (Llaubères et al. 1990). In contrast, β-glucan, produced by molds, especially the noble or gray rot fungus *B. cinerea*, is particularly deleterious to filtration. *B. cinerea* produces a high-molecular-weight glucan termed cinerean of up to 800 kDa that consists of a β-1,3-glucan backbone with single glucose monomers connected via β-1,6 linkages to every third monomer in the backbone (Montant and Thomas 1977; Dubourdieu et al. 1981). Cinerean's high gelling capacity hampers clarification and causes filtration problems, especially in the presence of alcohol due to partial denaturation. It is resistant to degradation by the majority of endogenous and exogenous enzymes and calls for specific β-1,3/1,6 glucanases (Martin et al. 2007). Primarily glucan exo-β-1,3-glucosidase (E.C.3.2.1.58) in conjunction with accessory exo-acting enzymes is the key enzyme in cinerean degradation. The successful use of fungal glucanases for the improvement of wine filtration has already been shown more than 30 years ago (Villettaz

et al. 1984). Surprisingly, the only efficient "*Botrytis* glucanases" available for winemaking today are derived from very limited amount of production strains. *Trichoderma longibrachiatum* and *Trichoderma harzianum* were a long time the only sources for products like Glucanex (Novozymes); meanwhile other organisms like *Talaromyces versatilis* (former systematic name *Penicillium funiculosum*) have emerged as potent producers. The conditions prevailing during winemaking do not significantly affect this class of enzymes. Tannins, low pH, and ethanol content have been shown to only exert a varying but in general minor reduction of enzyme activity, depending on the origin of the enzyme (Humbert-Goffard et al. 2004; Zinnai et al. 2010; Villettaz et al. 1984). However, the slow exo-acting glucan degradation causes prolonged processing times. To compensate this drawback, recommended dosages tend to be relatively high, and the application before alcoholic fermentation can help to avoid ethanol inhibition. The development of an effective endo-acting glucanase could significantly improve the efficacy of this type of enzyme.

The determination of activity for this specific enological treatment has been the subject of extensive debate. To evaluate the effectiveness of glucanases for the degradation of cinerean, the original substrate is indispensable, but cinerean is very hard to produce in reasonable amounts with reasonable effort. Therefore, the structural analogue schizophyllan, a polysaccharide from basidiomycete fungus *Schizophyllum commune*, was proposed as a substrate for an official OIV activity assay. This glucan is far easier to produce and constitutes an ideal substitute.

Sur Lie Treatment The aging of wines on the yeast deposit or lees has a long tradition and is referred to as sur lie method or bâtonnage. The traditional application was in white wine and sparkling winemaking, but it has developed into a popular practice for all types including red and rosé wines. This post-fermentative treatment aims to influence the organoleptic character of the wine, i.e., increasing the body, mouthfeel, and creaminess but also effects stability and aging potential. During aging, the yeast cells undergo autolysis, and cell wall fragments and intracellular components are released into the wine (Feuillat 2003). Mannoproteins and glucans are the major macromolecular substances released by endogenous yeast enzymes, and also smaller peptides and amino acids are liberated. Mannoproteins are considered as most influential for the desired sur lie effects (Caridi 2006). This class of heterogeneous glycoproteins consists of diverse protein components linked with branched mannan chains of up to 200 sugars (Herscovics and Orlean 1993). Via β-1,6-glucan linkers, the proteins are connected to the β-1,3-glucan network of the yeast cell wall, which constitutes 30–60% of the dry matter (Lesage and Bussey 2006; Orlean 2012). This essential structural and functional element is the point of attack of enological sur lie enzymes. The degradation of β-glucan is significantly enhanced by using exogenous β-glucanases; thus the considerable time needed for the natural autolysis process, which can range from several months to over a year, can be drastically reduced. This not only saves expensive storage time/capacity but also reduces the risk of the formation of off-flavors, microbial spoilage, or other detrimental changes like

oxidation. β-glucanases have been used in winemaking for several years for this purpose (Rodriguez-Nogales et al. 2012). Commercial enzymes are a mixture of different glucanolytic enzyme fractions that act on the fungal substrate, mostly combined with other wine-relevant enzyme activities like pectinases. Most importantly laminarinase (glucan endo-1,3-β-glucosidase, EC 3.2.1.39), glucan 1,3-β-glucosidase (EC 3.2.1.58), and pustulanase (glucan endo-1,6-β-glucosidase, EC 3.2.1.75) are part of sur lie enzyme preparations. In synergistic action, these enzymes degrade the branched polymer to glucose, thereby releasing the mannoproteins. These enzymes are frequently produced with fungal strains like *T. longibrachiatum* and *T. harzianum*, but also several other production organisms express useful biocatalysts for this purpose, like, for example, *T. versatilis* (formerly *P. funiculosum*).

While the organoleptic benefit of increased mannoprotein levels in wines is undisputable, the technological implications are often less evident. For example, the effect on tartaric acid stabilization has been a continuing matter of debate (Guise et al. 2014). Also proposed beneficial effects for color stabilization could not be induced by increased mannoproteins levels by using exogenous β-glucanase preparations (Palomero et al. 2009). In general, commercial β-glucanase enzymes do not contain relevant amounts of β-1,4-glucosidase; thus the destabilization of anthocyanins will not occur in red wine applications (see Sect. 26.3.2).

Urease Ethyl carbamate is a potent carcinogen that can develop in wine during storage and aging. Especially in the production of baked sherry, high levels can occur. It is formed from the reaction of ethanol with urea, which is produced by wine yeast as the end product of arginine metabolism (An and Ough 1993). Several other minor synthesis routes are possible, for example, by conversion of citrulline or carbamyl phosphate stemming from lactic acid bacteria during malolactic fermentation (Ough et al. 1988). As preventing measure to reduce ethyl carbamate formation, the use of urease (urea amidohydrolase EC 3.5.1.5) was originally first used in the production of sake. Urease cleaves the carcinogen into carbon dioxide and ammonia and is commonly produced by *Lactobacillus fermentum*. Since several years, it is also an approved enological practice with Nagapsin (Nagase) as commercial enzyme. This is a rare example of the use of a bacterial enzyme in winemaking, due to the exceptionally good activity at wine pH.

Lysozyme *N*-acetylmuramide glycanhydrolase (EC 3.2.1.17) or muramidase is an enzyme serving as antibacterial defense mechanism in mammalian body fluids like saliva, tears, or milk, which was identified by Alexander Fleming in 1922 (Sim and Nakai 1994; Liburdi et al. 2014). Commercial enzymes are almost exclusively isolated from chicken egg white. It cleaves the β-1,4-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine in bacterial peptidoglycan and is therefore effectively destabilizing the gram-positive cell wall, leading to cell lysis. In winemaking, the use of lysozyme is an OIV-approved technique to prevent the growth of lactic acid bacteria particularly from genera *Lactobacillus* and *Pediococcus* in wine but has no inhibitory effect on wine yeast (Bartowsky 2009; Liburdi et al. 2014). The lactic acid bacteria can produce substances leading to

quality defects like mousy taint, volatile acidity, ropy exopolysaccharides, and overt buttery characters. *Oenococcus oeni* is also susceptible to lysozyme action; therefore the addition should be done after the malolactic fermentation has ended—or before in case it should be avoided. Classically, lysozyme is added in the final wine. The dosages need to be quite high due to limited activity in wine conditions. The optimal pH is in the neutral range, so distinctly above the pH in wines, especially white varieties (Davies et al. 1969). Therefore, dosages of 250–500 ppm are frequently used, which means an introduction of considerable amounts of enzyme protein into the wine. There have been several reports of high reactivity with wine polyphenols and associated negative effects like color loss and haze formation (Gerbaux et al. 1999; Bartowsky et al. 2004). Furthermore, the classification of lysozyme preparations from hen egg white as food allergen with the implication of mandatory labeling has further complicated the application in winemaking; therefore the use of this antibacterial enzyme remains a niche. Meanwhile, the search for efficient bacteriolytic enzymes of microbial origin continues (Callewaert et al. 2011). Up to now, reports of promising microbial alternatives to hen egg white lysozyme did not result in commercially available enzymes yet (Blättel et al. 2009; Sebastian et al. 2014).

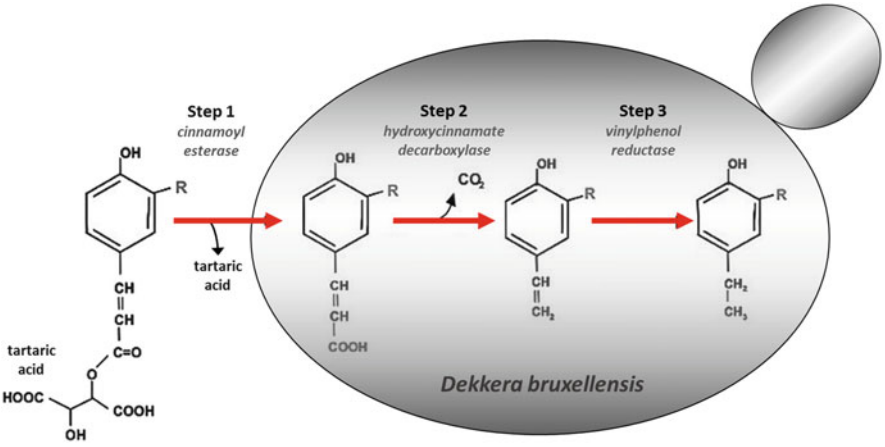
26.3 Enzymes with Negative Effect on Wine Quality

26.3.1 Cinnamoyl Esterase

Enzyme preparations produced by *A. niger* very often contain enzymatic side activities termed cinnamoyl esterase or "depsidase" that are known to cause negative sensorial effects in the final wine in interaction with microbial wine-associated organisms (Hasselbeck 1997). The off-flavors are described as barnyard, smoky, or medicinal taints and are caused by volatile phenols that are derivatives of hydroxycinnamic acids naturally present in the grapes. Therefore, the absence of cinnamoyl esterase is generally viewed as an important quality criterion of enological enzymes (OIV/OENO 6/2007), and much effort has been invested by enzyme manufacturers to separate this enzyme fraction from the main activity. The purification processes increase the costs of enzyme production considerably. Nowadays most enological enzymes contain reduced levels of cinnamoyl esterase, but there are frequent exceptions. Especially when cheaper enzyme products for fruit juice treatment are used in winemaking, the chance is high that cinnamoyl esterases end up in the process.

Several types of esterases, named cinnamoyl esterases, are found in high concentrations in commercial enzymes, especially bulk pectinases (Burkhardt 1976; Barbe and Dubourdieu 1998). Cinnamoyl esterases are assigned to the classes chlorogenic acid hydrolase (EC 3.1.1.42) and ferulic acid esterase (EC 3.1.1.73). For the fungal organism, this group of enzymes is an auxiliary means for the

utilization of plant substrates like xylan or lignocellulose (de Vries et al. 2002). In grape juice, they act on tartaric acid esters of the phenolic acids caffeic, p-coumaric, and ferulic acid present in considerable concentrations of up to several 100 mg/L in the grape (Ali et al. 2010; Ong and Nagel 1978; Singleton 1961). This esterase action is only the first step in a sequence of reactions (see Fig. 26.2). The liberated hydroxycinnamic acids are prone to conversion into volatile phenols by wine-associated microorganisms. Numerous strains of phenolic off-flavor positive (POF+) *S. cerevisiae* and also non-*Saccharomyces* yeasts like *Dekkera/Brettanomyces bruxellensis* are able to produce the corresponding vinylphenols by a hydroxycinnamic acid decarboxylase activity, but also wine-associated lactic acid bacteria have been demonstrated to express the relevant enzymes (Harris et al. 2009; Silva et al. 2011; de las Rivas et al. 2009; Edlin et al. 1998). The perception threshold for the products 4-vinylphenol, 4-vinylguaiacol, and 4-vinylcatechol is quite low, generally below 100 µg/L (Parker et al. 2012). Small amounts can be perceived as a positive spicy, smoky aroma impression, but the threshold to a negative impact is small. Vinylphenols can be reduced to the corresponding ethyl derivatives either by chemical reaction or by action of vinylphenol reductase of *Dekkera bruxellensis* (Chatonnet et al. 1992b; Tchobanov et al. 2008). The resulting 4-ethylphenol, 4-ethylguajacol, and 4-ethylcatechol have slightly higher detection thresholds, but more negative sensory attributes (Table 26.2). Barnyard, horse sweat, and phenolic are common, predominantly negative, descriptors. Red



Ester	Hydroxycinnamic acid	Hydroxy styrene	Ethyl derivates
R = H: *p*-coutaric acid	*p*-coumaric acid	4-vinylphenol	4-ethylphenol
R = OCH: fertaric acid	ferulic acid	4-vinylguajacol	4-ethylguajacol
R = OH: caftaric acid	caffeic acid	4-vinylcatechol	4-ethylcatechol

Fig. 26.2 Generation of volatile phenols from hydroxycinnamic acid esters. A sequence of reactions involving cinnamoyl esterase from enzyme preparations and subsequent microbial transformations

Table 26.2 Volatile phenols commonly found in wines

Phenol	Odor impact	Sensory threshold in wine or water (μg/L)	Content in wines (μg/L)
4-Vinylphenol	Pharmaceuticals, gouache paint and "Band-Aids"	180	White 73–1150 Red 0–111
4-Vinylguajacol	Carnation	40	White 15–496 Red 0–57
4-Ethylphenol	Phenolic, barnyard, sweaty saddles	605	White 0–28 Red 1–6047
4-Ethylguajacol	Smoke, spicy	110	White 0–7 Red 0–1561

Data derived from Chatonnet et al. (1992a, b, 1993), Curtin et al. (2007), Culleré et al. (2004), and Guth (1997)

wines display in general a higher ratio of ethylphenols to vinylphenols, about 10:1, while white wines are characterized by low levels in ethylphenols and high levels of vinylphenols. The increased maceration as compared to white wines is one factor explaining the higher hydroxycinnamic acid content in red wines (Goldberg et al. 1998). Even at concentrations below the sensory threshold (Table 26.2), volatile phenols can mask the fruity scent of white wine and therefore reduce the aroma impression. The velocity and extent of volatile phenol release are mainly determined by the action of cinnamoyl esterase in the first step of the reaction sequence. CE activity is seldomly found in wine-associated microorganisms. Very recently, this bottleneck activity was demonstrated for certain strains of *O. oeni* (Chescheir et al. 2015), but the practical implications remain elusive. Therefore the addition of exogenous enzymes, especially pectinases, during winemaking is the most important cause of significantly increased volatile phenol content of final wines (Chatonnet et al. 1992a; Dugelay 1993). Besides being potent aroma substances in their free form, volatile phenols and also their precursor hydroxycinnamic acids frequently combine with anthocyanins in copigmentation reactions to form pyranoanthocyanins like pinotin or portisin (He et al. 2012; Ferment et al. 2009). The concept of combined use of cinnamoyl esterase-containing enzyme with a POF + yeast strain has been introduced by the company DSM (Rapidase® Maxifruit/Fermicru® XL) to achieve improved color stability right after fermentation. However, the formed pyranoanthocyanins display predominantly an orange hue, which is generally an undesired color for young red wines.

