CHAPTER 1

Yeasts

1.1 Introduction

Man has been making bread and fermented beverages since the beginning of recorded history. Yet the role of yeasts in alcoholic fermentation, particularly in the transformation of grapes into wine, was only clearly established in the middle of the 19th century. The ancients explained the boiling during fermentation (from the Latin *fervere*, to boil) as a reaction between substances that come into contact with each other during crushing to produce effervescence. In 1680, a Dutch cloth merchant, Antonie van Leeuwenhoek, first observed yeasts in beer wort using a microscope that he designed and produced. He did not, however, establish a relationship between these corpuscles and alcoholic fermentation. It was not until the end of the 18th century that Antoine Lavoisier began the chemical study of alcoholic fermentation. Joseph Louis Gay-Lussac continued Lavoisier's research into the next century. As early as 1785, Adam Fabroni, an Italian scientist, was the first to provide an interpretation of the chemical composition of the ferment responsible for alcoholic fermentation, which he described

Handbook of Enology, Volume 1: The Microbiology of Wine and Vinifications, Third Edition.
Pascal Ribéreau-Gayon, Denis Dubourdieu, Bernard B. Donèche and Aline A. Lonvaud.

as a plant–animal substance. According to Fabroni, this material, comparable to the gluten in flour, was located in special utricles, particularly on grapes and wheat, and alcoholic fermentation occurred when it came into contact with sugar in the must. In 1837, a French physicist named Charles Cagnard de La Tour proved for the first time that yeast was a living organism. According to his findings, it was capable of multiplying and belonged to the plant kingdom; its vital activities were the basis for the fermentation of sugar-containing liquids. The German naturalist Theodor Schwann confirmed his theory and demonstrated that heat and certain chemical products were capable of stopping alcoholic fermentation. He named the beer yeast *zuckerpilz*, which means sugar fungus—*Saccharomyces* in Latin. In 1838, Franz Meyen used this nomenclature for the first time.

This vitalist or biological conception of the role of yeasts in alcoholic fermentation, obvious to us today, was not readily embraced. Justus von Liebig and certain other organic chemists were convinced that chemical reactions, not living cellular activity, were responsible for the fermentation of sugar. In his famous works, *Studies on Wine* (1866) and *Studies on Beer* (1876), Louis Pasteur gave definitive credibility to the vitalist view of alcoholic fermentation. He demonstrated that the yeasts responsible for spontaneous fermentation of grape must or crushed grapes came from the surface of the grape; he isolated several races and species. He even conceived the notion that the nature of the yeast carrying out the alcoholic fermentation could influence the taste characteristics of wine. He also demonstrated the effect of oxygen on the assimilation of sugar by yeasts and proved that the yeast produced secondary products such as glycerol in addition to alcohol and carbon dioxide.

Since Pasteur, yeasts and alcoholic fermentation have incited a considerable amount of research, making use of progress in microbiology, biochemistry, and now genetics and molecular biology.

In taxonomy, scientists define yeasts as single-celled fungi that reproduce by budding and binary fission. Certain multicellular fungi have a single-celled stage and are also grouped with yeasts. Yeasts form a complex and heterogeneous group found in three classes of fungi, characterized by their reproduction mode: Ascomycetes, Basidiomycetes, and the imperfect fungi (Deuteromycetes). However, yeasts found on the surface of the grape and in wine belong only to Ascomycetes and the imperfect fungi. The haploid spores or ascospores of the Ascomycetes class are contained in the ascus, a type of sac made from vegetative cells. Asporogenous yeasts, incapable of sexual reproduction, are classified with the imperfect fungi.

In this chapter, the morphology, reproduction, taxonomy, and ecology of grape and wine yeasts will be discussed. Cytology is the morphological and functional study of the structural components of the cell (Rose and Harrison, 1991).

Yeasts are the most simple of the eukaryotes. The yeast cell contains cell envelopes, a cytoplasm with various organelles, and a nucleus surrounded by a membrane and enclosing the chromosomes (Figure 1.1). Like all plant cells, the yeast cell has two cell envelopes: the cell wall and the plasma membrane. The periplasmic space is the space between the cell wall and the membrane. The cytoplasm and the membrane make up the protoplasm. The terms protoplast and spheroplast designate a cell whose cell wall has been “artificially” removed in full or in part, respectively. Yeast cell envelopes play an essential role: they contribute to a successful alcoholic fermentation of must and release certain constituents that add to the resulting wine’s composition. To take advantage of these properties, the winemaker or enologist must have a profound knowledge of these organelles.

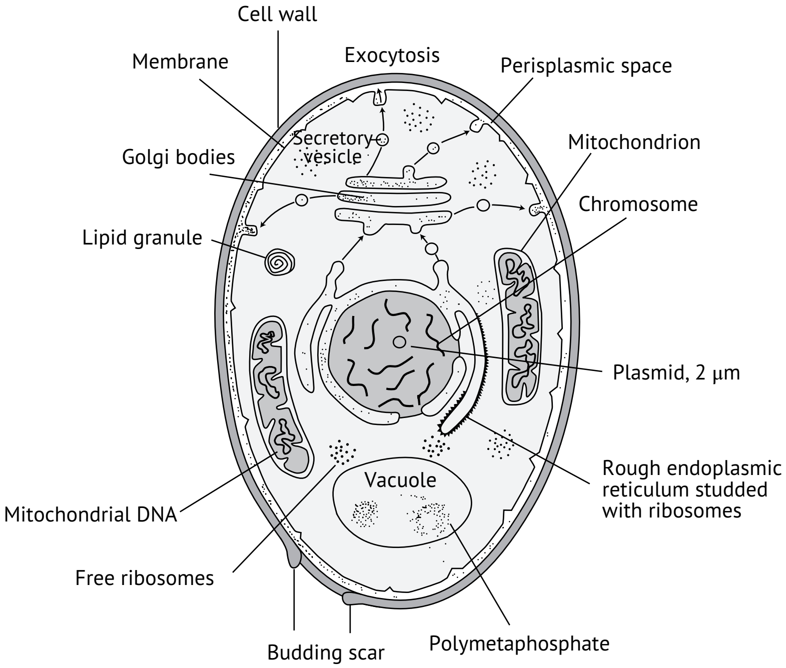


FIGURE 1.1 A yeast cell (Gaillardin and Heslot, 1987).

1.2 The Cell Wall

1.2.1 The General Role of the Cell Wall

The works of various authors (Fleet, 1991; Klis, 1994; Stratford, 1994; Klis *et al.*, 2002) have greatly expanded our knowledge of the yeast cell wall, which represents 15–25% of the dry weight of the cell. The yeast cell wall essentially consists of polysaccharides. It is a rigid envelope, yet endowed with a certain elasticity.

Its first function is to protect the cell. Without its wall, the cell would burst under the internal osmotic pressure, which is determined by the composition of the cell's environment. Protoplasts placed in pure water are immediately lysed in this manner. Cell wall elasticity can be demonstrated by placing whole yeasts, sampled during their log growth phase, in a hypertonic (NaCl) solution. Their cell volume decreases by approximately 50%. The cell wall appears thicker, while the cytoplasmic membrane practically does not detach from it at all. The cells regain their initial form after being placed back into an isotonic medium.

Yet the cell wall cannot be considered an inert, semirigid "armor." On the contrary, it is a dynamic and multifunctional organelle. Its composition and functions evolve during the life of the cell, in response to environmental factors. In addition to its protective role, the cell wall gives the cell its particular shape through its macromolecular organization. It is also the site of molecules that determine certain cellular interactions such as sexual union, flocculation, and the killer factor, which will be examined in detail in Section 1.7. Lastly, a number of enzymes, generally hydrolases, are connected to the cell wall or situated in the periplasmic space. Their substrates are nutritive substances of the environment and the macromolecules of the cell wall itself, which are constantly reshaped during cell morphogenesis.

1.2.2 The Chemical Structure and Function of the Cell Wall Constituents

The yeast cell wall is made up of two principal constituents: β-glucans and mannoproteins. Chitin represents a small part of its composition. The most detailed work on the yeast cell wall has been carried out on *Saccharomyces cerevisiae*—the principal yeast responsible for the alcoholic fermentation of grape must.

Glucan represents about 60% of the dry weight of the cell wall of *S. cerevisiae*. It can be chemically fractionated into three categories:

1. A β-1,3-glucan that is insoluble in water, acetic acid, and alkalis. It has very few branches. The branch points involve β-1,6 linkages. Its degree of polymerization is 1,500. Under an electron microscope, this glucan appears fibrous. It ensures the shape and the rigidity of the cell wall. It is always associated with chitin.
2. A β-1,3-glucan, with about 1,500 glucose units, that is insoluble in water but soluble in alkalis. It has very few branches, like the preceding glucan. In addition to these few branches, it has a small number of β-1,6 glycosidic linkages. It has an amorphous appearance under the electron microscope. It gives the cell wall its elasticity and acts as an anchor for mannoproteins. It can also constitute an extraprotoplasmic reserve substance.
3. A β-1,6-glucan that is obtained from alkali-insoluble glucans by extraction in acetic acid. The resulting product is amorphous, water-soluble, and extensively branched with β-1,3-glycosidic linkages. Its degree of polymerization is 140. It links the different constituents of the cell wall together. It is also a receptor site for the killer factor (Section 1.7).

The fibrous β-1,3-glucan (alkali-insoluble) probably results from the incorporation of chitin in the amorphous β-1,3-glucan.

Mannoproteins constitute 25–50% of the cell wall of *S. cerevisiae*. They can be extracted from the whole cell or from the isolated cell wall using chemical or enzymatic methods. Chemical methods make use of autoclaving in the presence of bases or a citrate buffer solution at pH 7. The enzymatic method frees the mannoproteins by digesting the glucan. This method does not denature the structure of the mannoproteins as much as chemical methods. Zymolyase, obtained from a bacterium (*Arthrobacter luteus*), is the enzymatic preparation most often used to extract the cell wall mannoproteins of *S. cerevisiae*. This enzymatic complex is effective primarily because of its β-1,3-glucanase activity. The action of protease contaminants in the zymolyase also combines with the aforementioned activity to liberate the mannoproteins. Glucanex, another industrial preparation of β-glucanase, produced by a fungus (*Trichoderma harzianum*), has been demonstrated to possess endo- and exo-β-1,3- and endo-β-1,6-glucanase activities (Dubourdieu and Moine, 1995). These activities also facilitate the extraction of the cell wall mannoproteins from the *S. cerevisiae* cell.

The mannoproteins of *S. cerevisiae* have a molecular weight between 20 and 450 kDa. Their degree of glycosylation varies. However, some of them, containing about 90% mannose and 10% peptides, are hypermannosylated.

Four forms of glycosylation are described (Figure 1.2) but do not necessarily exist at the same time in all mannoproteins.

The mannose in mannoproteins may form short, linear chains with one to five residues. They are linked to the peptide chain by O-glycosyl linkages on serine and threonine residues. These glycosidic side-chain linkages are α-1,2 and α-1,3.

The carbohydrate part of the mannoprotein can also be a polysaccharide. It is linked to an asparagine residue of the peptide chain by an *N*-glycosyl linkage. This

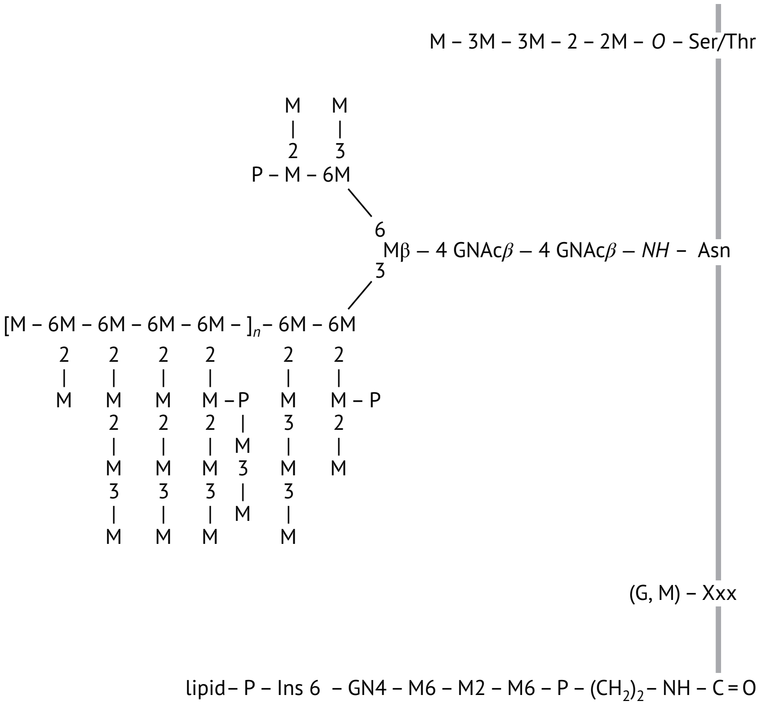


FIGURE 1.2 The four types of glucosylation of cell wall yeast mannoproteins (Klis, 1994). M, mannose; G, glucose; GN, glucosamine; GNAc, *N*-acetylglucosamine; Ins, inositol; Ser, Serine; Thr, threonine; Asn, asparagine; P, Phosphate; Xxx, the nature of the bond is not known.

linkage consists of a double unit of *β*-1,4-linked *N*-acetylglucosamine (chitobiose). The mannan linked in this manner to the asparagine includes an attachment region made up of a dozen mannose residues and a highly branched outer chain consisting of 150–250 mannose units. The attachment region beyond the chitobiose residue consists of an *α*-1,6-linked mannose skeleton with side branches possessing one, two, or three mannose residues with *α*-1,2 and/or *α*-1,3 bonds. The outer chain is also made up of a skeleton of *α*-1,6-linked mannose units. This chain bears short side chains composed of *α*-1,2-linked mannose residues and an *α*-1,3-linked terminal mannose. Some of these side chains possess a branch attached by a phosphodiester bond.

A third type of glycosylation was also described. It can occur in mannoproteins, which make up the cell wall of the yeast. It consists of a glucomannan chain containing essentially *α*-1,6-linked mannose residues and *α*-1,6-linked glucose residues. The nature of the glycan–peptide point of attachment is not yet clear, but it may be an asparaginyl–glucose bond. Moreover, this type of glycosylation characterizes the proteins freed from the cell wall by the action of a *β*-1,3-glucanase. Therefore, *in vivo*, the glucomannan chain may also comprise *β*-1,3-linked glucose residues.

The fourth type of glycosylation of yeast mannoproteins is the glycosylphosphatidylinositol (GPI) anchor. This attachment between the terminal carboxylic group of the peptide chain and a membrane phospholipid permits certain mannoproteins, which cross the cell wall, to anchor themselves in the plasma membrane. The region of attachment is characterized by the following sequence (Figure 1.2):

ethanolamine-phosphate-6-mannose-α-1,2-mannose-α-1,6-mannose-α-1,4-glucosamine-α-1,6-inositol-phospholipid. The presence of such an anchor in certain mannoproteins does not mean that these remain bound to the membrane. They may detach via enzymatic cleavage of the phospholipids. A phospholipase C specific to phosphatidylinositol (PI) and therefore capable of completing this cleavage has been demonstrated in *S. cerevisiae* (Flick and Thorner, 1993). Several GPI-type anchor mannoproteins have been identified in the cell wall of *S. cerevisiae*.

Chitin is a linear polymer of β-1,4-linked *N*-acetylglucosamine and is not generally found in large quantities in yeast cell walls. In *S. cerevisiae*, chitin constitutes 1–2% of the cell wall and is found for the most part (but not exclusively) in bud scar zones. These zones are a type of raised crater easily seen on the mother cell under an electron microscope (Figure 1.3). This chitin scar is formed essentially to ensure cell wall integrity and cell survival. Yeasts treated with Polyoxin D, an antibiotic inhibiting the synthesis of chitin, are not viable; they burst after budding.

The presence of lipids in the cell wall has not been clearly demonstrated. It is true that cell walls prepared in the laboratory contain some lipids (2–15% for *S. cerevisiae*), but this is most likely contamination by the lipids of the cytoplasmic membrane, adsorbed by the cell walls during their isolation. The cell wall can also adsorb lipids from its external environment, especially the various fatty acids that activate and inhibit fermentation (Section 3.6.2).

Several enzymes are connected to the cell wall or situated in the periplasmic space. One of the most characteristic is invertase or β-fructofuranosidase. This enzyme catalyzes the hydrolysis of sucrose into glucose and fructose. It is a thermostable mannoprotein anchored to a β-1,6-glucan of the cell wall. Its molecular weight is 270,000 Da. It contains approximately 50% mannose and 50% protein. Periplasmic acid phosphatase is also a mannoprotein.

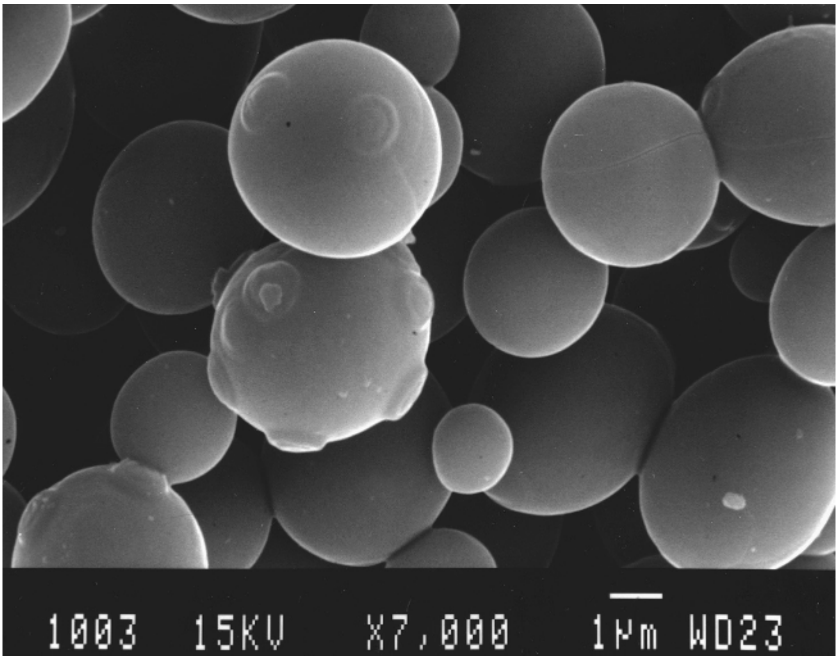


FIGURE 1.3 Scanning electron microscope photograph of proliferating *S. cerevisiae* cells. The budding scars on the mother cells can be observed. (***Source***: Photograph from M. Mercier, Department of Electron Microscopy, Université de Bordeaux I.)

Other periplasmic enzymes that have been noted are β-glucosidase, α-galactosidase, melibiase, trehalase, aminopeptidase, and esterase. Yeast cell walls also contain endo- and exo-β-glucanases (β-1,3 and β-1,6). These enzymes are involved in the reshaping of the cell wall during the growth and budding of cells. Their activity is at a maximum during the exponential growth phase of the population and diminishes notably afterward. However, cells in the stationary phase and even dead yeasts contained in the lees still retain β-glucanase activity in their cell walls several months after the completion of fermentation. These endogenous enzymes are involved in the autolysis of the cell wall during the lees aging of wines. This aging method will be covered in the Chapter 13.

1.2.3 General Organization of the Cell Wall and Factors Affecting Its Composition

The cell wall of *S. cerevisiae* is made up of an outer layer of mannoproteins. These mannoproteins are connected to a matrix of amorphous β-1,3-glucan, which covers an inner layer of fibrous β-1,3-glucan. The inner layer is connected to a small quantity of chitin (Figure 1.4). β-1,6-glucan probably acts as a cement between the two layers. The rigidity and the shape of the cell wall are due to the internal framework of the fibrous β-1,3-glucan. Its elasticity is due to the outer amorphous layer. The intermolecular structure of the mannoproteins of the outer layer (hydrophobic bonds and disulfide bonds) equally determines cell wall porosity for micromolecules (molecular weights less than 4,500) and impermeability to macromolecules. This impermeability can be affected by treating the cell wall with certain chemical agents, such as β-mercaptoethanol. This substance breaks the disulfide bonds, thus destroying the intermolecular network between the mannoprotein chains.

The composition of the cell wall is strongly influenced by nutritive conditions and cell age. The proportion of glucan in the cell wall increases with respect to the amount of sugar in the culture medium. Certain deficiencies (for example, of mesoinositol) also result in an increase in the proportion of glucan compared with mannoproteins. The cell walls of older cells are richer in glucans and in chitin and less rich in mannoproteins than younger ones. For this reason, they are more resistant to physical and enzymatic agents used to break them down. Finally, the composition of cell walls is profoundly modified by morphogenetic alterations (conjugation and sporulation).

1.3 The Plasma Membrane

1.3.1 Chemical Composition and Organization

The plasma membrane is a highly selective barrier controlling exchanges between the living cell and its external environment. This organelle is essential to the life of the yeast.

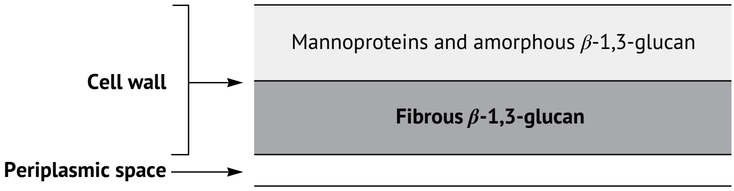


FIGURE 1.4 Cellular organization of the cell wall of *S. cerevisiae*.

Like all biological membranes, the yeast plasma membrane is principally made up of lipids and proteins. The plasma membrane of *S. cerevisiae* contains about 40% lipids and 50% proteins. Glucans and mannans are only present in small quantities (a few percent).

The lipids of the membrane are essentially phospholipids and sterols. They are amphiphilic molecules, i.e. possessing a hydrophilic and a hydrophobic part.

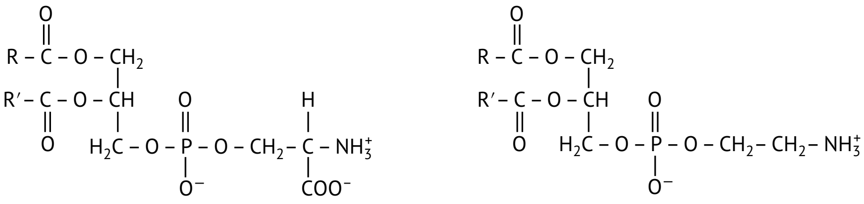
The three principal phospholipids (Figure 1.5) of the plasma membrane of yeast are phosphatidylethanolamine (PE), phosphatidylcholine (PC), and PI, which represent 70–85% of the total. Phosphatidylserine (PS) and diphosphatidylglycerol or cardiolipin (PG) are less prevalent. Free fatty acids and phosphatidic acid are frequently reported in plasma membrane analysis. They are probably extraction artifacts caused by the activity of certain lipid degradation enzymes.

The fatty acids of the membrane phospholipids contain an even number (14–24) of carbon atoms. The most abundant are C16 and C18 acids. They can be saturated, such as palmitic acid (C16) and stearic acid (C18), or unsaturated, as with oleic acid (C18, double bond in position 9), linoleic acid (C18, two double bonds in positions 9 and 12), and linolenic acid (C18, three double bonds in positions 9, 12, and 15). All membrane phospholipids share a common characteristic: they possess a polar or hydrophilic part made up of a phosphorylated alcohol and a nonpolar or hydrophobic part comprising two more-or-less parallel fatty acid chains. Their symbolic representation is shown in Figure 1.6. In an aqueous medium, the phospholipids spontaneously form bimolecular films or a lipid bilayer because of their amphiphilic nature. The lipid bilayers are cooperative but non-covalent structures. They are maintained in place by mutually reinforced interactions: hydrophobic interactions and van der Waals forces between the hydrocarbon tails, and hydrostatic interactions and hydrogen bonds between the polar heads and water molecules. The examination of cross-sections of yeast plasma membranes under an electron microscope reveals a classic lipid bilayer structure with a thickness of about 7.5 nm. The membrane surface appears sculpted with creases, especially during the stationary phase. However, the physiological meaning of this anatomical characteristic remains unknown. The plasma membrane also has a depression under the bud scar.

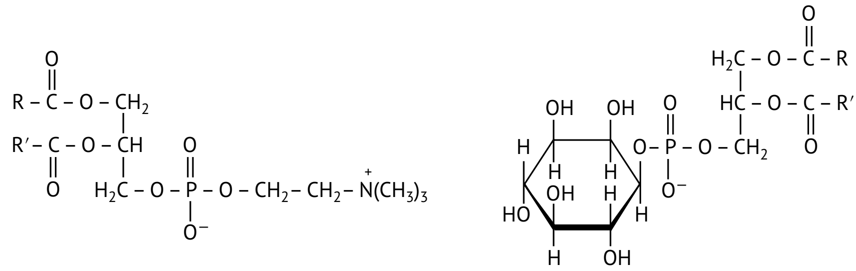
Ergosterol is the primary sterol of the yeast plasma membrane. In addition, 24(28)-dehydroergosterol and lesser amounts of zymosterol are present (Figure 1.7). Sterols are exclusively produced in the mitochondria under aerobic conditions during the yeast log phase. As with phospholipids, membrane sterols are amphipathic. The hydrophilic part is composed of the hydroxyl group in the C3 position, while the rest of the molecule is hydrophobic, especially the flexible hydrocarbon tail.

The plasma membrane also contains numerous proteins or glycoproteins presenting a wide range of molecular weights (from 10,000 to 120,000). The available information indicates that the organization of the plasma membrane of a yeast cell resembles the fluid mosaic model. This model, proposed for biological membranes by Singer and Nicolson (1972), consists of two-dimensional solutions of proteins and oriented lipids. Certain proteins penetrate the membrane; they are called integral proteins (Figure 1.6). They interact strongly with the nonpolar part of the lipid bilayer. The peripheral proteins are linked to the integral ones by hydrogen bonds. Their location is asymmetrical, at either the inner or the outer side of the plasma membrane. The molecules of proteins and membrane lipids, constantly in lateral motion, are capable of rapidly diffusing in the membrane.

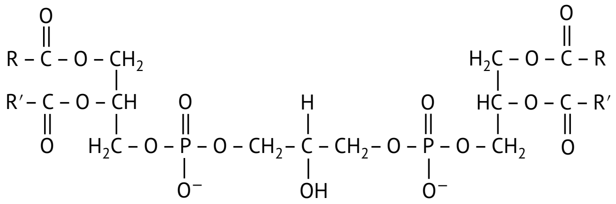
Some of the yeast membrane proteins have been studied in greater depth. These include adenosine triphosphatase (ATPase), solute (sugars and amino acids) transport proteins, and enzymes involved in the production of glucans and chitin of the cell wall.



Phosphatidylserine **Phosphatidylethanolamine**



Phosphatidylcholine **Phosphatidylinositol**



Diphosphatdiylglycerol (cardiolipin)

FIGURE 1.5 Yeast membrane phospholipids.

