

Fermentation

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Modern winemaking is almost unrecognizable from its accidental and hesitant inception some 7500–9000 years ago. Improvements, once sporadic, have come at an ever-increasing pace, reflecting scientific understanding and technologic advancements. Major improvements in glass production and the rediscovery of cork as a closure during the 1600s favored the development of wine styles that benefitted from aging. These advances also permitted the perfection and consistent production of sparkling wine. In the mid-1800s, Pasteur initiated studies that began our understanding of the microbial nature of fermentation. He established not only that yeasts initiated fermentation, but also that some bacteria could cause wine spoilage. Only later were

some lactic acid bacteria found to be beneficial in reducing excessive wine acidity. Investigations are still ongoing on the microbial ecology of wine, with DNA sequencing techniques discovering a growing number of microbes present during and after fermentation. What is uncertain is whether they are metabolically active in a sensory significant way or just present.

Subsequent work by innumerable researchers and winemakers has honed winemaking to its current sophisticated state. Further fine-tuning should permit the seamless production of premium wines, showing the quality connoisseurs expect and deserve. In addition, distinctive features based on varietal, regional, or stylistic differences should become more discernible and

controllable. Dr. Richard Peterson, a highly respected winemaker in California, has commented that Mother Nature is “a nasty old lady, who must be controlled.” Modern enologic and viticultural science is increasingly providing the means by which many of the vicissitudes of Mother Nature’s effects can be mollified, but not controlled. One of the more recent innovations involves real-time monitoring of fermentation, permitting the rapid detection of developing problems and implementation of remedial action (e.g., [Kioroglou et al., 2018](#); [Roberts et al., 2018](#)).

Although science can provide explanations and guidance, winemakers still need to make individual judgment calls about which technique or option to use. In addition, major philosophical differences exist concerning how wine should be made. Some producers claim (at least in public) that “wine is made in the vineyard”—the winemaker being only a handmaiden, facilitating the grapes’ unique attributes to manifest themselves. Others feel that grapes are simply “putty” in the hands of skilled artisans (e.g., “flying winemakers”), and capable of being molded as desired. Production procedures certainly can direct wine development (within limits) to possess the characteristics desired by the cellar master or possess the attributes desired by target consumers. These differences are the enologic equivalent of the nature–nurture debate in human development. Superimposed on these viewpoints may be limitations imposed by traditional styles or appellation control regulations. Fundamentally, no decisions are inherently right or wrong, just choices that are more or less appropriate or required under particular circumstances. Because grape characteristics vary from vintage to vintage, no set production formula is possible. What is crucial is that the winemaker be fully aware of the benefits and shortcomings of the techniques available, selecting the optimal (or most judicious) procedure under the circumstances. In the following chapters, the advantages and disadvantages of alternative procedures are presented to facilitate making rational decisions.

Wine production

Vinification formally begins when the grapes or juice reaches the winery. The basic steps involved in the production of table wines are outlined in [Fig. 7.1](#).

The first step involves removing leaves and other extraneous material that were inadvertently collected with the fruit. The grapes are typically crushed (or

pressed) to release the juice, and if desired, to initiate maceration. Maceration facilitates the extraction of nutrients, flavorants, and other constituents from the pulp, skins, seeds, and occasionally stems. Hydrolytic enzymes released from ruptured cells promote this liberation. The cytotoxic action of pectic enzymes further promotes the release of cellular constituents into the juice. Enzymes released or activated by cell death may also synthesize flavor compounds and hydrolyze macromolecules into forms utilizable by yeast and bacteria.

For white wines, maceration is kept to a minimum and seldom lasts more than a few hours. The juice that runs freely from the crushed grapes (free-run) is usually combined with that released by pressing. Because of their different attributes, the free-run and first pressings are usually clarified separately before combining and joint fermentation. Subsequent pressings are typically fermented separately. This avoids contaminating the free- and first press-runs with undesirably high tannin levels. It can also avoid the generation of off-odors, as in the case of Sauvignon blanc grapes ([Nikolantonaki and Darriet, 2011](#)).

For red wines, maceration is prolonged and occurs simultaneously with the inception and action of alcoholic fermentation. The alcohol generated by yeast metabolism favors anthocyanin extraction, but most solubilization occurs early during fermentation when the alcohol content is still low ([Canals et al., 2005](#)). Although anthocyanin absorption is reflected in the dynamics of total phenol extraction, seed tannin uptake is more influenced by alcohol content. Anthocyanin extraction is also a function of the cultivar ([Romero-Cascales, 2005](#)), fruit ripeness ([Canals et al., 2005](#)), and fermentation temperature and duration. Once extracted, retention and color depend on another host of factors, such as the pH, presence of soluble proteins, copigment and tannin content, carbonyls, and other reactants.

The solubilized phenolic compounds give red wines their basic appearance, taste, and flavor. They also give red wines most of their aging and mellowing attributes. In addition, ethanol promotes the liberation of relatively water-insoluble aromatic ingredients from the pulp and skins. After partial or complete fermentation, the free-run is collected as it flows away from the pomace (grape solids). Subsequent pressings extract most remaining juice (press fractions). These are incorporated with the free-run fraction to the degree desired by the winemaker as well as for the type and style of wine intended.

Rosé wines are made from red grapes typically given a comparatively brief prefermentative maceration. This may

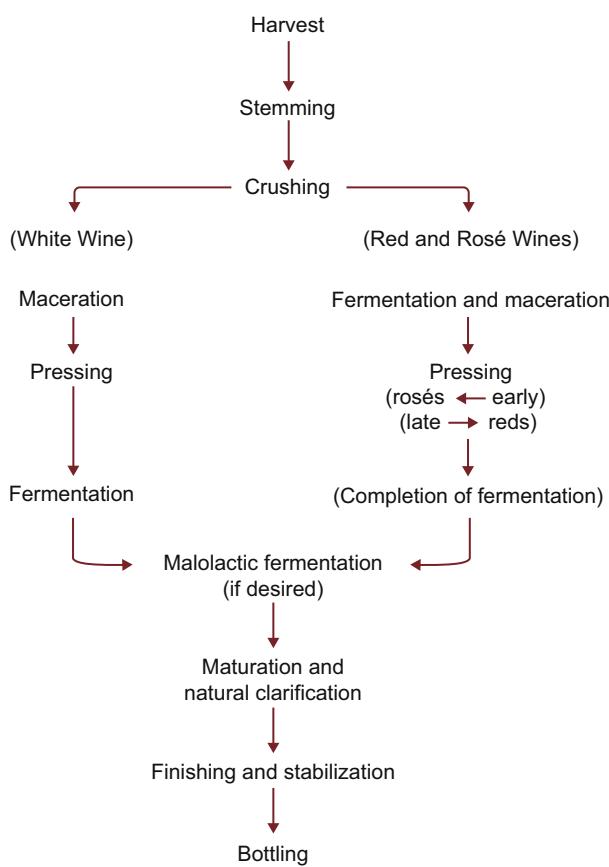


FIGURE 7.1 Flow diagram of winemaking.

involve grapes being crushed or gently broken with the juice left in contact with the pomace at cool temperatures until sufficient color has been extracted ($\sim 12\text{--}24\text{ h}$). The free-run juice is subsequently drawn off and fermented similarly to that of a white wine. Alternatively, the grapes may be pressed whole, a process that limits color extraction. The free-run and some of the first press-run juice is fermented without further contact with the skins or seeds. Where grape coloration is low, the fruit may be crushed and fermented with the pomace until sufficient pigment has been extracted. Subsequent fermentation of the free-run juice occurs without further pomace contact. When color depth is used to time pressing, consideration must be taken of both pigmentation loss (associated with precipitation of yeast cells) and bleaching due to any sulfur dioxide added. Because of the short (or incidental) maceration, alcoholic fermentation often begins in earnest only after the juice is separated from the pomace.

Fermentation may start spontaneously due to endemic yeasts derived from the grapes and winery equipment. Standard practice now, however, is to inoculate the juice/must with one or more yeast strains of

known characteristics. Yeasts not only produce the alcohol but also generate the general bouquet and flavor attributes that typify wines.

After completing alcoholic fermentation, the wine may be treated to foster a second, malolactic fermentation. This is particularly valuable in cool climatic regions, where a reduction in acidity ameliorates the wine's taste. Although most red wines undergo malolactic fermentation, fewer white wines profit from its occurrence. The milder fragrance of most white wines makes them more susceptible to accompanying sensory modifications. Acidity retention is essential to the fresh taste of white wines. In warm viticultural regions, malolactic fermentation can be undesirable. Its development is usually discouraged by practices such as the addition of sulfur dioxide, early clarification, and storage at cool temperatures.

Newly fermented wine is protected from or given only minimal exposure to oxygen during maturation. This limits oxidation and microbial spoilage. During storage, excess carbon dioxide escapes, yeasty odors dissipate, and suspended material precipitates. Changes in aroma and the initiation of processes leading to an aged bouquet may begin during maturation. Exposure to air is usually limited to events such as racking or *bâtonnage* (during *sur lies* maturation). Such slow or limited exposure can help oxidize hydrogen sulfide and favor color stability in red wines.

After several weeks to months, the wine is racked. Racking separates the wine from solids that settle out during spontaneous or induced clarification. The sediment consists primarily of yeast and bacterial cells, grape cell debris, and precipitated tannins, proteins, and potassium tartrate crystals. If left in contact with wine, they could lead to the production of off-odors as well as favor microbial spoilage.

Before bottling, the wine may be fined to remove suspended proteins and other materials. Otherwise, they could generate haziness, especially if the wine is subsequently exposed to heat. Fining may also be used to soften the wine's taste by removing excess tannins. Wines are commonly chilled and filtered to further enhance clarification and stability.

At bottling, wines are generally given a small dose of sulfur dioxide to limit oxidation and microbial spoilage (about 0.8–1.5 mg/L free molecular SO₂). Sweet wines are usually sterile-filtered as a further protection against microbial spoilage.

Newly bottled wines are normally aged at the winery for several months or years before release. This permits wines, blended shortly before bottling, to "harmonize." In addition, it allows acetaldehyde, produced following bottling (as a consequence of incidental oxygen uptake), to become

nonvolatile (chemically bound to other wine constituents). Thus “bottle sickness,” usually attributed to free acetaldehyde, has vanished before the wine reaches the consumer.

Prefermentation practices

Sorting

Although stemmers effectively reject stems and most leaf parts, they do not remove smaller material, such as trellis clips, staples, wood splinters, snails, slugs, or insects, although magnets can retain metal objects. Stemmers also do not segregate out immature, diseased, raisined, or other forms of substandard berries. As even small quantities of inferior fruit can negatively impact wine quality, limiting their incorporation is important. This has usually required manual sorting. Due to labor costs (or unavailability), automatic sorters are becoming more common.

Automatic sorters can differentiate and selectively remove undesirable material from harvested grapes (Falconer and Hart, 2005). Rejection can be selected to function on color and/or size categories. As the fruit passes under the detector, located above a conveyer belt, the color intensity of the grapes in the green, red, and infrared parts of the spectrum is assessed. Depending on instructions supplied by the operator, a computer determines whether the sample should be rejected. A jet of air expels the undesired material. Although not inexpensive, automatic sorters are more rapid and less expensive (amortized over several years) than manual sorting. Whether such an investment is merited will depend on the economic return based on need of use and improved wine quality.

Prefermentative drying

In some ways, this is a more controlled means of achieving effects similar to those that occur naturally with delayed harvest. Traditionally, it has been used to achieve dehydration when producing sweet and fortified wines (see Mencarelli and Tonutti, 2013). However, interest in the technique has expanded in the desire to produce a new wine style. The technique usually goes under its Italian name, *appassimento*. Although historically used to produce sweet (and some sparkling) wines, its current fame rests primarily with the production Amarone della Valpolicella. The occasional association of some grapes with a form of noble rotting during the drying period was unsuspected and generated a unique flavor (see Recioto-style wines, Chapter 9). However, in most current applications, the grapes remain healthy, only experiencing those chemical changes associated with overmaturation (and slight dehydration). Storage temperatures have traditionally been cool, but experimentation with warmer temperatures is being assessed.

If juice concentration (and the accompanying higher alcohol content of the wine) is undesired and enhanced anthocyanin accumulation is wanted, mid-season basal leaf removal may be practiced.

Formerly, grape clusters were arranged in single layers on mats or porous trays and placed either in the sun (sweet white wines) or in well-ventilated warehouses (red grapes). The current trend is to use temperature-regulated drying chambers to achieve a quicker, more regulated level of overmaturation/dehydration. Depending on the style desired, avoiding sun exposure (and its associated heating) limits the browning of white wines and enhances the color depth of red wines. A wide range of metabolic adjustments occurs during drying, affecting ethylene production, flavor development, and the type and accumulation of phenolics (Bonghi et al., 2012).

Quality assessment

Shortly after the grapes reach the winery, they are subjected to a series of chemical and quality checks. At a minimum, their soluble solids ($^{\circ}$ Brix), acidity, and phenolic contents are assessed. Such data are important not only because adjustment may be necessary before fermentation, but also because the final price supplied to the grower is frequently based on these attributes. In addition, acceptance, rejection, and price for the crop are affected by the proportion of diseased grapes. For example, the degree of *Botrytis* infection has often been assessed relative to gluconic acid content. Gluconic acid is not a fully reliable measure, however, since it may arise from other sources. More specific measures of *Botrytis* infection include PTA-ELISA (Obanor et al., 2004), tube immunoassays (Dewey and Meyer, 2004), or microfluidic immunosensor with micromagnetic beads coupled with carbon-based screen-printed electrodes (Fernández-Baldo et al., 2010).

Stemming and crushing

Stemming and crushing are typically conducted as soon as possible after harvesting. During the harvest, some grapes are unavoidably broken and their juice released, whereas others may be bruised. Thus, oxidative browning often begins before the grapes reach the winery and crushing begins. The escaped juice also becomes field-inoculated with epiphytic yeast and bacteria. If the berries are harvested during the heat of the day, undesirable microbial contamination can rapidly ensue. To minimize this occurrence, grapes may be sulfited at harvest or preferably harvested during cooler parts of the day.

Left in containers, harvested fruit quickly warms due to the metabolic activity of grapes and the insulating

influence of the volume. This can aggravate contamination by speeding microbial activity. In addition, warming may necessitate the expense of cooling to bring the temperature down to an acceptable, prefermentation value.

Stemming

The current trend is to separate the processes of stemming and crushing. The removal of stems, leaves, and grape stalks (termed MOG—material other than grapes) before crushing has several advantages. Notably, it minimizes the uptake of phenolics and lipids from these materials. The extraction of stem phenols may be of potential value when dealing with red varieties low in phenol content, such as Pinot noir. Stem phenols are intermediate in astringency and bitterness relative to the less strident tastes of skin tannins and the more assertive seed tannins. The phenolics extracted from stems include catechins, flavonols (notably quercetin), and cafataric acid (Sun et al., 1999).

In the past, stems were often left with the must throughout fermentation, especially in the production of red wines (to generate channels through which juice or wine could more easily escape, facilitating pressing). In poor vintages, the enhanced tannin content also gave the wines extra body and improved color density. Stems can also augment the supply of long-chain unsaturated fatty acids (Bréchot et al., 1971), improving the ability of yeasts to complete fermentation under cool cellar conditions.

Leaf removal before crushing limits the formation and uptake of C₆ ("leaf") aldehydes and alcohols. They are produced during the enzymatic oxidation of linoleic and linolenic acids extracted from the leaf cuticle. Leaf aldehydes and alcohols can taint wine with a grassy to herbaceous odor. Nonetheless, they may contribute to the typical aroma of some wines in small amounts. High leaf content in the must may also result in excessive uptake of quercetin. This can lead to the formation of a yellowish haze in white wines (Somers and Ziemelis, 1985). If the wines are matured sufficiently, much of the quercetin precipitates before bottling. High flavonol contents derived from leaves can also generate white wine bitterness.

For convenience and efficiency, the same equipment often performs both stemming and crushing. Stemmers often possess an outer perforated cylinder that permits berry exit but restricts the passage of stems, stalks, and leaves (Fig. 7.2). Rotation of a series of flexible paddles situated on the ends of spirally arranged arms on a central shaft draws the grape clusters into the stemmer. The fruit is forced through the perforations in the outer cylinder, and the stems and leaves are expelled via a terminal outlet. When stemmer-crushers are working optimally, the fruit is separated from the leaves and



FIGURE 7.2 Internal view of a crusher-stemmer. Photo courtesy of the Wine Institute.

stems with minimal breakage. Expelling the stems and leaves in as dry a state as possible avoids juice loss and facilitates disposal. Stems and other vine remains may be chopped for composting and subsequent soil incorporation.

Crushing

Since time immemorial, crushing involved the action of human feet in a relatively shallow container, often slotted to aid juice escape and collection. The ancient Egyptians put horizontally positioned rods over these containers, from which short ropes dangled. They were used by the crusher(s) to remain upright. More commonly, the crushers linked arms to remain standing, as they danced within the slurry of juice, skins, seeds, and berries. It was only in the early part of the 1800s that mechanical crushers began to replace treading. Although still used in a few situations, as for some quinta ports, the old procedure has otherwise been relegated to history. It was doomed more by labor costs and the movement of farm workers to the cities than the queasy stomachs of citified consumers.

Stemming usually precedes crushing. Because some berries are unavoidably broken during stemming, leading to oxidative browning, crushing often occurs immediately after or simultaneously in a stemmer-crusher.

Crushing may be accomplished by several processes. Those generally preferred involve pressing the fruit against a perforated wall or passing them through a set of rollers. In the former, the berries are broken open and the juice, pulp, seeds, and skins collected and pumped to a retaining tank or vat. In the latter process, berries are

crushed between a pair of rollers rotating toward each other. The rollers usually have spiral ribbing or contain grooves with interconnecting profiles. These draw the grapes between the rollers, crushing the grapes. Spacing between the rollers can be adjusted to accommodate cultivar or yearly variation in berry size. This avoids seed rupture and contaminating the must with seed oils. Their oxidation could lead to the development of rancid odors.

Crushing can also be achieved by centrifugal force. In centrifugal crushers, the fruit is spun against the sides of the crusher, which tends to turn the fruit into a pulpy slurry. Because this complicates juice clarification and may rupture seeds, centrifugal crushers are generally eschewed.

Although grapes are customarily crushed before vinification, there are exceptions, notably sparkling wine. In this instance, whole grape clusters are pressed versus "crushed." Special broad shallow presses have historically been used to extract the juice to minimize pigment and tannin extraction. This is particularly important when white sparkling wine is made from red-skinned grapes. These ancient presses are being replaced by more efficient pneumatic presses, which also take up much less space and involve less labor. Pressing intact grape clusters has also become popular with some table wine producers. It reduces the extraction of soluble solids by up to 60%–70% and phenolic content by 10%–35% (Seckler, 1997), thereby reducing the need for clarification.

Botrytized grapes are also frequently pressed whole versus crushed. The gentler juice separation minimizes the liberation of fungal dextran (β -glucans) polymers. These glucans can quickly plug filters used in clarification. In the production of Tokaji Eszencia, even pressing is avoided. Only the juice that drains away freely from the heavily infected grapes is used.

In the production of wines employing full or partial carbonic maceration, such as *Vino Novello* and Beaujolais, it is essential that most fruit remains uncrushed until grape cell fermentation has ended. It donates the characteristic fragrance shown by these wines. After a variable period of autofermentation, berries that have not broken under their own weight during this initial phase are pressed to release their juice. Fermentation is completed by yeast action.

In the past, inefficient crushing often left a portion of the grapes remaining uncrushed (Henderson, 1824). Thus, many wines in former times would have experienced partial carbonic maceration.

While the means of berry rupture before (or during) pressing is critical to the characteristics of the juice/must obtained, how the grapes were harvested and their state of health significantly influence its properties (Fig. 7.3). How it is transferred to fermentation tanks can also affect juice/must quality (Seckler et al., 2001).

Cryoextraction and supraextraction

An alternative to crushing is cryoextraction (Defranoux et al., 1989). It involves cooling the grapes to -4°C , freezing those low in sugar content. Thus, only unfrozen grapes release most of their juice during subsequent pressing. It helps to augment the sugar content of juice obtained. A variant of the technique (supraextraction) allows the grapes to warm to about 10°C before pressing. Freezing causes both grape cell rupture and skin splitting, facilitating the escape of juice during pressing. Although increasing the extraction of sugars and phenolics, supraextraction reduces total acidity and raises the pH—likely the result of crystallization and precipitation of tartrate salts. Whether these features are desirable will depend on the intention of the winemaker and characteristics of the grapes. A comparison with other techniques is provided by Busse-Valverde et al. (2010).

Maceration (skin contact)

Maceration refers to a period during which constituents diffuse out of the pomace (grape solids) into the juice. Its effects depend primarily on its duration but also on factors such as concentration differentials, solvent and solute chemistry (molecular size and polarity), surface area, membrane permeability (if cells are not ruptured), hydrolytic enzyme action, temperature, agitation, and exposure to ethanol (if occurring simultaneously with fermentation).

With red wines, the duration of maceration can be as long as or longer than fermentation. In contrast, maceration is short with rosé wines and may be almost nonexistent with some white wines. When the maceration period is short, a pectinase preparation is often added to the must immediately after crushing. This speeds tissue disintegration, facilitating juice release during pressing, and speeds settling during clarification. It also promotes the rapid release of flavorants from the skins. Enzyme preparations are discussed more fully later in the chapter.

White wines

With the shift to light fruity white wines in the 1970s, maceration was reduced to a minimum. This trend was encouraged by the widespread adoption of mechanical harvesting. Stemming and crushing could occur in the field, with only the juice transported to the winery. Where whole grapes were delivered to the winery, there was always some grape rupture and associated maceration before arrival—the extent depending on the cultivar, maturity, machine operation, time separating harvest and crushing/pressing, and temperature.

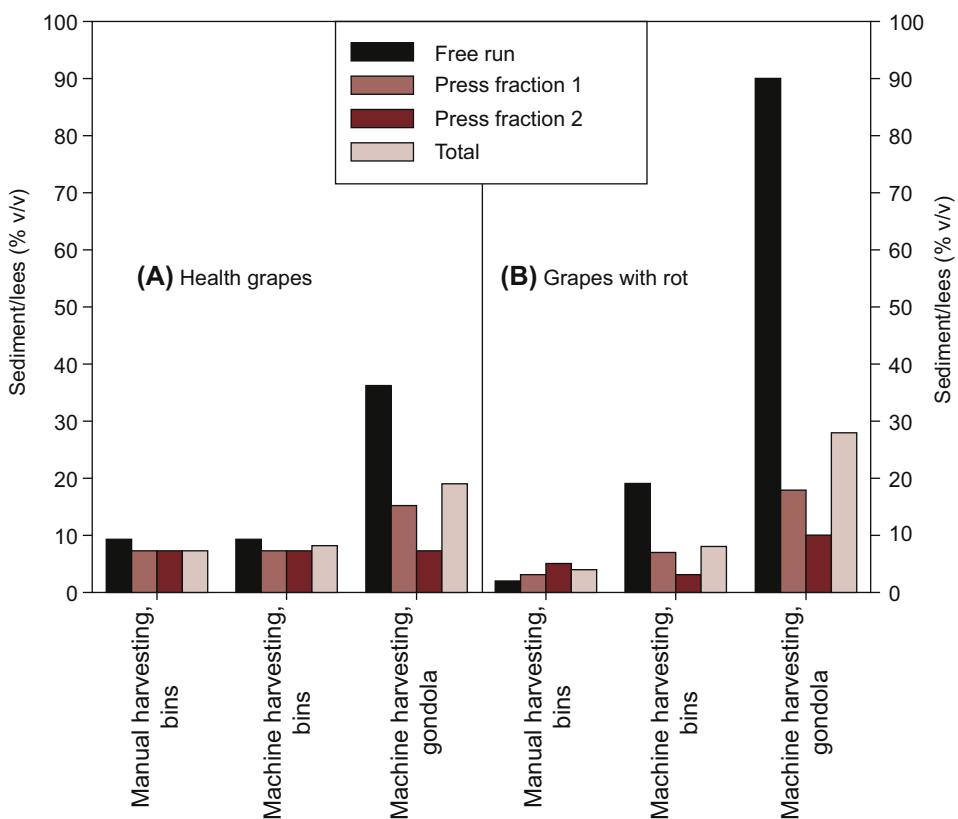


FIGURE 7.3 Sediment relative to harvesting technique and berry firmness. From Christmann, M., Freund, M., 2010. *Advances in grape processing equipment*. In: Reynolds, A.G. (Ed.), *Managing Wine Quality*. Vol. I. Viticulture and Wine Quality, pp. 547–558. Woodhead Publishing Ltd., Cambridge, UK. Data from Seckler, J., 1997. *Ganztraubenpressung*, ATW-Forschungsbericht Nr. 88. Darmstadt, Germany, reproduced by permission by Elsevier.

Minimizing these influences reduced the uptake of heat-unstable proteins, thereby decreasing the need for protein stabilization products such as bentonite.

Because most varietal flavorants are located in the skins (Fig. 7.4), minimizing maceration reduced uptake—the degree depending on the flavorant, cultivar, and grape maturity (Fig. 7.5). For wines depending on skin-derived aromatics (e.g., Gewürztraminer and Muscat cultivars), this became increasingly significant with the adoption of gentler pressing methods (e.g., pneumatic and whole-grape pressing) (Fig. 7.6). This deficiency can be partially offset by increasing the use of press-run fractions. For example, the uptake of varietal thiol precursors is markedly enhanced by using fractions derived at the end of the press cycle (Patel et al., 2010; Roland et al., 2011). Adjusting the proportion of late free- and press-run fractions is often easier to manipulate than maceration time due to the complexities of temperature and duration on extraction, precipitation, and degeneration of compounds during maceration. Nevertheless, increasing reliance on the use of press fractions augments the wine's phenolic content. Pectinase addition could speed the liberation of skin-derived aromatics but may have undesirable sensory effects due to unsuspected

enzymatic actions. Thus, there is renewed interest in extended maceration, occasionally up to 3–7 days.

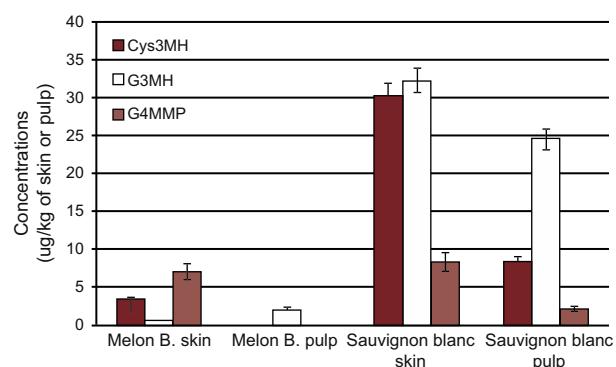


FIGURE 7.4 Mean concentration of cysteinylated and glutathionylated precursor concentrations for S-3-(1-hexanol cysteine) (Cys3MH and G3MH) and S-4-(4-methyl-4-mercaptop-2-pentanone) (G4MMP) in Melon B. and Sauvignon blanc skin and pulp (results are expressed in μg of precursor per kg of initially fresh skin or pulp of the corresponding grapes). Reprinted from Roland, A., Schneider, R., Charrier, F., Cavelier, F., Rossignol, M., Razungles, A., 2011. Distribution of varietal thiol precursors in the skin and the pulp of Melon B. and Sauvignon blanc grapes. *Food Chem.* 125, 139–144, with permission from Elsevier.

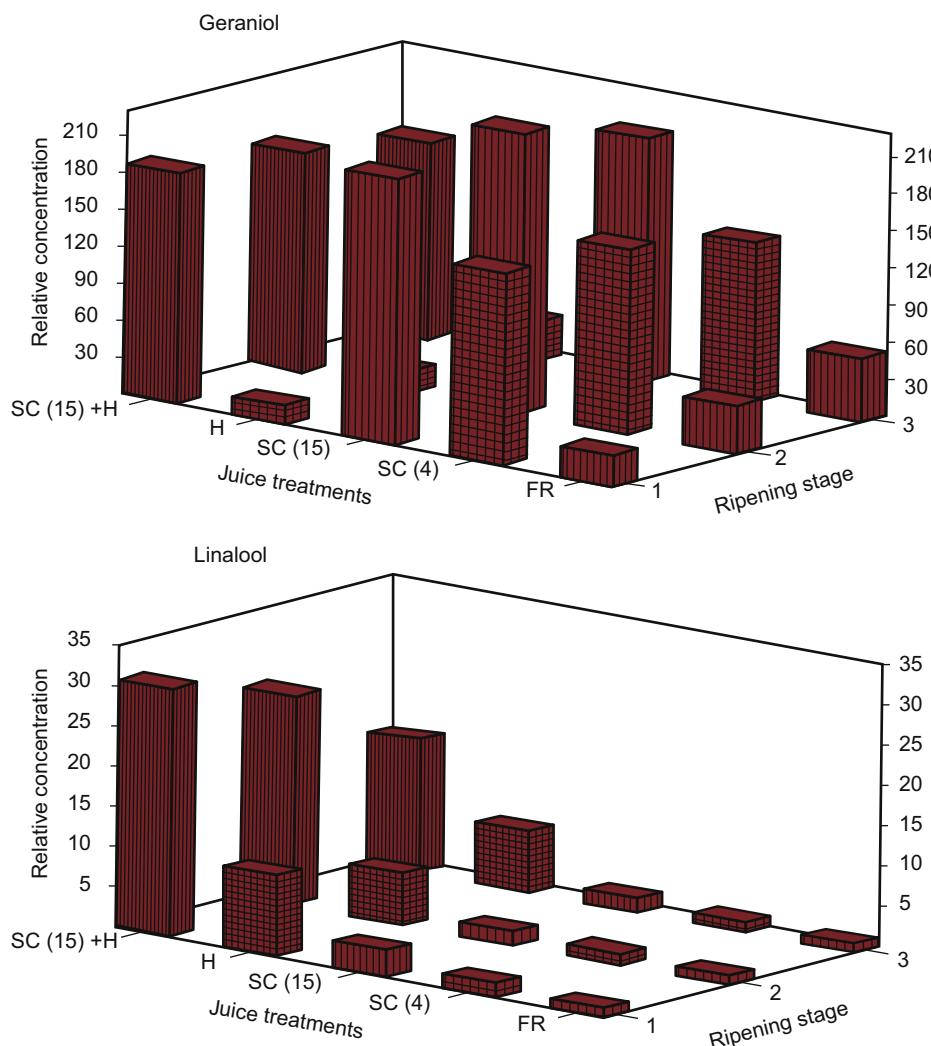


FIGURE 7.5 The effect of grape maturity and juice treatment on the relative concentration of geraniol and linalool in Gewürztraminer juice. Ripening stage: 1 = 22.0°Brix, 2 = 23.0°Brix, 3 = 24.0°Brix; FR=Free-run, SC(4) = Skin contact for 4 h, SC(15) = Skin contact for 15 h, H = heat treatment (70°C for 15 min), SC(15)+H = Combined skin contact for 15 h and heat treatment. *From Mass Marais, J., 1987. Terpene concentrations and wine quality of *Vitis vinifera* L. cv. Gewürztraminer as affected by grape maturity and cellar practices. Vitis 26, 231–245, reproduced by permission.*

However, increasing varietal and fruit flavors by extending the duration of skin contact may also result in a deterioration in mouthfeel (Tamborra, 1992). The latter may be a consequence of increased potassium uptake (reduced acidity) and/or enhanced phenolic extraction (increased bitterness) (Sokolowsky et al., 2015). Greater phenolic uptake could also augment astringency and browning potential. Although these effects have been observed in some studies, they have not been consistently found.

Procedures that may diminish the negative effects of extended maceration include the use of cool

temperatures (5–8°C) and hyperoxygenation (Cejudo-Bastante et al., 2011a). Hyperoxidation likely has its effect by promoting the oxidation and precipitation of flavonoid phenolics during fermentation. Regrettably, hyperoxidation may undesirably modify the wine's fragrance (Cejudo-Bastante et al., 2011b). Another alternative is to freeze some or all of the grapes before crushing (cryogenic maceration). Freezing ruptures cell membranes, facilitating the escape of flavorants. Another option is to retain a fraction of the crop for addition as whole grapes during fermentation (Bavčar et al., 2011). Both procedures may enhance the fruity/floral fragrance

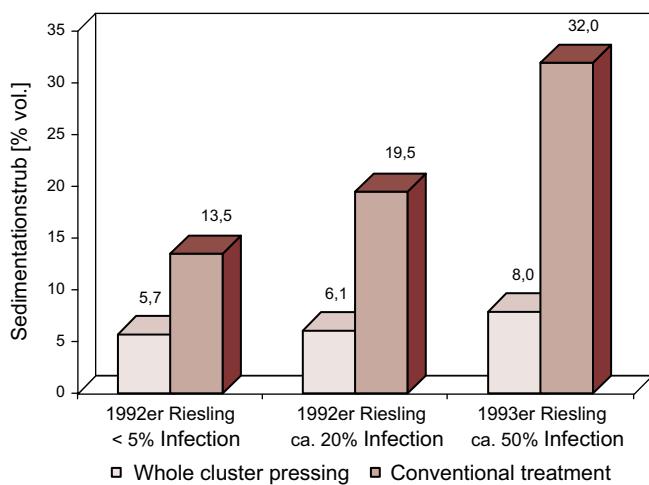


FIGURE 7.6 Influence of method of pressing (whole cluster vs. conventional destemming and crushing) as well as the degree of grape botrytization on the amount of soluble solids. Modified from Seckler, J., 1997. *Ganztraubenpressung, ATW-Forschungsbericht Nr. 88*. Darmstadt, Germany, reproduced by permission.

of the wine. Like most choices in winemaking, each decision has its pros and cons and needs to be investigated, preferably experimentally at the winery, before adoption.

As noted, adding macerating (pectinase) enzymes to the must is frequently used to facilitate the release of flavorants. This effect continues after pressing. Because cell fragments are often extracted with the juice, the enzymes can continue to release aromatics during settling before juice clarification.

What the winemaker needs to estimate is the relative importance of skin-derived flavorants relative to the ease and extent of phenolic extraction. These properties are largely cultivar-dependent but also vary with vineyard and vintage conditions, grape health and maturity, and conditions during maturation. Determining the relative extraction of seed versus skin phenolic has been made easier with the development of a technique that can directly differentiate between them (Peyrot des Gachons and Kennedy, 2003).

If the situation were not complicated enough, in the case of the varietal thiols, the concentration of precursors in the juice is not directly correlated with the presence of the volatile (free) thiols. Their release appears to be more dependent on liberation during harvesting than during fermentation (Allen et al., 2011).

Although maceration enhances phenolic extraction, it does not lead to the same astringency that characterizes red wine. This not only reflects the much longer maceration of red wines and its association with fermentation

(accumulation of ethanol) but also the absence of anthocyanins. Anthocyanins, although tasteless, bind with catechins and flavonoid tannins (see Chapter 6), increasing their solubility. In white wines, most extracted phenolics precipitate during fermentation, limiting their effects on the wine's sensory attributes.

Grape varieties differ considerably in the phenolics released during crushing or extracted during maceration. For example, few flavonoids accumulate in the musts of Palomino and Sauvignon fdb blanc; moderate amounts accumulate in the musts of Riesling, Sémillon, and Chardonnay; whereas extensive extraction occurs with Muscat Gordo, Colombard, Trebbiano, and Pedro Ximénez (Somers and Pocock, 1991). Increased extraction favors subsequent in-bottle browning. This feature may be partially offset by hyperoxygenation (Cejudo-Bastante et al., 2011b).

The major physical factors influencing phenol extraction are temperature and duration. Extraction is often linearly related to both factors. Cool temperatures and short duration reduce the uptake of flavonoids (Fig. 7.7), limiting potential bitterness and astringency but also limiting their antioxidant benefits. This can be an important concern for cultivars such as Sauvignon blanc that depend on thiols for much of their varietal character. In this regard, techniques such as semicarbonic maceration (see Chapter 9), possibly combined with cryogenic techniques, are being investigated to enhance the concentration of antioxidant phenolics while still maintaining a traditional mouthfeel (Olegar et al., 2015) (instead of adding press fractions to the wine).

Occasionally, the concentration of extracted compounds decreases with prolonged maceration, presumably due to precipitation and/or degradation. Extraction also varies markedly with the relative solubility of the class of compound, its deposition within the cell (storage vacuole vs. bound to cell-wall constituents), tissue (seed, skin, stem), and berry ripeness. Although many nonflavonoids quickly escape into the juice, subsequent extraction of flavonoid phenolics occurs more readily than nonflavonoids (Fig. 7.7).

As with phenolics, the concentration of flavorants and nutrients in the juice is markedly influenced by the duration of maceration. For example, skin contact augments the uptake of monoterpenes (Marais, 1996). The content of amino acids, fatty acids, and higher alcohols may rise, whereas total acidity falls (Soufleros and Bertrand, 1988; Guitart et al., 1997). The decline in acidity appears to be caused by the increased release of potassium. The latter induces tartrate salt formation and precipitation. Other changes result from indirect effects on yeast metabolism. For example,

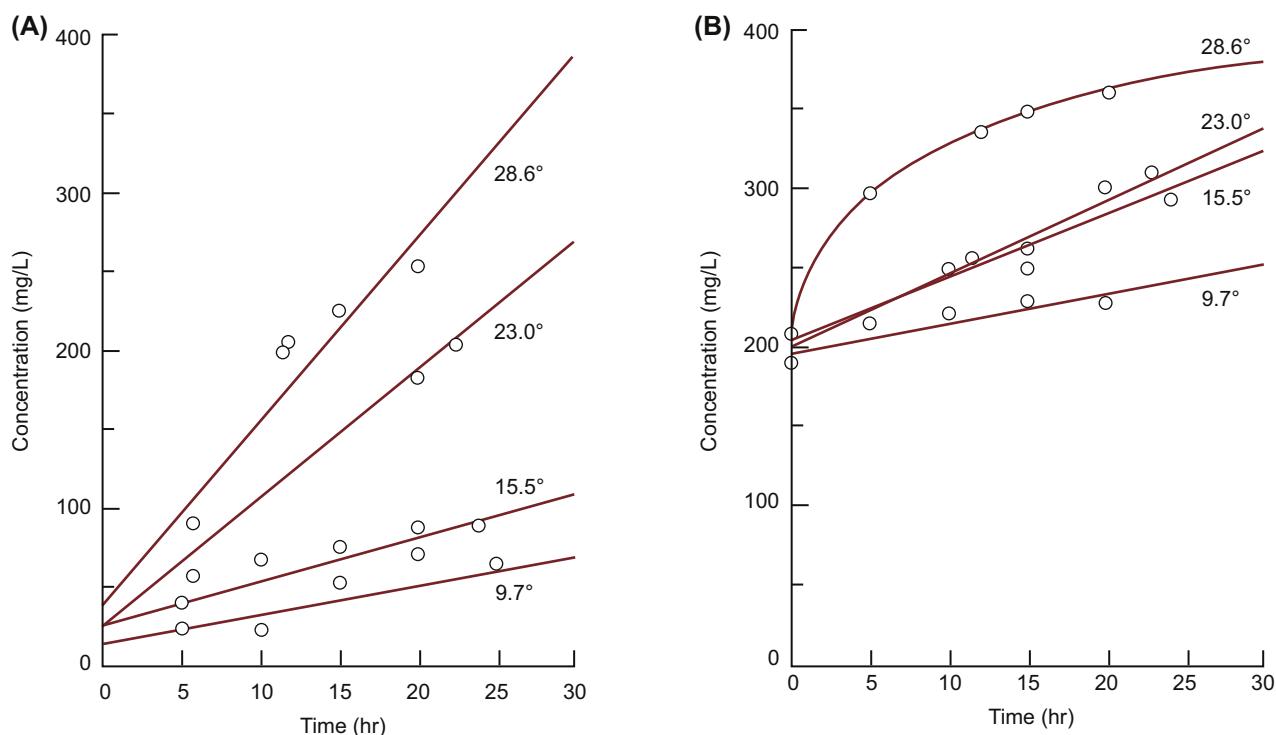


FIGURE 7.7 Flavonoid (A) and nonflavonoid (B) phenol content in Chardonnay must during skin contact. Temperatures are in °C. From Ramey, D., Bertrand, A., Ough, C.S., Singleton, V.L., Sanders, E., 1986. Effects of skin contact temperature on Chardonnay must and wine composition. *Am. J. Enol. Vitic.* 37, 99–106, reproduced by permission.

increased amino acid extraction has been correlated with reduced hydrogen sulfide (Vos and Gray, 1979).

Occasionally, a short exposure (15 min) to high temperatures ($\sim 70^{\circ}\text{C}$) greatly increases the release of volatile compounds, such as monoterpenes (Marais, 1996; see Fig. 7.5). Although the concentration of most monoterpenes increases on short-term exposure to high-temperature maceration, not all follow this trend. For example, the concentration of geraniol decreases.

Generally, maceration is conducted at cool temperatures. This not only has the advantage of suppressing the growth of potential spoilage organisms before the onset of active fermentation but also affects the subsequent synthesis of yeast flavorants during fermentation. For example, the synthesis of volatile esters may increase with a rise in maceration temperature up to 15°C but decrease thereafter. The synthesis of most alcohols (apart from methanol) is reduced following maceration at warmer temperatures (Fig. 7.8). Although methanol content increases due to the action of grape pectinases (releasing methyl groups from pectins), this effect is insignificant even with cultivars

requiring pectinase enzyme addition (Lee et al., 1975). Methanol concentrations are usually well below limits set by the OIV.

The sensory influence of maceration can also be influenced by the degree of oxygen exposure. This can occur during crushing or via intentional exposure (hyperoxygenation). In either case, the oxygen content rapidly declines to almost zero within a few minutes after crushing or the termination of hyperoxygenation (Dubernet and Ribéreau-Gayon, 1974; Silva and Lambri, 2006).

Oxygen promotes the enzymatic oxidation of the primary phenolics in white must (nonflavonoid *o*-diphenols, notably caftaric acid). Although their polymerization causes juice browning, the polymers usually precipitate during fermentation. This tends to leave the wine less sensitive to subsequent in-bottle oxidation as well as lower in bitterness.

Hyperoxygenation seemingly would be ill-advised with some cultivars, such as Sauvignon blanc. It can reduce (oxidize) important varietal aroma compounds. Nonetheless, the presence of oxygen during maceration can promote the formation of thiol precursors (Larcher

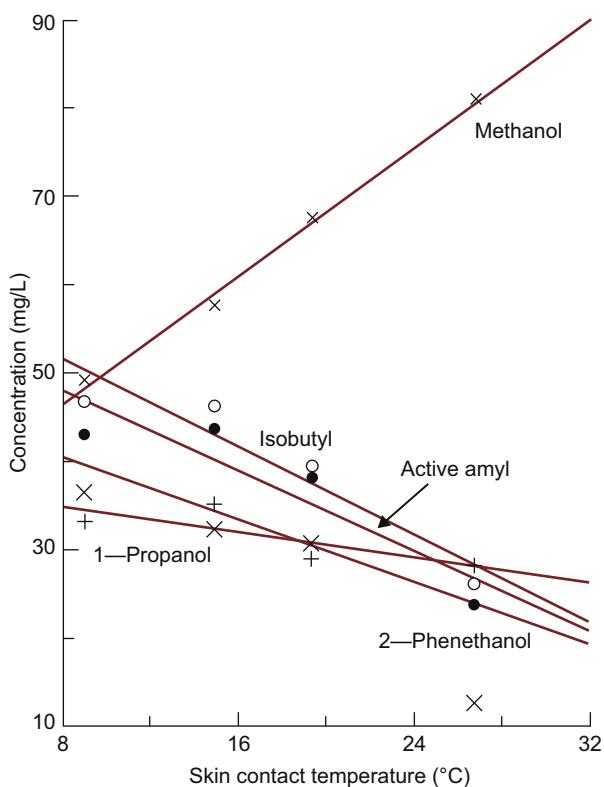


FIGURE 7.8 Concentration of various alcohols in Chardonnay wine as a function of skin contact temperature. *From Ramey, D., Bertrand, A., Ough, C.S., Singleton, V.L., Sanders, E., 1986. Effects of skin contact temperature on Chardonnay must and wine composition. Am. J. Enol. Vitic. 37, 99–106, reproduced by permission.*

et al., 2013). Hyperoxidation may also be ill-advised with cultivars low in glutathione contents (important antioxidant limiting browning potential) but may be offset by the addition of glutathione along with sulfur dioxide. Alternatively, natural glutathione presence may be enhanced by extended skin contact and pressing under nitrogen (Pons et al., 2015).

