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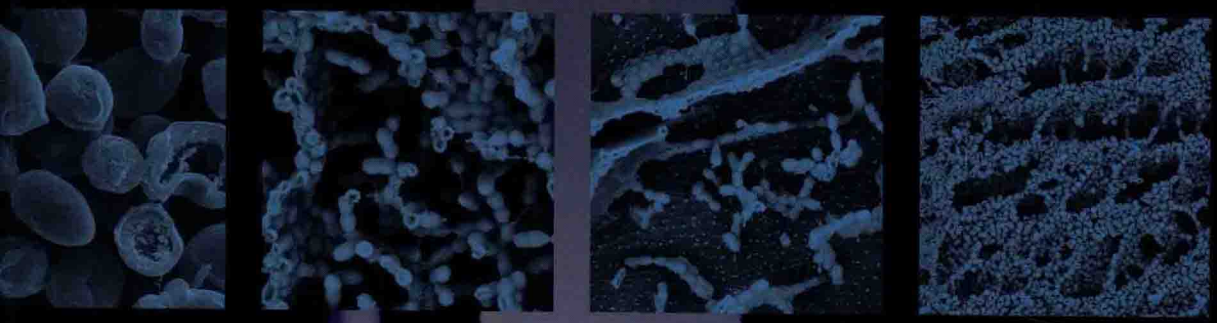

Molecular Wine Microbiology

MOLECULAR WINE MICROBIOLOGY

Edited by

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Academic Press is an imprint of Elsevier

Academic Press is an imprint of Elsevier
32 Jamestown Road, London, NW1 7BY, UK
30 Corporate Drive, Suite 400, Burlington, MA 01803, USA
525 B Street, Suite 1800, San Diego, CA 92101-4495, USA

First edition 2011

Translation by Anne Murray and Iain Patten

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

ISBN: 978-0-12-375021-1

Typeset by TNQ Books and Journals

Printed and bound in the United States of America

11 12 13 14 15 10 9 8 7 6 5 4 3 2 1

MOLECULAR WINE MICROBIOLOGY

Preface

The publication of Louis Pasteur's *Mémoire sur la fermentation alcoolique* in 1857 has come to represent a milestone in the history of science and its applications, as it marked the beginning of a growing fascination with the biology of wine microorganisms among researchers worldwide. Since then, unprecedented improvements in winemaking processes have gone hand in hand with the development of modern microbiology, and it would now be impossible to understand the continuing progress made in the wine industry without taking into account the impact of advances in microbiological research.

A greater understanding of the microbiology of wine holds the key to critical issues affecting the industry, such as the management of safety and quality. For instance, by identifying and gaining a better understanding of the molecular mechanisms underlying the growth of microorganisms that cause wine spoilage or pose a threat to consumer health, winemakers will be better positioned to control and even eradicate them during the production process.

It is hoped that *Molecular Wine Microbiology* will be a useful tool for researchers and educators working in both the private and public sectors. Above all, however, it will be a valuable resource for those starting out on their fascinating journey through the world of wine microbiology.

Coordinated by Alfonso V. Carrascosa, Rosario Muñoz, and Ramón González from the Spanish National Research Council (CSIC), this book brings together contributions from a range of experts on the microbiology of wine working in universities, research centers, and industry.

Translation by Anne Murray and Iain Patten

The editors would like to acknowledge the excellent translation of the Spanish text. The translators have been able to capture all the nuances of the original, using accurate winemaking English terms.

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CHAPTER

1

Saccharomyces Yeasts I: Primary Fermentation

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1. YEASTS OF INTEREST IN WINE PRODUCTION

1.1. Yeast Flora on the Grape, in the Winery, and in the Must

The fermentation of grape must is a complex microbiological process that involves interactions between yeasts, bacteria, and filamentous fungi (Fleet, 2007; Fugelsang & Edwards, 2007). Yeasts, which play a central role in the winemaking process, are unicellular fungi that reproduce by budding. Most yeasts belong to the phylum Ascomycota on the basis of their sexual development. In these organisms, the zygote develops within a sac-like structure, the ascus, while the nucleus undergoes two meiotic divisions, often followed by one or more mitotic divisions. A wall forms around each daughter nucleus and its surrounding cytoplasm to generate four ascospores within the ascus. The ascus then ruptures and releases the ascospores, which can germinate and produce new vegetative cells. Although thousands of yeast species have been identified, only 15 correspond to wine yeasts (Ribéreau-Gayon et al., 2006).