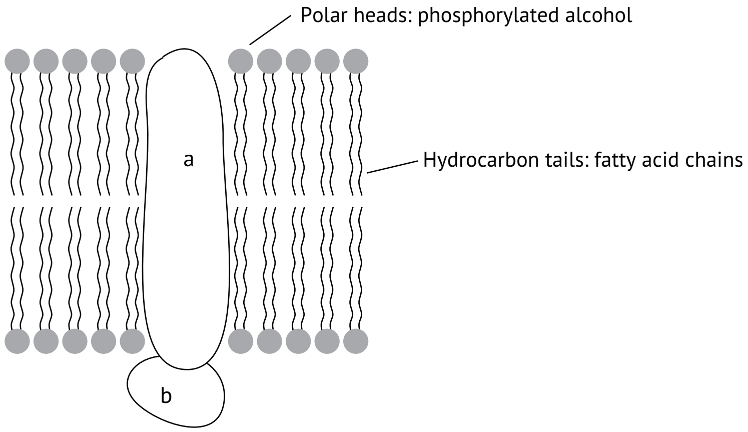
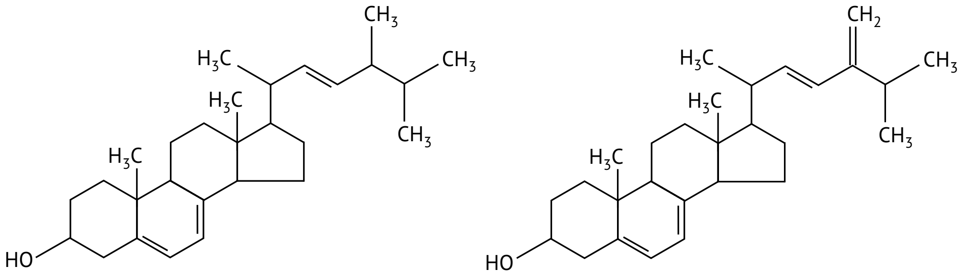
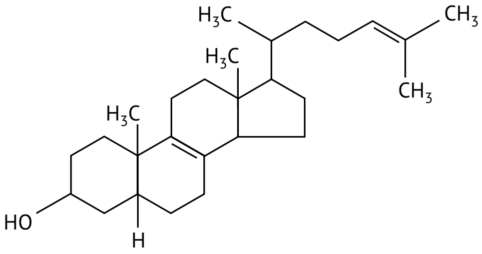


FIGURE 1.6 Diagram of a membrane lipid bilayer. The integral proteins (a) are strongly associated to the hydrocarbon region of the bilayer. The peripheral proteins (b) are linked to the integral proteins.



Ergosterol

24(28)-Dehydroergosterol



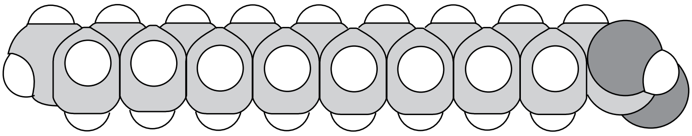
Zymosterol

FIGURE 1.7 Principal yeast membrane sterols.

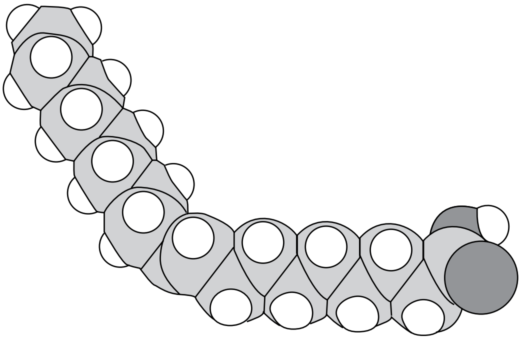
Yeast possesses three ATPases: one each in the mitochondria, the vacuole, and the plasma membrane. The plasma membrane ATPase is an integral protein with a molecular weight of around 100,000 Da. It catalyzes the hydrolysis of adenosine triphosphate (ATP), which furnishes the necessary energy for the active transport of solutes across the membrane. (Note: active transport moves a compound against the concentration gradient.) Simultaneously, the hydrolysis of ATP creates an efflux of protons toward the exterior of the cell.

The penetration of amino acids and sugars into the yeast activates membrane transport systems called permeases. General amino acid permease (GAP) contains three membrane proteins and ensures the transport of a number of neutral amino acids. The cultivation of yeasts in the presence of an easily assimilated nitrogen-based nutrient such as ammonium represses this permease.

The fatty acid composition of the membrane and its sterol content control its fluidity. The hydrocarbon chains of fatty acids of the membrane phospholipid bilayer can be in a rigid and orderly state or in a relatively disorderly and fluid state. In the rigid state, all of the carbon bonds of the fatty acids are *trans*. In the fluid state, some of the bonds become *cis*. The transition from the rigid state to the fluid state takes place when the temperature rises beyond the melting point. This transition temperature depends on the length of the fatty acid chains and their degree of unsaturation. The straight hydrocarbon chains of the saturated fatty acids interact strongly. These interactions intensify with their length. The transition temperature therefore increases as the fatty acid chains become longer. The double bonds of the unsaturated fatty acids are generally *cis*, giving a curvature to the hydrocarbon chain (Figure 1.8). This curvature breaks the orderly stacking of the fatty acid chains and lowers the transition temperature. Like cholesterol in the cells of mammals, ergosterol is also a fundamental regulator of membrane fluidity in yeasts.



Stearic acid (C18, saturated)



Oleic acid (C18, unsaturated)

FIGURE 1.8 Molecular models representing the three-dimensional structure of stearic and oleic acids. The *cis* configuration of the double bond of oleic acid produces a curvature of the carbon chain.

Ergosterol is inserted in the bilayer perpendicularly to the plane of the membrane. Its hydroxyl group is bound by hydrogen bonds with the polar head of the phospholipid, and its hydrocarbon tail is inserted in the hydrophobic region of the bilayer. Thus, the membrane sterols insert themselves between the phospholipids. In this manner, they inhibit the crystallization of the fatty acid chains at low temperatures. Conversely, in reducing the movement of these same chains by steric encumbrance, they regulate any excessive membrane fluidity when the temperature rises.

1.3.2 Functions of the Plasma Membrane

The plasma membrane constitutes a stable, hydrophobic barrier between the cytoplasm and the environment outside the cell, owing to its phospholipids and sterols. This barrier presents a certain impermeability to solutes as a function of osmotic properties.

Furthermore, through its system of permeases, the plasma membrane also controls the exchanges between the cell and the medium. The functioning of these transport proteins is greatly influenced by its lipid composition, which affects membrane fluidity. In a defined environmental model, the supplementing of membrane phospholipids with unsaturated fatty acids (oleic and linoleic) promoted the penetration and accumulation of certain amino acids as well as the expression of GAP (Henschke and Rose, 1991). On the other hand, membrane sterols seem to have less influence on the transport of amino acids than the degree of unsaturation of the phospholipids. The synthesis of unsaturated fatty acids is an oxidative process and requires the aeration of the culture medium at the beginning of alcoholic fermentation. Under semi-anaerobic winemaking conditions, the amount of unsaturated fatty acids in the grape, or in the grape must, probably favors the membrane transport mechanisms of amino acids.

The transport systems of sugars across the membrane are far from being completely elucidated. There exists, however, at least two kinds of transport systems: a high affinity one for glucose and a low affinity system with one-tenth of the affinity of the former (Bisson, 1991). The low-affinity transport system is essential during the log growth phase, and its activity decreases during the stationary phase. The high-affinity system is, in contrast, repressed by high concentrations of glucose, as in the case of grape must (Salmon *et al.*, 1993) (Figure 1.9).

The activity of sugar transport is dependent on the protein synthesis activity of cells. As soon as this activity slows down or stops, we observe a reduction in the sugar transport activity (phenomenon called catabolite inactivation) (Busturia and Lagunas, 1986; Salmon *et al.*, 1993). Under winemaking conditions, the protein synthesis rate starts to decrease very early during alcoholic fermentation at the end of cell growth. Thus, the catabolite inactivation process of sugar transport systems intervenes and is accelerated in case of yeast-assimilable nitrogen deficiencies (Salmon, 1989).

The amount of sterols in the membrane, especially ergosterol, as well as the degree of unsaturation of the membrane phospholipids favor the penetration of glucose in the cell. This is especially true during the stationary and decline phases. This phenomenon explains the decisive influence of aeration on the successful completion of alcoholic fermentation during the yeast multiplication phase (Section 3.7.2).

The presence of ethanol, in a culture medium, slows the penetration speed of arginine and glucose into the cell and limits the efflux of protons resulting from membrane ATPase activity (Alexandre *et al.*, 1994; Charpentier, 1994). Simultaneously, the presence of ethanol increases the synthesis of membrane phospholipids and their percentage of unsaturated fatty acids (especially oleic). Temperature and ethanol act in synergy to affect membrane ATPase activity. The concentration of ethanol required to cancel the proton efflux decreases as temperature rises. However, this modification of membrane ATPase activity by ethanol may not be the source of the decrease in plasma membrane permeability in an alcohol medium. The role of

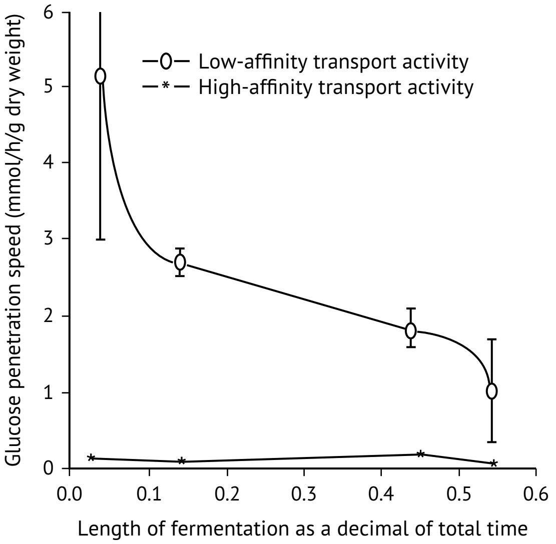


FIGURE 1.9 Evolution of glucose transport system activity of *S. cerevisiae* fermenting a model medium (Salmon *et al.*, 1993).

membrane ATPase in yeast resistance to ethanol has not been clearly demonstrated.

The plasma membrane also produces cell wall glucan and chitin. Two membrane enzymes are involved: β-1,3-glucan synthase and chitin synthase. These two enzymes catalyze the polymerization of glucose and *N*-acetyl-glucosamine, derived from their activated forms (uridine diphosphate or UDP). Mannoproteins are essentially produced in the endoplasmic reticulum (ER; Section 1.4.2). They are then transported by vesicles that fuse with the plasma membrane and deposit their contents at the exterior of the membrane.

Finally, certain membrane proteins act as specific cell receptors. Therefore, the yeast can react to various external stimuli such as sexual hormones or changes in the concentration of external nutrients. The activation of these membrane proteins triggers the release of compounds such as cyclic adenosine monophosphate (cAMP) into the cytoplasm. These compounds serve as secondary messengers that set off other intercellular reactions. The consequences of these cell mechanisms in the alcoholic fermentation process merit further study.

1.4 The Cytoplasm and Its Organelles

Bounded by the plasma membrane and the nuclear membrane, the cytoplasm contains a basic cytoplasmic substance or cytosol. The organelles (ER, Golgi apparatus, vacuole, and mitochondria) are isolated from the cytosol by membranes.

1.4.1 Cytosol

Cytosol is a buffered solution, with a pH between 5 and 6, containing soluble enzymes, glycogen, and ribosomes.

Glycolysis and alcoholic fermentation enzymes (Sections 2.2.1 and 2.2.2) as well as trehalase (an enzyme catalyzing the hydrolysis of trehalose) are present. Trehalose is a reserve disaccharide, also cytoplasmic, that ensures yeast viability during the dehydration and rehydration phases by maintaining membrane integrity.

The lag phase, preceding the log growth phase in a sugar-containing medium, is marked by a rapid breakdown of trehalose, linked to an increase in trehalase activity. This activity is itself closely related to an increase in the amount of cAMP in the cytoplasm. This compound is produced by a membrane enzyme, adenylate cyclase, in response to the stimulation of a membrane receptor by an environmental factor.

Glycogen is the principal yeast carbohydrate reserve substance. Similar in structure to animal glycogen, it accumulates during the stationary phase in the form of spherical granules of about 40 μm in diameter.

When observed under the electron microscope, the yeast cytoplasm appears rich in ribosomes. These tiny granulations, made up of ribonucleic acids and proteins, are the center of protein synthesis. Associated with polysomes, several ribosomes migrate along the length of the messenger RNA. They translate it simultaneously so that each one produces a complete polypeptide chain.

1.4.2 The ER, the Golgi Apparatus, and the Vacuoles

The ER is a double membrane system partitioning the cytoplasm. It is linked to the cytoplasmic membrane and nuclear membrane. It is, in a way, an extension of the latter. Although less developed in yeasts than in exocrine gland cells of higher eukaryotes, the ER has the same function. It ensures the routing of the proteins synthesized by the attached ribosomes. As a matter of fact, ribosomes can be either free in the cytosol or bound to the ER. The proteins synthesized by free ribosomes remain in the cytosol, as do the enzymes involved in glycolysis. Those produced in the ribosomes bound to the ER have three possible destinations: the vacuole, the plasma membrane, and the external environment (secretion). The presence of a signal sequence (a particular chain of amino acids) at the N-terminal extremity of the newly formed protein determines the association of the initially free ribosomes in the cytosol with the ER. The synthesized protein crosses the ER membrane by an active transport process called translocation. This process requires the hydrolysis of an ATP molecule. Having reached the inner space of the ER, the proteins undergo certain modifications including the necessary excising of the signal peptide by signal peptidase. In many cases, they also undergo a glycosylation. The yeast glycoproteins, in particular the structural or enzymatic cell wall mannoproteins, contain carbohydrate side chains (Section 1.2.2). Some of these are linked to asparagine by *N*-glycosidic bonds. This oligosaccharidic linkage is constructed in the interior of the ER by the sequential addition of activated sugars (in the form of UDP derivatives) to a hydrophobic lipid transporter called dolichol phosphate. The entire unit is transferred in one piece to an asparagine residue of the polypeptide chain. Thus, the dolichol phosphate is regenerated.

The Golgi apparatus consists of a stack of membrane sacs and associated vesicles. It is an extension of the ER. Transfer vesicles transport the proteins issued from the ER to the sacs of the Golgi apparatus. The Golgi apparatus has a dual function. It is responsible for completing the glycosylation of proteins and then it sorts them so as to direct them via specialized vesicles either into the vacuole or into the plasma membrane. An N-terminal peptide (propeptide) sequence determines the routing of proteins toward the vacuole. This sequence is revealed in the precursors of two cytoplasm-to-vacuole targeting enzymes in the yeast: carboxypeptidase Y and proteinase A. However, the vesicles that transport the proteins of the plasma membrane or the secretion granules, like those that transport periplasmic invertase, are still the default destinations.

The vacuole is a spherical organelle, 0.3–3 μm in diameter, surrounded by a single membrane. Depending on the stage of the cellular cycle, yeasts have one or several vacuoles. Before budding, a large vacuole splits into small vesicles. Some penetrate into the bud, while others gather at the opposite end of the cell and fuse to form one or two large vacuoles. The vacuolar membrane, or tonoplast, has the same general structure (fluid mosaic) as the plasma membrane, but it is more elastic and its chemical composition is somewhat different. It is less rich in sterols and contains less protein and glycoprotein but more phospholipids with a higher degree of unsaturation. The vacuole stores some of the cell hydrolases, in particular carboxypeptidase Y, proteases A and B, aminopeptidase I, X-prolyl-dipeptidylaminopeptidase, and alkaline phosphatase. In this respect, the yeast vacuole can be compared to an animal cell lysosome. Vacuolar proteases play an essential role in the turnover of cellular proteins. In addition, protease A is indispensable in the maturation of other vacuolar hydrolases. It excises a small peptide sequence and thus converts precursor forms (proenzymes) into active enzymes. The vacuolar proteases are also responsible for autolysis, after cell death, when aging white wine on its lees.

Vacuoles also have a second principal function: they store metabolites before their use. In fact, they contain a quarter of the pool of amino acids of the cell, including a lot of arginine as well as *S*-adenosyl methionine. In this organelle, there is also potassium, adenine, isoguanine, uric acid, and polyphosphate crystals. These are involved in the fixation of basic amino acids. Specific permeases ensure the transport of these metabolites across the vacuolar membrane. An ATPase linked to the tonoplast furnishes the necessary energy for the movement of stored compounds against the concentration gradient. It is different from the plasma membrane ATPase, but also produces a proton efflux.

The ER, Golgi apparatus, and vacuoles must therefore be considered as different components of an internal system of membranes, called the vacuome, participating in the flux of glycoproteins to be excreted or stored.

1.4.3 The Mitochondria

Distributed on the periphery of the cytoplasm, the mitochondria (mt) are spherical or rod-shaped organelles surrounded by two membranes. The inner membrane is highly folded to form cristae. The general organization of mitochondria is the same as in higher plants and animal cells. The membranes delimit two compartments: the intermembrane space and the matrix. Mitochondria are true respiratory organelles for yeasts. Under aerobic conditions, the *S. cerevisiae* cell contains about 50 mitochondria. Under anaerobic conditions, these organelles degenerate, their inner surface decreases, and the cristae disappear. Supplementing the culture medium with ergosterol and unsaturated fatty acids limits the degeneration of mitochondria under anaerobic conditions. In any case, when cells formed under anaerobic conditions are placed under aerobic conditions, the mitochondria regain their normal appearance. Even in aerated grape must, the high sugar concentration represses the synthesis of respiratory enzymes. As a result, the mitochondria no longer function. This phenomenon is called catabolite repression by glucose (Section 2.3.1).

The mitochondrial membranes are rich in phospholipids—principally PC, PI, and PE (Figure 1.5). PG, a minority component in the plasma membrane, is predominant in the inner mitochondrial membrane. The fatty acids of the mitochondrial phospholipids are C16:0, C16:1, C18:0, and C18:1. Under aerobic conditions, the unsaturated residues predominate. When the cells are grown under anaerobic conditions, without lipid supplements, the short-chain saturated residues become predominant; cardiolipin and PE diminish, whereas the proportion of PI increases. Under aerobic conditions, the temperature during the log growth phase influences the degree of unsaturation of the phospholipids—which becomes higher as the temperature decreases.

The mitochondrial membranes also contain sterols, as well as numerous proteins and enzymes (Guerin, 1991).

The two membranes, inner and outer, contain enzymes involved in the synthesis of phospholipids and sterols. The ability to synthesize significant amounts of lipids, characteristic of yeast mitochondria, is not limited by respiratory-deficient mutations or glucose catabolite repression.

The outer membrane is permeable to most small metabolites coming from the cytosol, since it contains porin, a 29 kDa transmembrane protein possessing a large pore. Porin is present in the mitochondria of all eukaryotes as well as in the outer membrane of bacteria.

The intermembrane space contains adenylate kinase, which ensures interconversion of ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP).

Oxidative phosphorylation takes place in the inner mitochondrial membrane, while the matrix, on the other hand, is the center of the reactions of the citric acid cycle and of the oxidation of fatty acids.

The majority of mitochondrial proteins are coded by the genes of the nucleus and are synthesized by the free polysomes of the cytoplasm. The mitochondria, however, also have their own machinery for protein synthesis. In fact, each mitochondrion possesses a circular 75 kb (kilobase pairs) molecule of double-stranded DNA and ribosomes. The mtDNA is extremely rich in A (adenine) and T (thymine) bases. It contains a few dozen genes, which code in particular for the synthesis of certain pigments and respiratory enzymes, such as cytochrome *b*, and several subunits of cytochrome oxidase and of the ATP synthase complex. Some mutations affecting these genes can result in the yeast becoming resistant to certain specific mitochondrial inhibitors such as oligomycin. This property has been applied in the genetic marking of wine yeast strains. Some mitochondrial mutants are respiratory deficient and form small colonies on solid agar media. These "*petite*" mutants are not used in winemaking because it is impossible to produce them industrially by respiration.

1.5 The Nucleus

The yeast nucleus is spherical. It has a diameter of 1–2 μm and is barely visible using a phase-contrast optical microscope. It is located near the principal vacuole in non-proliferating cells. The nuclear envelope is made up of a double membrane attached to the ER. It contains many ephemeral pores, whose locations are continually changing. These pores enable the exchange of small proteins between the nucleus and the cytoplasm. Contrary to what happens in higher eukaryotes, the yeast nuclear envelope is not dispersed during mitosis. In the basophilic part of the nucleus, the crescent-shaped nucleolus can be seen by using a nuclear-specific staining method. As in other eukaryotes, it is responsible for the synthesis of ribosomal RNA. During cell division, the yeast nucleus (Williamson, 1991) also contains rudimentary spindle threads composed of microtubules of tubulin, some discontinuous and others continuous (Figure 1.10). The continuous microtubules are stretched between the two spindle pole bodies (SPB). These corpuscles are permanently included in the nuclear membrane and correspond to the centrioles of higher organisms. The cytoplasmic microtubules depart from the SPB toward the cytoplasm.

The size of the yeast genome, about 12,800 kb, is low compared to that of higher eukaryotes (about 10 times lower than that of *Arabidopsis*). It has a genome almost three times larger than in *Escherichia coli*, but its genetic material is organized into true chromosomes. Each one contains a single molecule of linear double-stranded DNA associated with basic proteins known as histones, to form chromatin that contains repetitive units called nucleosomes. Because of their small size and their weak condensation, yeast chromosomes cannot be observed under the microscope.

Pulsed-field electrophoresis (Carle and Olson, 1984; Schwartz and Cantor, 1984) enables the separation of the 16 chromosomes in *S. cerevisiae*, whose sizes range from 200 to 2,000 kb. This species has a very large degree of chromosomic polymorphism. This characteristic has made karyotype analysis one of the main criteria for the identification of *S. cerevisiae* strains (Section 1.9.3).

The full chromosomal DNA sequence of *S. cerevisiae* (S288C) was established in 1996. It has 6,275 genes, including 23% in common with humans (Goffeau *et al.*, 1996). In 2009, the EC1118 diploid genome of a yeast strain in wine was fully sequenced. It reveals the gene transfer mechanisms between *Saccharomyces* and non-*Saccharomyces*. These works show that the genome of winemaking yeast may be constantly remodeled by the addition of exogenous genes (Novo *et al.*, 2009). This detailed knowledge of the yeast genome will constitute a powerful tool, both for the molecular understanding of its physiology and for the selection and improvement of winemaking strains. Current research done in the field of synthetic biology, as well as in enology, aims to create and assemble a full artificial genome of *S. cerevisiae* yeast with a number of potential medical and industrial applications, including in the field of enology (Richardson *et al.*, 2017).

The yeast chromosomes contain relatively few repeated sequences. Most genes are only present in a single copy in the haploid genome, but the ribosomal RNA genes are highly repeated (about 100 copies).

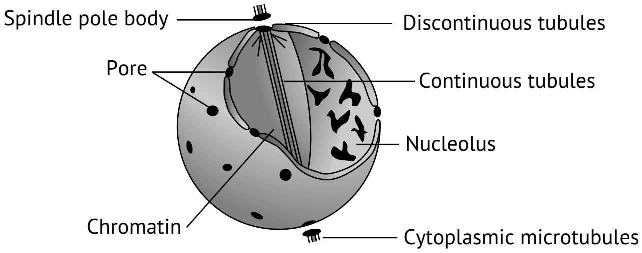


FIGURE 1.10 The yeast nucleus (Williamson, 1991).

The genome of *S. cerevisiae* also contains transposable elements or transposons—specifically, transposon yeast (Ty) elements. These comprise a central ε region (5.6 kb) framed by a direct repeated sequence called the δ sequence (0.25 kb). The δ sequences have a tendency to recombine, resulting in the loss of the central region and a δ sequence. As a result, there are about 100 copies of the δ sequence in the yeast genome. The Ty elements code for noninfectious retrovirus particles. This retrovirus contains Ty messenger RNA as well as a reverse transcriptase capable of copying the RNA into complementary DNA. The latter can reinsert itself into any site of the chromosome. The random excision and insertion of Ty elements in the yeast genome can modify the genes and play an important role in strain evolution.

A single plasmid, called the 2 μm plasmid, has been identified in the yeast nucleus. It is a circular molecule of DNA, containing 6 kb, and there are 50–100 copies per cell. Its biological function is not known. However, it is a very useful tool, used by molecular biologists to construct artificial plasmids and genetically transform yeast strains.

1.6 Reproduction and the Yeast Biological Cycle

Like other spore-forming yeasts belonging to the Ascomycetes class, *S. cerevisiae* can multiply either asexually by vegetative reproduction or sexually by forming ascospores. By definition, yeasts belonging to the imperfect fungi class can only reproduce by vegetative reproduction.

1.6.1 Vegetative Reproduction

Most yeasts undergo vegetative reproduction by a process called budding. Some yeasts, such as species belonging to the genus *Schizosaccharomyces*, reproduce by binary fission.

Figure 1.11 (Tuite and Oliver (1991)) represents the life cycle of *S. cerevisiae* divided into four phases: M, G1, S, and G2. M corresponds to mitosis; G1 is the period preceding S, which is the synthesis of DNA; and G2 is the period before mitosis. As soon as the bud emerges, at the beginning of S, the splitting of the SPB can be observed in the nuclear membrane by electron microscopy, while the cytoplasmic microtubules orient themselves toward the emerging bud. These microtubules seem to guide numerous vesicles that appear in the budding zone and are involved in the reshaping of the cell wall. As the bud grows larger, discontinuous nuclear microtubules begin to appear. The longest microtubules form the mitotic spindle between the two SPBs. At the end of G2, the nucleus begins to push and pull apart in order to penetrate the bud. Some of the mitochondria also pass with some small vacuoles into the bud, whereas a large vacuole is formed at the other pole of the cell. The expansion of the latter seems to push the nucleus into the

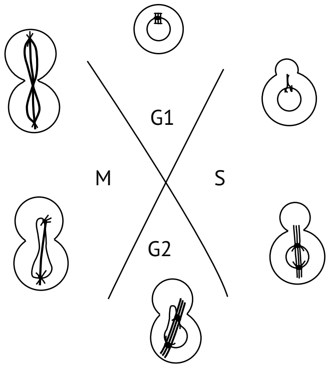


FIGURE 1.11 *Saccharomyces cerevisiae* cell cycle (vegetative reproduction) (Tuite and Oliver, 1991). M, mitosis; G1, period preceding DNA synthesis; S, DNA synthesis; G2, period preceding mitosis.

bud. During mitosis itself, the nucleus stretches to its maximum, and the mother cell separates from the daughter cell. This separation takes place only after the construction of the separation cell wall and the depositing of a ring of chitin on the bud scar of the mother cell. The movement of chromosomes during mitosis is difficult to observe in yeasts, but a microtubule–centromere link almost certainly guides the chromosomes. In grape must, the duration of budding is approximately one to two hours. As a result, the cell population doubles in two hours during the active yeast growth phase of fermentation.