Partially to facilitate early phenolic oxidation and removal, sulfur dioxide addition at crushing is generally avoided where possible, as it limits the action of grape polyphenol oxidase. In addition, sulfur dioxide can undesirably enhance the production of acetaldehyde and several other aldehydes during fermentation (Frivik and Ebeler, 2003), augment phenolic extraction, and retard the initiation of malolactic fermentation (if desired). Adding sulfur dioxide at or just after crushing is now largely limited to situations where a significant proportion of the crop is diseased or damaged or where the interval between harvesting and crushing is protracted. Sulfur dioxide retards the multiplication of bacteria or other potentially undesirable microbes. When

added, sulfur dioxide is applied several hours before yeast inoculation. This allows time for the sulfur dioxide to inhibit contaminant organisms and bind with other wine constituents. This reduces the free molecular SO₂ content, thereby limiting any disruption to the activity and multiplication of the inoculated yeast.

Maceration has been observed to improve juice fermentability (Ollivier et al., 1987) and enhance yeast viability. Part of these effects is due to the greater release of particulate matter, lipids, and soluble nitrogen compounds into the juice. Particulate matter is well known to increase microbial growth. The solids provide surfaces on which microbes can adhere and grow; nutrients adhere and/or be released; toxic C₁₀ and C₁₂ carboxylic fatty acids absorb; and nucleation of carbon dioxide bubbles initiate. The associated effervescence increases must agitation, encouraging uniform nutrient distribution.

Skin contact facilitates the extraction of long-chain (C₁₆ and C₁₈) saturated and unsaturated fatty acids, such as palmitic, oleanolic, linolenic, and linoleic acids. These reduce the synthesis of toxic, mid-chain (C₁₀ and C₁₂) fatty acids (Guilloux-Benatier et al., 1998). The former lipids are important in permitting multiplying yeast cells to generate the steroids needed to construct new cell membranes under the anaerobic conditions of active fermentation (Deytieux et al., 2005). In addition, the small amounts of oxygen absorbed during crushing and other prefermentation cellar activities permit the synthesis of essential sterols by yeasts.

Extended skin contact also improves (more than doubles) the production of extracellular mannoproteins formed during alcoholic fermentation. The effects of increased mannoprotein content and reduced synthesis of C₁₀ and C₁₂ fatty (carboxylic) acids combine to facilitate malolactic fermentation (Guilloux-Benatier et al., 1998).

In general, minimal maceration at cool temperatures is often preferred for the production of young, fresh, fruity wines. Longer, warmer maceration favored the production of Chardonnay wine deeper in color and fuller flavored. The latter also tends to mature more quickly during barrel aging and develop a more complex character than wines produced with minimal skin contact (Ramey et al., 1986). Longer maceration times also produced Chenin Blanc wines with a shift from fresh and tropical flavors (due to terpenes, esters, and thiols) to riper fruit notes (Aleixandre-Tudo et al., 2015). Thus, varietal characteristics (Singleton et al., 1980), fruit quality, equipment availability, and market demands can all play a role in the decision of a winemaker to use maceration, and to what extent.

Cell-cracking is a comparatively new procedure proposed as a complement to or replacement for maceration (Bach et al., 1990). The procedure involves forcing the

must through narrow gaps, separating steel balls positioned in a small bore. It is reported to speed the extraction of flavorants.

Rosé wines

Occasionally, rosé wines are made directly from juice released by pressing whole grape clusters. Nevertheless, the more common practice is to gently stem and crush the grapes before pressing. This may or may not be followed by a period of prefermentative maceration lasting up to 24 h. If this occurs at $\leq 20^{\circ}\text{C}$, microbial action tends to be retarded. Data from [Murat and Dumeau \(2005\)](#) suggest that the upper portion of the temperature range might be preferable due to improved extraction of the precursor of 3-mercaptopropan-1-ol located in the skin. Its metabolic conversion to the fruity smelling 3-mercaptopropan-1-ol is also favored during fermentation at about 20°C .

Short maceration limits both tannin and anthocyanin uptake, respectively, to about 25% and 10% of that of red wines—generating the wine's pinkish coloration. With few tannins, rosé wines tend to show poor color stability—much of the color being derived from free anthocyanins or their self-association or copigment complexes. Despite their relatively low anthocyanin content, they still act as important antioxidants. For example, they retard the oxidation of 3-mercaptopropan-1-ol (and the rapid loss of its desired aroma). In addition, low levels of catechin uptake are important in limiting other volatile thiols from forming nonvolatile complexes with oxidized phenolics ([Nikolantoni et al., 2010](#)). Sulfur dioxide also helps to protect aromatic thiols from oxidation but may contribute to muting their perception by forming nonvolatile sulfonates. Phenethyl acetate and isoamyl acetate are also significant contributors to the fruity flavor of many rosé wines ([Murat, 2005](#)).

It is important that maceration occurs under anaerobic conditions. This not only limits oxidation of important volatile thiols but equally protects anthocyanins from oxidative discoloration. Somewhat surprisingly, oxygen ingress after bottling reportedly favors the floral and fruity attributes of Grenache rosé wines ([Wirth et al., 2012](#)).

Adding pectolytic enzymes during maceration can improve anthocyanin extraction, enhancing color stability and improving flavor development ([Salinas et al., 2003](#)). Both features are important to the shelf life of rosé wines.

Typically, only free-run juice is used in rosé production. Unless the grapes were comparatively immature (before full coloration), the anthocyanin content of the press-run is often too high for use in rosé production. The anthocyanin content for rosé wines is generally in

the range of 20–50 mg/L. Tannins in the press-run juice can also donate excessive bitterness. The press-run juice may be added to the must used to produce a red wine or other wine products.

Occasionally a portion of the juice is drawn off from a red wine fermentation to produce a rosé. The remaining must is used to produce the red wine. The technique, called *saignée*, has particular value in years or with cultivars where color extraction is likely to be less than that desired for the production of a red wine.

To decrease the likelihood of oxidative browning and color change during aging, sulfur dioxide is usually supplied at bottling at levels equivalent to those of white wines. In addition, enologic tannins may also be added to increase color stability.

In a novel solution to grape waste in the juice industry, [Pedroza et al. \(2011\)](#) have investigated the addition of dehydrated red grape skins to white wine to produce a rosé. Apparently, the results have attributes equivalent to those of more traditionally produced rosé wines.

Red wines

Assessing the conditions and duration of maceration are no less difficult for red wines than for other wines. The major problem relates to the shifting states of the various phenolics extracted and their subsequent retention (e.g., bonding and precipitation with cell remnants and yeasts after fermentation). Correspondingly, extending the duration of maceration (before and during fermentation) may not necessarily increase wine coloration but can enhance color stability. In addition, anthocyanins and other flavonoids begin to interact in complex ways with each other and other wine constituents during fermentation, maturation, and aging. These changes are partially dependent on the increasing alcohol content of the must, the production of yeast metabolites, and slow nonenzymic reactions. Such modifications have significant influences on the wine's color stability as well as its ultimate gustatory, tactile, and flavor characteristics. These parameters vary with the cultivar, conditions during grape development, and the method of crushing as well as temperature and agitation during fermentation. Thus, general recommendations, in the sense of a paint-by-numbers approach, are impossible. Furthermore, it is becoming clearer that what may be an appropriate procedure for *vinifera* cultivars may not be applicable to hybrid and non-*vinifera* grapes ([Manns et al., 2013](#)).

One of the properties of French-American hybrids and other non-*vinifera* cultivars is their greater disease resistance. This relates to their propensity to produce pathogenesis-related proteins in response to biotic and

abiotic stresses. In wine, though, they can bind and precipitate phenolic compounds (Burtsch et al., 2017), thus limiting the wine's color stability and aging potential. These may not be disadvantages where the wine is consumed (young) but does limit the wine's appeal to those who consider a wine's long-aging potential as critical to quality.

Most maceration studies have focused on pigment and tannin rather than flavorant extraction. Anthocyanins, being more soluble, are the first extracted. As fermentation becomes active, ethanol production not only enhances solubility but also facilitates anthocyanin escape by increasing membrane porosity. Smaller oligomeric tannins (proanthocyanidins) are also comparatively water soluble, whereas larger polymeric tannins are more dependent on increasing ethanol content and must agitation for their extraction. The dynamics of tannin extraction, precipitation, and binding with cell constituents (primarily cell-wall polysaccharides) throughout the course of fermentation are still incompletely understood.

Both the style and consumer acceptance of wine can be dramatically affected by how the duration and conditions of maceration affect phenolic extraction. Thus, maceration provides one of the principal means by which winemakers can adjust the character of their wines. Short macerations (<24 h) typically produce a rosé. For the production of early maturing reds, maceration times are limited to about 3–5 days by pressing the fermenting must. Fermentation continues to completion off the pomace. This provides good coloration and avoids the uptake of excessive amounts of harsh-tasting seed tannins but extracts sufficient skin tannins to favor color stability.

Although often viewed negatively by some wine critics, the desirability and promotion of techniques producing early-drinking wines is not new, being strongly advocated for the best Médoc wines in Bordeaux as early as the 1870s (Thudichum and Dupré, 1872, page 324). However, would 4 years in barrel and 2 in bottle be interpreted as "early drinking" today?

Other options influencing the relative proportion of skin to seed tannins extracted involve grape maturity (Hanlin et al., 2010; Bindon and Kennedy, 2011), adding grape cell-wall polysaccharides to the must (Bindon et al., 2010), maceration temperature, and pumping-over and pressing methods (Bindon and Kennedy, 2011). Additional factors affecting the ratio of seed to skin tannins include the number of seeds per berry (Harbertson et al., 2002) and seed cuticle thickness. The latter, however, are largely beyond the winemaker's control other than by cultivar choice.

Most flavonoids reach peak extraction within about 5 days. A second extraction phase may begin after 15 days (Soleas et al., 1998). The latter is correlated with increased uptake of higher-molecular-weight tannins. It may also be associated with enhanced extraction of undesirable flavorants, such as methoxypyrazines from some cultivars (Kotseridis et al., 1999). Wines for long aging have often been macerated on the pomace for as long as 3 weeks. Although it results in a decline in free anthocyanin content, color stability is augmented by encouraging early polymerization with proanthocyanidins. However, whether the prolonged skin contact, traditional in some parts of France and Italy, is required for wines to age well is a moot point. Little agreement exists among winemakers or enologists.

Although the correlation between maceration, pumping over, and wine style is well known, the ease with which anthocyanins are extracted varies with the cultivar. Romero-Cascales et al. (2005) promote the idea of determining extractability and seed maturity indices to facilitate determining the timing (and method) of maceration. Their extractability index involves measuring anthocyanin content at two pH values—3.6 and 1. Assessment of the seed maturity index is equally important, as it markedly affects color stability. It is obtained using a method described by Saint-Criq et al. (1998).

An old technique influencing phenolic extraction, and undergoing renewed interest, is cold maceration (Feuillat, 1996). It has long been used in Burgundy but is now being experimented with worldwide. It involves a prefermentation maceration period (3–4 days) at cool (15°C) to cold (4°C) temperatures—somewhat equivalent to the cooling that often occurred in small, unheated Burgundian cellars at harvest time. Initiating cool maceration with the addition of dry ice (Alezandré-Tudó et al., 2016a) in combination with pulsed electric field (El Darra et al., 2013) and the addition of sulfur dioxide (Casassa et al., 2016) may be even more effective. Where fewer proanthocyanins are desired, heating the must at the end of the cold maceration treatment may be desirable (Koyama et al., 2007).

The procedure has been reported to slow but facilitate the extraction of phenolics, notably anthocyanins (Gil-Muñoz et al., 2009). This may be cultivar specific, as the observation has not been consistently reported (Casassa et al., 2016). In addition, flavor development is reported to be more complex and intense. For example, Heatherbell et al. (1996) note that cool maceration temperatures generated Pinot noir wines with more of a peppery or bitter aspect, whereas cold temperatures tended to accentuate a sweet blackberry attribute.

Differential flavor effects have also been noted using Airen and Macabeo (Peinado et al., 2004). Beneficial effects have occasionally, but not consistently, been reported with cultivars as different as Pinotage, Sangiovese, and Syrah. Some of these sensory effects may result from influences on the must flora (Maturano et al., 2015), possibly contributing to yeasts with higher β -glucosidase activity (Hall et al., 2017).

However, the improved color of red wines shown early in maturation may be short-lived. On aging, color intensity may approach that of those employing traditional treatments (Heatherbell et al., 1996). Cold maceration has some of the same benefits as enzyme treatment in improving color development.

A potential disadvantage of cold maceration is an increased risk of early *Brettanomyces* spoilage (Renouf et al., 2006). It also provides time for adaptation of the indigenous grape flora. Thus, growth of *Kloeckera* spp. may be enhanced when the must is warmed and fermentation begins. This can result in a depletion of the amino acid and micronutrient content, potentially increasing H₂S production during fermentation.

Typically, maceration occurs at ambient (warm) temperatures, extraction largely occurring by diffusion. As it increases relatively linearly with temperature, heating may be used to speed anthocyanin uptake, limit tannin absorption (extracted more slowly), and/or inactivate the laccase present in diseased grapes. For more details on thermovinification, see later in this chapter ("Color enhancement").

Adding sulfur dioxide promotes anthocyanin and phenolic extraction (Bakker et al., 1998), especially at cooler temperatures. Sulfite-addition products are more soluble in aqueous alcohol solutions than free anthocyanins. However, it causes anthocyanin bleaching (reversible) and delays early anthocyanin–tannin polymerization.

Another technique for improving color (and/or flavor) extraction is the addition of supplementary skins or seeds and skins during fermentation (Revilla et al., 1998). The procedure is partially based on an old Spanish technique termed *double pasta*. It involves the addition of extra pomace during fermentation. Enriching the must with grape skins or seeds may enhance the varietal character of the wine. The process also promotes color stabilization. To avoid producing an excessively tannic wine due to the increased uptake of catechins and dimeric proanthocyanidins, supplementation is usually limited to about one-third the pomace volume in the original must.

Except for cold maceration, little attention has been given to the extraction of aromatic compounds during skin contact. In one of the few studies on the subject, the "berry" aspect of Cabernet Sauvignon (potentially β -damascenone) increased during maceration, and the less desirable canned bean–asparagus aspect (presumably methoxypyrazines) was diminished (Schmidt and

Noble, 1983). In another study, extraction of the cysteinylated precursor of 3-mercaptop-1-hexanol (3 MH) increased in Merlot and Cabernet Sauvignon in relation to the duration of maceration (Murat et al., 2001). The precursor is preferentially (60%) localized in skin. Rotundone is another significant red wine odorant known to be extracted during maceration. However, similarly to anthocyanins, extending maceration may eventually result in a loss of aromatics if they bind and precipitate with yeasts and cell remnants. This again shows the significance of frequent assessment and experimentation by the winemaker.

Dejuicing

Dejuicers are particularly valuable when dealing with large volumes of must. By removing most of the free-run, press capacity can be used more efficiently and economically to extract the pressings.

Batch dejuicers consist of a columnar tank possessing a perforated basket at one end. The mass of must forces much of the liquid from the crushed grapes into the basket, from which it flows to a receiving tank (sump). Carbon dioxide pressure may be used to speed separation. When drainage is complete, the basket is raised to discharge the pomace for transport to a press.

Continuous dejuicers consist of a sloped central cylinder containing perforations that permit the juice to escape but retain the pomace. The pomace is moved up the cylinder by rotation of a central screw. The dejuiced material is directly dumped into a hopper for loading into a press. The upward flow of the crush generates the gravitational force needed to expedite juice release. The main disadvantage of dejuicers is the additional clarification that may be required due to the increased uptake of suspended solids.

Pressing

Presses of various designs have been used for at least five millennia. The earliest illustrations occur in ancient Egyptian tombs. Their initial function was simply to facilitate separating the juice from the seeds and skins. Only later did technological advances permit presses to become a sophisticated tool by which winemakers could influence wine attributes.

The earliest presses must have been both exceptionally inefficient and incredibly cumbersome to use. Crushed grapes were placed in a cloth sac held by poles at each end. Stylized drawings show men twisting the poles in opposite directions. Subsequently, the "press" was stretched by pulling on one end of the poles, while another horizontally positioned worker pushed the

poles apart in the center with his arms and legs (see [Darby et al., 1977](#)).

By the Third Dynasty (2650–2575 BC), presses are shown as being held fixed at one end while workers twist at the other, shortening the press and bringing the cloth taut against the crushed fruit. There seems little if any evidence of further advancement until Classical times. The beam-press is thought to have been developed by the Mycenaeans, possibly about 1600 BC. In this type of press, a large beam, affixed at one end and weighted down at the other by a heavy object, applied pressure on grapes in a sack or other flexible porous container. The next advance appears to have involved the use of a screw to control the application of pressure by the beam on grapes held in a slatted holder. It appears to have been invented in Greece by at least the second century BC. A wall fresco discovered in Pompeii illustrates its use in Roman times. The next advance eliminated the need for the beam, affixing the screw to a U-shaped support, with the pressure applied to a rounded plate positioned over the grapes in a slotted wooden container ([Forbes, 1957](#); [White, 1984](#)). Its advantages were discussed by Pliny the Elder (*Historia Naturalis*) and Vitruvius (*Architectura*).

The next major advance in press design seems to have been the application of hydraulic force in the thirteenth century. It replaced muscle power associated with a massive screw and lever action ([Plate 7.1](#)). Incorporation of a removable bottom permitted easier pomace discharge. Previously, presses had to be dismantled or the pomace shoveled out at the end of each press cycle. Both tasks were unpleasant, time-consuming, and labor-intensive.

Increasing the drainage area involved was another momentous development. It facilitated juice release by increasing the surface over which pressure was applied while reducing the flow path for fluid escape.



PLATE 7.1 Old vertical wine press in Kloster Erbach, Rheingau, Germany. Photo courtesy R. Jackson.

Diminishing the force required for juice extraction also produced juice or wine fractions of improved quality.

Placing the press on its side (horizontally) was another astute development, again increasing the surface area for liquid escape. It permitted the length (former height) of the press to be increased without difficulty. The horizontal orientation also permitted a section of the press to be hinged, providing access for both convenient filling and emptying. By suspending the press on heavy gears, the press could both be rotated for pomace crumbling (tumbling) as well as inverted for emptying ([Plate 7.2](#)). Crumbling breaks the compacted pomace produced during pressing and helps entrapped juice escape on subsequent pressings. Previously, chains or manual mixing were used to achieve crumbling. These had the disadvantages of potentially crushing the seeds and increasing juice clouding (due to the greater release of solids with the juice).

Another significant innovation was the development of the continuous screw press. By permitting uninterrupted operation, time-consuming filling and emptying cycles could be avoided. This is especially valuable when large volumes of must or wine need to be pressed in a short period. Its principal disadvantage involves an increase in suspended solids, requiring additional fining or clarification.

Because the free- and various press-run fractions possess different physicochemical properties, wine-makers can use these and how they are derived to influence the wine's character. Free-run fractions are clearer and possess lower amounts of skin-derived phenolics and flavorants but higher suspended solids (turbidity). Subsequently, press-run fractions contain decreasing amounts of suspended solids but higher pigment, tannin, and flavorant contents. Press-run fractions also are more



PLATE 7.2 Horizontal press showing chains used to crumble the press cake (rice hulls were added to aid juice extraction for frozen grapes in ice wine production). Photo courtesy of E. Brian Grant, CCOVI, Brock University, St Catharines, Canada.

likely to oxidize (possess more polyphenol oxidase); possess lower acidity (higher potassium contents); and have higher concentrations of polysaccharides, gums, and soluble proteins. Most wines are a judicious blending of both free-run and the first press-run fractions. Depending on the intentions of the winemaker and characteristics of the grapes, a portion of the second and possibly third pressing may be incorporated.

Not surprisingly, views vary considerably on the relative merits of using press-run fractions and the various types of presses. Until more is known about the dynamics of flavor extraction during pressing and how to predict its sensory consequences, the choice of press is likely to remain based more on subjective and anecdotal percepts than objective data.

Brief descriptions of the major types of presses are given below. Fig. 7.9 compares the operational characteristics of equivalent volumes of wine or juice pressed in vertical, horizontal, and pneumatic presses.

Vertical (basket) presses

Vertical presses generally consist of a series of concentrically arranged vertical slats between which the juice can escape. Pressure is applied hydraulically from above. The plate that presses the grapes is usually retracted before the sides are removed to extract or crumble the press cake. Alternatively, the bottom may be lowered.

Vertical presses are principally used with small lots of fruit or where use of horizontal or pneumatic presses may be impractical or uneconomic. In addition, they have a particular advantage in the pressing of frozen grapes, as in the production of ice wine. Breaking the press cake is achieved by direct access.

A variant of the vertical press is what was traditionally used in producing sparkling wine. It is much broader than standard versions. Although relatively slow in operation, maximizing the surface area-to-volume ratio increased its effectiveness in pressing whole grape clusters.

Horizontal (moving head) presses

A diagrammatic representation of a horizontal press is illustrated in Fig. 7.10. Both crushed and uncrushed grapes as well as fermented juice can effectively be pressed in horizontal presses.

Loading occurs through an opening in the upper portion of the press. Pressing is conducted by hydraulically forcing end plate(s) inward from one or both ends. The rate at which pressure is applied can be modified to suit the needs of the grape variety and characteristics of the press fractions desired. Fluid escape occurs between slats of, or slits in, the pressing cylinder. Chains and/or rotation of the press breaks the pomace cake between successive pressings. Once

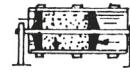
Press type	Vertical	Horizontal	Pneumatic
Size of the basket (cm)	113 x 90	215 x 73	215 x 73
Volume (m^3)	0.9	0.9	0.9
Pressure area (m^2)	1	0.42	4.95
Pressure per 1 cm^2 (MPa)	1.25 – 1.6	1.2	0.6
Pressure over the whole area (Mpa)	12,500 – 16,000	5000	29,700
Pressure per 1 dm of pomace (Mpa)	13.9 – 17.8	5.6	33.0
Average size of the cake (cm) at one half of the original volume	113 x 18	73 x 43	215 x 239 x 3.3
Shape of the cake			
Flowing out of the must (time)	long	Short	very short
Time of one pressing (min)	100 – 120	100 – 120	50 – 90
Number of pressings	2	1	1
Total time of pressing (hr)	3 – 4	2	1

FIGURE 7.9 Comparison of various types of presses. From Farkaš, J., 1988. *Technology and Biochemistry of Wine*, vols. 1 and 2. Gordon & Breach, New York, NY, reproduced by permission.

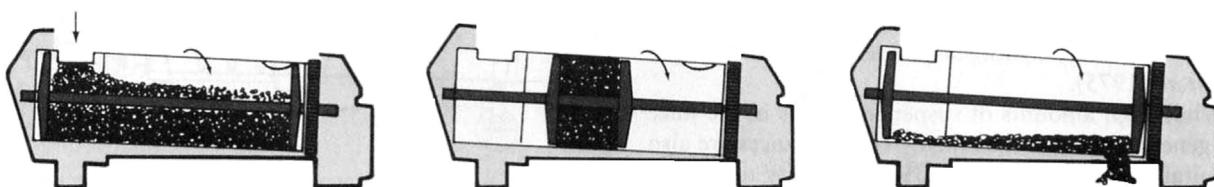


FIGURE 7.10 Diagram of the operation of a horizontal press. Courtesy of CMMC, Chalonnes-sur-Loire, France.

pressing is complete, retracting the end plate(s) and inversion permit dumping.

The primary drawback to most vertical and horizontal presses is the progressive reduction in drainage surface during pressing. Thus, the force required to maintain an adequate discharge rate increases during pressing. This correspondingly increases the extraction of suspended solids and tannins as pressing progresses. To maintain juice quality, the last portion of the juice may need to be sacrificed. The presence of grape stems can improve this situation. They create channels for easier juice escape. Slower pressing is also another option favoring juice quality. It provides more time for juice escape (and the release of aromatics from the skins and pulp—a form of maceration under increasing pressure).

Pneumatic (tank, bladder, or membrane) presses

Pneumatic presses avoid some of the deficiencies of vertical and horizontal presses as well as effectively press crushed or whole grapes and fermented must. The press is filled through an elongated opening in the top. Once filled and closed, the press is inverted to allow juice or wine (free-run) to escape. Compressed gas entering between a plastic bladder and solid outer cylinder wall compresses the grape mass against perforated plates that project along the central cavity (Fig. 7.11). Alternative models possess a central or

side-positioned bladder that forces the must or grapes against a perforated outer cylinder. Presses with a central bladder tend to be more efficient in extracting juice at lower pressures due to the must (or grapes) being pressed more uniformly against the whole surface area of the cylinder wall. Lower pressures exerted over a larger surface area liberate juice more quickly and with reduced extraction of suspended solids and phenolics. This is often of particular concern when extracting juice with little or minimal color, as with white or rosé wines. Crumbling the pomace cake between successive presses is achieved by rotating the press cylinder. Opening the filling trap and inversion discharge the pomace.

Small, medium, and large volume (5–22 hL) versions are commercially available. Smaller versions are of particular value when dealing with limited quantities of select lots of high-quality juice or wine.

Both horizontal and pneumatic presses yield high-quality pressings. They are relatively low in suspended solids, and pressing neither crushes the seeds nor extracts undue amounts of tannins. A common drawback involves the time associated with their repeated filling and emptying and comparatively fixed press cycle (about 1–2 h).

A recent development has been the design of presses extracting juice by negative (vacuum) rather than positive pressure.

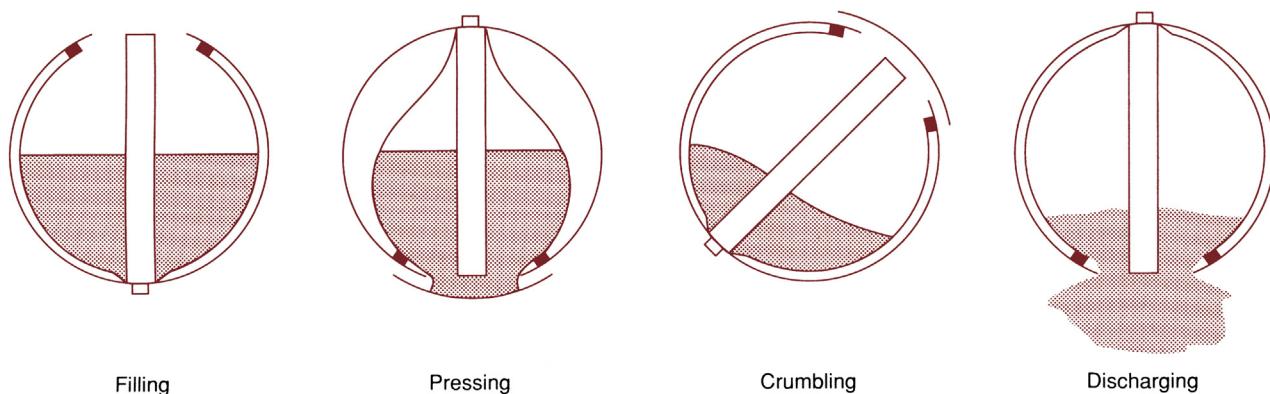


FIGURE 7.11 Diagram of the operation of a pneumatic press. Note the centrally located perforated plates for drainage and the inward-moving bladder membrane. Courtesy of Willmes.

Continuous screw press

Continuous-type presses have the advantage of running uninterruptedly. They avoid the time and labor costs associated with cyclical filling and emptying. Although they work best with fermented must, they can be adjusted to handle crushed, nonpulpy grapes. They do not function adequately with uncrushed grapes.

Crushed grapes as well as fermenting or fermented must are pumped into the press via a hopper at one end of the press (Fig. 7.12). A fixed helical screw forces the material into the pressing chamber, whose perforated wall allows the juice or wine to escape. Pressed pomace accumulates at the end of the pressing cylinder, where it periodically is discharged through an exit portal.

The primary disadvantage of the continuous press is the poorer quality of the juice or wine liberated. For example, the production of fruit esters during fermentation tends to be lower in juice derived from continuous screw presses. This is particularly noticeable in older models, in which separation of different press fractions was impossible. Newer models permit such a separation. The first fractions (closest to the intake) possess characteristics similar to free-run fractions. Fractions obtained approaching the end of the pressing cylinder progressively resemble the first, second, and third pressings of conventional presses. Slower pressing rates decrease the incorporation of suspended solids that diminish juice or wine quality but also reduce one of the principal advantages of continuous-type presses, namely speed.

Pressing aids, such as cellulose and rice hulls (Plate 7.2), may be added to improve extraction. The use of such preparations can increase free-run yields by up to 5%–15%. Occasionally, however, their addition may influence subsequent fragrance development. In addition, pectinases may be added to facilitate juice release—especially with slip-skin (*V. labrusca*) or other pulpy cultivars. Pectinase also eases filtering and improves clarity.

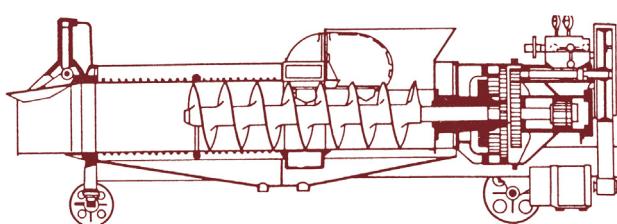


FIGURE 7.12 Schematic representation of a continuous press with hydraulic control. Courtesy of Diemme.

Must clarification

For white wine production, the juice is clarified before fermentation, partially to favor the retention of a fruity character. It could be masked by production of fusel alcohols associated with juice containing high levels of suspended solids. The largest particles seem particularly active in inducing fusel alcohol synthesis (Klingshirn et al., 1987). High levels of suspended solids have also been reported to increase hydrogen sulfide production (Singleton et al., 1975). In addition, much of the remaining polyphenol oxidase activity is associated with this fraction. Thus, early removal is desired to limit excessive enzyme-catalyzed oxidation.

Although large amounts of suspended solids are undesirable, excessive clarification is equally inappropriate. Filtration and centrifugation can remove more than 90% of the fatty acids from the must (Bertrand and Miele, 1984) as well as much of its sterol content (Delfini et al., 1993). This loss can slow alcoholic fermentation; retard malolactic fermentation; diminish yeast viability; and provoke excessive acetic acid production during alcoholic fermentation (Fig. 7.13). The last of these favors the formation of ethyl acetate, which has a much lower sensory threshold than acetic acid. Part of this tendency relates to polyphenol removal as well as unsaturated fatty acids that can limit yeast synthesis and the release of acetic acid (Delfini et al., 1992).

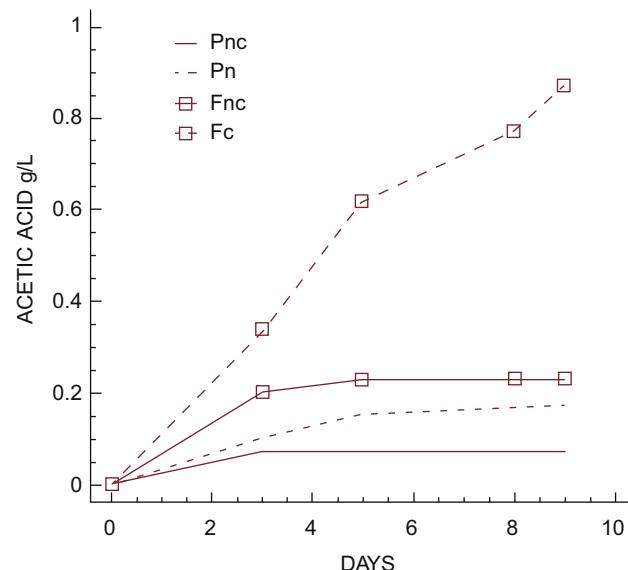


FIGURE 7.13 Acetic acid production by yeasts in free- (F) and press-run (P) grape juice, with (c) or without clarification (nc). From Delfini, C., Cervetti, F., 1991. Metabolic and technological factors affecting acetic acid production by yeasts during alcoholic fermentation. Wein-Wiss 46, 142–150.

In addition to influencing juice nutrient status, clarification procedures can variably reduce the indigenous microbial population derived from grapes. Both features can influence the dynamics of yeast growth and the number and types of species/strains that develop, not only in spontaneous but also in induced fermentation (Mora and Mulet, 1991).

Because suspended solids favor early malolactic fermentation, retention of small amounts can be beneficial in this regard. Concentrations between 0.1% and 0.5% seem desirable for several white wines (Groat and Ough, 1978). Nicolini et al. (2011) found similar results but noted that yeast strain had more influence on wine flavor than turbidity within the normal range. Suspended solids are usually measured in turbidity units, for which appropriate values range between 50–150 and 100–250 NTU (nephelometric turbidity units) depending on wine color, cultivar, and winemaker intentions. Within the range noted, juice fermentation usually goes to completion and is associated with synthesis of desirable fruit esters and higher alcohols. The precise reasons for these varied influences are poorly understood but may involve factors such as the adsorption of toxic carboxylic acids produced during fermentation; release of grape lipids (precursors for yeast cell membranes); and increased availability (concentration) of essential minerals and other nutrients adhering to the particulate matter. The colloidal content of the juice is also inversely related to the production and release of extracellular macromolecules during the early stages of fermentation, notably mannoproteins (Guilloux-Benatier et al., 1995).

White juice is frequently allowed to settle spontaneously for several hours (12–24 h). Settling is usually at cool temperatures (5–15°C), again depending on the preferences of the winemaker. Settling at the lower end of the temperature range is associated with the extraction of fewer flavonoids and more effectively limits any indigenous microbial activity. Nonetheless, it takes longer and retards the activity of enzyme additives. Particulate settling is primarily a function of size and the distance traveled for deposition. Butzke (2010) notes that larger particles, such as sections of grape skin (~100 µm), fall at about 1 m/15 min, whereas yeast cells (10 µm) settle at about 1 m/24 h.

Bentonite or potassium caseinate may be added to facilitate settling and subsequent protein stability. If used, bentonite is added after an initial period of spontaneous settling. This minimizes production of a voluminous loose sediment and the associated loss of juice. Occasionally, a portion of the precipitate may be left with the juice during alcoholic fermentation. This has been interpreted as permitting vital nutrients to remain available (e.g., sterols and unsaturated fatty acids).

Although bentonite is the most commonly used clarifying agent, its effects on wine quality remain contentious (Groat and Ough, 1978; Weiss and Bisson, 2002). Part of this may relate to extensive variation in the timing and rate of application, vintage conditions, and cultivar specificity. In addition, the removal of important fatty acids, such as palmitic, oleic, linoleic, and linolenic acid as well as squalene, stigmastanol, and β-sitosterol, is difficult to predict or control. Controversy about accelerated clarification also applies to alternative treatments (Moio et al., 2004; Armada and Falqué, 2007).

Other aspects that may influence the outcome of clarification include the removal of amino acids by bentonite. This could influence their involvement in flavor development, notably higher alcohol synthesis and their various esters. In contrast, potassium caseinate tends to remove primarily polyphenolics.

Another alternative clarification technique is centrifugation. Because particles are removed based on size (mass), centrifugation can be adjusted as seems appropriate to limit its effect on juice chemistry. Although centrifuges are expensive, minimal juice loss and speed have made them particularly popular. In contrast, an alternate technique, termed vacuum filtration, tends to remove solids excessively, resulting in longer fermentation times and generating more volatile acidity (Ferrando et al., 1998). Diatomaceous earth may be added as a filter aid in some forms of prefermentative clarification.

Flotation is another clarification technique (Ferrarini et al., 1995). It involves the implosion of microbubbles of gas (air, nitrogen, or oxygen) into the juice under pressure (5–6 ATM). As the gas rises to the surface, suspended solids adhere to the bubble surface. Adherence may be assisted by the addition of adjuvants, such as gelatin or bentonite. The flocculated solids are subsequently skimmed off. The procedure has several advantages, relative to other clarification techniques, notably better control over the degree of clarification as well as limited fatty acid removal (Cocito and Delfini, 1997). In addition, clarification can be achieved in less time and at ambient temperatures (avoiding the typical refrigeration associated with static settling). The technique also permits simultaneous hyperoxygenation if desired. In addition, it can be used as a pretreatment to improve the efficiency of procedures such as cross-flow filtration.

Adjustments to juice and must

Acidity and pH

Juice and must that lack the desired acidity or are too high in pH ideally should be adjusted before

fermentation. Acidification at this time has the benefit of limiting microbial spoilage and favoring better flavor development. In addition, postfermentative acidification is illegal in some jurisdictions. Acidification may be required, especially in warm to hot viticultural regions, due to extensive malic acid degradation near the end of grape maturation.

In contrast, deacidification typically occurs after fermentation. At this point, fermentative changes in wine acidity are complete. Deacidification can thus be based on actual rather than predicted need. In addition, postfermentative deacidification may be delayed until spring, when other winery activities are less exigent.

No precise recommendations for optimum acidity or pH values are possible. "Ideal" values are too dependent on wine style and winemaker preferences. Nevertheless, the acceptable range for total acidity in most wines generally falls between 5.5 and 8.5 mg/L. White wines are generally preferred at the higher end of the scale, whereas red wines are more appreciated at the lower end. For pH, a range between 3.1 and 3.4 is generally preferred for white wines, and between 3.3 and 3.6 for most red wines. Somewhat lower values are usually desired in the juice or must because pH often increases slightly during or after fermentation (frequently as a result of tartrate crystallization). Adequate acidity not only is important for generating a clean fresh taste and favoring color stability but also is central to optimal aging and limiting microbial spoilage.

One of the oldest pH adjustment procedures is plastering (Illustration 7.1). On addition, gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) converts some of the potassium bitartrate to the free acid form

This ancient form of acidification is rarely, if ever, used today due to undesirably increasing the wine's sulfur and calcium contents. In addition, organic acids for acidification are readily available, are relatively inexpensive, and do not similarly disrupt wine chemistry.

The high pH associated with low total acidity is most simply corrected by direct acidification—the addition of organic acids (Buechsenstein and Ough, 1979). Tartaric acid is typically used because of its relative insensitivity to microbial decomposition. It also has the advantage of being a natural wine constituent. It decreases the pH by

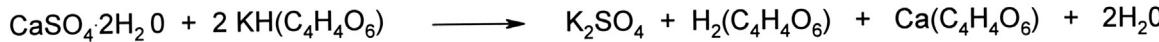
inducing the precipitation of excess potassium (as a bitartrate salt). Another alternative is lactic acid. It not only increases the wine's fresh acidic taste but is also considered by some to enhance the perception of "body." Although citric acid may be substituted (it also facilitates iron stabilization), it is susceptible to microbial degradation by lactic acid bacteria. This could lead to undesirably high concentrations of diacetyl.

Cases of excessive acidity may be rectified by blending in juice of lower acidity (where available). Alternatively, some of the acid may be neutralized with calcium carbonate, potassium carbonate, or Acidex. If calcium carbonate is used, it is usually added before malolactic fermentation to limit the production of significant quantities of calcium lactate—which can generate a bitter taste (Lawless et al., 2003).

The most difficult situation occurs when the juice shows both high total acidity and high pH. This seeming anomaly is particularly common in cool climatic regions, where grapes may possess both high malic acid and high potassium content. Nagel et al. (1988) suggest adding tartaric acid to adjust the malic/tartaric ratio to unity. This is followed by precipitation of the excess potassium by Acidex. Subsequent addition of tartaric acid can provide both desirable acidity and pH.

Amelioration achieves deacidification by dilution with water. Because it simultaneously reduces the sugar content, the addition of sugar is required to readjust the °Brix. Although amelioration is illegal in most countries, it has the property of having little effect on juice pH. This results from the dicarboxylic nature of tartaric acid and its low dissociation constant. The dilution of H^+ , which results from the addition of water, is counterbalanced by increased dissociation of tartaric acid. Thus, total acidity falls, but the pH is only marginally affected. Although reduced color, body, and flavor may be consequences of amelioration, these effects may actually be desirable in intensely flavored varieties. Nevertheless, the greatest disadvantage of amelioration is consumer image. The addition of sugar, and especially water, is commonly viewed by connoisseurs as unprofessional if not unscrupulous.

Postfermentation adjustments to acidity and pH are discussed in Chapter 8.



Calcium sulfate + Potassium dihydrate

Potassium + Tartaric + Calcium + Water
sulfate acid tartrate

ILLUSTRATION 7.1 The chemistry of wine plastering.

Sugar content and alcohol potential

Sugar content is usually assessed with a hydrometer at the beginning of fermentation. It provides a quick-and-easy method of assessing specific gravity, an indicator of total soluble solids and sugar content (at values above 18 °Brix) (Crippen and Morrison, 1986). In the United States, these units are measured in °Brix, whereas in Europe, the units are usually measured as Balling, Baumé, or Oechsle (Appendix 6.1). More precise measurements are available, but hydrometer determinations are sufficiently precise early in the winemaking process. In the field, refractometer readings are used to measure juice sugar content.

As fermentation progresses, hydrometer readings become increasingly imprecise measures of sugar content. This results from alcohol's effect on specific gravity readings. Nevertheless, specific gravity can still be used to adequately indicate the termination of fermentation in dry table wines. For sweet fortified wines, correction tables are necessary for this purpose (Amerine and Ough, 1980).

Following the completion of fermentation, a precise chemical analysis of the wine's residual sugar content is usually required (Zoecklein et al., 1995). Even small amounts of residual fermentable sugars can affect microbial stability and therefore how the wine should be treated up to and including bottling.

If the sugar content of the must is insufficient to generate the desired alcohol content, chaptalization may be employed. This involves the addition of a concentrated sugar solution. It was advocated in 1801 by Dr. Chaptal as a means of improving the stability and character of wines produced from immature or rain-swollen grapes. The increased fermentative capacity of the juice not only increases the alcohol content but also extends the fermentation process, modifying and improving the wine's fragrance. Although honey or concentrated grape juice has been added to wine since ancient Greek and Roman times (to increase sweetness), adding these before fermentation is not mentioned. The connection between alcoholic strength and sugar content was noted by the end of the 1600s (Houghton, 1699–1700), but its relevance to winemaking seemingly was unrecognized until much later.

Chaptalization is typically illegal in regions or countries where warm growing conditions obviate its need but is usually permissible where cool climates may limit full grape ripening. Nonetheless, even where legitimate, chaptalization is permissible only with specific governmental approval.

Although many factors influence the conversion of sugar to alcohol (Jones and Ough, 1985), 17 g sucrose typically yields about 10 g ethanol. For chaptalization, the sugar is first dissolved in grape juice and added near the end of the exponential phase of yeast growth

(about 2–4 days after the commencement of active fermentation) (Ribéreau-Gayon et al., 1987). By this time, yeast multiplication is essentially complete, and the sugar does not disrupt fermentation. The disaccharide sucrose is quickly converted into equimolar amounts of glucose and fructose. Concurrent aeration of the fermenting juice or must is typically recommended.