Traditionally, wine has been produced using yeast strains found on the surface of grapes and in the winery environment. The yeasts reach the grapes by wind and insect dispersal and are present on the wines from the onset of fruit ripening (Lafon-Lafourcade, 1983). The predominant species on the grape is *Kloeckera apiculata*, which can account for more than 50% of the flora recovered from the fruit (Fugelsang & Edwards, 2007). Other species of obligate aerobic or weakly fermentative yeasts with very limited alcohol tolerance may also be found in lesser proportions. These belong to the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Hansenula*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, and *Rhodotorula* (Fleet & Heard, 1993; Ribéreau-Gayon et al., 2006). The fermentative species *Saccharomyces cerevisiae* and *Saccharomyces bayanus* are present in limited numbers. This microflora can be affected by a wide variety of factors, principally temperature, rainfall, altitude, ripeness of the crop, and use of fungicides (Boulton et al., 1996). The flora associated with winery equipment is largely made up of *S. cerevisiae* (Fleet & Heard, 1993; Fleet, 2007; Martini & Vaughan-Martini, 1990), though species of the genera *Brettanomyces*, *Candida*, *Hansenula*, *Kloeckera*, *Pichia*, and *Torulaspora* have also been isolated.

The yeasts present in the must during the first few hours after filling the tanks belong to the same genera as those found on the grapes, predominantly *Hanseniaspora/Kloeckera*. In these spontaneous vinification conditions, *Saccharomyces* yeasts (mainly *S. cerevisiae*) begin to develop after around 20 h and are present alongside the grape-derived yeast flora. After 3 or 4 d of fermentation, *Saccharomyces* yeasts predominate and are ultimately responsible for alcoholic fermentation (Ribéreau-Gayon et al., 2006). This change in the yeast population is linked to the increasing presence of ethanol, the anaerobic conditions, the use of sulfites during harvesting and in the must, the concentration of sugar, and the greater tolerance of high temperatures shown by *S. cerevisiae* compared with other yeasts (Fleet & Heard, 1993; Fleet, 2007). *S. cerevisiae* comprises numerous strains with varying biotechnological properties (Ribéreau-Gayon et al., 2006). The importance of using genetic techniques to identify and characterize the different species and strains of yeast that participate in fermentation should not be underestimated. This is considered further in Chapter 5, which addresses the taxonomy of wine yeasts.

Currently, the usual strategy employed in winemaking involves inoculation of the must with selected yeasts in the form of active dried yeast. This practice, which emerged in the 1970s, shortens the lag phase, ensures rapid and complete fermentation of the must, and helps to create a much more reproducible final product (Bauer & Pretorius, 2000; Fleet & Heard,

1993). The selection of wine yeasts with specific genetic markers provides a system for the precise monitoring of the growth of particular strains during fermentation. Analyses of this type have shown that fermentation is driven mainly by inoculated yeasts (Delteil & Aizac, 1988), although these sometimes become only partially established (Esteve-Zarzoso et al., 1999). Given that the growth of the natural flora is not completely suppressed during the initial days of vinification, these strains can make substantial contributions to certain properties of the wine (Querol et al., 1992; Schütz & Gafner, 1993). Consequently, there is increasing interest in the use of mixed starter cultures in which non-*Saccharomyces* yeasts contribute desirable characteristics—particularly in terms of the organoleptic quality of the wine—that complement the fermentative capacity of *Saccharomyces* yeasts (Fleet, 2008).

The inoculated yeast strain must obviously be very carefully selected on the basis of certain necessary characteristics (Degré, 1993; Fleet, 2008). For instance, it must produce vigorous fermentation with short lag phases and little residual sugar, have reproducible fermentation characteristics, be tolerant of high pressure, ethanol, and suboptimal temperatures, and produce glycerol and β-glucosidases in adequate quantities to achieve a good aroma. Other valuable properties include fermentative capacity at low temperatures, low foaming, killer activity (Barre, 1980), certain levels of specific enzymatic activities (Darriet et al., 1988; Dubourdieu et al., 1988), and resistance to the adverse growth conditions present during winemaking (Zuzuarregui & del Olmo, 2004a). It is particularly important in the secondary fermentation of some sparkling wines for the yeast to be flocculent or easily separated from the medium (Degré, 1993; Zaworski & Heimsch, 1987). Autochthonous strains that meet these criteria have been increasingly used in recent years in an effort to obtain wines that maintain the sensory characteristics associated with specific wine-growing regions (Lafon-Lafourcade, 1983; Snow, 1983).

1.2. Morphology and Cellular Organization of Yeasts

Saccharomyces yeast cells have a rigid cell wall that allows them to resist the changes in osmotic pressure that can occur in the extracellular environment. Inside the cell wall, there is a periplasmic space and a plasma membrane surrounding the cytoplasm. Various transport mechanisms control the permeability of these structures and maintain their role as barriers.