1.6.2 Sexual Reproduction

When the diploid cells of spore-forming yeast are in a hostile nutrient medium (for example, depleted of fermentable sugar, poor in nitrogen, and very aerated), they stop multiplying. Some transform into a kind of sac with a thick cell wall. These sacs are called asci. Each one contains four haploid ascospores arising from meiotic division of the nucleus. Grape must and wine are not propitious to yeast sporulation and, in principle, it never occurs in this medium. Yet Mortimer *et al.* (1994) observed the sporulation of certain wine yeast strains, even in sugar-rich media. Our researchers have often observed asci in "old" agar culture media stored for several weeks in the refrigerator or at ambient temperatures (Figure 1.12). The natural

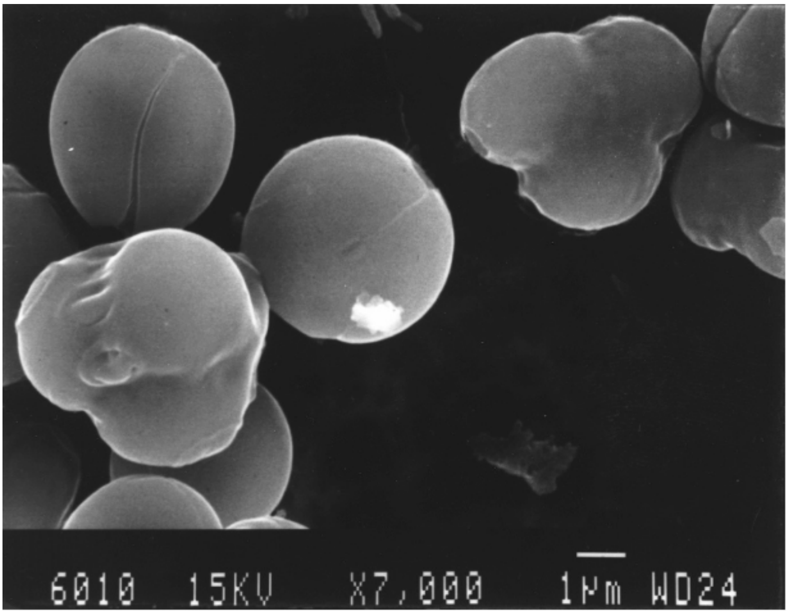


FIGURE 1.12 Scanning electron microscope photograph of *S. cerevisiae* cells kept on a sugar-agar medium for several weeks. Asci containing ascospores can be observed. (***Source***: Photograph from M. Mercier, Department of Electron Microscopy, Université de Bordeaux 1.)

conditions under which wild wine yeasts sporulate and the frequency of this phenomenon are not known. In the laboratory, the agar or liquid media conventionally used to provoke sporulation have a sodium acetate base (1%). In *S. cerevisiae*, aptitude for sporulation varies greatly from strain to strain. Wine yeasts, both wild and selected, do not sporulate easily, and when they do, they often produce nonviable spores.

Meiosis in yeasts and in higher eukaryotes (Figure 1.13) has some similarities. Several hours after the transfer of diploid vegetative cells to a sporulation medium, the SPB splits during the S phase of DNA replication. A dense body (DB) appears simultaneously in the nucleus near the nucleolus. The DB evolves into synaptonemal complexes—structures permitting the coupling and recombination of homologous chromosomes. After eight to nine hours in the sporulation medium, the two SPBs separate and the spindle begins to form. This stage is called metaphase I of meiosis. At this stage, the chromosomes are not yet visible. Then, while the nuclear membrane remains intact, the SPB divides. At metaphase II, a second mitotic spindle stretches itself while the ascospore cell wall begins to form. Spindle stretching and cell wall development of ascospores go hand in hand. Nuclear buds, cytoplasm, and organelles migrate into the ascospores. At this point, edification of the cell wall is completed. The spindle then disappears when the division is completed.

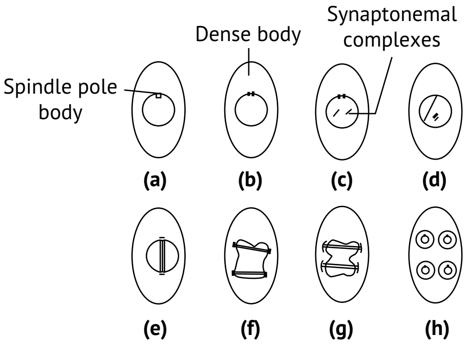


FIGURE 1.13 Meiosis in *S. cerevisiae* (Tuite and Oliver, 1991). (a) Cell before meiosis; (b) dividing of SPB; (c) synaptonemal complexes appear; (d) separation of SPBs; (e) constitution of spindle (metaphase I of meiosis); (f) dividing of the SPBs; (g) metaphase II of meiosis; (h) end of meiosis; formation of ascospores.

Placed under favorable conditions, i.e. sugar-enriched nutrient media, the ascospores germinate, breaking the cell wall of the ascus, and begin to multiply. In *S. cerevisiae*, the haploid cells have two mating types: **a** and *α*. The ascus contains two **a** ascospores and two *α* ascospores (Figure 1.14). Type **a** (*MAT***a**) cells produce a mating pheromone **a**. This peptide made up of 12 amino acids is called mating factor *α*. In the same manner, type *α* cells produce mating factor *α*, a peptide made up of 13 amino acids. The **a** factor, emitted by the *MAT***a** cells, stops the reproduction of *MATα* cells in G1, and reciprocally, the *α* factor produced by *α* cells stops the biological cycle of type **a** cells. Mating occurs between two cells of the opposite mating type. Their agglutination enables cellular and nuclear fusion and makes use of cell wall glycoproteins, called **a** and *α* agglutinins. The vegetative diploid cell that is formed (**a**/*α*) can no longer produce mating pheromones and is insensitive to their action; it reproduces by budding.

Some strains, from a monosporic culture, can be maintained in a stable haploid state. Their mating type remains constant for many generations. They are heterothallic. Others change mating type during cell division, causing diploid cells to appear in the descendants of an ascospore. They are homothallic and have an *HO* gene that inverses mating type at a high frequency during vegetative division. This interconversion (Figure 1.15) occurs in the mother cell at the G1 stage of the biological cycle, after the first budding but before the DNA replication phase. In this manner, a type *α* M ascospore divides to produce two *α* cells (M and the first daughter cell, D1). During the following cell division, M produces two cells (M and D2) that have become **a** cells. In the same

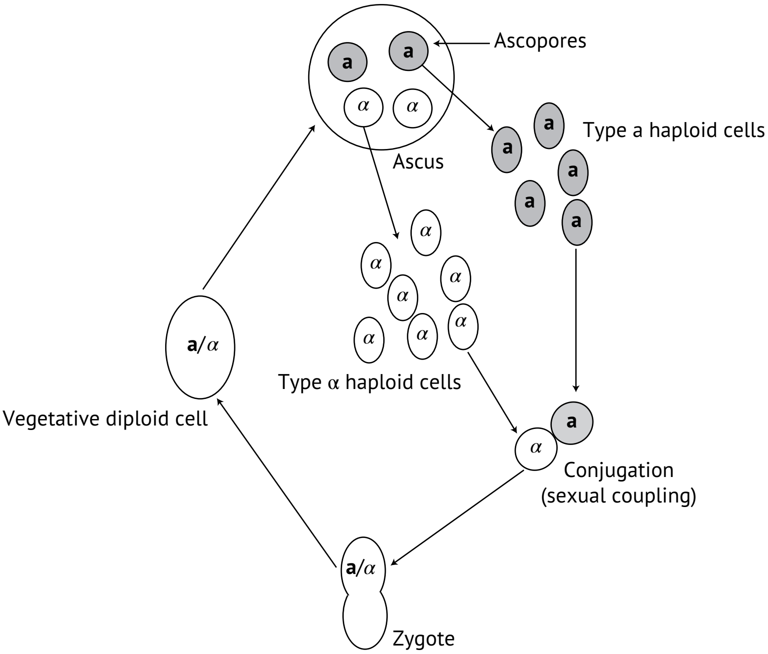


FIGURE 1.14 Reproduction cycle of a heterothallic yeast strain. **a**, *α*: spore mating types.

manner, the D1 cell produces two *α* cells after the first division and two **a** cells during its second budding. Laboratory strains that are deficient or missing the *HO* gene have a stable mating type. Heterothallism can therefore be considered the result of a mutation of the *HO* gene or of genes that control its functioning (Herskowitz *et al.*, 1992).

Most wild and selected winemaking strains that belong to the *S. cerevisiae* species are diploid and homothallic. This is also true of almost all of the strains that have been isolated in vineyards of the Bordeaux region. Moreover, recent studies carried out by Mortimer *et al.* (1994) in Californian and Italian vineyards have shown that the majority of strains (80%) are homozy-

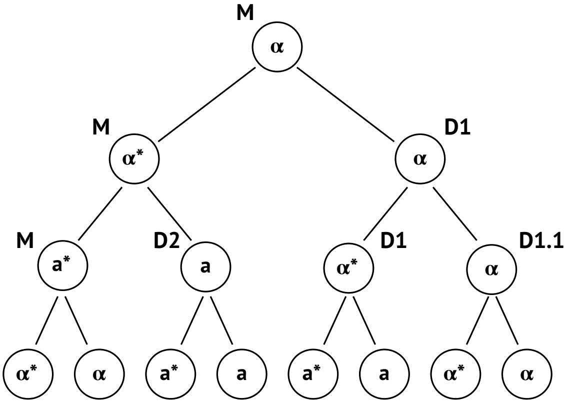


FIGURE 1.15 Mating type interconversion model of haploid yeast cells in a homothallic strain (Herskowitz *et al.*, 1992). * designates cells capable of changing mating type at the next cell division or cells already having undergone budding. M, initial cell carrying the *HO* gene; D1, D2, daughter cells of M; D1.1, daughter cell of D1.

gous for the *HO* character (*HO*/*HO*); heterozygosis (*HO*/*ho*) is in the minority. Heterothallic strains (*ho*/*ho*) are rare (less than 10%). We have made the same observations for yeast strains isolated in the Bordeaux region; for example, the F10 strain, which is fairly prevalent in spontaneous fermentations in certain Bordeaux wines, is *HO*/*HO*. In other words, the four spores arising from an ascus give monoparental diploids, capable of forming asci when placed in a pure culture. This generalized homozygosis for the *HO* character of wild winemaking strains is probably an important factor in their evolution, according to the genome renewal phenomenon proposed by Mortimer *et al.* (1994) (Figure 1.16). According to this author, the continuous reproduction of a yeast strain in its natural environment is accompanied by the accumulation of heterozygotic damage to the DNA. Certain slow-growth or functional loss mutations of certain genes decrease strain vigor in the heterozygous state. Sporulation, however, produces haploid cells containing various combinations of these heterozygotic characters. All of these spores will become homozygous diploid cells with a series of genotypes because of the homozygosity of the *HO* character. Certain diploids that prove to be more vigorous than others will in time supplant the parents and less vigorous collaterals. This very persuasive model is supported by the characteristics of the wild winemaking strains analyzed. In these, the spore viability rate is an inverse function of the heterozygosis rate for a certain number of mutations. The completely homozygous strains present the highest spore viability and vigor.

In conclusion, we can question whether sporulation of strains under natural conditions is indispensable to ensure their growth and fermentation performance. We can also raise the question of conserving selected strains of active dry yeasts (ADYs) for use as yeast starters. It may be necessary to regenerate them periodically to eliminate possible mutations from their genome, which could diminish their vigor.

1.7 The Killer Phenomenon

1.7.1 Introduction

Certain yeast strains, known as killer strains (K), secrete protein toxins into their environment that are capable of killing other, sensitive strains (S). The killer strains are not sensitive to their own toxin but can be killed by a toxin that they do not

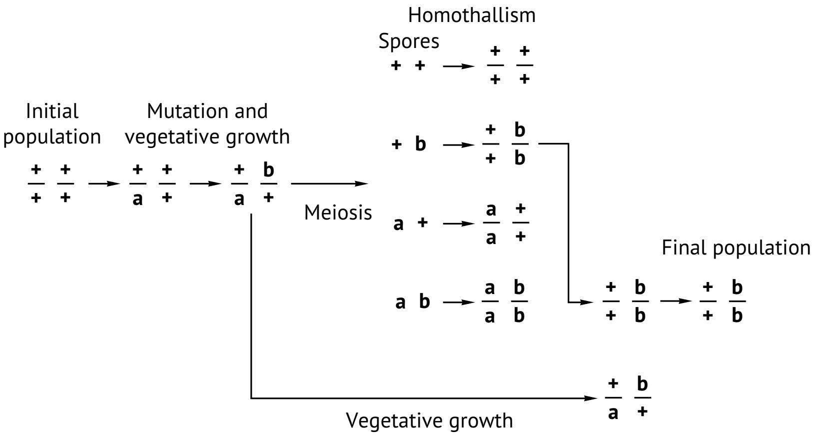


FIGURE 1.16 Genome renewal of a homozygote yeast strain for the *HO* gene of homothallism, having accumulated recessive mutations during vegetative reproduction (Mortimer *et al.*, 1994). (a and b), mutation of certain genes.

produce. Additionally, neutral strains (N) do not produce a toxin but are resistant. The action of a killer strain on a sensitive strain is easy to demonstrate in the laboratory on an agar culture medium at pH 4.2–4.7 at 20°C. The sensitive strain is inoculated into the mass of agar before it solidifies; then the strain to be tested is inoculated in streaks on the solidified medium. If it is a killer strain, a clear zone in which the sensitive strain cannot grow encircles the inoculum streaks (Figure 1.17).

This phenomenon, known as the killer factor, was discovered in *S. cerevisiae*, but killer strains also exist in other yeast genera such as *Hansenula*, *Candida*, *Kloeckera*, *Hanseniaspora*, *Pichia*, *Torulopsis*, *Kluyveromyces*, and *Debaryomyces*. Killer yeasts have been classified into 11 groups according to the sensitivity reaction between strains as well as the nature and properties of the toxins involved. The killer factor is a cell interaction model mediated by the excreted protein toxin. It has given rise to much fundamental research (Tipper and Bostian, 1984; Young, 1987). Barre (1984, 1992), Radler (1988), and Van Vuuren and Jacobs (1992) have described the technological implications of this phenomenon for wine yeasts and the fermentation process.

1.7.2 Physiology and Genetics of the Killer Phenomenon

The determinants of the killer factor are both cytoplasmic and nuclear. In *S. cerevisiae*, the killer phenomenon is associated with the presence of double-stranded RNA particles, called virus-like particles (VLP), in the cytoplasm. They are in the same category as noninfectious mycoviruses. There are two kinds of VLP: M and L. The M genome (1.3–1.9 kb) codes for the toxin (K) and for the immunity factor (R). The L genome (4.5 kb) codes for an RNA polymerase and the protein capsid that encapsulates the two genomes. Killer strains (K^+R^+) secrete the toxin and are immune to it. The sensitive cells (K^-R^-) do not possess

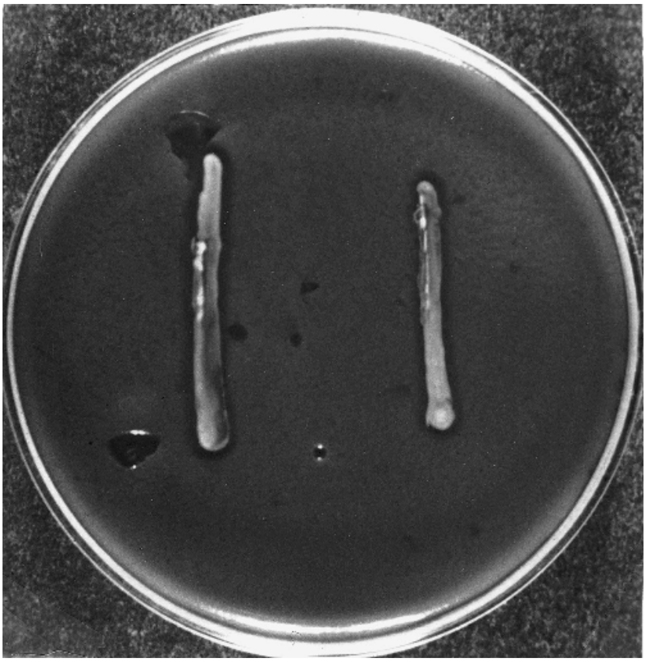


FIGURE 1.17 Identification of the K2 killer phenotype in *S. cerevisiae*. The presence of a halo around the two streaks of the killer strain is due to the death of the sensitive strain cultivated in the medium.

M VLP, but most of them have L VLP. The two types of viral particles are necessary for the yeast cell to express the killer phenotype (K^+R^+), since the L mycovirus is necessary for the maintenance of the M type.

There are four types of killer activity in *S. cerevisiae* strains, corresponding to K1, K2, K28, and Klus toxins. They are, respectively, coded by M1, M2, M28, and Mlus VLPs with sizes of 1.8, 1.7, 2.1, and 2.3 kb. The K2 activity group is by far the most widespread in the *S. cerevisiae* strains encountered in wine (Rodríguez-Cousiño *et al.*, 2011). Neutral strains (K^-R^+) are insensitive to a given toxin without being capable of producing it. They possess M VLPs of normal size that code only for the immunity factor, but they either do not produce toxins or are inactive because of mutations affecting the M-type RNA.

Many chromosomic genes are involved in the maintenance and replication of L and M RNA particles as well as in the maturation and transport of the toxin produced.

The K1 toxin is a small protein made up of two subunits (9 and 9.5 kDa); it is active and stable in a very narrow pH range (4.2–4.6). It is therefore inactive in grape must. The K2 toxin, a 16 kDa glycoprotein produced by homothallic strains of *S. cerevisiae* encountered in wine, is active between pH 2.8 and 4.8 with a maximum activity between 4.2 and 4.4. It is therefore active at the pH of grape must and wine.

K1 and K2 toxins attack sensitive cells by attaching themselves to a receptor located in the cell wall—a β-1,6-glucan. Two chromosomal genes, *KRE*1 and *KRE*2 (*k*iller *re*sistant), determine the possibility of this linkage. The *KRE*1 gene produces a cell wall glycoprotein that has β-1,6-glucan synthase activity. *KRE*1 mutants are resistant to K1 and K2 toxins because they are deficient in this enzyme and devoid of a β-1,6-glucan receptor. The *KRE*2 gene is also involved in the fixation of toxins to the cell wall receptor; *KRE*2 mutants are also resistant. The toxin linked to a glucan receptor is then transferred to a membrane receptor site by a mechanism requiring energy. Cells in the log phase are therefore more sensitive to the killer effect than cells in the stationary phase. When the plasma membrane of the sensitive cell is exposed to the toxin, it manifests serious functional alterations after a lag phase of about 40 minutes. Serious functional alterations include the interruption of the coupled transport of amino acids and protons, the acidification of the cell contents, and potassium and ATP leakage. The cell dies in two to three hours after contact with the toxin because of the above damage, due to the formation of pores in the plasma membrane.

The killer effect exerts itself exclusively on yeasts, and the pharmacological tests reported on killer toxins are negative.

1.7.3 The Role of the Killer Phenomenon in Winemaking

Depending on the authors and viticultural regions studied, the frequency of the killer character varies a lot among wild winemaking strains isolated on grapes or in fermenting grape must. In a work by Barre (1978) studying 908 wild strains, 504 manifested the K2 killer character, 299 were sensitive, and 95 neutral. Investigating the killer phenomenon among winemaking yeasts from French vineyards, Cuinier and Gros (1983) reported a high frequency (65–90%) of K2 strains in Mediterranean and Beaujolais vineyards, whereas none of the strains analyzed in the Touraine region manifested the killer effect. In the Bordeaux region, the K2 killer character is extremely widespread. Thus, in a study carried out in 1989 and 1990 on the ecology of native strains of *S. cerevisiae* in several tanks of red must in a Pessac-Léognan vineyard, all of the isolated strains manifested K2 killer activity, i.e. about 30, differentiated by their karyotype (Frezier, 1992). Rossini *et al.* (1982) reported an extremely varied frequency (12–80%) of killer strains in spontaneous fermentations in Italian wineries. Some K2 killer strains were also isolated in the Southern Hemisphere (Australia, South Africa, and Brazil). On the other hand, most of the killer strains isolated in Japan presented the K1 characteristic, and K2 strains are the minority. The Klus characteristic was highlighted in wine yeasts isolated in Spain (Rodríguez-Cousiño *et al.*, 2011). Most research on the killer character of wine yeasts concerns the species *S. cerevisiae*. Recent works have reported data on the killer effect of non-*Saccharomyces* species, which make up the great proportion of grape microflora. Killer strains of *Hanseniaspora uvarum* and *Pichia kluyveri* have been identified by Zorg *et al.* (1988) and those of *Torulaspora delbrueckii* by Velázquez *et al.* (2015). Two toxins (PMKT and PMKT 2) have also been described in *Pichia membranifaciens* (review by Belda *et al.*, 2017). Certain non-*Saccharomyces* species are described for their capacity to produce an active killer toxin that affects the spoilage yeast *Brettanomyces bruxellensis*. For example, *Candida pyralidae* (Mehlomakulu *et al.,* 2017) may constitute a potential biocontrol agent to fight against *B. bruxellensis*.

In *S. cerevisiae,* Barre (1992) studied the activity and stability of the K2 killer toxin under winemaking conditions (Figure 1.18). The killer toxin only manifests pronounced activity on cells in the log phase. Cells in the stationary phase are relatively insensitive. The amount of ethanol or SO_2 in the wine has practically no effect on killer toxin activity. On the other hand, it is quickly destroyed by heat, since its half-life is around 30 minutes at 32°C. It is also quickly inactivated by the presence of phenolic compounds. Lastly, it is easily adsorbed by bentonite.

The role of the killer factor in the competition between species during alcoholic fermentation merits further study. However, its role in the competition between *S. cerevisiae* strains during grape must fermentation has been diversely reported in the literature. In an example given by Barre (1992), killer cells inoculated at 2% can completely supplant the sensitive strain during the alcoholic fermentation of must (Heard and Fleet, 1987). In other works, the killer yeast/sensitive yeast ratio able to affect the implantation of sensitive yeasts during winemaking varies between 1/1,000 and 100/1, depending on the author. This considerable discrepancy can probably be attributed to the implantation and fermentation speeds of the strains present. The killer phenomenon seems more important to interstrain competition when the killer strain implants itself quickly and the sensitive strain slowly. In the opposite situation, a high percentage of killer yeasts would be necessary to eliminate the sensitive population. Some authors have observed spontaneous fermentations dominated by sensitive strains despite a non-negligible proportion of killer strains (2–25%). In Bordeaux, we have always observed that certain sensitive strains establish themselves well in red wine fermentation, despite a strong presence of killer yeasts in the wild microflora (for example, 522M, an active dry yeast [ADY] starter). In white winemaking, the neutral yeast VL1 and sensitive strains such as EG8, a slow-growth strain, also successfully establish themselves. The wild killer population does not appear to compete with a sensitive yeast starter, and therefore is not a significant cause of fermentation difficulties in real-life applications.

As such, in light of the high frequency of killer strains among native yeasts in many viticultural regions, there is little advantage conferred on a selected killer strain in terms of implantation capacity. In other words, this character is not sufficient to guarantee the implantation of a certain strain during winemaking over a wild strain that also has killer activity. On the other hand, under certain conditions, inoculating with a sensitive strain will fail because of the killer effect of a wild population. Therefore, resistance to the K2 toxin (killer or neutral phenotype) should be included among the selection criteria

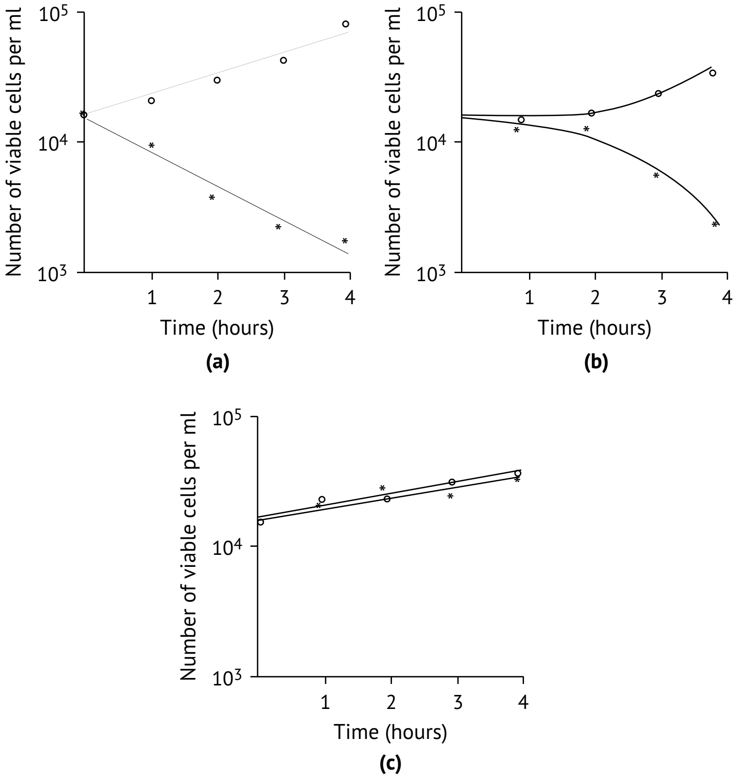


FIGURE 1.18 Yeast growth and survival curves in a grape juice medium containing killer toxin (Barre, 1992). (a) White juice at pH 3.4; cells in exponential growth phase introduced at t_0. (b) Same juice, cells in stationary phase introduced at t_0. (c) Red juice extracted by hot maceration, pH 3.4; cells in exponential growth phase introduced at t_0. *, 10% of an active supernatant of a K2 strain culture; o, 10% of supernatant inactivated by heating.

for winemaking strains. The high frequency of the K2 killer character in native wine yeasts greatly facilitates this strategy.

1.8 Classification of Yeast Species

1.8.1 General Remarks

As mentioned in the introduction to this chapter, yeasts constitute a vast group of single-celled fungi that are taxonomically heterogeneous and very complex. Hansen's first classification at the beginning of the 20th century only distinguished between sporogenous and asporogenous yeasts. Since then, yeast taxonomy has been the subject of considerable research. This research has been gathered in successive works, thus progressively creating the classification known today. The previous enological textbook from the University of Bordeaux (Ribéreau-Gayon *et al.*, 1975) was based on Lodder's (1970) classification. Between the last edition of that book and the previous classification (Lodder and Kregger-Van Rij, 1952), the designation and classification of yeasts had already changed profoundly. In this book, we use the latest classification, given by Kurtzman *et al.* (2011), relying on the recent methods of molecular biology and genome analysis for the demarcation of species and genera.

Rules concerning the taxonomy of yeasts and other fungi fall under the International

Code of Nomenclature for algae, fungi, and plants. Its most recent version was adopted in 2005, during the 17th International Botanical Congress in Vienna, Austria (http://ibot.sav.sk/icbn/main.htm). According to the current taxonomy, genera are classified in alphabetical order according to four groups: teleomorphic ascomycetes, anamorphic ascomycetes, teleomorphic basidiomycetes, and anamorphic basidiomycetes. The terms "teleomorphic" and "anamorphic" refer, respectively, to the sexual and asexual forms.

Taking into account synonymy and physiological races (varieties of the same species), at least 4,000 names for yeasts have been used since the 19th century. However, the species of yeasts likely to be highly present in the grape and in wine, intervening as an agent of alcoholic fermentation or responsible for wine spoilage, are more limited. Table 1.1 presents the classification of the main order *Saccharomycetales*, to which grape and wine yeasts belong. Basidiomycetes are not found in this table, as none of the main genera of grapevine and grape yeasts belong to that phylum.