26.3.2 Anthocyanase

The detrimental effect of some enzyme preparations on colored fruit products has been known for a long time (Huang 1955). Anthocyanins, responsible for the color of red wines, and also most other plants with red-blue color, are composed of the

aglycone anthocyanidin that is connected to a sugar moiety, predominantly glucose. If the sugar is removed, the aglycone is prone to conversion to brown or colorless derivatives. The so-called aroma enzymes (β-glycosidases) from *A. niger* have been reported multiple times to exert a decolorization effect in red wines by deglycosylation of anthocyanins and were termed "anthocyanase" (Fumian et al. 1994; Wightman et al. 1997). It could be demonstrated that different beta-glucosidase isoenzymes from *A. niger* had differing specificity for anthocyanins and had thus different impact on color (Le Traon-Masson and Pellerin 1998). Therefore, the choice of enzyme should be carefully made if color loss has to be avoided. For some applications, where a lighter color is requested, the use of anthocyanase is a potential solution.

26.3.3 Polyphenol Oxidase

Polyphenol oxidases (PPO) cause oxidative browning of phenolic compounds of the grape (Macheix et al. 1991). Two types of PPO are commonly found in grape juice. Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme of grape origin that shows quick inactivation by sulfite and negligible activity in the presence of carbon dioxide in red wine mash fermentation. Therefore, tyrosinase is not of great importance during winemaking. In contrast, laccase (EC 1.10.3.2) is a copper-dependent enzyme of fungal origin that is introduced into the juice by mold-infected grape material. Prominent producer is *B. cinerea*. It is not inhibited by sulfite and highly active at vinification temperatures. To achieve complete inactivation, harsh heat treatment with temperatures above 90 °C and sufficient holding time must be applied. Therefore, stringent quality control of the harvested grapes is a common measure to prevent browning effects effectively. In modern wineries, monitoring of the grade of mold infection is a common practice.

26.4 Recent Developments and Future Options

Real innovation in the development of new wine enzymes is rare, despite many interesting problems and obvious demand. The reasons for this are on the one hand the general rejections of enzymes from genetically modified microorganisms, which provide tremendous potential that remains unusable. On the other hand, the list of OIV-approved enzyme activities in winemaking is restricted, so that new enzymes need to pass a long and cumbersome approval process. However, several topics are currently discussed. As mentioned before, a microbial lysozyme or equivalent antibacterial enzyme would provide a solution for the allergenic and animal-derived egg lysozyme, which is also not allowed for vegan winemaking. Furthermore, enzymatic solutions for preventing or curing off-flavors or other negative organoleptic impressions like cork taint, astringency, pinking, and others

are interesting. The tremendous progress in molecular genetics to screen for candidate enzymes in genomes and also metagenomes (Sathya and Khan 2014) opens up a whole new world for enzyme development. Additionally, the efficient genetic engineering of productions strains has vastly improved. However, increasing regulatory demands for toxicological and safety assessment of food enzymes, and the resulting costs for enzyme registration, will lower the enthusiasm for cutting-edge future developments.

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Chapter 27
Mass Spectrometry: A Powerful Tool for the Identification of Wine-Related Bacteria and Yeasts

Birte Meyer, Andreas Rabenstein, and Jan Kuever

27.1 Introduction

Historically, traditional methods for identifying microorganisms in microbiology laboratories are based on microscopic and biochemical methods (phenotyping) and gene sequencing identification techniques (genotyping). Bacterial phenotypes can be determined by assessing the morphology of bacterial colonies on solid media surfaces, gram staining, biochemical/metabolic patterns, immunology-based assays, and antibiotic susceptibility. However, these procedures take considerable time and have been shown to suffer from error-prone results, indistinct reactions of closely related strains, and limited or outdated databases. As a new reference standard, discrimination of strains based on comparison of genetic variation is now widely used to classify bacteria by techniques of DNA fingerprinting, DNA sequence information, and microarrays. DNA fingerprint-based methods analyze patterns of DNA bands (fragments), which are generated by digestion of genomic DNA using restriction enzymes, amplification of DNA, or by a combination of both (e.g., pulsed-field gel electrophoresis, PFGE; restriction fragment length polymorphism, RFLP; multilocus variable number tandem repeat analysis, MLVA, etc.). These genotyping methods can provide accurate, quantitative information about the unknown microorganism, but they are time-consuming, laborious, technically demanding, and expensive (e.g., microarrays) and require expert knowledge. Indeed, 16S rRNA and 18S rRNA gene sequencing have become the new gold standard for universal molecular identification of bacteria and fungi, respectively, with their discriminatory power for species-level determination. However, this method is employed primarily by large clinical and reference laboratories for

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confirmatory testing and in research as they require specialized instrumentation and dedicated laboratory space and staff. Therefore, the use of automated instruments for the phenotypic analysis of bacterial isolates (e.g., VITEK 2, BioMérieux, France) predominated as the basis for routine microbial identification particularly in routine clinical diagnostics until recently.

In the past decade, the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been established as a new reliable, rapid, and inexpensive automatable method for identifying a wide array of bacteria, archaea, fungi, dermatophytes, and even viruses (for an overview of the workflow, see Fig. 27.1). Noteworthy, the use of MALDI with biological macromolecules by Tanaka and coworkers was awarded with a shared Nobel Prize in Chemistry in 2002, and whole-cell protein profiling by MALDI-TOF MS was merited the designation of the "revolutionary technique" in 2009 (Seng et al. 2010; Posterano et al. 2013). Its "molecular-phenotypic"-based methodology is based on the reproducible detection of protein mass patterns (proteomic profile) obtained from whole cells, cell lysates, or crude bacterial extracts. Microbial MALDI-TOF MS mass spectra can be regarded as snapshots of the protein composition of the strains under study. Many of the mass spectral signals have been assigned as high-abundance proteins with housekeeping functions, such as basic ribosomal proteins or

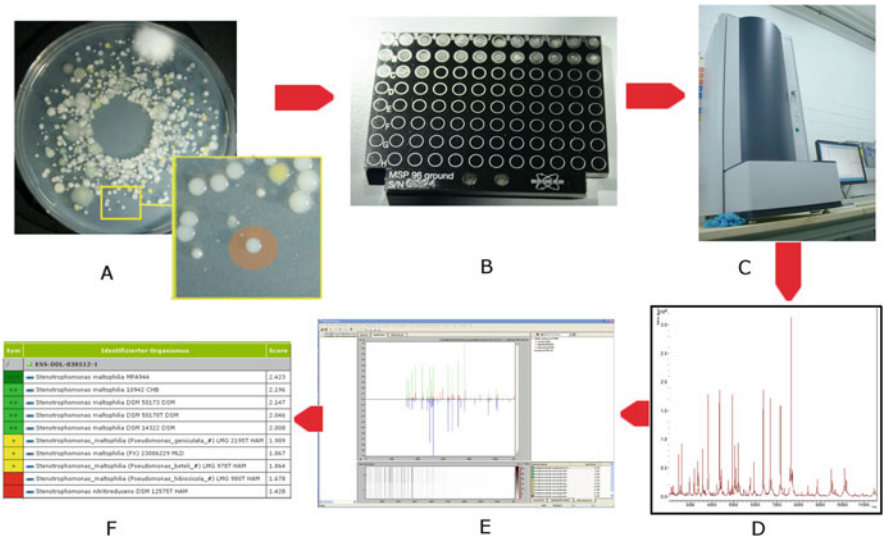

Fig. 27.1 Routine workflow for MALDI-TOF MS microbial identification: (**A**) cell material (e.g., single colony) is directly deposited on target plate (alternatively, cell lysate is transferred); (**B**) sample is overlaid with matrix solution and air dried; (**C**) measurements conducted in the MALDI-TOF MS instrument; (**D**) a sample-specific mass spectrum is obtained; (**E**) microbial identification through automatic matching of the measured mass spectrum with reference spectra in the database; (**F**) output of identification results as species lists with scores of confidence

DNA-binding proteins. These proteins are highly conserved and consistently expressed under nearly all growth conditions. They can thus be regarded as robust biomarker candidates of the respective microorganism and their identification. As ribosomal protein genealogies mirror the rRNA genes reconstructed phylogenies, the mass spectra show congruency with the genealogy of microorganisms, and hence, can be implemented as valuable analytical tool for polyphasic approaches in microbial systematics.

For microbial characterization by MALDI-TOF MS, the protein mass spectra can be analyzed in two principally different ways which are accompanied by different types of sample preparation, instrumentation, and required data processing (illustrated in Fig. 27.2). In the library- or database-based approaches, mass peak tables from unknown bacterial strains are matched against libraries with validated microbial reference spectra. These methods are popular because of their ease of use and the high speed of data collection (in its simplest form only a small amount of intact cells, typically a fraction of a single colony from culture plates, is required for

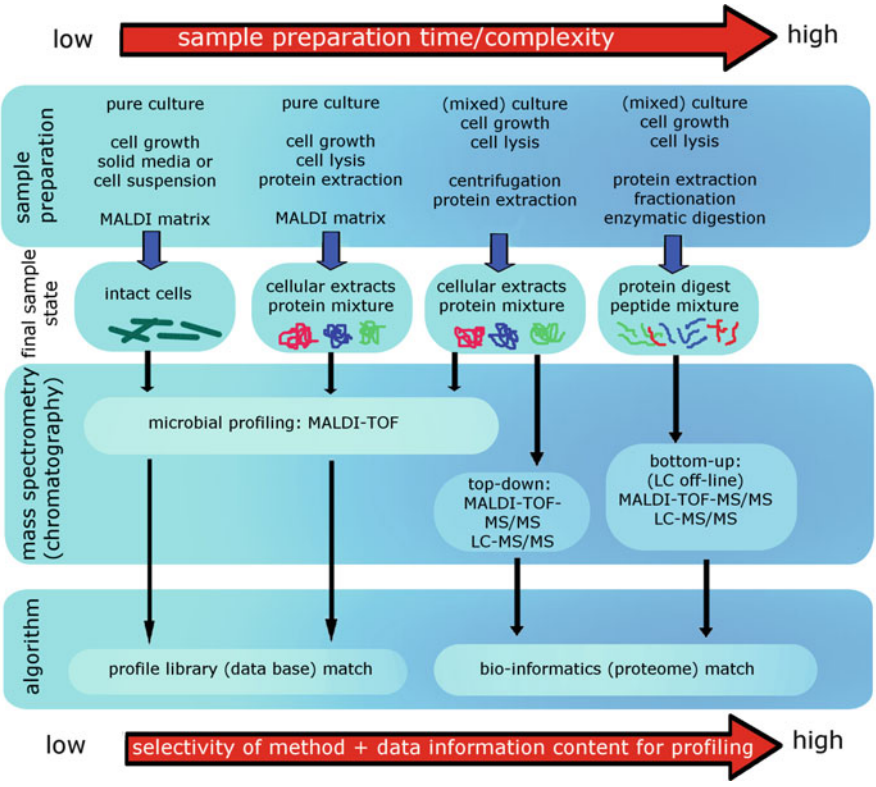

Fig. 27.2 MS-based approaches for microbial proteome profiling (relationship between each method, its sample preparation complexity, and resulting mass spectrum data information for microbial typing is shown)

analysis and deposited directly on metal plate); such methods do not require the precise knowledge of biomarker identities; however, they require the use of rigorously standardized experimental conditions during analysis to ensure for accuracy and reproducibility of the results. Alternatively, if standard mass spectral databases are not available or incomplete, experimentally obtained protein masses from pure microbial samples may be identified by matching to a proteome database predicted from genome sequence data. In contrast to library-based methods, these bioinformatics-enabled approaches identify peaks in MALDI profiles as particular proteins from publicly available databases with genome sequence data to characterize unknown bacteria. Two strategies have been used: a top-down and a bottom-up method. For the top-down method, intact (undigested) proteins are introduced into the mass spectrometer after cell lysis and protein extraction. The intact proteins are fragmented into smaller peptides by gas phase fragmentation (tandem MS or MS/MS) and the observed ions in the tandem mass spectrum are then matched to expected fragmentation patterns of proteins contained in a proteome database. The selectivity of this proteomics approach can be enhanced (i.e., more proteins detected) by the use of a liquid chromatography (LC) separation/fractionation step followed by MS/MS of the intact protein ions. However, this comes with additional sample preparation steps and the need for specialized MS instrumentation allowing for the fragmentation of large protein ions and their analysis to provide meaningful database search results. The second, bottom-up method involves site-specific enzymatic digestion of the proteins to create complex peptide mixtures that are analyzed by LC-MS/MS. The acquired tandem mass spectrum for each peptide is then matched via database search to the protein originating this peptide, and the identified proteins can be used as biomarkers to identify bacteria. As long as the ions of biomarkers are consistent with the sequences in the database, the spectral reproducibility is not critical in bioinformatics-based approaches. Thus, strictly standardized experimental protocols to the extent required by library-based methods are not needed. Indeed, the increased level of complexity for the sample preparation and/or analysis steps for top-down and bottom-up approaches results in the highest degree of selectivity of all MS-based methods for proteomic profiling, as mixtures of microbial strains can, in principle, be identified, regardless of growth conditions. However, bioinformatics-enabled approaches cannot be applied to microorganisms that do not have fully sequenced genomes and readily available protein/peptide databases, limiting currently their utility for microbial typing of unknown microorganisms. In addition, they have significantly greater hardware and software demands (typically, expensive TOF/TOF or other MS/MS capable instruments) and require more time, labor, and sophisticated training.