Yeasts have multiple subcellular organelles characteristic of eukaryotic cells. These include a nucleus surrounded by a nuclear envelope, a smooth and a rough endoplasmic reticulum, a Golgi apparatus, mitochondria, and vacuoles. The cytoplasm contains numerous enzymes involved in the metabolic events described below, such as the enzymes responsible for alcoholic fermentation. Although some *Saccharomyces* strains lack mitochondria (respiration-deficient or "petite" mutants), these organelles play a fundamental role in metabolism. During fermentation, the high concentration of glucose in the medium inhibits synthesis of enzymes involved in the citric acid cycle and cytochromes from the respiratory chain through an effect known as glucose repression (Gancedo, 2008; Santangelo, 2006 and references therein). As a result, mitochondrial oxidative metabolism is limited under these conditions. However, aerobic metabolism, which is dependent upon mitochondria, does occur during the production of commercial yeasts for must inoculation and during some phases of the winemaking process.

Vacuoles are important for homeostasis, since enzymes that participate in the degradation and recovery of cell constituents are exclusively or predominantly localized to these structures. They also accumulate metabolites such as basic amino acids, S-adenosylmethionine, polyphosphates, allantoin, and allantoate at much higher

concentrations than those found in the cytoplasm.

More in-depth reviews of the cellular organization of yeasts can be found in *The yeasts* (1991), edited by Rose and Harrison, and in *The molecular biology of the yeast Saccharomyces cerevisiae* (1982), edited by Strathern, Jones, and Broach.

1.3. Genetic Characteristics of Wine Yeasts

Unlike their counterpart laboratory strains, wine strains of *S. cerevisiae* are prototrophs, meaning that they do not require amino acids or nucleotides for their growth. This has important consequences for genetic manipulation, since genes conferring resistance to antibiotics, such as cycloheximide (del Pozo et al., 1991) or geneticin (Hadfield et al., 1990), must be used or auxotrophies introduced prior to transformation of these yeasts.

Wine strains of yeast are usually diploid, polyploid, or even aneuploid (Bakalinsky & Snow, 1990; Codón et al., 1995). Chromosome length in these yeasts is highly polymorphic (Bidenne et al., 1992; Rachidi et al., 1999), and this results in extensive variability in sporulation capacity and spore viability. This characteristic also influences the options for gene manipulation, since at least two copies of a gene need to be eliminated to obtain a deletion mutant. The ploidy of wine yeasts may provide them with advantages in adapting to changeable environments or, perhaps, represent a way of increasing the dose of genes that are important for fermentation (Bakalinsky & Snow, 1990; Salmon, 1997).

Finally, wine yeasts are predominantly homothallic (HO), meaning that following sporulation the daughter cells can change mating type, conjugate with a cell of the opposite mating type, and ultimately form a cell with 2n DNA content that is homozygous for all genes except the MAT locus (Thornton & Eschenbruch, 1976). In contrast, in heterothallic (ho) strains, the MAT locus is stable and cells remain in a haploid state until they encounter a cell of the opposite mating type with which to fuse (reviewed in Sprague, 1995). Wine strains also exhibit a high degree of heterozygosity (Barre et al., 1993; Codón et al., 1995), including for the HO locus (Guijo et al., 1997; Mortimer et al., 1994), and they can undergo mitotic recombination (Longo & Vézinhet, 1993; Puig et al., 2000), a characteristic that is not observed in haploid laboratory strains. This capacity for extensive genomic change means that wine yeasts do not display genetic stability (Pretorius, 2000; Snow, 1983). These factors and their relationship with evolutionary processes are discussed in detail by Pérez-Ortín et al. (2002) and are also considered in Chapter 6.

As in all eukaryotes, the mitochondria of *S. cerevisiae* have a circular mitochondrial DNA (mtDNA) (Christiansen & Christiansen, 1976; Hollenberg et al., 1970). This is usually located in the mitochondrial matrix but may occasionally be bound to the inner mitochondrial membrane. The mtDNA contains genes encoding proteins essential for mitochondrial function and, in yeasts, exhibits a high degree of polymorphism due to variability in the presence of certain introns and differences in the size of intergenic regions (Clark-Walker et al., 1981). This variability has been used in taxonomic studies, as discussed in Chapter 5.

2. GROWTH CHARACTERISTICS OF *SACCHAROMYCES* YEASTS DURING FERMENTATION

2.1. Must Composition

Grape must is a complex medium containing all of the nutrients necessary for the growth of *S. cerevisiae*. However, the varying composition of different musts, in addition to being crucial for the characteristics of the final product,

influences the growth dynamics of the yeast. Vinification is a discontinuous, batch-type fermentation process in which all of the nutrients are present in the culture medium from the outset and the concentration of the nutrients declines as they are consumed by the yeast. As a result, the availability of some nutrients may act as a limiting factor for growth. Below we describe the main components of the must and their effect on the process of alcoholic fermentation.