In addition to elevating the alcohol content, chaptalization slightly augments the production of certain compounds—for example glycerol, succinic acid, and 2,3-butanediol (see Figs. 7.22 and 7.23). The synthesis of some aromatically important esters may also be increased, whereas others are decreased (see Fig. 7.39). In some varietal wines, such as Riesling, chaptalization can diminish the green or unripe taste derived from immature fruit (Bach and Hess, 1986). However, these influences cannot compensate for the lack of varietal character found in immature grapes or those engorged by rains.

Another technique used to improve the character of wines produced in poor vintages, without the addition of sugar, is reverse osmosis (Duitschaeffer et al., 1991). Although first designed as a means of obtaining freshwater from seawater, reverse osmosis has found many applications in other industries from sewage treatment to fruit-juice concentration. It is the latter application that attracted the attention of enologists. In addition to offsetting problems, such as juice dilution associated with preharvest rains, reverse osmosis can concentrate fruit flavors. Reverse osmosis operates by forcing water out of the juice through a membrane that retains most of the sugars, nutrients, and flavorants. Regrettably, membranes are not fully impermeable to varietal aromatics. Thus, important aroma components may be lost (Mietton-Peuchot et al., 2002). Small, highly volatile water-soluble compounds, such as esters and aldehydes, are the most likely to be lost. Thankfully, most of these are fermentation by-products produced after treatment. Any varietal character loss has the potential of being rectified by the addition of untreated juice. Concentration of volatiles removed with the water and their reintroduction into the treated juice constitute another option. Development of filters with improved selective permeability may eliminate this issue. Another potential problem is augmentation in total acidity, but where marked, it can be rectified with deacidification.

Cryoextraction is an alternative technique that can overcome deficiencies in sugar and flavor content (Chauvet et al., 1986). As with reverse osmosis, cryoextraction can be used with immature grapes or berries swollen with water after rains. It may also be used to augment the sugar and flavor content of grapes in the production of sweet table wines. Cryoextraction is the technical equivalent of ice wine production, except

that overmature grapes are not used. Cryoextraction involves freezing the grapes and their subsequent pressing while frozen. As water in the grapes freezes, dissolved substances become increasingly concentrated in the remaining liquid. Because berries of greater maturity (and thus greater sugar content) freeze more slowly than immature grapes, cryoextraction tends to favor juice concentration in immature grapes. Although temperatures down to -15°C increase solute concentration, temperatures between -5 and -10°C are generally sufficient to remove unwanted water (which remains as ice with the pomace after pressing). Cryoextraction appears not to produce undesirable sensory consequences. Probably its most significant limitation is the cost/benefit ratio, especially if the equipment is needed rarely. The same limitation also applies to the Entropie concentrator. This is another juice concentration procedure that uses vacuum but at ambient temperatures ($\sim 20^{\circ}\text{C}$) (Froment, 1991).

Reducing alcohol content

Although sugar adjustment is usually designed to increase °Brix, there is also a growing market for low-alcohol wines. Reduced alcohol content is usually generated by some form of dealcoholization following the completion of fermentation (see Dealcoholization, Chapter 8). However, the joint action of glucose oxidase and peroxidase can diminish the capacity of juice to support ethanol production. Glucose oxidase converts glucose to gluconic acid, a nutrient that yeasts cannot ferment. Hydrogen peroxide, produced as a by-product of glucose oxidation, is converted to water by peroxidase. Two reactions are involved (Illustration 7.2)

With glucose oxidase, alcohol production can be reduced by about 50%, equivalent to the juice glucose content. Thus, ethanol production is dependent on the remaining fructose. Because of the oxygen required, the juice becomes oxidized and turns brown. Although most oxidized compounds precipitate during fermentation, the wine is still left with a distinct golden tint. The generally undesirable sensory aspects of this process are discussed in work by Pickering et al. (1999). Nonetheless, limited use (to slightly reduce the alcohol-

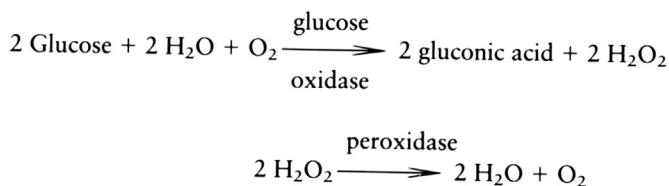


ILLUSTRATION 7.2 The reactions involved in the use of glucose oxidase and peroxidase to reduce the ethanol potential of grape juice.

producing potential of high °Brix red musts) may be beneficial (Biyela et al., 2009). The degree of oxygen involved can resemble that used in hyperoxidation. This could offset modern tendencies of harvesting grapes later in the belief that this achieves higher flavor and coloration.

Alternatively, particular *Saccharomyces cerevisiae* strains can divert more fermentation by-products to compounds, such as glycerol, rather than alcohol (Kutyna et al., 2010). This could also be useful in generating a smoother mouthfeel. However, a potential disadvantage of the excess NAD⁺ generated in glycerol synthesis is the increased likelihood of acetaldehyde being oxidized as acetic acid. Although this might be minimized by using a strain of yeast deficient in aldehyde dehydrogenase, it would result in an undesirable increase in acetaldehyde content. Adjusting a metabolic pathway to solve one problem may unexpectedly generate others (Varela et al., 2012). Alternatively, the use of non-*Saccharomyces* yeasts (e.g., *Candida zemplinina* and *Metschnikowia pulcherrima*), either alone (Quirós et al., 2014) or in combination with *S. cerevisiae* (Contreras et al., 2015), can limit ethanol production. This requires limited aeration to favor respiratory metabolism.

In a novel approach, unripe grapes (harvested during cluster thinning) are fermented to produce a low-alcohol, acidic wine (Kontoudakis et al., 2011)—somewhat equivalent to producing a *verjus*. The wine is treated with bentonite and charcoal to produce a flavorless wine that is added to wine made later from fully ripened grapes. It increases the wine's acidity while reducing its alcohol content. The resultant blend has a more moderate (traditional) alcohol content, and the acidity enhances color intensity and freshness. The procedure apparently does not negatively affect fragrance. To date, trials have involved Cabernet Sauvignon, Merlot, and Bobal as well as Verdelho and Petit Verdot grapes (Longo et al., 2018). Another alternative, now permissible in some jurisdictions, is diluting the juice/must with water. It is used to compensate for the juice concentration that results from grape shriveling (Schelezki et al., 2018). The process seemingly does not negatively affect the wine's sensory attributes (Bindon et al., 2014).

Other procedures under investigation involve vacuum distillation or stripping with carbon dioxide halfway through fermentation (Aguera et al., 2010). Both can reduce alcohol content by a moderate amount ($\sim 2\%$). Although seemingly a stress for the yeasts, fermentation came to completion normally. The reduction in alcohol content had only minor influences on the wine's sensory attributes. Another approach is to directly reduce the sugar content of the must via its selective removal with nanofiltration (Mihnea et al., 2012).

Color enhancement

Cultivars such as Pinot noir rarely produce dark red wines. This is exacerbated by standard vinification procedures that may extract little more than 30% of a grape's anthocyanin content. Most anthocyanins remain with the pomace or precipitate with cellular remains in the lees.

Where poor coloration is typical, several techniques may enhance anthocyanin extraction and/or color development. Some have already been noted (cold maceration, pressing variations, *saignée*) or are discussed later (enzyme addition, various pumping-over options, choice of yeast strain, or the addition of enologic tannins). In many instances, their relative benefits depend on whether deficiencies involve copigmentation cofactors or the extraction of flavonoids active in polymeric pigment formation.

Thermovinification refers to a range of procedures where grapes (or must) are heated prior to fermentation. Alternatively, heating may occur at the end of fermentation, before pressing, as occasionally done with several cultivars in southern France.

Thermovinification is primarily used to enhance the extraction of anthocyanins in cultivars relatively low in their content (Fig. 7.14) or with diseased grapes (contaminated laccases). Rapid (a few seconds) heating to between 50 and 80°C inactivates these enzymes. With moderate *Botrytis* infection, red grapes need to be heated to above 60°C.

Other versions of thermovinification entail exposing whole grapes to steam or boiling water (flash heating). Such treatments are typically short in duration (~1 min) and heat only the outer pigment-containing layers of the fruit to ~80°C. This inactivates phenol oxidases and kills skin cells, facilitating the quick release of anthocyanins during subsequent maceration (~45°C for 6–10 h). Other versions consist of heating some or all of

the pomace or both the pomace and the juice. The juice and pomace are typically rapidly heated (in a tubular heat exchanger) to between 55 and 70°C. Heating may occur with or without continuous stirring.

A more recent variation, termed *flash détente* (Price, 2013; Sebastian and Nadeau, 2002), involves short heating at 80–85°C. The must is then piped to a vacuum flash cooling system, where the temperature rapidly drops 30–32°C. This causes cell rupture, favoring anthocyanin extraction, without the potential flavor modifications that may accompany conventional thermovinification.

Maceration times vary inversely with the temperature (the higher the temperature, the shorter the duration) (Wiederkehr, 1997) and the desires of the winemaker. If treatment is likely to damage subtle varietal aromas, temperatures as low as 50°C may be used. For especially delicate varieties, such as Pinot noir, heating may be as low as 32°C for 12 h (Cuénat et al., 1991). Subsequent vinification may be conducted in the presence or absence of the seeds and skins. Alternately, the must may be pressed immediately after heating (leading to the term "hot pressing"). The juice is then cooled to an appropriate temperature and fermentation initiated. Each variation influences the wine attributes generated.

Possibly because thermovinification has little effect on tannin extraction, the procedure is also useful in producing wines for early consumption. In addition to generating a rich color, thermovinification improves juice fermentability (both alcoholic and malolactic); produces wines low in astringency; reduces vegetal odors; and may enhance fruit flavor development. Certain varietal aromas, such as those from *Vitis labrusca* cultivars, tend to be diminished and made more acceptable to consumers habituated to European wines. In addition, it may reduce the vegetative, grassy aromas that may mar wines made from some red French-American hybrids. The procedure allows the natural fruity fragrances of most grapes to express themselves. This has led to a

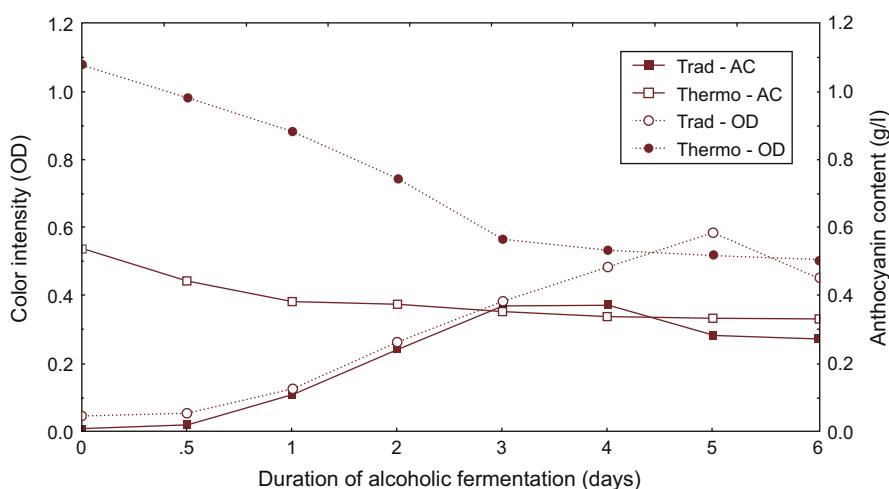


FIGURE 7.14 Development of color intensity (OD) and level of anthocyanins (mg/L) during fermentation. AC, anthocyanin content; OD, optical density; Thermo, thermovinification; Trad, traditional fermentation. Fermentation ended after 5 days with a traditional fermentation and 3 days for thermovinification. Reproduced from data in Ribéreau-Gayon, J., Peynaud, E., Ribéreau-Gayon, P., Sudraud, P., 1976. *Traité d'Oenologie, Sciences et Techniques du Vin*, vol. 3. Dunod, Paris.

trend in the eastern United States of blending red wines fermented traditionally with those derived from thermovinified musts.

The rapid fermentation that follows thermovinification has several benefits including the efficient use of fermentor capacity. However, the associated rapid heat buildup increases the need for effective fermentor temperature control.

Occasionally, thermovinification generates undesirable bluish colors and cooked flavors. These usually can be avoided by adjustments, such as strict exclusion of oxygen and keeping the duration of heating as short as possible. Surprisingly, thermovinification provides a richer red color (less blue) with red French-American hybrids. Difficulties with clarification and filtering may also be experienced with thermovinification due to the denaturation of grape pectinases. This can be corrected by posttreatment addition of pectinase.

An alternative to thermovinification is a pulsed electric field (PEF), where millisecond, high-voltage (10–60 kV) pulses are applied at the onset of maceration. It disrupts cell membranes, favoring the release of cellular constituents. It is particularly useful in liberating phenolic compounds, notably anthocyanins (Plate 7.3). As such, the need for extended fermentative maceration is reduced as well as the expense associated with thermovinification or enzyme treatments. For example, PEF increased the release of anthocyanins from Aglianico grapes by more than 75%, whereas polyphenolic content was increased by about 20% (Donsi et al., 2010). However, the same treatment had little effect on anthocyanin release from Piedirosso grapes. Differential varietal effectiveness has been found with other cultivars (Lopez et al., 2008). PEF may be most useful with cultivars where extraction is the most difficult. A distinctly different use for PEF is nonthermal microbial sterilization. For this, considerably higher voltages are required. For a review see Puértolas et al. (2010).

Accentuated cut edges is another new technique designed to enhance anthocyanin extraction. It was initially developed to enhance the color depth and stability of Pinot noir wines (a cultivar with thin skins, low pigment content, and limited color stability). The process involves increasing the number (by reducing the size) of skin particles relative to that generated during crushing (Sparrow et al., 2016a). Correspondingly, the surface area over which anthocyanins can escape is increased. By facilitating anthocyanin extraction, earlier pressing is possible, limiting the uptake of bitter, astringent tannins while still favoring the release of sufficient skin tannins to stabilize the wine's color. The technique also marginally enhances the wine's fruity character (Sparrow et al., 2016b).

Red wines are often treated to microoxidation (see Chapter 8) to enhance color stability. Its rationale is based on the production of acetaldehyde and its promotion of tannin–anthocyanin polymerization. Nonetheless, this can equally result in undesirable oxidative reactions and the activation of microbial contaminants. An alternative suggestion is the direct addition of acetaldehyde to fermenting must. Its addition also can result in reduced astringency and other sensory attributes (Aleixandre-Tudo et al., 2016b). Some of these benefits are equally achieved with the slow oxygen uptake of oak barrels (Sheridan and Elias, 2015).

Enzyme addition

Advancements in microbiology and chemical purification have permitted the isolation of enzymes in commercial quantities. Their use is now commonplace in many industries including wine production. Filamentous fungi, notably *Aspergillus* and *Trichoderma* spp., are the primary sources of those authorized for wine use. They have the advantage of being active within the pH range and SO₂ conditions found in wine.

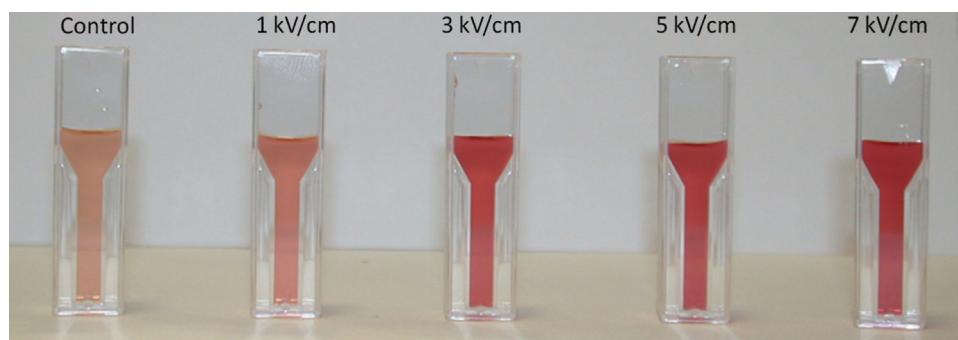


PLATE 7.3 Visual aspect of the Garnacha must after 1 h of maceration with grapes untreated and treated by PEF (50 exponential decay pulses; 1–7 kV/cm; 0.4–4.1 kJ/kg. Photo courtesy Dr. Raso; reprinted from Puértolas, E., López, N., Condón, S., Álvarez, I., and Raso, J., 2010. Potential applications of PEF to improve red wine quality. Trends Food Sci. Technol. 21, 247–255., with permission from Elsevier.

Enzyme preparations may be added to facilitate the release of free-run juice; aid wine clarification, filtration, decoloration, and dealcoholization; enhance flavor development; or augment anthocyanin liberation. Regrettably, most commercial enzyme preparations are not fully purified (Fig. 7.15), possessing collateral enzymic activities. In addition, isozymic forms have different pH and temperature optima. Consequently, their effects can vary depending on the supplier, wine composition, and temperature. This can occasionally result in unanticipated or undesired effects (Fia et al., 2016). As usual, it is judicious to conduct small trials with any new product to ascertain its effects under local conditions. This situation has been partially simplified with a comparative study of the enzymic properties of many preparations (Guérin et al., 2009). For additional details see Ugliano (2010) and O'Kennedy and Canal-Llaubères (2013a, b).

Most wine grapes lose their pulpy texture and become juicy as they ripen. In some situations, though, juice extraction is improved by the addition of

commercial pectolytic enzyme preparations. Most are derived from *Aspergillus* (usually *A. niger*). Polygalacturonase preparations are particularly valuable with slip-skin (*Vitis labrusca*) cultivars. They may also be useful if added after pressing to improve juice clarity and filterability. Degrading colloidal pectins limits their tendency to clog filters. In addition, degraded pectins possess more negatively charged sites, facilitating their association with positively charged suspended particles. As the complexes possess greater mass and are less hydrophilic, they are more likely to precipitate spontaneously, thereby minimizing clarification issues.

Special pectolytic preparations are also available to assist color and flavor release (Wightman et al., 1997; Revilla and González-San José, 2003) or limit the astringency of red wines (Ducasse et al., 2010). These actions partially relate to their macerating influence (inducing cell death and tissue disintegration). The benefit is often variety specific and primarily useful with lightly colored red or slip-skin cultivars. In the former, the principal

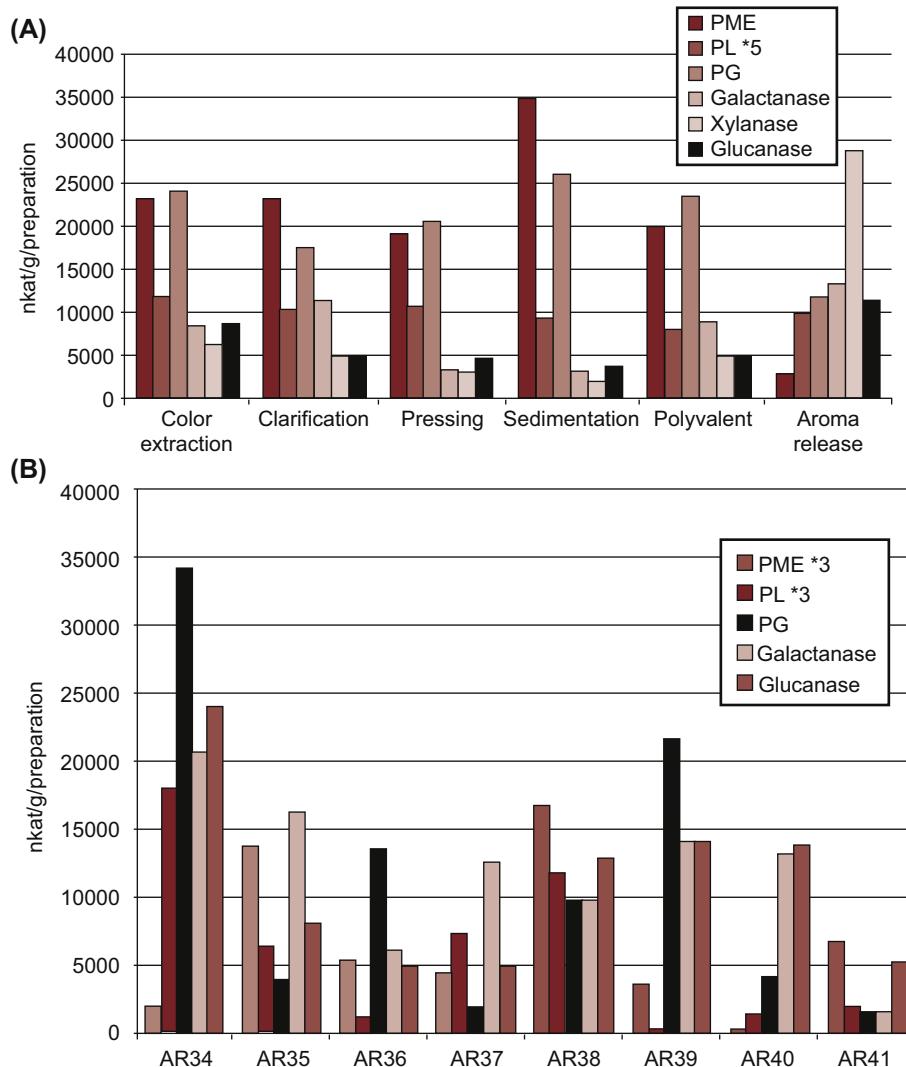


FIGURE 7.15 Pectolytic (pectin methyl esterase [PME], polygalacturonase [PG], and pectin lyase [PL]), cellulolytic (glucanase), and hemicellulolytic (galactanase and xylanase) activities (expressed in nanokatal/g preparation) of (A) 41 commercial enzyme preparations, sorted by technological interest, and (B) aroma release (AR) preparations (1 nkatal = 1 nmol/s enzymatic reaction product). From Guérin, L., Sutter, D.-H., Demois, A., Chereau, M., Trandafir, G., 2009. Determination of activity profiles of the main commercial enzyme preparations used in winemaking. Am. J. Enol. Vitic. 60, 322–331, reproduced with paid permission, conveyed through the Copyright Clearance Center.

benefit may accrue from the release of additional color-stabilizing phenolics (Bucelli et al., 2006); in the latter, degradation of pectins facilitates anthocyanin liberation.

Although commercial pectinase preparations primarily contain pectin lyase, they often possess additional enzymic attributes. Pectin lyase degrades methylated pectins (releasing methanol), whereas preparations with polygalacturonase and pectin methyl esterase activities release less methanol (Revilla and González-San José, 1998). Additional actions (such as hemicellulase activity) may be incorporated to further assist color extraction and filterability.

In addition, preparations may possess β -glucanase activities. They typically come from *Trichoderma harzianum*. These are generally designed to enhance juice or wine clarification—especially with grapes possessing significant amounts of viscous glucans (e.g., due to *Botrytis* infection). In this situation, the enzyme preparation is best applied immediately after juice release before phenolic extraction and subsequent ethanol accumulation can disrupt enzymic activity (Zinnai et al., 2010). Other glucanase preparations may be used after fermentation to promote earlier yeast autolysis, releasing manoproteins and other cellular constituents.

Most preparations now possess little cinnamoyl (cinnamyl) esterase activity. Cinnamoyl esterase breaks the bonding between hydroxycinnamates and tartaric acid (notably caffeoyl tartrate, but also fertaric acid). Deesterification converts hydroxycinnamates into forms decarboxylated to vinylphenols by many strains of *Brettanomyces*. Some strains of *Saccharomyces cerevisiae* can also deesterify hydroxycinnamates. At above-threshold values, vinylphenols can generate an undesirable phenolic odor (Chatonnet et al., 1992). More significantly, further metabolism by *Brettanomyces* generates barnyard-like ethyl phenols. This is primarily a problem with red wines—their being more frequently contaminated with *Brettanomyces* due to maturation in oak. One potential benefit, though (in the absence of *Brettanomyces*) is the polymerization between vinylphenols and anthocyanins (Morata et al., 2007). Vinyl pyrananthocyanins are much more resistant than free anthocyanins to oxidation and sulfur dioxide decoloration.

Some pectinase preparations possess β -glycosidase activity, effective in releasing aromatics glycosidically bound in nonvolatile sugar complexes. Glycosidic linkages rupture naturally under acidic conditions (Mateo and Jimenez, 2000). However, this is slow, and heating to speed the process induces flavor damage. Thus, most attention has been directed toward enzymatic hydrolysis, especially white wines designed for early consumption. Addition occurs at the end of fermentation, because sugars in the juice inhibit their catalytic action (Canal-Llaubères, 1993). Although liberating free terpenes and

norisoprenoids may be desirable, this does not apply to volatile phenols. Thus, β -glycosidase preparations may not be desirable, notably for red musts (Lao et al., 1997).

Of enzyme preparations, β -glucosidases have been the most studied, even though preparations containing activities affecting α -arabinosides, α -rhamnosides, β -xylosides, and β -apiosides improve their effectiveness. This results from flavorants being occasionally bound to sugars other than glucose. These sugars may need to be removed before glucosidase can have its effect. Thus, preparations intended to enhance flavor release should be assessed in trials. Accentuated aroma release may disrupt the wine's traditional characteristics (e.g., Sauvignon blanc and Chardonnay) or sacrifice aging potential (the slow release of aromatics can replace or compensate for those that oxidize or escape via the closure during aging).

Inactivation of most enzyme preparations can be achieved by the addition of agents such as bentonite. Nonetheless, immobilization on plates, and regulating the rate of wine passage over these plates, has the greatest potential to modulate enzymatic activity (Caldini et al., 1994).

Considerable concern was aroused several years ago when it was discovered that wines, especially those that were heated during processing, possessed a suspected carcinogen—ethyl carbamate (urethane). Ethyl carbamate can also form spontaneously as a reaction by-product between ethanol and urea. Urea can occur in wine as a result of arginine metabolism; be supplied directly as nitrogen supplement during fermentation; or be a residue from its uptake by roots following vineyard fertilization. Urea presence can be influenced by the choice of yeast strain (Dahabieh et al., 2009); by avoiding urea addition during fermentation; or by the addition of urease (Ough and Trioli, 1988).

One of the major developments in enzyme application involves immobilization on or within an inert support. This limits activity to only the time and portion of wine passing across (or through) the support. The technique has several distinct advantages. It permits better control over the degree of modification; increases use efficiency; and avoids adding protein to the wine (possibly complicating protein stability). Although more costly, the advantages may outweigh the cost differential.

Other adjustments

The addition of nitrogen (typically ammonium salts) and vitamins is usually unnecessary but can significantly improve the fermentability of botrytized and highly clarified white juice. When required, addition is more effective when supplied periodically rather than in a single dose at the onset of fermentation. Nitrogen is often supplied as diammonium phosphate (DAP) or ammonium hydrogen phosphate. Addition beyond established need can undesirably enhance phosphate contents and

off-odor production (see [Vilanova et al., 2015](#)). Nitrogen addition is rarely needed with red wines, as adequate supplies are available from the pomace.

Other additions may include yeast extract (to favor malolactic fermentation) and tannins (to must from red grapes low in phenolic content) ([Alcalde-Eon et al., 2014](#)).

Blending

In white wine production, it is common to combine free-run juice with the first pressing. Occasionally, the second pressing may also be added. Other pressings usually are too tannic and complicate clarification to justify their use. However, where warranted, several finings and centrifugations can permit their incorporation with the other fractions. Alternatively, late pressings and the pomace may be fermented to obtain alcohol for distillation or used in vinegar production.

For grape varieties such as Riesling, pressings may contain two to five times the concentration of terpenes found in the free-run juice ([Marais and van Wyk, 1986](#)). Because of their importance to the distinctive aroma of certain cultivars, the addition of pressings may enhance wine quality. The distribution of individual monoterpenes within the fruit varies with the cultivar, grape maturity, and the relative amounts of free and bound terpenes ([Park et al., 1991](#)). Thus, the addition of pressings may affect both the quantitative and qualitative aspects of a wine's aroma. Their use has some of the same benefits as maceration but offers the winemaker greater control.

For red wines, blending usually occurs at the end of fermentation. However, when it occurs prior to fermentation, it usually involves must from grapes derived from different sites or vineyards. Occasionally, though, it may involve must from several red varieties (a procedure termed mixed winemaking or co-winemaking). Although uncommon, it can enhance copigmentation and color stability ([Lorenzo et al., 2005](#)). The procedure may also result in flavor synergy ([García-Carpintero et al., 2010](#)). Occasionally, even the juice or pomace from white grapes may be added (to supply additional copigments) ([Gordillo et al., 2014](#)).

Decoloration and reducing browning potential

Although most white wines come from white grapes, some clones of "white" grapes, such as Gewürztraminer, synthesize small amounts of anthocyanins in their skins. In addition, as most cultivars possess colorless juice, even red cultivars can be used to produce white wines. Because anthocyanin levels typically decline spontaneously during fermentation, decoloration, if necessary, is usually performed after fermentation. However, if

experience indicates that this is likely to be inadequate for the style desired, preparations with anthocyanase activity may be added before fermentation. The enzyme removes the anthocyanin's sugar moiety, reducing solubility and favoring precipitation. Loss of the sugar moiety also makes anthocyanins more susceptible to oxidative decoloration. Because anthocyanase is inactivated by sulfur dioxide, ethanol, and high temperatures, treatment normally follows juice clarification and before the start of fermentation. At this point, the free sulfur dioxide content (if added) will have diminished, and little ethanol will have been generated.

Another color removal/browning prevention technique uses laccase. The fungal polyphenol oxidase has a wider range of substrates than grape polyphenol oxidases and acts over a wider range of conditions. It discolors anthocyanins and oxidizes other phenolics. These can be subsequently removed with fining agents. Unfortunately, laccase can oxidize colorless glutathionyl caftaric acid complexes, enhancing subsequent browning potential. In addition, because laccase is not a permitted wine additive, it must be immobilized ([Brenna and Bianchi, 1994](#)). In this form, laccase remains attached to a reactor through which wine is passed during treatment.

There are several means of reducing the browning potential of white wines including gentle pressing, short maceration, phenolic removal with fining agents, adding sulfur dioxide before bottling, and acid addition to musts of high pH. Roughly nine times more highly oxidizable phenolate flavonoids are present at a pH of 4.0 than at a pH of 3.0 ([Singleton, 1987](#)). Catechin-type phenolics have the strongest correlation with browning potential ([Simpson, 1982](#)). Of these, epicatechin appears to be most active in the production of yellowish xanthium pigments ([Clark et al., 2010](#)). Contributing factors in pigment accumulation include iron (a catalyst) and caffeic acid (an inhibitor) ([George et al., 2006](#)).

Another technique limiting browning potential involves removing readily oxidized phenolics before fermentation. This can vary from permitting air exposure during crushing to hyperoxygenation (bubbling air through the must) ([Schneider, 1998](#)). It may take from one to three saturations (9–30 mg/L) to protect the wine against premature oxidative browning (depending on the must's flavonoid content). Without maceration, oxygen uptake during crushing is usually inadequate to induce sufficient phenolic oxidation. Most oxidized phenols precipitate during fermentation, leaving the wine bright and with a reduced tendency to brown. The removal of oxidized phenolics is partially due to their adherence to yeast cells that precipitate postfermentation. Hyperoxidation is usually conducted

immediately after crushing and before clarification and SO₂ addition. Sulfur dioxide reduces one of the primary oxidation products (caftaric acid quinone). Hyperoxidation also reduces the bitterness and astringency of flavonoids extracted during long maceration. Clarification after hyperoxidation is usually required to remove precipitated phenolics to prevent their resolubilization. Although the clarified juice appears brownish, it subsequently clears during fermentation.

Whether hyperoxidation has positive, negative, or neutral effects on wine aroma, depends principally on the variety employed, their potential for oxidative browning, and the type of clarification involved. The effects on aroma development are highly variable and no generalities seem apparent.

Addition of sulfur dioxide

Sulfur dioxide supplied at about 50–100 mg/L juice (or must) used to be habitual, the precise amount depending on the health of the fruit and the maceration temperature. This practice was recommended on the belief that it controlled the growth and metabolism of indigenous members of the grape flora as well as provided needed protection from oxidation. Research has thrown this practice into question, especially with healthy grapes chilled and macerated at cool temperatures. Inoculation with *Saccharomyces cerevisiae* may itself be adequate to suppress other yeasts (Henick-Kling et al., 1998). Although sulfur dioxide limits the activity of indigenous yeasts before *S. cerevisiae* takes over and completes fermentation (Fig. 7.16), the degree of suppression depends on the species and strain. With yeast inoculation, rapid growth of the added strain speeds the development of anaerobic conditions; reduces the availability of limiting nutrients; and generates levels of ethanol that are often inhibitory or toxic to most yeasts and bacteria at wine pH values. In spontaneous fermentations, sulfur dioxide tends to retard the onset of active fermentation, by suppressing not only non-*Saccharomyces* spp. but also most endemic strains of *Saccharomyces* (Suzzi and Romano, 1982).

Sulfur dioxide is particularly effective against bacteria—its action being accentuated by the juice's low pH. It favors the presence of the most toxic form of sulfur dioxide (molecular SO₂). Thus, where an early onset of malolactic fermentation is desired, the addition of sulfur dioxide before fermentation is best avoided. However, avoiding the addition of sulfur dioxide in musts of high pH could favor the activity of spoilage lactic acid bacteria, notably *Pediococcus* and *Lactobacillus* spp. (Davis et al., 1986a, b). In addition, sulfur dioxide use can occasionally facilitate malolactic fermentation, by inhibiting endemic lactic acid bacteria that might infect *Oenococcus oeni* with bacteriophage (Davis et al., 1985). This is another

example where successful winemaking is a combination of science, art, experience, and good luck.

If added to juice/must, sulfur dioxide is usually supplied several hours before yeast inoculation (either at harvest or before crushing). During the settling period, the free SO₂ content declines rapidly (as it binds with sugars, carbonyls, and phenolics or is taken up by microbes). Thus, the antimicrobial effect of sulfur dioxide is much reduced when and if the must is inoculated with one or more cultured yeast strains. For musts derived from partially moldy grapes, the traditional dose of sulfur dioxide is increased. Not only are the numbers of microbial contaminants much higher, but the presence of microbial by-products (e.g., glucuronic acid and galacturonic acid), can bind sulfur dioxide, reducing its antimicrobial effects.

While there is doubt about the need for sulfur dioxide's antimicrobial action in juice, there is even more incertitude concerning the effectiveness and need for its inhibition of grape polyphenol oxidases. Depending on the variety, between 25 and 100 mg SO₂/liter may be required to inhibit the early (enzymatic) oxidation of phenolics (White and Ough, 1973). As a result, these phenolics may remain in the juice, leading to increased potential for in-bottle browning. Regrettably, the undesirable oxidation induced by fungal laccases (found in moldy grapes) is not controlled by commercially acceptable additions of sulfur dioxide. In the past, ascorbic acid was added along with sulfur dioxide to limit early phenolic oxidation. Although effective in this regard, ascorbic acid induces even further delays in oxidation and precipitation of readily oxidized phenolics, potentially postponing the process until after bottling (Peng et al., 1998). Ascorbic acid addition to white wine after crushing now tends to be discouraged. This may apply more to wine of higher alcohol contents—ascorbic acid degradation (and subsequent browning reactions) being directly correlated with alcohol concentration (Hsu et al., 2012).

With red wines, sulfur dioxide can bleach anthocyanins. Although reversible, sulfur dioxide also binds to flavonoids, delaying as well as limiting the formation of stable, colored, anthocyanin–tannin complexes. In addition, by binding with acetaldehyde and pyruvic acid, sulfur dioxide can delay the formation of vitisins (Morata et al., 2006).

The effects of sulfur dioxide on yeast-derived flavorants (Herraiz et al., 1990) as well as their imparting of a metallic taste are additional points of concern. Thus, whether the antimicrobial and antioxidant effects of sulfur dioxide are more beneficial or detrimental applied before fermentation depends on grape health and maturity, the cultivar involved, and the wine style desired.

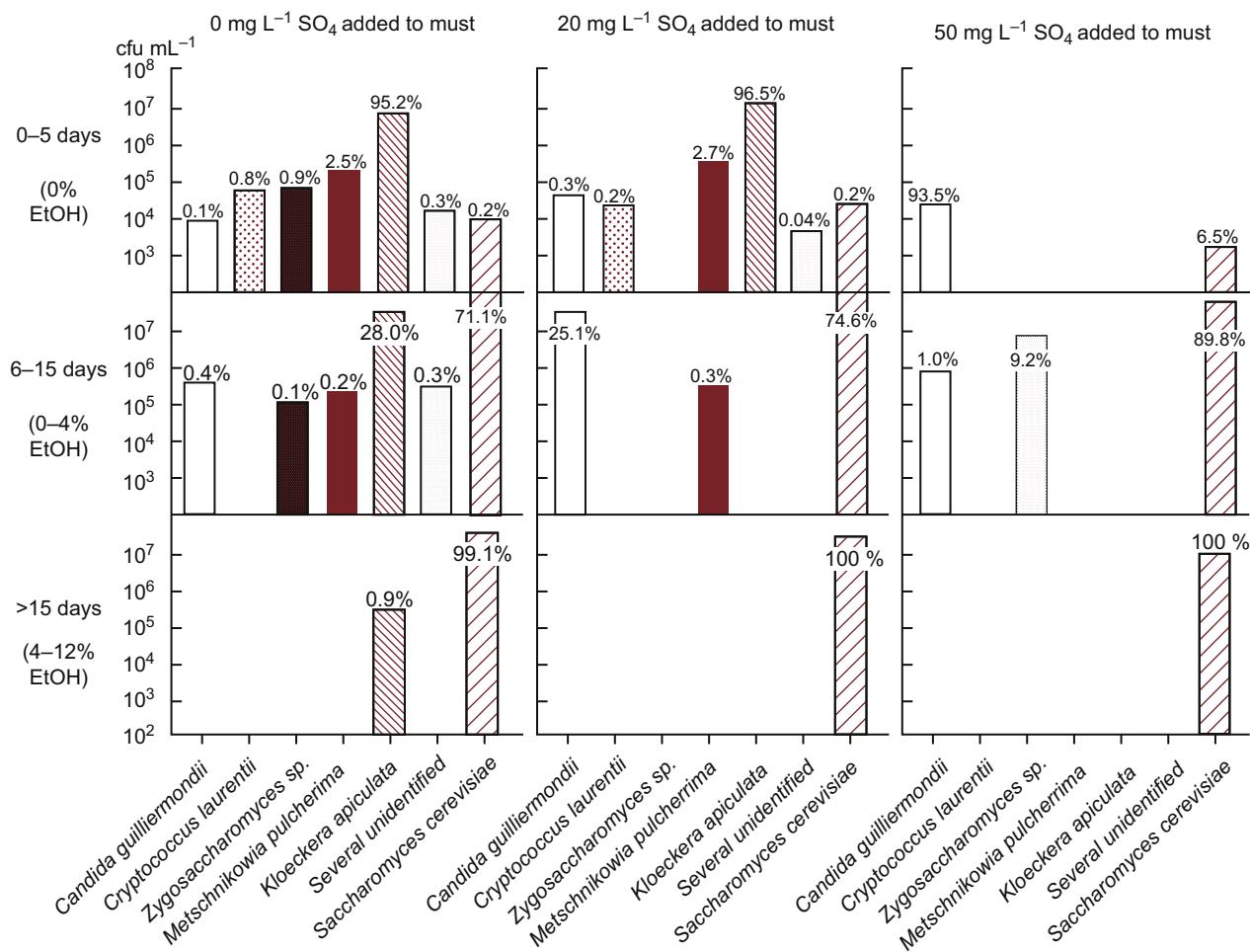


FIGURE 7.16 Predominance (% colonies recovered) and total cell numbers (cfu/mL) of all yeasts during uninoculated fermentations at 16°C with three sulfite treatments. From Henick-Kling, T., Edinger, W., Daniel, P., Monk, P., 1998. Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. *J. Appl. Microbiol.* 84, 865–876, reproduced by permission.

Because of some of the potentially detrimental aspects of sulfur dioxide use, extensive studies have investigated how to reduce or eliminate its use. One of the more interesting techniques involves substituting dry ice for sulfur dioxide (Corona, 2010). It avoids the tendency of sulfur dioxide to enhance phenolic extraction while still reducing polyphenol oxidase activity. It also enhances desirable flavorant extraction.

Alcoholic fermentation

Fermentors

Fermentors come in a wide variety of shapes, sizes, and technical designs. Most differ little in design from those used centuries ago. However, some are complex and fashioned for specific purposes. Most fermentors are straight-sided (Plate 7.4) or have the form of slightly inverted cones. Tanks are differentiated from vats by

being closed, whereas vats have open tops. Tanks have the potential of being used as storage cooperage, whereas vats are limited to their role as fermentors.

Batch-type fermentors

During the fermentation of red wines, some carbon dioxide released during yeast metabolism becomes entrapped in the pomace. This causes the pomace to rise to the top, forming a cap. This severely restricts contact between the pomace and most of the juice, retarding the extraction of anthocyanins and other compounds from the skins and pulp. Many design features of modern fermentors are intended to solve this problem.

With vats, periodic submerging of the cap into the fermenting must (punching down) often achieved adequate extraction and temperature equilibration. Automatic methods of achieving the same benefits only became widely available in the later part of the



PLATE 7.4 Winery with large stainless steel fermentation tanks.
Photo courtesy of Gary Pickering, Brock University, St Catharines, Canada.

20th century. Since their development, tanks have almost completely replaced vats. Because they have closed tops, exposure to airborne contaminants and oxygen can largely be avoided.

Since the 1950s, there has been a move away from wooden fermentation tanks (e.g., oak, chestnut, red-wood) to more impervious and inert materials. Cement has been used in some regions, but stainless steel is the preferred material. Fiberglass tanks also have an appeal due to their lighter weight and lower production costs.

Modern adjustments to oak tanks have again reintroduced them as a viable option for those desiring a more traditional fermentor. They can be produced with a Plexiglas "window" replacing one of staves, permitting the progress of the fermentation to be observed. They can be fitted with cooling coils and are far easier to empty and clean than in the past (Plate 7.5). Nevertheless, stainless steel has one distinct advantage over all other materials—rapid heat transfer. This facilitates maintenance of desired fermentation temperatures.

Years ago, when water seemed limitless and inexpensive, rapid heat transfer from stainless steel tanks could be achieved by flushing water over the sides. Evaporation acted as the coolant. However, the development of double-jacketed tanks, with a coolant circulating between the inner and outer walls, provided more versatile and precise temperature regulation. Temperature control in tanks constructed of other materials usually requires the insertion of cooling coils or plates in the fermentor or pumping the fermenting juice through external cooling coils. Adequate temperature control avoids the need for defoaming agents. Otherwise, these may be required to prevent excessive froth development and wine loss associated with its discharge through overflow valves. Common commercial defoaming



PLATE 7.5 Oak fermentor (Tonnellerie Seguin Moreau, Merpins, Cognac, France). Photo courtesy Ronald S. Jackson.

agents consist of a mixture of mono- and diglycerides of oleic acid and polydimethylsiloxane.

As a construction material, cement has some initial advantages. They are less expensive to build than equivalently sized stainless-steel tanks. However, cement is difficult to surface-sterilize. Epoxy coatings help but require frequent maintenance. Another option is lining the tank with ceramic tiles or vitrification.