Considering the more widespread application of library-based approaches to profile microorganisms at the species and even subspecies level, this review will focus on challenges and limits associated with the library-based MS profiling technique and its real-world application of identifying microbes involved in the winemaking process.

27.2 MS and MALDI-TOF MS: A Brief History

Laser ionization MS has been extensively studied and used for several decades in chemistry, but in 1975, Anhalt and Fenselau (1975) employed pyrolysis-MS to pioneer the use of mass spectrometry in microbial identification (Anhalt and Fenselau 1975) (Fig. 27.3). By directly inserting lyophilized cells into a double-focusing mass spectrometer, they noticed that unique mass spectra were produced from the bacterial extracts of different genera and of different species. However, these experiments suffered from irreproducible results due to variabilities caused by growth conditions and media. In the 1980s, the development of desorption/ionization techniques such as plasma desorption (PD), laser desorption (LD), and fast bombardment (FAB) allowed the generation of molecular biomarker ions from microorganisms leading to bacterial profiling (Heller et al. 1987; Platt et al. 1988). In early experiments, only biomarker molecules of low molecular masses, such as bacterial lipids, were analyzed (Heller et al. 1988), as the processes used for ionization of biomolecules were too energy-rich to avoid unpredictable analyte decomposition. In 1987, Karas et al. (1987) demonstrated that the use of an appropriate matrix for absorbing the laser energy could reduce the required photon intensity for desorption by an order of magnitude (thereby, eliminating the issues of fragmentation during the process) and named the process "matrix-assisted laser desorption/ionization" or MALDI (Karas et al. 1987). The introduction of this "soft

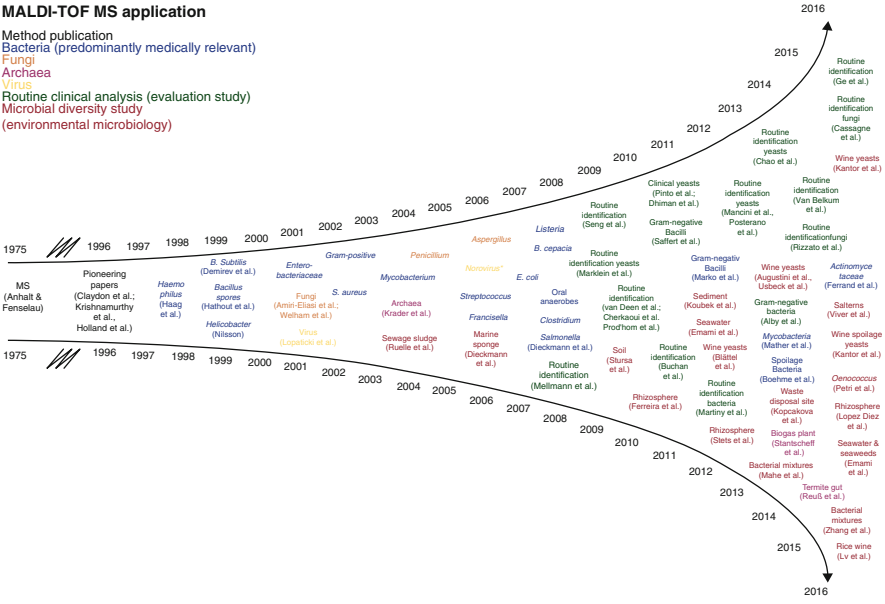

Fig. 27.3 Timeline for MALDI-TOF MS development in microbial profiling: increasing number of publications related to its application in medical and environmental microbiology (highlighted in *different colors*)

ionization" technique finally removed the volatility barrier for MS, since it allowed the analysis of large biomolecules such as intact ribosomal proteins. The new potential of MS triggered the development of improved time-of-flight (TOF) instrumentation particularly tailored for the MALDI ionization technique, which subsequently enabled the observation of proteins of masses >100 kDa in the late 1980s (Hillenkamp 1989). This catalyzed new interest in the application of MS to microbial identification. However, the resolving power obtained in this initial work was rather low due to ion fragmentation in flight. Shortly after the discovery of MALDI, the first practical MALDI-TOF mass spectrometer for high mass-to-charge ratios (m/z) was built by Beavis and Chait (1989) and included a simple linear time-of-flight (TOF) as mass analyzer which offered the advantage that the ions dissociating in flight arrive at nearly the same time. The further development of TOF-MS has been driven by advances in hardware and electronics that improved resolving power and allowed high-speed data acquisition over a broad mass range. In 1994, Cain et al. (1994) reported that MALDI-TOF MS could be used to differentiate selected bacteria by analysis of protein profiles from disrupted cells. Two years later, Claydon et al. (1996), Holland et al. (1996), and Krishnamurthy et al. (1996) demonstrated for the first time that MALDI-TOF spectral fingerprints could be obtained from whole bacterial cells without pretreatment before the MS analysis. This approach was then used to identify diverse medically and environmentally relevant bacteria at the genus and species levels by multiple research teams [e.g., (Haag et al. 1998; Hathout et al. 1999; Nilsson 1999) and others summarized in Seng et al. (2010) and Croxatto et al. (2012)] resulting also in the description of several group-specific protocols for sample preparation and experimental conditions. Since 2000, its applications were further extended to characterization of archaea (Krader and Emerson 2004), fungi (Welham et al. 2000; Amiri-Eliasi and Fenselau 2001), viruses (Lopaticki et al. 1998; Kim et al. 2001), and even multicellular organisms including nematodes (Perera et al. 2005). Until today, the capability of MALDI-TOF MS to rapidly characterize microbes favored its use in multiple areas including medical diagnostics, biodefense, environmental monitoring, and food quality control (Giebel et al. 2010; Seng et al. 2010; Croxatto et al. 2012; Posterano et al. 2013; van Belkum et al. 2015; Santos et al. 2016; Spitaels et al. 2016). In several clinical studies, MALDI-TOF MS has been demonstrated to be suitable for high-throughput and rapid microbial identification at low cost (Seng et al. 2009; Cherkaoui et al. 2010; Dhiman et al. 2011; Ge et al. 2016), with equal, if not even superior, performance when compared to conventional biochemical and molecular identification systems (Marklein et al. 2009; Seng et al. 2009; Cherkaoui et al. 2010; van Veen et al. 2010; Buchan et al. 2011; Dhiman et al. 2011; Pinto et al. 2011; Saffert et al. 2011; Chao et al. 2014) and is now used worldwide as routine diagnostic tool especially in medical microbiology laboratories.

27.3 MALDI-TOF MS Instrumentation and Technique

A mass spectrometer is composed of three functional units: (1) an ion source to ionize and transfer sample molecules ions into a gas phase, (2) a mass analyzer that separates ions according to their *m*/*z* ratio, and (3) a detector to monitor separated ions (Fig. 27.4). The first two components define the capabilities of any MS instrument by the type of ionization and the mass analysis device (Sauer and Kliem 2010; Croxatto et al. 2012; Clark et al. 2013; Basile and Mignon 2016).

The method of ionization is determined according to the nature of the sample and the goal of MS analysis; thus, several ionization methods have been developed (including PD, FAB, chemical ionization, etc.). MALDI (as well as ESI) is a soft ionization technique that allows ionization and vaporization of large nonvolatile biomolecules such as intact proteins without inducing fragmentation. In the "intact cell MS" (ICMS) method, the microorganisms can be directly processed without pretreatment since vegetative bacteria are lysed following exposure to water, an organic solvent, and/or strong acids. Samples are prepared for MALDI-MS by adding a saturated solution of low-mass, acidic organic compounds, termed the matrix, in excess to the analyte molecules, and the mixture is then spotted onto a conductive metal plate (termed the "target plate") for analysis. Selection of the matrix influences the specific biomarkers that are detected (Table 27.1; SA, FA, and CHCA used preferentially for proteins). Upon drying, the organic compound forms

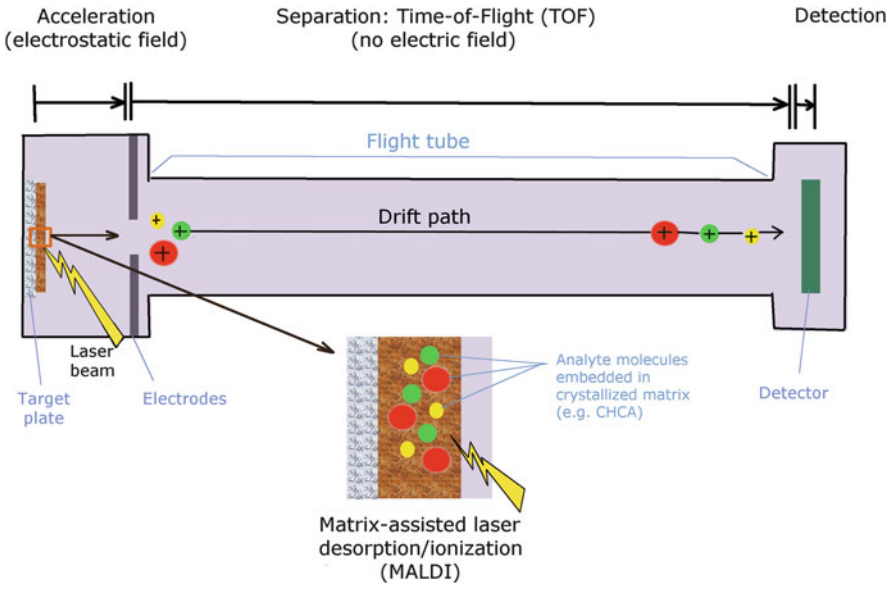

Fig. 27.4 Principle of MALDI-TOF MS identification of bacteria and fungi in schematic diagram

Table 27.1 List of common matrices used for UV-MALDI MS methods

Chromophore matrix		Sample type(s) analyzed
Picolinic acid 3-hydroxypicolinic acid 3-aminopicolinic acid	PA HPA APA	Oligonucleotides, DNA, biopolymers
2,5-dihydroxybenzoic acid	DHB	Oligosaccharides
α-cyano-4-hydroxycinnamic acid	CHCA	Proteins, peptides
3,5-dimethoxy-4-hydroxycinnamic acid	SA	Proteins
Ferulic acid	FA	Proteins
2-(4-hydroxyphenylazo)benzoic acid	HABA	Peptides, proteins, glycoproteins
2-mercaptobenzothiazole	MBT	Peptides, proteins, synthetic polymers
2,6-dihydroxyacetophenone	DHAP	Glycopeptides, phosphopeptides
2,4,6-trihydroxyacetophenone	THAP	Oligonucleotides

a heterogeneous crystalline matrix that surrounds and isolates individual analyte molecules in the original sample. It acts both as a scaffold by which ionization can occur and as a supplier of protons for ionization. After loading the metal plate into the instrument, the mixture is irradiated by a pulsed laser beam with wavelenghs ranging from UV to infrared light. UV lasers are most commonly used in commercial instruments and include those from nitrogen lasers (337 nm), followed by excimer lasers, neodymium-doped yttrium aluminum garnet (Nd:YAG) lasers (355 nm), and (more recently) infrared lasers such as erbium-doped yttrium aluminum garnet (Er:YAG) lasers (2.49 μm) and transversely excited atmospheric (TEA-CO_2) lasers (10.4 μm). Laser pulse rates have increased to 1 kHz in many commercial instruments with some being now available with rates of up to 5 kHz. These have the advantage of higher sample throughput, with increased sensitivity and dynamic range, and better sample utilization. The laser beam is rastered over the sample spot on the target plate while each laser shot is focused for 3–4 ns on a small spot on the matrix-analyte crystalline surface (typically 0.05–0.2 mm in diameter). At least 10,000 laser shots are typically summed to obtain a spectrum, and as many as 200,000 laser shots can be used if necessary to completely ionize a sample. This provides reproducible mass spectra by reducing background noise caused by variations in the amount and distribution of sample on the target plate. Upon irradiation with the pulsed laser, the photon energy is absorbed predominantly by the matrix compound, and this electronic excitation is converted into thermal (vibrational) and translational energy, which provokes both the matrix, as well as analyte molecules, to rapidly sublimate from the solid phase into the gas phase (without passing through a liquid phase), forming a plume containing ions from the matrix and the sample. The MALDI ionization process is very complex and depends on the type of analyte, matrix used, and laser fluence; however, two models have been suggested: (1) charge separation during the desorption step of preformed ions embedded in the crystalline matrix and (2) gas phase protonation via ion-molecule reactions during the desorption step. As the MALDI process

Table 27.2 Common mass analyzers and their properties

Mass analyzer	Separation property	Resolution[a]	Mass accuracy (Da)	m/z range (Da)
Quadrupole	Ion trajectory stability	1000–2000	0.1	200–4000
Time-of-flight	Drift velocity	2000–100,000	0.001	Up to 10^6
Quadrupole ion trap	Ion trajectory stability	1000–5000	0.1	200–4000
Ion cyclotron resonance	Orbital frequency	5000–5,000,000	0.0001	200–20,000

[a]A unitless measure used to describe resolution of peptides or proteins

generates mostly singly charged sample ions ($z = 1$), MALDI derived spectra may include larger numbers of proteins (mass spectrum).