2.1.1. Sugars

With the exception of water, monosaccharides are the most abundant component of grape must. Glucose and fructose are the main hexose sugars and are present in approximately equimolar concentrations. Other monosaccharides present as minor components include arabinose (0.2–1.5 g/L) and xylose (0.03–0.1 g/L); low concentrations of the disaccharide sucrose, which is generally hydrolyzed at the low pH found in must, are also present (Ough, 1992). Although polysaccharides such as pectins, gums, and dextrin are present at concentrations of around 3 to 5 g/L, they are not assimilable by wine yeasts. The total concentration of sugars is generally between 170 and 220 g/L (Ribéreau-Gayon et al., 2006). In musts with sugar concentrations of more than 200 g/L, there is a slowing of fermentation. Sugar concentrations between 250 and 300 g/L can inhibit yeast growth as a result of the high osmotic pressure and the elevated intracellular concentration of ethanol (Nishino et al., 1985). However, the low sugar concentrations typical of northerly wine-growing areas do not limit yeast growth and only affect the final alcohol concentration.

2.1.2. Organic Acids

The second most abundant compounds, organic acids, are present at concentrations of between 9 and 27 g/L (Ough, 1992). Tartaric and malic acid together account for 90% of the fixed acidity (Jackson, 1994); citric and ascorbic acid are found at lower concentrations. Tartaric acid predominates in must from warmer climates, where it reaches concentrations of 2 to 8 g/L, whereas, in cooler climates, malic acid concentrations may exceed those of tartaric acid, depending on the ripeness of the grapes. These acids have no direct effect on yeast growth but do play a decisive role in the pH of the must (see Section 2.2.3).

2.1.3. Nitrogenous Compounds

Nitrogen content is important since it tends to be limiting for the growth of *S. cerevisiae* (Ingledew & Kunkee, 1985) and the principal cause of stuck fermentation (Bisson, 1999). The concentration of soluble nitrogen varies between 0.1 and 1 g/L (Henschke & Jiranek, 1993). The composition of nitrogen sources in the must depends on a large number of factors, such as the grape variety, infection with *Botrytis cinerea* (which eliminates large quantities of the nutrients that can be assimilated by *Saccharomyces* yeasts), the timing of harvest, use of fertilizers, addition of supplements in the winery, and the extent of clarification of the musts, particularly in white grape musts (Lagunas, 1986). Variations in the quantity and form of the nitrogen sources in the must influence yeast cell growth, fermentation rate, and ethanol tolerance. The main compounds are ammonia (3–10%), amino acids (25–30%), polypeptides (25–40%), and proteins (5–10%). In addition, smaller quantities of nitrates, nucleotides, amines, and vitamins may be present. Nucleotides are only present at very low concentrations in the must (e.g., adenine and uracil nucleotides are found at concentrations of 4–15 mg/L and 4–8 mg/L, respectively). These are taken up by the yeast and incorporated into their nucleic acids, although yeast can also synthesize their own nucleotides (Monteiro & Bisson, 1992).

Saccharomyces yeasts cannot assimilate inorganic nitrogen sources such as nitrates and nitrites. They are also unable to assimilate proteins and polypeptides present in the

medium, since they do not have a system for extracellular digestion of these types of compound. As a result, they are essentially dependent on the concentrations of ammonia and amino acids, their preferred nitrogen sources (Ough & Amerine, 1988). The most abundant amino acids in the must tend to be proline and arginine, and their concentrations vary in different musts. Proline cannot be metabolized by yeast under the low-oxygen conditions associated with alcoholic fermentation and should therefore not be taken into account when considering nitrogen availability. It has been reported that concentrations of assimilable nitrogen below 140 mg/L impair fermentation at normal sugar concentrations (Bely et al., 1990), and a concentration of ammonium ions below 25 mg/L is generally considered to be undesirable. However, outcomes can vary according to the individual strain. Cases have been described in which normal fermentation occurred in the presence of 120 mg/L assimilable nitrogen (Carrasco et al., 2003), while strains that require a minimum of 267 mg/L to complete the process have also been reported (Mendes-Ferreira et al., 2004). Since *Saccharomyces* yeasts can synthesize their own amino acids, the simplest solution to the problem of nitrogen deficiencies is to provide ammonium salt supplements, usually in the form of diammonium sulfate or phosphate (Ribéreau-Gayon et al., 2006). Addition of up to 30 g/hl of diammonium phosphate (DAP) is permitted in the European Union (EU), whereas in the United States up to 96 g/hl is allowed (Fugelsang & Edwards, 2007). Excessive nitrogen supplementation can alter the microbiological stability of the wine (providing nutrients for spoilage organisms) and its aroma (in many cases derived from deamination of amino acids). The timing of nitrogen addition is also important. Although reductions in fermentation time have been reported to occur independently of the timing of addition, better results are obtained when nitrogen is added during the exponential growth phase (Beltrán et al., 2005). Because ethanol impedes the uptake of nitrogenous compounds during later stages, it has been proposed that nitrogen should be added prior to or during the initial phases of fermentation, to coincide with aeration of the must (Sablayrolles et al., 1996). More recent data have shown poorer recovery of fermentation activity with addition of ammonia alone than with addition of amino acids or a combination of the two (Jiménez-Martí et al., 2007).