1.8.2 Evolution of the General Principles of Yeast Taxonomy and Species Delimitation

Yeast taxonomy (from the Greek *taxis*: putting in order), and the taxonomy of other microorganisms for that matter, includes classification and identification. Classification groups organisms into *taxa* according to their similarities and/or their ties to a common ancestor. The basic taxon is species. A species can be defined as a collection of strains having a certain number of morphological, physiological, and genetic characteristics in common. This group of characteristics constitutes the standard description of the species. Identification compares an unknown organism to individ-

TABLE 1.1

Classification of Grape and Wine Yeast Genera (Kurtzman *et al.*, 1994)

Phylum	Subphylum	Order	Families	Genera
Ascomycota	*Saccharomycotina*	*Saccharomycetales*	*Saccharomycetaceae*	*Saccharomyces*
				Kluyveromyces
				Torulaspora
				Zygosaccharomyces
				Kazachstania
				Lachancea
			Pichiaceae	*Pichia*
				Brettanomyces
				Dekkera
			Metschnikowiaceae	*Metschnikowia*
			Debaryomycetaceae	*Debaryomyces*
				Lodderomyces
			Saccharomycodaceae	*Hanseniaspora*
				Kloeckera
				Saccharomycodes
		Saccharomycetales Incertae sedis	*Wickerhamomycetaceae*	*Candida*

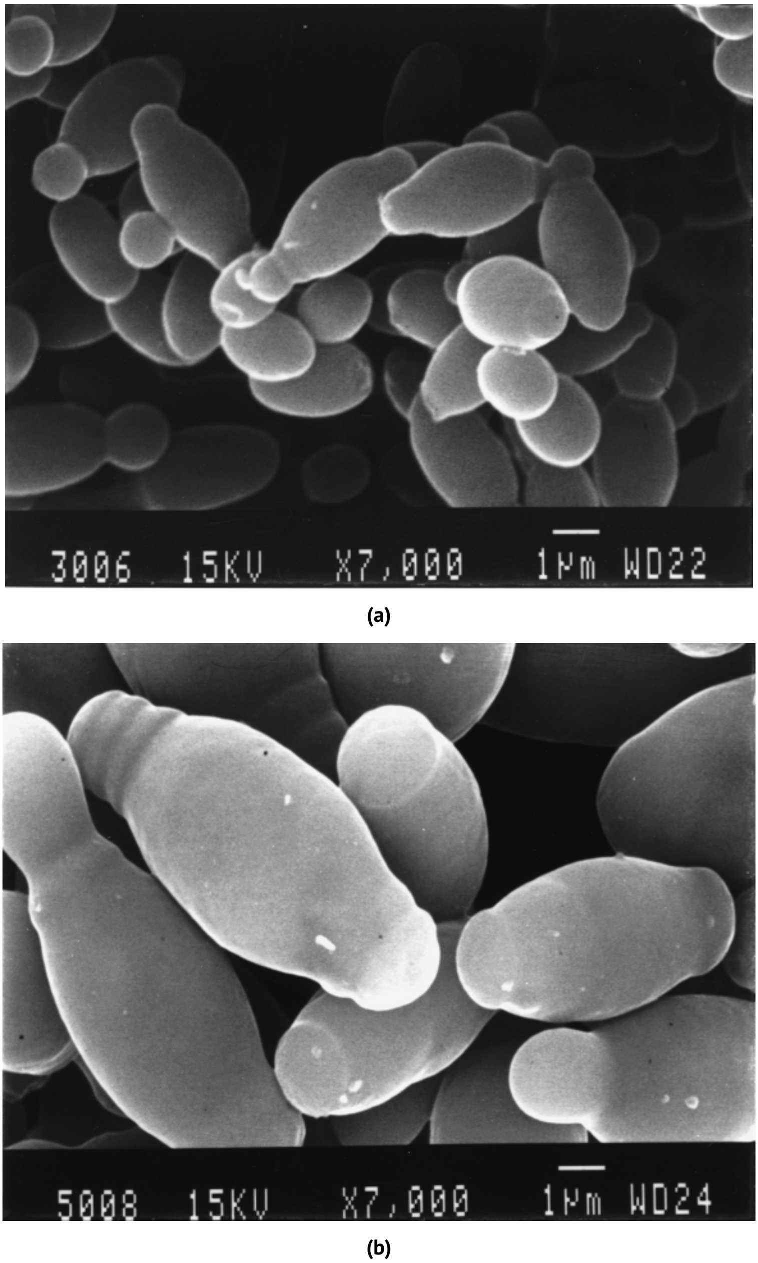


FIGURE 1.19 Observation of two winemaking yeast species having an apiculate form (***Source***: Photographs from M. Mercier, Department of Electron Microscopy, Université de Bordeaux 1.) (a) *H. uvarum*. (b) *S. ludwigii*.

uals already classified and named that have similar characteristics.

Taxonomists first delimited yeast species using morphological and physiological criteria. The first classifications were based on the phenotypic differences between yeasts: cell shape and size, spore formation, cultural characteristics, fermentation and assimilation of various sugars, assimilation of nitrates, growth-factor needs, and resistance to cycloheximide. Ribéreau-Gayon *et al.* (1975) described the application of these methods to wine yeasts in detail. Since then, many rapid, ready-to-use diagnostic kits

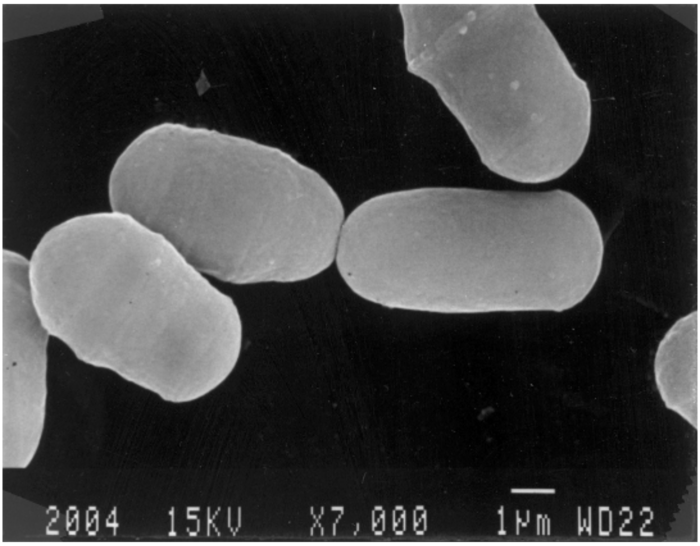


FIGURE 1.20 Binary fission of *S. pombe*. (***Source***: Photographs from M. Mercier, Department of Electron Microscopy, Université de Bordeaux 1.)

have been proposed to determine yeast response to different physiological tests. Lafon-Lafourcade and Joyeux (1979) and Cuinier and Leveau (1979) designed the API 20 C system for the identification of winemaking yeasts. It contains eight fermentation tests, 10 assimilation tests, and a cycloheximide resistance test. For a more complete identification, the API 50 CH system contains 50 substrates for fermentation (under paraffin) and assimilation tests. Heard and Fleet (1990) developed a system that uses the various tests listed in the work of Barnett *et al.* (1990).

Due to the relatively limited number of yeast species significantly present on grapes or in wine, these phenotypic tests identify winemaking yeast species in certain genera without difficulty. Certain species can be identified by observing growing cells under the microscope alone. Small apiculate cells, with small lemon-like shapes, are indicative of the species *H. uvarum* and its imperfect form *Kloeckera apiculata* (Figure 1.19). *Saccharomycodes ludwigii* is characterized by much larger (10–20 μm) apiculate cells. Since most yeast multiply by budding, the genus *Schizosaccharomyces* can be recognized because of its vegetative reproduction by binary fission (Figure 1.20). In modern taxonomy, this genus only contains the species *Schizosaccharomyces pombe*. Finally, the budding of *Candida stellata* (formerly known as *Torulopsis stellata*) occurs in the shape of a characteristic star.

According to Barnett *et al.* (1990), the physiological characteristics listed in Table 1.2 can be used to distinguish between the principal grape and wine yeasts. Yet some of these characteristics (for example, fermentation profiles of sugars) vary within the species and are even unstable for a given strain during vegetative reproduction. Taxonomists have realized that they cannot differentiate species based solely on phenotypic discontinuity criteria. They have progressively established a delimitation founded on the biological and genetic definition of a species.

In theory, a species can be defined as a collection of interfertile strains whose hybrids are themselves fertile—capable of producing viable spores. This biological definition runs into several problems when applied to yeasts. First of all, many

TABLE 1.2

Physiological Characteristics of the Principal Grape and Wine Yeasts (Barnett *et al.*, 1990)

	Fl D-Glucose fermentation	F2 D-Galactose fermentation	F3 Maltose fermentation	F4 α-D-glucoside fermentation	F5 Sucrose fermentation	F6 α,α-Trehalose fermentation	F7 Melibiose fermentation
Candida stellata	+	–	–	–	+	–	–
Candida vini	v	–	–	–	–	–	–
Candida famata	v	v	v	–	v	v	v
Dekkera anomala	+	v	v	v	+	v	–
Dekkera bruxellensis	v	v	v	+	+	+	–
Hanseniaspora uvarum	+	–	–	–	–	–	–
Melschnikowia pulcherrima	+	v	–	–	–	–	–
Pichia anomala	+	v	v	v	+	v	–
Pichia fermentans	+	–	–	–	–	–	–
Pichia membranifaciens	v	–	–	–	–	–	–
Saccharomyces cerevisiae	+	v	v	v	v	v	v
Saccharomycodes ludwigii	+	–	–	–	+	–	–
Kluyveromyces thermolerens	+	v	v	v	+	v	–
Schizosaccharomyces pombe	+	v	v	v	+	–	v
Zygosaccharomyces bailii	+	–	–	–	v	v	–

	C30 Galactitol growth	C31 myo-Inositol growth	C32 D-Glucono-1, 5-lactone growth	C33 2-Keto-D-gluconate growth	C34 5-Keto-D-gluconate growth	C35 D-gluconate growth	C38 DL-Lactate growth
Candida stellata	–	–	–	–	–	–	–
Candida vini	–	–	–	v		–	v
Candida famata	v	–	v	+		v	v
Dekkera anomala	–	–	v	–	–	v	v
Dekkera bruxellensis	–	–	v	v	–	–	v
Hanseniaspora uvarum	–	–	+	+	–	v	–
Melschnikowia pulcherrima	–	–	+	+		+	v
Pichia anomala	–	–	+	–	–	v	+
Pichia fermentans	–	–	v	–	–	–	+
Pichia membranifaciens	–	–	v	–	–	–	v
Saccharomyces cerevisiae	–	–	v	–	–	–	v
Saccharomycodes ludwigii	–	–	v	–	–	–	v
Kluyveromyces thermolerens	–	–	v	v	–	v	–
Schizosaccharomyces pombe	–	–	v	v	–	v	–
Zygosaccharomyces bailii	–	–	v	v	–	–	–

(Continued)

TABLE 1.2 (CONTINUED)

	F8 Lactose fermentation	F9 Cellobiose fermentation	F10 Melezitose fermentation	F11 Raffinose fermentation	F12 Inulin fermentation	F13 Starch fermentation	C1 D-Glucose growth
Candida stellata	–	–	–	v	–	–	+
Candida vini	–	–	–	–	–	–	+
Candida famata	–	v	v	v	–	–	+
Dekkera anomala	v	v	v	v	–	–	v
Dekkera bruxellensis	–	v	v	–	–	–	v
Hanseniaspora uvarum	–	v	–	–	–	–	+
Melschnikowia pulcherrima	–	–	–	–	–	–	+
Pichia anomala	–	v	–	v	–	v	+
Pichia fermentans	–	–	–	–	–	–	+
Pichia membranifaciens	–	–	–	–	–	–	+
Saccharomyces cerevisiae	–	–	v	v	–	v	+
Saccharomycodes ludwigii	–	–	–	v	–	–	+
Kluyveromyces thermolerens	–	–	v	+	v	–	+
Schizosaccharomyces pombe	–	–	–	v	v	v	+
Zygosaccharomyces bailii	–	–	–	v	–	–	+

	C39 Succinate growth	C40 Citrate growth	C42 Ethanol growth	N1 Nitrate growth	N2 Nitrite growth	N3 Ethylamine growth	N4 L-Lysine growth
Candida stellata	–	–	–	–	–	–	+
Candida vini	+	–	+	–	–	+	+
Candida famata	+	+	+	–	v	+	+
Dekkera anomala	v	–	v	v	+	+	+
Dekkera bruxellensis	–	–	v	v	v	+	+
Hanseniaspora uvarum	–	–	–	–	–	+	+
Melschnikowia pulcherrima	+	+	+	–	–	+	+
Pichia anomala	+	+	–	+	+	+	+
Pichia fermentans	+	+	–	–	–	+	+
Pichia membranifaciens	v	–	+	–	–	+	+
Saccharomyces cerevisiae	v	–	v	–	–	–	–
Saccharomycodes ludwigii	–	–	v	–	–	+	+
Kluyveromyces thermolerens	v	–	v	–	–	+	+
Schizosaccharomyces pombe	–	–	–	–	–	v	v
Zygosaccharomyces bailii	–	–	+	–	–	+	+

	C2 D-Galactose growth	C3 D-Sorbose growth	C4 D-Glucosamine growth	C5 D-Ribose growth	C6 D-Xylose growth	C10 Sucrose growth	C11 Maltose growth
Candida stellata	–	v	–	–	–	+	–
Candida vini	–	–	–	–	–	–	–
Candida famata	+	v	v	v	+	+	+
Dekkera anomala	v	–	v	–	–	v	v
Dekkera bruxellensis	v	–	v	v	–	v	v
Hanseniaspora uvarum	–	–	–	–	–	–	–
Melschnikowia pulcherrima	+	v	v	v	v	+	+
Pichia anomala	v	–	–	v	v	+	+
Pichia fermentans	–	–	v	–	+	–	–
Pichia membranifaciens	–	v	v	–	v	–	–
Saccharomyces cerevisiae	v	–	–	–	–	v	v
Saccharomycodes ludwigii	–	–	–	–	–	+	–
Kluyveromyces thermolerens	v	+	–	–	–	+	+
Schizosaccharomyces pombe	v	–	–	–	–	+	+
Zygosaccharomyces bailii	v	v	–	–	–	v	–
	N5 Cadaverine growth	**N6 Creatine growth**	**N7 Creatinine growth**	**N8 Glucosamine growth**	**V1 Growth W/O vitamins**	**V2 Growth W/O myo-Inositol**	**V3 Growth W/O Pantothenate**
Candida stellata	–	–	–	–	–	–	v
Candida vini	+	–	–	–	–	+	+
Candida famata	+	v	–	–	v	+	+
Dekkera anomala	+	–	–	–	–	v	–
Dekkera bruxellensis	+	–	–	–	–	+	+
Hanseniaspora uvarum	+	–	–	–	–	–	–
Melschnikowia pulcherrima	+	–	–	–	–	+	+
Pichia anomala	+	–	–	–	+	+	+
Pichia fermentans	+	–	–	+	–	+	+
Pichia membranifaciens	+	–	–	v	v	+	+
Saccharomyces cerevisiae	–	–	–	–	v	v	v
Saccharomycodes ludwigii	+	–	–	+	–	+	–
Kluyveromyces thermolerens	+	–	–	–	–	–	v
Schizosaccharomyces pombe	v	–	–	–	–	–	v
Zygosaccharomyces bailii	+	–	–	–	–	+	+

TABLE 1.2 (CONTINUED)

	C12 α,α-Trehalose growth	C13 Me α-D-Glucoside growth	C14 Cellobiose growth	C15 Salicin growth	C16 Arbutin growth	C17 Melibiose growth	C18 Lactose growth
Candida stellata	–	–	–	–	–	–	–
Candida vini	–	–	–	–	–	–	–
Candida famata	+	+	v	v	v	v	v
Dekkera anomala	v	v	v	v	v	–	v
Dekkera bruxellensis	v	v	v	v	v	–	–
Hanseniaspora uvarum	v	–	+	+	+	–	–
Melschnikowia pulcherrima	+	+	+	+	+	–	–
Pichia anomala	v	+	v	+	+	–	–
Pichia fermentans	–	–	–	–	–	–	–
Pichia membranifaciens	–	–	–	–	–	–	–
Saccharomyces cerevisiae	v	v	–	–	–	v	–
Saccharomycodes ludwigii	–	–	+	+	+	–	–
Kluyveromyces thermolerens	+	+	–	–	–	–	–
Schizosaccharomyces pombe	v	v	–	–	–	v	–
Zygosaccharomyces bailii	v	–	–	–	–	–	–
	V4 Growth W/O Biotin	**V5 Growth W/O Thiamin**	**V6 Growth W/O Biotin and Thiamin**	**V7 Growth W/O Pyridoxine**	**V8 Growth W/O Pyridoxine and Thiamin**	**V9 Growth W/O Niacin**	**T2 Growth at 30°C**
Candida stellata	–	–	–	+	–	v	+
Candida vini	v	v	v	+		+	v
Candida famata	v	+	v	+		+	+
Dekkera anomala	–	–	–	+	–	+	+
Dekkera bruxellensis	–	–	–	+	–	+	+
Hanseniaspora uvarum	v	v	–	–	–	–	+
Melschnikowia pulcherrima	–	+	–	+		+	v
Pichia anomala	+	+	+	+	+	+	+
Pichia fermentans	+	–	–	+	–	+	+
Pichia membranifaciens	v	v	v	+	v	+	+
Saccharomyces cerevisiae	v	v	v	v	v	+	+
Saccharomycodes ludwigii	–	v	–	–	–	–	+
Kluyveromyces thermolerens	v	v	v	v		v	+
Schizosaccharomyces pombe	v	v	–	v	–	–	+
Zygosaccharomyces bailii	–	+	–	+		+	+

	C19 Raffinose growth	C20 Melezitose growth	C21 Inulin growth	C22 Starch growth	C23 Glycerol growth	C24 Erythritol growth	C25 Ribitol growth
Candida stellata	+	–	–	–	–	–	–
Candida vini	–	–	–	–	v	–	v
Candida famata	+	+	v	v	+	v	+
Dekkera anomala	v	v	–	–	v	–	–
Dekkera bruxellensis	v	v	v	–	v	–	–
Hanseniaspora uvarum	–	–	–	–	–	–	–
Melschnikowia pulcherrima	–	+	–	–	+	–	v
Pichia anomala	v	v	–	+	+	v	v
Pichia fermentans	–	–	–	–	+	–	–
Pichia membranifaciens	–	–	–	–	v	–	–
Saccharomyces cerevisiae	v	v	–	v	v	–	–
Saccharomycodes ludwigii	v	–	–	–	v	–	–
Kluyveromyces thermolerens	+	+	v	–	+	–	v
Schizosaccharomyces pombe	+	–	v	v	–	–	–
Zygosaccharomyces bailii	v	–	–	–	v	–	v

	T3 Growth at 35°C	T4 Growth at 37°C	T5 Growth at 40°C	O1 0.01% Cycloheximide growth	O3 1% Acetic acid growth	O4 50% D-Glucose growth	M2 Acetic acid production
Candida stellata	v	–	–	–	–	v	–
Candida vini	–	–	–	–	–	–	–
Candida famata	v	v	–	v	–	+	–
Dekkera anomala	+	v	–	+	–	–	+
Dekkera bruxellensis	v	v	v	+	v	–	+
Hanseniaspora uvarum	–	–	–	+	–	v	–
Melschnikowia pulcherrima	v	–	–	–	–	v	–
Pichia anomala	v	v	–	–	–	v	–
Pichia fermentans	+	+	–	–	–	v	–
Pichia membranifaciens	v	v	–	–	–	v	–
Saccharomyces cerevisiae	v	v	v	–	–	v	–
Saccharomycodes ludwigii	+	v	–	–	–	–	–
Kluyveromyces thermolerens	+	v	–	–	–	+	–
Schizosaccharomyces pombe	+	+	v	v	+	+	–
Zygosaccharomyces bailii	v	v	–	–	+	+	–

TABLE 1.2 (CONTINUED)

	C26 Xylitol growth	C28 D-Glucitol growth	C29 D-Mannitol growth
Candida stellata	–	–	–
Candida vini	v	+	+
Candida famata	+	+	+
Dekkera anomala	–	–	–
Dekkera bruxellensis	–	–	–
Hanseniaspora uvarum	–	v	–
Melschnikowia pulcherrima	+	+	+
Pichia anomala	v	+	+
Pichia fermentans	–	–	–
Pichia membranifaciens	v	–	–
Saccharomyces cerevisiae	–	v	v
Saccharomycodes ludwigii	–	–	–
Kluyveromyces thermolerens	+	v	v
Schizosaccharomyces pombe	–	–	–
Zygosaccharomyces bailii	v	v	v
		M3 Urea hydrolysis	
Candida stellata		–	
Candida vini		–	
Candida famata		–	
Dekkera anomala		–	
Dekkera bruxellensis		–	
Hanseniaspora uvarum		–	
Melschnikowia pulcherrima		–	
Pichia anomala		–	
Pichia fermentans		–	
Pichia membranifaciens		–	
Saccharomyces cerevisiae		–	
Saccharomycodes ludwigii		–	
Kluyveromyces thermolerens		–	
Schizosaccharomyces pombe		+	
Zygosaccharomyces bailii		–	

+, test positive; –, test negative; v, variable result.

[a] Cannot be differentiated with these tests from *S. bayanus*, *S. paradoxus*, and *S. pastorianus*.

Ascomycetes yeasts are homothallic; hybridization tests are thus especially tedious and difficult for routine identification. Finally, certain wine yeast strains have little or no sporulation aptitude, which makes the use of strain infertility criteria even more difficult.

To overcome these difficulties, researchers have developed a molecular taxonomy based on the following tests: DNA–DNA reassociation, the similarity of DNA base composition, the similarity of enzymes, ultrastructure characteristics, and cell wall composition. The development of DNA sequence analysis has profoundly changed the way in which yeasts are identified. More specifically, the comparison of gene sequences coding for ribosome RNA now resolves the taxonomic position of closely related species, as well as the most distantly related taxa. The sequence analysis of variable domains 1 and 2 (D1/D2) has been successfully applied to the identification of ascomycetous yeasts (Kurtzman and Robnett, 1998). Then, Fell *et al.* (2000) developed the method by bringing in an additional database to identify basidiomycetous yeasts. Moreover, analysis of internal transcribed spacer (ITS) sequences is often used to identify species (Granchi *et al.*, 1999). Currently, the sequencing of all genomes is used to refine the taxonomy of yeasts in phylogenomic studies.

1.8.3 Successive Classifications of the Genus *Saccharomyces* and the Position of Wine Yeasts in the Current Classification

Due to many changes in yeast classification and nomenclature since the beginning of taxonomic studies, wine-related yeast names and their positions in the classification have often changed. This has inevitably resulted in some confusion for winemakers. Even the most recent enological textbooks (Fleet, 1993; Delfini, 1995; Boulton *et al.*, 1995) use a number of species names (*cerevisiae*, *bayanus*, *uvarum*, etc.) attached to the genus name *Saccharomyces* to designate yeasts responsible for alcoholic fermentation. Although still in use, this enological terminology is no longer accurate to designate the species currently delimited by taxonomists.

The evolution of species classification for the genus *Saccharomyces* since the early 1950s (Table 1.3) has created this difference between the naming of wine yeasts and current taxonomy. By taking a closer look at this evolution, the origin of the differences may be understood.

In Lodder and Kregger-Van Rij (1952), the names *cerevisiae*, *oviformis*, *bayanus*, *uvarum*, etc., referred to a number of the 30 species of the genus *Saccharomyces*. Ribéreau-Gayon and Peynaud (1960) considered that two principal fermentation species were found in wine: *S. cerevisiae* (formerly called *ellipsoideus*) and *Saccharomyces oviformis*, which was encountered especially toward the end of fermentation and was considered more ethanol resistant. The difference in their ability to ferment galactose distinguished the two species: *S. cerevisiae* (Gal^+) fermented galactose, whereas *S. oviformis* (Gal^-) did not. According to the same authors, the species *Saccharomyces bayanus* was rarely found in wines. Although it possessed the same physiological fermentation and sugar assimilation characters as *S. oviformis*, its cells were more elongated, its fermentation was slower, and it displayed specific behavior toward growth factors. As for the species *Saccharomyces uvarum*, identified in wine by many authors, it differed from *S. cerevisiae*, *S. oviformis*, and *S. bayanus* because it could ferment melibiose.

In Lodder's following edition (1970), the number of species of the genus *Saccharomyces* increased from 30 to 41. Some species formerly grouped with other genera were integrated into the genus *Saccharomyces*. Moreover, several species names were considered to be synonyms and disappeared altogether. Such was the case of *S. oviformis*, which was moved to the species *S. bayanus*.

TABLE 1.3

Evolution of the Nomenclature for the *Saccharomyces* Genus, 1952–2011 (Libkind *et al.* 2011)

1952	1970	1984	2011
Saccharomyces cerevisiae ⇒	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*	*Saccharomyces arboricolus*
Saccharomyces pastorianus	*Saccharomyces aceti*		*Saccharomyces bayanus*[a]
Saccharomyces bayanus ⇒	*Saccharomyces bayanus*		*Saccharomyces cariocanus*
Saccharomyces oviformis	*Saccharomyces capensis*		*Saccharomyces cerevisiae*
Saccharomyces logos	*Saccharomyces prostoserdovii*		*Saccharomyces eubayanus*
Saccharomyces chevalieri ⇒	*Saccharomyces chevalieri*		*Saccharomyces kudriavzevii*
Saccharomyces fructuum	*Saccharomyces coreanus*		*Saccharomyces mikatae*
Saccharomyces lactis	*Saccharomyces diastaticus*		*Saccharomyces paradoxus*
Saccharomyces elegans	*Saccharomyces globosus*		*Saccharomyces pastorianus*[a]
Saccharomyces heterogenicus	*Saccharomyces heterogenicus*		*Saccharomyces uvarum*
Saccharomyces fermentati	*Saccharomyces hienipiensis*		
Saccharomyces mellis	*Saccharomyces inusitatus*		
Saccharomyces italicus ⇒	*Saccharomyces italicus*		
Saccharomyces steineri	*Saccharomyces norbensis*		
Saccharomyces pastori	*Saccharomyces oleaceus*		
Saccharomyces carlsbergensis	*Saccharomyces oleaginosuss*		
Saccharomyces uvarum ⇒	*Saccharomyces uvarum*		

1952	1970	1984	2011
	Saccharomyces incompspicuus		
Saccharomyces acidifaciens	*Saccharomyces amurcae*		
Saccharomyces bailii ⇒	*Saccharomyces bailii*		
Saccharomyces fragilis	*Saccharomyces cidri*		
	Saccharomyces dairensis⇒	*Saccharomyces dairensis*	
Saccharomyces delbrueckii ⇒	*Saccharomyces delbrueckii*		
Saccharomyces marxianus	*Saccharomyces eupagycus*		
Saccharomyces exiguus ⇒	*Saccharomyces exiguus* ⇒	*Saccharomyces exiguus*	
Saccharomyces veronae	*Saccharomyces fermentati*		
Saccharomyces florentinus ⇒	*Saccharomyces florentinus*		
Saccharomyces bisporus ⇒	*Saccharomyces bisporus*		
Saccharomyces willianus	*Saccharomyces kloeckerianus*		
	Saccharomyces kluyveri ⇒	*Saccharomyces kluyveri*	
Saccharomyces microellipsodes ⇒	*Saccharomyces microellipsodes*		
	Saccharomyces montanus		
	Saccharomyces mrakii		
	Saccharomyces pretoriensis		

TABLE 1.3 (CONTINUED)

1952	1970	1984	2011
Saccharomyces rosei ⇒	*Saccharomyces rosei*		
Saccharomyces rouxii ⇒	*Saccharomyces rouxii*		
	Saccharomyces saitoanus		
	Saccharomyces telluris ⇒	*Saccharomyces telluris*	
	Saccharomyces transvaalensis		
	Saccharomyces unisporus ⇒	*Saccharomyces unisporus*	
	Saccharomyces vafer	*Saccharomyces servazzii*	

Source: From Barnett *et al*. (2000), Lodder and Kregger-Van Rij (1952), Lodder (1970), Kregger-Van Rij (1984), and Kurtzman *et al*. (2011).

[a] Interspecific hybrids.