Following laser bombardment, the analyte ions generated are analyzed by the mass analyzer component of the mass spectrometer to determine their respective masses and identities. All mass analyzers are operated under vacuum (~10^{-4} to 10^{-12} Torr; their magnitude depends on the mode of operation) required to avoid collisions of the analyte gas phase ions with neutral molecules present in air which increases signal sensitivity. A variety of mass analyzers exists for measuring ionized proteins from biological samples (Table 27.2); however, no single analyzer type is ideal for all applications. For microbial diagnostics, a TOF mass analyzer is usually applied in commercial systems. It is suitable to measure the m/z distribution of discrete pulsed ion sources, unlike a continuous stream of ion, and for this reason it is usually coupled with a MALDI, a pulsed ion source. In a TOF-MS, a discrete packet of ions with different m/z's (generated via MALDI) are first accelerated through an electrostatic field to the same kinetic energy by applying a constant voltage (10–25 kV) to the target plate. These ions are then ejected into the field-free region of the flight tube (no voltage or magnetic fields) where all parameters except for mass, charge, and time are constant. Therefore, the time of flight of an ion (t_{TOF}) is proportional to the square root of m/z ratio. Analytes with small m/z's travel faster than those with large m/z's, and the different times to travel the predefined distance to the detector located at the end of the tube form the basis for their mass separation. Thus, the singly charged analytes with different m/z ratio previously composing a complex sample are separated according to their velocity and create a mass spectrum that is characterized by both the m/z and the intensity of the ions, which is the number of ions of a particular m/z that hit the detector. The results of a spectral signature are composed of spikes usually ranging from 1 to 20 kDa. In principle, the TOF-MS does not have an upper mass limit, however, in practice they are limited by the efficiency of the multichannel plate (MCP) detector in converting low kinetic energy ions (i.e., large m/z's) into a detectable electrical current and the ability of the MALDI to produce singly charged ions of virtually unlimited mass range (high m/z ratio). Operationally, the relationship between m/z and t_{TOF} is established by calibration with a set of standard compounds of known m/z values for their ions. This calibration is dependent on matrix type and laser intensity (each

affects the initial ion velocity during desorption) and the sample position on the MALDI plate (affecting the distance traveled, and the effective accelerating voltage experienced by the desorbed ion).

The aforementioned linear TOF type forms part of most, if not all, of the commercially available MALDI-TOF MS microorganism identification systems that are based on matching a sample mass spectrum ("mass fingerprint") to a mass spectral library of microorganisms which is the profile-based MALDI-TOF MS that is discussed here in this chapter. The linear TOF method has high sensitivity and high efficiency, with the ability to analyze molecules in femtomolar (10^{-15} mol/l) and attomolar (10^{-18} mol/l) concentrations. However, as a limitation it provides a poor resolution due to peak broadening caused by spatial distribution of analyte molecules on the surface and unequal distribution of energies from the laser pulse. This results in ions with the same m/z having different kinetic energies. The additional application of a reflectron device (not shown here) as a focusing element at the end of the TOF instrument greatly improves mass signal resolution although sacrificing sensitivity. It uses a static electric field to reverse the direction of ions and assures that ions of the same m/z, but different kinetic energy arrive simultaneously at the detector, usually consisting of an ion conversion dynode and an electron multiplier device. A single sample can be analyzed in less than a minute, meaning that a fully loaded target plate with ~100 spots can be analyzed in $<$2 h time.

27.4 Biomarkers

For a reliable profile-based microbial identification of unknown isolates, the stability of a microbial mass fingerprint is essential with the latter depending highly on the selected mass range used in MALDI-TOF MS analysis (Fig. 27.5). Mass fingerprints must meet two additional major conditions: (1) high interspecies variability to enable species differentiation and (2) high intraspecies similarity to allow for the establishment of a species-specific mass fingerprint consisting of multiple, reproducible peaks ("consensus spectrum") (Welker 2011). As demonstrated in several studies (Demirev et al. 1999; Holland et al. 1999; Rhyzov and Fenselau 2001; Dieckmann et al. 2008), biomolecules desorbed from whole unfractionated cells being detected above 4 kDa are intact proteins. Most of these proteins detected as biomarkers in the ICMS spectra have molecular masses below 15 kDa and correspond to cytosolic proteins with housekeeping functions that are abundant, basic, and of intermediate hydrophobicity. In lower mass ranges, i.e., 0.5–2.5 kDa, the variability between MS profiles of different species can be either too low for species identification or MS profiles of closely related isolates can be very dissimilar, e.g., in microbial groups that produce a high diversity of secondary metabolites. In the mass range above 20 kDa, only a limited number of distinct peaks has been recorded in ICMS despite of the large diversity of cellular proteins. This is explained by signal suppression caused by the generally low abundance of

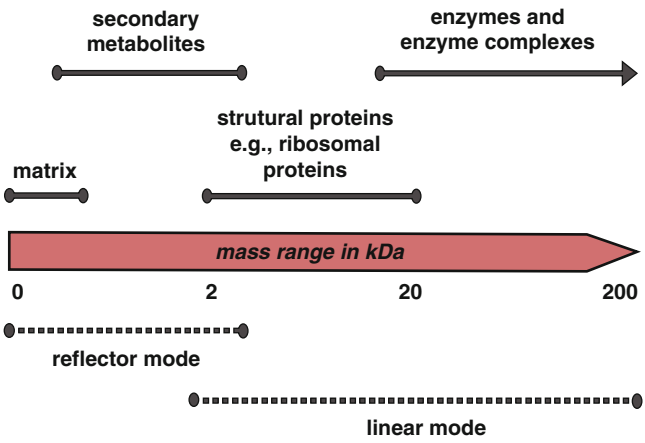

Fig. 27.5 Types of cellular compounds detected in different mass ranges and TOF modes by intact cell MS (ICMS) (modified after Welker (2011))

individual proteins in crude cell extracts, limiting their use as specific biomarkers (Welker 2011). Thus, for the ICMS method-based microbial identification by MALDI-TOF, a mass range of 2–20 kDa is generally selected.

Among the MALDI MS detected proteins, about half have been experimentally determined to be ribosomal proteins (Holland et al. 1999; Rhyzov and Fenselau 2001; Dieckmann et al. 2008) because of their high abundance (more than 20% of total cell proteins) and basic nature which is a biochemical trait favorable for efficient ionization during the MALDI process (Krause et al. 1999). Additionally, the lysis of bacterial cells in organic solvents and acidic conditions of the matrix favors their extraction, explaining why the majority of peaks in a MALDI-TOF spectrum correspond to ribosomal proteins. However, several biomarker peaks of additional groups of "structural proteins" (without catalytic activity but constituents of cell structure and function) were also identified in the mass range below 20 kDa. These include abundant nucleic acid-binding proteins, cold-shock and heat-shock proteins, translation initiation and ribosome modulation factors, nucleoid-associated proteins, RNA chaperones, carbon storage regulators, as well as phosphocarrier protein HPr (Holland et al. 1999; Rhyzov and Fenselau 2001; Dieckmann et al. 2008); all of which are characterized by a high isoelectric point >9 (Dieckmann et al. 2008). Indeed, large variations were seen in ICMS spectra of the same bacterial species obtained under different experimental conditions (Wang et al. 1998; Valentine et al. 2005; Dieckmann et al. 2008). The reproducibility of a MALDI mass spectrum was demonstrated to be dependent on the instrument, the matrix used, the age of microorganism, the sample-to-matrix ratio, the sample concentration, the culture medium, and growth conditions (Wang et al. 1998; Valentine et al. 2005; Liu et al. 2007; Toh-Boyo et al. 2012). However, several studies have also shown that a subset of peaks from vegetative bacteria is conserved in spectra obtained under different growth conditions when using exponentially

grown cells. The conserved, invariant peaks correspond mainly to the ribosomal proteins which are highly expressed under high division rates, irrespective of experimental growth conditions. These selected specific, conserved biomarker proteins lead to the feasibility of using MALDI-TOF MS for microbial identification at the genus and species level irrespective of the growth condition-influenced changes in other biomarkers (Valentine et al. 2005; Dieckmann et al. 2008; Wieme et al. 2014). Since a proteome mass fingerprint is related to the genomic sequence of a microorganism which is in turn one of the key properties to defining the microbial species itself, MS profile-based grouping and taxonomic grouping, e.g., by 16S rRNA gene phylogeny, are congruent.

Studies investigating intra- and interlaboratory reproducibility of MS species identification showed that variability in mass spectra using the same MALDI-TOF MS system was low when analysis was performed with high-quality deposits and similar sample preparation techniques (Saenz et al. 1999; Mellmann et al. 2009; Croxatto et al. 2012). However, interlaboratory reproducibility was significantly negatively affected by variation in MS instruments (Wang et al. 1998; Wunschel et al. 2005). Wunschel et al. (2005) found, in analyses of aliquots from an identical bacterial culture by three independent laboratories, each using a different commercial MALDI-TOF MS instrument, that only 25% of the biomarkers were similar, while more than 50% of the peaks were detected in spectra from only one laboratory. Interestingly, when using the mass spectra collected from the instrument of one laboratory for analysis in the other two laboratories, only 70% of the mass fingerprints could be correctly identified which, as a result, underlines the importance of the instrument in the establishment of bacterial fingerprint databases (Wunschel et al. 2005). Therefore, as emphasized in many studies (Liu et al. 2007; Freiwald and Sauer 2009; Giebel et al. 2010; Welker 2011; Croxatto et al. 2012; Posterano et al. 2013; van Belkum et al. 2015; Basile and Mignon 2016; Zhang and Sandrin 2016), to maximize the reproducibility and usefulness of MALDI-TOF spectra for species identification (and even more for subspecies level differentiation, see Sect. 27.7), standardized protocols for cultivation, sample preparation (e.g., choice of matrix, concentrations, solvent, and crystallization conditions), data processing, and analysis by the different commercially available instruments must be established and strictly followed in microbiology laboratories.

27.5 Sample Preparation

In early studies, the intact-cell MS (ICMS) method was reported as a simple, uniform method to prepare samples directly from isolates of bacteria and yeasts for MALDI-TOF MS species identification (Claydon et al. 1996; Fenselau and Demirev 2001). For this "on-plate" approach of bacteria inactivation and protein extraction, a single colony (limit of detection is 10^5 cells) freshly grown on defined agar medium is picked with a sterile tip and smeared as a thin film onto a spot of the stainless steel MALDI target plate which is followed by the addition of 1 µl of

formic acid (for cell wall disruption). The microbial film is then overlaid with 1 µl MALDI matrix selected as recommended by the manufacturers (typically, α-cyano-4-hydroxycinnamic acid, CHCA), air dried, and subsequently introduced into the MALDI-TOF instrument for analysis. However, as identification of microorganisms by MALDI-TOF MS became more commonplace, it became apparent that the IC method, in spite of its relative simplicity, was not appropriate for all specimen types (e.g., *Mycobacteria*, *Nocardia*, and fungal species) because different microbial groups vary fundamentally in their cellular composition and architecture. These differences have been demonstrated to affect the quality of spectra generated during MS experiments and, thus, the accuracy of MALDI-TOF MS-derived identifications. To improve spectral generation and be compliant with biosafety regulations, several modified sample preparation methods have been developed in the past for the different groups of microorganisms (Table 27.3), ranging from on-plate inactivation to full-scale protein extraction procedures (Liu et al. 2007; Sauer et al. 2008; Freiwald and Sauer 2009; Clark et al. 2013; Posterano et al. 2013).

Although there is no single "gold standard" protocol for MALDI-TOF MS-based microbial identification, many laboratories use the "formic acid extraction" procedure (Sauer et al. 2008; Freiwald and Sauer 2009) (see Table 27.4) that has been shown to produce reliable results for a wide range of microorganisms (Clark et al. 2013). As a starting material, aliquots of liquid cultures (centrifuged or filtered) or single colonies from solid medium are suspended in a small volume of 70% ethanol (for microbial inactivation), briefly vortexed, and then concentrated by centrifugation. The supernatant is discarded (removal of media contamination), the cell pellet is resuspended in 50 µl of 70% formic acid (for cell wall disruption), an equal amount of acetonitrile (ACN) is added (for protein extraction), the sample is vortexted, and again concentrated by centrifugation. One µl of the supernatant with the extracted proteins is spotted on the target plate, air dried, overlaid with 1 µl matrix consisting of a saturated solution of CHCA in 50% ACN and 2.5% trifluoroacetic acid (TFA) (ACN as a solvent and TFA as an organic acid to ensure the solubility of CHCA), and dried before being introduced into the MS instrument. With this protocol, sufficient spectra are obtained for microbial identification, if the initial suspension contains a minimum of 5–10 $\times$ 10^6 cells/ml (Demirev et al. 1999; Freiwald and Sauer 2009). Other matrix compounds may also be used such as 2,5-dihydroxybenzoic acid (DHB), sinapic acid (SA), or ferulic acid (FA), resulting in slight differences in mass spectral patterns, primarily varying in the relative sizes and intensities of individual protein peaks but generally with equal performance with respect to identification. DHB and CHCA are usually optimal for the detection of lower mass ions with a detection up to 10 kDa when the proper solvent is used (DHB is the best choice for detection of glycoproteins). Both SA and FA have been shown to improve the detection of higher mass ions (above 15 kDa) but provide a lower sensitivity than CHCA (Wang et al. 1998; Demirev et al. 1999; Wahl et al. 2002; Ruelle et al. 2004; Sedo et al. 2011).

After the analysis, the used target plate is removed from the MS instrument and cleansed for repeated use. For regular workup, a quick cleansing protocol with a

Table 27.3 Recommended MALDI-TOF MS sample preparation procedures for use with different groups of microorganisms; summarized after Clark et al. (2013)

Microbial group	Sample preparation procedure
Gram-positive and gram-negative bacteria	1. Select colony of target organism 2. Spot cell material on MALDI plate, overlay with 1 µl formic acid or 2. Full extraction with ethanol and formic acid 3. Add matrix to sample, co-crystallization, and analyze
Non-fermenting gram-negative bacteria	1. Select colony of target organism 2. Spot cell material on MALDI plate (optional formic acid overlay) 3. Add matrix to sample, co-crystallization, and analyze
Anaerobic bacteria	1. Select colony of target organism 2. Spot cell material on MALDI plate, overlay with 1 µl formic acid or 2. Full extraction with ethanol and formic acid/acetonitrile 3. Add matrix to sample, co-crystallization, and analyze
Mycobacteria	1. Select colony of target organism 2. Transfer cell material to screw cap tube containing water and detergent (Tween) 3. Heat at 95 °C for 1 h to inactivate bacteria 4. Lysis with glass beads 5. Spot lysate on MALDI plate, add formic acid-acetonitrile mixture 6. Add matrix to sample, co-crystallization, and analyze
Nocardia, actinomycetes	1. Liquid culture 2. Boil large amounts of bacteria (turbid suspension) 3. Extract ethanol, dry pellets, and resuspend in formic acid 4. Spot lysate on MALDI plate 5. Add matrix to sample, co-crystallization, and analyze
Yeasts	1. Culture 24–72 h depending upon fungal species and media type 2. Select colony of target organism 3. Spot cell material on MALDI plate, overlay with 1 µl formic acid or 3. Full extraction with ethanol and formic acid 4. Add matrix to sample, co-crystallization, and analyze

short incubation in 70% ethanol, followed by treatment with a small amount of TFA, and subsequent mechanical cleaning with water and cloth, is sufficient. More aggressive protocols as recommended by the manufacturer, which include several mechanical cleansing steps (e.g., sonication), can be performed on weekly/monthly basis (Freiwald and Sauer 2009; Croxatto et al. 2012).