Among the nitrogenous compounds, vitamins deserve special mention. Wine yeasts are able to synthesize all of their own vitamins except for biotin (Ough, 1992) and nicotinic acid under anaerobic conditions (Panozzo et al., 2002), meaning that they are not as dependent as more complex organisms on the availability of these cofactors. Nevertheless, the presence of vitamins in the must stimulates the growth and metabolic activity of yeasts via the vitamins' participation as coenzymes in numerous biochemical reactions, and as a result they can be considered as growth factors (Ribéreau-Gayon et al., 2006). Must is generally rich in vitamins, but the concentrations of some are suboptimal. As a result, addition of vitamins can stimulate growth, particularly when the grapes have been subject to fungal infection, which always reduces the total concentration of vitamins. Thiamine is also an important component of the must. However, it is partially degraded by the sulfite added to prevent the appearance of spoilage organisms (Jackson, 1994) and is also consumed by the yeasts over the course of the fermentation. Consequently, it is advisable to add it to the must. The amount recommended by the EU is 50 mg/hl, whereas the maximum permitted level in the United States is 60 mg/hl (Fugelsang & Edwards, 2007). Deficiencies in other vitamins, such as pantothenic acid and pyroxidine, should also be avoided as they can lead to generation of undesirable compounds such as acetic acid and hydrogen sulfide (Wang et al., 2003).

2.1.4. *Polyphenols*

The many and varied phenolic compounds present in the must are essential elements in determining the organoleptic character of the wine (Waterhouse, 2002). Although it has been reported that the anthocyanins in red grape musts and the procyanidins in white grape musts can stimulate and inhibit growth, respectively (Cantarelli, 1989), these compounds have no relevant influence on the growth of wine yeasts. Their most noteworthy effect is as antioxidants, particularly in the case of quinones. It has recently been described that resveratrol and other polyphenols with recognized preventive effects in cardiovascular disease (Fremont, 2000) can extend the replicative lifespan of *Saccharomyces* yeasts (Howitz et al., 2003).

2.1.5. *Mineral Salts*

Inorganic elements are necessary for normal metabolism and maintenance of pH and ion balance in yeasts. Potassium, sodium, calcium, and magnesium are the predominant cations in the must, and chlorates, phosphates, and sulfates the main anions (Ough & Amerine, 1988). Must generally provides the inorganic elements required for yeast growth, but, if the concentration of one of these elements is limited, normal progression of fermentation can sometimes be affected (Bisson, 1999). Phosphate ions are particularly important given their vital metabolic role, as hexose sugars must be phosphorylated in order to be metabolized. Deficiencies in this anion can be compensated along with those of nitrogen by supplementation with DAP (see Section 2.1.3).

2.1.6. *Lipids*

Under the anaerobic conditions associated with wine fermentation, yeasts cannot synthesize sterols or long-chain unsaturated fatty acids. Synthesis of these compounds will only occur if oxygen is added during fermentation to increase yeast cell viability and the quality of fermentation (Sablayrolles, 1996). The lack of these types of lipid (especially ergosterol, the principal sterol in the plasma membrane of *Saccharomyces* yeasts) affects the structure and function of the plasma membrane and leads to increased effects of ethanol and poor glucose uptake (Jackson, 1994). These compounds are referred to as survival factors, since their presence is necessary for cell viability but their addition does not increase growth (Ribéreau-Gayon et al., 2006). Generally, the presence of these types of lipid in the must is guaranteed given their abundance in grape skins. Problems are only encountered in excessively clarified white wines, since up to 90% of unsaturated fatty acids may be lost under these conditions (Bertrand & Miele, 1984). In such situations, it is appropriate to supplement the must with yeast extract or lysed yeast (Muñoz & Ingledew, 1990). In other situations, the use of dried yeast grown under aerobic conditions usually guarantees the presence of sufficient lipids in the cell wall for fermentation of the must to take place.

2.1.7. *Inhibitors*

This section covers exogenous compounds added to the grapes and must to prevent the appearance of undesirable microorganisms that can also influence the growth of wine yeasts.