Ribéreau-Gayon *et al.* (1975) considered, however, that the distinction between *S. oviformis* and *S. bayanus* was of enological interest because of the different technological characteristics of these two yeasts. Nevertheless, by the early 1980s, most enology texts had abandoned the name *S. oviformis* and replaced it with *S. bayanus*.

The new classification by Kregger-Van Rij (1984), based on Yarrow's work on percentages of guanine and cytosine bases in yeast DNA, brought forth another important change in the designation of *Saccharomyces* species. Only seven species continued to exist, while 17 names became synonyms of *S. cerevisiae*. As with the preceding yeast classification, these yeast were differentiated by their sugar utilization profile (Table 1.4), and some authors considered them to be races or physiological varieties of *S. cerevisiae*. However, this was nothing more than an artificial taxonomy without biological significance. Enologists took the habit of adding the varietal name to *S. cerevisiae* to designate wine yeasts: *S. cerevisiae* var. *cerevisiae*, var. *bayanus*, var. *uvarum*, var. *chevalieri*, etc. In addition, two species, *bailii* and *rosei*, were removed from the genus *Saccharomyces* and integrated into two other genera to become *Zygosaccharomyces bailii* and *T. delbrueckii*, respectively.

Based on recent advances in genetics and molecular taxonomy, the latest yeast classification (Kurtzman *et al.*, 2011) and recent studies (Libkind *et al.*, 2011) have again thrown the species delimitation of the genus *Saccharomyces* into confusion. There are now 10 species (Table 1.3). The species *Saccharomyces paradoxus*, *Saccharomyces arboricolus*, *Saccharomyces mikatae*, and *Saccharomyces eubayanus* include strains initially isolated from tree exudates, insects, and soil (Naumov *et al.*, 1998, 2000a; Wang and Bai, 2008; Libkind *et al.*, 2011). Using genome analysis, Redzepovic has nevertheless identified a high percentage of *S. paradoxus* in Croatian grape microflora in Redzepovic *et al.* (2002).

TABLE 1.4

Physiological Races of *S. cerevisiae* Grouped Under a Single Species *S. cerevisiae* by Yarrow and Nakase (1975)

	Fermentation					
	Ga	Su	Ma	Ra	Me	St
Saccharomyces						
aceti	–	–	–	–	–	–
bayanus	–	+	+	+	–	–
capensis	–	+	–	+	–	–
cerevisiae	+	+	+	+	–	–
chevalieri	+	+	–	+	–	–
coreanus	+	+	–	+	+	–
diastaticus	+	+	+	+	–	+
globosus	+	–	–	–	–	–
heterogenicus	–	+	+	–	–	–
hienipiensis	–	–	+	–	+	–
inusitatus	–	+	+	+	+	–
norbensis	–	–	–	–	+	–
oleaceus	+	–	–	+	+	–
oleaginosus	+	–	+	+	+	–
prostoserdovii	–	–	+	–	–	–
steineri	+	+	+	–	–	–
uvarum	+	+	+	+	+	–

Ga, D-galactose; Su, sucrose; Ma, maltose; Ra, raffinose; Me, melibiose; St, soluble starch.

TABLE 1.5

DNA/DNA Reassociation Percentages Between the Four Species Belonging to the *Saccharomyces* Genus in the Strict Sense (Vaughan Martini and Martini, 1987)

	Saccharomyces cerevisiae	Saccharomyces bayanus	Saccharomyces pastorianus	Saccharomyces paradoxus
Saccharomyces cerevisiae	100			
Saccharomyces bayanus	20	100		
Saccharomyces pastorianus	58	70	100	
Saccharomyces paradoxus	53	24	24	100

Saccharomyces pastorianus replaces the former name *Saccharomyces carlsbergensis*, given to brewery yeast strains, used for low-temperature fermentations (lager) and until then included in the *cerevisiae* species. The placement of *S. bayanus, S. uvarum*, and *S. pastorianus* within the *Saccharomyces* genus was the subject of controversies among yeast scientists for years. The latest works, based on DNA sequencing and the recent discovery of the *S. eubayanus* species, have definitively determined the taxonomic positions of *S. bayanus* and *S. pastorianus* (Tables 1.4 and 1.5; Nguyen and Gaillardin, 2005; Libkind *et al*., 2011; Nguyen and Boekhout, 2017). It has been clearly established that *S. bayanus* and *S. pastorianus* refer to hybrid individuals, composed of *S. eubayanus*, *S. uvarum*, and *S. cerevisiae* genomes. On the other hand, *S. uvarum* and *S. eubayanus* are considered as being of genetically pure lineage.

Thus, *S. bayanus* is now considered a distinct hybrid species of *S. cerevisiae* and *S. uvarum* according to taxonomists. Nevertheless, enologists and winemakers use the name of *bayanus (ex oviformis)* to designate a physiological race of *S. cerevisiae* that does not ferment galactose. It possesses a greater resistance to ethanol than *S. cerevisiae*. The implementation of molecular biology methods, based on DNA analysis, has helped establish the position of the winemaking yeasts formerly designated as var. *bayanus* and var. *uvarum* in agreement with the current taxonomy.

For some 30 years, fragment amplification of the genome by the polymerase chain reaction (PCR) has provided an excellent discrimination tool for winemaking yeast species.

Since its discovery by Saiki *et al*. (1985), PCR has often been used to identify different plant and bacteria species. This technique consists in enzymatically amplifying one or several gene fragments *in vitro*. The reaction is based on the hybridization of two oligonucleotides that frame a target region on a double strand of DNA or template. These oligonucleotides have different sequences and are complementary to the DNA sequences that frame the strand being amplified. Figure 1.21 illustrates the various stages of the amplification process. The DNA is first denatured at a high temperature (95°C). The reaction mixture is then cooled to a temperature between 37 and 55°C, enabling the hybridization of these oligonucleotides on the denatured strands. The strands serve as primers from which a DNA polymerase enables the step-by-step addition of deoxyribonucleotide units in the 5′–3′ direction. The DNA polymerase (Figure 1.22) requires four deoxyribonucleoside-5Π-triphosphates (dATP, dGTP, dTTP, and dCTP). A

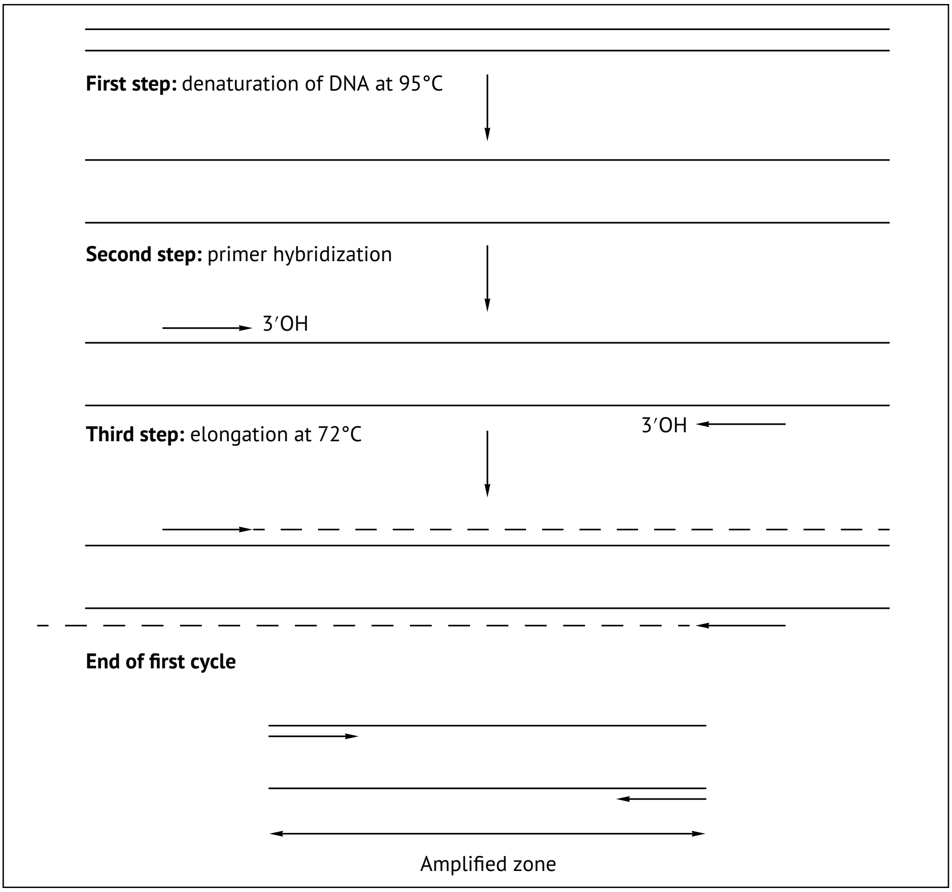


FIGURE 1.21 Principle of the polymerization chain reaction (PCR).

phosphodiester bond is formed between the 3П–OH end of the primer and the innermost phosphorus of the activated deoxyribonucleoside. Pyrophosphate is thus released. The newly synthesized strand is elongated on the template. A heat-resistant enzyme, TAQ DNA polymerase, comes from the heat-resistant bacteria *Thermus aquaticus*. It is used to conduct a large number of amplification cycles (25–40) *in vitro* without having to add DNA polymerase after each denaturation. In this manner, the DNA fragment amplified during the first cycle serves as the template for the following cycles. In consequence, each successive cycle doubles the target DNA fragment, which is thus amplified by a factor of 10^5 to 10^6 during 25–30 amplification cycles.

Hansen and Kielland-Brandt (1994) proposed *MET*2 gene PCR amplification to differentiate between *S. cerevisiae* and *S. uvarum* (formerly *S. bayanus*), when working on strain types of these two species. This gene, which codes for the synthesis of homoserine acetyltransferase, has different sequences in the two species. Part of the gene is initially amplified by using two complementary oligonucleotides of the sequences bordering the fragment to be amplified. The fragment obtained (about 600 bp) is the same size for typical strains of the *S. cerevisiae* and *S. uvarum* species. Different restriction endonucleases, which recognize certain specific DNA sequences, then digest the amplified fragment. Figure 1.23 gives an example of the mode of action of the *Eco*R1 restriction endonuclease. This enzyme recognizes the base sequence GAATTC and cuts at the location indicated by the arrows. Electrophoresis is

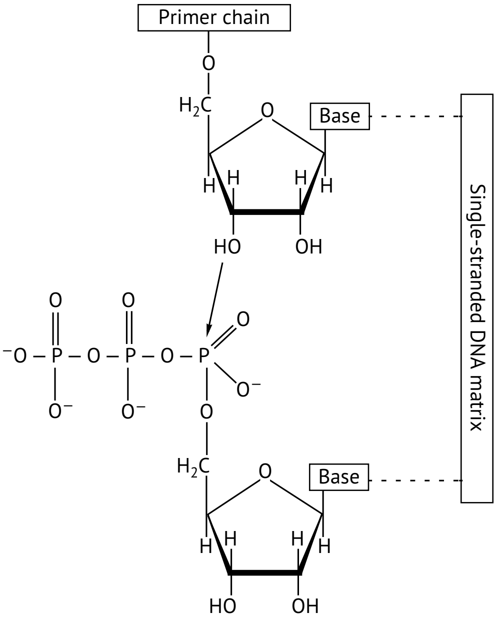


FIGURE 1.22 Mode of action of DNA polymerase.

used to separate the obtained fragments. As a result, restriction fragment length polymorphism (RFLP) can be assessed. The restriction profiles obtained differ between *S. cerevisiae* and *S. uvarum*.

This PCR-RFLP technique associated with the *MET*2 gene has been developed and adapted for rapid analysis. The whole cells are simply heated in water to 95°C for 10 minutes before amplification. Only two restriction enzymes are used: *Eco*R1 and *Pst*1 (Figure 1.24; Masneuf *et al.*, 1996a,b).

By applying this relatively simple and quick technique to different wine yeast strains studied by Naumov *et al.* (1993), we obtained perfect concordance between the *MET*2 gene PCR-RFLP and hybridization tests in order to delimit *S. cerevisiae* and *S. uvarum* species.

We extended this type of analysis by PCR-RFLP of the *MET*2 gene to different yeast strains selected from the market and often used in winemaking. Depending on their ability to ferment galactose or not, they are still sometimes called *S. cerevisiae* or *S. bayanus* by wine professionals around the world at the time this handbook is being written (Table 1.6). For all of these strains,

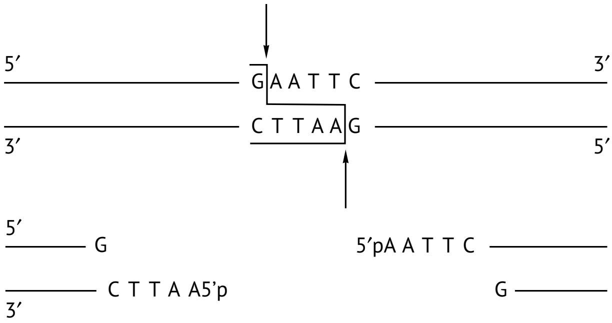


FIGURE 1.23 Recognition site and cutting mode of an *Eco*R1 restriction endonuclease.

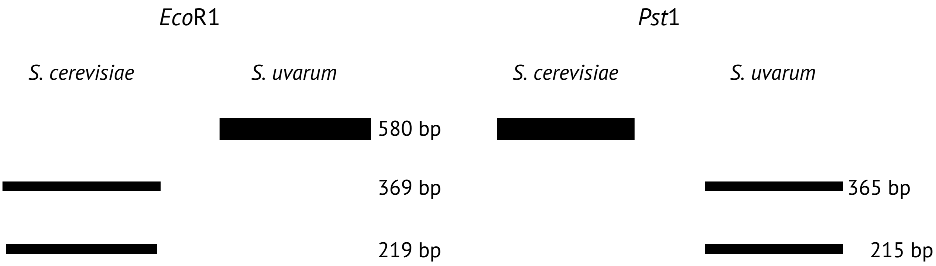


FIGURE 1.24 Identification principles for the *S. cerevisiae* and *S. uvarum* species by the *MET*2 gene PCR-RFLP technique, after cutting the amplified fragment with *Eco*R1 and *Pst*1 restriction enzymes.

TABLE 1.6

Characterization by PCR-RFLP of the *MET2* Gene of Various Commercial Strains of the *S. cerevisiae* Species Used in Winemaking (Masneuf, 1996)

Strains	Commercial brand	Origin	Enological designation	Species
VL1	Zymaflore VL1	FŒB	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*
VL3c	Zymaflore VL3	FŒB	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*
WET 136	Siha levactif 3	Dormstadt	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*
71B	Actiflore primeur	INRA Narbonne	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*
F10	Zymaflore F10	FŒB	*Saccharomyces bayanus*	*Saccharomyces cerevisiae*
R2	Vitlevure KD	NA	*Saccharomyces bayanus*	*Saccharomyces cerevisiae*
BO213	Actiflore bayanus	Institut Pasteur	*Saccharomyces bayanus*	*Saccharomyces cerevisiae*
CH158	Siha levactif 4	NA	*Saccharomyces bayanus*	*Saccharomyces cerevisiae*
QA23	Lalvin QA23	UTM	*Saccharomyces bayanus*	*Saccharomyces cerevisiae*
IOC182007	IOC 182007	IŒC	*Saccharomyces bayanus*	*Saccharomyces cerevisiae*
DV10	Vitlevure DV10	CIVC	*Saccharomyces bayanus*	*Saccharomyces cerevisiae*
O16	Lalvin O16	UB	*Saccharomyces bayanus*	*Saccharomyces cerevisiae*
Epernay2	Uvaferm CEG	NA	*Saccharomyces bayanus*	*Saccharomyces cerevisiae*

FŒB: Faculté d' Œnologie de l'Université de Bordeaux II, Talence, France. UTM: Université de Tras os Montes, Portugal. ŒC: Institut Œnologique de Champagne, France. CIVC: Comité Interprofessionnel des Vins de Champagne (Interprofessional Champagne Committee), Epernay, France. UB: Université de Bourgogne, Dijon, France. NA: not available.

TABLE 1.7

Characterization by PCR-RFLP of the *MET2* Gene of Various Species of Native *Saccharomyces* Isolated on the Grape and in Wine (Masneuf, 1996)

Number of different strains analyzed	Origin	Collection	Enological designation	Species
8	Sauternes wines	FŒB	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*
2	Dry white Bordeaux wines	FŒB	*Saccharomyces bayanus*	*Saccharomyces cerevisiae*
9	Sauternes wines	FŒB	*Saccharomyces bayanus*	*Saccharomyces cerevisiae*
2	Dry white Bordeaux wines	FŒB	*Saccharomyces chevalieri*	*Saccharomyces cerevisiae*
1	Sauternes wines	FŒB	*Saccharomyces capensis*	*Saccharomyces cerevisiae*
36	Unknown	Lallemand	*Saccharomyces bayanus*	*Saccharomyces cerevisiae*
11	Sauternes wines	FŒB	*Saccharomyces uvarum*	*Saccharomyces uvarum*
1	Sauternes wines	FŒB	*Hanseniaspora uvarum*	*Saccharomyces cerevisiae*
10	Sancerre and Pouilly Fumé (Loire) wines	FŒB	*Hanseniaspora uvarum*	*Saccharomyces uvarum*
1	Sancerre grapes	FŒB	*Hanseniaspora uvarum*	*Hanseniaspora uvarum*
2	Unknown	Lallemand	*Hanseniaspora uvarum*	*Saccharomyces cerevisiae*

FŒB: Faculté dOEnologie de l'Université de Bordeaux II, Talence, France. Lallemand: Lallemand Inc., Montreal, Quebec, Canada.

we have obtained the same characteristic restriction profiles of the species *S. cerevisiae*.

Moreover, we have determined the species of 82 strains of native *Saccharomyces* isolated from fermenting wine or from grapes (Table 1.7). For the eight Gal$^+$ Mel$^-$ strains analyzed, as for the 47 Gal$^-$ Mel$^-$, respectively, called *S. cerevisiae* and *S. bayanus* by enologists, restriction profiles of the amplified fragment of the *MET*2 gene are characteristic of *S. cerevisiae*. The same goes for the two *S. chevalieri* strains, which ferment galactose but not maltose (Ma$^-$), as well as for the *S. capensis* strain (Gal$^-$ Ma$^-$). As for Mel$^+$ strains, called *S. uvarum* until now, most of them (11 out of 12 for Sauternes isolates and 11 out of 11 for Sancerre isolates) belong to the *S. uvarum* species. However, some Mel$^+$ strains are *S. cerevisiae* (one strain from Sauternes and two strains from the Lallemand collection). In short, regarding the classification of the main winemaking yeasts (Section 1.8.3), we can distinguish between three stages. First, we considered them to be several species: *S. cerevisiae*, *S. bayanus*, and/or *S. oviformis*, *S. uvarum*. Then, we decided they were different races of the *S. cerevisiae* species. The current taxonomy, based on molecular biology results, has made substantial changes. It has defined three species: *S. cerevisiae*, *S. uvarum*, and *S. paradoxus*. The involvement of *S. paradoxus* in the fermentation microflora of grapes remains to be confirmed.

All of the results of molecular taxonomy presented above show that the former phenotypic classifications, based on physiological identification criteria, are not suitable even for delimiting the small number of fermentative species of the *Saccharomyces* genus found in winemaking. Moreover, specialists have long known about the instability of the physiological properties of *Saccharomyces* strains. Rossini *et al.* (1982) reclassified a thousand strains from the yeast collection of the Microbiology Institute of Agriculture at the University of Perugia. They observed that 23 out of 591 *S. cerevisiae* strains conserved on malt agar had lost the ability to ferment galactose. The use of genetic methods is thus essential to identify winemaking yeasts.

1.8.4 Interspecific Hybrids

PCR-RFLP associated with the *MET*2 gene can be used to demonstrate the existence of hybrids between the species *S. cerevisiae* and *S. uvarum*. This method has been used to prove the existence (Masneuf *et al.*, 1998) of one such natural hybrid (strain S6U) among commercial dry yeasts sold by Lallemand Inc. (Montreal, Canada). Ciolfi (1992, 1994) isolated this yeast in an Italian winery, and it was selected for certain enological properties, in particular its aptitude to ferment at low temperatures, its low production of acetic acid, and its ability to preserve must acidity. The *MET*2 gene restriction profiles of this strain by *Eco*R1 and *Pst*1, composed of three bands, are identical (Figure 1.25). In addition to the amplified fragment, two bands characteristic of *S. cerevisiae* with *Eco*R1 and two bands characteristic of the species *S. bayanus* with *Pst*1 are obtained. The bands are not artifacts due to an impurity in the strain, because the amplification of the *MET*2 gene carried out on subclones (obtained from the multiplication of unique cells isolated by a micromanipulator) produces identical results. Hansen from the Carlsberg laboratory (Denmark) sequenced two of the *MET*2 gene alleles from this strain. The sequence of one of the alleles is identical to that of the *S. cerevisiae* *MET*2 gene, with the exception of one nucleotide. The sequence of the other allele is 98.5% similar to that of *S. uvarum*. The presence of this allele is thus probably due to an interspecific cross.

Subsequently, more recent research (Naumov *et al.*, 2000b) has shown that the S6U strain is, in fact, a tetraploid hybrid. Indeed, the percentage germination of spores from 24 tetrads, isolated using a micromanipulator, was very high (94%), whereas it would have been very low for a

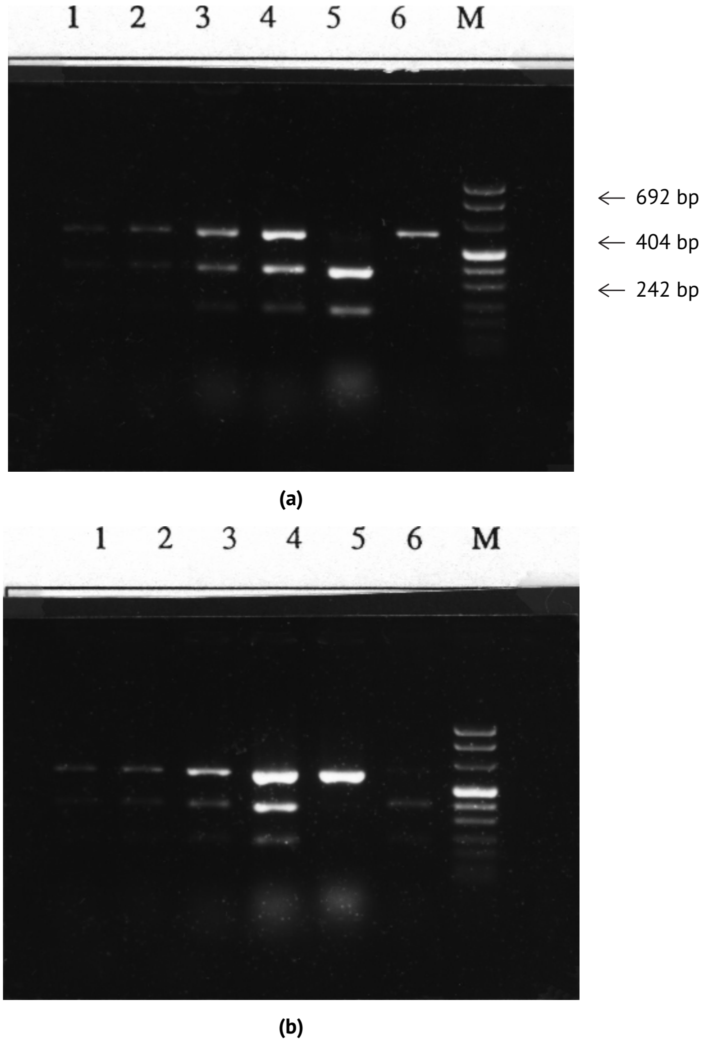


FIGURE 1.25 Electrophoresis in agarose gel (1.8%) of (a) *Eco*R1 and (b) *Pst*1 digestions of the amplified fragments of the *MET*2 gene of the hybrid strain. Bands 1, 2, 3, subclones of the hybrid strain; band 4, hybrid strain; band 5, *S. cerevisiae* control; band 6, *S. uvarum* control; M, molecular weight marker.

"normal" diploid interspecific hybrid. The monospore clones in this first generation (D1) were all capable of sporulating. However, none of the ascospores of the second-generation tetrads was viable. The hybrid nature of the monospore clones produced by D1 was confirmed by the presence of the *S. cerevisiae* and *S. uvarum MET*2 gene, identified by PCR/RFLP. Finally, measuring the DNA content per cell using flux cytometry estimation confirmed that the descendants of S6U were interspecific diploids and that S6U itself was an allotetraploid.

The molecular characterization of wine yeasts isolated on grapes and in must undergoing spontaneous fermentation revealed the existence of many natural hybrids between *S. cerevisiae* and *S. uvarum* (Le Jeune *et al.*, 2007). This is also true between *S. cerevisiae* and *Saccharomyces kudriavzevii* (Sipiczki, 2008; Arroyo-López *et al.*, 2009; Erny *et al.*, 2012). Interspecific hybridization leads to new gene combinations in a given cell and may confer a selective advantage with respect to parental strains. Interspecific hybrids possess interesting technological properties for winemaking. For example, *S. uvarum* and *S. kudriavzevii* are better adapted to low-temperature growth, and *S. cerevisiae* presents high tolerance to ethanol. Natural hybrids between these species are more adapted to growth in a large range of temperatures and at high ethanol concentrations, thanks to the genetic heritage of one or both parents, thus yielding properties of interest (Da Silva *et al.*, 2015). This mechanism for acquiring superior phenotypic characteristics in hybrid descendants during cross-breeding of parental strains is well described in plants. This is called a heterosis effect.

1.9 Identification of Wine Yeast Strains

1.9.1 General Principles

The principal yeast species involved in grape must fermentation, particularly *S. cerevisiae* and *S. uvarum*, comprise a very large number of strains with extremely varied technological properties. The yeast strains involved during winemaking influence fermentation speed, the nature and quantity of secondary products formed during alcoholic fermentation, and the aroma characteristics of the wine. The ability to differentiate between the different strains of *S. cerevisiae* is required for the following fields: the ecological study of wild yeasts responsible for the spontaneous fermentation of grape must, the selection of strains presenting the best enological qualities, production and marketing controls, the verification of the implantation of selected yeasts used as yeast starter, and the constitution and maintenance of wild or selected yeast collections.

The initial research on infraspecific differentiation within *S. cerevisiae* attempted to distinguish strains by electrophoretic analysis of their exocellular (Bouix *et al.*, 1981) or intracellular (Van Vuuren and Van Der Meer, 1987) proteins or glycoproteins. Other teams proposed identifying the strains by the analysis of long-chain fatty acids using gas chromatography (Tredoux *et al.*, 1987; Augustyn *et al.*, 1991; Bendova *et al.*, 1991; Rozes *et al.*, 1992). Although these different techniques differentiate between certain strains, they are irrefutably less discriminating than genetic differentiation methods. They also present the major drawback of depending on the physiological state of the strains and on cultural conditions, which must always be identical.

In the late 1980s, owing to the development of genetics, certain techniques from molecular biology were successfully applied to characterize wine yeast strains. They are based on the clonal polymorphism of the mitochondrial and genomic DNA of *S. cerevisiae* and *S. uvarum*. These genetic methods are independent of the physiological state of the yeast, unlike the previous techniques based on the analysis of metabolism by-products.

1.9.2 Mitochondrial DNA Analysis

The mtDNA of *S. cerevisiae* has two remarkable properties: it is extremely polymorphic, depending on the strain; and stable (it mutates very little) during vegetative reproduction. Restriction endonucleases (such as *Eco*R5) cut this DNA at specific sites. This process generates fragments of variable size that are few in number and can be separated by electrophoresis on agarose gel.