Table 27.4 Variations of the "formic acid protocol" for MALDI-TOF MS identification of bacteria as described by Sauer et al. (2008) (steps in column 1 represent the standard workflow, while the variations of the individual steps are listed in column 3; exemplary references for the methodical variations are listed in column 4)

Step in "formic acid protocol"	Feature	Variation	Reference
Bacterial colony	Sample of microbial isolate	Liquid culture (cell harvest by centrifugation or filtration)	Freiwald and Sauer (2009)
Adding of ethanol/water	Microbial inactivation/ precipitation	Inactivation of pathogenic bacteria by concentrated TFA solution	Lasch et al. (2008)
		Inactivation by boiling	Verroken et al. (2010)
Adding of FA/ACN mixture	Cell wall disruption and protein extraction	Sample solvents (chloroform/ methanol/propanol mixtures)	Liu et al. (2007)
		Affinity extraction	Lin et al. (2005)
		Bead beating	Mather et al. (2014)
		Cell fractionation	Hu et al. (2015)
		Solid phase extraction (SELDI)	Shah et al. (2011)
		Proteolytic digest	Schmidt et al. (2009)
Extract deposition as dried droplet	Transfer to the target plate	Direct deposition/smear	Claydon et al. (1996), Holland et al. (1999)
		Premix and spray	Toh-Boyo et al. (2012)
Stainless steel target plate	Target plate choice	Titanium chips and special surface pretreatment (selective capture)	Hasan et al. (2014)
		Teflon-precoated spots	Schuerenberg et al. (2000)
Adding of CHCA	Matrix choice	Matrix solvents (methanol/ ethanol/propanol mixtures)	Liu et al. (2007)
		SA	Huber et al. (2011)
		FA	Wahl et al. (2002)
		DHB	Benagli et al. (2011)
Overlaying extract with matrix	Matrix preparation	Premix and spray	Toh-Boyo et al. (2012)
		Addition of detergents	Meetani and Voorhees (2005)
		Low TFA concentrations	Munteanu et al. (2012)
Protein mass profiles	Targeted biomolecules	Nucleic acids	Von Wintzingerode et al. (2002)
		Lipids	Park et al. (2015)
Spectrum 2–20 kDa	Mass range	>20 kDa	Dieckmann et al. (2008), Meetani and Voorhees (2005)
TOF in linear positive mode	Spectrum acquisition	Application of "matrix blast"	Munteanu et al. (2012)
		Reflectron mode	Schmidt et al. (2009)

27.6 MALDI-TOF MS Platforms, Software, Databases, and Data Processing

Currently, four different MALDI-TOF MS benchtop identification platforms that are ready-to-use for routine identification of bacteria and fungi are available on the market: Andromas (Andromas SAS, Paris, France), the MALDI Biotyper (Bruker Daltonics GmbH, Bremen, Germany), the Axima@Saramis (Shimadzu/AnagnosTec, Duisburg, Germany), and the VITEK MS (BioMérieux, Marcy-l'Étoile, France). These platforms and respective spectral databases are marketed as part of a proprietary system, as opposed to a publicly accessible open platform, and are constructed and maintained by their representative manufacturers. They operate with different MALDI-TOF MS instruments, commercialized by the three MS companies Bruker Daltonics, Scientific Analysis Instrument (Manchester, UK), and Shimadzu (Tokyo, Japan), with no substantial differences in any functional unit of the hardware and general analytical methodology (e.g., ionized analyte separation, signal detection, and amplification). However, there are large differences between these platforms, including sample preparation recommendations, the software for automatic (in silico) spectra preprocessing, the reference databases, and reference creation algorithms, as well as the interpretive criteria and algorithms for microbial identification of unknown spectra. As a result, numerical data (i.e., spectral scores) from the different systems are not directly comparable. Comparative analysis among MS systems is therefore usually performed by using final identifications in the context of each system's interpretive algorithm. Most of the spectral databases can be expanded by adding new spectral entries not included in the marketed version allowing users to construct custom ("in-house") databases. Currently, the market is dominated by two commercial systems: (1) Bruker Daltonics with its Flex line series of benchtop MS instruments and the Bruker-owned Biotyper platform and (2) BioMérieux offering a Shimadzu Axima MS device and the BioMérieux-maintained VITEK MS system with the recently integrated Saramis platform (previously sold along with the Shimadzu MS device as the Axima-iD Plus system, before being purchased by BioMérieux) (Welker 2011; Clark et al. 2013; Posterano et al. 2013; Cassagne et al. 2016). This implies that most, if not all, medical and environmental MS proteome profile-based studies and platform comparisons have been performed with these two instrument brands [see (Giebel et al. 2010; Seng et al. 2010; Welker 2011; Clark et al. 2013; van Belkum et al. 2015; Zhang and Sandrin 2016) and references therein]. Therefore, the differences in reference databases and query methods as well as performances of the two platforms are discussed in the following paragraphs along with a general description of the spectra preprocessing and identification steps.

The output data of the mass spectrometer is referred to as a "raw spectrum" which consists of 20,000–30,000 sets of values—the m/z and relative intensity. The latter is a combination of three components: the real signal, the baseline (derived from the impact signal of the cluster of matrix and small fragments of degraded large molecules), and the random noise (background detector signals). The

automatic preprocessing of a "raw spectrum" reduces the amount of data, rendering them interpretable, and is generally composed of three steps. An initial baseline subtraction step that estimates the "blank" sample (i.e., the matrix spectrum with a bell curve shape in the low m/z region) is followed by a denoising step to filter out the random electronic background noise (as signals recorded from an empty spot) from the set of independent peaks reflecting the microbial sample proteome (species-specific mass profile). In the last step, signal peaks that exceed a defined signal-to-noise ratio are selected from the preprocessed unknown spectrum, listed in peak tables, and, finally, in postprocessing compared to known references included in the databases for microbial identification. The reference databases and database query methods differ significantly between the platforms, since each manufacturer has developed its own peak-matching algorithms which directly impact ability and performance of the commercial platforms (Welker 2011; Clark et al. 2013; Posterano et al. 2013; Cassagne et al. 2016; Zhang and Sandrin 2016).

MALDI Biotyper uses MainSpectra as the reference spectra, i.e., consensus spectra computed from multiple spectra derived from a single sample of a unique strain grown under different conditions (Welker 2011; Cassagne et al. 2016). After preprocessing each of about 20 raw spectra, 20 matrices of peak lists and their corresponding intensities are obtained. The reference mass spectrum consists of a maximum of 100 prevalent peaks with a minimum frequency of 25% of a peak within the 20-spectrum set. The identification result of an unknown spectrum is linked to a score computed by counting the prevalent peaks in the mass peak lists of sample mass spectrum that match with peak lists of reference mass spectra and vice versa and correlating signal intensities of matched peak signals of mass spectra. The three scores obtained are multiplied, normalized to a value of 1000, and eventually transformed to a log score-based classification system ranging from 0 to 3. A score above 2.0 is considered a reliable criterion for a species-level identification (Freiwald and Sauer 2009; Posterano et al. 2013; Zhang and Sandrin 2016); however, the identification thresholds have been shown to vary for bacterial and fungal species identification (Seng et al. 2009; Cherkaoui et al. 2010).

In contrast, the VITEK MS uses SuperSpectra as reference spectra which are calculated from a minimum number of strains (at least 25–20 isolates obtained from different locations and grown under different conditions) from the same species to build the reference peak signature for the given species (Welker 2011; Cassagne et al. 2016). Based on a "mass binning" algorithm, a preprocessed spectrum is divided into 1300 predefined intervals, called bins, on a mass range from 3 to 17 kDa. After only the peak with the highest intensity is retained for each bin, the data are transformed into a list of bins with a corresponding intensity, and each peak is weighted based on its specificity at the species, genus, or other taxonomic level by the "Advanced Spectra Classifier" algorithm. If a peak is frequently found exclusively in the spectra corresponding to strains of the same species, a high positive weight is attributed to that peak for the specific species and, vice versa, a high negative weight is attributed to this peak for all other species. As each SuperSpectrum is constructed with the spectral information of numerous strains, the reference database contains only a matrix of 1300 bins with species-specific

weights (the identifier matrix). For the identification of a sample, the bin scores of an unknown spectrum are multiplied with the weight of each species-specific weighted bin included in the reference matrix, and the sum of the weighted bins is calculated for each species contained in the reference matrix which are then transformed into percentage-based confidence values of identity with the reference spectra, with 90% recommended for species-level identification (Posterano et al. 2013; Cassagne et al. 2016; Zhang and Sandrin 2016).

In conclusion, the reference mass spectra databases of commercial systems are based on two opposite approaches for database architecture: the isolate-specific reference approach (Biotyper, Andromas) and the taxonomical group-specific approach (VITEK MS, Saramis) (Cassagne et al. 2016). Isolate-specific references, which are issued from spectral data acquired from replicates of the same isolates, are not linked to one another and do not influence other references. The isolate-reference approach, therefore, facilitates the addition of new references while also enabling subspecies typing by peak differentiation between the similar spectra of isolates within a species. A major disadvantage is its vulnerability for sample identification errors when a reference is misidentified. Group-specific references, in contrast, are issued of the analyses of all spectral data acquired from all isolates regardless of the species, as the databases are organized as a matrix in which a weight is attributed to all existing peaks based on the species. This ensures a high robustness of identification due to the low impact of including misidentified references in the weighted peak matrix as well as in silico space gain and short calculation times. The drawback of this approach, however, is the need to recalculate the entire matrix for each new database entry. In fact, the VITEK MS platform has now included a second, isolate-specific reference database to circumvent the aforementioned limitations (Cassagne et al. 2016).

Numerous studies have investigated the performance of the different MALDI-TOF MS platforms concerning species-level identification of bacteria and fungi particularly in the medical microbiology field. Overall, the MS proteome-profile-based characterization outperformed the biochemical and conventional identification methods due to its identification accuracy (Marklein et al. 2009; Seng et al. 2009; Cherkaoui et al. 2010; van Veen et al. 2010; Buchan et al. 2011; Dhiman et al. 2011; Pinto et al. 2011; Saffert et al. 2011; Chao et al. 2014), time gain, and low cost (Seng et al. 2009; Cherkaoui et al. 2010; Dhiman et al. 2011; Ge et al. 2016). The identification rates for bacteria and fungi were generally above 90% and even higher when additional in-house reference databases were used besides the manufacturer's databases included in the commercially available platforms (Mellmann et al. 2008; Marklein et al. 2009; Seng et al. 2009; Cherkaoui et al. 2010; Prod'hom et al. 2010; van Veen et al. 2010; Buchan et al. 2011; Dhiman et al. 2011; Pinto et al. 2011; Saffert et al. 2011; Clark et al. 2013; Chao et al. 2014; Rizzato et al. 2015; van Belkum et al. 2015; Cassagne et al. 2016; Ge et al. 2016). Comparative studies between the Biotyper and VITEK MS system showed no significant differences in the efficiency as species identification levels were essentially indiscriminate between both platforms (Seng et al. 2009; Cherkaoui et al. 2010; Buchan et al. 2011; Marko et al. 2012; Martiny et al. 2012; Alby et al. 2013; Mancini et al. 2013; Chao et al. 2014; Mather et al. 2014; Cassagne et al. 2016; Ferrand et al. 2016).

27.7 Microbial Typing: Profile-Based Subspecies Level Identification by MALDI-TOF MS

The current "gold standard" for microbial taxonomy is to systematically classify microorganisms in a polyphasic approach on the basis of genome sequence data and experimentally determined phenotypic traits. As MALDI-TOF MS mass fingerprints provide information about multiple protein components that characterize a microorganism, it can be used as an additional tool for phenotype analysis in polyphasic taxonomy. The mass profiles provide data of both the presence (conservation/divergence) and the intensity (expression) level that, together, compose a two-dimensional taxonomical asset which offers better discriminative resolution for microorganism classification. Indeed, genus- and species-level characterization of bacteria and fungi using MALDI-TOF MS with library-based methods has been successfully supplied in medical, environmental, and food industry microbiology (Liu et al. 2007; Giebel et al. 2010; Seng et al. 2010; Welker 2011; Croxatto et al. 2012; Posterano et al. 2013; van Belkum et al. 2015; Basile and Mignon 2016; Santos et al. 2016; Spitaels et al. 2016; Zhang and Sandrin 2016), with identification rates equivalent to those obtained with 16S rRNA sequencing (Mellmann et al. 2008; Seng et al. 2009; Boehme et al. 2013) and can be regarded as a relatively robust technique. However, several studies reported limited success or even inability to characterize bacteria below the species level with MALDI-TOF MS [(Croxatto et al. 2012; Kolecka et al. 2013; Sandrin et al. 2013; Basile and Mignon 2016) and references therein] which demonstrated that the requirements for library-based microbial typing (differentiation of genetically different strains belonging to the same species) are different and more complex compared to higher taxonomic ranks. Relatively few biomarkers (5–10 peaks) are usually required for the identification of isolates at the species level, whereas a much larger number of reproducible peaks are needed for strain identification. Since members of a single species tend to yield remarkably similar proteomic mass profiles, the detected number of unique and characteristic strain-specific biomarkers decreases, representing only a small portion of the MS profile, and differentiation of bacteria at the subspecies level by MS becomes challenging (Fig. 27.6). As a result, MS measurement requires a higher level of selectivity, mass spectral profile reproducibility (relative peak intensity), and mass accuracy to increase the discriminatory power and resolution of the method allowing reliable strain identification. For MS-based measurements, this may imply strategies involving rigorous optimization of experimental parameters (e.g., amount of cell material, type of matrix, sample to matrix ratio, concentration of acid to the matrix, etc.), additional sample preparation steps (e.g., protein extraction, digestion), the inclusion of separation step (fractionation, LC), extending the mass range of the analysis (detection of higher molecular weight biomarkers), increasing the selectivity of the MS measurement (MS/MS), improved bioinformatic approaches for data analysis (improved curve-based algorithms; additional software), and/or use of extended in-house reference databases (Croxatto et al. 2012; Sandrin et al. 2013; Basile and Mignon 2016; Lasch et al. 2016; Zhang and Sandrin 2016). As mentioned previously in this review (see Sect. 27.4), growth