White wine fermentors are generally of simple design. The primary technical requirements are prevention of oxygen exposure and efficient temperature control. The first goal is easily achieved with a tank. For temperature regulation, if the juice is not sufficiently cool to begin with, it is usually chilled to an appropriate temperature before yeast inoculation. This is normally achieved with cooling coils but can be more rapidly obtained by adding food-grade dry ice or liquid nitrogen. The ferment is usually maintained within a relatively narrow temperature range throughout fermentation. The desired temperature can vary considerably depending on the desires and preferences of the winemaker. Cooler temperatures (10–15°C) tend to encourage the production and retention of fruit esters, whereas warmer temperatures favor the development of varietal fragrances in certain cultivars—for example, Sauvignon blanc (Masneuf-Pomarède et al., 2006).

Historically, fermentors for red wine production were vats of simple design. Cap formation, vigorous carbon dioxide production, and a higher phenol content often provided the fermenting juice with adequate protection from oxygen exposure. Also, if the cellars were cool, and

the volumes relatively limited (50–100 hL), fermentation was sufficiently restrained that cooling was unnecessary.

Fermentation often occurred at or was allowed to rise to 25–28°C. Periodically punching the cap down into the fermenting must partially equilibrated the temperate throughout the vat. In addition, it facilitated the release and dispersion of potassium (extracted from the skins) throughout the must. This helped limit both an excessive rise in cap pH and the growth of spoilage microbes. At the same time, it submerged most potential spoilage organisms into the inhibitory anaerobic conditions of the fermenting juice. Punching down also promoted the more effective extraction of anthocyanins, favoring optimal color development. Finally, the procedure partially aerated the fermenting must, facilitating yeast growth.

However, when a shift from vats to large tanks began, the development of mechanical substitutes for manual punching down became necessary. This spawned an incredible array of ingenious inventions. One of the first was devised by [Gervais \(1820\)](#). It consisted of one cover or a series of covers that floated in the must at different levels. Carbon dioxide trapped in the cap caused the cover(s) to rise periodically. As they rose, they were trapped at one (or more) positions against the inward sloping sides of the tank. The publication also included plans for an ingenious device to collect alcohol and other volatiles lost from barrels during fermentation for readition to the fermented wine.

One of the more novel designs is the *pileage* fermentor. It possesses mechanical cap plungers to simulate the action of manual punching down ([Fig. 7.17](#)). Open vat versions are available if exposure to air is deemed desirable. This is often viewed as valuable with musts of high °Brix values to encourage fermentation going to completion.

Other modern solutions include automatic periodic or continuous pumping of juice over the cap. This may involve rotating sprinklers or a spout that submerges the cap as it moves over the cap. The flow of juice may be combined with temperature control by passing the must through cooling coils. Oxygen uptake can be limited during pumping over by filling the headspace with inert gas (N₂ or CO₂). This may be important at the beginning and end of fermentation, especially if it occurs slowly. During both stages, flushing of the headspace with carbon dioxide released during fermentation is limited. A stable cap, exposed to oxygen, is particularly susceptible to uncontrolled microbial spoilage.

An alternative procedure for encouraging color and flavor extraction as well as must cooling and limited aeration (to favor yeast growth) is *délestage*. The procedure has several variants but usually consists of the following. After fermentation has become sufficiently vigorous to produce a cap, the juice is drained into a

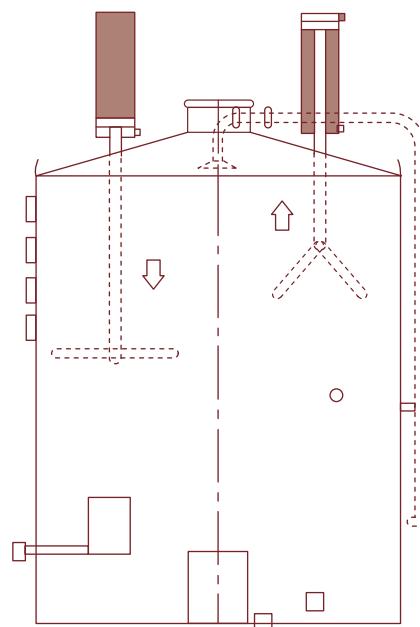


FIGURE 7.17 Diagram of a *pileage* fermentor showing the punching-down action of a system of stainless steel plungers and flaps on the cap of fermenting red must. Modified from *Anonymous, 1983. Steel feet punch the cap at Buena Vista. Wines Vines 64 (2), 52*, reproduced by permission.

holding tank. The juice often passes through a mesh to withhold seeds suspended in the juice (termed *déportation*). The juice is pumped and sprayed into a second tank, while the cap in the fermentor completes draining. The juice is then pumped back into the fermentor, achieving a second aeration while also breaking up the pomace (cap). The procedure may be repeated several times during fermentation. The procedure improves anthocyanin extraction and the early formation of polymeric pigments ([Bossu et al., 2001](#); [Zoecklein et al., 2004](#)). It is also reported to be particularly valuable with incompletely ripened grapes, as it provides an opportunity to remove most of the immature ("green") seeds (see [Canales et al., 2008](#)). They possess a high proportion of extractable phenolics.

Autofermentors achieve the same advantages as *délestage*, but automatically. They generally possess two superimposed chambers. The lower (main) chamber contains two traps into a smaller upper chamber. An elongated, cone-shaped cylinder may descend from the upper into main chamber. In the Ganimede model, carbon dioxide generated during fermentation partially accumulates between the cone and sides of the fermentor. This increases must volume, pushing a portion of the juice and pomace cap into the upper chamber. At a certain point, the increasing pressure opens a bypass into the upper chamber, and a portion of the carbon dioxide and fermenting juice gushes into the upper chamber. This mixes the juice with the extract-concentrated cap.

The disrupted cap and juice flow back into the lower chamber and the bypass closes. As more carbon dioxide is generated (and is trapped between the cone and sides of the fermentor), it forces the cap and juice back into the upper chamber. Repetitions of the cycle accentuate extraction of anthocyanins and flavorants. Seeds settle to the bottom of the fermentor. Comparisons with more traditional winemaking techniques are given in Vázquez et al. (2010) and Bai et al. (2013).

In another design, perforations in the cylinder prevent the pomace from escaping into the upper chamber, where the fermenting juice cools slightly. Its weight forces a second, downward-directing trap to open. The flush of juice back in the main chamber ruptures the cap and temporarily disperses it into the fermenting must. In this version, the cap is normally submerged. A simpler system for achieving a submerged cap involves a grill located below the surface of the must. Although these autofermentors avoid the need of punching down, additional agitation is required to achieve adequate mixing of the must for color extraction.

Rotary fermentors are another solution designed to improve and automate color and flavor extraction (Fig. 7.18). The horizontal position of the fermentor increases the surface contact between the juice and the pomace. Rotation of spirally shaped paddles gently, but continuously, mixes the fermenting juice with the pomace. Alternatively, the fermentor may be set to produce a gentle rocking back-and-forth. Although the fermentors principal advantage comes from the rapid extraction of flavor and anthocyanins (Catania et al., 2011), it also avoids temperature stratification between the cap and fermenting juice (see Fig. 7.46). In addition, by permitting earlier pressing, the juice can be separated from the pomace before most bitter/astringent polyphenolics are extracted. This facilitates the production of red wine designed for early consumption or to permit early transfer of the ferment to barrel for the completion of fermentation.

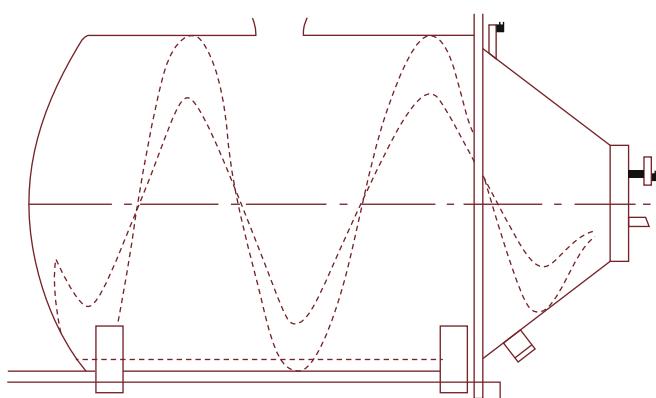


FIGURE 7.18 Diagram of a rotary fermentor. Courtesy of CMMC.

Rotary fermentors, and other automated replacements for punching down, have the distinct advantage of reducing the likelihood of microbial spoilage developing in the cap. Nonetheless, the associated reduced oxygen uptake by the must may increase the incidence of sluggish or stuck fermentation. Where a concern, techniques for microoxygenation are available to supply the amount of oxygen considered appropriate.

Although rotary fermentors are most frequently used for red wines, they may be used to shorten the maceration time needed to achieve flavor extraction from several white varieties, notably Chardonnay, Gewürztraminer, and Riesling.

The major disadvantage of most automatic fermentors comes from the increased investment required in their purchase. In addition, if the ferment is separated too early, reduced phenolic extraction can compromise the formation of stable, colored, anthocyanin–tannin polymers. Nevertheless, shorter holding periods can achieve cost-saving by limiting the number of fermentors needed to process large volumes of must.

When investigating any new procedure, it is important to make assessments relative to the development of attributes over at least several years. For example, a comparison between standard floating-cap and submerged-cap fermentations of a Barbera wine initially indicated reduced color and phenolic content in the submerged-cap wine. However, during aging, the submerged-cap version retained higher values of both (Bossò et al., 2011).

Modern fermentors typically include systems to ease pomace discharge. For this purpose, sloped bottoms with trap doors are often used. Removable tank bottoms are another solution. With rotary fermentors, rotation can position the opening to facilitate discharge.

In a few regions, wine is still fermented (and matured) in large (500 to >1000 L) clay vessels, resembling those used by the ancient Greeks (*pithoi*) and Romans (*dolia*). Examples of their use include traditionally produced Georgian *kvevri* wines, Montilla-Moriles sherry-like wines (produced in *tinaja*), and some wines from Alentejo, Portugal (produced in *talhas de barro*) (e.g., Martins et al., 2018). The vessels may be submerged in soil up to their neck (as in Georgia) or partially or totally positioned above ground (e.g., in Spain and Portugal). Impermeability may be achieved with pine pitch (as in antiquity and still with *talha*) or with bees wax (*kvevri*). This is distinct from modern experimentation with the use of amphoras for maturation. It is being adopted by some producers wishing to be associated with the “natural wine movement” (Baiano et al., 2014). A few producers are also experimenting with maturing wine in amphorae submerged in the sea.

Continuous fermentation and related procedures

Most fermentors are of the batch type; that is, separate volumes (batches) of juice/must are individually fermented. In contrast, most industrial fermentations are continuous. Substrate is added at a relatively constant rate or at frequent intervals, while equivalent volumes of the ferment are removed, maintaining a constant volume. Continuous fermentors may operate uninterrupted for weeks or months. For the industrial production of single metabolic products, synthesized primarily during a particular phase of colony growth, continuous fermentation is ideal. It can maintain most cells in a phase where synthesis of the desired metabolic product is at its optimum, resulting in superior cost-efficiency. The technique is less compatible with enologic practice, especially due to wine quality depending on subtle and complex associations of dozens of compounds produced at various stages during fermentation.

Despite the cost-savings associated with continuous fermentation, it is rarely used in winemaking, even to produce bulk wines. Their expense and complexity means that continuous fermentors are economically feasible only when used almost year-round. This, in turn, demands a steady supply of must. With the seasonal character of the grape harvest, this requires the storage of must under sterile, nonoxidizing conditions. These requirements necessitate more sophisticated storage than would be needed to store the corresponding volume of wine. Thus, technical and financial concerns generally outweigh the benefits of product uniformity, and the easier alcoholic and malolactic fermentations achieved. Nevertheless, yeast immobilization (Iconomou et al., 1996; Verbelen et al., 2006) may make continuous fermentation more applicable as a means of reducing production costs. Even with traditional batch-type fermentations, immobilized yeasts have shown sensory benefits (Tsakiris et al., 2004).

Another technique is cell-recycle-batch fermentation (Rosini, 1986). After each fermentation, the yeasts are collected and used to initiate subsequent fermentations. Collection may involve filtration, centrifugation, or spontaneous sedimentation. In addition to reducing inoculation costs, fermentation is shortened and the efficiency of sugar conversion to ethanol is slightly improved. There is also a reduction in the synthesis of sulfur dioxide by yeast cells, but an increase in volatile acidity. Because cell division continues, but at progressively reduced rates, frequent monitoring for contamination with undesirable yeasts and bacteria is necessary. In addition, periodic assessment is required to assess that the genetic characteristics of the yeast

population have not changed. Both requirements may be reduced using immobilization in calcium alginate beads (Suzzi et al., 1996).

Immobilization, involving the entrapment of cells within alginate beads, has several potential advantages (Diviès et al., 1994). Its use could significantly reduce the production costs associated with disgorging in sparkling wine production. In addition, encapsulation appears to give cells enhanced resistance to low temperatures and high concentrations of ethanol and acetic acid (Krisch and Szajáni, 1997). Entrapment may also modify the composition of fermentation by-products. For example, glycerol, propanol, and isoamyl alcohol production may be increased, whereas acetaldehyde generation decreased. None of these methods are used in traditional wine producing regions.

Fermentor size

Optimal fermentor size relates primarily to the volumes of juice or must normally fermented. Only with small lots of juice/must, possessing unique qualities, do tanks specifically designed to accentuate these features become economic.

Small juice/must volumes may result from limited vineyard holdings; desires to keep the grapes from different clones or varieties separate; and selective harvesting to collect fruit at distinct states of maturity. An example of the latter is the selection involved in producing higher-level Prädikat and botrytized wines. Separate fermentation maintains the individuality of unique lots. In any of these situations, fermentation may be conducted in small oak cooperage. Although in popular perception small is equated with better, there is no inherent linkage between the two.

In addition to maintaining the individual attributes of small lots of juice or must, in-barrel fermentation possesses additional advantages as well as disadvantages. Because cooling occurs only by passive heat radiation, fermentation may occur at higher temperatures than currently preferred for white wine. Fruit-smelling acetate esters, formed by yeasts during fermentation, dissipate (sparge) more readily at warmer temperatures along with escaping carbon dioxide (see Fig. 7.44). This may achieve a clearer varietal expression with cultivars possessing distinctive aromas (less association with a general fruity background). Otherwise, it is a disadvantage.

Wine fermented in small fermentors is usually left on the lees longer than in large fermentors. This favors earlier onset of malolactic fermentation, but also increases the risk of off-odor production. This may be

reduced by the use of yeast strains synthesizing little hydrogen sulfide (Cordente et al., 2007, 2009). In addition, periodic mixing of the lees and must (*bâtonnage*) provides aeration that further decreases the likelihood of reduced-sulfur odor development in the lees. Yeast viability is also enhanced by slight aeration. This is anecdotally credited with contributing to better integration of oak flavors and tannins in the wine.

On the negative side, more effort is involved in topping, racking, cleaning, sterilizing, and maintaining small wood fermentors. There is also an increased risk of oxidation as well as yeast and bacterial spoilage (Stuckey et al., 1991). In small amounts, acetaldehyde and acetic acid production, and the uptake of oak flavors, can increase wine complexity. Nevertheless, excess amounts can mar subtle wine flavors. The risk of microbial contamination increases with barrel reuse.

For many premium wines, fermentors range in size from 50 to 100 hL. Such volumes appear to provide a judicious balance between economics and ease of operation, and the desire to maintain individuality. For most wines, though, the economies of size favor fewer but larger fermentors. In this case, fermentors with capacities from 200 to more than 2000 hL (~50,000 gal) are practical. Computers have permitted the monitoring and regulation required with gargantuan fermentors. Advances in spectroscopy and other analytic techniques permit real-time analysis of multiple chemical parameters during fermentation (Dubernet, 2010; Cozzolino et al., 2011). Quantitative nuclear magnetic resonance is also being investigated as a potential procedure for profiling metabolite production during fermentation (López-Rituerto et al., 2009).

Such technical advances should facilitate the development and use of computer fermentation algorithms designed to produce wines possessing precise sensory attributes. In conjunction with real-time, on-site data, models could anticipate the development of potential problems, predict their likely consequences as well as suggest solutions—winemaking on autopilot.

Associated with enlarged fermentor volumes is increased temperature and dispersion control issues. In large fermentors, passive heat dissipation via the surface is insufficient to prevent excessive heat buildup, resulting in a premature termination of fermentation. This requires the instillation of sophisticated temperature control systems. Large must volumes also increase the likelihood of excessive foaming and wine loss (and the potential need for defoamers). Sedimentation of large amounts of grape solids and yeasts can delay the onset of active fermentation. Without intentional mixing, fermentation begins principally at the base, with the yeast population slowly rising upwards through the fermentor volume, becoming uniform only when cell division ceases and carbon dioxide production is pronounced (Vlassides and Block, 2000).

Temperature stratification is generally limited by pumping-over, requiring half the volume being transferred twice per day (Lerno et al., 2018). Despite the potential problems associated with colossal fermentors, the economics of size often more than compensates for their disadvantages.

Fermentation

Chemically, fermentation is an energy-releasing form of metabolism in which the substrate (initial electron donor) and end product (final electron acceptor) are organic compounds. It differs fundamentally from respiration in not requiring the involvement of molecular oxygen. Although many fermentative pathways exist, *S. cerevisiae* possesses the most common—alcoholic fermentation. In it, ethanol acts as the final electron acceptor (end product), whereas glucose is the preferred electron donor (substrate). Although *S. cerevisiae* possesses the ability to respire, it predominantly ferments, even in the presence of oxygen.

Although most organisms are able to ferment sugars, they do so only when oxygen is lacking or deficient. This partially results from the toxic action of the usual by-products of fermentation, ethanol, or lactic acid. In addition, fermentation is inherently an inefficient mode of energy release. For example, alcoholic fermentation converts only about 6%–8% of the chemical-bond energy of glucose into readily available metabolic energy as adenosine triphosphate (ATP). Most of the energy remains bound in the terminal electron acceptor—ethanol. Although seemingly a disadvantage, yeasts utilize limited oxidative phosphorylation (Vander Heiden et al., 2009). Fermentation supplies the carbon skeletons necessary for growth as well as sufficient reducing power in the form of nicotinamide adenine dinucleotide phosphate (NADPH). In the short term, fermentative metabolism is adequate.

The two main organisms involved in vinification, *Saccharomyces cerevisiae* and *Oenococcus oeni*, are somewhat unusual in selectively employing fermentative metabolism. *S. cerevisiae* is so well adapted to fermentative metabolism that it can generate as many ATP/sec as would normally be generated by respiration (Pfeiffer et al., 2001). These properties are partially based on the presence of a highly efficient alcohol dehydrogenase (ADH1), oxidizing acetaldehyde to ethanol; a high titer of glycolytic enzymes in the cytoplasm; and a mitochondrion that only produces respiratory enzymes in the presence of a preponderance of nonfermentable substrates (Ihmels et al., 2005). In addition, both *S. cerevisiae* and *O. oeni* can withstand moderately high ethanol concentrations.

In addition to preferential alcoholic fermentation (suppression of respiration by glucose) as well as alcohol and

acid tolerance, *Saccharomyces cerevisiae* is osmo-tolerant and can multiply several times in the absence of oxygen. Thus, it is amazingly preadapted for growing in must. By rapidly producing and secreting large amounts of ethanol, wine yeast soon exclude most other potential competitors from growing in grape juice (Hagman and Piškur, 2015). *S. cerevisiae* also has the highest cardinal temperature-growth parameters of any *Saccharomyces* or non-*Saccharomyces* spp. (Salvadó et al., 2011)—appropriate for fermentation in large cooperage before refrigeration.

Oenococcus oeni is less well adapted to growing in grape juice or must than *S. cerevisiae*. It typically grows slowly in juice, developing most commonly in wine after *S. cerevisiae* has completed alcoholic fermentation. The production of lactic acid, the major by-product of *O. oeni* metabolism, limits the growth of other potential bacterial competitors. Lactic acid bacteria are one of the few acid-tolerant bacterial groups. However, the acidity of grape juice and wine actually retards or inhibits the growth of most lactic acid bacteria. Thus, in this instance, converting malic acid to lactic acid, a weaker acid, has the benefit of increasing the pH, favoring the growth of *O. oeni*. It also can improve microbial stability, by consuming residual fermentable substrates, and generate flavorants often viewed as desirable. Incidentally, the conversion makes excessively acidic wines more acceptable to the human palate.

Because wine is typically batch-fermented, nutrient availability is maximal at the beginning of fermentation, and declines progressively thereafter. By the end of fermentation, most sugars have been metabolized, leaving the wine “dry” and most other nutrients significantly depleted.

Batch fermentations generally show a growth pattern consisting of four, clearly distinguishable phases—lag, log, stationary, and decline. Immediately following inoculation, cells need to adjust to the conditions of the new substrate. Because some cells do not acclimate successfully, there is an initial period in which cells have yet to begin to divide and/or the number of dividing cells approximates the number that die. This is called the lag phase.

Once adapted, the cells begin to multiply at a steady rate, until conditions become unfavorable. Because most microbes are unicellular, the growth curve approximates an exponential equation, and the phase is correspondingly called the exponential or log (logarithmic) phase. During this period, the population of viable cells rapidly increases to its maximum value.

As the nutrient content declines, there is an accompanying accumulation of toxic metabolic by-products. Thus, after a period of rapid growth, the rate of cell division (growth) declines. The colony enters a state in which the number of cells dying (or become metabolically inactive) and dividing is equal. The culture is said to have now entered the stationary phase. This involves

considerable transcriptional modification by the cell (Rossignol et al., 2003). As nutrient conditions continue to deteriorate, and the concentration of toxic metabolites keeps increasing, more cells die (or become dormant) than divide. At this point, the culture enters a decline phase. Because most viable cells are not replaced, the colony eventually perishes or becomes dormant.

Although similar, the population growth pattern displayed by yeast growth in must shows several variations from the norm (Fig. 7.19). The lag phase is abnormally short or undetectable; the exponential phase is relatively short (seldom involving more than eight cell divisions when the must is inoculated); the stationary phase may be short (commencing long before nutrients become limiting); and the decline phase is atypically long (with the viable cell population remaining high for up to several months). As much as 40% of the sugar fermented to alcohol may occur during the decline phase (Ribéreau-Gayon, 1985).

The brevity or apparent absence of a lag phase may result from the preadapted state of the cells initiating fermentation. Active dry yeast, commonly used for inoculation, comes from cultures grown exponentially in aerated media. Although possessing mitochondria capable of respiration, their cytoplasm contains a full complement of fermentative enzymes. Thus, little time is required for a conversion from respiratory to fermentative metabolism. Similarly, the epiphytic yeast population of grapes requires little enzymatic adaptation to commence rapid cell growth. Endemic yeast cells are commonly bathed in the juice released from broken grapes during harvesting and may pass through the lag phase before fermentation “officially” begins in the winery. Thus, the absence of a noticeable lag period may simply be an artifact. Even dormant yeast inocula, derived from winery equipment, may contain a full complement of enzymes and be prepared for a rapid initiation of growth.

Although physiological adjustment to growth in grape juice appears minimal, a lag phase may be observed when conditions are less than optimal. Conditions such as low temperature ($\leq 10^{\circ}\text{C}$) and excessive protection of the juice from oxygen during crushing may disadvantage yeast cells. Active dry yeast cells are often leaky, and initially may lose vital nutrients (Kraus et al., 1981). In addition, nitrogen deficiency and low juice pH can prolong any lag phase. The latter probably results from the enhanced antimicrobial action of any added sulfur dioxide (Ough, 1966a). High °Brix values or ethanol contents (for example, the second fermentation in sparkling wine production) also suppress yeast growth and fermentation rate (Ough, 1966a, 1966b).

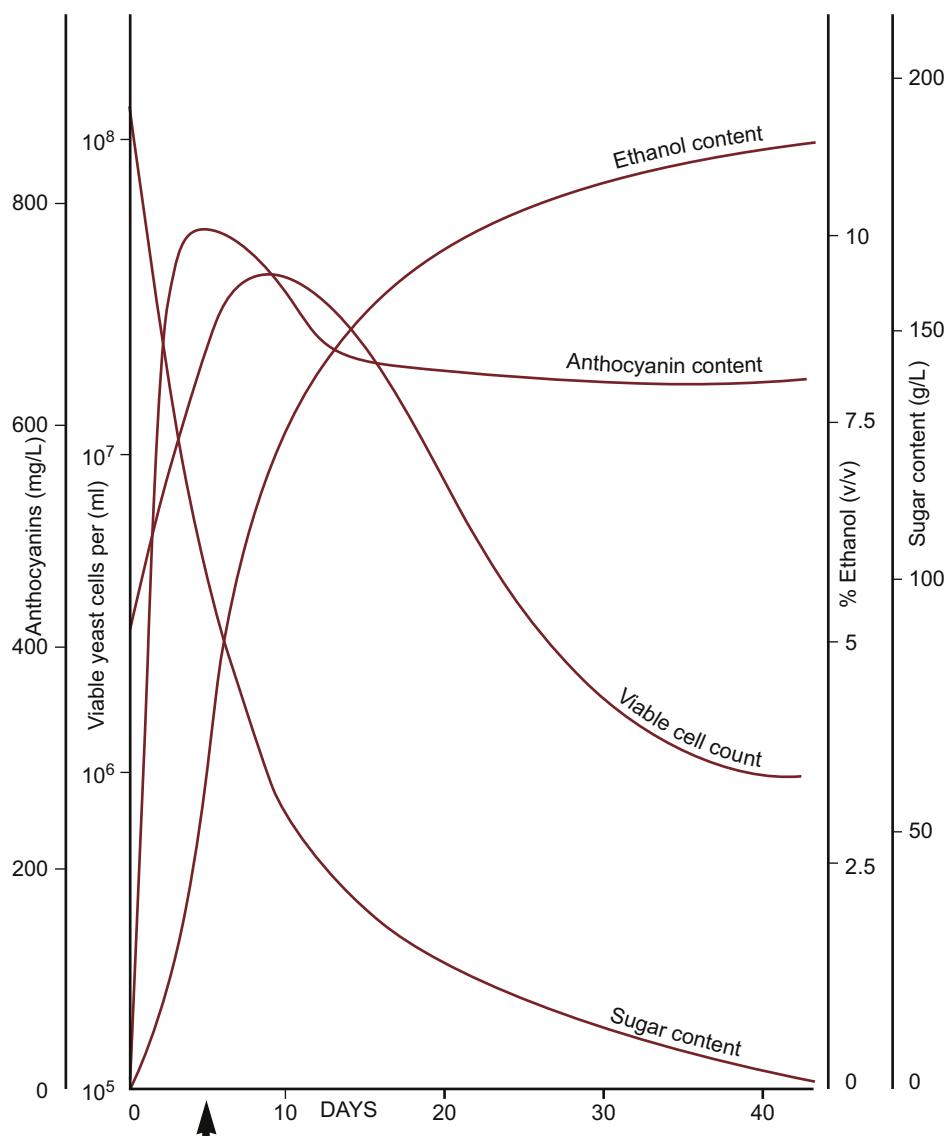
During the exponential phase, cells grow and reproduce at the maximal rate permitted by the prevailing conditions. The presence or absence of oxygen does

not appear to affect the rate (Schulze et al., 1996). The protein content of the cytoplasm approaches 60% (w/w), with the RNA content reaching about 15% (w/w). Little storage carbohydrate (glycerol and trehalose) accumulates.

Early termination of exponential growth may partially be the result of the initial inoculation (at about 10^5 – 10^6 cells/mL), rapidly rising to 10^8 – 10^9 viable cells/mL. Thus, the slowing and termination of cell division (while there are still ample nutrients in the juice/must) is likely an expression of quorum sensing (Avbelj et al., 2015). Many more cell divisions occur during spontaneous fermentations, where the grape epiphytic population is reduced as a result of clarification, and the population of *Saccharomyces cerevisiae* is initially low. Increasing sensitivity to the accumulating ethanol concentration during fermentation may also partially

explain why the viable cell count seldom rises above 10^8 cells/mL. Ethanol eventually disrupts glucose uptake by affecting membrane function. However, other factors appear to be involved. Populations can still reach 10^6 – 10^8 cells/mL in juice initially fortified to 8% ethanol. Other limiting factors may include the inability of yeasts to synthesize essential sterols and long-chain unsaturated fatty acids in the absence of oxygen, the accumulation of toxic, mid-size, carboxylic acids (by-products of yeast metabolism); and reduction in the nitrogen supply may accentuate catabolic repression by glucose (Bely et al., 1994). One factor clearly not involved is a lack of fermentable substrate. Cells enter the stationary phase with approximately half the fermentable sugar content still present. The remaining sugars are slowly metabolized during the stationary and decline phases—constituting up to 80% of the total

FIGURE 7.19 Growth cycle of yeasts and fermentation kinetics in grape must with a high sugar content. Diagram courtesy of Herman Castelein.



fermentation period (Ribéreau-Gayon, 1985), and between 50% and 80% of the sugars metabolized.

As yeast cells enter the stationary phase, their enzyme complement changes, several heat-shock proteins (HSPs) are produced (Riou et al., 1997), and trehalose and glycerol accumulate. Trehalose helps stabilize membrane fluidity (Iwahashi et al., 1995); limits ethanol toxicity (Lucero et al., 2000); and restricts protein denaturation (Hottiger et al., 1994). HSPs also protect structural and enzymic proteins from denaturation (Parsel et al., 1994). In addition, HSPs may play important roles in prolonging cell viability during the decline phase.

Initiation of the decline phase probably results from membrane dysfunction becoming progressively disruptive to cellular function. Membrane disorganization results from the combined effects of ethanol (Hallsworth, 1998), mid-chain fatty (carboxylic) acids (Viegas et al., 1998), and a shortage in sterol precursors. The absence of oxygen may be an additional factor. Its presence is required for the synthesis of nicotinic acid, a vital component of the electron carriers NAD⁺ and NADP⁺. However, why the decline initially stabilizes at a viable population of approximately 10⁵–10⁶ cells/mL for several weeks is unknown. Cell viability is improved by extended maceration before (white wines) or during fermentation (red wines).

Subsequently, the cells progressively autolyze, dying over the next several weeks to months. As a consequence, yeast nutrients and cellular constituents are released into the wine. This is also associated with an increased synthesis of HSP12. It interacts with membrane lipids, stabilizing its structure. As the membrane breaks down, peptides released enhance wine sweetness (Marchal et al., 2015).

Another distinction between industrial and wine fermentations is its microbially mixed status. Most industrial fermentations occur in a sterilized nutrient medium. Except for continuous wine fermentations, grape juice/must is not sterilized. Traditionally, endemic non-*Saccharomyces* yeasts are active at the start of fermentation, but are usually soon superceded by indigenous *Saccharomyces cerevisiae*. These typically continue and complete fermentation. However, this sequence is less common than in the past, due to inoculation with selected yeast strain(s). Although sulfur dioxide may be added to limit indigenous (wild) yeasts, it is only partially effective in that regard (Martínez et al., 1989; Henick-Kling et al., 1998).

Previously, there was no adequate means of assessing whether wild *S. cerevisiae* were controlled by sulfur dioxide. With techniques such as mitochondrial DNA sequencing (Dubourdieu et al., 1987) and gene marker

analysis (Petering et al., 1991), it is now possible to identify the strain(s) conducting fermentation. Although species and strains occurring on grapes or winery equipment may occasionally dominate the fermentation of inoculated juice (Bouix et al., 1981), inoculated yeasts appear to be the primary, if not the only strain(s) detectable by the end of fermentation (see Figs. 7.15 and 7.27).

Red wine vinifications routinely occur in the presence of high concentrations of epiphytic yeasts, regardless of yeast inoculation. In contrast, white grapes, which are pressed shortly after crushing, cold settled, and quickly clarified, usually contain a diminished endemic yeast population. Nevertheless, white juice still possesses sufficient endemic yeasts to initiate and conduct alcoholic fermentation. Although indigenous yeasts may be present and remain viable throughout a fermentation dominated by *S. cerevisiae*, they are typically viewed as being metabolically inactive. Admittedly, though, unequivocal evidence for this is absent.

Biochemistry of alcoholic fermentation

Wine fermentation involves the metabolism of glucose and fructose to ethanol via glycolysis (Embden–Meyerhof pathway) (Fig. 7.20). Although ethanol is the primary end product, additional yeast metabolites donate the basic aromatic attributes of wine. Yeast action may also influence the development of a varietal aroma by hydrolyzing nonvolatile aroma precursors. This can release terpenes, phenols, norisoprenoids, and thiols as free, volatile compounds. In addition, the changing physicochemical conditions produced during fermentation progressively modify yeast metabolism, generating the dynamically changing range of compounds released. This reflects adjustments in cellular energy and nutrient status, due to changes in the nutrients absorbed and by-products both released and reabsorbed throughout fermentation. Thus, much of a wine's fragrance can be interpreted in terms of adjustments in primary and secondary yeast metabolism.

Energy balance and the synthesis of metabolic intermediates

During the changing phases of colony growth, yeasts have differing requirements for ATP and reducing power (NADH and NADPH). These energy-carrying compounds are required to activate cellular functions and maintain an acceptable ionic and redox balance. Ionic balance refers to the maintenance of a subtle disequilibrium between various ions on either side of cellular membranes. Redox balance refers primarily to the equilibrium between the oxidized and reduced forms of the

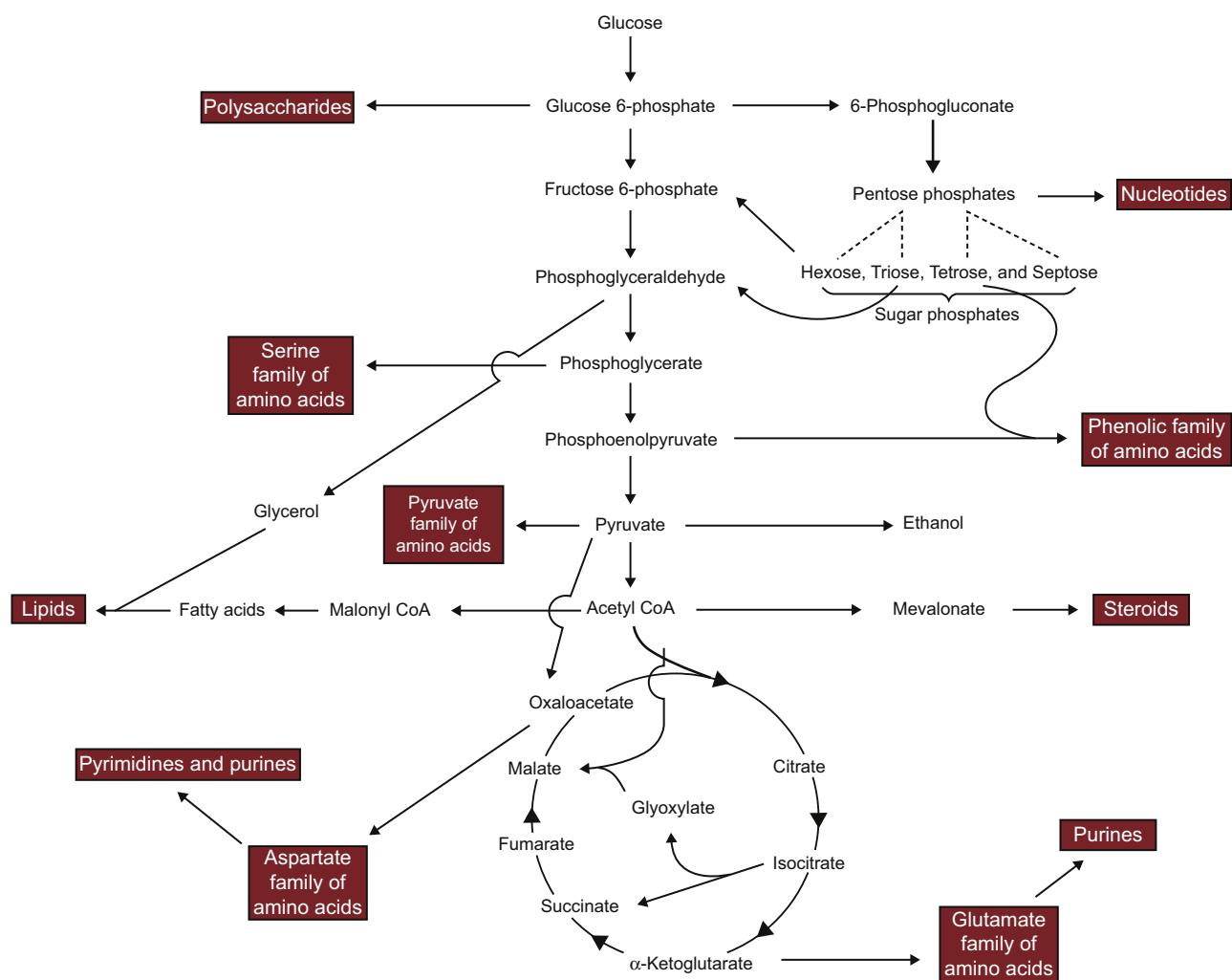


FIGURE 7.20 Core reactions of metabolism showing the main energy-yielding pathways (**bold arrows**) and the major biosynthetic products derived from central metabolism (boxes). The central pathway is the Embden–Meyerhof pathway of glycolysis, the top right shows a highly schematic pentose phosphate pathway, and the bottom is the TCA (tricarboxylic acid) cycle. Each pathway has been simplified for clarity by the omission of several intermediates. The directions of the reactions are shown as being unidirectional, although several are reversible. Energy transformations and the loss or addition of carbon dioxide are not shown. Under the anaerobic conditions of vinification, the TCA cycle does not function. However, except for the enzyme involved in the conversion of succinate to fumarate, those TCA enzymes present appear to be active only in the cytoplasm. In addition, decarboxylation of pyruvate to acetyl CoA is inactive and the glyoxylic acid pathway is suppressed (by glucose).

two major pyridine nucleotides (NAD^+ /NADH and NADP^+ /NADPH).

As glucose and fructose are oxidized to pyruvate, electrons are transferred to NAD^+ (nicotinamide adenine dinucleotide), reducing it to NADH. Pyruvate is subsequently decarboxylated to acetaldehyde, which is subsequently reduced to ethanol with electrons derived from NADH. In the process, redox balance is maintained and the electron transfer cycle complete.

The net synthesis of only ATP from sugar fermentation is inherently much less efficient than respiration. Most chemical energy associated with the sugars remains bound in the end product, ethanol. The energy

associated with the electrons transferred to NAD^+ during fermentation is unavailable as they are transferred to acetaldehyde in its reduction to ethanol. There is no option, as in respiration, for the energy associated in NADH to perform metabolic activity or to be transferred to ATP via the oxidative phosphorylation of ADP (adenosine diphosphate). Under the anaerobic conditions of fermentation, regeneration of oxidized NAD^+ requires the reduction of an organic molecule. Because cells contain only a limited supply of NAD, without the regeneration of NAD^+ in acetaldehyde reduction, the fermentation of sugars would quickly cease. Correspondingly, alcoholic fermentation generates only about two molecules of ATP per sugar.

molecule, in contrast to the potential 24–34 ATPs derived via respiration. Most ethanol produced during fermentation escapes from the cell to accumulate in the surrounding medium.

The low respiratory capacity of *S. cerevisiae* reflects its limited ability to produce the requisite enzymes in the presence of high sugar concentrations (glucose repression). The high proportion of glycolytic enzymes in yeast cytoplasm (about 50% of the soluble protein content) clearly denotes the importance of fermentation to wine yeasts (Hess et al., 1969). Yeasts show high rates of glycolysis, usually about 200–300 μmol glucose/min/g cell weight (de Deken, 1966). It is estimated that about 85% of the sugars incorporated by *S. cerevisiae* are used in energy (ATP) production (and correspondingly the release of ethanol), whereas only about 15% are incorporated in biosynthetic reactions. Specific values vary depending on the prevailing conditions during fermentation, and the number of cell divisions involved in reaching a stationary population.

Although most fermentable sugars in juice or must are metabolized via glycolysis, some are channeled through the pentose phosphate pathway (PPP) (Fig. 7.20, upper right). This diversion is important in the production of pentose sugars needed for nucleic acid synthesis. The PPP also generates the NADPH, required to activate certain cellular functions such as amino acid synthesis (Gancelos and Serrano, 1989). Thus, amino acid availability in the juice can decrease the need for, and activity of, PPP intermediates.

During alcoholic fermentation redox balance is maintained, but no NADH accumulates. To obtain the reducing power needed for growth and division, yeast use the PPP (yielding NADPH) and oxidize pyruvic acid to acetic acid (yielding NADH). Additional supplies come directly from NADH generated in glycolysis, circumventing the recycling of NADH to NAD^+ involved in the reduction acetaldehyde to ethanol.

The changing needs of yeasts for reducing power during the phases of colony growth during fermentation probably explains why compounds such as acetaldehyde and acetic acid are initially released into the juice, but subsequently reincorporated (Figs. 7.21 and 7.22). Early in fermentation, growth and cell division require reducing power. In contrast, in the decline phase, NADH and NADPH may accumulate. This could suppress fermentation by diminishing the supply of the requisite NAD^+ and NADP^+ (see Fig. 7.20). The reincorporation and reduction of compounds such as acetaldehyde and acetic acid would help to oxidize these reducing compounds, balancing the redox potential and permitting continued fermentation.

The metabolic intermediates needed for cell growth and maintenance are generally synthesized from components of the glycolytic and PPP pathways, and the TCA (tricarboxylic acid) cycle (see Fig. 7.20). However, during vinification, most TCA-cycle enzymes in mitochondrion are inactive. Isozymic versions of most of these enzymes (located in the cytoplasm) take over the

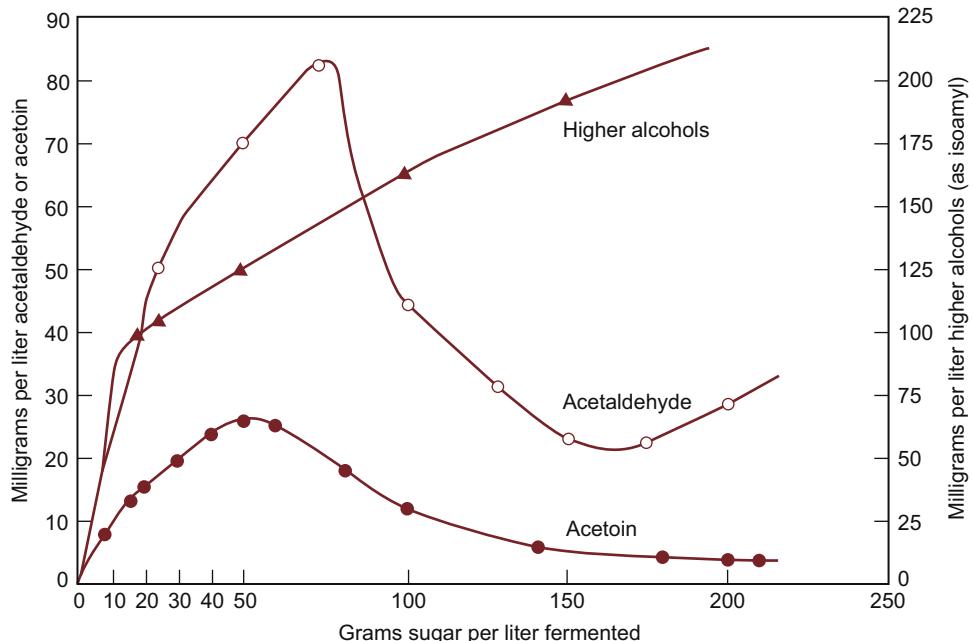


FIGURE 7.21 Formation of acetaldehyde, acetoin, and higher alcohols during alcoholic fermentation. The dynamics of the production of these compounds varies considerably with the yeast strain. From Amerine, M.A., Joslyn, M.A., 1970. *Table Wines, the Technology of Their Production*, second ed. University of California Press, Berkeley, CA, reproduced by permission.