Aigle *et al.* (1984) first applied this technique to brewer's yeasts. Since 1987, it has been used for the characterization of enological strains of *S. cerevisiae* (Dubourdieu *et al.*, 1987; Hallet *et al.*, 1988).

The extraction of mtDNA comprises several stages. The protoplasts obtained by enzymatic digestion of the cell walls are lysed in a hypotonic buffer. The mtDNA is then separated from the chromosomal

DNA by ultracentrifugation in a cesium chloride gradient, in the presence of bisbenzimide, which acts as a fluorescent intercalating stain. This agent amplifies the difference in density between chromosomal DNA and mtDNA. The mtDNA has a high number of adenine and thymine base pairs, for which bisbenzimide has a strong affinity. Finally, the mtDNA is purified by a phenol–chloroform extraction and an ethanol precipitation.

Defontaine *et al.* (1991) and Querol *et al.* (1992) simplified this protocol by separating the mitochondria from the other cell constituents before extracting the DNA. In this manner, they avoided the ultracentrifugation step. The coarse cellular debris is eliminated from the yeast lysate by centrifuging at 1,000 g. The supernatant is then recentrifuged at 15,000 g to obtain the mitochondria. The mitochondria are then lysed in a suitable buffer to liberate the DNA.

Unlike the industrial brewer's yeast strains analyzed by Aigle *et al.* (1984), which have the same mtDNA restriction profile, implying that they are of common origin, the winemaking yeast strains have a large degree of mtDNA diversity. This method easily differentiates between most of the selected yeasts used in winemaking as well as wild strains of *S. cerevisiae* found in spontaneous fermentations (Figure 1.26). This method may also help differentiate *S. uvarum* strains (Naumova *et al.*, 2010).

This technique is very discriminating and not too expensive, but it is long and requires several complex manipulations. It is useful for the subtle characterization of a small number of strains. Inoculation effectiveness can also be verified by this method. In the laboratory, the lees, sampled during or toward the end of alcoholic fermentation, are cultured in a liquid medium. The mtDNA restriction profiles of this total biomass and of the yeast starter strain are compared. The absence of any extra bands, with respect to the yeast starter strain restriction profile, demonstrates that the yeast starter has been properly implanted, with an accuracy of 90%. In fact, in the case of a binary mixture, the minority strain must represent around 10% of the total population to be detected (Hallet *et al.*, 1989).

1.9.3 Karyotype Analysis

Saccharomyces cerevisiae has 16 chromosomes with a size range between 250 and 2,500 kb. Its genomic DNA is very polymorphic; thus, it is possible to differentiate

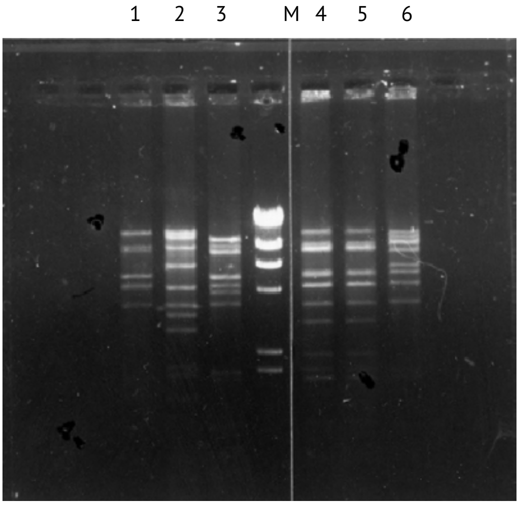


FIGURE 1.26 Restriction profile by *Eco*R5 of mtDNA of different strains of *S. cerevisiae*. Band 1, FIO; band 2, BO213; band 3, VLI; M, marker; band 4, 522; band 5, Sita 3; band 6, VL3c.

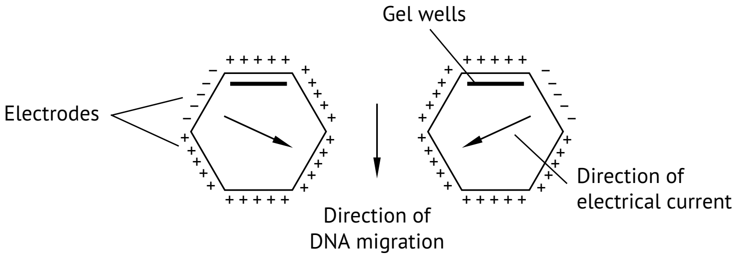


FIGURE 1.27 CHEF pulsed-field electrophoresis device (*contour clamped electrophoresis field*).

strains of the species according to the size distribution of their chromosomes. Pulsed-field electrophoresis is used to separate *S. cerevisiae* chromosomes and to compare karyotypes of the strains. This technique uses two electric fields oriented differently (90–120°). The electrodes placed on the sides of the apparatus apply the fields alternately (Figure 1.27).

The user can define the duration of the electric current that will be applied in each direction (pulse). With each change in the direction of the electric field, the DNA molecules reorient themselves; the smaller chromosomes reorient themselves more quickly than the larger ones (Figure 1.28).

Blondin and Vezhinet (1988), Petering *et al.* (1988), and Dubourdieu and Frezier (1990) applied this technique to identify wine yeast strains. Sample preparation is relatively easy. The yeasts are cultivated in a liquid medium, collected during the log phase, and then placed in suspension in a warm agarose solution that is poured into a partitioned mold to form small plugs.

Figure 1.29 gives an example of the identification of *S. cerevisiae* strains isolated from a grape must undergoing spontaneous fermentation. Vezhinet *et al.* (1990) have shown that karyotype analysis can distinguish between strains of *S. cerevisiae* as well or better than the use of mtDNA restriction profiles. Furthermore, karyotype analysis is much quicker and easier to use than mtDNA analysis. In the case of ecological studies of spontaneous fermentation microflora, pulsed-field electrophoresis of chromosomes is extensively used today to characterize strains of *S. cerevisiae* (Frezier and Dubourdieu, 1992; Versavaud *et al.*, 1993, 1995).

Very little research on the chromosomal polymorphism in other species of grape and wine yeasts is currently available. Naumov *et al.* (1993) suggested that *S. uvarum* and *S. cerevisiae* karyotypes can be easily distinguished. Other authors (Vaughan Martini and Martini, 1993; Masneuf, 1996) have confirmed their results. In fact, a specific chromosomal band systematically appears in *S. uvarum*. Furthermore, there are only two chromosomes whose sizes are less than 400 kb in *S. uvarum* but generally more in *S. cerevisiae*, in all of the strains that we have analyzed.

Non-*Saccharomyces* species, in particular apiculate yeasts (*H. uvarum* and

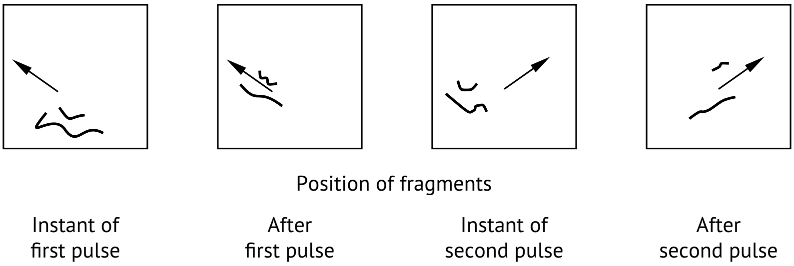


FIGURE 1.28 Mechanism of DNA molecule separation by pulsed-field electrophoresis.

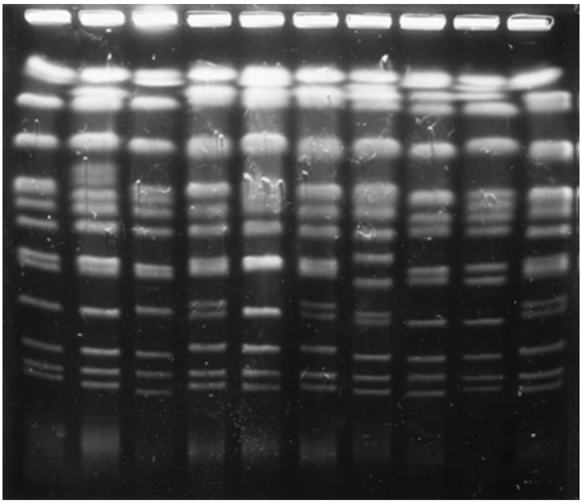


FIGURE 1.29 Example of electrophoretic (pulsed field) profile of *S. cerevisiae* strain karyotypes.

K. apiculata), are present on grapes and are sometimes found at the beginning of fermentations. These species have fewer polymorphic karyotypes and fewer bands than in *Saccharomyces*. Versavaud *et al.* (1993) differentiated between strains of apiculate yeast species and *Candida famata* by using restriction endonucleases at rare sites (*Not*1 and *Sfi*1). The endonucleases cut the chromosomes into a limited number of fragments, which were then separated by pulse-field electrophoresis.

1.9.4 Genomic DNA Restriction Profile Analysis Associated with DNA Hybridization by Specific Probes (Fingerprinting)

The yeast genome contains DNA sequences that repeat from dozens to hundreds of times, such as the δ sequences or Y1 elements of the chromosome telomeres. The distribution, or more specifically, the number and location of these elements, has a certain intraspecific variability. This genetic fingerprint is used to identify strains (Pedersen, 1986; Degre *et al.*, 1989).

The yeast strains are cultivated in a liquid medium and are sampled during the log phase, as in the preceding techniques. The entire DNA is isolated and digested by restriction endonucleases. The generated fragments are separated by electrophoresis on agarose gel and then transferred to a nylon membrane (Southern, 1975). Complementary radioactive probes (nucleotide sequences taken from δ and Y1 elements) are used to hybridize with fragments having homologous sequences. The result gives a hybridization profile containing several bands.

Genetic fingerprinting after hybridization is a more complicated and involved method than mtDNA or karyotype analysis. It is, however, without doubt the most discriminating strain identification method and may even discriminate too well. It has correctly indicated minor differences between very closely related strains. In fact, in the Bordeaux region (Frezier, 1992), *S. cerevisiae* clones isolated from spontaneous fermentations in different wineries have been encountered, which have the same karyotype and the same mtDNA restriction profile. Yet their hybridization profiles differ depending on sample origin. These strains, probably descendants of the same mother strain, have therefore undergone minor random modifications, maintained during vegetative reproduction.

1.9.5 PCR Associated with δ Sequences

This method consists of using PCR to amplify certain sequences of the yeast genome (Section 1.8.4), occurring between the repeated δ elements, whose separation distance does not exceed a certain value (1 kb). This method was developed (Ness *et al.*, 1992; Masneuf and Dubourdieu, 1994; Legras and Karst, 2003) to characterize *S. cerevisiae* strains. The amplification is carried out directly on whole cells. They are simply heated to make the cell envelopes permeable. The resulting amplification fragments are separated according to their size by agarose gel electrophoresis and viewed using ultraviolet fluorescence (Figure 1.30).

This analysis can distinguish between most *S. cerevisiae* ADY strains used in winemaking (Figure 1.31). Out of the 26 selected commercial yeast strains analyzed, 25 can be differentiated by their PCR profile associated with δ sequences. Lavallée *et al.* (1994) also observed excellent discriminating power with this method while analyzing industrially produced commercial strains from Lallemand Inc. (Montreal, Canada). In addition, this

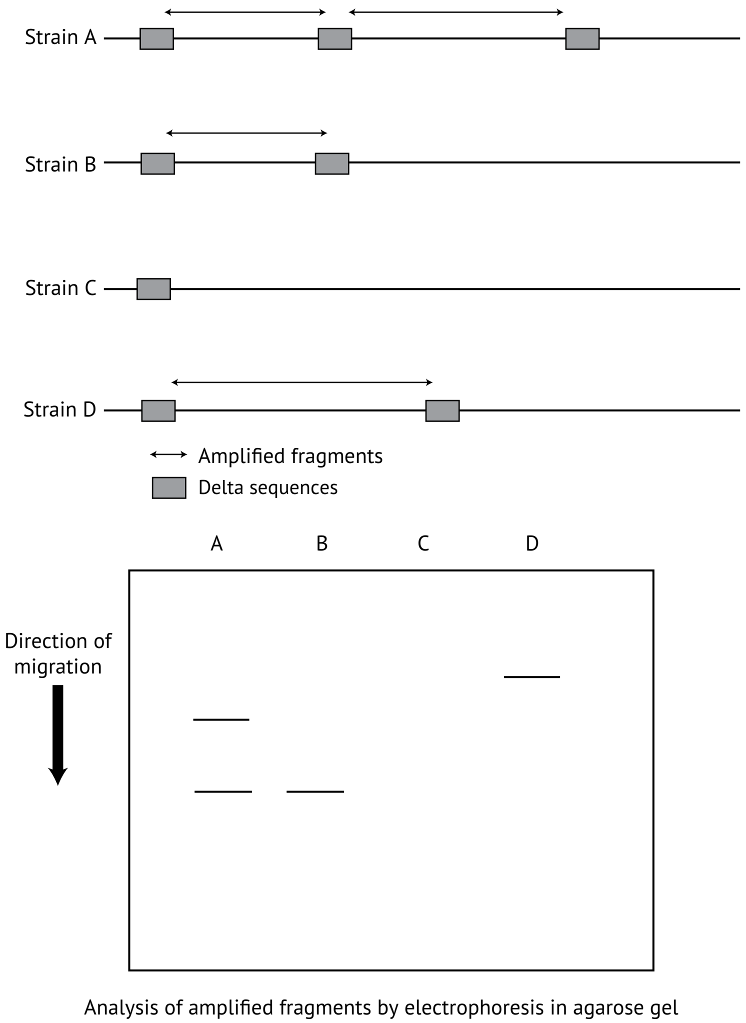


FIGURE 1.30 Principle of identification of *S. cerevisiae* strains by PCR associated with δ elements.

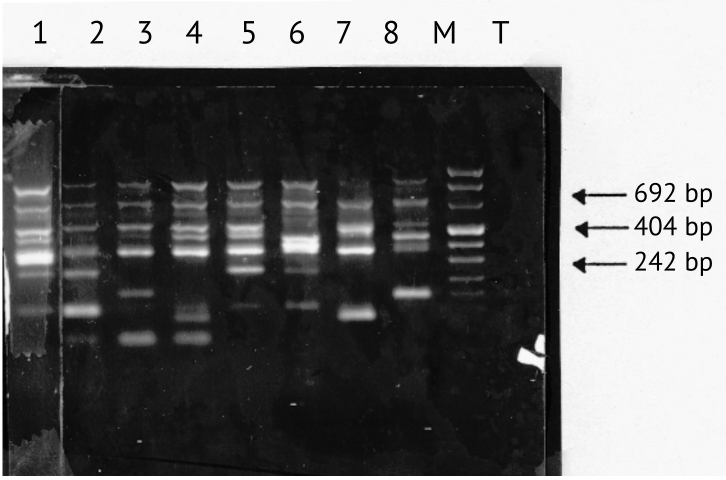


FIGURE 1.31 Electrophoresis in agarose gel (at 1.8%) of amplified fragments obtained from various commercial yeast strains. Band 1, F10; band 2, BO213; band 3, VL3c; band 4, UP30Y5; band 5, 522 D; band 6, EG8; band 7, L-1597; band 8, WET 136; M, molecular weight marker; T, negative control.

method enables the identification of 25–50 strains per day; it is the quickest of the different strain identification techniques currently available. When used for native yeast strain identification in a given viticultural region, however, it seems to be less discriminating than karyotype analysis. PCR profiles of wild yeasts isolated in a given location often appear similar. They have several constant bands and only a small number of variable discriminating bands. Certain strains have the same PCR amplification profile while having different karyotypes. In a given location, the polymorphism witnessed by PCR associated with δ sequences is lower than that of the karyotypes. This method is therefore complementary to other methods for characterizing winemaking strains. PCR enables a rapid primary sorting of a native yeast population. Karyotype analysis refines this discrimination.

Saccharomyces uvarum strains cannot be distinguished by this technique because their genome contains only a few Ty elements.

Lastly, because of its convenience and rapidity, PCR associated with δ sequences facilitates verification of the implantation of yeast starters used in winemaking. The analyses are conducted on the entire biomass derived from lees, placed beforehand in a liquid medium in a laboratory culture. The amplification profiles obtained are compared with inoculated yeast strain profiles. They are identical with a successful implantation, and different if the inoculation fails. Figure 1.32 gives examples of successful (yeasts B and C) and unsuccessful (yeasts A, D, and E) implantations. Contaminating strains have a different amplification profile than the yeast starter.

The detection threshold of a contaminating strain was studied in the laboratory by analyzing a mixture of two strains in variable proportions. In the example given in Figure 1.33, the contaminating strain is easily detected at 1%. In winery fermentations, however, several native strains can coexist with the inoculated strain in small proportions. When must undergoing fermentation or lees is analyzed by PCR, the yeast implantation rate is at least 90% when the amplification profiles of the lees and the yeast starter are identical.

1.9.6 PCR with Microsatellites

Microsatellites are tandem repeat units of short DNA sequences (1–10 nucleotides),

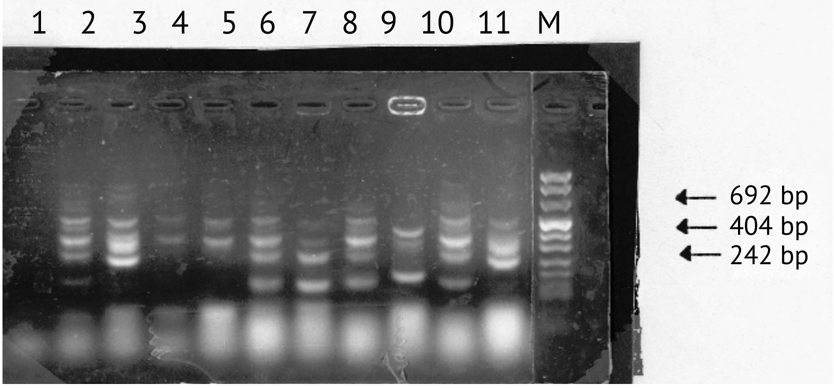


FIGURE 1.32 Electrophoresis in agarose gel (1.8%) of amplified fragments illustrating examples of yeast implantation tests (successful: yeasts B and C; unsuccessful: yeasts A, D, and E). Band 1, negative control; band 2, lees A; band 3, ADY A; band 4, lees B; band 5, ADY B; band 6, lees C; band 7, ADY C; band 8, lees D; band 9, ADY D; band 10, lees E; band 11, ADY E; M, molecular weight marker.

i.e. in the same direction and dispersed throughout the eukaryote genome. The number of motif repetitions is extremely variable from one individual to another, making these sequences highly polymorphic in size. These regions are easily identified, thanks to knowledge of the full sequence of the *S. cerevisiae* genome. Approximately 275 sequences have been listed, mainly AT dinucleotides and AAT and AAC trinucleotides (Field and Wills, 1998; Hennequin *et al.*, 2001; Perez *et al.*, 2001). Furthermore, these sequences are allelic markers, transmitted to the offspring in a Mendelian fashion. Consequently, these are ideal genetic markers for identifying specific yeast strains, making it possible not only to distinguish between strains but also to arrange them in related groups. This technique has many applications in humans: paternity tests, forensic medicine, etc. In viticulture, this molecular identification method has already been applied to *Vitis vinifera* grape varieties (Bowers *et al.*, 1999).

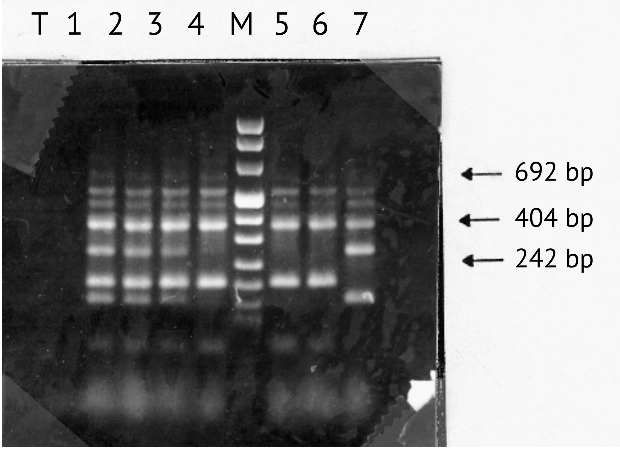


FIGURE 1.33 Determination of the detection threshold of a contaminating strain. T, negative control; band 1, strain A 70%, strain B 30%; band 2, strain A 80%, strain B 20%; band 3, strain A 90%, strain B 10%; band 4, strain A 99%, strain B 1%; M, molecular weight marker; band 5, strain A 99.9%, strain B 0.1%; band 6, strain A; band 7, strain B.

The technique consists in amplifying the region of the genome containing these microsatellites, and then analyzing the size of the amplified portion to a level of detail of one nucleotide by capillary electrophoresis. This size varies by a certain number of base pairs (approximately 8–40) from one strain to another, depending on the number of times the motif is repeated. A yeast strain may be heterozygous for a given locus, giving two different-sized amplified DNA fragments. Using six microsatellites, Perez *et al.* (2001) were able to identify 44 different genotypes within a population of 51 strains of *S. cerevisiae* used in winemaking. Other authors (Gonzalez Techera *et al.*, 2001; Hennequin *et al.*, 2001, Klis *et al.*, 2002) have shown that the strains of *S. cerevisiae* used in winemaking are weakly heterozygous for the loci studied. However, interstrain variability of the microsatellites is very high. The results are expressed in numerical values for the size of the microsatellite in base pairs or the number of repetitions of the motifs on each allele. These digital data are easy to interpret, unlike the karyotype images on agarose gel, which are not really comparable from one laboratory to another. Based on 41 microsatellites, Legras *et al.* (2005) selected six very discriminating and reproducible loci. They highlight the relationships between strains from different geographical zones or industrial environments.

Microsatellite analysis has also been used to identify the strains of *S. uvarum* (Masneuf-Pomarède *et al.*, 2007, 2016) and of *S. kudriavzevii* (Erny *et al.*, 2012) used in winemaking. As the *S. uvarum*, *S. kudriavzevii*, and *S. cerevisiae* microsatellites have different amplification primers, this method provides an additional means of distinguishing between these species and their hybrids.

The development of new-generation sequencing methods for yeast genomes has made sequences available for non--*Saccharomyces* species. A typing method of yeast strains by microsatellite marker analysis is now offered for *B. bruxellensis*, *T. delbrueckii*, *H. uvarum*, and *Starmerella bacillaris* (Albertin *et al.*, 2014a,b, 2016; Masneuf-Pomarede *et al.*, 2015, 2016). When applied to the study of a great number of yeast isolates, these methods help to better describe the genetic diversity and the population structure of winemaking yeasts. Factors influencing this structure as well as their life cycle and reproduction mode have also been described. From an applied point of view, this molecular typing method is a useful tool in winemaking yeast strain identification, ecological surveys, and quality control of industrial production batches.

1.9.7 Genome Sequencing

With the development of new-generation sequencing methods, new approaches to yeast characterization have been suggested. The multilocus sequence typing (MLST) method is a standardized approach to full or partial sequence analysis of certain gene expressions in yeast. These genes are characterized by a slow accumulation of mutations, which help differentiate between individuals, as well as deduce phylogenetic relationships between strains. Applied to *S. cerevisiae*, the results obtained do not indicate a superior ability to discriminate among yeast strains when compared with analysis by repetitive-element PCR or microsatellite marker polymorphism (Ayoub *et al.*, 2006). However, studies have revealed the specific population structure of wine yeasts, confirming the domestication of these yeasts (Fay and Benavides, 2005). Other approaches consist in establishing sequences of regions located between randomly selected restriction sites in the genome (restriction site-associated DNA sequencing or RAD-seq). Many positions of variation of a base, or single nucleotide polymorphism (SNP), can thus be used for phylogenetic analyses. The RAD-seq method has established the diversity and genetic structure of *S. cerevisiae* strains from a variety of ecological niches (Cromie *et al.*, 2013; Hyma and Fay, 2013).

1.10 Ecology of Grape and Wine Yeasts

1.10.1 Succession of Grape and Wine Yeast Species

A large amount of research was focused on the description and ecology of wine yeasts. It concerned the distribution and succession of species found on the grape and then in wine during fermentation and conservation (Ribéreau-Gayon *et al.*, 1975; Lafon-Lafourcade, 1983).

The ecological study of grape and wine yeast species represents a considerable amount of research. De Rossi began his research in the 1930s (De Rossi, 1935). Castelli (1955, 1967) pursued the study of yeast ecology in Italian vineyards. Peynaud and Domercq (1953) and Domercq (1956) published the first results on the ecology of wine yeasts in France. They described not only the species found on the grape and during alcoholic fermentation but also contaminating and spoilage yeasts. Among the many publications on this topic since the 1960s in viticultural regions around the world, the following works are worth noting: Brechot *et al.* (1962), Sapis-Domercq (1970), Barnett *et al.* (1972), Minarik (1971), Cuinier and Guerineau (1976), Park (1975), Soufleros (1978), Belin (1979, 1981), Poulard *et al.* (1980), Poulard and Lecocq (1981), Bureau *et al.* (1982), Rossini *et al.* (1982), Fleet *et al.* (1984), Mills *et al.* (2002), Baleiras Couto *et al.* (2005), Hierro *et al.* (2006), and Nisiotou and Nychas (2007).

Yeasts are widespread in nature and are found in soils, on the surface of plants, and in the digestive tract of animals. Wind, insects, and birds disseminate them. Yeasts are distributed irregularly on the surface of the grapevine; found in small quantities on leaves, the stems, and unripe grapes, they colonize the grape skin during maturation. During the winter season, the main natural reservoir of yeasts is the trunk, while during the vegetative growth phase of the vine, it is found in the ground and in berries (Cordero-Bueso *et al.*, 2011). Observations under a scanning electron microscope have identified the location of yeasts on the grape. They are rarely found on the bloom, but multiply preferentially on exudates released from microlesions in zones situated around the stomatal apparatus. *Botrytis cinerea* spores and lactic acid and acetic acid bacteria also develop in the proximity of these peristomatic fractures (Figure 1.34).

The number of yeasts on the grape berry, just before harvest, is between 10^3 and 10^5, depending on the geographical location of the vineyard, weather conditions during maturation, the healthiness of the harvested grapes, and pesticide treatments applied to the vine. The most abundant yeast populations are obtained under warm weather conditions (lower latitudes and higher temperatures). Insecticide treatments and certain fungicidal treatments can make the native grape microflora less populous. Quantitative results available on this subject, however, are few. After the harvest, transport, and crushing of the crop, the number of cells capable of forming colonies on an agar medium generally reaches 10^6 cells/ml of must.