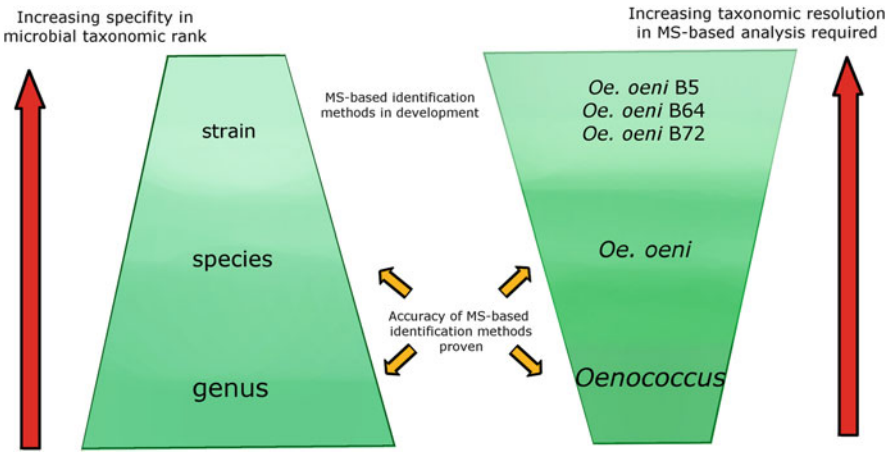

Fig. 27.6 Increasing taxonomic resolution is required to reliably characterize/identify bacteria with increasing microbial taxonomic rank from genus (e.g. *Oenococcus*) to subspecies (e.g., *O. oeni* K12)

medium and cultivation conditions exert a noticeable influence on the protein expression of the microorganisms under study and, hence, have a significant impact on the MALDI-TOF spectral profiles (Valentine et al. 2005; Dieckmann et al. 2008; van Veen et al. 2010; Sandrin et al. 2013; Usbeck et al. 2013; Wieme et al. 2014). Furthermore, spectra signal and reproducibility of filamentous fungi are strongly influenced by the phenotype of the fungus (hyphal or conidial). The latter may even differ in the same organism as vegetative mycelium on agar may show multiple zones that correspond to different ages or development stages. A comprehensive database of filamentous fungi has therefore been suggested to include MS fingerprints of several different development forms to guarantee high yields and accuracy of identification (Posterano et al. 2013). Indeed, failures to identify a bacterial or fungal isolate on subspecies- and even on species-level in the past were due to incomplete reference spectra database, mislabeling of species, or poor spectral quality (Table 27.5).

27.8 Parameters Influencing MALDI-TOF MS Microbial Typing: Successes, Challenges, and Strategies to Improve Its Discriminative Power and Spectra Reproducibility

As demonstrated by several studies, rigorous standardization of the cultivation conditions, sample preparation, and MALDI measurement parameters as well cautious optimization of the entire MALDI-TOF MS workflow (Fig. 27.7) is crucial to obtain a sufficient number of reproducible peaks with specificities below species-level specificity.

Table 27.5 Problems commonly found in strain typing by MALDI-TOF MS; after Croxatto et al. (2012)

Problems	Examples
Insufficient protein signal	Yeasts require a protein extraction procedure to be correctly identified
Difficult to lyse cell wall structures	Pneumococci and most strains of *Haemophilus influenzae* possess a caspule which prevents efficient lysis and results in poor spectral quality
Small amount of material sample	*Actinomyces* and *Nocardia* spp. usually display weak protein signals
Limit of resolution of the MALDI-TOF MS method	*Shigella* spp. identified as *E. coli*
Database discordances	*Propionibacterium acnes* ssp. identified as *Eubacterium brachy* ssp.
Errors in reference spectra	Due to incorrect reference spectra in database
Similarities of spectra present in database	Incomplete reference libraries for viridans streptococci and pneumococci
Absence or insufficient reference spectra in database	No reference of non-*Clostridium* anaerobes in the database Insufficient number of reference spectra for archaeal species
Taxonomical discordances	*Agrobacterium tumefaciens* is synonymous for *Rhizobium rhizogenes*

27.8.1 Culture Conditions

To allow for high reproducibility in microbial strain characterization, the use of standardized growth conditions (e.g., medium form, medium composition, incubation time, etc.) has been recommended as the growth conditions significantly influence the expression patterns of proteins other than ribosomal and hence alter the mass spectrum (Ruelle et al. 2004; Valentine et al. 2005; Dieckmann et al. 2008; Sandrin et al. 2013; Wieme et al. 2014; Zhang and Sandrin 2016); however, these effects seem to be bacterium-specific (Giebel et al. 2010; Sedo et al. 2011; Clark et al. 2013). The medium type (whether cells were grown on solid medium or in liquid broth) was reported to affect MALDI-TOF MS profiles with greater cell heterogeneity observed in plate culture. Because of the homogeneous populations of cells synchronized in their growth phase, samples from liquid cultures generally have a higher discriminatory power in MALDI-TOF MS compared to plate cultures with older, senescent cells in the center and newer, more actively growing cells at the perimeter (Sandrin et al. 2013). Medium compounds have been reported to interfere with the ionization of microbial biomolecules, especially if the microbes have the tendency to adhere onto the culture medium surface (Dieckmann et al. 2008; Alispahic et al. 2010), and culture media that do not sustain optimal growth can strongly affect the mass spectra generated (Wieme et al. 2014). To maximize taxonomic resolution for strain level identification, the potential effects should be thoroughly investigated, and databases containing multiple reference strains grown

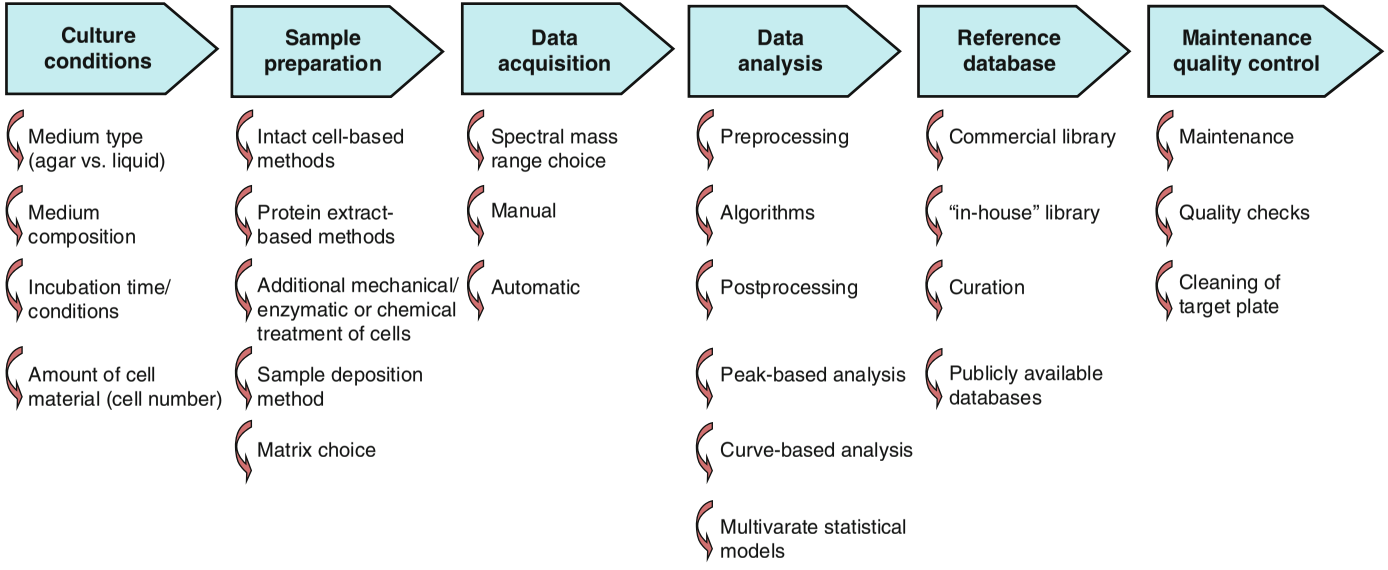

Fig. 27.7 General workflow to identify microorganisms by MALDI-TOF MS

on different culture media may need to be established. It has further been demonstrated that the rate of successful identification is directly linked to the amount of cell material used and age of cell culture at the time of analysis (Demirev et al. 1999; Freiwald and Sauer 2009; Giebel et al. 2010; Croxatto et al. 2012). When applied manually onto the sample spot of the target plate, sample and matrix are generally not homogeneously dispersed on the surface. As only a fraction of the sample spot is analyzed spectrometrically during MS measurement, a bacterial cell number of at least 10^6 has been shown to be necessary to obtain consistent spectral signals of sufficient quality for strain identification. A lower amount of bacteria (minimally, 5×10^3 cells from a pure strain) may, however, be identified by MALDI-TOF MS using particular analysis approaches (hierarchical clustering analysis) (Croxatto et al. 2012). Optimally, cells from colonies or liquid cultures in the exponential phase are used for MS analysis, since weaker and less distinguished peaks will appear in the spectra with increasing cultivation time as a result of protein degradation (Freiwald and Sauer 2009).

27.8.2 Sample Preparation

Sample preparation methods have been suggested to affect the taxonomic resolution of MALDI-based approaches to bacterial characterization (Sedo et al. 2011; Croxatto et al. 2012; Sandrin et al. 2013; Basile and Mignon 2016; Zhang and Sandrin 2016). As previously described, two standard approaches to prepare samples for library-based analysis have been employed: intact cell-based methods (ICMS) and protein extract-based methods (see Sect. 27.5) with the former depositing suspensions of intact cells directly on the MALDI target plate and the latter placing cell extracts alone on the target. Although strain-level resolution has been reported by several groups applying the simple and rapid ICMS approach (e.g., Dieckmann et al. 2008), efforts to optimize a set of standard sample preparation methods for microbial profiling have led to the more common use of protein extract-based protocols with the ethanol-formic acid extraction procedure (Sauer et al. 2008; Freiwald and Sauer 2009). Indeed, several studies have suggested that protein extract-based methods afford higher taxonomic resolution than intact cell-based methods [(Bizzini et al. 2010; Clark et al. 2013; Sandrin et al. 2013; Zhang and Sandrin 2016) and references therein]. However, failures to obtain spectra of sufficient quality for microbial typing from several fungal and bacterial groups (e.g., *Mycobacteria*) by the standard protocol (as a result of different cell wall architecture) have led to many modifications of the initial procedure (including additional pretreatments like heating steps, enzymatic digestion, or mechanical cell disruption) to allow for efficient protein extraction (see Table 27.4). As the protein extraction protocol and matrix choice both have a noticeable effect on the mass spectra (Freiwald and Sauer 2009; Giebel et al. 2010; Sedo et al. 2011; Clark et al. 2013; Zhang and Sandrin 2016), separate databases may have to be constructed for samples that have been extracted and/or measured with different protocols. Matrix

sample deposition onto the target plate particularly by the dried-droplet method (i.e., sample deposited and dried first followed by matrix) has been shown to lead to inhomogeneous distribution of the analyte within the sample-matrix preparation resulting in so-called "hot" (or "sweet") signal spots within a MALDI sample (Basile and Mignon 2016). As only a fraction of the sample spot surface is being analyzed spectrometrically, this heterogeneity directly affects the mass spectrum quality and, hence, microbial typing rates. The intrasample variance can be mitigated through an increase in the number of MS measurements, either by averaging a large number of laser shots (technical replicates) and/or increasing the number of samples being analyzed (biological replicates). Indeed, these strategies are already being incorporated in most manufacturer's protocols, as 20–40 replicates for each sample being analyzed are generally collected, and 200–400 laser shots/spectrum averaged reaching the limit of reproducibility that can be achieved with standard manual sample preparation methods (Basile and Mignon 2016). However, a new sample deposition approach using a spray-based method to evenly distribute sample material onto the target surface resulted in homogeneous sample-matrix preparations with highly reproducible mass spectral profiles (90% reduction of variance of the measurement compared to the manual dried-droplet method), regardless of the spatial coordinates of the laser shot on the sample (Toh-Boyo et al. 2012). Notably, the same study showed that a 60% reduction in the variance could be achieved by depositing premixes of microbial suspension in matrix solution, when compared to the dried-droplet method (Toh-Boyo et al. 2012). This improvement in reproducibility would also be expected to be observed in protein extract-based protocols, adding only a single dilution step in the overall procedure (1:1, sample:matrix). Its implementation in standard protocols would enhance the detection of small peak signals and and/or peak differences between closely related microorganisms (caution: not suitable for clinical samples).

27.8.3 Data Acquisition

It has been argued that increasing the selectivity in protein biomarker detection by extension of the molecular mass range in MALDI-TOF MS could improve differentiation of isolates at the strain level (Meetani and Voorhees 2005; Dieckmann et al. 2008; Paauw et al. 2014). The mixed success at strain-level identification in the past stems in part from the fact that under the current experimental condition of the commercial systems, ribosomal proteins are serendipitously detected in profile-based MALDI-TOF MS measurements. The latter represent only a small fraction of the possible protein pool in microorganisms [e.g., in *E. coli* ~2.8% of the total detectable proteins in the mass range up to 100 kDa which are estimated to be 1600 proteins (Basile and Mignon 2016)]. An extended mass range in MS in order to cover proteins with higher molecular weight and hence a wider range of the microbial proteome has been shown to enable differentiation of closely related microorganisms, as proteins responsible for unique genotypic traits could be

detected. For this purpose, matrices like SA or FA are available that efficiently ionize larger proteins during the MALDI process (Meetani and Voorhees 2005; Dieckmann et al. 2008; Paauw et al. 2014).