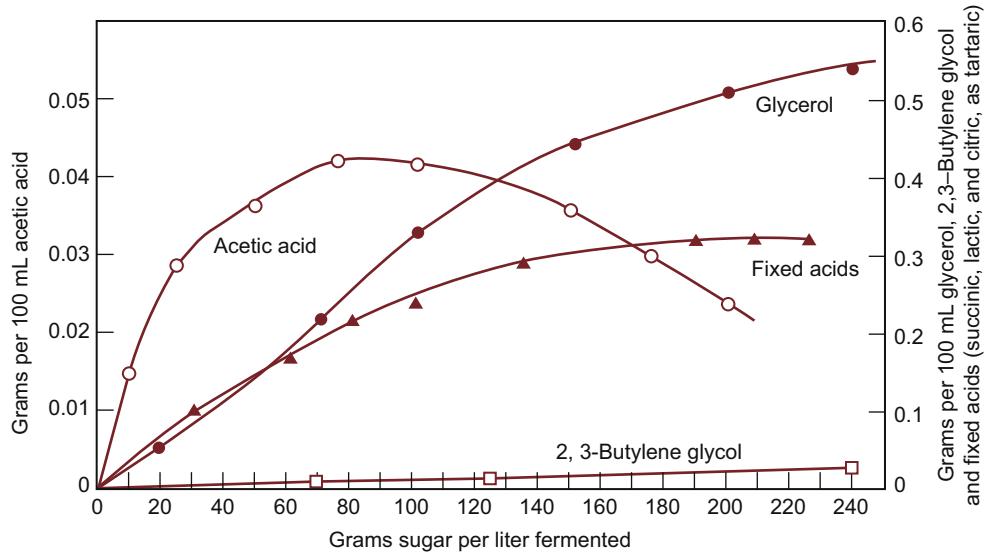


FIGURE 7.22 Formation of acetic acid, glycerol, 2,3-butylene glycol, and fixed acids during alcoholic fermentation. The dynamics of the production of these compounds varies with the strain. From Amerine, M.A., Joslyn, M.A., 1970. *Table Wines, the Technology of Their Production*, second ed. University of California Press, Berkeley, CA, reproduced by permission.

function of generating the necessary metabolic intermediates used in the biosynthesis of amino acids and nucleotides (see Fig. 7.20). Full operation of the TCA cycle would produce an excess of NADH, disrupting the redox balance. This is avoided because NADH, produced during the oxidation of citrate to succinate (the “right-hand” side of the TCA cycle), can be oxidized back to NAD⁺ by reducing oxaloacetate to succinate (the “left-hand” side of the TCA cycle). The result is redox balance. In addition, NADH generated in glycolysis may be oxidized in the reduction of oxaloacetate to succinate, rather than oxidize acetaldehyde to ethanol. In both scenarios, excess succinate is generated. This probably explains why succinate is one of the major by-products of yeast fermentation. In yeasts, the PPP functions primarily in the generation of particular amino acids and pentose sugars involved in nucleotide synthesis.

The replacement of TCA-cycle intermediates lost to biosynthesis probably comes from pyruvate. Pyruvate may be directly channeled through acetate carboxylated to oxaloacetate or indirectly routed via the glyoxylate pathway. The last pathway, if active, is probably functional only near the end of fermentation as glucose suppresses the glyoxylate pathway. The involvement of biotin in the carboxylation of pyruvate to oxaloacetate probably accounts for its primary requirement by yeast cells.

The accumulation of another major by-product of fermentation, glycerol, also has its origin in the need to maintain a favorable redox balance. Its production is also valuable as an osmoticum. In some strains, glycerol

accumulation appears to enhance the synthesis of another osmoticum, trehalose (Li et al., 2010). The importance of glycerol synthesis to redox balance is suggested by the inability of mutants, defective in glycerol synthesis, to grow under anaerobic conditions (Nissen et al., 2000). In addition, Rouston and Sablayrolles (2002) present evidence supportive of the role of glycerol synthesis (at least during the stationary phase) in eliminating excess reducing power. The reduction of dihydroxyacetone phosphate to glycerol 3-phosphate can oxidize the NADH generated in the oxidation of glyceraldehyde 3-phosphate in glycolysis (Fig. 7.23). However, the coupling of these two reactions does not generate ATP and is therefore an energy-neutral form of glucose fermentation. This

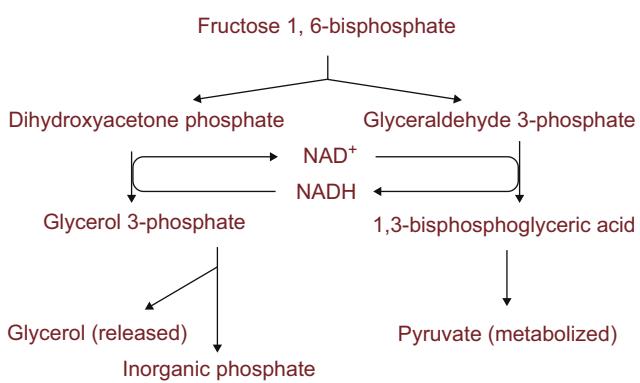


FIGURE 7.23 Simplified pathway showing how NADH derived from the oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglyceric acid is used in the reduction of dihydroxyacetone phosphate to glycerol. As a consequence, NADH is unavailable to reduce acetaldehyde to ethanol.

is in contrast to the net production of two ATP molecules, and release of CO₂, during the fermentation of glucose to ethanol. The separate functions of redox balance and osmotolerance appear to be regulated by different isozymes of glycerol-3-phosphate dehydrogenase, GPD1 and GPD2 (Ansell et al., 1997).

The presence of sulfur dioxide increases the production of glycerol. This probably comes as an indirect effect of bonding with acetaldehyde. This limits its reduction to ethanol, blocking the usual means by which alcoholic fermentation regenerates NAD⁺.

Because of the changing conditions throughout batch fermentation, yeast cells need to adjust their levels of ATP and reducing power to synthesize necessary metabolic intermediates, while maintaining favorable redox and ionic balances. Consequently, the concentration of yeast by-products in the cytoplasm and juice changes continuously throughout fermentation (see Figs. 7.22 and 7.23). Because several metabolic intermediates are aromatic, for example acetic acid, acetoin (primarily by conversion from diacetyl), and succinic acid, their presence can affect bouquet development. The accumulation of acetyl CoA (as a result of limited activity of Krebs Cycle enzymes) may explain the accumulation and release of acetate esters during fermentation. The alcoholysis of acetyl CoA (and other acyl SCoA complexes) during esterification would release CoA for other metabolic functions. In addition, the formation of other aromatics, notably higher alcohols, reflects the relative availability of amino acids and other nitrogen sources in the juice. Adequate availability permits amino acids to be used as an energy source or generates organic acids, fatty acids, and reduced-sulfur compounds.

Although all strains of *S. cerevisiae* possess the same set of enzymes, their catalytic activities may vary due to allelic differences. In addition, slight differences in regulation or gene copy number mean that any two strains are unlikely to respond identically under the same conditions. This variability undoubtedly accounts for many of the subtle (and not so subtle) differences between fermentations conducted by different strains. For example, overexpression of cytoplasmic malate dehydrogenase increases not only the accumulation of malic acid, but also fumaric and citric acids (Pines et al., 1997). In addition, overexpression of glycerol 3-phosphate dehydrogenase not only results in marked increases in glycerol production, but also augments the accumulation of acetaldehyde, pyruvate, acetate, 2,3-butanediol, succinate, and especially acetoin (Michnick et al., 1997). This is the mechanism by which glycerol production may be enhanced by heat shocking reactivated yeast before inoculation (Berovic and Herga, 2007). The shift in metabolism toward glycerol synthesis is of enologic interest as it could result in more traditional alcohol contents in wines produced from grapes having high °Brix

values. This is becoming more common, as prolonged growing seasons, associated with delayed harvest, can lead to table wines possessing up to 15% ethanol.

Influence on grape constituents

Yeasts have their major effect on the sugar content of juice or must. If fermentation goes to completion, only minute amounts of fermentable sugars remain (≤ 1 g/L). Small amounts of nonfermentable sugars, such as arabinose, rhamnose, and xylose, also remain (~ 0.2 g/L). These small quantities have no sensory significance, leaving the wine tasting "dry."

Yeasts may increase the pH by metabolizing malic acid to lactic acid. However, the proportion converted is highly variable, differing among strains by 3%–45% (Rankine, 1966). Some strains of *Saccharomyces paradoxus* can degrade malic acid up to 40% (Orlic et al., 2007). In contrast, some strains of *S. cerevisiae* synthesize malic acid (more so at warmer temperatures) (Farris et al., 1989). This is more common a feature of *S. bayanus* var. *uvarum*.

Schizosaccharomyces pombe, a common member of the grape epiphytic flora, can completely decarboxylate malic acid to lactic acid (Benito et al., 2013). It also has the potential to limit urea and alcohol accumulation. The former reduces the potential for ethyl carbamate production and could limit alcohol accumulation in wines made from overmature grapes. Nonetheless, this yeast has been little used because its sensory impact has generally, but not consistently, been viewed as negative. The chemical reasons for this are unclear. Delaying its inoculation until after *S. cerevisiae* has been active for several days or has completed fermentation apparently reduces its potential negative sensory impact (Carre et al., 1983). Immobilization appears to be an alternative and effective approach that does not spoil the wine's character (Silva et al., 2003). It also permits greater control over the degree of deacidification.

During fermentation, the release of alcohols and other organics helps dissolve compounds from seeds and skins. Quantitatively, the most significant chemicals extracted are anthocyanins and various flavonoid phenolics, notably tannins. The latter are partially dependent on the solubilizing action of ethanol. Anthocyanin extraction often reaches a maximum within 3–5 days, when the alcohol content has reached about 5%–7% (Somers and Pocock, 1986). As the alcohol concentration continues to rise, color intensity may begin to fall. This can result from the coprecipitation of anthocyanins with grape and yeast cells to which they may bind. Nevertheless, the primary reason for color loss appears to be disruption of weak anthocyanin complexes present in the juice. Freed anthocyanins may convert into uncolored states. Although extraction of tannins occurs more slowly, their content often reaches higher values than anthocyanins.

Tannin extraction from stems (rachis), if present, may reach a plateau after about 7 days. Seed tannins are the slowest to be liberated; their accumulation may still be active after several weeks (Siegrist, 1985).

Ethanol also aids the solubilization of certain aromatic compounds from grape cells. Unfortunately, little is known about the dynamics of this process. Conversely, ethanol decreases the solubility of other grape constituents, notably pectins and other carbohydrate polymers. The pectin content may fall by upward of 70% during fermentation.

As noted, the metabolic action of yeasts produces many important wine volatiles, notably higher alcohols, fatty acids, and esters. Yeast metabolism may also degrade some grape aromatics, notably aldehydes. This potentially could limit the expression of the herbaceous odor generated by C₆ aldehydes and alcohols produced as a consequence of oxidation during the grape crush. Yeasts can also influence wine flavor by decarboxylating hydroxycinnamic acids to their equivalent vinylphenols. More significantly, fermentation may play a major role in the liberation of varietal aromatics, notably those bound in complexes with glycosides (Williams et al., 1996) or cysteine (Tominaga et al., 1998). Because yeast strains differ significantly in these attributes (Howell et al., 2004), strain choice can either enhance, diminish, or modify varietal expression.

Indirect effects of yeast action include wine color modification (Eglinton et al., 2004; Medina et al., 2005). This may involve pigment loss, by adherence to grape cell remnants and yeast mannoproteins, or color stabilization by the release of carbonyls, such as pyruvic acid and acetaldehyde, and the generation of vinylphenols. Because yeast strains vary in these characteristics, strain selection can influence color depth and stability in red wines (Bartowsky et al., 2004b; Morata et al., 2006).

Yeast

Classification and life cycle

Yeasts are a diverse group of fungi characterized by possessing a unicellular growth habit. Cell division may involve budding (extrusion of a daughter cell from the mother cell) (Plate 7.6) or fission (division of the mother cell into one or more cells by localized ingrowths). Occasionally, yeasts may form short chains. Yeasts are also distinguished by possessing a single nucleus—in contrast to the frequently variable number of nuclei found most filamentous fungi. In addition, the composition of their cell walls is unique. The major fibrous cell-wall component of most fungi, chitin, occurs only as a minor component in yeast cell walls. It is

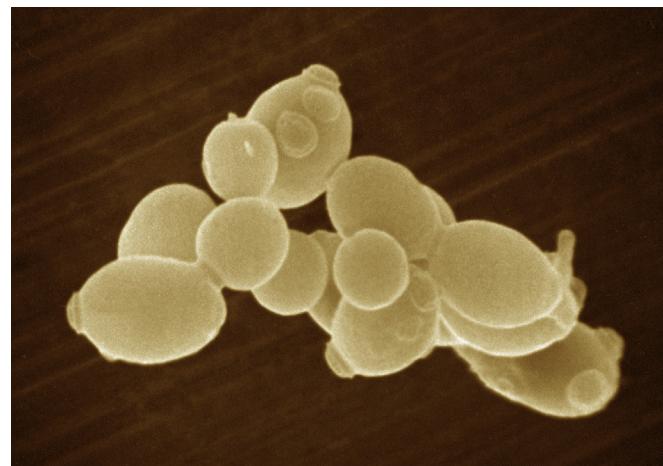


PLATE 7.6 Scanning electron micrograph of *Saccharomyces cerevisiae*. Photo courtesy Lallemand.

localized to the bud scar, the site where new (daughter) cells originate. The major constituent of yeast cell walls (β -1,3-D-glucans) consists of chains of glucose molecules. Also present are mannoproteins, complex polymers of the sugar, mannose and proteins. These are covalently linked to either β -1,3-D-glucans or smaller amounts of β -1,6-D-glucans.

Although characterized by a distinctive set of properties, yeasts are not a single evolutionary group. A yeastlike growth habit has evolved independently in at least three major fungal taxa—the Zygomycota, Ascomycota, and Basidiomycota. Only yeast members of the Ascomycota (and related imperfect forms) are significant in wine production. Most imperfect yeasts (those that have lost the ability to undergo sexual reproduction), are derived from ascomycete yeasts. Under appropriate conditions, most ascomycete yeast cells differentiate into ascospores—the structures in which haploid spores are produced through meiosis and cytoplasmic division.

In *Saccharomyces cerevisiae* and related species, four haploid spores are produced as a consequence of meiosis (Fig. 7.24). After rupture of the ascospore wall (originally the mother cell wall), spores typically germinate to produce haploid vegetative cells. Those of opposite

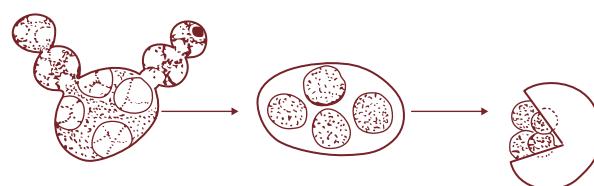


FIGURE 7.24 Stages of yeast development. Left to right: budding vegetative cells, ascospore development, spore release from ascus. A yeast cell can reproduce vegetatively about 20 times before it dies.

mating type usually fuse shortly after germination to reestablish the diploid state. This fusion may occur even before rupture of the ascus wall. Although individual cells only have the capacity to grow and bud about eight times before dying, newly budded cells have the same capacity as the mother cell—to generate (bud) eight new cells. Cellular death apparently results from disruption caused by the accumulation of circular copies of rDNA in the nucleus (Sinclair and Guarente, 1997). Although possible, most *Saccharomyces cerevisiae* wine strains rarely express this potential in must or wine. Ascus development appears to be suppressed by high concentrations of glucose, ethanol, and CO₂.

If sporulation is desired, as in breeding experiments, nutrient starvation, the addition of sodium acetate, or both, can induce ascospore production in *S. cerevisiae*. Bi-carbonate accumulation in the growth medium also acts as a meiosis-promoting factor (Ohkuni et al., 1998).

Until the late 1970s, yeast classification was, by necessity, based on physiological properties and the few morphological traits readily observable under the light microscope. These have now been supplemented or replaced with genomic analysis. These procedures will hopefully usher in a period of more stable classification, based on evolutionary relationships.

In most recent taxonomic treatments (e.g., Kurtzman et al., 2011), many named species of *Saccharomyces*

have been reduced to synonymy. This does not refute the differences that formerly were used to distinguish "species," but rather indicates that they were either minor genetic variants or genetically unstable. Most of these former species are viewed as physiological races of recognized species or occasionally members of different genera. For example, *S. fermentati* and *S. rosei* are considered strains of *Torulaspora delbrueckii*. Fig. 7.25 illustrates a modern interpretation of the relatedness among *Saccharomyces* and related genera, species, and strains.

S. cerevisiae and related species (*Saccharomyces sensu stricto*) apparently evolved from an ancient chromosome doubling (autopolyploidy) (Wolfe and Shields, 1997; Wong et al., 2002). This was followed by inactivation or loss of most duplicate chromosomes (diploidization). Although this is estimated to have occurred millions of years ago, hybridization and polyploidy still occur within the genus (de Barros Lopes et al., 2002; Naumova et al., 2005). For example, *S. pastorianus* is an allopolyploid hybrid between *S. cerevisiae* and *S. eubayanus* (Libkind et al., 2011). Appendix 7.1 provides a list of accepted names and synonyms for yeasts commonly found on grapes or in wine. Differences between some of the physiological races of *S. cerevisiae* (formerly given species status) are noted in Appendix 7.2.

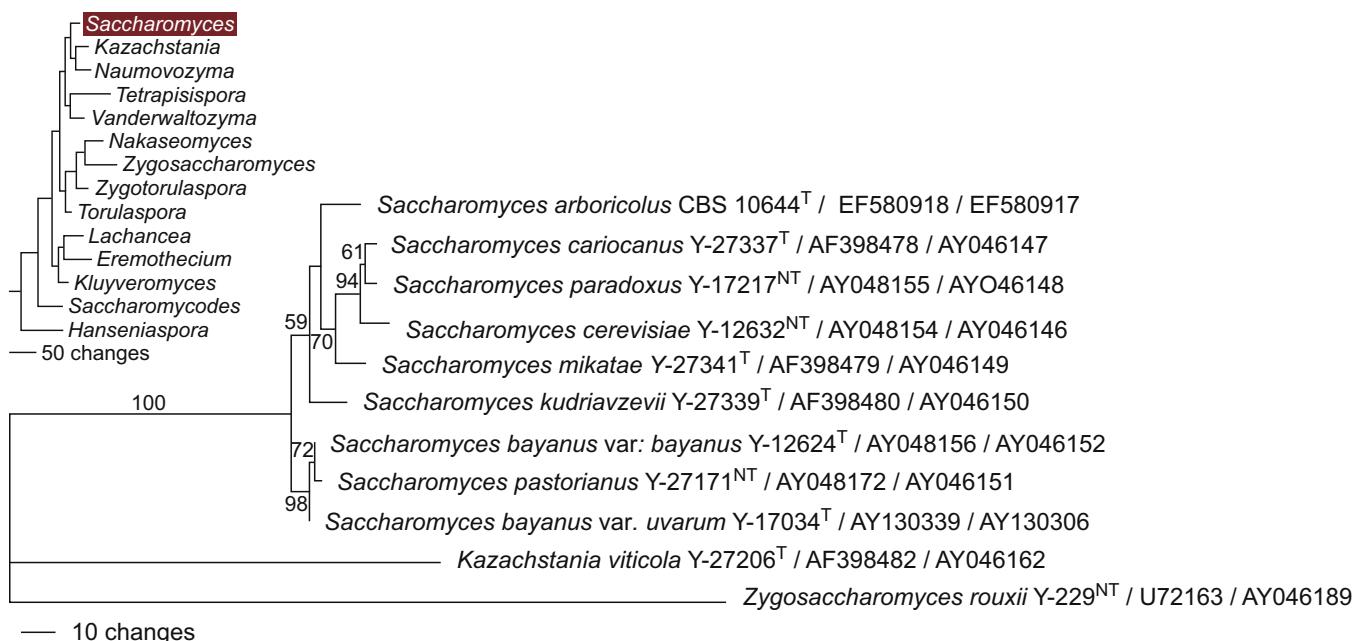


FIGURE 7.25 Phylogenetic relationships among *Saccharomyces* spp. determined from maximum parsimony analysis of the combined sequences of the D1/D2 LSU rRNA gene and ITS. Species names are followed by CBS or NRRL strain accession numbers, and respectively the GenBank accession numbers for D1/D2 and ITS. Bootstrap values given at the nodes are from 1000 replicates. T = type strain, NT = neotype strain. The small tree, which shows relationships among genera, was determined from maximum parsimony analysis of combined sequences of the nearly entire LSU rRNA, SSU rRNA and translation elongation factor-1 α genes. Reprinted from Vaughan-Martini, A., Martini, A., 2011. *Saccharomyces Meyen ex Reess* (1870). In: Kurtzman, C.P., Fell, J.W., Boekhout, T. (Eds.), *The Yeasts, a Taxonomic Study*, fifth ed. Elsevier, Amsterdam, pp. 733–746, with permission from Elsevier.

Yeast identification

Identification procedures based on genomic analysis permit the rapid (hours vs. days) identification of species, even strains from small samples (Cocolin et al., 2000; Martorell et al., 2005). The procedures are based primarily on ribosomal (rRNA) genes. These possess conserved unique segments—termed primers in polymerase chain reaction (PCR) procedures. rRNA genes have the advantage over single-copy genes in that they occur in multiples (50–100 copies/cell), making the test more sensitive. Real-time PCR procedures have the added advantage that they can be used to both identify and quantify species/strains from a site (Martorell et al., 2005). Thus, investigations of yeast origin and activity are now possible that were once too onerous (e.g., determining what strain(s) are conducting fermentation at any point during the process as well as the dynamics of their population fluctuations). These techniques have not only been a boon to academic research, but also have spawned renewed interest in endemic strains as potential sources of regional typicity or site-specific (*terroir*) specificity (Bokulich et al., 2014; Comitini et al., 2017).

Molecular techniques are also particularly useful in the early detection of potential spoilage organisms. They are also being applied to assessing the dynamics of physiological changes during fermentation. Regrettably, these procedures are costly, technically demanding, and currently only available in research centers, commercial laboratories, or the largest of wineries.

Alternative techniques for the differentiation between fermentative and spoilage yeasts involve free fatty acid analysis, gas chromatography, or pulsed-field electrophoresis. However, these are equally unavailable to the vast majority of wineries. Computer-based analysis of data via synoptic keys, in contrast to structured dichotomous keys (Payne, 1998), would facilitate identification using standard culture techniques. In their absence, specialized culturing procedures, such as those given by Cavazza et al. (1992), or the standard methods noted in Kurtzman et al. (2011), are effective but not speedy.

Traditional identification required the isolation of single cells, and their reproduction on culture media. This typically involved dilution from the source, usually containing upward of millions of cells/mL. Selective media also often was required for the isolation and culture of different species. Identification was based primarily on the capacity to grow with or without particular nutrients. Depending on how isolation is done, estimates of the number of viable (culturable) cells of each species and strain could be obtained. However, just because an organism is isolated does not necessarily mean that it was growing or physiologically active in the juice, must, or wine. Conversely, inability to culture an

organism does not necessarily mean it was not present in a viable state or was metabolically inactive. Yeasts may survive for extended periods in a dormant (unculturable) state (Cocolin and Mills, 2003).

Because of the time, equipment, and experience required in the effective use of even traditional identification techniques, this was often beyond the capacity of most wineries. Except for large wineries, yeast identification has typically been contracted out to commercial laboratories.

Another complexity re identification, unrelated to isolation or identifying issues, relates to how yeast (or other microbial) species are delimited. Because it is, and may always remain, a taxonomic conundrum, shifts in yeast nomenclature may continue to be perplexing and frustrating forever. Contributing to confusion for the newcomer is the alternative use of a yeast's teleomorph (sexual) and anamorphic (asexual) name (e.g., Dekkera vs. *Brettanomyces*).

Yeast evolution and grape flora

Saccharomyces cerevisiae is undoubtedly the most important yeast species. In various forms, it functions as the wine yeast, brewer's yeast, distiller's yeast, and baker's yeast (Fay et al., 2019). In addition, laboratory strains are extensively used in industry and in fundamental studies on genetics, biochemistry, and molecular biology. For all its importance, the natural habitat of *S. cerevisiae* is only beginning to be suspected with some certainty. Current evidence indicates that its indigenous niche is the sap, bark and acorns of oak trees, other members of the Fagaceae, and adjacent soil (Peter et al., 2018). In these sites, it may co-inhabit with its most closely related species, *S. paradoxus*. The latter has often been viewed as the progenitor of *S. cerevisiae*. This view is supported by their extensive genetic and physiological similarities, equivalent natural habitats, and ability to ferment wines to dryness (Redžepović et al., 2002). Nonetheless, the two species appear not to exchange genes due to DNA mismatch repair systems. Other *Saccharomyces* species that may occur sympatrically with *S. cerevisiae* and *S. paradoxus* are *S. kudriavzevii* and *S. uvarum* (Sampaio and Gonçalves, 2008).

Wild strains of *Saccharomyces cerevisiae* and *S. paradoxus* differ primarily by possessing genomic sequence divergence; showing different thermal growth profiles; and being reproductively isolated (i.e., having an inability to mate under natural conditions). They also vary in that wild strains of *S. cerevisiae* demonstrate limited genetic distinction across continents, versus partial reproductive isolation between dispersed populations of *S. paradoxus* (Sniegowski et al., 2002; Kuehne et al., 2007). Wild strains of *S. cerevisiae* are

distinguishable from wine strains by being prototrophic and sporulation-proficient. In contrast, wine strains exhibit less genetic diversity, show variation in ploidy, aneuploidy, and genomic introgressions (Peter et al., 2018) (Almeida et al., 2015; Fig. 9.36). Attributes adapting wine strains to being effective fermentative organism apparently arose from insertions derived from other yeasts (Fig. 7.26 (e.g., *S. uvarum* and *S. eubayanus*). These properties include more rapid growth, improved nitrogen utilization, and enhanced resistance to copper and sulfites (see Marsit and Dequin, 2015). They also differ in the aromatic aspects they donate to wines: wild versions generating earthy and sulfurous attributes, whereas wine strains yield fruity/floral characteristics (Hyma et al., 2011).

Although *Saccharomyces cerevisiae* had been isolated from fruit flies, bees and wasps, their importance in the yeast life cycle of yeast was unknown. Recent studies have demonstrated that they may have been instrumental in some of the genome introgressions noted above. In the case of social wasps, they act as a site for sexual gene transfer (Stefanini et al., 2016). Insects as well as birds can act as dispersal agents.

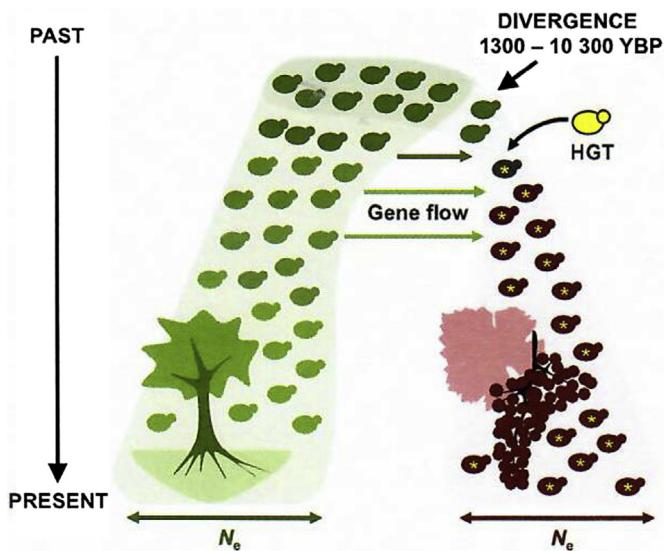


FIGURE 7.26 The emergence of domesticated *Saccharomyces cerevisiae* wine strains (red) from wild Mediterranean oak populations (green), according to Almeida et al. (2015). Domestication probably took place 1000–11,000 YBP, creating a bottleneck event (effective population size, N_e , reduction) and initiating the divergence between the two populations. Wine strains inherited genomic elements providing enhanced wine characteristics (yellow stars) by horizontal gene transfer (HGT) from other fungi. Progressive divergence between wild and wine populations was accompanied by limited gene flow, mostly from the wild into wine populations (green arrows) followed by continuous population size increase (proportional to branch width). From Eberlein, C., Leducq, J.-B., Landry, C.R., 2015. The genomics of wild yeast populations sheds light on the domestication of man's best (micro) friend. *Molec. Ecol.*, 24, 5309–5311, reproduced with permission of John Wiley & Sons.

In spite of the association of *S. cerevisiae* with wine, it is usually absent or rare on healthy grapes. Even in long-established vineyards, the isolation of *S. cerevisiae* (in small numbers) occurs only near the end of ripening. Mortimer and Polsonelli (1999) estimate about one healthy berry per 1000 carries wine yeasts. However, on surface-damaged fruit, the frequency may rise to one in four (1×10^5 to 1×10^6 yeast cells/berry). In Croatia, Redžepović et al. (2002) found that *S. paradoxus* was more frequently found on grapes than *S. cerevisiae*. A comparison of the attributes contributed to wine by *S. paradoxus* versus *S. cerevisiae* is provided by Majdak et al. (2002) and Orlić et al. (2010).

A related species, *Saccharomyces bayanus*, can also conduct effective alcoholic fermentations. Nevertheless, they are less encountered, and their use typically associated with special winemaking situations. For example, *S. bayanus* var. *bayanus* has properties especially well adapted to the production of sparkling wines and fino sherries. *S. bayanus* is also well adapted to fermenting white wines from relatively neutral flavored grapes grown in warm climates. It tends to produce little volatile acidity, augments glycerol as well as malic and succinic acid contents, and generates more aromatic alcohols and ethyl esters (Castellari et al., 1994; Antonelli et al., 1999). For cool fermentations (below 15°C), cryotolerant *S. bayanus* var. *uvarum* is of particular value. It is often involved in the production of tokaji, amarone, and sauternes wines. In some regions, such as Alsace, it has been reported to be the predominant fermentative yeast (Demuyter et al., 2004). The origin of the species (often considered subspecies of *S. cerevisiae*) is unknown, as are their natural habitats. Wild strains have occasionally been isolated from the caddis fly, some mushroom species, and hornbeam tree exudate (Carpinus).

Attribute tendencies of different species, subspecies, and strains have been investigated, but these are often significantly influenced by the conditions of fermentation and the chemical composition of the juice/must. Thus, precise prediction of their actions under specific conditions of a particular vintage is impossible. Individual experimentation under in-house conditions and over several years is the only way of assessing the suitability of any strain or strains.

Although wine yeasts rarely occur in significant numbers on the fruit, leaves, or stems of grapevines, other yeasts are frequently found. These include species of *Hanseniospora* (Kloeckera), *Candida*, *Pichia*, *Hansenula*, *Metschnikowia*, *Sporobolomyces*, *Cryptococcus*, *Rhodotorula*, and *Aureobasidium*. Their population numbers change during fruit ripening (Renouf et al., 2005), increasing markedly during the last few weeks of maturation. Endemic yeasts may be found on the fruit pedicel, but occur more frequently on its callused terminal ends, the receptacle. Yeasts are most frequently isolated from

around stomata on the fruit or next to cracks in the cuticle, where they form small colonies (Belin, 1972). They presumably grow on nutrients seeping out of openings in the fruit, receptacle, and pedicel. Yeasts do not grow on the plates of wax that cover much of the berry surface, the matte-like bloom. In fact, yeasts cease to grow where they come in contact with the waxy cuticular plates.

Commonly, the cells are dormant or only slowly reproducing. This unquestionably results from the typically dry state of the cuticle. Microbes require at least a thin coating of water to be metabolically active. This occurs only during rainy spells or when fog or dew condenses on grape surfaces. In contrast, damaged fruit surfaces, where juice may escape, may be slightly hygroscopic, providing a more favorable, albeit highly osmotic, site for yeast (and bacterial) growth.

As noted, grapes do not appear to have been the natural (ancestral) habitat for *S. cerevisiae* (or its progenitor). Nonetheless, winery equipment, and the winery itself, can act as a significant inoculum source for spontaneous fermentations (Ciani et al., 2004; Santamaría et al., 2005). This is suggested by the repeat isolation of one or a few dominant strain(s) from particular wineries. Outside the winery, strain spread seems limited, and occurs only over short distances (Valero et al., 2005). A possible exception occurs where pomace is spread as a vineyard soil conditioner. In addition, regional strains isolated from vineyards often differ (Khan et al., 2000). Thus, this lends credence to the view that endemic yeast populations could contribute to a site's regional character.

The most frequently occurring yeast species on mature grapes is *Kloeckera apiculata*. In warm regions, the perfect state of *Kloeckera* (*Hanseniaspora*) tends to replace the asexual form. Filamentous fungi, such as *Aspergillus*, *Botrytis*, *Penicillium*, *Plasmopara*, and *Uncinula*, are rarely isolated, except from diseased or damaged fruit. Similarly, acetic acid bacteria are usually found in significant numbers only on diseased or damaged fruit.

Under most conditions, especially where must is inoculated with a particular strain of *S. cerevisiae*, the indigenous yeast flora has often been viewed as of little significance. The acidic, highly osmotic conditions of grape juice were thought to retard the growth of most epiphytic yeasts, fungi, and bacteria. Also, the rapid development of anaerobic conditions and the accumulation of ethanol compromise the growth of most potentially competitive species. Nevertheless, in musts low in sugar content, the activity of yeasts less tolerant of high sugar and ethanol contents may be important (Ciani and Picciotti, 1995). Diseased grapes also have significantly modified and enhanced populations of the epiphytic flora. These can markedly affect the outcome of fermentation, even with inoculation.

Even on healthy grapes, other yeasts may occur at concentrations approaching those typically used in inoculated fermentations (10^5 – 10^6 cells/mL) (Fig. 7.28). Molecular evidence indicates that they may continue to persist in a viable, but unculturable state, throughout fermentation (Cocolin and Mills, 2003). At present, it is not established how commonly or for how long these species remain metabolically active (Millet and Lonvaud-Funel, 2000). Thus, their significance to vinification remains uncertain, and possibly underestimated. However, in some instances, they maintain or increase their numbers during fermentation (Mora and Mulet, 1991; Fig. 7.27). This appears to be more common when fermentation temperatures are low (Heard and Fleet, 1988), or yeast inoculation is delayed (Petering et al., 1993). Why this occurs is uncertain, but may relate to the influence of killer yeasts in the grape or winery flora (Maqueda et al., 2012). Only in diseased or damaged grapes is the microbial grape flora clearly known to play an important role in vinification.

In addition to the epiphytic yeast inoculum, juice and must may become inoculated from winery equipment (notably crushers, presses, and sumps) and the winery environment (Ocón et al., 2010). This is especially true in old wineries, in which the equipment and buildings are thinly covered with wine yeasts. In addition, *Hansenula anomala* and *Pichia membranaefaciens* commonly occur in wineries, with *Brettanomyces* and *Aureobasidium pullulans* often being isolated from the walls of moist cellars. The hygienic operation of present-day wineries greatly limits juice and must inoculation or contamination from the winery and its equipment. In such situations, the vineyard is presumably the main source of *S. cerevisiae* in spontaneous fermentations (Török et al., 1996).

Succession during fermentation

In spontaneous fermentations, there is a rapid and early succession of yeast species. Initially, *Kloeckera apiculata* and *Candida stellata* may occur in the range of 10^3 – 10^6 cells/mL. This number increases rapidly if grapes or must are left at warm temperatures. Although these endemic yeasts may grow at the beginning of fermentation, most strains soon pass into decline, with culturable numbers becoming but a minor component of the yeast population. This has frequently been interpreted as a result of the increasing concentration of ethanol produced by *Saccharomyces*, and/or the addition of sulfur dioxide. Nonetheless, other inter-strain and interspecies effects and influences may also play a role. Examples may involve the selective action of acetaldehyde (Cheraiti et al., 2005), and their relative abilities to translocate glucose into the cell (Nissen et al., 2004).

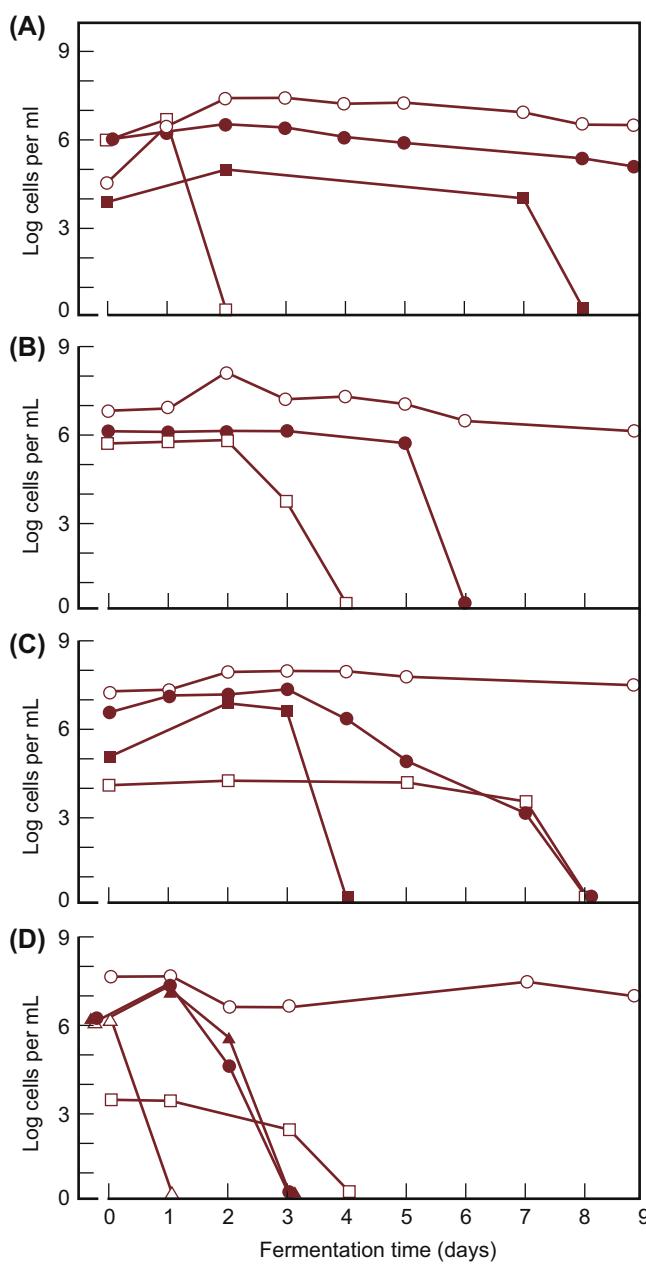


FIGURE 7.27 Yeast numbers during fermentation of white (A–B) and red (C–D) wines. ○, *Saccharomyces cerevisiae*; ●, *Kloeckera apiculata*; □, *Candida stellata*; ■, *C. pulcherrima*; ▲, *C. colliculosa*; △, *Hansenula anomala*. The initial population of *S. cerevisiae* comes predominantly from the inoculation conducted in the fermentations. From Heard, G.M., Fleet, G.H., 1985. Growth of natural yeast flora during the fermentation of inoculated wines. *Appl. Environ. Microbiol.* 50, 727–728, reproduced by permission.

During the initial stage of fermentation, the activities of non-*Saccharomyces* yeasts contribute to the production of compounds such as acetic acid, glycerol, and various esters (Ciani and Maccarelli, 1998; Romano et al., 2003). This can be sufficiently significant to influence the wine's aroma (Eglinton et al., 2000; Soden et al., 2000). For example, some strains of *Kloeckera apiculata* can

produce up to 25 times the amounts of acetic acid typically produced by *S. cerevisiae*. *K. apiculata*, along with other members of the grape epiphytic flora, may also produce above-threshold amounts of 2-aminoacetophenone. This compound has been associated with the naphthalene-like odor characteristic of untypical aging (Sponholz and Hühn, 1996). In addition, *K. apiculata* may inhibit some *S. cerevisiae* strains from completing fermentation (Velázquez et al., 1991), possibly due to its production of acetic acid, octanoic and decanoic acids, or "killer" factors. In addition, the indigenous grape flora may enhance amino acid availability by their proteolytic activities (Dizy and Bisson, 2000). At cool fermentation temperatures (10°C), yeasts such as *K. apiculata* may remain active (or at least viable) throughout alcoholic fermentation (Heard and Fleet, 1988; Erten, 2002).

Occasionally, strains of *Candida stellata* persist in fermenting juice (Fleet et al., 1984). Their ability to produce high concentrations of glycerol could play a role in enhancing a wine's smooth mouthfeel. Jolly et al. (2003) report that the perceived quality of Chenin Blanc wine was increased when *Candida pulcherrima* was combined with *Saccharomyces cerevisiae*. *Torulaspora delbrueckei* also has the potential to positively influence the sensory properties of wine, due to its low production of acetic acid and synthesis of succinic acid. Although *Kloeckera apiculata* and *C. stellata* typically ferment only up to approximately 4% and 10% alcohol, respectively, they are able to survive much higher alcohol concentrations (Gao and Fleet, 1988).

Of equal or possibly greater significance is the property of some *Hanseniaspora*, *Debaryomyces*, and *Dekkera* spp. to produce glycosidases (Villena et al., 2007). Their action in releasing terpenoids and other volatiles could positively affect the development of the varietal character of a wine. The role of their other enzymatic activities on flavor development is largely unknown, except for the negative influence of *Dekkera* spp.—its decarboxylation of hydroxycinnamic acids generating 4-ethylphenol and 4-ethylguaiacol.

Nonetheless, most members of the grape flora are either slow growing or typically suppressed by the low pH, high initial osmolarity of the juice/must, the ferment's augmenting ethanol content, oxygen deficiency, and/or sulfur dioxide. Their relative inability to incorporate glucose under vinous conditions may also play a significant role in their early demise (Nissen et al., 2004). Consequently, most species of *Candida*, *Pichia*, *Cryptococcus*, and *Rhodotorula* probably do not contribute significantly to fermentation. Their populations seldom rise above 10^4 cells/mL, and the species usually disappear quickly from the ferment. However, if warm conditions prevail, and active fermentation is delayed, these yeasts may initiate severe spoilage.

Under such conditions, *Pichia guilliermondii* has the potential to produce sufficient 4-ethylphenol to generate odors variously described as burnt beans, band-aid, wet dog, horse sweat, and barnyard (Barata et al., 2006).

Because of their potential to affect wine quality (Egli et al., 1998), there is increasing interest among winemakers in using endemic yeasts to give their wines distinctiveness (Fig. 7.28). Nevertheless, as with any technique, it needs to be used with discretion and tempered with experience. For example, cool fermentation without sulfur dioxide runs the risk that non-*Saccharomyces* yeast may dominate fermentation, generating sufficient acetic acid and fusel alcohols to mask grape varietal aromas. In addition, other yeasts can lead to stuck fermentation, especially under stressful vinifications (Ciani et al., 2006).

The few bacteria able to grow in juice or must are usually inhibited by *Saccharomyces cerevisiae*, with the exception of lactic acid bacteria. Thus, *S. cerevisiae* (or occasionally other *Saccharomyces* spp.) find few if any organisms capable of competing with them in grape must. Not surprisingly, *S. cerevisiae* tends to dominate and complete fermentation, even when its initial presence in must is rare (less than 1/5000 colonies), as in many spontaneous fermentations (Holloway et al., 1990). Subsequently, however, other yeasts may multiply in association with lactic acid bacteria during or following malolactic fermentation, notably *Pichia* spp. (Fleet et al., 1984).