The number of yeast species significantly present on the grape is limited. From fruit set to maturity, *Aureobasidium pullulans*, *Cryotococcus*, and *Rhodotorula* are the main genera and species encountered (Prakitchaiwattana *et al.*, 2004; Martins *et al.*, 2014). Their proportion then decreases in favor of Ascomycetes. Among the latter, the apiculate species (*K. apiculata* and its sporogenous form *H. uvarum*) are the most common. They comprise up to 99% of the yeasts isolated in certain grape samples. The following are generally found but in lesser proportions: *Metschnikowia pulcherrima*, *C. famata*, *C. stellata*, *P. membranifaciens*, *Pichia fermentans*, and *Hansenula anomala*. The increase of grape exudate sugar content

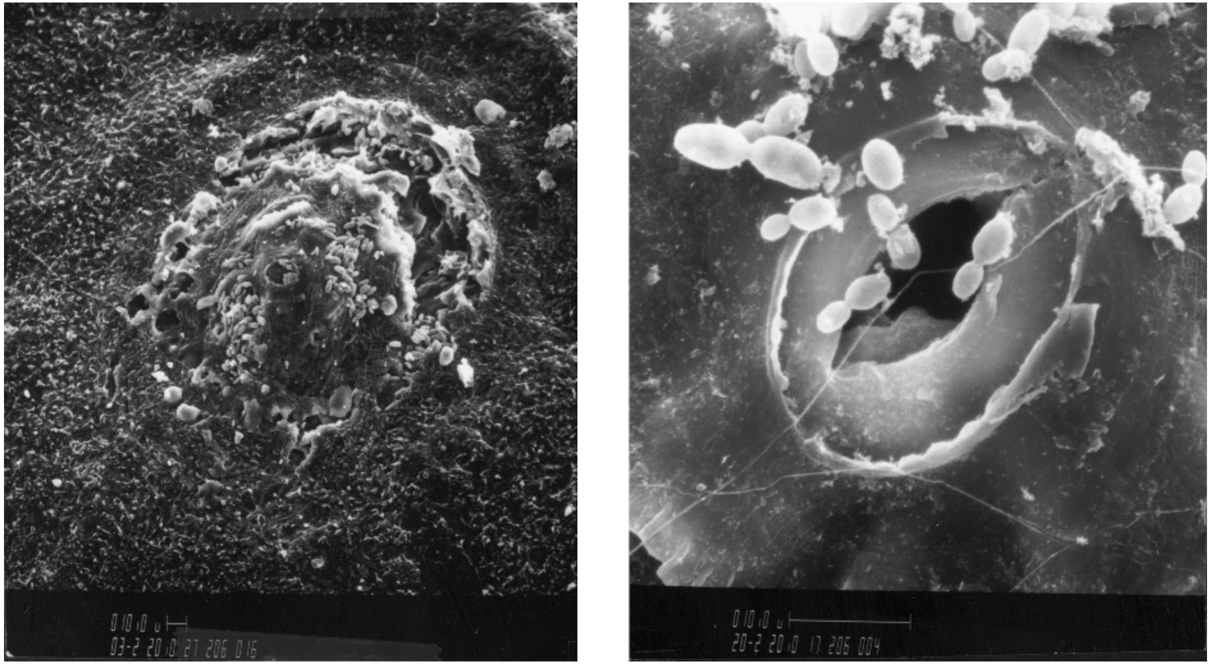


FIGURE 1.34 Grape surface under scanning electron microscope, with detail of yeast peristomatic zones. (***Source***: Photographs from B. Pucheu-Plante and M. Mercier, Department of Electron Microscopy, Université de Bordeaux 1.)

may partly explain these changes in yeast populations (Martins, 2012).

All research confirms the extreme rarity of *S. cerevisiae* on grapes (Mortimer and Polsinelli, 1999). However, these yeasts are not totally absent. Their existence cannot be proven by streaking diluted must on a solid medium prepared under aseptic conditions, but their presence on grapes can be proven by analyzing the spontaneous fermentative microflora of grape samples placed in sterile bags, then aseptically crushed and vinified in the laboratory in the absence of all contamination. Red and white grapes from the Bordeaux region were treated in this manner. At mid-fermentation in the majority of cases, *S. cerevisiae* represented almost all of the yeasts isolated. In some rare cases, no yeast of this species developed and non-*Saccharomyces* yeasts began the fermentation. Nevertheless, growth of *S. cerevisiae* under these conditions from healthy grapes is not guaranteed. Thus, based on 134 samples of grapes collected in the Bordeaux region, 31% of samples were positive for *S. cerevisiae* at the two-thirds mark of alcoholic fermentation (Börlin, 2015). Under the same operating conditions, 28–38% of positive fermentation was obtained for grape samples from the Douro region in Portugal (Schuller *et al.*, 2012). The frequency of isolation of *S. cerevisiae* may approach 100% on damaged grapes, which are a very favorable environment for the development of fermentation yeasts (Mortimer and Polsinelli, 1999).

Ecological surveys carried out at the Bordeaux Faculty of Enology from 1992 to 1999 (Naumov *et al.*, 2000a) demonstrated the presence of *S. uvarum* yeasts on grapes and in spontaneously fermenting white musts from the Loire Valley, Jurançon, and Sauternes. The frequency of the presence of this species alongside *S. cerevisiae* varies from 4 to 100%. On one estate in Alsace, strains of *S. uvarum* were identified on grapes, in the press, and in tanks, where they represented up to 90% of the yeasts involved throughout fermentation in three consecutive years (Demuyter *et al.*, 2004). More recently, other authors (Naumov *et al.*, 2002; Zhang *et al.*, 2015) have shown that *S. uvarum*, identified on grapes and in fermenting must, is involved in making Tokaji and New Zealand wines.

The adaptation of *S. uvarum* to relatively low temperatures (6–10°C) certainly explains its presence in certain ecological niches: northerly vineyards, late harvests, and spontaneous "cold" fermentation of white wines. In contrast, this strain is sensitive to high temperatures and has not been found in spontaneous fermentations of red Bordeaux wines.

Between two harvests, winery walls, floors, equipment, and sometimes even the winery building itself are colonized mostly by the various non-fermentation species previously cited. Winemakers believe, however, that spontaneous fermentations are more difficult to initiate in new tanks than in tanks that have already been used. This empirical observation leads to the supposition that *S. cerevisiae* can also survive in the winery between two harvests. Moreover, this species was found in non-negligible proportions in the wooden fermentors of some of the best vineyards in Bordeaux during the harvest, just before they were filled.

Recent studies relying on global approaches to sequencing of DNA extracted from a biological sample demonstrate the presence of the main yeast species in fermenting must (*H. uvarum*, *S. bacillaris*, and *S. cerevisiae*) in the various zones of winemaking cellars (pressing, fermentation, and storage) before, during, and after the harvest period (Bokulich *et al.*, 2013).

In the first hours of spontaneous fermentations, the first tanks filled have a very similar microflora to that of the grapes, with a large proportion of *H. uvarum*, *S. bacillaris* (formerly known as *Candida zemplinina*), and *M. pulcherrima* species. After about 20 hours, *S. cerevisiae* develops and coexists with the grape yeasts (Zott *et al.*, 2008). Non-*Saccharomyces* yeasts quickly disappear at the start of

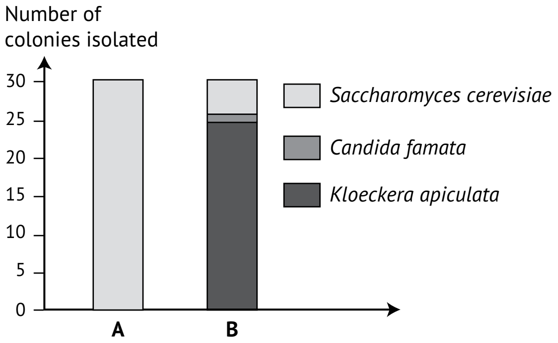


FIGURE 1.35 Comparison of yeast species present at the start of alcoholic fermentation ($d = 1.06$). A, in a tank of sulfited red grapes in Bordeaux (Frezier, 1992); B, in a tank of unsulfited white must, for the production of Cognac (Versavaud, 1994).

spontaneous fermentation (Figure 1.35). The microflora of tanks inoculated with yeast is very similar to that of tanks undergoing spontaneous fermentation (Figure 1.36). The major difference resides in the nature of *S. cerevisiae* strains that conduct fermentation: there is a dominant clone in the case of adding yeast. Meanwhile, in the case of spontaneous fermentation, several clones coexist (Section 1.10.2). Thus, the practice of adding yeast does not eliminate populations of non-*Saccharomyces* yeasts at the start of alcoholic fermentation. In red winemaking in the Bordeaux region, as soon as must specific gravity drops below 1.070–1.060, the colony samples obtained by plating diluted must on a solid medium generally isolate *S. cerevisiae* (10^7–10^8 cells/ml) exclusively. This species plays an essential role in the alcoholic fermentation process. Environmental conditions influence its selection. This selection pressure is exhibited by four main parameters: anaerobic conditions, must or grape sulfiting, sugar concentration, and the increasing presence of ethanol. The increase in temperature, especially in the case of red winemaking, also favors the development of *S. cerevisiae* to the detriment of non-*Saccharomyces* yeasts (Goddard, 2008). In winemaking, where no sulfur dioxide is used, such as white wines for the production of spirits, the dominant grape microflora can still be found. It is largely present at the beginning of alcoholic fermentation (Figure 1.35). Even in this type of winemaking, the presence of apiculate yeasts is limited at the midpoint of alcoholic fermentation.

During dry white winemaking, the separation of the pomace after pressing, combined with clarification by racking, greatly reduces yeast populations, at least in the first few days of the harvest. The yeast population of a severely racked must rarely exceeds 10^4–10^5 cells/ml.

A few days into the harvest, the *S. cerevisiae* yeasts colonize the harvest equipment, grape transport machinery, and especially the grape receiving equipment, the crusher, stemmer, the wine press, and cellar atmosphere (Grangeteau *et al.*, 2015). For this reason, *S. cerevisiae* is already widely present at the time of filling the tanks (around 50% of yeasts isolated during the first homogenization pump-over of a red grape tank). Fermentations are initiated more rapidly as harvest goes on. In fact, the last tanks filled often complete their fermentations before the first ones. Similarly, static racking in dry white winemaking becomes more and more difficult to achieve, even at low temperatures, from the second week of the harvest onward, especially in hot years. The entire facility inoculates the must with a sizeable fermentation yeast population. General weekly disinfection of the pumps, piping,

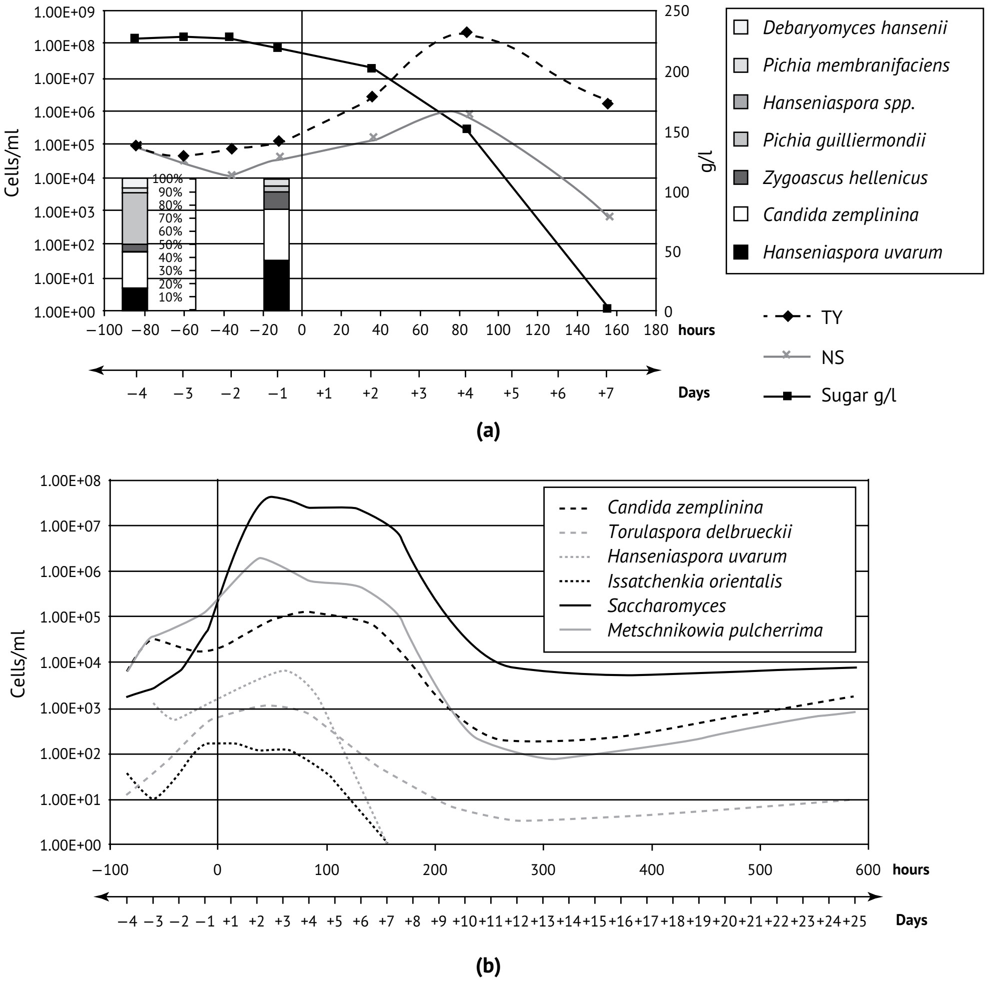


FIGURE 1.36 Dynamics of total yeasts and non-*Saccharomyces* yeasts during red winemaking monitored by (a) culture/RFLP-ITS-PCR and (b) specific quantitative PCR applied on a DNA pellet extracted directly from fresh must. Hour 0/day 0, time of inoculation with commercial yeast; – hours/days, cold soaking; + hours/days, alcoholic fermentation (Zott *et al.*, 2010).

wine presses, settling tanks, etc., is therefore strongly recommended.

During the final part of alcoholic fermentation (the yeast decline phase), the population of *S. cerevisiae* progressively decreases while still remaining greater than 10^6 cells/ml. Under favorable winemaking conditions, characterized by a rapid and complete exhaustion of sugars, no other yeast species significantly appears at the end of fermentation. Under poor conditions, spoilage yeasts can contaminate the wine. One of the most frequent and most dangerous contaminations is due to the development of *B. bruxellensis*, which is responsible for serious off-odors (Volume 2, Section 8.4.5).

In the weeks that follow the completion of alcoholic fermentation, the viable populations of *S. cerevisiae* drop rapidly, falling below a few hundred cells/ml. In many cases, other yeast species (spoilage yeasts) can develop in wines during bulk or bottle aging. Some yeasts have an oxidative metabolism of ethanol and form a veil on the surface of the wine, such as *Pichia* or *Candida*, or even certain strains of *S. cerevisiae*—sought after in the production of specialty wines. By topping up regularly, the development of these respiratory metabolism yeasts can be prevented. Some other yeasts, such as *Brettanomyces* or *Dekkera*, can develop under anaerobic conditions, consuming trace amounts of sugars that have been incompletely or not fermented by *S. cerevisiae*. Their population can attain 10^4–10^5 cells/ml in a contaminated red wine in which alcoholic fermentation is otherwise completed normally. These contaminations can also occur in the bottle. Lastly, refermentation yeasts can develop significantly in sweet or botrytized sweet wines during aging or bottle storage. The principal species found are *S. ludwigii*, *Z. bailii*, and also some strains of *S. cerevisiae* that are particularly resistant to ethanol and sulfur dioxide.

1.10.2 The Ecology of *S. cerevisiae* Strains

The ecological study of the clonal diversity of yeasts, and in particular of *S. cerevisiae* during winemaking, was inconceivable for a long time because of a lack of means to distinguish yeast strains from one another. Such research has become possible with the development of molecular yeast strain identification methods (Section 1.9). This section focuses on recent advances in this field.

The alcoholic fermentation of grape must or grapes is essentially carried out by a single yeast species, *S. cerevisiae*. Therefore, an understanding of the clonal diversity within this species is much more important for the winemaker than investigations on the partially or non-fermentative grape microflora.

The analysis of *S. cerevisiae* strains under practical winemaking conditions in particular is intended to answer the following questions:

- Is spontaneous fermentation carried out by a dominant strain, a small number or a very large number of strains?
- Can the existence of a succession of strains during alcoholic fermentation be proven? If so, what is their origin: grapes, harvest equipment, or winery equipment?
- During winemaking and from one year to another in the same winery or even the same vineyard, is spontaneous alcoholic fermentation carried out by the same strains?
- Can the practice of inoculating with selected strains modify the wild microflora of a vineyard?

During recent research conducted in the Bordeaux region (Dubourdieu and Frezier, 1990; Frezier, 1992; Masneuf, 1996), many samples of yeast microflora were taken in the vineyard and the winery from batches of white and red wines spontaneously fermenting or inoculated with ADYs. Several conclusions can be drawn from this research, carried out on several thousand wild strains of *S. cerevisiae*.

In the majority of cases, a small number of major strains (one to three) representing up to 70–80% of the colonies isolated, carry out the spontaneous fermentations of red and dry white wines. These dominant strains are found in comparable proportions in all of the fermentation tanks from the same winery from the start to end of alcoholic fermentation. This phenomenon is illustrated by the example given in Figure 1.37, describing the native microflora of several tanks of red must from a Pessac-Léognan vineyard in 1989. The strains of *S. cerevisiae*, possessing different karyotypes, are identified by an alphanumeric code comprising the initial of the vineyard, the tank number, the time of the sampling, the isolated colony number, and the year of the sample. Two strains, Fzlb1 (1989) and Fzlb2 (1989), are encountered in all of the tanks throughout the entire alcoholic fermentation process.

The spontaneous fermentation of dry white wines from the same vineyard is also carried out by the same dominant yeast strains in all of the barrels.

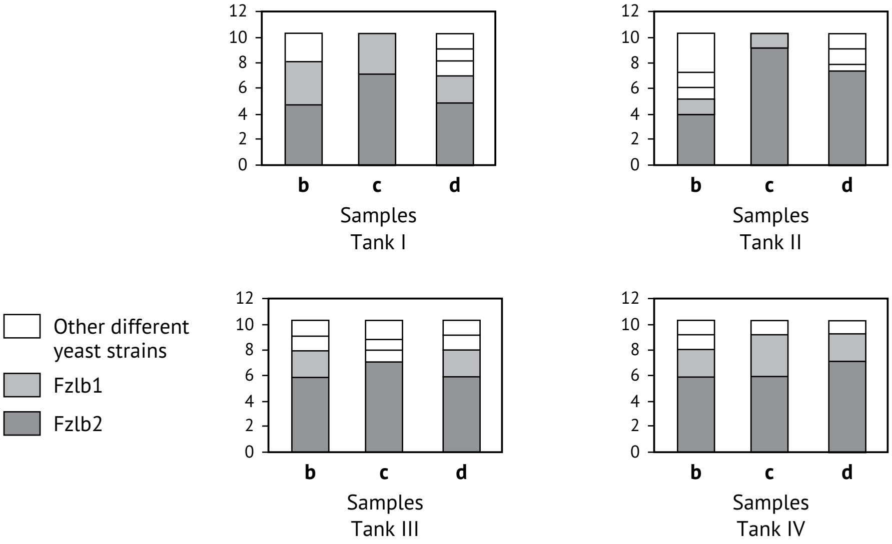


FIGURE 1.37 Breakdown of *S. cerevisiae* karyotypes during alcoholic fermentation in red grape tanks in the Fz vineyard (Pessac-Léognan, France) in 1989 (Frezier, 1992). b, c, and d designate the start, middle, and end of alcoholic fermentation, respectively. Tanks I and II (Merlot) and III and IV (Cabernet Sauvignon) were filled on the 1st, 3rd, 17th, and 23rd day of the harvest, respectively.

The tank filling order and the grape variety have little effect on the clonal composition of the populations of *S. cerevisiae* spontaneously found in the winery. The daily practice of pumping over the red grape must, with pumping equipment used for all of the tanks, and the contact of must with the atmosphere in the cellar enriched with *S. cerevisiae* populations probably ensure the dissemination of the same strains in the winery. In white winemaking, the wine press facility plays the same role as an inoculator.

The same major strain is frequently encountered for several consecutive vintages in the same vineyard in spontaneously fermenting red must tanks. In 1990, one of the major strains was the same as the previous year in the red must tanks of the Fz vineyard. Other strains appeared, however, which had not been isolated in 1989.

When sterile grape samples are taken, pressed sterilely, sulfited at winemaking levels, and fermented in the laboratory in sterile containers, one or several dominant strains responsible for spontaneous fermentations in the winery exist in some samples. These strains are therefore present in the vineyard. In practice, they probably begin to multiply as soon as the grapes arrive at the winery. A few days into the harvest, they infest the winery equipment, which in turn ensures a systematic inoculation of the fresh grape crop.

The presence each year of the same dominant strain in the vineyard is not systematic (Table 1.8). In the Fz vineyard, the Fzlb2-89 strain could not be isolated in 1991, although it was present in certain vineyard samples in 1990, 1992, and 1994. In 1993, another strain proved to be dominant in spontaneous fermentations of sterile grape samples.

The spontaneous microflora of *S. cerevisiae* seems to fluctuate. At present, the factors involved in this fluctuation have not been identified. In a given vineyard, spontaneous fermentation is not systematically carried out by the same strains each year, and there is thus no specific strain that is one of the vineyard's characteristics. Ecological observations do not confirm the notion of a vineyard-specific yeast. Furthermore, some native strains, dominant in a given vineyard, have been found in other nearby or distant vineyards. For example,

TABLE 1.8

Rate of Occurrence of the Dominant Fzlb2-89 Karyotype in Microvinifications Carried Out on Sterile Grape Samples (I, II, and III) in the Fz Vineyard (The Number of Clones Analyzed is Indicated in Parentheses)

	1990 (30 clones)	1991 (60 clones)	1992 (85 clones)	1993 (74 clones)	1994 (79 clones)
Sample 1	—	—	25%	—	87%
Sample 2	70%	—	31%	—	—
Sample 3	—	—	3%	—	40%

the Fzlb2-89 strain, isolated for the first time in a vineyard in Pessac-Léognan, was later identified not only in the spontaneous fermentation of dry white and red wines of other vineyards in the same appellation but also in relatively distant wineries as far away as the Médoc. This strain has since been selected and commercialized under the name Zymaflore F10.

In some cases (Figure 1.38), *S. cerevisiae* populations with a large clonal diversity carry out spontaneous must fermentation. Many strains coexist. Their proportions differ from the start to the end of fermentation and from one tank to another. Is it more favorable for the sensory characteristics of wine to have a fermentation with low or large clonal diversity? Few scientific works have addressed this question until now. Howell *et al.* (2006) showed that analytical profiles of wines obtained from mixed fermentations are different from those obtained with yeast monocultures. These cannot be reproduced by blending different monoculture wines. Evidently, metabolic interactions explain the differences observed between mixed fermentation and blending of monocultures. The contribution of a yeast clone to the aroma profile of a wine resulting from polyclonal fermentation cannot simply be predicted by this clone's population. Thus, the direct relationship between the diversity of yeast strains and wine complexity has not received until now any scientific demonstration and must be considered with care. In the Bordeaux region, the coexistence of a large number of yeast strains is often associated with slow fermentations, and sometimes even stuck fermentations before full depletion of sugars. No single strain seems to be capable of asserting itself. On the other hand, the presence of a small number of dominant strains generally characterizes complete and rapid spontaneous fermentations. These dominant strains are found from the start to the end of fermentation.

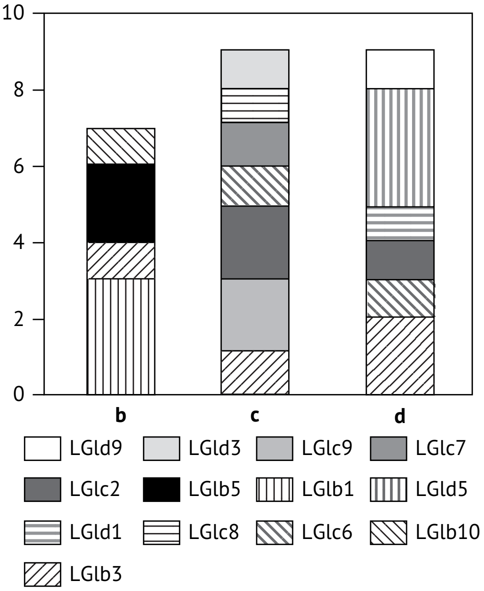


FIGURE 1.38 Breakdown of *S. cerevisiae* karyotypes in tank I of red grapes from the LG vineyard (Pomerol, France) in 1989 (Frezier, 1992). b, c, and d designate the start, middle, and end of alcoholic fermentation, respectively.

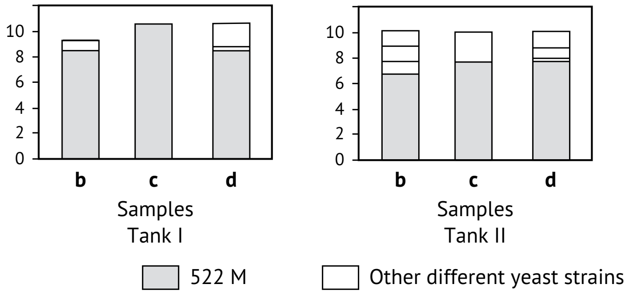


FIGURE 1.39 Breakdown of karyotypes for 10 strains analyzed in tank I and tank II from vineyard (P) in 1990. Tank I was inoculated with 522M dry yeast and tank II underwent spontaneous fermentation. b, c, d: start, middle, and end of alcoholic fermentation, respectively.

Under normal red winemaking conditions, the inoculation of the first tanks in a winery influences the wild microflora of non-inoculated tanks. The strain(s) used for inoculating the first tanks are frequently found as the predominant strain(s) in the latter. Figure 1.39 provides an example comparing the microflora of a tank of Merlot from Pomerol, inoculated with an ADY strain (522M) on the first day of the harvest, with a non-inoculated tank filled later. From the start of alcoholic fermentation, the selected strain is successfully established in the inoculated tank. Even in the non-inoculated tank, the same strain is equally established throughout the fermentation. It is therefore difficult to select dominant wild strains in red winemaking tanks when some of the tanks have been inoculated. An early and massive inoculation of the must, however, enables the successful establishment of different selected yeasts in several tanks at the same winery (Figure 1.40).

In white winemaking, inoculating rarely influences the microflora of spontaneous fermentations in wineries. For the most part, dominant native strains in non-inoculated barrels of fermenting dry white wine are observed, even though in the same wine cellar, other batches have been inoculated with different selected yeasts. The absence of pump-overs probably hinders the dissemination of the same yeasts in all of the fermenting barrels. This situation means the fermentative behavior

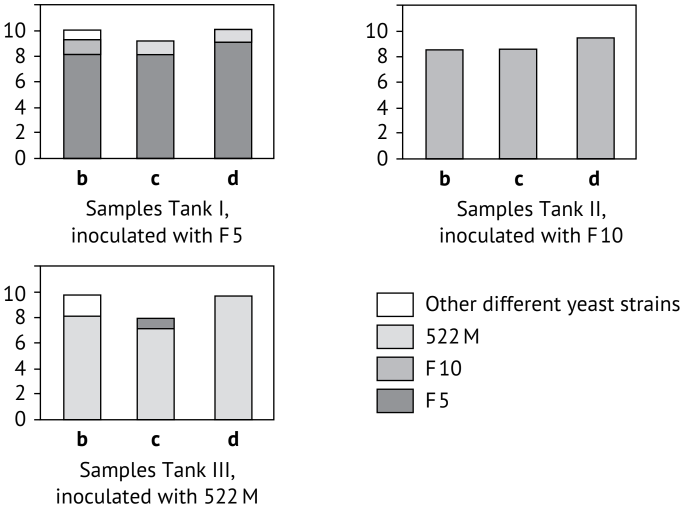


FIGURE 1.40 Breakdown of karyotypes of 10 strains analyzed in tanks I, II, and III from vineyard (F) in 1990, with massive early inoculation with F5, F10, and 522M, respectively (Frezier, 1992). b, c, and d: see Figure 1.39.

and enological interest of different selected strains can be easily compared with each other and with native strains from a given vineyard. The barrels are filled with the same must; some are inoculated with the yeast to be compared. A sample of the biomass is taken at mid-fermentation. The desired implantation is then verified by PCR combined with δ sequences. Due to the ease of use of this method, information on the characteristics of selected strains and their influence on wine quality can be gathered at the winery.