MALDI-TOF mass spectrometers are operated with software that allow several parameters of data acquisition to be user-defined, e.g., laser power, peak evaluation strategies, mass spectra accumulation, and laser movement on each sample spot. Notably, it has been reported that the mode of data acquisition (manual or automated) affects the taxonomic resolution of MALDI-TOF MS profiling technique particularly at the strain level, where minor differences in profiles can have profound effects on the ability of the method to reliably resolve closely related microbial strains (Sandrin et al. 2013). Although manual data acquisition yielded more reproducible and higher-quality mass spectra, automation in MS is still desirable for high-throughput applications with large quantities of analyses as, e.g., in clinical laboratories. Using a statistical design of experiments approach, Zhang and Sandrin (2016) were able to optimize automated data acquisition to yield spectra with reproducibility comparable to those obtained manually.

27.8.4 Data Analysis

The general workflow in MS data analysis is preprocessing, postprocessing, and model validation. The objective of preprocessing of raw mass spectra is signal noise reduction. Within the context of subspecies identification, a strict workflow with routine tests for spectral quality, smoothing, baseline correction, and intensity normalization has been suggested (Lasch et al. 2016). The quality of microbial mass spectra can be assessed first visually with regard to the criteria of the signal-to-noise ratio (SNR), presence of sufficient peak number, a flat spectral baseline, and absence of interfering or confounding peaks. With quality outliers being removed, the remaining spectra are denoised by applying a smoothing filter [e.g., Savitzky-Golay, 17–25 smoothing points, or Kaiser (Zhang and Sandrin 2016)] to average data points with neighboring points in a time-series of data. The baseline is corrected by subtracting an estimate of a background from the unprocessed spectra [e.g., rolling disk algorithm with a user-defined radius; other subtraction algorithms include monotone minimum, moving bar, and binning (Zhang and Sandrin 2016)], and the data are normalized (vector normalization) to enable comparison of MS intensity values as an essential step for the subsequent use of so-called pseudo-gel views. This results in a spectrum in which the sum of the squared intensities over all m/z is a constant (Lasch et al. 2016). As a final step in preprocessing, peak detection is used to separate real peaks from false peaks representing noise which is automatically done by the software applied with user-defined SNRs. Notably, it has been reported that smaller minimal SNRs support higher taxonomic resolution (Zhang and Sandrin 2016). During peak detection, the mass spectra are transformed by a specific algorithm into peak tables which reduces the amount of data significantly (factor of >100), facilitating further analysis. Several complex peak

detection algorithms have been developed by diverse groups [(Lasch et al. 2016) and references therein], but are generally not implemented in the software of commercial systems (e.g., Flex Analysis from Bruker) for routine microbial identification due to practical aspects. The use of additional custom-designed software (e.g., Matlab-based MicrobeMS) has been reported to be helpful for peak detection and subsequent classification analysis of closely related microorganisms (Lasch et al. 2016). In curve-based approaches that take the entire MS profile into consideration, postprocessing includes the comparison of the peak tables of an unknown isolate with reference peak tables of a database for which similarity coefficients are calculated by pattern matching algorithms. It has been noted that major limitations of MALDI-TOF MS-based microbial typing are due to insufficient algorithmic methods, as single, seemingly minor software features can have a profound effect on the analysis. Some of the algorithms calculate similarity coefficients from binary peak lists that contain only the presence or absence of peaks (e.g., Dice similarity coefficient), whereas others take also the peak intensities into consideration (e.g., Pearson similarity coefficient). The chosen similarity coefficient affects the reproducibility and the discriminatory power of the method. Several studies demonstrated the Pearson coefficient to be more adequate for correct classification of microbial isolates at subspecies level [(Croxatto et al. 2012; Sandrin et al. 2013; Zhang and Sandrin 2016) and references therein]. If routine profile-based identification with curve-based algorithms is insufficient to discriminate closely related isolates, a spectra evaluation for the presence of single or multiple reliable strain-specific MS signals has been shown to be useful as a marker-based identification strategy. The implementation of decision tree algorithms was shown to facilitate automated marker-based classification if several strains and, hence, more masses need to be considered (Croxatto et al. 2012). Unfortunately, single or small sets of unique strain-specific peaks are scarce and usually masked in complex mass profiles that require expanded statistical evaluation of the existing standard spectra ("weighted pattern matching"), e.g., by application of multivariate statistics. The latter can be performed with additional software offered by MS manufacturer platforms (e.g., ClinPro Tools software, Bruker), but it is also possible to export peak tables to other softwares for statistical analysis (e.g., Matlab, The Mathworks Inc.) or the freely available statistical programming language R (with the MALDIquant package). Statistical models can be deduced based on the entire protein mass profile or a subset of strain-specific biomarkers and then used to predict the class of an unknown isolate. However, a prerequisite of the procedure is that a sufficient number of isolates are available for every group and have been classified using an accepted method so that the models can be validated, e.g., by repeated division into "reference samples" for model generation and "test sample" for model-based identification. The successful implementation of artificial neural networks (ANN) for MS-based microbial identification at strain level has also been demonstrated (Lasch et al. 2016). But ANN model development demands a lengthy and tedious training process, and the validation of ANNs necessitates relatively large sample numbers limiting a broad application in routine MALDI-TOF MS analysis.

27.8.5 Reference Databases

The result of microbial identification at the strain level strongly depends on the reference spectrum database that is used. Unfortunately, the taxonomic coverage of the microbial diversity in the databases of the commercial MS systems is still insufficient (e.g., for anaerobic bacteria and archaeal species) and highly biased toward medically relevant species (Bizzini et al. 2010). In addition, mislabeling of species or taxonomical discordances in these databases can cause misidentifications by MALDI-TOF MS (Seng et al. 2009). Therefore, most studies rely on user-generated in-house reference spectra libraries that are more comprehensive in terms of possible variations of the spectral phenotype of the microbial groups of interest, to improve identification rates at species and strain level [(Giebel et al. 2010; Seng et al. 2010; Croxatto et al. 2012; Stantscheff et al. 2014; Basile and Mignon 2016; Lasch et al. 2016; Santos et al. 2016; Spitaels et al. 2016; Zhang and Sandrin 2016) and references therein]. However, uncritical inclusion of all available isolates into an in-house database may be counterproductive, and the set of references must be carefully chosen. Addressing the lower reproducibility of mass spectra derived from strains of certain microbial groups (especially filamentous fungi with highly heterogeneous phenotypes), it has been shown that MALDI-TOF MS-based identification rates could be significantly improved by increasing the number of both reference meta-spectra per strain (biological replicates) and strains for a given species used for later establishment of reference database entries (Normand et al. 2013). Considering the widespread application of the library-based approach for species and strain identification, it has been suggested to create a publicly available, dynamic database of MALDI-TOF MS profiles similar to the CDC's Pulsenet which houses PFGE profiles. However, different sample preparation techniques, MS experimental conditions, and MS instruments in MALDI-TOF MS reference spectra generation may limit the compatibility of such a spectra library. Currently, spectra can be made available in public online repositories, such as SpectraBank (Boehme et al. 2012), Spectra, an initiative of the public health agency of Sweden (http://spectra.folkhalsomyndigheten.se), or via a private laboratory database shared online.

27.8.6 MS Maintenance and Quality Control

Appropriate maintenance is also essential to warrant accurate microbial identification by MALDI-TOF MS (Croxatto et al. 2012). Vacuum failure and, thus, MS functional disturbance might be observed due to the presence of dust on plastic joints or their aging. Dust exposure of the instrument can be reduced by placing the mass spectrometer in a separate room without drafts. Carbonization of microbial cell material embedded in the matrix following each laser shot is also a source of concern, as the laser source may be soiled. According to the manufacturer's

recommendations, maintenance should normally be done four to five times a year if a workload of three to five target plates is analyzed per day. Laboratory-internal quality controls to check the performance of the extraction step and the mass spectrometer can be done by routinely testing selected species for which spectra are available in the database (recommended once a week). A systematic control of the reusable target plates (Bruker) including the cleaning procedure has also been advised. Calibration controls (consisting, e.g., of lyophilized *E. coli* extracts and supplementary proteins for Bruker devices) to identify technical problems and to recalibrate the MS instrument are directly available from the manufacturers.

27.9 MALDI-TOF MS Applications in Environmental Microbiology

Determining the number and identity of all cultivable species of an ecosystem, and, if possible, distinguishing among different strains or ecotypes of each of these species, is a major challenge in microbial diversity studies from environmental samples. As a standard approach, 16S rRNA gene amplification and sequencing, has been commonly used; however, this gene lacks taxonomic resolution to allow for differentiation at the subspecies and even the species level (Fröhlich et al. 2009). Accurate (sub)species-level identification is therefore based on polyphasic methodical approaches in a stepwise manner with an initial screening and selection to reduce the number of sequences to a smaller, nonredundant set ("dereplication") which is subsequently followed by identification of a representative for each unique sequence cluster. In the past, $(GTG)_5$-PCR has been frequently used as dereplication tool, but the introduction of MALDI-TOF MS in routine clinical microbiology provided a technically appealing alternative (Santos et al. 2016; Spitaels et al. 2016). Today, MALDI-TOF MS is used as a powerful tool in environmental microbiology for the rapid screening/dereplication and identification of bacteria at the species level based on proteomic mass fingerprints as well as for untargeted metabolomics, more specifically in metabolic profiling and fingerprinting (Santos et al. 2016; Spitaels et al. 2016). Previous ecological studies described its application with a diverse range of environmental samples, e.g., sewage sludge, termite guts, marine sponges, solar saltern sediments, water, (contaminated) soil, fermenters of biogas plants, and the rhizosphere (Ruelle et al. 2004; Dieckmann et al. 2005; Stursa et al. 2009; Ferreira et al. 2011; Emami et al. 2012; Koubek et al. 2012; Stets et al. 2013; Kopcakova et al. 2014; Stantscheff et al. 2014; Reuß et al. 2015; Viver et al. 2015; Emami et al. 2016; Lopez Diez et al. 2016). However, this technique still presents some drawbacks as most studies reported low overall identification rates (20–65%) by commercial reference databases which could only be improved by construction of in-house databases for the underrepresented microbial groups (Stursa et al. 2009; Ferreira et al. 2011; Emami et al. 2012; Koubek et al. 2012; Kopcakova et al. 2014; Santos et al. 2016). This highlights

the need for a comprehensive and environmentally focused high-quality reference database (including high intraspecies diversity) to improve the ability of MALDI-TOF MS to successfully identify environmental bacteria at species and even subspecies level. These environmentally oriented databases may require special considerations relative to clinical databases, since the range of environmental stresses a microorganism is exposed to and that alter its protein profile due to the production of stress-related proteins is more variable.

To overcome the limitations of incomplete databases, several work groups have successfully attempted to identify environmentally relevant bacteria by detecting and identifying (strain-) specific biomarkers in the proteome mass profiles of the respective groups (Ruelle et al. 2004; Dieckmann et al. 2005). There has even been progress to directly infer the species composition of polymicrobial samples on the basis of a single mixture mass spectrum without prior pure culture isolation (Mahe et al. 2014; Zhang et al. 2015). Using novel biomarker- as well as similarity coefficient-based methods, model mixtures containing up to six environmental bacteria could be characterized. However, varying amounts of individual bacteria in mixtures affected the representation of component bacteria in the mixture spectra (Mahe et al. 2014; Zhang et al. 2015). Thus, though promising, both identification strategies still need to be examined and optimized prior to application to particular mixtures to maximize performance.

As it is known, $<1\%$ of microbial species in the environment are able to grow in rich growth media under laboratory conditions; since only cultivable bacteria can be identified by MALDI-TOF MS analysis, this might be seen as major limitation of the method. However, there has been renewed interest in cultivation-based analyses of microbial diversity to complement other "-omics" studies (Lagier et al. 2012). Recent high-throughput cultivation approaches ("culturomics") aimed at isolating the whole microbial community from environmental samples are using more than 200 different isolation conditions (media composition and growth conditions) for a maximal recovery of the cultivable microbiota and yielded several thousands of isolates. This necessitates the application of a fast and cost-effective dereplication and identification technique to rapidly process these isolates in order to reduce the risk of losing part of them. Not surprisingly, MALDI-TOF MS is currently used in such culturomics studies (Lagier et al. 2012; Dubourg et al. 2013). Indeed, MALDI-TOF MS has become an essential analytical tool in the detection and description of several new species in a range of genera (Spitaels et al. 2016), and mass spectral data are increasingly added to novel species descriptions. Highlighting its increasing importance in microbial taxonomy, microbial identification, and diversity studies, the journal *Systematic and Applied Microbiology* published a special issue on this subject in 2011 (vol. 34).

27.10 Microorganisms Involved in Winemaking Process

Wine grapes harbor a complex microbial ecosystem consisting of a wide variety of filamentous fungi, yeasts, and bacteria (associated with the berry skin) that have different physiological/metabolic characteristics and effects upon wine production (Swiegers et al. 2005; Bartowsky and Pretorius 2009; Russo et al. 2016). Some species are only found in grapes, such as parasitic fungi and environmental bacteria, while others have the ability to survive and grow in wines, constituting the wine microbial consortium which covers yeasts, lactic acid bacteria, and acetic acid bacteria. The proportion of these microorganisms depends on the grape ripening stage and on the availability of nutrients (Renouf et al. 2005; Barata et al. 2011; Setati et al. 2012; Pinto et al. 2014; Bargeri et al. 2015; Setati et al. 2015; del Carmen Portillo et al. 2016).