2.1.7.1. SULFITES

Sulfites are added to control the appearance of spoilage organisms in the must. Industrial yeasts have been selected to be resistant to the quantities of sulfites used in wineries, and their growth is not usually affected by the concentrations of between 0.8 and 1.5 mg/L that are normally used. Concentrations above 1.5 mg/L, however, can inhibit growth (Sadraud & Chauvet, 1985). This inhibition is dependent upon the pH of the must; SO_2, the active molecular species, is generated at lower pH, and as a result the toxicity of a given concentration of the compound increases under those conditions

(Farkaš, 1988). Sulfite toxicity is also increased by the richness of methionine in the must, whereas it is reduced by higher concentrations of adenine (Aranda et al., 2006). Nevertheless, sulfite normally only delays the onset of fermentation and does not affect the rate or completion of the process.

2.1.7.2. PESTICIDES

Chemical compounds applied to the vines to prevent parasite infection can sometimes affect the growth of *Saccharomyces* yeasts during vinification. Folpet and captan, traditionally the most commonly used fungicides, have a substantial antiseptic effect on yeasts (Cabras & Angioni, 2000). New-generation fungicides are only marketed if they have been shown to have no effect on yeasts. For instance, metalaxyl, cymoxanil, famoxadone, fenhexamid, fluquinconazole, kresoxim-methyl, quinoxyfen, and trifloxystrobin have no effect on yeast growth (Jackson, 1994; Oliva et al., 2007). In addition, clarification of the must eliminates most of the pesticides present on the surface of the grapes, and many are degraded spontaneously under the acidic conditions of the must. As with sulfites, traces of fungicide in the must tend to inhibit the onset of fermentation rather than interfere with fermentation rate or completion.

2.2. Physical Parameters of Fermentation

The main physicochemical factors that affect the growth of *Saccharomyces* yeasts during alcoholic fermentation are described below.

2.2.1. Temperature

Temperature is the most important physical factor in the growth of yeasts and the progression of fermentation (Fleet & Heard, 1993). Although *S. cerevisiae* has an optimal growth temperature of around 30°C, it can adapt to a wide range of temperatures up to a maximum of 40°C, at which point viability begins to decline (Watson, 1987). Although there is a linear increase in the rate of fermentation between 10 and 32°C (doubling every 10°C), this does not mean that higher temperatures are the most appropriate for fermentation of the must. Ethanol toxicity increases with temperature, and higher temperatures lead to evaporation of ethanol and other volatile compounds that are essential to the organoleptic properties of the wine (Torija et al., 2003), particularly in the case of white wines. Excessively low temperatures are also not recommended, since they can cause stuck fermentation when yeast membrane fluidity begins to be affected (Bisson, 1999). It is also not economically viable to maintain fermentations under these conditions for extended periods. Consequently, controlling fermentation temperature is an essential element of modern wine production. White wines are generally fermented at between 10 and 18°C to improve the retention of aromas, whereas red wines tend to be fermented at higher temperatures (between 18 and 29°C) to achieve good extraction of phenolic compounds (Fugelsang & Edwards, 2007). Nevertheless, an initial fermentation temperature of 20°C is recommended in both cases in order to stimulate initiation of yeast growth (Jackson, 1994). Low temperatures may favor the growth of non-*Saccharomyces* yeasts during the initial stages of fermentation.

2.2.2. Aeration

Saccharomyces yeasts are facultative anaerobes, able to consume sugars in the absence of oxygen more effectively than non-*Saccharomyces* yeasts (Visser et al., 1990). In fact, excess oxygen can inhibit fermentation, a phenomenon known as the Pasteur effect. Nevertheless, a certain amount of oxygen is beneficial for the growth of wine yeasts since it is required for the synthesis of sterols (mainly ergosterol) and unsaturated fatty acids. A more oxygenated environment may be helpful in musts with nitrogen deficiencies, as this will allow the

amino acid proline to be metabolized (Ingledew & Kunkee, 1985). It is also advisable to add exogenous nitrogen sources during aeration of the must (Sablayrolles et al., 1996). The oxygen captured by the must during pressing is usually sufficient to reach saturation, and is therefore generally adequate for normal progression of fermentation. In red wines, oxygen consumption due to oxidation of phenols is compensated by the aeration created during pump-over, resulting in oxygen concentrations of around 10 mg/L. This effect is most beneficial at the end of the exponential growth phase. Nevertheless, excessive aeration may lead to undesirable production of acetaldehyde and hydrogen sulfide, and reduced production of aromatic esters (Nykänen, 1986).