Several investigations indicate that local populations of *S. cerevisiae* are heterogeneous, consisting of many

strains, differing considerably even among regional wineries (Bokulich et al., 2014). Although their numbers are usually low, they may occasionally reach 10^4 – 10^5 cells/mL, possibly derived from winery equipment or as residents on damaged grapes. There may also be shifts in their dominance from year to year (Bokulich et al., from site to site (Guillamón et al., 1996), and across the vine (Polsinelli et al., 1996).

If the must is inoculated, the strain typically comes to dominate fermentation. Nonetheless, members of the grape flora and winery may influence the wine's character (Bokulich et al., 2013), even if they remain metabolically active for only a few days (Querol et al., 1992). However, occasionally they remain at high populations throughout fermentation (Fig. 7.27A). Shifts in population numbers may also occur throughout fermentation when must is inoculated with several strains (Schütz and Gafner, 1993a; Sipiczki et al., 2004). Because of the low incidence of sexual recombination, infrequent ascospore production, and poor ascospore viability, it is suspected that mitotic recombination and mutation are the primary sources of this variability (Puig et al., 2000). However, strain selection in an existing heterogenous population could be at work as well.

Must inoculation

During inoculation, sufficient *Saccharomyces cerevisiae* is added to reach a population of about 10^5 – 10^6 cells/mL. With active dry yeasts, this is equivalent to about 0.1–0.2 g/L of juice/must. Active dry yeast often contains about 20 – 30×10^9 cells/g. In its production, the culture is treated so that the cells possess adequate amounts of protein, ergosterol, unsaturated fatty acids, and reserve materials for several divisions. Principal among the latter is trehalose, important in providing resistance to both drying and rehydration.

Inoculation is particularly common in the production of white wine, due to its lower nutrient status and yeast population (short maceration followed by clarification), and cool fermentation temperature. The different processes involved in red wine production mean inoculation is less necessary (i.e., fermentation with the pomace, warmer fermentation temperatures, possibility of some aeration during pumping over). Nonetheless, inoculation still has significant benefits in assuring a clean, predictable fermentation. With stuck fermentations, inoculation is essential.

Before addition, the inoculum is placed in water or dilute juice. Rehydration is recommended to occur at about 37°C for 20 min (details depending on the strain and supplier). Supplementation of the rehydration medium with small amounts of sterols (often in the form of yeast cell fragments) (Soubeyrand et al., 2005) increases both yeast viability and improves their

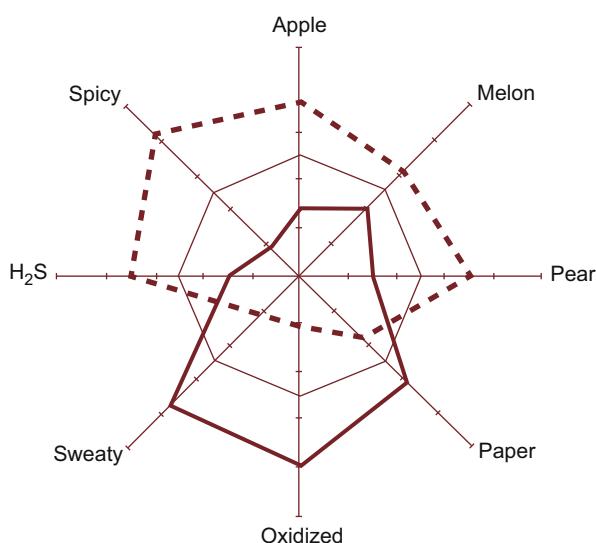


FIGURE 7.28 The effect of spontaneous (dashed line) and induced (thick solid line) fermentation on the sensory characteristics of Riesling wine; thin solid line, mean score. From Henick-Kling, T., Edinger, W., Daniel, P., Monk, P., 1998. Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. *J. Appl. Microbiol.* 84, 865–876, reproduced by permission.

fermentative ability. A short interval at 25°C before inoculation also improves survival, during which damaged cytoplasmic and membrane structures (Beker and Rapoport, 1987) are repaired and metabolism adjusts to the new conditions. This is important before the cells are exposed to the full osmotic potential of grape must. Readaptation occurs progressively (Fig. 7.29) and is estimated to involve about 2000 genes (Rossignol et al., 2006). Inoculation of cool musts requires even longer and progressive adaptation. Fractional addition of further juice, before final incorporation of the inoculum into the must, avoids rapid temperature changes and promotes retention of high cell viability.

Specific strains of *S. cerevisiae* first became commercially available in the 1950s, with development of active dry by the mid-sixties. They possess a wide range of characteristics, suitable for almost any winemaking situations. These include those that enhance varietal flavorants (e.g., releasing thiols from glycosidic or cysteinylated/glutathionylated complexes) (Holt et al., 2011); secrete acid proteases (potentially valuable in achieving protein stability) (Younes et al., 2011); synthesize varying amounts of HSPs; produce an abundance of fruit esters (especially valuable in fermenting neutral flavored cultivars); are of neutral character (allowing the varietal character to be highlighted); or possess high hydroxycinnamate decarboxylase activity (enhancing the formation of stable pyranoanthocyanin pigments). Strains are also available that are notable for their production of low levels of acetic acid, hydrogen sulfide, or urea. Others may be chosen because of their relative fermentation speed; their ability to synthesize or degrade malic or lactic acid; their ability to augment the concentration of glycerol; their ability to restart stuck fermentations; or their known value in

producing particular wine styles, notably carbonic maceration, late-harvest, or early- versus late-maturing reds. Yeast strain can also affect anthocyanin uptake and color stability (e.g., Pinot noir, Carew et al., 2013). In addition, there are locally selected strains that are reported to produce regionally distinctive wines.

Despite all the apparent phenotypic differences, most commercial strains are almost genetically identical (Borneman et al., 2016). Admittedly, unsuspected epistatic modifications may be generating known differences (e.g., competitiveness under winemaking conditions, García-Ríos et al., 2014). Nonetheless, the winemaker's choice is far from simple. In addition, much information is anecdotal. Suppliers provide data and suggestions on the various species and strains they provide, but cross-comparison between strains is fraught with difficulty, combined with the knowledge that local conditions often are pivotal in feature expression. Thus, it is again up to the winemaker to do their own experimentation to determine what best suits their needs and situations. Figs. 7.30 and 7.31 give an indication of sensory differences yeast strains may engender.

In only a few instances is inoculation absolutely essential. With thermovinification or pasteurized juice, most of the endogenous yeast population has been killed, requiring inoculation to achieve rapid initiation of fermentation. In addition, inoculation is necessary to restart "stuck" fermentations, and to promote fermentation of juice containing a significant number of moldy grapes. Moldy grapes generally possess various inhibitors, such as acetic acid, that slow yeast growth and metabolism. Finally, inoculation is required to assure the initiation of the second fermentation in sparkling wine production. However, the predominant reason for using a specific yeast strain or strains is to avoid

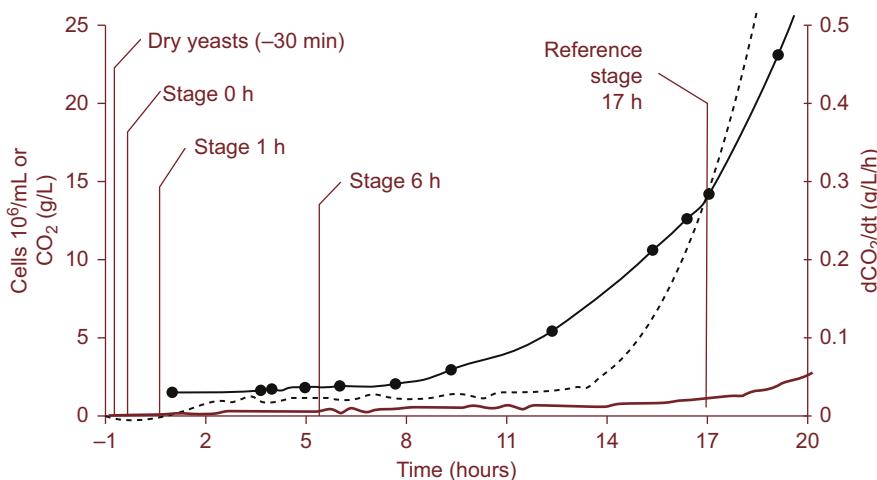


FIGURE 7.29 Evolution of cell population and fermentation parameters during and after rehydration of active dry yeasts: dashed line, cell population; burgundy solid line, fermentation rate; ●, cumulated CO₂ released. Reprinted from Rossignol, T., Postaire, O., Storai, J., Blondin, B., 2006. Analysis of the genomic response of a wine yeast to rehydration and inoculation. *Appl. Microbiol. Biotechnol.* 71, 699–712, with kind permission of Springer Scientific and Business Media.

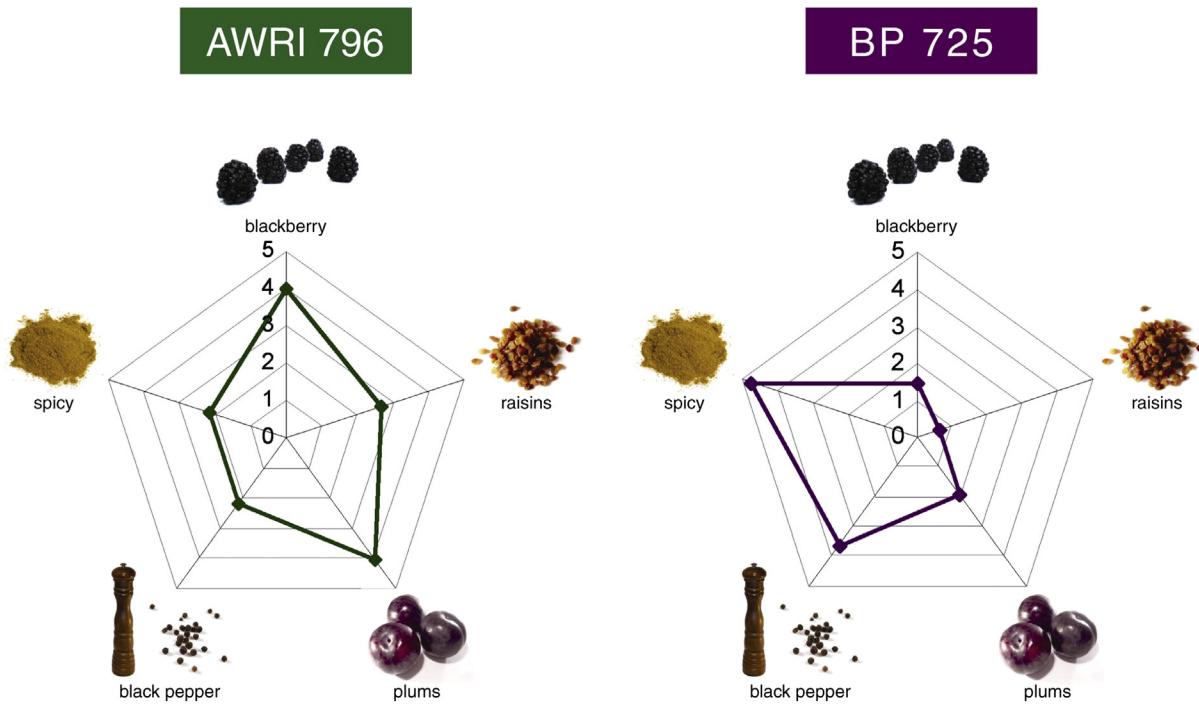
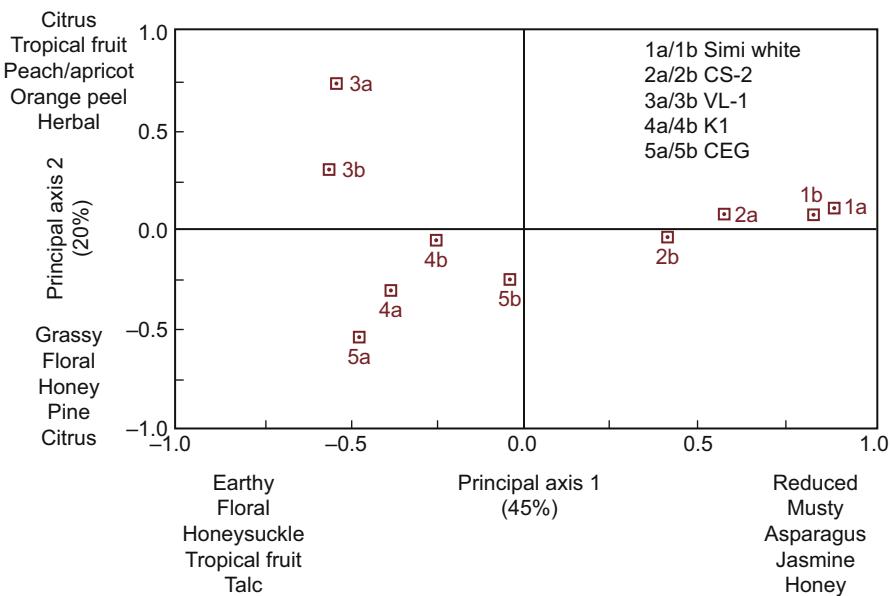


FIGURE 7.30 Comparative aromas of Shiraz wines after fermentation with AWRI 796 and BP 725. The ranking system was designed with one being the lowest intensity and six being the highest. *From Walsh, T., Heinrich, A., Skurray, G., 2006. Yeast contributes to Shiraz aroma and flavour. Aust. NZ Grapeseeker Winemaker 513, 78–80, reproduced with permission.*

FIGURE 7.31 Profile of aroma of a Riesling wine (after 20 months) fermented with different yeast strains. *From Dumont, A., Dulau, L., 1996. The role of yeasts in the formation of wine flavors. In: Henick-Kling, T. (Ed.), Proc. 4th Int. Symp. Cool Climate Vitic. Enol. New York State Agricultural Experimental Station, Geneva, NY. pp. VI–24–28, reproduced by permission.*



the production of undesirable flavors occasionally associated with spontaneous fermentations.

Spontaneous versus induced fermentation

There has been much discussion over the years concerning the relative merits of spontaneous versus induced fermentation. That different strains of

Saccharomyces cerevisiae supply distinctive sensory attributes is indisputable. This is particularly important for aromatically neutral cultivars. Their use may also play an increasingly important role in restricting the undesirably higher alcohol potentials of late-harvested grapes (Donaldson, 2008). Yeast strains differ significantly in ethanol production under

identical conditions. In addition, strain choice can equally affect the varietal character of aromatically distinctive cultivars, by influencing the liberation or synthesis of specific grape flavorants (Ugliano et al., 2006). These influences not only affect the sensory attributes of young wines, but are still detectable after 3 years (King et al., 2011). This may be even more significant with non-*Saccharomyces* yeasts. They appear to have greater activity in breaking glycosidic bonds (Mendes-Ferreira et al., 2001). These effects can be further modified or enhanced by *sur lies* maturation. Regrettably, our knowledge of the chemical origin of their sensory effects is insufficient to permit prediction of results. Nevertheless, using established strains provides the winemaker with the greatest confidence that fermentation will initiate rapidly, go to completion cleanly (diminishing the possibility of undesirable microbial activity, disruption from killer factors, or must oxidation), and possess relatively predictable flavor and quality attributes.

In contrast, spontaneous fermentations may accentuate yearly variations in character. Capitalizing on the mystic of vintage and *terroir* uniqueness is part of the marketing strategy of many producers. Spontaneous fermentations are often promoted as a means of generating greater aromatic complexity. Whether this is valid or not depends on the perceiver. It is commonly viewed by wine writers, and some wine professionals, to be of significance to the consumer. Nonetheless, for such yeast-derived attributes to be of legitimate relevance, their influence should be consistent, distinguishable, and recognizable by the consumer (a dubious conjecture). Implying uniqueness may have sales value at the winery or for wines produced at sites with a well-established international reputation. However, it is probably unrealistic to expect that most consumers can detect, appreciate, or value such nebulous differences. Even if statistically significant, they may be sensorially irrelevant. What may be more important is that wine critics *think* they can and promulgate that impression. Perception of reality is often more important than reality itself.

Although spontaneous fermentation can influence a wine's sensory character (Hernández-Orte et al., 2008; Varela et al., 2009), there is risk that these may be off odors and other undesirable sensory attributes. For example, the activity of *Kloeckera* and *Hanseniaspora* can increase the incidence of nonprotein haze formation (Dizy and Bisson, 2000). Spontaneous fermentations associated with oxidative yeasts are also frequently associated with higher concentrations of volatile acidity and ethyl acetate (Salvadores et al.,

1993). They also tend to possess noticeable lag periods (most likely due to the low *S. cerevisiae* inoculum) and thus be more susceptible to disruption by killer factors (see below) or suppression by other yeast species.

Those who favor spontaneous fermentation believe that the endemic grape flora supplies a desired subtle or regional character (Mateo et al., 1991), supposedly missing with induced fermentations. For large-scale wineries, where brand-name consistency is important, the risks of spontaneous fermentation outweigh its potential advantages. Even induced fermentations are not pure-culture fermentations, and correspondingly run the hazards of an indigenous microbial flora. Juice and must always contain sizable populations of epiphytic yeasts and bacteria, unless pasteurized or treated to thermovinification.

An alternative to either spontaneous or inoculated fermentation is coinoculation with a mix of local and commercial yeast strains. The combination may diminish individual differences, producing a more consistent but still distinctive character (Fig. 7.32). Alternatively *Saccharomyces cerevisiae* may be jointly inoculated with species, such as *Candida stellata* (Soden et al., 2000), *Hanseniaspora vineae* (Medina et al., 2013), *Debaryomyces vanriji* (Garcia et al., 2002), *Kluyveromyces marxianus* (Rollero et al., 2018), or *Pichia kluyveri* (Anfang et al., 2009). Each has unique metabolic attributes that may provide specific benefits to the resultant wine. For example, *K. marxianus* possesses pectinase activity, while *P. kluyveri* facilitates the liberation of the aromatically significant thiol, 3-mercaptophexyl. Because the interaction between species is often strain specific (Wang et al., 2016), individual testing is required to determine how they influence each other under local conditions.

The activity and survival of members of a mixture is highly dependent on a variety of factors, notably species and temperature (Fig. 7.33). Aeration of the must also favors the prolonged activity of non-*Saccharomyces* yeasts (Hansen et al., 2001). Because joint inoculation may result in partial suppression of one or more of the strains or species, sequential inoculation of non-*Saccharomyces* species, followed by later inoculation with *Saccharomyces cerevisiae*, may be required (Langue et al., 2005; Raynal et al., 2011). This generates, under semicontrolled conditions, a sequence that may resemble spontaneous fermentation.

As noted earlier, on-site experimentation is the only sure means of determining the value of any practice. The metabolic products released by any strain, species, or their combination depends largely on the specific fermentation conditions (Thorngate, 1998; Regodón Mateos et al., 2006).

FIGURE 7.32 Descriptive analysis of wines using the three fermentation treatments with a trained panel. Descriptors are shown as mean rating of aroma attributes with significant differences for secondary tier descriptors (shown in upper case) and tertiary tier descriptors (shown in lower case) for commercial, spontaneous and *Hanseniaspora vineae* cofermentation. From Medina, K., Boido, E., Fariña, L., Bioia, O., Gomez, M.E., Barquet, M. et al., 2013. Increased flavor diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae*. *Food Chem.* 141, 2513–2521, reproduced by permission of Elsevier.

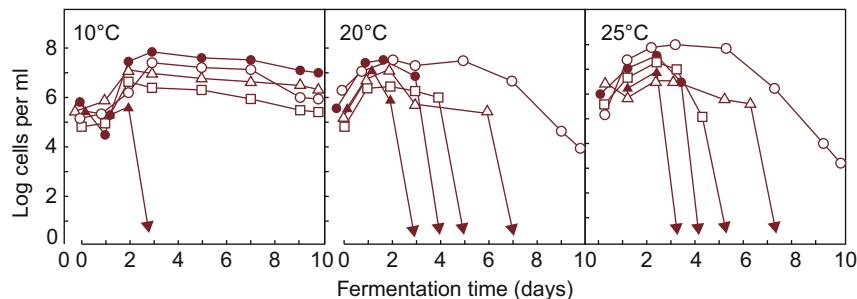
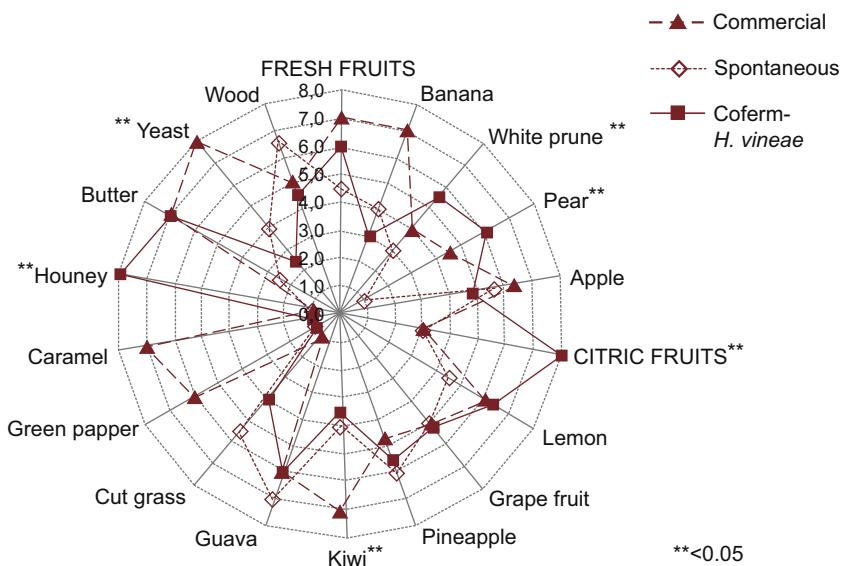


FIGURE 7.33 Growth of yeast species during mixed culture fermentation of Chardonnay grape juice (pH 3.5) at 10°C, 20°C, and 25°C: ○, *Saccharomyces cerevisiae*; ●, *Kloeckera apiculata*; △, *Candida stellata*; □, *Candida krusei*; ▲, *Hansenula anomala*. From Fleet, G.H., Heard, G.M., Gao, C., 1989. The effect of temperature on the growth and ethanol tolerance of yeasts during wine fermentation. Yeast 5, S43–S46, reproduced by permission.

Another choice for winemakers searching to add a distinctive aspect to their wine is to use cryotolerant yeasts, notably *S. bayanus* var. *uvarum*. It is characterized not only by its ability to ferment at cold temperatures (Fig. 7.34), but also by its potential to direct the development of desirable sensory characteristics. For example, cryotolerant yeasts generally produce higher concentrations of glycerol, succinic acid, 2-phenethyl alcohol, and isoamyl and isobutyl alcohols; synthesize malic acid; and produce less acetic acid than many mesophilic *S. cerevisiae* strains (Castellari et al., 1994; Massoutier et al., 1998).

Although different strains, species, and their combination can affect the sensory attributes of wines, until recently the question of whether these differences

affected consumer preference had not been studied. Under laboratory conditions, a relatively recent study obtained an affirmative answer (King et al., 2010). Whether such investigations reliably predict consumer purchase behavior is unestablished.

Yeast breeding

Genetically, *Saccharomyces cerevisiae* is the best understood eukaryote, having been used for decades as a favorite laboratory organism. It is easily cultured and has a comparatively small genome (~13,000 kb), possessing relatively little repetitive DNA and few introns. The complete genome of a laboratory strain was deciphered in 1997, indicating that it possessed about 5800 protein-coding genes, located on 16

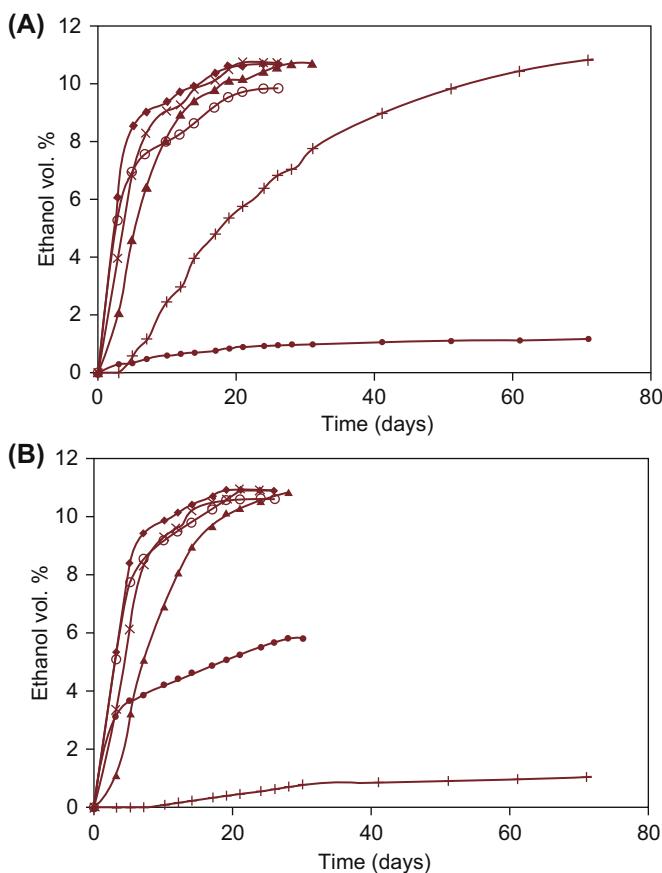


FIGURE 7.34 Fermentation curves at six different temperatures (+, 6°C; ▲, 12°C; ×, 18°C; ◆, 24°C; ○, 30°C; ●, 36°C) for (A) cryotolerant and (B) noncryotolerant strains. From Castellari, L., Magrini, A., Passarelli, P., Zambonelli, C., 1995. Effect of must fermentation temperature on minor products formed by cryo- and non-cryotolerant *Saccharomyces cerevisiae* strains. Ital. J. Food Sci. 7, 125–132, reproduced by permission.

chromosomes. Furthermore, its proteome (full set of proteins) may be defined in the near future. In addition, recent developments in DNA microarray technology may make it easier to understand the intricacies and interconnections of its metabolic pathways (see Cavalieri et al., 2000). These advances should permit a shift from the traditional cross-and-select breeding to one based on the selective elimination, modification, or introduction of specific genes.

Although *S. cerevisiae* is admirably suited to its role as the predominant fermenter of grape must, and there exists a wide diversity of strains possessing distinctive and useful properties, improved expression or new properties would be useful. Such modifications can vary from subtle variations in aromatic synthesis (Fig. 7.35) to the incorporation of properties such as malolactic fermentation.

Genetic modification

In contrast to industrial fermentations, winemaking has made little use of genetic engineering (Cebollero et al., 2007). This is partially explained by the complex chemical origin of wine quality, which makes delineating specific improvements difficult. In addition, other factors such as grape variety, fruit maturity, and fermentation temperature are generally, but not necessarily, considered of greater importance. In addition, more is known about the negative influences of certain yeast properties than about the positive sensory attributes they may donate. Finally, public suspicion of genetic engineering is regrettably, but undoubtedly, a prime reason for its minimal use.

Features controlled by one or a few genes are the most easily modified. For example, inactivating the gene that encodes sulfite reductase limits the conversion of sulfite to H₂S. Improvement in other attributes, such as flocculation, has been more difficult. Flocculation is regulated by several genes, epistatic (modifier) genes, and possibly cytoplasmic genetic factors (Teunissen and Steensma, 1995). The major locus involved encodes cell-surface proteins, notably lectins and lectin receptors. These are not constitutively present on the cell surface, but appear later in colony growth. This may result from the proteins being selectively deposited where budding has occurred, and at the tips of buds (Bony et al., 1998). In addition, the flocculation mechanism employed by different yeast strains can differ, as is the case with top-versus bottom-fermenting brewing yeasts (Dengis and Rouxhet, 1997).

In a similar manner, most important enologic properties appear to be under multigenic control (Marullo et al., 2004). Alcohol tolerance, and the ability to ferment steadily and cleanly at low temperatures, are undoubtedly multigenic; individual genes possessing slight or synergistic effects. Genetic improvement is possible, but probably will take considerable effort and time to achieve. Chances of improvement are significantly enhanced if the biochemical basis of such factors is understood. This knowledge can pinpoint the genes most likely worth modifying.

Even more convoluted problems arise from our inability to predict the consequences of changing the direction of metabolic pathways (Prior et al., 2000). Modifying the regulation of one pathway can have important and unforeseen consequences on another (Varela et al., 2012). Unanticipated metabolic disruptions are less likely when the compound concerned is the end by-product of a metabolic pathway. Thus, terpene synthesis has been incorporated into a wine strain, by the transfer of farnesyl diphosphate synthetase from a laboratory strain, without apparent

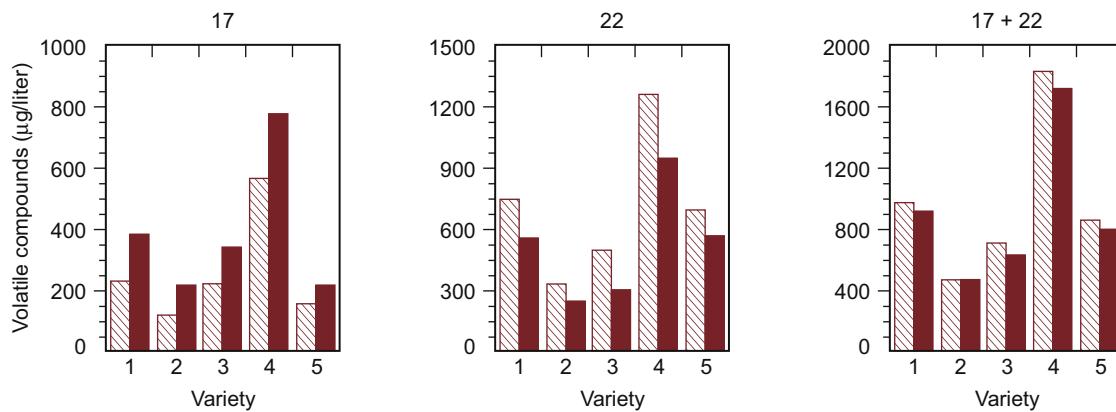


FIGURE 7.35 Concentration of the volatile components hexyl acetate (17), hexanol (22), and the sum (17 + 22) in wines of five cultivars (1, Chenin Blanc; 2, Sémillon; 3, Muscat of Alexandria; 4, Cape Riesling; 5, Colombar) with the yeast strains WE 14 (crosshatched bars) and WE 452 (filled bars). From Houtman, A.C., du Plessis, C.S., 1985. *Influence du cépage et de la souche de levure*. Bull. O.I.V. 58, 236–246, reproduced by permission.

undesirable side-effects (Javelot et al., 1991). Use of the modified yeast gives the wine a Muscat-like attribute.

Many techniques are available to the researcher interested in improving wine yeasts. The most direct approach involves simple selection and is often initially effective. This method is much facilitated if a selective culture medium can be devised to permit only cells containing the desired trait to multiply. Otherwise, cells must be laboriously isolated and individually studied for the presence of the desired trait.

Alternatively, adaptive evolution has had a long and successful history in developing industrial microbes. Although little used in wine-yeast breeding, its applicability has been illustrated by McBryde et al. (2006) and Tilloy et al. (2014). It avoids problems associated with the reticence of many winemakers and consumers to accept wine produced using genetically engineered strains. However, for certain changes, more direct means are required to modify the genetic makeup. This can involve procedures such as hybridization, backcrossing, and mutagenesis or newer techniques such as somatic fusion and genetic engineering.

Interspecific hybridization has occurred naturally in the past within *Saccharomyces sensu stricto* species (de Barros Lopes et al., 2002)). It has the potential to augment (Steenseis et al., 2014) as well as introduce novel flavor and aroma diversity (Bellon et al., 2011). This may be facilitated by passage of the spores through the gut of *Drosophila*, a known vector of *S. cerevisiae* (Reutter et al., 2007), and occurs in the guts of social wasps (Stefanini et al., 2016). Nonetheless, wine yeast breeding has proven difficult. One of the principal constraints results from the early self-fertilization (intraascus fusion of spores of opposite mating-type) (Cubillos et al., 2009).

This results from the tendency of ascospores to switch mating type shortly after germination, precluding designed mating.

Crossing difficulties can occasionally be avoided by tetrad analysis—the early physical isolation and separation of ascospores. Positioning spores from desired strains adjacent to one another can favor fusion and successful generic exchange. Selective mating can also be facilitated if the strains are first made heterothallic (Bakalinsky and Snow, 1990b). Most wine strains are homothallic (self-fertile), with haploid nuclei fusing within the ascus or shortly thereafter, obviating the possibility of desired crosses. Introduction of a recessive allele of the *HO* gene prevents switching of the mating-type gene, and correspondingly increases the probability of successful breeding. An alternative technique has been described by Ramírez et al. (1998). Regrettably, techniques that work successfully with well-characterized, haploid, laboratory strains do not necessarily work equivalently with industrial strains, such as wine yeasts.

Breeding wine strains is also exacerbated by the low frequency of sexual reproduction and spore germination (Bakalinsky and Snow, 1990a). Many strains undergo meiosis infrequently, a clear precondition for sexual reproduction. Of the haploid spores produced, infertility is frequent, probably due to the high incidence of aneuploidy (unequal numbers of similar chromosomes) (Bakalinsky and Snow, 1990a). Wine strains may also show chromosomal polymorphism, associated with rearrangement. It has been suggested that aneuploidy and other chromosomal abnormalities, due to their frequent occurrence, may possess unsuspected selective value under winemaking conditions.

Even in successful matings, the typical diploid state of wine yeasts can mask the presence of potentially desirable recessive alleles. In haploid organisms, the phenotype of recessive genes is expressed—there being no corresponding dominant (usually functional) allele. Although a complication in crossing experiments, diploidy permits “genome renewal” by allowing the selective elimination of growth-retarding alleles. These slowly accumulate due to mutation (Ramírez et al., 1999).

If only a single dominant genetic trait needs incorporating, backcross breeding can be effective. After incorporation of the desired trait, continued selection for the trait during repeated backcrossing with the desired strain can rapidly eliminate undesired donor genes, unintentionally incorporated in the original cross.

Although knowing the genetic nature of a desired trait is usually considered essential, breeding can occur when it is unknown or under complex genetic control. However, it requires crossing seemingly suitable strains and growing out tens of thousands of progenies. These must be individually assessed for their respective characteristics. Because this is so arduous, time-consuming, and expensive, breeders may incorporate selective techniques that permit only desirable progeny to grow.

In situations where standard procedures are not applicable (existing strains do not or are not known to possess the desired trait), “unconventional” techniques may be sought. For example, somatic fusion may permit the incorporation of traits from yeast species with which traditional mating is impossible. Somatic fusion requires enzymatic, cell-wall dissolution, and subsequent mixing of the protoplasts. This usually occurs in the presence of polyethylene glycol or some other agent that facilitates protoplast survival and fusion. Fusion permits the possibility of gene exchange by combining the genes of both species in a single cytoplasm.

A serious limitation and disadvantage with somatic fusion is the frequent instability of the association. Fused cells often revert to one of the original species. The incorporation of foreign genes can also interfere with expression of existing traits.

Less disruptive is the incorporation of one or only a few genes from a donor organism. The procedure is called transformation or more popularly, genetic engineering. It has the advantage that the donor and recipient need not be closely related, as required for crossing or somatic fusion. However, for it to be effective, its true genetic basis must be known. This may be established with a test-cross (crossing strains with and without the feature). Where the genes function in a dominant/recessive manner, obtaining one of the standard generic ratios provides clear evidence. However, where the alleles function in a codominant manner or

the phenotype is under multigenic control, a test-cross is likely to prove inconclusive. In this situation, mutant screening can be useful. Knowing this information is usually essential before attempting to locate the gene(s) and detecting markers to assist in their isolation for genetic procedures such as clustered regularly interspaced short palindromic repeat (CRISPR), TALEN, or ZFN.

In transformation, yeast protoplasts may be bathed in a solution containing DNA from the donor organism. These DNA segments also require appropriate initiator and termination sequences for proper function when incorporated. Upon uptake, the gene needs to be transported into the nucleus and inserted into a chromosome. Without this, it is likely to be unstable and lost in subsequent generations. An alternative procedure uses short, single-stranded oligonucleotides for allelic replacement (DiCarlo et al., 2013).

Alternatively, the desired gene may be spliced into a plasmid before uptake. Plasmids are circular, cytoplasmic DNA segments that partially control their own replication. Thus, even without chromosomal insertion, the gene may be replicated, along with the plasmid in the cytoplasm. Although most eukaryotic cells do not possess plasmids, wine yeasts frequently carry copies of a 2-μm plasmid. Despite their presence, the plasmid is nonessential to the host cell.

Using transformation, the malolactic gene from *Lactococcus lactis* has been transferred to *S. cerevisiae*, along with the malate permease transport gene from *Schizosaccharomyces pombe* (Bony et al., 1997). The possession of several copies of the malolactic enzyme facilitates the almost complete conversion of malic acid to lactic acid within 4 days. Recently, a commercial strain (ML01) of this type has been released, based on a construct of genes from *S. pombe* and *Oenococcus oeni* (Husnik et al., 2007). The strain conducts simultaneous alcoholic and malolactic fermentations. This permits rapid decacidification, without the development of the buttery and other flavors usually associated with bacterial malolactic fermentation. Wine stabilization procedures can commence immediately after alcoholic fermentation, without waiting (sometimes for months) for bacterial malolactic fermentation to come to completion.

Other genes of enologic interest that have been incorporated are β-(1,4)-endoglucanase (to increase flavor by hydrolyzing glycosidically bound aromatics), and alcohol acetyltransferase (to increase fruity flavors). Both features might benefit wines produced from aromatically neutral varieties. Yeasts have also been transformed with polysaccharide genes so that they can degrade glucans and xylans (Louw et al., 2006). They can increase the proportion of free-run wine and enhance the color intensity and stability of red wines.

Other examples involve combining the flocculation gene (*FLO1*) with a late-fermentation, HSP promotor (*HSP30*). Exposing the yeast to heat-shock can induce flocculation “on demand.” In another case, a homozygous strain, recessive for one of the aldehyde dehydrogenase genes (*ALD6*), was transformed with a high-copy 2- μ m plasmid containing the glycerol 3-phosphate dehydrogenase gene (*GPD2*). This diverted more sugar to glycerol production, without an overproduction of acetic acid (Eglinton et al., 2002). This has the potential benefits of reducing ethanol production (producing more balanced wines from fully mature grapes), increasing the fermentation rate (by enhancing osmotolerance) (Remize et al., 1999) as well as enhancing the wine’s smooth mouthfeel.

However, with characteristics based on many unlinked genes, it is a moot point whether genetic engineering has a selective advantage. In addition, genetic engineering is still complex and expensive. Part of this involves the long and complicated set of tests required before getting approval from the US Food and Drug Administration or equivalent regulatory agencies. There is also considerable controversy about the safety of releasing genetically modified organisms into the environment (Grossmann et al., 2011; Pérez-Torrado et al., 2015) as well as reluctance by a segment of consumers to consume products derived from genetically modified organisms, be they plant or microbial. Although caution is always prudent, imprudent caution impedes progress. When reflecting on the incredible modification that has occurred historically during crop domestication (Zohary and Hopf, 2000), notably with corn (Mangelsdorf, 1974), the modifications involved in genetic engineering seem almost inconsequential.

In contrast to the methods previously used in producing genetically modified organisms, CRISPR is far more precise and less expensive (Stovicek et al., 2017; Raschmanová et al., 2018). It usually involves the silencing or activation of individual genes, versus the insertion of a gene from another organism. RNA segments are used to locate the desired gene for modification (Fig. 2.27). An associated DNA endonuclease produces double-stranded breaks in the DNA segment recognized by CRISPR. Subsequent repair may involve processes such as nonhomologous knitting (end-joining) or homologous recombination. Such repair mechanisms (editing) may result in the excision of a gene segment (eliminating or changing the gene’s function), facilitate the insertion of a donor DNA segment (adding new functionality), or upregulate (increase) the gene’s transcription rate.

A requirement for all useful strains, no matter how they are derived, is genetic stability. Although a property of most traits, genetic stability is not a universal characteristic (Ambrona et al., 2005). For instance,

flocculant strains often lose their ability to form large clumps of cells, settling out as a powdery sediment. Genetic instability may arise due to aneuploidy, mutation, or epigenetic modification. The latter is a previously unsuspected but major factor in gene regulation. Being often influenced by environmental conditions or transposon movement, prediction seems impossible.

A final caveat relates to the difference between conditions in the laboratory, microvinification, and commercial production conditions. Not only do vinification procedures vary from winery to winery, but the indigenous yeasts and bacterial flora of grapes can significantly influence the attributes expressed by an inoculated strain. These differences frequently modulate the characteristics of the added strain, to such a degree that its intended effects may be largely negated or unpredictably qualified. Occasionally, indigenous yeasts even displace inoculated strains. As always, experience under actual winery conditions for several years is essential to have relative confidence in the actual value of “new and improved” strains.

Because of the oft mentioned reticence of consumers (at least in some countries) to consume genetically modified crops, and the international trade of wine, many wine producers prefer to avoid any association with their techniques. Where deemed of value, they consider it more judicious to use selective co-fermentation. It may occasionally achieve some of the same goals derived by genetic modification (see above, Spontaneous vs. Induced Fermentation).

Environmental factors affecting fermentation

Carbon and energy sources

The major carbon and energy sources for fermentation are glucose and fructose. Other nutrients may be used, but they are present either in small amounts (amino acids), poorly incorporated into the cell (glycerol), or metabolized (respired) only in the presence of oxygen (acetic acid and ethanol). Sucrose can be fermented, but it is seldom present in significant amounts in grapes. It may be added, however, during processes such as chaptalization and amelioration or as a nutrient for the second fermentation in sparkling wine production. For its metabolism, sucrose must be enzymatically split into its components, glucose and fructose. This occurs under the action of one of several invertases. Hydrolysis usually occurs external to the cell membrane by an invertase located between the cell wall and plasma membrane (periplasm). Most other sugars cannot be fermented by *Saccharomyces cerevisiae* due to the absence of the requisite enzymes and/or transport proteins.

Significantly, though, other grape sugars can be utilized as an energy source by several spoilage microorganisms.

Glucose is transported across the plasma membrane using several mechanisms (Kruckeberg, 1996). *Saccharomyces cerevisiae* shows an extraordinary duplication and diversification of its HXT (hexose transport) gene family. Each of the approximately 18 variants possesses distinct regulatory and transport-kinetic properties. Some are low-affinity systems, typically working at high substrate concentrations. Low-affinity systems appear to function by facilitated diffusion (without direct expenditure of metabolic energy) and most effective at high sugar concentrations. For example, the HXT1 carrier is expressed only at the beginning of fermentation (Perez et al., 2005), HXT2 (an intermediate carrier) is active during the lag phase, whereas HXT3 is functional throughout fermentation (but maximally at the onset of the stationary phase). In contrast, high-affinity systems require metabolic energy for transport to occur but are particularly valuable at low glucose concentrations. They tend to be activated by a membrane protein, Snf3p, when sugar levels decline. Despite this, the major high-affinity systems of wine yeasts (HXT6 and HXT7) become active when the yeast colony enters the stationary phase (when about half the sugar still remains in the must). Although these transport proteins are named because of their transport capabilities, deletion mutants suggest that they have additional, regulatory roles that are not fully understood.

Glucose concentration not only differentially affects the activation of sugar transport mechanisms, but also regulates expression of enzymes in the TCA and glyoxylate pathways. Nevertheless, continued expression of selected chromosomal (vs. mitochondrial) genes results in some TCA enzymes being found at higher sugar concentrations in the cytoplasm. They catalyze biosynthetic reactions essential for growth.