Vezhinet *et al.* (1992) and Versavaud *et al.* (1995) have also studied the clonal diversity of yeast microflora in other wine regions. Their results confirm the polyclonal character of fermentative populations of *S. cerevisiae*. The notion of dominant strains (one to two per fermentation) is obvious in the work carried out in the Charentes region. As in Champagne and the Loire Valley, some Charentes strains are found for several years in a row in the same winery. The presence of these dominant strains on the grape has been confirmed before any contact with winery equipment during several harvests.

Why do some *S. cerevisiae* strains coming from a very heterogeneous population become dominant during spontaneous fermentation? Why can they be found two to three years in a row in the same vineyard and wine cellar? Despite their practical interest, these questions have not often been studied, and there are no definitive responses. It seems that these strains rapidly start and complete alcoholic fermentation and have good resistance to sulfur dioxide (up to 10 g/hl). Furthermore, during mixed inoculations in the laboratory of either non-fermented musts or partially fermented musts (8% ethanol by vol.), these strains rapidly become dominant when placed in the presence of other wild non-dominant strains of *S. cerevisiae* isolated at the start and end of fermentation. This subject merits further research.

Studies conducted in the Bordeaux region were recently revisited by using the analysis of microsatellite markers, in order to integrate genetic approaches to populations in the genetic diversity results for the strains (Börlin, 2015). Results confirm the very large genetic diversity of *S. cerevisiae* strains in the vineyard and in spontaneous fermentation in the Bordeaux region. Of 1,374 isolates from grapes and 1,078 isolates from fermenting must, 75% have different genotypes. During spontaneous fermentation, the presence of yeasts related to commercial yeasts, i.e. that share at least 75% of genetic markers, is low (7%). This result, which mainly concerns organic wine cellars, merits confirmation in conventional cellars. However, a higher percentage (25%) of strains related to industrial yeast is obtained for the population of *S. cerevisiae* isolated from grapes in the vineyard. Therefore, this result highlights the importance, underestimated until now, of cellar yeasts being returned to the vineyard environment. Prior work showed that the detection of strains in the vineyard was possible in a radius of 10–200 m around the cellar where they were used (Valero *et al.*, 2007; Schuller *et al.*, 2007). Our data show a dissemination of winery yeasts in the vineyard over a maximum distance of 400 m from the cellar.

In Bordeaux vineyards, structural analyses of *S. cerevisiae* yeast populations on grapes have highlighted a significant genetic difference of these populations depending on the appellation. At the estate scale, while the populations involved in spontaneous fermentations are not very distinct over two to three consecutive years, they are clearly different over a longer period of time (>20 years). This confirms that in a given vineyard, populations of yeast fluctuate over time (Börlin *et al.*, 2016). Moreover, there are clearly exchanges of populations between neighboring vineyards. This is probably due to the fact that yeast can disseminate in the environment through various vectors (insects, birds, and humans) to arrive in neighboring parcels of land.

References

Aigle M., Erbs D. and Moll M. (1984) Am. Soc. Brew. Chem., 42, 1, 1.

Albertin W., Chasseriaud L., Comte G., Panfili A., Delcamp A., Salin F., Marullo P. and Bely M. (2014a) PLoS One, 9, 4, e94246.

Albertin W., Panfili A., Miot-Sertier C., Goulielmakis A., Delcamp A., Salin F., Lonvaud-Funel A., Curtin C. and Masneuf-Pomarede I. (2014b) Food Microbiol., 42, 188.

Albertin W., Setati M.E., Miot-Sertier C., Mostert T.T., Colonna-Ceccaldi B., Coulon J., Girard P., Moine V., Pillet M., Salin F., Bely M., Divol B. and Masneuf-Pomarede I. (2016) Front. Microbiol., 20, 6, 1569.

Alexandre E.H., Rousseaux I. and Charpentier C. (1994) Biotechnol. Appl. Biochem., 19.

Arroyo-López F.N., Orlié S., Querol A. and Barrio E. (2009) Int. J. Food Microbiol., 31, 663.

Augustyn O.P.H., Kock J.L.F. and Ferreira D. (1991) Syst. Appl. Microbiol., 15, 105–115.

Ayoub M.J., Legras J.L., Saliba R. and Gaillardin C. (2006) J. Appl. Microbiol., 100, 699.

Baleiras Couto M.M., Reizinho R.G. and Duarte F.L. (2005) Int. J. Microbiol., 102, 49.

Barnett J.A., Delaney M.A., Jones E., Magson A.B. and Winch B. (1972) Arch. Microbiol., 83, 52–55.

Barnett J.A., Payne R.W. and Yarrow D. (1990) *Yeast: Characteristics and Identification*, 2nd edition. Cambridge University Press, Cambridge.

Barnett J.A., Payne R.W. and Yarrow D. (2000) *Yeasts: Characteristics and Identification*, 3rd edition. Cambridge University Press, Cambridge.

Barre P. (1978) *Killer factor activity under vinification conditions*, *Vth International Symposium on Yeasts*, Montpellier.

Barre P. (1984) Bull. OIV, 57, 635–643.

Barre P. (1992) Le facteur killer. In *Les acquisitions récentes de la microbiologie du vin*, pp. 63–69 (Ed B. Donèche). Tec & Doc, Lavoisier, Paris.

Belda I., Ruiz J., Alonso A., Marquina D. and Santos A. (2017) Toxins, 23, 9.

Belin J.M. (1979) Mycopathologia, 67, 2, 67–81.

Belin J.M. (1981) *Biologie des levures liées à la vigne et au vin*, Thèse Docteur ès Sciences Naturelles, Université de Dijon.

Bendova O., Richter V., Janderova B. and Haüsler J. (1991) Appl. Microbiol. Biotechnol., 35, 810–812.

Bisson L.F. (1991) Yeasts–metabolism of sugars. In *Wine Microbiology and Biotechnology*, pp. 55–75 (Ed G.H. Fleet). Harwood Academic Publishers, Chur.

Blondin B. and Vezhinet F. (1988) Rev. Fr. Oenol., 115, 7.

Bokulich N.A., Ohta M., Richardson P.M. and Mills DA. (2013) PLoS One, 8, 6, e66437.

Börlin M. (2015) *Diversité et structure de population des levures Saccharomyces cerevisiae à l'échelle du vignoble bordelais: Impact de différents facteurs sur la diversité*, Thèse de Doctorat, Université de Bordeaux 2.

Börlin M., Venet P., Claisse O., Salin F., Legras J.L. and Masneuf-Pomarede I. (2016) Appl. Environ. Microbiol., 82, 2909–2918.

Bouix M., Leveau J.Y. and Cuinier C. (1981) Œno One, 15, 41–52.

Boulton R.B., Singleton V.L., Bisson L.F. and Kunkee R. (1995) *Principles and Practices of Winemaking*. Chapman & Hall Enology Library, New York.

Bowers J., Boursiquot J.M., This P., Chu K., Johansson H. and Meredith C. (1999) Science, 285, 1562–1565.

Brechot P., Chauvet J. and Girard H. (1962) Ann. Technol. Agric., 11, 3, 235–244.

Bureau G., Brun O., Vigues A., Maujean A., Vesselle G. and Feuillat A. (1982) Œno One, 16, 1, 15.

Busturia A. and Lagunas R. (1986) J. Gen. Microbiol., 132, 379.

Carle G.F. and Olson M.V. (1984) Nucleic Acids Res., 12, 5647–5664.

Castelli T. (1955) Am. J. Enol. Vitic., 6, 18–20.

Castelli T. (1967) *Ecologie et systématique des levures du vin*, *2éme Symposium International d'Enologie*, Bordeaux-Cognac, INRA, Paris.

Charpentier C. (1994) Rev. Oenol., 73S, 25–28.

Ciolfi G. (1992) L'enotechnico, 11, 87.

Ciolfi G. (1994) L'enotechnico, 71.

Cordero-Bueso G., Arroyo T., Serrano A. and Valero E. (2011) FEMS Microbiol. Ecol., 77, 429.

Cromie G.A., Hyma K.E., Ludlow C.L., Garmendia-Torres C., Gilbert T.L., May P., Huang A.A., Dudley A.M. and Fay J.C. (2013) G3 (Bethesda), 3, 12, 2163–2171.

Cuinier C. and Gros C. (1983) Vigne Vin, 318, 25–27.

Cuinier C. and Guerineau L. (1976) Vigne Vin, 269, 29–41.

Cuinier C. and Leveau J.Y. (1979) Vigne Vin, 283, 44–76.

Da Silva T., Albertin W., Dillmann C., Bely M., la Guerche S., Giraud C., Huet S., Sicard D., Masneuf-Pomarede I., de Vienne D. and Marullo P. (2015) PLoS One, 10, 5, e0123834.

De Rossi G. (1935) *Il lieviti della fermentazione nella regione umbra*, *IVème Congrès International de la Vigne et du Vin*, Lausanne.

Defontaine A., Lecocq F.M. and Hallet J.N. (1991) Nucleic Acids Res., 19, 1, 185.

Degre R., Thomas D.Y., Frenette J. and Mailhot K. (1989) Rev. Fr. Oenol., 119, 23–26.

Delfini C. (1995) *Scienza e technica di microbiologia enologica*. Il Lievito, Asti.

Demuyter C., Lollier M., Legras J.L. and Le Jeune C. (2004) J. Appl. Microbiol., 97, 6, 1140–1148.

Domercq S. (1956) *Etude et classification des levures de vin de la Gironde*, Thèse de Docteur-Ingénieur de l'Université de Bordeaux.

Dubourdieu D. and Frezier V. (1990) Rev. Fr. Oenol., 30, 37–40.

Dubourdieu D. and Moine V. (1995) *Actualités oenologiques 95*, *Compte-rendus du 5ème Symposium International d'Enologie de Bordeaux*, Bordeaux.

Dubourdieu D., Sokol A., Zucca J., Thalouarn P., Datee A. and Aigle M. (1987) Œno One, 4, 267.

Erny C., Raoult P., Alais A., Butterlin G., Delobel P., Matei-Radoi F., Casaregola S. and Legras J.L. (2012) Appl. Environ. Microbiol., 78, 3256.

Fay J.C. and Benavides J.A. (2005) PLoS Genet., 1, 1.

Fell J.W., Boekhout T., Fonseca A., Scorzetti G. and Statzell-Tallman A. (2000) Int. J. Syst. Evol. Microbiol., 50, 3, 1351–1371.

Field D. and Wills C. (1998) Proc. Natl. Acad. Sci., 95, 1647.

Fleet G.H. (1991) Cell walls. In *The Yeasts, Vol. 4: Yeast Organelles*, p. 199 (Eds A.H. Rose and J.S. Harrison). Academic Press, London.

Fleet G.H. (1993) *Wine Microbiology and Biotechnology*. Harwood Academic Publishers, Chur.

Fleet G.H., Lafon-Lafourcade S. and Ribereau-Gayon P. (1984) Appl. Environ. Microbiol., 48, 1034–1652.

Flick J.S. and Thorner J. (1993) Mol. Cell. Biol. 13, 5861–5876.

Frezier V. (1992) *Recherche sur l'écologie des souches de Saccharomyces cerevisiae au cours des vinifications bordelaises*. Thèse de Doctorat de l'Université de Bordeaux II.

Frezier V. and Dubourdieu D. (1992) Am. J. Enol. Vitic. 43, 375–380.

Gaillardin C. and Heslot H. (1987) La Recherche, 188, 18, 586.

Goddard M.R. (2008) Ecology, 89, 2077.

Goffeau A., Barrell B.G., Bussey H., Davis R.W., Dujon B., Feldmann H., Galibert F., Hoheisel J.D., Jacq C., Johnston M., Louis E.J., Mewes H.W., Murakami Y., Philippsen P. and Tettelin H. (1996) Science, 274, 563–567.

Gonzalez Techera A., Jubany S., Carrau F.M. and Gaggero C. (2001) Lett. Appl. Microbiol., 33, 71.

Granchi L., Bosco M., Messini A. and Vincenzini M. (1999) J. Appl. Microbiol., 87, 949.

Grangeteau C., Gerhards D., Rousseaux S., von Wallbrunn C., Alexandre H. and Guilloux-Benatier M. (2015) Food Microbiol., 50, 70.

Guerin B. (1991) Mitochondria. In *The Yeasts, Vol. 4: Yeast Organelles*, p. 541 (Eds A.H. Rose and J.S. Harrison). Academic Press, London.

Hallet J.N., Craneguy B., Zucca J. and Poulard A. (1988) Prog. Agric. Vitic., 105, 328.

Hallet J.N., Craneguy B., Daniel P. and Poulard A. (1989) *Actualités oenologiques 89*, *Comptes rendus du 4ème Symposium d'oenologie de Bordeaux*, Dunod.

Hansen J. and Kielland-Brandt M.C. (1994) Gene, 140, 33–40.

Heard G.M. and Fleet G.H. (1987) Appl. Environ. Microbiol. 51, 539–45.

Heard G.M. and Fleet G. (1990) J. Appl. Bacteriol., 68, 445.

Hennequin C.A., Thierry G.F., Richard G., Lecointre H., Nguyen V., Gaillardin C. and Dujon B. (2001) J. Clin. Microbiol., 39, 551.

Henschke P.A., and Rose A.H. (1991) Plasma membrane. In *The Yeasts, Vol. 4: Yeast Organelles*, p. 297 (Eds A.H. Rose and J.S. Harrison). Academic Press, London.

Herskowitz I., Rine J. and Strathern J.N. (1992) Mating-type determination and mating-type interconversion in *Saccharomyces cerevisiae*. In *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression*, p. 583 (Eds E.W. Jones, J.R. Pringle, and J.R. Broach). Cold Spring Harbor Laboratory Press, New York.

Hierro N., Esteve-zarzozo B., Mas A. and Guillamon J.M. (2006) FEMS Yeast Res., 6, 102.

Howell K.S., Cozzolino D., Bartowsky E., Fleet G.H. and Henschke P.A. (2006) FEMS Yeast Res., 6, 91.

Hyma K.E. and Fay J.C. (2013) Mol. Ecol., 22, 2917.

Klis F.M. (1994) Yeast, 10, 851.

Klis F.M., Moll P., Hellingwerf K. and Brul S. (2002) FEMS Microbiol. Rev., 26, 3, 239–256.

Kregger-Van Rij N.J.W. (1984) *The Yeasts, a Taxonomic Study*. Elsevier, Amsterdam.

Kurtzman C.P. and Robnett C.J. (1998) Antoine Van Leeuwenhoek, 73, 4, 331.

Kurtzman C., Fell J.W. and Boekhout T. (Eds) (1994) *The Yeasts: A Taxonomic Study*, Vol. 1, 5th edition, 2354 pp. Elsevier, Amsterdam.

Kurtzman C.P., Fell J.W. and Boekhout T. (2011) *The Yeasts, a Taxonomic Study*, 5th edition, Elsevier: Amsterdam.

Lafon-Lafourcade S. (1983) Wine and brandy. In *Biotechnology Vol. V: Food and Feed Production with Microorganisms*, pp. 100–109 (Eds H.J. Rehm and G. Reed). Verlag Chemie, Weinheim.

Lafon-Lafourcade S. and Joyeux A. (1979) Œno One, 4, 295.

Lavallée F., Salves S., Lamy S., Thomas D.Y., Degre R. and Dulau L. (1994) Am. J. Enol. Vitic., 45, 86.

Le Jeune C., Lollier M., Demuyter C., Erny C., Legras J.L., Aigle M. and Masneuf-Pomarède I. (2007) FEMS Yeast Res., 7, 4, 540–549.

Legras J.L. and Karst F. (2003) FEMS Microbiol. Lett., 221, 2, 249–255.

Legras J.L., Ruh O., Merdinoglu D. and Karst F. (2005) Int. J. Food Microbiol., 102, 1, 73–83.

Libkind D., Hittinger C.T., Valério E., Gonçalves C., Dover J., Johnston M., Gonçalves P. and Sampaio J.P. (2011) Proc. Natl. Acad. Sci. U. S. A., 108, 35, 14539–14544.

Lodder J. (1970) *The Yeasts, a Taxonomic Study*. Elsevier, Amsterdam.

Lodder J. and Kregger-Van Rij N.J.W. (1952) *The Yeasts, a Taxonomic Study*. Elsevier, Amsterdam.

Martins, G.M. (2012) *Communautés microbiennes de la baie de raisin: Incidence des facteurs biotiques et abiotiques*, Thèse de Doctorat, Université de Bordeaux 2.

Martins G., Vallance J., Mercier A., Albertin W., Stamatopoulos P., Rey P., Lonvaud A. and Masneuf-Pomarède I. (2014) Int. J. Food Microbiol., 2, 177, 21–28.

Masneuf I. (1996) *Recherches sur l'identification génétique des levures de vinification. Applications oenologiques*, Thèse de Doctorat de l'Université de Bordeaux II.

Masneuf I. and Dubourdieu D. (1994) Œno One, 28, 2, 153.

Masneuf I., Aigle M. and Dubourdieu D. (1996a) FEMS Microbiol. Lett., 138, pp. 239–244.

Masneuf I., Aigle M. and Dubourdieu D. (1996b) Œno One, 30, 1, 15.

Masneuf I., Hansen J., Groth C., Piskpur J. and Dubourdieu D. (1998) Applied. Environ. Microbiol., 64, 3887.

Masneuf-Pomarède I., Le Jeune C., Durrens P., Lollier M., Aigle M. and Dubourdieu D. (2007) Syst. Appl. Microbiol., 30, 1, 75–82.

Masneuf-Pomarede I., Juquin E., Miot-Sertier C., Renault P., Laizet Y., Salin F., Alexandre H., Capozzi V., Cocolin L., Colonna-Ceccaldi B., Englezos V., Girard P., Gonzalez B., Lucas P., Mas A., Nisiotou A., Sipiczki M., Spano G., Tassou C., Bely M. and Albertin W. (2015) FEMS Yeast Res., 15, 5.

Masneuf-Pomarede I., Salin F., Börlin M., Coton E., Jeune C.L. and Legras J.L. (2016) FEMS Yeast Res., 16, 2, fow002.

Mehlomakulu N.N., Prior K.J., Setati M.E. and Divol B. (2017) J. Appl. Microbiol., 122, 747–758.

Mills D.A., Johansen E.A. and Cocolin L. (2002) Applied Env. Microbiol., 68, 4884.

Minarik E. (1971) Œno One, 2, 185–198.

Mortimer R.K. and Polsinelli M. (1999) Res. Microbiol., 150, 199.

Mortimer R.K., Romano P., Suzzi G. and Polsinelli M. (1994) Yeast, 10, 1543.

Naumov G., Naumova E. and Gaillardin C. (1993) Syst. Appl. Microbiol., 16, 274.

Naumov G.I., Naumova E.S. and Sniegowski P.D. (1998) Can. J. Microbiol., 44, 1045.

Naumov G.I., Masneuf I., Naumova E.S., Aigle M. and Dubourdieu D. (2000a) Res. Microbiol., 151, 683.

Naumov G.I., Naumova E.S., Masneuf I., Aigle M., Kondratieva V.I. and Dubourdieu D. (2000b) Syst. Appl. Microbiol., 23, 442.

Naumov G.I., Naumova E.S., Antunovics Z. and Sipiczki M. (2002) Appl. Microbiol. Biotechnol., 59, 6, 727.

Naumova E.S., Naumov G.I., Barrio E. and Querol A. (2010) Mikrobioligiia, 79, 543.

Ness F., Lavallee F., Dubourdieu D., Aigle M. and Dulau L. (1992) J. Sci. Food Agric., 62, 89.

Nguyen H.V. and Boekhout T. (2017) FEMS Yeast Res., 17, 2. Doi: 10.1093/femsyr/fox014.

Nguyen H.V. and Gaillardin C. (2005) FEMS Yeast Res., 5, 471.

Nguyen H.V., Lepingle A. and Gaillardin C.A. (2000) Syst. Appl. Microbiol., 23, 1, 71.

Nisiotou A.A. and Nychas G.J.E. (2007) Applied Env. Microbiol., 73, 6705.

Novo M., Bigey F., Beyne E., Galeote V., Gavory F., Mallet S., Cambon B., Legras J.L., Wincker P., Casaregola S. and Dequin S. (2009) Proc. Natl. Acad. Sci. U. S. A., 106, 38, 16333–16338.

Park Y.H. (1975) Œno One, 3, 253.

Pasteur L. (1866) *Etudes sur le vin*. Imprimerie Impériale, Paris.

Pasteur L. (1876) *Etudes sur la bière*. Gauthier-Villars, Paris.

Pedersen M.B. (1986) Carlsb. Res. Commun., 51, 163.

Perez M.A., Gallego F.J., Martinez I. and Hidalgo P. (2001) Lett. Appl. Microbiol., 33, 461.

Petering J., Langridge P. and Henschke P. (1988) Aust. N. Z. Wine Ind. J., 3, 48.

Peynaud E. and Domercq S. (1953) Ann. Technol. Agric., 4, 265.

Poulard A. and Lecocq M. (1981) Rev. Fr. Oenol., 82, 31.

Poulard A., Simon L. and Cuinier C. (1980) Œno One, 14, 219.

Prakitchaiwattana C.J., Fleet G.H. and Heard G.M. (2004) FEMS Yeast Res., 4, 8, 865.

Querol A., Barrio E. and Ramon D. (1992) Syst. Appl. Microbiol., 15, 439.

Radler F. (1988) *Application à l'oenologie des progrès récents en microbiologie et en fermentation*, p. 273. Office International de la Vigne et du Vin, Paris.

Redzepovic S., Orlic S., Sikora S., Madjak A. and Pretorius I.S. (2002) Lett. Appl. Microbiol., 35, 305.

Ribéreau-Gayon J. and Peynaud E. (1960) *Traité d'oenologie*, 2nd edition. Librairie Polytechnique Ch. Béranger, Paris.

Ribéreau-Gayon J., Peynaud E., Ribéreau-Gayon P. and Sudraud P. (1975) *Traité d'oenologie. Sciences et techniques du vin*, Vol. 2. Dunod, Paris.

Richardson S.M., Mitchell L.A., Stracquadanio G., Yang K., Dymond J.S., DiCarlo J.E., Lee D., Cheng L., Huang C.L.V., Chandrasegaran S., Cai Y., Boeke J.D., Joel S. and Bader J.S. (2017) Science, 355, 1040.

Rodríguez-Cousiño N., Maqueda M., Ambrona J., Zamora E., Esteban R. and Ramírez M. (2011) Appl. Environ. Microbiol., 77, 1822.

Rose A.H. and Harrison J.S. (1991) *The Yeasts, Vol. 4: Yeasts Organelles*. Academic Press, London.

Rossini G., Federici F. and Martini A. (1982) Microb. Ecol., 8, 83.

Rozes N., Garcia-Jares C., Larue F. and Lonvaud-Funel A. (1992) J. Sci. Food Agric., 59, 351.

Saiki R., Sharf S., Falcona F., Mullis K., Horn G., Erlich H. and Arnheim N. (1985) Science, 230, 1350.

Salmon J.M. (1989) Appl. Environ. Microbiol., 55, 953.

Salmon J.M., Vincent O., Mauricio J.C. and Bely M. (1993) Am. J. Enol. Vitic., 44, 1, 56.

Sapis-Domercq S. (1970) Œno One, 4, 45.

Schuller D., Pereira L., Alves H., Cambon B., Dequin S. and Casal M. (2007) Yeast, 24, 625.

Schuller D., Cardoso F., Sousa S., Gomes P., Gomes A.C., Santos M.A. and Casal M. (2012) PLoS One, 7, 2, e32507.

Schwartz D.C. and Cantor C.R. (1984) Cell, 37, 67.

Singer S.J. and Nicolson G.L. (1972) Science, 175, 720–731.

Sipiczki M. (2008) FEMS Yeast Res., 8, 996.

Soufleros E. (1978) *Les levures de la région viticole de Naoussa, Grèce, Identification et classification. Etude des produits volatils formés au cours de la fermentation*, Thèse Docteur-Ingénieur Université de Bordeaux II.

Southern E. (1975) J. Biol. Chem., 98, 503.

Stratford M. (1994) Yeast, 10, 1741.

Tipper D.J. and Bostian K.A. (1984) Microbiol. Rev. 48, 2, 125.

Tredoux H.G., Kock J.L.F., Lategan P.L. and Muller H.B. (1987) Am. J. Enol. Vitic., 38, 161.

Tuite M.F. and Oliver S.G. (1991) *Saccharomyces Biotechnology Handbooks*. Plenum Press, New York, London.

Valero E., Cambon B., Schuller D., Casal M. and Dequin S. (2007) FEMS Yeast Res., 7, 317.

Van Vuuren H.J.J. and Jacobs C.J. (1992) Am. J. Enol. Vitic., 43, 2, 119.

Van Vuuren H.J.J. and Van Der Meer L. (1987) Am. J. Enol. Vitic., 38, 49.

Vaughan Martini A. and Martini A. (1987) Antonie Van Leeuwenhoek, 53, 77.

Vaughan Martini A. and Martini A. (1993) Syst. Appl. Microbiol., 16, 113.

Velázquez R., Zamora E., Alvarez M.L., Hernández L.M. and Ramírez M. (2015) Front. Microbiol., 3, 1222.

Versavaud A. (1994) *Analyse de la diversité génétique de la microflore levurienne de la région des Charentes: application à la sélection de souches œnologiques*, Thèse de Doctorat, Université de Nantes.

Versavaud A., Dulau L. and Hallet J.N. (1993) Rev. Fr. Oenol., 142, 20.

Versavaud A., Courcoux P., Roulland C., Dulau L. and Hallet J.N. (1995) Appl. Environ. Microbiol., 61, 10, 3521.

Vezhinet F., Blondin B. and Hallet J.N. (1990) Appl. Microbiol. Biotechechnol., 32, 568.

Vezhinet F., Hallet J.N., Valade M. and Poulard A. (1992) Am. J. Enol. Vitic., 43, 1, 83.

Wang S.A. and Bai F.Y. (2008) Int. J. Syst. Evol. Microbiol., 58, Pt 2, 510.

Williamson D.H. (1991) Nucleus, chromosomes and plasmids. In *The Yeasts, Vol. 4: Yeast organelles*, p. 433 (Eds A.H. Rose and J.S. Harrison). Academic Press, London.

Yarrow D. and Nakase T. (1975) Antonie Van Leeuwenhoek, 41, 81.

Young T.W. (1987) Killer yeasts. In *The Yeasts, Vol. 2, Yeasts and the Environment*, p. 131 (Eds A.H. Rose and J.S. Harrison). Academic Press, New York.

Zhang H., Richards K.D., Wilson S., Lee S.A., Sheehan H., Roncoroni M. and Gardner R.C. (2015) Front. Microbiol., 46, 92.

Zorg J., Kilian J. and Radler F. (1988) Arch. Microbiol., 149, 261.

Zott K., Miot-Sertier C., Claisses O., Lonvaud-Funel A. and Masneuf-Pomarede I. (2008) Int. J. Food Microbiol., 125, 197.

Zott K., Claisse O., Lucas P., Coulon J., Lonvaud-Funel A. and Masneuf-Pomarede I. (2010) Food Microbiol., 27, 559.