2.2.3. *pH*

The typical pH of grape must is between 2.75 and 4.2 (Heard & Fleet, 1988). These pH values do not have a negative effect on the growth of *Saccharomyces* yeasts, and problems only begin to present themselves at a pH below 2.8. The toxic effects of low pH are due to the increased effects of ethanol (Pampuhla & Loereiro-Dias, 1989) and sulfite (Farkaš, 1988). Tolerance of acidic pH depends on the abundance of potassium ions in the must (Kudo et al., 1998). Low pH favors the hydrolysis of disaccharides and, therefore, fermentation. In addition, the acidic character of the must prevents the appearance of spoilage microorganisms. Consequently, acids such as tartaric acid are sometimes added (addition of 1 g/L, for example, reduces the pH by 0.1 units). However, addition of excess tartaric acid can lead to undesirable precipitation.

2.2.4. *Clarification*

Elimination of solid particles from the must is an important element in the production of white wines. However, elimination of the nutrients that are associated with them, particularly nitrogenous compounds, can impair yeast growth (Ayestaran et al., 1995). Furthermore, solid particles act as nuclei for the formation of carbon dioxide bubbles and favor dissipation of the gas, which at high levels can inhibit the growth of *Saccharomyces* yeasts (Thomas et al., 1994). On the other hand, the final products obtained from clearer musts have better organoleptic characteristics. The extent of clarification must therefore be optimized to produce better wines without affecting the fermentation process.

2.2.5. *Carbon Dioxide*

Alcoholic fermentation of hexose sugars generates carbon dioxide, which can reach volumes equivalent to 56 times that of the fermented must (Boulton et al., 1996). The release of this gas contributes to the dissipation of some heat and produces convection currents within the must that aid the diffusion of nutrients. However, its evaporation also favors loss of ethanol and volatile compounds (Jackson, 1994). Furthermore, if produced in excess, carbon dioxide affects the viability of *Saccharomyces* yeasts, mainly due to membrane damage.

2.3. Yeast Growth and Fermentation Kinetics

Yeast growth during wine fermentation differs from that occurring in other industrial processes such as brewing, since the high concentration of sugars leads to the production of ethanol at concentrations that inhibit growth. Fermentation begins rapidly with inoculums containing approximately 10^6 cells/mL. The typical growth cycle of *Saccharomyces* yeasts consists of three phases and begins following a short lag period (Lafon-Lafourcade, 1983). The first phase is the limited growth phase and lasts between 2 and 5 d, generating a population of up to 10^7 or 10^8 cells/mL. Fermentation during this phase occurs at a constant, maximal rate, and it tends to consume between a third and half of the initial sugar content (Castor & Archer, 1956). Next, growth enters a quasi-stationary

phase that lasts around 8 d. During this time, there is no increase in the number of cells in the population. However, the cells are metabolically active and the rate of fermentation remains maximal. Finally, the culture enters the death phase, which is poorly characterized and highly variable. Whereas some authors claim that death does not occur until all of the sugars have been consumed (Boulton et al., 1996), others have assigned greater importance to this phase. According to this view, the death phase is estimated to be three or four times longer than the growth phase and still involves consumption of a considerable quantity of sugar (Ribéreau-Gayon et al., 2006). The loss of viability is accompanied by a reduction in the rate of fermentation, due not only to a reduction in the number of viable cells but also to inhibition of the metabolic activity of the nonproliferative cells. The loss of fermentative capacity of the cells in this final phase has been linked to the depletion of adenosine triphosphate (ATP) and the accumulation of ethanol, which have negative effects on membrane transport. It has been observed that, under these conditions, cellular enzyme systems are functional but the intracellular concentration of sugars decreases progressively.

Yeast growth is monitored by microscopic counts of the cells in diluted samples of fermenting must. The number of cells can also be estimated by measuring the optical density at 600 to 620 nm following the generation of standard curves for the inoculated strain. In both cases, estimations of the numbers of cells present in the fermenting must do not differentiate between viable and dead cells, a very important distinction when monitoring the progression of wine fermentation. To differentiate between the two, plate counts can be performed with solid nutrient media, on which only viable cells will be able to produce colonies; however, this type of analysis is slow, as the colonies take 3 to 4 d to grow. Other more rapid techniques based on the use of fluorescent reagents or bioluminescent quantification of ATP are available for estimation of the number of viable cells, but they are less reliable.

Another parameter that is analyzed in wine yeasts is vitality; that is, the capacity of the cells to achieve complete metabolic activity. There is a relationship between this metabolic activity and the time necessary to reach maximum fermentation rate. This is usually measured by indirect impedance; in other words, the reduction in impedance due to a solution of potassium hydroxide that reacts with the carbon dioxide produced by the metabolic activity of the yeast (Novo et al., 2007).