At maturity, the sugar concentration of most wine grapes ranges between 20% and 25%. At this concentration, the osmotic influence of sugar can delay the onset of fermentation. The resulting partial plasmolysis of yeast cells may be one of the causes of a lag period before active fermentation commences (Nishino et al., 1985). In addition, cell viability may be reduced; cell division retarded; and sensitivity to alcohol toxicity enhanced. At sugar concentrations above 25%–30%, the likelihood of fermentation terminating prematurely increases markedly. Strains of *Saccharomyces cerevisiae* differ greatly in their sensitivity to sugar concentration.

The nature of the remarkable tolerance of wine yeasts to the plasmolytic action of sugar is unclear, but appears to be related to increased synthesis of glycerol and its reduced permeability through the cell membrane (see

Brewster et al., 1993). These responses to increased environmental osmolarity permit glycerol to equilibrate the osmotic potential of the cytoplasm to that of the surrounding medium. The accumulation of trehalose may also be involved.

Sugar content affects the synthesis of several important aromatic compounds. High sugar concentrations increase the production of acetic acid (Fig. 7.36 and its esters. However, as indicated in Fig. 7.37, the effect of total soluble solids on esterification is not solely due to sugar content. For example, the synthesis of isoamyl acetate and 2-phenethyl acetate during fermentation decreases with increasing maturity (associated with a decreased synthesis of their corresponding fatty acids), but increases in juice from immature grapes, augmented with sugar to achieve the same °Brix. The importance of precursors in the must have been directly confirmed by Dennis et al. (2012). They found that the concentrations of C₆ alcohols and aldehydes directly influence the synthesis of acetates such as benzyl, hexyl, and octyl acetates during fermentation.

Over a wide range of sugar concentrations, ethanol production is directly related to sugar content. However, above 30%, ethanol production per gram sugar begins to decline (Fig. 7.36).

Alcohols

All alcohols are toxic to varying degrees. Because *Saccharomyces cerevisiae* shows considerable insensitivity to ethanol toxicity, much effort has been spent attempting to understand the nature of this tolerance, and why it breaks down at high concentrations. Several factors appear to be associated with ethanol tolerance. These include activation of glycerol and trehalose synthesis (Hallsworth, 1998; Lucero et al., 2000); accumulation of HSPs (e.g., HSP104 and HSP12) (Sales et al., 2000); and modification of the plasma membrane (e.g., activating membrane ATPase, substitution of ergosterol

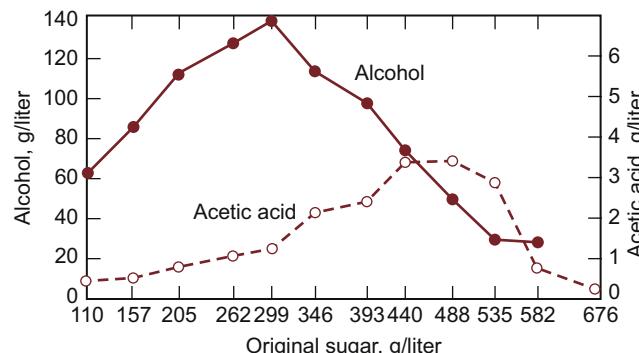


FIGURE 7.36 Effect of sugar concentration on alcohol and volatile acid production. After unpublished data of C. von der Heide, from Schanderl, H., 1959. Die Mikrobiologie des Mostes und Weines, second ed. Ulmer, Stuttgart, Germany, reproduced by permission.

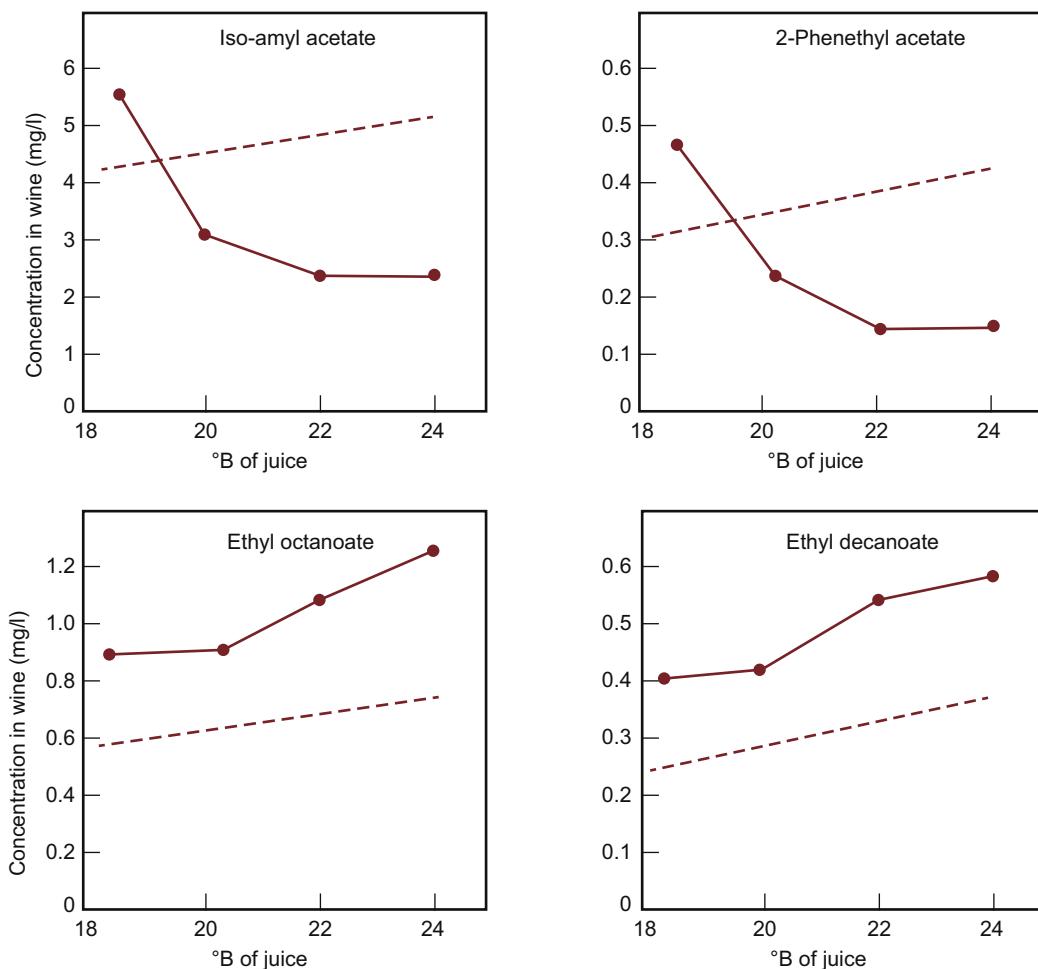


FIGURE 7.37 Ester concentration of wines made from grapes of varying maturity and with chaptalized must. Wines for musts of 4° of maturity (—); average slope from wines produced from chaptalized must (— — —). From Houtman, A.C., Marais, J., du Plessis, C.S., 1980. *Factors affecting the reproducibility of fermentation of grape juice and of the aroma composition of wines, I. Grape maturity, sugar, inoculum concentration, aeration, juice turbidity and ergosterol*. Vitis 19, 37–84, reproduced by permission.

for lanosterol, increasing the portion of phosphatidyl inositol vs. phosphatidyl choline, and augmenting the incorporation of palmitic acid) (Aguilera et al., 2006). These membrane changes decrease permeability (Mizoguchi and Hara, 1998), minimizing the loss of nutrients and cofactors from the cell, notably magnesium and calcium. That these factors may disrupt cellular redox potential is suggested by the growth stimulation provided by acetaldehyde (increasing the availability of NAD⁺) (Vriesekoop et al., 2007). Vacuolar membrane function is also crucial for the retention of toxic substances stored in vacuoles (Kitamoto, 1989). In addition to disrupting membrane function, ethanol can inactivate some enzymes by inducing structural modifications.

Although alcohol buildup eventually inhibits fermentation, it begins disrupting yeast metabolism at much lower concentrations. For example, suppression of sugar uptake can begin at 2% ethanol (Dittrich, 1977). This

property is partially dependent on the fermentation temperature, and is reflected in yeast growth potential (Casey and Ingledew, 1986), possibly through its effect on membrane fluidity. Disruption of ammonium transport and inhibition of general amino acid permeases also occurs as alcohol content increases. These influences can be partially offset by enrichment with unsaturated fatty acids (e.g., adding yeast hulls). Although higher (fusel) alcohols are more inhibitory than ethanol, their much lower concentration substantially limits their toxic influence.

Although most strains of *S. cerevisiae* can ferment up to 13%–15% ethanol, there is wide variation in this ability. Cessation of growth routinely occurs at concentrations below those that inhibit fermentation. It is generally believed that this results from disruption of the semifluid nature of the cell membrane, thus lowering water activity (Hallsworth, 1998). This destroys the ability of the cell to control cytoplasmic function, leading to

nutrient loss and disruption of the electrochemical gradient across the membrane. The latter is vital for nutrient transport (see Cartwright et al., 1989). Lowered water activity also disrupts hydrogen bonding, essential to enzyme function. High sugar contents enhance ethanol toxicity.

Ethanol is occasionally added to fermenting must or wine, usually in the form of distilled wine spirits (unmatured brandy), to arrest or prevent yeast and other microbial activity. This property is used selectively in sherry, port, and madeira production. In port, the brandy is added early during fermentation to retain about half the must's sugar content. This leaves the wine with the aromatic attributes typical of early fermentation. For example, young port is likely to be higher in acetic acid, acetaldehyde, and acetoin content, but lower in glycerol, fixed acids, and higher alcohols than had it fermented to dryness (see Figs. 7.21 and 7.22). In sherry production, wine spirits are added at the end of fermentation to inhibit the growth of acetic acid bacteria ($>15\%$ ethanol), or *flor* yeasts ($>18\%$ ethanol), during solera aging.

The accumulation of alcohol during fermentation has an important dissolving action on phenolic compounds. This effect is most pronounced at the beginning of fermentation. Given sufficient maceration time, though, phenolic compounds dissolve in water (Hernández-Jiménez et al., 2012). Most of the distinctive taste of red wines depends on the facilitated extraction of flavanols by ethanol during fermentation. Ethanol aids but is less critical to the extraction of anthocyanins. Phenolic extraction can be further enhanced in the presence of sulfur dioxide (Oszmianski et al., 1988).

Nitrogenous compounds

Next to sugars, nitrogenous compounds are quantitatively the most important yeast nutrients. Under most circumstances, juice and must contain sufficient nitrogen for fermentation. Nitrogen contents can, however, vary considerably—values reported for juice in California can range from 60 to 2400 mg/L. In addition, some cultivars have a tendency to experience nitrogen deficiency more frequently than others (e.g., Chardonnay and Colombard). The situation can be exacerbated if the juice has been given a prefermentation centrifugation or filtration.

Nitrogen is required not only for the synthesis of structural, transport, and enzymatic proteins (essential for growth and metabolism) but is also an essential element in information molecules (nucleic acids) and electron transport compounds. The importance of a ready availability of nitrogen for synthesis is illustrated by the rapid turnover in sugar transport proteins. These essential molecules have a half-life of about 6 h.

Production of reduced sulfur odors can also commence within 30 min of the onset of nitrogen starvation.

Most nitrogen in grape must is in the form of free amino acids, notably proline and arginine. The proline content can be disregarded as it is poorly incorporated under anaerobic conditions. Thus, most of the assimilable nitrogen available to yeasts consists of arginine, in addition to any ammonium ions present. Because arginine is largely localized in the skins, the duration of maceration and the method of pressing (with or without prior crushing) can significantly influence its liberation. With vineyard fertilization, the content of other available amino acids, notably glutamine, is increased.

Uptake is highly correlated with the dynamics of yeast growth (Fig. 7.38), being initially stored before use. Optimum levels suggested by Henschke and Jiranek (1993) are in the range of 400–500 mg/L, specific values depending on the demands of the yeast species and strain as well as fermentation conditions (e.g., temperature and must sugar content). Significantly higher

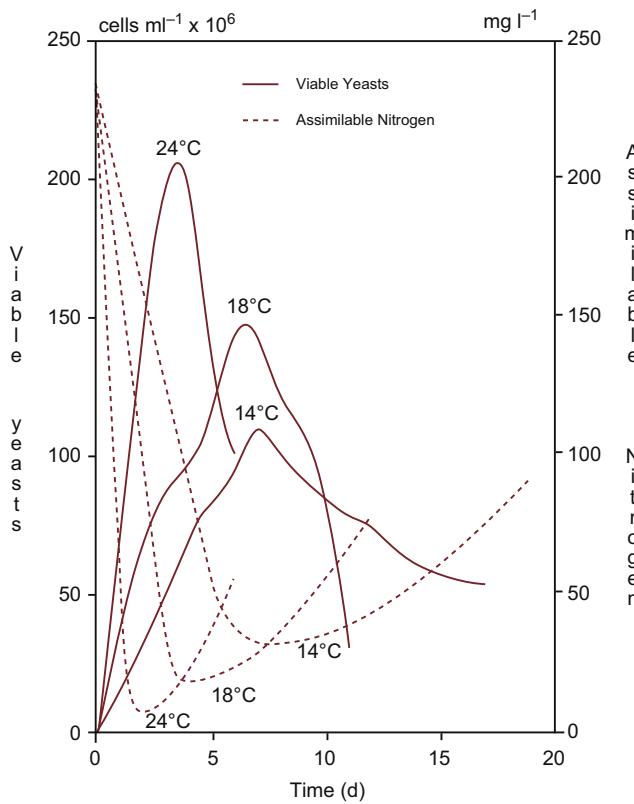


FIGURE 7.38 Evolution of viable yeast cells (10^6 CFU/mL) and assimilable nitrogen (mg/L): amino acids, apart from proline, plus ammonia during fermentation at 14°C, 18°C, and 24°C. From Lopez, R., Santamaria, P., Gutierrez, A.R., Iñiguez, M., 1996. Changes in amino acids during the alcoholic fermentation of grape juice at different temperatures. *Sci. Aliments* 16, 529–535, reproduced by permission.

nitrogen concentrations can be a disadvantage. They promote unnecessary cell multiplication and correspondingly reduce the conversion of sugar to alcohol. In contrast, low values or amino acid imbalance can enhance the release of higher alcohols; the accumulation of reduced sulfur off-odors; and an increased likelihood of sluggish or stuck fermentation.

When nitrogen is required, it is usually supplied as DAP, although glutamine appears to be an effective alternative (Sturgeon et al., 2013). Ammonia is the least energy-demanding form of nitrogen available to yeasts. Amino acid uptake is associated with simultaneous hydrogen uptake. This eventually requires the expenditure of ATP for the expulsion of H^+ , to avoid lowering the pH of the cytoplasm. As ethanol content rises, its disruption of the cell membrane results in the spontaneous diffusion of H^+ into the cytoplasm, and uptake of amino acids declines or ceases.

If added, DAP is usually supplied halfway through fermentation, near the onset of the stationary phase (Bely et al., 1990). As such, it does not interfere with the early synthesis of sugar transport proteins (Bely et al., 1994). Earlier addition reduces yeast uptake of amino acids early during fermentation, when alcohol accumulation is still low. This has the potential to influence flavor development, due to the involvement of amino acids in higher alcohol and thereby ester formation and other sensory attributes (Fig. 7.39). Late addition is ill-advised as residual amounts can favor microbial spoilage.

In situations where nitrogen deficiency is severe, significant amounts of hydrogen sulfide are likely to be released as a consequence of restricted amino acid synthesis and degradation. This is most marked if nitrogen becomes limiting during the exponential phase. It is countered by the availability of ammonia (or most amino acids, with the notable exception of cysteine and proline). The breakdown of cysteine has the potential to increase the release of H_2S and mercaptans. Under such conditions, earlier addition of DAP is advisable (Jiranek et al., 1995). Avoiding nitrogen deficiency also minimizes arginine degradation, and the subsequent release of urea. This in turn reduces the likelihood of ethyl carbamate generation, especially in wine exposed to heating. Ethyl carbamate is a suspected carcinogen.

Although a valuable fermentation aid, DAP addition is best minimized or avoided where possible. Excessive nitrogen can predispose some strains to high hydrogen sulfide production, as much as low nitrogen availability.

Rapid methods of estimating assimilable nitrogen (Dukes and Butzke, 1998) permit wineries to better assess actual need (Gardner et al., 2002). Fermentation temperature, sugar content, yeast strain (Brice et al., 2014), and flavor preferences need to be considered when estimating the “ideal” nitrogen supply (O’Kennedy and Reid, 2008). Because of the multiplicity of

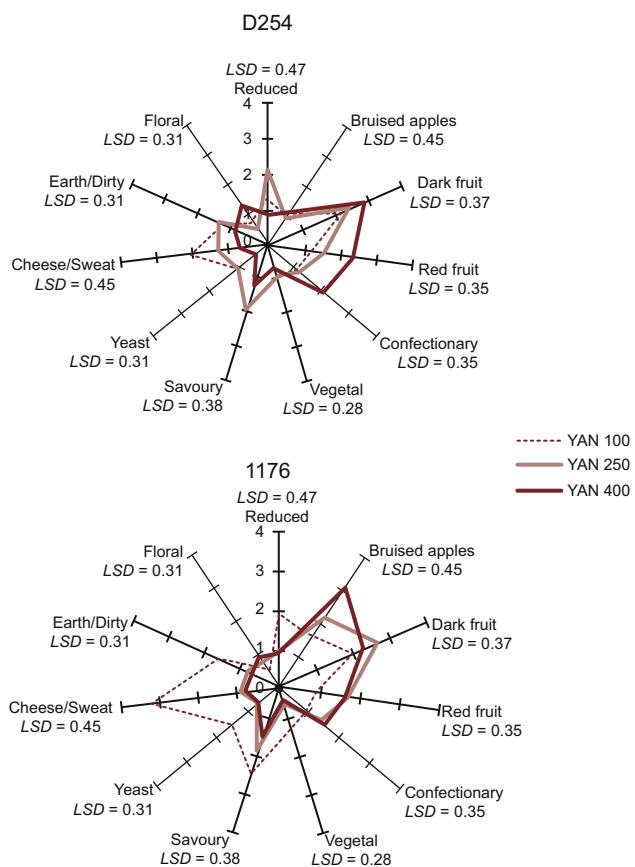


FIGURE 7.39 Sensory profiles of experimental Shiraz wines fermented with *Saccharomyces cerevisiae* (D254) or *S. bayanus* (AWRI 1176)—100 mg/L yeast assimilable nitrogen (YAN) has no nitrogen addition; 250 mg/L, same must as 100 mg/L but initial YAN increased until 250 mg/L by means of DAP addition; 400 mg/L, same must as 100 mg/L but initial YAN increased until 400 mg/L by means of DAP addition. From Ugliano, M., Travis, B., Francis, I.L., Henschke, P.A., 2010. Volatile composition and sensory properties of Shiraz wines as affected by nitrogen supplementation and yeast species: rationalizing nitrogen modulation of wine aroma. *J. Agric. Food Chem.* 58, 12417–12425. Copyright 2010, American Chemical Society, reproduced by permission.

factors affecting the beneficial/detrimental aspects of nitrogen supplementation, it is often recommended that when conditions change (such as yeast strain or other vinicultural practices), microvinification should be conducted prior to full-scale fermentation. It is possible to detect nitrogen deficiency during fermentation by the high expression of several genes in yeast cells (Mendes-Ferreira et al., 2007). Regrettably, this is not practical method for the vast majority of wineries.

Several conditions can reduce must nitrogen content. Nitrogen deficiency in the vineyard and must clarification can limit or diminish the assimilable nitrogen content. If sufficiently marked, inadequate nitrogen levels slow fermentation and are one potential cause of “stuck” fermentations. This may result from the partially irreversible inactivation of sugar transport by ammonia

starvation (Lagunas, 1986). The half-life of the main glucose transport system is short, with complete inactivation occurring within about 50 h (Schulze et al., 1996). This results from both the cessation of protein synthesis and enzyme degradation. The lack of ammonia can also negate the activation of crucial glycolytic enzymes, such as phosphofructokinase and pyruvic kinase. This in turn further inhibits glucose uptake (Bely et al., 1994).

Juice nitrogen content may be markedly decreased as a consequence of *Botrytis cinerea* infection (Rapp and Reuther, 1971). Nitrogen deficiency is also a frequent problem in the second fermentation of sparkling wines, resulting both from consumption during the initial fermentation and clarification of the *assemblage*. It is avoided by the addition of ammonium salts, notably DAP.

Nitrogen demand and yeast incorporation occurs most rapidly during fermentation's exponential phase. This correlates with the period when cell growth and division are most active. Subsequently, there is a slow release of nitrogen-containing compounds back into the fermenting juice/must (Fig. 7.40).

Of inorganic nitrogen sources, ammonia is incorporated preferentially. Wine yeasts tend to overexpress ammonia transport genes (*MEP2*) (Cavalieri et al., 2000). The oxidized state of ammonia permits its direct incorporation into organic compounds. Although ammonia is potentially capable of repressing the uptake of amino acids, its concentration in grape juice is usually insufficient to exert this influence.

Saccharomyces cerevisiae has several amino acid transport systems (Cartwright et al., 1989). One is nonspecific and directs the uptake of all amino acids, with the exception of proline. The other systems are more selective, transporting only particular amino acids. These properties probably explain why certain amino acids are preferentially incorporated. Their selective uptake seems to be consistent between strains, some being rapidly absorbed (lysine), followed asparagine, glycine, leucine, leucine, isoleucine, while alanine, arginine, valine, and tryptophan tend to be the last utilized (Crépin et al., 2012).

The principal amino acids available in grape must are proline and arginine. Because proline metabolism requires molecular oxygen for its metabolism (Tomenchok and Brandriss, 1987), arginine is the primary amino acid nitrogen source. Unfortunately, arginine may be degraded to ornithine and urea. If the ammonia derived from the breakdown of urea is not incorporated by cell metabolism, urea may accumulate and be secreted

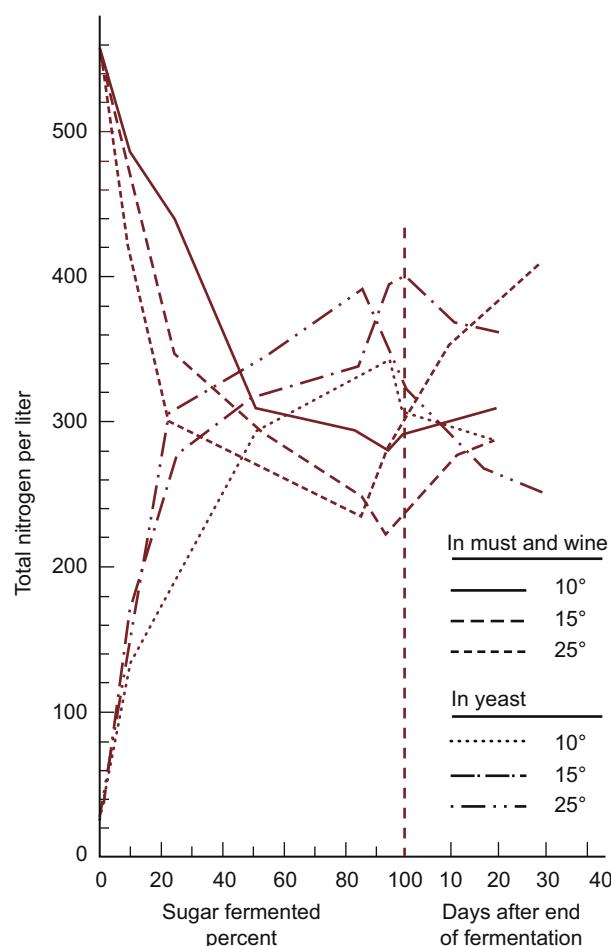


FIGURE 7.40 Changes in total nitrogen during and after fermentation in musts and in yeasts fermenting them. After Nilov, V.I., Valuiko, G.G., 1958. Changes in nitrogen during fermentation (in Russian). Vinodel. Vinograd. S.S.R. 18, 4–7, in Amerine, M.A., Berg, H.W., Kunkee, R.E., Ough, C.S., Singleton, V.L., Webb, A.D., 1980. *The Technology of Wine Making*. Avi, Westport, CT, reproduced by permission.

from the cell. This, as noted, could participate in the generation of ethyl carbamate (Ough et al., 1990). Although proline is not metabolized under fermentative conditions, its presence may be important in stabilizing the yeast-cell membrane.

Amines and peptides may also be incorporated as nitrogen sources, but not protein nitrogen. Wine yeasts are incapable of either transporting proteins into the cell or enzymatically degrading them to amino acids outside the plasma membrane.

Yeast cells generally synthesize their own amino acid and nucleotide requirements from inorganic nitrogen and sugar. Consequently, most yeast strains do not need these metabolites in the medium. Nevertheless, several can and are assimilated from the medium when

available. Assimilation avoids the diversion of metabolic intermediates and energy to their biosynthesis.

Nitrogen content can influence the synthesis and release of aromatic compounds during fermentation. Noticeable in this regard is the reduction in fusel alcohol content in the presence of ammonia or urea. The effect can be reversed by the presence and assimilation of certain amino acids. These opposing effects are due to fusel alcohol use in amino acid biosynthesis, and their release during amino acid deamination, respectively. Under nitrogen starvation, there is a dramatic increase in the production of glycerol and trehalose (Schulze et al., 1996).

During, and especially after fermentation, cellular constituents are slowly released into the wine. This is associated with the autolysis of dead and dying yeast cells (see Fig. 7.42). Because this may activate undesirable microbial activity, the first racking typically occurs shortly after fermentation. When malolactic fermentation is desired, however, racking is delayed until the bacterial conversion of malic to lactic acid is complete. Racking is also delayed if *sur lies* contact is desired. When the liberation of assimilable nitrogen into the wine is desired, small cooperage is preferred as it provides better contact between the wine and the lees (larger surface area/volume ratio).

Large-molecular-weight nitrogen-containing compounds are also released during yeast autolysis at the end of fermentation. This is particularly notable in relation to mannoproteins during *sur lies* maturation. The amount released often reaches 150–200 mg/L, but actual amounts depend on the yeast strain, agitation, duration of contact, and the ambient temperature.

Lipids

Lipids are the basic constituents of cell membranes (phospholipids and sterols), function in energy storage (oils), act as pigments (carotenoids), and are components in proteins (lipoproteins) and carbohydrates (glycolipids).

Yeasts synthesize their own lipid requirements when grown aerobically, but are unable to produce long-chain, unsaturated fatty acids and sterols under anaerobic conditions. This is less significant in red wine production, where limited supplies of precursors are derived from the pomace during fermentation. The anaerobic limitation of lipid synthesis can, however, cause sluggish fermentation in highly clarified white juice. Clarification can remove more than 90% of the fatty acid content. This is particularly marked with unsaturated fatty acids, notably oleic, linoleic, and linolenic acids (Bertrand and Miele, 1984). Sterols are probably removed as well. Nevertheless, yeasts may possess sufficient reserves of these vital nutrients to initiate fermentation and complete several cell divisions (typically four to five when

a yeast inoculum is used). However, in spontaneous fermentation, up to 16 or more cell divisions may be involved in reaching the typical stationary population. Thus, a deficit in sterol and unsaturated fatty acid content can aggravate ethanol-induced reduction in glucose uptake, resulting in stuck fermentation.

Wine yeasts are sensitive to mid-chain (C₈ and C₁₀) saturated fatty acids, such as octanoic and decanoic acid. As by-products of yeast metabolism, they accumulate during fermentation. Because they increase membrane fluidity, proton influx increases, acidifying the cytoplasm. Cytoplasmic acidification favors the ethanol-induced leakage of nutrients, such as amino acids (Sá Correia et al., 1989), and can inhibit both high- and low-affinity sugar-transport systems (Zamora et al., 1996). Because toxicity increases with a decrease in pH, injury is likely associated with the undissociated form of these fatty acids. Toxic effects are limited or reversed by the addition of ergosterol and long-chain unsaturated fatty acids (Fig. 7.41) or the addition of various absorptive substances, such as activated charcoal, bentonite, silica gel, or yeast hulls (consisting primarily of cell-wall remnants following controlled autolysis). By removing octanoic and decanoic acids (via absorption), their potential for membrane disruption is reduced. In addition, yeast hulls may act as a source of required sterols and unsaturated fatty acids (see Muñoz and Ingledew, 1990).

Another possible solution to problems associated with low sterol and unsaturated fatty acid content is the use of yeasts with high sterol contents. Sterol synthesis is commonly suppressed in the presence of glucose, but some strains do not show this effect. Growing yeast under highly aerobic conditions, as in active dry yeast production, also generates cells elevated in unsaturated fatty acids. They may contain up to three times the sterol content of cells grown semiaerobically (Tyagi, 1984). Musts inoculated with strains possessing enhanced sterol contents frequently ferment more sugar than strains possessing low sterol contents.

Phenols

The phenolic content of must can have various effects on the course of fermentation. In red grapes, anthocyanins stimulate fermentation, whereas proanthocyanidins in white grapes can be slightly inhibitory (Cantarelli, 1989). Nonetheless, they appear to favor fermentation after an initial inhibitory period (Li et al., 2014). By contrast, phenolic aldehydes may be inhibitory or influence the direction of yeast metabolism (Cao et al., 2014). Phenolic compounds are also a determining factor in the activation of film formation, important in *fino* sherry production (Cantarelli, 1989). Certain phenolics, notably the esters of gallic acid, are toxic, whereas

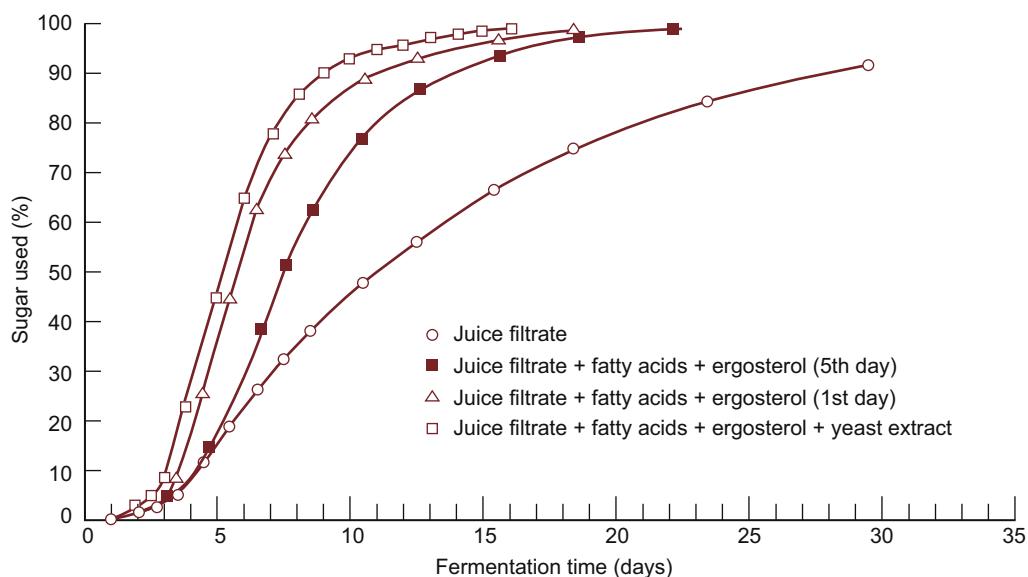


FIGURE 7.41 Fermentation curves of juice filtrates with the addition of yeast extract, unsaturated fatty acids, and ergosterol at inoculation and on the fifth day thereafter. From Houtman, A.C., du Plessis, C.S., 1986. Nutritional deficiencies of clarified white grape juices and their correction in relation to fermentation. *S. Afr. J. Enol. Vitic.* 7, 39–46, reproduced by permission.

others, such as chlorogenic and isochlorogenic acids, stimulate fermentation. The principal situation in which phenols suppress yeast metabolism is during the second fermentation of sparkling wine production. This is one of the primary reasons why few red sparkling wines are produced using standard procedures.

In addition to affecting fermentation, phenols may also be modified by yeast action—the most sensory significant example being the decarboxylation of ferulic and *p*-coumaric acids to aromatic vinylphenols (4-vinyl guaiacol and 4-vinylphenol, respectively) (Chatonnet et al., 1989). Other phenolic constituents in the must may influence this conversion.

Sulfur dioxide

Sulfur dioxide is one of the most common wine additives. However, sulfur dioxide is also a by-product of amino acid metabolism. In most instances, yeast-derived sulfur dioxide is rapidly bound to organic compounds in yeast cells or the fermenting must. Thus, its antimicrobial potential is unlikely to play a significant role in the success in *S. cerevisiae* in out-competing other microbes during fermentation. Interestingly, sulfur dioxide addition favors not only the growth of strains resistant to sulfur dioxide, but also appears to select strains that produce greater amounts of sulfur dioxide.

The differential action of sulfur dioxide against the grape epiphytic flora can be used to selectively control the action of indigenous yeasts and bacteria (Bokulich et al., 2015). As our understanding of their role increases in wine fragrance development, their modulation versus

inhibition is being viewed as an important tool in adjusting wine character.

At the concentrations typically used (less than 50 ppm total with healthy grapes), sulfur dioxide is not known to affect the fermentation rate. However, it can slow the onset of fermentation. The presence of 15–20 ppm can reduce the viability of a yeast inoculum from 10^6 to 10^4 cells/mL or less (Lehmann, 1987). Although sulfur dioxide limits the growth of indigenous yeasts and bacteria, this may be unnecessary as inoculated yeasts tend to rapidly come to dominate fermentation, even in the absence of sulfur dioxide (Petering et al., 1993; Henick-Kling et al., 1998).

Yeast resistance to sulfur dioxide is correlated with several factors. For example, the *SSU1-R* gene that controls sulfite efflux is overexpressed in wine yeasts (Hauser et al., 2001). This apparently results from its translocation to a position where it is under the control of the *ECM34* promotor (Pérez-Ortíz et al., 2002). In addition, resistance appears to be influenced by the presence of various nutrients, for example adenine increases tolerance, whereas methionine decreases it (Aranda et al., 2006).

In addition to its antimicrobial activities, sulfur dioxide can significantly influence yeast metabolism. Sulfur dioxide readily binds with several carbonyl compounds, notably acetaldehyde, pyruvic acid, and α -ketoglutaric acid. Binding increases their biosynthesis and potential release into the fermenting must. Thus, their concentration in finished wine often correlates with the concentration of added sulfur dioxide. Sulfur dioxide also favors glycerol synthesis, whereas it tends to limit acetic acid production. Fixed acidity generally does not change,

partially because sulfur dioxide suppresses the metabolism of both lactic and acetic acid bacteria.

As sulfur dioxide binds to carbonyl compounds, it inadvertently increases the amount needed to suppress the action of spoilage microbes. Although molecular SO₂ is the most antimicrobial state of sulfur in wine, bound forms can be more significant because of their higher concentration (Wells and Osborne, 2011). This may involve uptake of bound form and the subsequent release of SO₂ inside the cell.

Sulfur dioxide favors the extraction of phenolic compounds including anthocyanins. However, it can also reversibly decolorize anthocyanins. In addition, anthocyanins bound to sulfur dioxide are unable to polymerize with proanthocyanidins. This can adversely reduce long-term color stability.

Although one of the most effective antimicrobial agents, many strains of *Saccharomyces ludwigii*, *Zygosaccharomyces bailii*, and *Brettanomyces* spp. are comparatively tolerant to sulfur dioxide (Hammond and Carr, 1976, see Fig. 8.11). Thus, other measures are required for their control.

Elemental sulfur, potentially present as a residue from its use as a fungicide, can be assimilated and used in the synthesis of sulfur-containing amino acids and coenzymes. It may also be oxidized to sulfate and sulfur dioxide or reduced to hydrogen sulfide. The reduction of sulfur to hydrogen sulfide may be a means, albeit aromatically unpleasant, of maintaining a favorable redox balance in yeast cells under anaerobic conditions.

Oxygen and aeration

The process of fermentation itself requires no oxygen. Even in the presence of oxygen, *S. cerevisiae* preferentially ferments. Nevertheless, trace amounts of oxygen can indirectly favor fermentation by permitting the biosynthesis of sterols and long-chain unsaturated fatty acids. The production and proper functioning of the yeast cell membrane require sterols (primarily ergosterol) as well as C₁₆ and C₁₈ fatty acids. Alternatively, essential sterols can be extracted from crushed grapes during maceration. Molecular oxygen is also required for the synthesis of the vitamin nicotinic acid. Anaerobic conditions favor the accumulation of toxic (C₈ and C₁₀) fatty carboxylic acids (Alexandre and Charpentier, 1995) because they are not acylated in the synthesis of required long-chain fatty acids.

Juice oxidation during stemming and crushing is usually sufficient to permit adequate yeast growth during vinification. The amount of oxygen absorbed depends

on the duration of skin contact. Because phenolic extraction increases with extended skin contact, the capacity of the juice to consume oxygen rises correspondingly. In removing free oxygen, conditions shift from oxidative to reductive. Aeration beyond that which occurs coincidentally during stemming and crushing (e.g., hyperoxygenation) is variously viewed as being beneficial (Cheynier et al., 1991) or detrimental (Dubourdieu and Lavigne, 1990). These different views may result from strain response to oxygen; the degree and timing of oxygen uptake; and increased synthesis of higher alcohols and esters (Valero et al., 2002). Nevertheless, hyperoxygenation does favor cell growth and promotes fermentation.

The initial browning associated with crushing is acceptable because the colored compounds that form are primarily lost during fermentation (attachment to and precipitation with yeast cells) or removed during subsequent clarification. It results in providing white wines a degree of resistance to oxidative browning (by eliminating readily oxidizable phenols early during vinification).

During red wine fermentation, oxygen may be absorbed during pumping over. The resulting incorporation of about 10 mg O₂/liter often speeds the process of fermentation. This is more marked when aeration occurs at the end of the exponential phase (Sablayrolles and Barre, 1986). The yeast population increases, and average cell viability is enhanced. Aeration also increases the production of acetaldehyde, thus favoring color stability by assisting the early formation of anthocyanin–tannin copolymers.

During the fermentation of white wines, winemakers tend to assiduously avoid oxygen exposure. It increases a tendency to synthesize fusel alcohols and acetaldehyde. Increased levels of volatile acidity (acetic acid) may also result from the activation of acetic acid bacteria. In addition, semiaerobic conditions can depress the synthesis of esters (Nykänen, 1986). However, the absence of oxygen can enhance the likelihood of hydrogen sulfide accumulation. Depending on the timing of aeration, oxygen uptake may either oxidize H₂S or enhance its synthesis (Houtman and du Plessis, 1981). To offset H₂S accumulation, short aeration at the beginning, or a few days after the commencement of fermentation (Bertrand and Torres-Alegre, 1984), has been recommended. The timing and extent of aeration can also influence urea accumulation and therefore the potential for ethyl carbamate production (Henschke and Ough, 1991). In situations where stuck fermentation has been a problem, the addition of nitrogen (as DAP) and slight aeration (1 mg/L) at the onset of the

stationary phase has been suggested (Julien et al., 2000). In the production of sparkling wine, aeration of the *assemblage* prior to the second fermentation is beneficial. Because the relative benefits of oxygen uptake vary with the yeast strain and cultivar, tests of its value need on-site verification.

Following fermentation, limited slow oxygen uptake (~ 40 mg O₂/liter) is considered to benefit red wine maturation. It favors color stability and reduces the typical reduction in color associated with malolactic fermentation (Pérez-Magaríño et al., 2007). In contrast, most white wines are painstakingly protected from air exposure following fermentation. The exception to this practice occurs when wines are given extended contact with the lees (*sur lies* maturation). In this situation, limited oxygen uptake is desired and achieved by periodically stirring the lees (*bâtonnage*). This limits the accumulation of an excessively reduced environment in the lees (and the associated development of reduced-sulfur off-odors) (Fornairon-Bonnefond et al., 2003).

Oxygen absorption is influenced by many factors including clarification, skin contact, phenol concentration, sulfur dioxide content, the presence of polyphenol oxidases, sugar concentration, temperature, pumping over, rate of fermentation, and of course protection from air before, during, and after fermentation.

Carbon dioxide and pressure

During fermentation, large volumes of carbon dioxide are generated—about 260 mL/g glucose. This equates to over 50 times the volume of the juice fermented. The escape of carbon dioxide can remove about 20% of the heat generated during fermentation (the rest largely associated with water evaporation or radiated to the environment).

Various other volatile compounds are also carried off with the carbon dioxide. Ethanol loss is estimated to be about 1%–1.5% of that produced (Williams and Boulton, 1983), but varies with sugar use and temperature. Higher alcohols and monoterpenes are lost to about the same degree ($\sim 1\%$). In contrast, significant dissipation of both ethyl and acetate esters can occur (Fig. 7.42). Depending on the variety, and especially the fermentation temperature, up to 25% of these aromatically important compounds may be lost (Miller et al., 1987). On average, more acetate esters may volatilize than ethyl esters, but not consistently (Morakul et al., 2013). This degree of escape could noticeably reduce the fruity character of a wine. Trapping these compounds for read-dition to the wine is an interesting concept (Guerrini et al., 2016; Lezaeta et al., 2018). The idea is not new, though, having been advocated almost two centuries ago (Gervais, 1820).

Volatileization from fermenting juice is a function of concentration; the rates of synthesis and degradation;

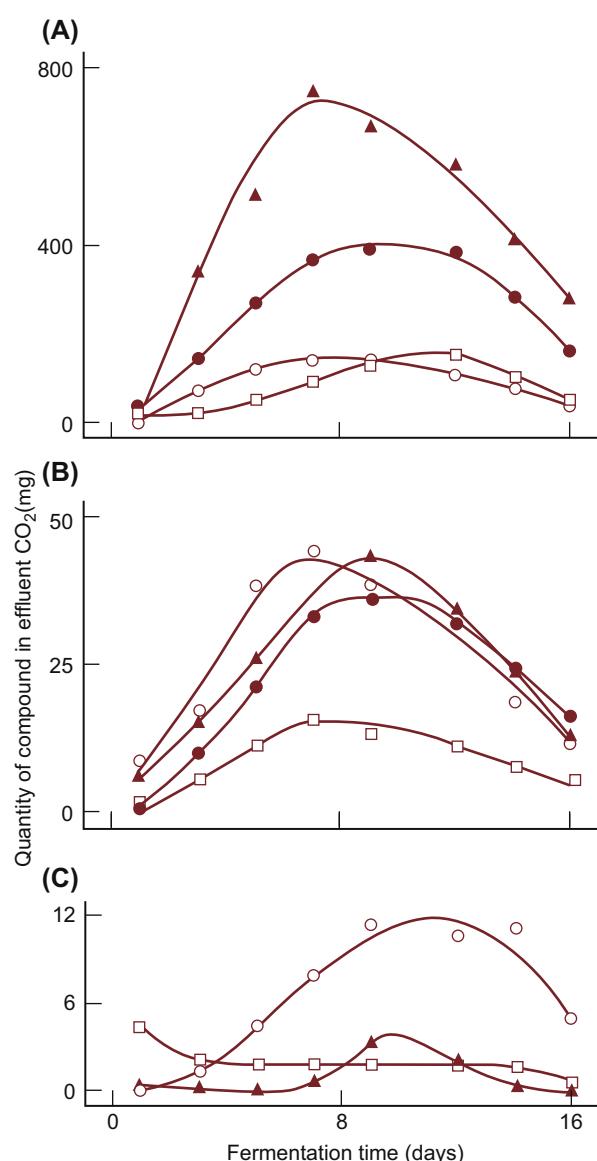


FIGURE 7.42 Yeast aromatics released with CO₂ during fermentation at 15°C. (A) ▲, Isoamyl acetate; ○, ethyl n-hexanoate; □, ethyl n-octanoate; ●, isoamyl alcohol; (B) □, Isobutyl acetate; ○, hexyl acetate; ●, ethyl n-butanoate; ▲, isobutanol; (C) ○, Ethyl n-decanoate; □, 1-hexanol; ▲, 2-phenylethanol. From Miller, G.C., Amon, J.M., Simpson, R.F., 1987. Loss of aroma compounds in carbon dioxide effluent during white wine fermentation. Food Technol. Aust. 39, 246–253, reproduced by permission.

weak associations with matrix constituents and their comparative solubilities in carbon dioxide and the increasingly alcoholic juice. Dissipation is further affected by fermentor size and shape. For example, small fermentors possess higher surface area/volume ratios and lower liquid pressures than larger fermentors, favoring volatility. In addition, although the reduction of vapor pressure at low temperatures tends to limit volatilization, the slower release of carbon dioxide could partially offset this factor by favoring incorporation and loss with CO₂.