Bunches of grapes are the main natural reservoir of indigenous wine yeasts and bacteria. So far, 93 yeasts and 50 bacterial species have been isolated from various grape varieties worldwide (Renouf et al. 2005, 2007; König and Fröhlich 2009; Barata et al. 2011; Setati et al. 2012, 2015; Bargeri et al. 2015). Despite their large number and taxonomic range (covering 30 genera), yeast species show relatively low population densities on grape berries with populations of 10^2–10^3 CFU/g on immature grapes which increase to 10^3–10^6 CFU/g at harvest time. More than 90% of these populations on the mature grape surface represent non-*Saccharomyces* species, e.g., basidiomycetous *Cryptococcus*, *Rhodotorula*, and *Rhodosporidium* spp., as well as ascomycetous *Aureobasidium pullulans* (Renouf et al. 2007; Barata et al. 2011; Pinto et al. 2014), whereas the most relevant fermentative wine yeast, *Saccharomyces cerevisiae*, only occurs at low cell numbers of 10–100 CFU/berry (Setati et al. 2012). The bacterial species isolated belong to two groups, the *Firmicutes* and *Proteobacteria*. *Firmicutes* present include the gram-positive *Lactobacillaceae* (*Lactobacillus* and *Pediococcus*) and *Leuconostocaceae* (*Leuconostoc*, *Weissella*, *Oenococcus*) that belong to the wine production-relevant group of lactic acid bacteria (LAB) as well as *Bacillaceae* and *Enterococcaceae*. Homofermentative *Lactobacillus* spp., *Pediococcus* spp., and heterofermentative *Leuconostoc mesenteroides*, *Weissella parameenteroides*, and *Oenococcus oeni* (*O. oeni*) have frequently been found on wine grapes. Among the isolated gram-negative *Proteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* are not often listed among oenological microbiota, in contrast to the *Alphaproteobacteria* (e.g., *Gluconobacter* spp.) that comprise the group of acetic acid bacteria (AAB) (Renouf et al. 2005; Barata et al. 2011; Pinto et al. 2014; del Carmen Portillo et al. 2016). At harvest time, averages of the different microbial populations were 10^3 CFU/berry for gram-negative aerobic or anaerobic bacteria (AAB) and 10^4 CFU/berry for gram-positive anaerobic bacteria (LAB) (Renouf et al. 2005), with *Lactobacillus* and *Pediococcus* spp. maximum populations of ~10^2 CFU/g (Barata et al. 2011). The frequency of detection of *O. oeni* on grapes is much lower and requires molecular methods to monitor these minority populations (Renouf et al. 2009). The grapevine microbiome is highly dynamic, revealing changes in the size

and structure of the population during the berry ripening process and in dependence on the grape's health status [e.g., AAB populations are stimulated by berry damage and grow to 10^6 CFU on rotten grapes (Barata et al. 2011)]. The microbial population levels rise gradually reaching their highest value when the berries are overripe with declining gram-negative and increasing gram-positive bacterial communities (Renouf et al. 2005; Barata et al. 2011; Pinto et al. 2014). Many of these grape surface-associated microorganisms cannot survive the extreme conditions of wine fermentation, but their metabolic activity on the grape surface has long-ranging consequences, as they are included in the initial fermentation steps that influence the flavor, color, and quality of the final product.

Recently, metagenomic approaches have become an important tool for assessment of the grape microbiome. It could be demonstrated that bacterial and fungal communities in vineyards not only differed by region but were also conditioned by climate, geographical orientation, grape cultivar, chemical treatments, and agronomic practices (Gayevskiy and Goddard 2012; Setati et al. 2012, 2015; Bokulich et al. 2013, 2016; Perez-Martin et al. 2014; Bargeri et al. 2015; del Carmen Portillo et al. 2016). These significant regional differences in vineyard biodiversity ("regional microbiome fingerprint") were hypothesized to be responsible for regional differences in wine style and character, commonly referred to as the microbial aspect of the *terroir* concept (*microbial terroir*) (Bokulich et al. 2013, 2016; Setati et al. 2015). Indeed, regionally, genetically differentiated populations of *Saccharomyces cerevisiae* have been experimentally demonstrated to differentially affect wine phenotype which is driven by a complex mix of chemicals (Knight et al. 2015).

This process of how the microbiome is contributing to the natural environment of grapes and identity of wine starts at the vineyards, at the harvest of the grapes, and then evolves along the different stages of fermentation. During spontaneous wine fermentation, the grape-indigenous microbiota metabolize the sugars from the grapes while showing a certain temporal succession in community structure with significant population shifts in distinct microbial groups. The natural diversity of metabolic pathways and the contribution of these different microorganisms involved on the fermentation process are well documented (Renouf et al. 2007; König and Fröhlich 2009; Piao et al. 2015; Pinto et al. 2015). Briefly, in the initial phase of winemaking, the grape-associated yeasts form the dominant consortium consuming the sugars; however, with increasing ethanol content, *Saccharomyces cerevisiae* strains start to gradually outnumber the other non-*Saccharomyces* yeasts and dominate the alcoholic fermentation process (AF). Subsequently, LAB, predominantly *O. oeni*, convert malic acid into lactic acid during the malolactic fermentation (MLF) which leads to deacidification, improvement of taste and flavor [production of various secondary metabolites that influence the aromatic quality (Swiegers et al. 2005)], and microbial stability of the wine. AAB, in contrast, cause a negative impact on the winemaking process due to the production of undesirable metabolites, such as acetic acid, and negatively affect the wine quality. Thus, they are considered spoilage bacteria. When both types of fermentation are completed, microbial populations must be reduced to prevent post-fermentation microbial

metabolisms in bottles or during storage in oak barrels which results in wine spoilage by impairment of its sensory characteristics. This is particularly true for volatile phenol synthesis by certain non-*Saccharomyces* yeasts which confers off-odors to wine, as well as causing exopolysaccharides, biogenic amines, and ethyl carbamate production by some LAB species of the grape skin-indigenous microbiome (Bartowsky and Pretorius 2009; Russo et al. 2016).

27.11 MALDI-TOF Analysis of Wine-Associated Microbiota

Several new molecular methods for rapid detection and identification of the wine-associated microbiota have been developed in the past (Fröhlich et al. 2009; Pozo-Bayon et al. 2009; Ivey and Phister 2011) because close monitoring of the microbiome in industrial winemaking is of high importance. To control the positive contribution of the MLF process to wine characteristics in spontaneous wine fermentations, wineries nowadays utilize commercial starter cultures of *O. oeni* as common oenological practice. Notably, there is a high phenotypical heterogeneity among the natural *O. oeni* populations associated with different types of wine. As the resistance to wine conditions is strictly strain-specific, *O. oeni* strains differ considerably in their MLF performance (Renouf et al. 2009; Cafaro et al. 2016; Virgentini et al. 2016). A correlation between strain diversity and the peculiarity of certain oenological niches has been reported with native *O. oeni* isolates being even vineyard-specific, thereby, contributing to the wine's unique organoleptic characteristics. Thus, there is considerable interest in the wine industry to identify and preserve these regionally specific strains by constant quality assessment of the starter cultures, while also searching for novel malolactic isolates from the grape-indigenous microbiota which may be more competitive and better adapted to the particular product and to the specific production technology due to new strain-specific traits. Besides routine quality checks of the *O. oeni* starter cultures, wineries also rely on real-time monitoring of the microbial populations throughout the vinification process to ensure consistent high wine quality and allow risk management to avoid contamination by spoilage bacteria or yeasts. The presence of certain LAB species from the genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus*, for example, which produce health-affecting biogenic amines, can be indicative of poor winemaking and bad sanitization practice. Thus, their early detection and identification during the vinification process is of high practical interest for wineries. Furthermore, the investigation of microbial communities from grapes will improve our knowledge concerning the emergence of sensorial defects in wine linked to the presence of certain fungi and bacteria species. A comprehensive knowledge about the regional grape-associated microbiome and its correlation with the chemical composition and sensory characteristics of wine ("*microbial terroir*" concept) may even allow winemakers to use postharvest

microbiota identification as an early predictor of metabolite presence and abundances in the finished product that determines its quality traits (referred to as *terroir*) (Knight et al. 2015; Bokulich et al. 2016). This knowledge might even be extended to agricultural practices that increase responsible environmental stewardship.

Various molecular methods suitable for the detection and identification of LAB and yeasts from wine are currently available (see Table 27.6 for an overview). These comprise DNA fingerprinting techniques, quantitative PCR (qPCR) (Kantor et al. 2016a), fluorescence in situ hybridization (FISH) (Blasco et al. 2003; Hirschhäuser et al. 2005; Röder et al. 2007), and denaturing gradient gel electrophoresis (DGGE) (Lopez et al. 2003; Renouf et al. 2006; Laforgue et al. 2009) analysis; however, most methods are only suitable for identification at species level (Fröhlich et al. 2009; Pozo-Bayon et al. 2009; Ivey and Phister 2011). Successful strain typing of LAB, AAB, and yeast isolates has been reported by, e.g., pulsed-field gel electrophoresis (REA-PFGE/karyotyping) (Rodas et al. 2005; Larisika et al. 2008; Ruiz et al. 2008), multilocus sequence typing (MLST) (Gonzalez-Arenzana et al. 2014; Wang et al. 2015), amplified fragment length polymorphism (AFLP) (Azumi and Goto-Yamamoto 2001), randomly amplified polymorphic DNA (RAPD)-PCR (Zavaleta et al. 1997; Ruiz et al. 2008), and (nested) specifically amplified polymorphic DNA ((n)SAPD)-PCR (Fröhlich and Pfannebecker 2007; Pfannebecker and Fröhlich 2008; Sebastian et al. 2011).

The use of MALDI-TOF MS as alternative analysis tool for fast and reliable identification of wine-relevant bacteria and yeasts at the strain level has been established over the last years. Petri and coworkers successfully differentiated eleven *O. oeni* strains originating from five different wine-growing regions with the MALDI-MS results, allowing better discrimination of the isolates when compared to nSAPD-PCR analysis (Petri et al. 2015). Recently, mass fingerprinting was implemented in a culture-dependent comprehensive survey of bacterial communities in traditional fermentation starter cultures for Chinese rice wine. Using the MALDI Biotyper 3.0 classification tool (Bruker), a total of 1314 isolates were investigated and shown to belong mainly to the *Bacillus* and LAB group (69% and 30% of species, respectively). Notably, profile-based MS analysis showed superior performance in terms of accurate differentiation between *Bacillus* spp. when compared to 16S rRNA (Lv et al. 2016). After establishing in-house reference mass spectra databases, successful strain typing of wine-relevant yeasts by MALDI-TOF MS was reported for 33 and 21 strains of the genus *Saccharomyces* (Blättel et al. 2013; Usbeck et al. 2014) as well as for wine-spoiling yeasts (Usbeck et al. 2013). Recently, MALDI mass fingerprinting was applied in high-throughput identification of environmental yeast isolates of grape must from different Brazilian vineyards (Agustini et al. 2014) as well as six unfiltered red wines and 15 still-fermenting wine ("Federweisser") from local Slovakian winemakers (Kantor and Kacaniova 2015; Kantor et al. 2016b) using the Biotyper manufacturer database and software (Bruker). While Kantor and coworkers reported successful strain typing of *Saccharomyces* and non-*Saccharomyces* yeast isolates obtained from the "Federweisser" samples (Kantor et al. 2016b), only species-level differentiation

Table 27.6 Molecular methods used in detection and identification of wine-associated microorganisms, summarized after Ivey and Phister (2011) and Fröhlich et al. (2009)

Type of identification method	Identification method applicable for	Taxonomic resolution level	Technique assessment
Hybridization			
FISH	Isolates and mixed cultures/environmental sample	Species	Laborious, expensive equipment, only group-specific probes for LAB or yeasts
Flow cytometry	Mixed cultures/environmental sample	Species	Limited coverage by commercially available antibodies
gDNA hybridization	Isolates	Strain	Expensive, limited to currently available genome sequences
Sequencing			
rRNA, functional gene	Isolates	Species	Rapid, inexpensive, limited resolution
MLST	Isolates	Species to strain	Rapid, inexpensive, limited discriminatory power with yeasts
Genome	Isolates	Strain	Expensive, laborious
Fingerprinting			
ITS-RFLP (ARDRA)	Isolates	Species	Rapid, inexpensive, identification limited by banding pattern database
16S rDNA-RFLP (ARDRA)	Isolates	Species (bacteria)	Rapid, inexpensive, identification limited by banding pattern database
26S rDNA-RFLP (ARDRA)	Isolates	Species (yeast)	Rapid, inexpensive, identification limited by banding pattern database
REA-PFGE	Isolates	Strain (bacteria)	Expensive, laborious, depends on selection of appropriate restriction enzymes, requires high level of training
Karyotyping (PFGE)	Isolates	Strain (yeast)	Expensive, laborious, discriminatory power limited in certain genera, requires high level of training
mt-RFLP	Isolates	Strain (yeast)	Rapid, inexpensive, limited discriminatory power in non-*Saccharomyces* yeasts
AFLP	Isolates	Strain (yeast)	Laborious, expensive
RAPD-PCR	Isolates	Strain	Rapid, inexpensive, rigorous standardization needed for band pattern reproducibility
(n)SAPD-PCR	Isolates	Strain	Rapid, inexpensive, rigorous standardization needed for band pattern reproducibility
δ-sequence amplification (RAPD-PCR technique)	Isolates	Strain (yeast)	Targets specific repetitive chromosomal regions, rigorous standardization needed for band pattern reproducibility

(continued)

Table 27.6 (continued)

Type of identification method	Identification method applicable for	Taxonomic resolution level	Technique assessment
Microsatellite amplification (RAPD-PCR technique)	Isolates	Strain (yeast)	Targets specific repetitive chromosomal regions, rigorous standardization needed for band pattern reproducibility
DGGE	Mixed cultures/ environmental sample	Species	Primer design critical for PCR-introduced bias, co-migration of gel bands, only group-specific primer sets for LAB or yeasts
PCR detection			
Bacterial and yeast targets (rRNA and functional genes)	Mixed cultures/ environmental sample	Species to strain	Rapid, inexpensive, only group-specific primer sets for LAB or yeasts, no quantitative analysis, no differentiating between viable and nonviable microbes
qPCR			
Bacterial and yeast targets (rRNA and functional genes)	Mixed cultures/ environmental sample	Species to strain	Rapid, sensitive, expensive, only group-specific primer sets for LAB or yeasts, no differentiating between viable and nonviable microbes, requires high level of training

was shown for the 152 and 854 environmental isolates in both other studies (Agustini et al. 2014; Kantor and Kacaniova 2015). Notably, only 67% of the strains could be differentiated at the species level by Agustini et al. (2014) as a consequence of insufficient taxonomic coverage of the commercially available database; the remaining 33% isolates could only be identified after addition of the missing reference spectra to a supplementary database (Agustini et al. 2014).

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