Because all of the methods for monitoring yeast growth are relatively difficult to implement in wineries, in practice, fermentation kinetics are analyzed using simpler techniques such as monitoring the reduction in sugar concentration, the increase in ethanol content, or the release of carbon dioxide (Ribéreau-Gayon et al., 2006). However, the simplest method to adapt to winery conditions is analysis of the density of the must, since measurement of the mass per unit volume provides an approximate measure of sugar content. During the course of fermentation, the sugar concentration decreases while ethanol content increases, and this leads to a reduction in density. The initial density of the must and the final density of the wine will depend on the initial sugar concentration, which will lead to a specific percentage of ethanol (approximately 1% [vol/vol] ethanol for every 17 g of sugars) (Ribéreau-Gayon et al., 2006).

2.4. Biochemistry of Fermentation

The biochemistry of wine production is also complex. The central metabolic process that takes place is alcoholic fermentation, a catabolic pathway involving the transformation of the hexose sugars present in the must into ethanol and carbon dioxide. Compounds are also generated that play a central role in yeast growth and in the organoleptic properties of the wine.

A more complete description of the biochemical processes that take place during wine production can be found elsewhere (Boulton et al., 1996; Rose & Harrison, 1991; Strathern et al., 1982). The aim here is to introduce readers to some of these pathways, in particular those that are most relevant in terms of yeast growth and the properties of the final product.

2.4.1. *Alcoholic Fermentation*

Carbon sources, in particular the hexose sugars glucose and fructose, allow cells to obtain energy by alcoholic fermentation. This metabolic pathway (Figure 1.1) occurs in the cytoplasm and can be expressed in terms of the following simplified equation:

$$C_6H_{12}O_6 + 2ADP + 2HPO_4^- \rightarrow 2C_2H_5OH + 2CO_2 + 2ATP + 2H_2O$$

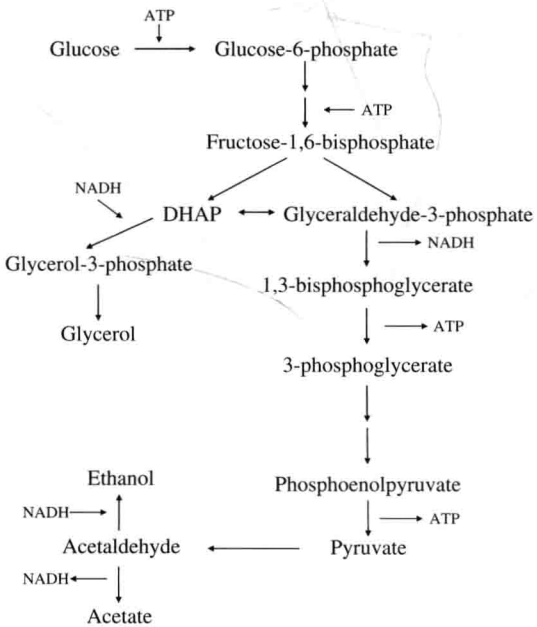

FIGURE 1.1 Schematic diagram of the conversion of glucose into ethanol during alcoholic fermentation by the yeast *Saccharomyces cerevisiae*. The figure also shows the relationship between energy production in this pathway and the processes linked to the redox state of the coenzyme NAD^+/NADH. The reactions in which consumption or synthesis of ATP and NADH occur are indicated. DHAP = dihydroxyacetone phosphate. *Figure adapted from Norbeck and Blomberg (1997).*

Alcoholic fermentation involves the Embden-Meyerhof-Parnas (EMP) pathway, which was described by Embden, Meyerhof, and Parnas around 1940 and is also known as glycolysis. The pathway involves 10 reactions. The first five reactions correspond to the energy investment phase, in which sugars are metabolically activated by ATP-dependent phosphorylation to give rise to a six-carbon sugar, fructose-1,6-bisphosphate, which is cleaved to produce two moles of triose phosphate. During the energy generation phase (reactions 6 to 10), the triose phosphates are reactivated, generating two compounds with a high phosphate-transfer potential: firstly 1,3-bisphosphoglycerate and then phosphoenolpyruvate. Each of these compounds transfers a high-energy phosphate group to adenosine diphosphate (ADP), thus producing ATP in a process known as substrate-level phosphorylation. The chemical energy of ATP can be subsequently transformed in the cell into other forms of energy necessary for cell growth. The first reaction in this energy generation phase is an oxidation reaction catalyzed by the enzyme glyceraldehyde-3-phosphate dehydrogenase. This enzyme requires nicotinamide-adenine dinucleotide (NAD^+) as a coenzyme to accept the electrons from the substrate being oxidized. As a consequence, this coenzyme is reduced to NADH.

After glycolysis, alcoholic fermentation is completed with two additional reactions used to reoxidize NADH to NAD^+ to guarantee the continuation of glycolysis. In the first reaction, the resulting pyruvate is decarboxylated to acetaldehyde and carbon dioxide by the enzyme pyruvate decarboxylase, which requires thiamine pyrophosphate as a coenzyme. Finally, the acetaldehyde is reduced to ethanol by the