The generation of carbon dioxide produces strong convection currents within the ferment. These help equilibrate the nutrient and temperature status throughout the juice. However, the presence of a floating or submerged cap disrupts equilibration in red musts (see Fig. 7.46).

In vats, and in most tank fermentors, the carbon dioxide produced during fermentation is allowed to escape into the surrounding air. When the gas is trapped, as during sparkling wine production, pressure rapidly rises (Fig. 7.43A). At pressures above 700 kPa (~7 atm), yeast growth ceases, although pressure-related effects have been reported to begin at much lower pressures. Low pH and high alcohol content increase yeast sensitivity to CO₂ pressure (Kunkee and Ough, 1966). This has its most significant influence during the production of sparkling wines. Even pressures of 20 kPa, which are easily found in young *assemblage* wines, can have a minor but noticeable effect in slowing fermentation and cell division (Fig. 7.43B). Pressures upward of 600 kPa are typically reached by the end of the second in-bottle fermentation. Nevertheless, the fermentative ability of yeasts may not be completely inhibited until about 3000 kPa. In addition, carbon dioxide accumulation may affect metabolism by influencing the balance between carboxylation and decarboxylation reactions (Table 7.1). The effect of pressure on the synthesis of aromatic compounds during vinification appears not to have been investigated.

Some of the consequences of high pressure on cell growth and metabolism may accrue from a decrease in water viscosity (Bett and Cappi, 1965). This could disrupt the intramolecular hydrogen bonding vital to protein structure and function. In addition, critical changes appear to involve damage to cellular organelles (Iwahashi et al., 2003). Synthesis of HSPs (such as HSP104) and trehalose can limit protein denaturation (Hottiger et al., 1994) and stabilize membrane fluidity (Iwahashi et al., 1995).

The pressure created by trapping the carbon dioxide produced during fermentation has occasionally been used to encourage a more constant rate of fermentation. It also has been used to induce the premature termination of fermentation, leaving wine with a sweet finish. However, care must be used with the latter. Spoilage yeasts, such as *Torulopsis* and *Kloeckera*, are less sensitive to high pressures than *S. cerevisiae*. Their production of acetic acid could generate a vinegary taint. Caution also needs to be taken because lactic acid bacteria (*Lactobacillus*) are little affected by the pressures that affect wine yeasts (Dittrich, 1977).

pH

The pH range normally found in juice and must has little effect on the rate of fermentation or on the synthesis and release of aromatic compounds. Only at abnormally

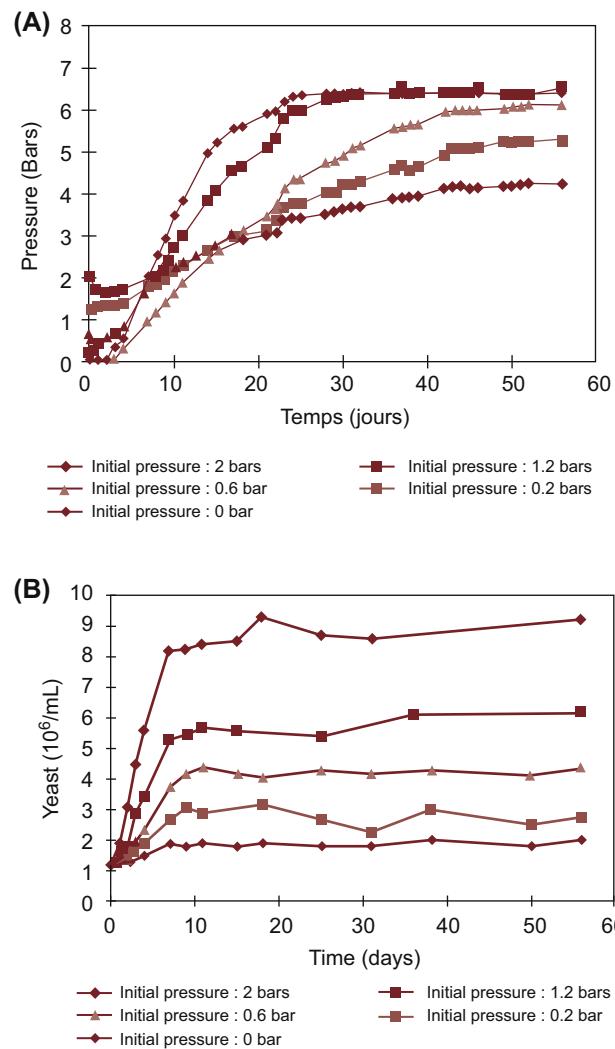
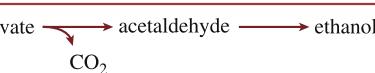
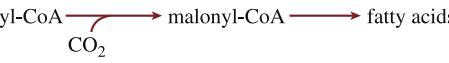
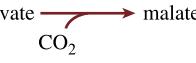
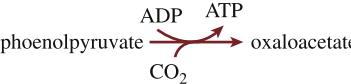
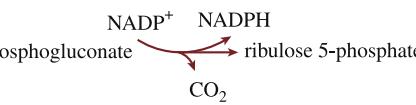


FIGURE 7.43 Increase in carbon dioxide pressure (A) and living yeast cell number (B) during the second, in-bottle fermentation of champagne, as a function of the initial concentration of carbon dioxide in the cuvée. Modified from Valade, M., Bunner, D., Tribaut-Sohier, I., Tusseau, D., Moncomble, D., 2011. *Le gaz carbonique et l'oxygène lors de l'élaboration du champagne*. Rev. Fr. Oenolog. 246 (avril/mai), 1–10, reproduced with the permission of the Comité interprofessionnel du vin de Champagne, France.

low pH values (<3.0) does fermentation begin to be impeded. However, low pH may assist in the uptake of some amino acids. It supplies protons used in activating transport across the cell membrane (Cartwright et al., 1989).

The most important effects of pH on fermentation are indirect, such as noted previously in discussing the antibiotic action of sulfur dioxide. The pH of juice also prevents many potentially competitive organisms from growing in must or wine. In addition, pH affects the stability of some fermentation by-products. The best-known example relates to the hydrolysis of ethyl and acetate esters, in which breakdown occurs more rapidly at

TABLE 7.1 Possible effects of carbon dioxide on key enzymes of *Saccharomyces cerevisiae*.

Reaction	Comment
Pyruvate 	Reduced production of ethanol
Pyruvate 	Stimulation, less available pyruvate for ethanol production
Acetyl-CoA 	Stimulation, less available pyruvate for ethanol production
Pyruvate 	Stimulation, less available pyruvate but malate enzyme level is not high
Phosphoenolpyruvate 	Stimulation, less available pyruvate but enzyme is repressed by glucose
6-Phosphogluconate 	Reduced production of biosynthetic precursors and thus cell yield will decrease; will reduce rate of production of ethanol

From Jones, R.P., Pamment, N., Greenfield, P.F., 1981. Alcohol fermentation by yeasts – the effect of environmental and other variables. *Process Biochem.* 16, 42–49, reproduced by permission.

low pH values. pH also has a marked effect on the coloration of anthocyanins and the relative oxidizability of phenolic compounds.

Vitamins

Vitamins play a crucial role in the regulation of yeast metabolism, functioning as coenzymes and enzyme precursors (Table 7.2). Although vitamins are not metabolized as energy sources, their concentrations decrease

markedly during fermentation (see Amerine and Joslyn, 1970). Yeast requirements typically are satisfied by either biosynthesis or assimilation from the juice. Certain conditions can, however, significantly reduce their concentration or availability. Fatty acids produced during fermentation can inhibit thiamine uptake; oversulfiting (or long-term storage of grape juice at high sulfur dioxide concentrations) degrades thiamine; and grape infection (notably by *Botrytis cinerea*) or contamination of

TABLE 7.2 The role of vitamins in yeast metabolism.

Vitamin	Active form	Metabolic role	Optimum conc. (mg/L)
Biotin	Biotin	All carboxylation and decarboxylation reactions	0.005–0.5
Pantothenate	Coenzyme A	Keto acid oxidation reactions; fatty acid, amino acid, carbohydrate, and choline metabolism	0.2–2.0
Thiamine (B ₁)	Thiamine-pyrophosphate	Fermentative decarboxylation of pyruvate; oxo acid oxidation and decarboxylation	0.1–1.0
Pyridoxine	Pyridoxal phosphate	Amino acid metabolism; deamination, decarboxylation, and racemization reactions	0.1–1.0
p-Aminobenzoic acid and folic acid	Tetrahydro-folate	Transamination; ergosterol synthesis; transfer of one-carbon units	0.5–5.0
Niacin (nicotinic acid)	NAD ⁺ , NADP ⁺	Dehydrogenation reactions	0.1–1.0
Riboflavin (B ₂)	FMN, FAD	Dehydrogenation reactions and some amino acid oxidations	0.2–0.25

From Jones, R.P., Pamment, N., Greenfield, P.F., 1981. Alcohol fermentation by yeasts – the effect of environmental and other variables. *Process Biochem.* 16, 42–49, reproduced by permission.

stored juice by fungi and wild yeasts can lower vitamin content. Under such conditions, a vitamin supplement may improve fermentation or be required to reinitiate stuck fermentation.

Adequate concentrations of thiamine reduce the synthesis of carbonyl compounds that bind to sulfur dioxide, thereby diminishing the amount of SO₂ needed to control spoilage organisms. In addition to limiting carbonyl synthesis, thiamine also reduces the concentration and relative proportions of higher alcohols produced during fermentation. Although seldom a problem, deficiencies in pyridoxine and pantothenic acid can disrupt yeast metabolism, resulting in increased hydrogen sulfide synthesis.

Occasionally, prophylactic vitamin addition is recommended in situations where sluggish or stuck fermentations are a recurring problem. Yeast extract, where permitted, can be a good source of missing vitamins as well as other nutrients. Although this is of value where deficiencies are known, such supplements can increase volatile acidity in some wines (Eglinton et al., 1993).

Inorganic elements

Inorganic elements are often essential components in the active (catalytic) sites of enzymes. They also play active roles in regulating cellular metabolism and maintaining cytoplasmic pH and ionic balance (Table 7.3). For example, magnesium is involved in the catalytic action of several key glycolytic enzymes and stabilizing membrane structure. As such, magnesium helps adapt yeast cells to rapidly increasing alcohol concentrations (Dasari et al., 1990), by limiting cell damage (Walker, 1998).

Because calcium tends to restrict magnesium uptake, it is important not to inadvertently augment calcium levels (Birch et al., 2003), such as the addition of calcium carbonate to neutralize excessive acidity.

Although inorganic elements are normally assumed to be in adequate supply, there is difficulty in accurately assessing yeast requirements and the available ion concentrations in grape juice and must. Not only do organic compounds, such as amino acids, sequester elements, thereby reducing their effective concentration, but ions can antagonize each other's uptake. Occasionally, as in the case of potentially toxic aluminum ions, this may be beneficial.

The abundance of potassium ions probably makes K⁺ the most significant metallic cation in juice and must. High potassium contents can interfere with the efficient uptake of amino acids, such as glycine (by limiting the excretion of potassium that may be needed to maintain an acceptable ionic balance). Protons (H⁺) are simultaneously incorporated with glycine (Cartwright et al., 1989). Abnormally high potassium contents may result in sluggish or stuck fermentation, possibly due to the joint uptake of potassium with glucose, and the subsequent decrease in external pH associated with the excretion of hydrogen ions to maintain cytoplasmic ionic equilibrium (Kudo et al., 1998). High potassium concentrations can also generate tartrate instability, associated with high juice and wine pH. High pH can also lead to microbial instability, increasing the tendency of white wines to brown, and inducing color instability in red wines.

TABLE 7.3 Major inorganic elements required for yeast growth and metabolism.

Ion	Role	Concentration (μM)
K ⁺	Enhances tolerance to toxic ions; involved in control of intercellular pH; K ⁺ excretion is used to counterbalance uptake of essential ions, e.g., Zn ²⁺ , Co ²⁺ ; K ⁺ stabilizes optimum pH for fermentation	20×10^3
Mg ²⁺	Levels regulated by divalent cation transport system; Mg ²⁺ seems to buffer cell against adverse environmental effects and is involved in activating sugar uptake	5×10^3
Ca ²⁺	Actively taken up by cells during growth and incorporated into cell wall proteins; Ca ²⁺ buffers cells against adverse environments; Ca ²⁺ counteracts Mg ²⁺ inhibition and stimulates effect of suboptimal concentrations of Mg ²⁺	1.5×10^3
Zn ²⁺	Essential for glycolysis and for synthesis of some vitamins; uptake is reduced below pH 5, and two K ⁺ ions are excreted for each Zn ²⁺ taken up	50
Mn ²⁺	Implicated in regulating the effects of Zn ²⁺ ; Mn ²⁺ stimulates synthesis of proteins	15
Fe ²⁺ , Fe ³⁺	Present in active site of many yeast proteins	10
Na ⁺	Passively diffuses into cells; stimulates uptake of some sugars	0.25
Cl ⁻	Acts as counterion to movement of some positive ions	0.1
Mo ²⁺ , Co ²⁺ , B ²⁺	Stimulates growth at low concentrations	0.5

From Jones, R.P., Pamment, N., Greenfield, P.F., 1981. Alcohol fermentation by yeasts – the effect of environmental and other variables. *Process Biochem.* 16, 42–49, reproduced by permission.

Temperature

Temperature is one of the most influential factors affecting fermentation. Not only does it directly and indirectly influence multiple aspects of yeast metabolism, but it is also one of the features over which the winemaker has the greatest control.

At the upper and lower limits, temperature can cause yeast death. However, inhibitory effects can be experienced well within these extremes. Relative tolerance to high temperatures appears to depend, at least partially, on production of a particular HSP—HSP104. This limits or reverses the aggregation of essential cellular proteins (Parsel et al., 1994). Increased fermentation temperatures also enhance the synthesis of HSP12, which helps stabilize cell membrane lipids.

The disruptive influences of high temperatures are increased under growth-limiting conditions, such as the presence of ethanol and C₈ to C₁₀ carboxylic acids. In contrast, low temperatures tend to diminish the toxic effects of ethanol. This may be partially a consequence of the higher proportion of unsaturated fatty acid residues in the plasma membrane (Rose, 1989). This property may help to explain the higher maximum viable cell count at the end of fermentations conducted at cooler temperatures (Ough, 1966a).

Yeast growth is particularly sensitive to the fermentation temperature during its exponential phase. For example, cell division was found to occur every 12 h at 10°C, every 5 h at 20°C, and every 3 h at 30°C (Ough, 1966a). Michelet et al. (2004) report that with the commercial yeast strains they tested, all strains completed fermentation without problems at either 12 or 20°C, the differences being a slower completion at the cooler temperature, and variation in their aromatic by-products. Although strain response to temperature can vary markedly, affecting strain proportions during fermentation, the highest yeast populations may be reached at 30°C (Torija et al., 2003).

At temperatures above 20°C, yeasts experience a rapid decline in viability at the end of fermentation, whereas at cooler temperatures, viability is enhanced. Cool temperatures also prolong any fermentative lag phase. For this reason, winemakers may warm white juice to 20°C before adding the yeast inoculum. Once fermentation has commenced, the juice may be cooled to a preferred temperature.

The temperature at which active dry yeast is rehydrated is critical, especially if cool fermentation temperatures are intended (Llauradó et al., 2005). It is important that rehydration occurs at the recommended temperature, with slow acclimation to the intended fermentation temperature. This minimizes the likelihood of sluggish or stuck fermentation. Growth and fermentation ability may also be assisted by the addition of ergosterol, inactive dry yeasts, or other constituents (Díaz-Hellín et al., 2014).

Alternatively, or in addition, cryogenic yeast strains or species may be chosen when cool fermentation temperatures are intended.

In addition to affecting growth and survival, temperature has many subtle, and not so subtle, effects on yeast metabolism. One of the most marked is its influence on fermentation rate. A quick onset and completion of fermentation have advantages. For example, it limits juice oxidation and the growth of epiphytic yeasts or those derived from the winery environment. Thus, the preferred fermentation temperature may have little to do with the optimum for ethanol production or yeast growth. Because yeast strains differ in their temperature response, the overall optimum temperature for vinification can vary widely. Equally, and potentially more significant, is the temperature's modulation of enzymatic pathways, and their effects on flavor production, both beneficial and detrimental.

The preference in most wine-producing regions is to conduct white wine fermentations within a range of 10–20°C. General consensus considers the wine's quality to be higher at the cooler end of this range (e.g., du Plessis, 1983). Nonetheless, some European regions still prefer fermentation temperatures within the range of 20–25°C. Most New World winemakers favor cooler temperatures because they yield fresher, more fruity wines. These properties are highly valued throughout much of the world. Freshness and fruitiness in young white wines is partially due to the increased synthesis and retention of fruit esters, such as isoamyl, isobutyl, and hexyl acetates (Fig. 7.44A). Fatty acid ethyl esters may also contribute to a fruity aspect, for example caproate and caprylate ethyl esters possess apple-like aspects. Many of these ethyl esters are produced more effectively at 15°C. In contrast, 2-phenethyl acetate achieves its highest concentration at 20°C (Fig. 7.44B). Production of ethanol and higher alcohols may also be enhanced at cool temperatures. Cooler fermentation temperatures also reduce the release of yeast colloids, facilitating clarification. On the negative side, cool temperatures slow fermentation, and may augment the likelihood of sluggish fermentation. Where rapid or continuous monitoring of fermentation is not possible, many small producers choose a temperature that generates about 1% alcohol per day.

Some of the effects noted above may result from the influence of temperature on the indigenous yeast flora. Cooler temperatures reduce both the growth rate and toxicity of ethanol (Heard and Fleet, 1988). As a result, species such as *K. apiculata* can remain active for a longer period during the extended fermentation period. For example, the indigenous flora appears to contribute significantly to the highly desired fruity-flora character of Riesling wines (Henick-Kling et al., 1998). Such

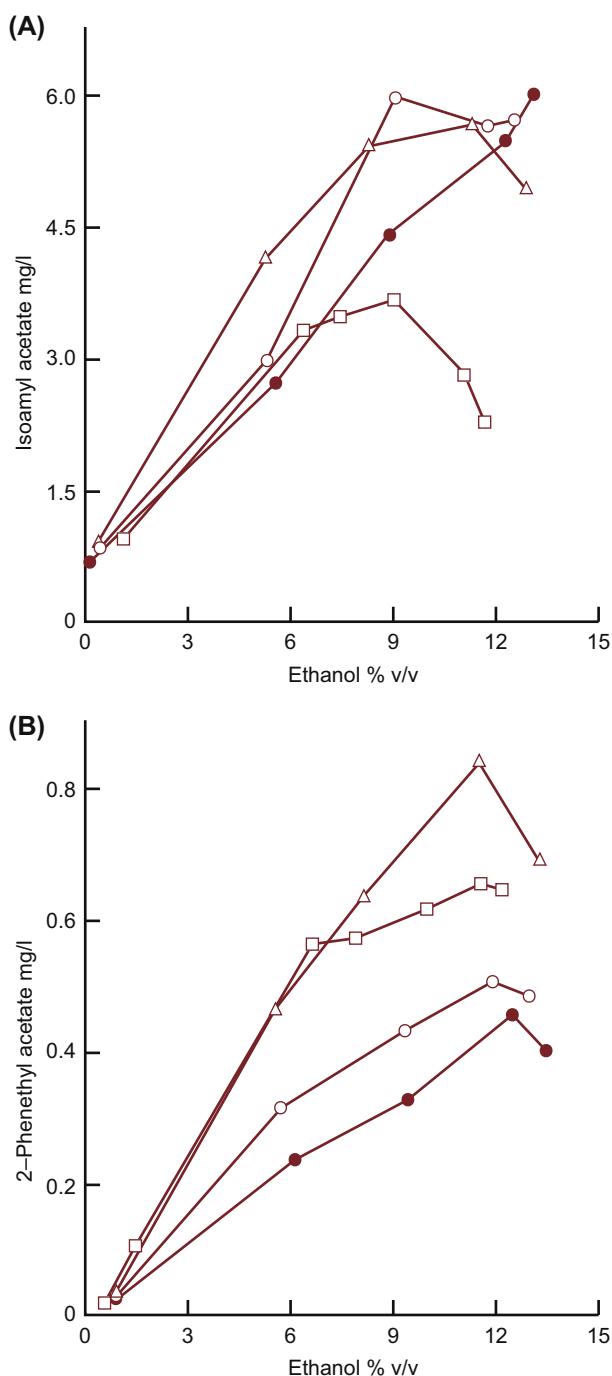


FIGURE 7.44 Effect of temperature and progress of fermentation on isoamyl acetate (A) and 2-phenethyl acetate (B) content. ●, 10°C; ○, 15°C; △, 20°C and □, 30°C. From Killian, E., Ough, C.S., 1979. Fermentation esters – formation and retention as affected by fermentation temperature. Am. J. Enol. Vitic. 30, 301–305, reproduced by permission.

findings have encouraged the use of cool fermentation temperatures and reduced sulfur dioxide addition.

The shift to cooler fermentation temperatures has increased interest in the use of *Saccharomyces bayanus* var. *uvarum*. Besides cryotolerance, these strains possess several additional desirable features. For musts low in

total acidity, *S. uvarum* strains typically increase the malic acid content. Thus, a flat taste can be avoided by biological acidification. *S. uvarum* may also augment the glycerol content; enhance the content of several aromatic esters and succinic acid; and generate a lower alcohol content (Massoutier et al., 1998).

Red wines are typically fermented at higher temperatures than white wines. Temperatures between 24 and 27°C are generally considered standard. Such temperatures favor not only anthocyanin but also tannin extraction. However, such temperatures are not universally preferred. For example, wines made from Pinotage are reportedly better when fermented at 15°C (du Plessis, 1983). The warmer temperatures generally preferred for red wine production probably relates more to its effect on phenol extraction than fermentation rate. Temperature and alcohol are the major factors influencing pigment and tannin extraction from seeds and skins. Both chemical groups dominate the characteristics of young red wines. The potentially undesirable consequences of higher fermentation temperatures, such as the production of increased amounts of acetic acid, acetaldehyde, and acetoin, and lower concentrations of some esters, are probably less noticeable against the more intense fragrance of red wines. The greater synthesis of glycerol at higher temperatures is often thought, probably unjustly, to give red wines a smoother mouthfeel.

Other important influences arise from factors not directly related to the effect of temperature on fermentation. For example, temperature affects the rate of ethanol loss during vinification (Williams and Boulton, 1983). The volatilization of hydrophobic, low-molecular-weight compounds such as esters is even more marked. Consequently, their dissipation has a greater potential impact on the sensory quality of wine than the loss of ethanol.

During fermentation, much of the chemical energy stored in grape sugars escapes as heat. It is estimated that this is equivalent to about 23.5 kcal/mol glucose (see Williams, 1982), resulting in a potential temperature rise of 1.3°C per 100 g sugar. This is sufficient for juice at 23 °Brix to potentially increase in temperature by about 30°C during fermentation. If this were to occur, yeast cells would die before completing fermentation. In practice, such temperature increases are not realized. Because heat is liberated over several days to weeks, some of the heat dissipates with escaping carbon dioxide and water vapor. Heat also radiates through the surfaces of the fermentor into the cellar environment. Nevertheless, the temperature rise can easily be in the range of 12–15°C, sufficient to be critical to yeast survival—if temperature-control measures are not implemented. Temperature control is also essential if the fermentation temperature is intended to remain within a narrow range.

Critical to the importance in any heat buildup is the initial temperature of the juice or must. This influences

the rate of fermentation and correspondingly the rate at which the temperature rises. Up to a point, the greater the initial rate of fermentation, the sooner a lethal temperature may be reached. Thus, cool temperatures at the beginning of fermentation can diminish the degree and sophistication of any temperature control required.

Also important to temperature control is the size and shape of the fermentor, and the presence or absence of a cap. The rate of heat lost is often directly related to the surface area/volume ratio of the fermentor. By retaining heat, the volume of juice can significantly affect the rate of fermentation—the larger the fermentor, the greater the heat retention and likelihood of overheating. This feature is illustrated in Fig. 7.45.

Another issue is the development and maintenance of a relatively uniform temperature throughout the fermentor. The turbulence induced by the tumultuous release of carbon dioxide during fermentation may be sufficient to achieve uniformity. This is often the case in producing white and rosé wines, in which vertical and lateral temperature variation is little more than 1°C. At cool fermentation temperatures, however, turbulence may be insufficient to equilibrate the temperature throughout the fermentor, and temperature stratification may develop.

With red wines, cap formation disrupts the effective circulation and mixing of the must. The cap-to-liquid temperature difference may reach 10°C (Fig. 7.46).

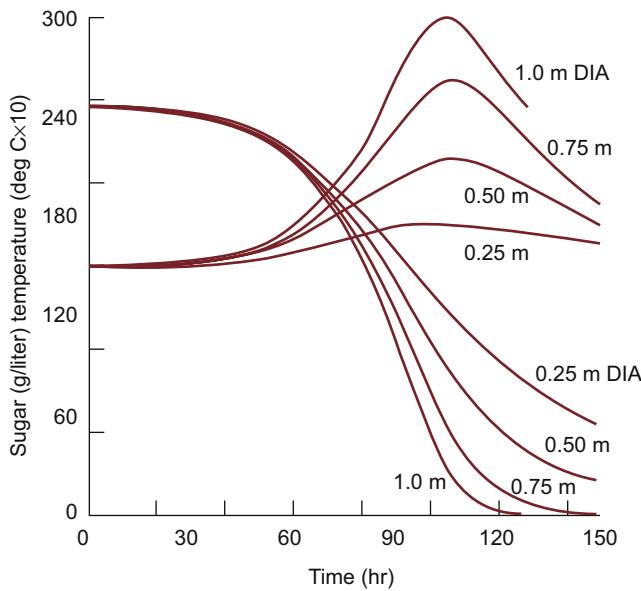


FIGURE 7.45 Effect of barrel diameter on fermentation rate and temperature rise during fermentation. Although the data are not presented in terms of cooperage capacity, barrels possessing maximum diameters of 0.5, 0.75 and 1.0 m could have capacities in the range 50–75, 225–500, and 800–1200 L, respectively, depending on barrel height and stave length. *From Boulton, R., 1979. The heat transfer characteristics of wine fermentors. Am. J. Enol. Vitic. 30, 152–156, reproduced by permission.*

Without mixing, the temperature differential may reach its maximum within 3 days, declining progressively thereafter to about 3.5°C after 6 days (Vannobel, 1986). Further details on the potential for vertically and horizontally temperature variations throughout the must are illustrated in Fig. 7.47. Periodic punching down produces only transitory temperature equilibration between the cap and the juice; full pumping over is required to reduce temperature differences to less than 5°C. In contrast, little temperature variation exists within the main volume of the must. Because high cap temperatures are a common feature of many traditional red wine fermentations, these vinifications may consist of two simultaneous but distinct phases—a liquid phase, in which the temperature is cooler and changes less during fermentation, and a largely uncontrolled high-temperature phase in the cap (Vannobel, 1986). Because the rate of fermentation is more rapid in the cap, the alcohol content rises quickly to above 10%. The higher temperatures found in the cap, plus the association of alcohol, probably increase the speed and efficiency of phenol extraction from the seeds and skins trapped in the cap. This feature is presumably absent or much diminished where the cap is submerged or where

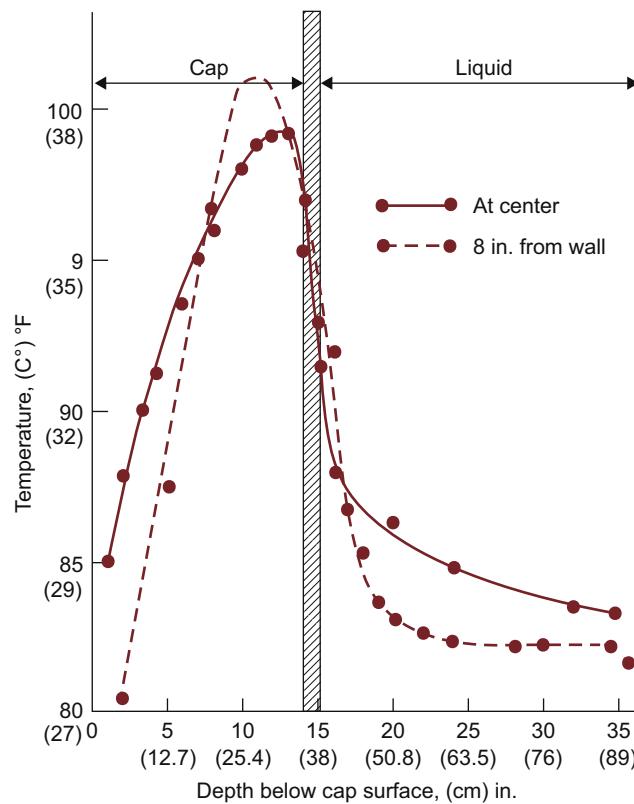


FIGURE 7.46 Vertical temperature profile through cap and liquid at 40 h. Crosshatching indicates that the boundary between the cap and liquid is not sharply defined. *From Guymon, J.F., Crowell, E.A., 1977. The nature and cause of cap-liquid temperature differences during wine fermentation. Am. J. Enol. Vitic. 28, 74–78, reproduced by permission.*

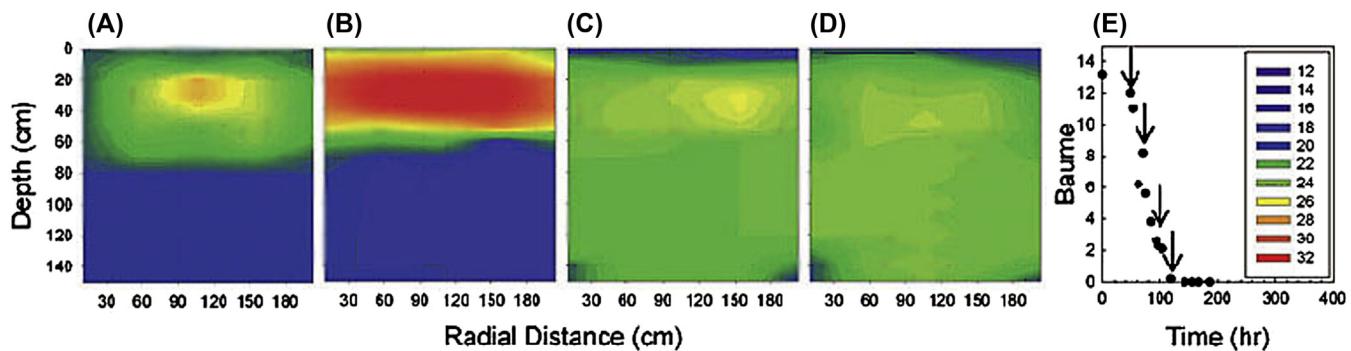


FIGURE 7.47 Spatial distribution of temperatures during a 3450-L Pinot noir fermentation. Temperature measurements were taken at approximately 24 h intervals at the time indicated (arrows) in the appropriate fermentation curve (E). From Schmid, F., Schadt, J., Jiranek, V., Block, D.E., 2009. Formation of temperature gradients in large- and small-scale red wine fermentations during cap management. *Aust. J. Grape Wine Res.* 15, 249–255, reproduced by permission.

automatic punching down, pumping over, or rotary fermentors are used to diminish the temperature and associated differences between cap and main must volume. There appears to have been little investigation of the sensory consequences of current cap submerging techniques. This also applies to older procedures, where incomplete and intermittent cap disruption resulted in fermentation occurring at varying temperatures within the cap and juice, respectively.

Temperature regulation is achieved by a variety of procedures. Selective harvest timing has the potential to provide fruit at a desired temperature, obviating at least the need for juice or must cooling. For centuries, relatively small fermentors, and vinification in cool cellars achieved a degree of natural temperature control. Pumping over in red wine vinification is another procedure that provides a degree of cooling, in addition to its main role of submerging the cap. However, the maintenance of fermentation temperatures within a select, narrow range requires direct cooling in all but small barrels in cool cellars. Although estimated to cost up to about 50% of a winery's electricity bill, cooling is often considered money well spent.

If heat transfer through the fermentor wall is sufficiently rapid, cooling the fermentor surface with water or by passing a coolant through an insulating jacket, can be effective. If thermal conductance is insufficient, fermenting must can be pumped through external heat exchangers, or cooling coils may be inserted directly into the fermentor. Alternatively, pumps providing additional agitation may avoid temperature stratification throughout the fermentor. In special fermentors, trapping carbon dioxide, and the associated pressure buildup, can be used to slow fermentation, minimizing heat accumulation.

Pesticide residues

Under most situations, no more than trace amounts of pesticide residues are found in juice or must. At such concentrations, they have little or no perceptible effect

on fermentation, wine quality, or human health. Used properly, pesticides help the fruit reach maximal quality, and reduce the risks of mycotoxin contamination. When used in excess or applied just before harvest, pesticides can negatively affect winemaking and potentially pose health risks.

Various factors influence pesticide residues on or in fruit. For example, heavy rains or sprinkler irrigation can wash contact pesticides off the fruit. Rain has less effect on systemic pesticides (those absorbed into plant tissues). Solar ultraviolet radiation degrades some pesticides, decreasing their residues. Epiphyte and plant metabolic decomposition are also possible.

Crushing, and especially maceration, can influence the incorporation of crop-protection chemicals into the must. Depending on the fungicide, extended maceration can either increase or decrease the amount found in the juice. Maceration generally has little effect on the content of systemic pesticides as they are already in the juice before crushing.

Clarification by cold settling, clarifying agents, or centrifugation significantly reduces the concentration of contact fungicides, such as elemental sulfur, but has little effect on most systemic pesticide residues (Fig. 7.48). The persistence of pesticide residues, once dissolved, depends largely on their stability under the physicochemical conditions found in the ferment and/or wine. For example, more than 70% of dichlofluanid residues are degraded under the acidic conditions found in juice and wine (Wenzel et al., 1980). In contrast, differences in racking, clarification, and filtration procedures did not markedly reduce the concentrations of chlorpyrifos, fenarimol, vinclozolin, metalaxyl, mancozeb, and penconazole (Navarro et al., 1999). Nevertheless, for many commonly used pesticides, degradation or precipitation reduce their levels in finished wine to trace or undetectable levels (Sala et al., 1996; Fernández et al., 2005). There are also investigations into the value of washing grapes with a 1% solution of citric acid before crushing

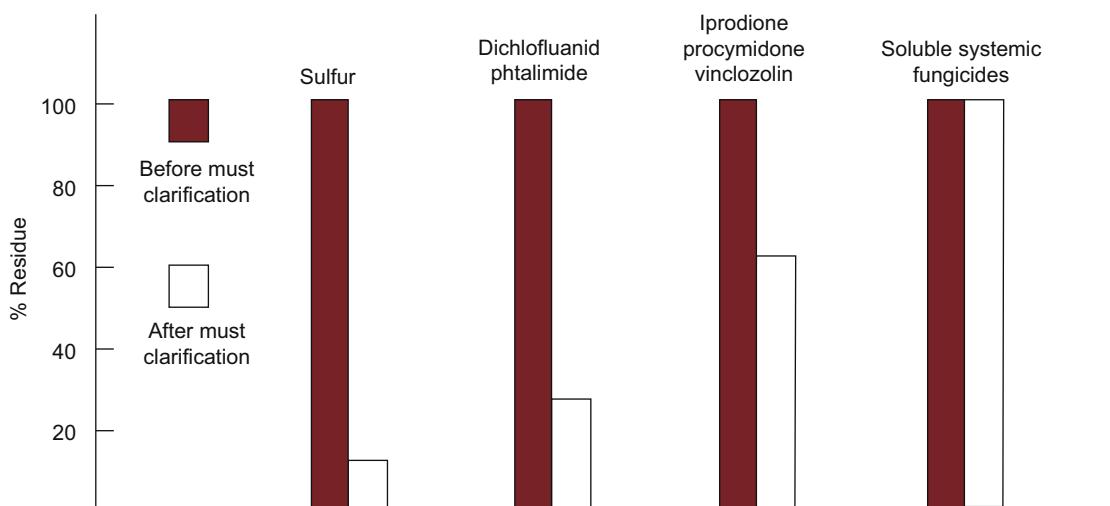


FIGURE 7.48 Influence of must settling on the elimination of various fungicides employed in viticulture. From Gnaegi, F., Aerny, J., Bolay, A., Crettenand, J., 1983. Influence des traitements viticoles antifongiques sur la vinification et la qualité du vin. *Rev. Suisse Vitic. Arboric. Hortic.* 15, 243–250, reproduced by permission.

(Cavazza et al., 2007). Initial results appeared encouraging.

Of pesticide residues, fungicides not surprisingly have the greatest effect on yeasts. Newer fungicides, such as metalaxyl (Ridomil) and cymoxanil (Curzate), do not appear to affect fermentation. In contrast, triadimefon (Bayleton) can depress fermentation, presumably by disrupting sterol metabolism. Older, broad-spectrum fungicides, such as dinocap, captan, mancozeb, and maneb, generally are toxic to yeasts. Copper residues from the application of copper sulfate fungicides increasingly suppress fermentation at concentrations above 10 mg/L (Sun et al., 2016). Residual elemental sulfur seldom have a significant effect, apart from at abnormally high concentrations (Conner, 1983). Tolerance to sulfur may be related to the relative insensitivity of wine yeasts to other sulfur compounds, notably sulfur dioxide.

Fungicides can have several direct and indirect effects on fermentation. Delaying the start of fermentation is probably the most common. As this primarily affects the lag phase, subsequent fermentation is unaffected. Increasing the inoculum size (5–10 g/hL active dry yeast) avoids most fungicide-induced suppression of fermentation (Lemperle, 1988). The increased yeast population reduces the amount of fungicide available to react with each cell. Occasionally, the onset of fermentation occurs normally, but the rate is depressed (Gnaegi et al., 1983). Such suppression may result in stuck fermentation.

Fungicides may affect the sensory qualities of wine by influencing the relative activities of various yeasts and their biosynthetic pathways. Where residues are sufficient, elemental sulfur can augment the synthesis of hydrogen sulfide in some yeast strains (Fig. 7.49), as can Bordeaux mixture, folpet, and zineb. Although hydrogen

sulfide favors the subsequent production of mercaptans, residual copper can limit mercaptan synthesis by forming insoluble cupric sulfide with H₂S. Vinclozolin (Ronilan) and iprodione (Rovral) occasionally appear to affect fermentation and may induce the development of off-flavors (San Romão and Coste Belchior, 1982). In addition, fungicides may occasionally react with varietal flavor constituents, reducing their impact. The primary example is copper (derived from Bordeaux mixture). It can reduce the content of 4-mercaptop-4-methylpentan-2-one (4-MMP) in Sauvignon blanc wines (Hatzidimitriou et al., 1996). Copper reacts with 4-MMP during alcoholic fermentation and may also interfere with the synthesis of its precursor during grape maturation. Demonstration that newer fungicides are inactive against yeasts is often a requirement for registration of use on grapes.

Fungicides may have selective effects on the endemic yeasts during vinification. For example, captan favors the growth of *Torulopsis bacillaris* by

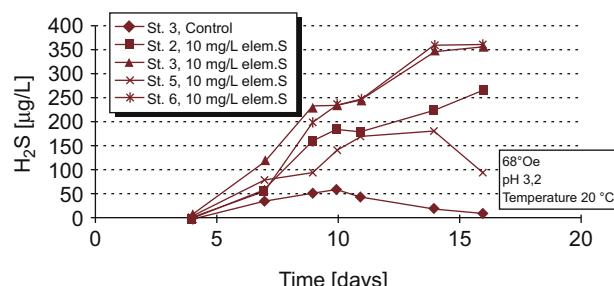


FIGURE 7.49 Influence of yeast strain on the production of H₂S during fermentation after the addition of elemental sulfur. Modified from Rauhut, D., Kürbel, H., 1994. Die Entstehung von H₂S aus Netzschwefel-Rückständen während der Gärung und dessen Einfluß auf die Bildung von böckerverursachenden schwefelhaltigen Metaboliten in Wein. *Wein-Wiss.* 29, 27–36, reproduced by permission.

suppressing the growth of most other yeast species (Minárik and Rágala, 1966). Several herbicides (2,4-dichlorophenoxyacetic acid [2,4-D] and simazine), and most insecticides do not disrupt yeast fermentation (Conner, 1983; Cabras et al., 1995).

Stuck and sluggish fermentation

Stuck fermentation refers to premature termination before all but trace amounts of fermentable sugars have been metabolized. Both stuck and sluggish fermentations have been problems since time immemorial. Historically, their occurrence was usually attributed to overheating during fermentation. In the absence of adequate cooling, fruit harvested and fermented under hot conditions can readily overheat and fermentation become stuck. The resulting wines are high in residual sugar and low in alcohol content, making them unacceptable as a table wine as well as highly susceptible to microbial spoilage. Instability is increased further if the grapes are low in acidity, high in pH, or both.

The current extensive use of temperature control has essentially eliminated overheating as a significant factor in stuck fermentation. Ironically, the application of cooling has favored the incidence of other causes of stuck and sluggish fermentations. The desire to accentuate the fresh, fruity character of white wines has encouraged the use of cool temperatures. This can limit yeast growth and potentially favor microbial contaminants that further retard growth. The search for enhanced freshness has also induced some winemakers to use excessive juice clarification, either with bentonite, centrifugation, or similar procedures. The resulting loss of sterols, unsaturated fatty acids, and nitrogenous nutrients can increase yeast sensitivity to the combined toxicity of ethanol and carboxylic acids, notably octanoic and decanoic acids and their esters. The latter enhance ethanol-induced leakage of amino acids and other nutrients (Sá Correia et al., 1989). Octanoic acid and other stress factors such as ethanol, high temperature, and nitrogen deficiency also cause a reduction in intracellular pH and associated enzymic and membrane dysfunction (see Viegas et al., 1998). Crushing, pressing, and other prefermentative activities scrupulously conducted in the absence of oxygen heighten these effects. Molecular oxygen is required for the biosynthesis of sterols and long-chain unsaturated fatty acids essential for cell membrane synthesis and function.

Juice from overmature and botrytized grapes generally has a very high sugar content. The resulting osmotic influence can partially plasmolyze yeast cells, resulting in slow or incomplete fermentation. In addition, overmature grapes may have an unusually low glucose/fructose ratio, a situation correlated with stuck fermentation in Switzerland (Schütz and Gafner, 1993b). It was solved by the addition of glucose to reestablish a more typical ratio. Overmaturity can also be associated with reduced

ammonia and amino acid content. This is further exacerbated in botrytized juice, which tends to have even lower available nitrogen as well as thiamine contents. The activity of indigenous yeasts can also reduce thiamine content, thereby suppressing the activity of *S. cerevisiae* (Bataillon et al., 1996). Nutrient depletion adds to the combined inhibitory effects of high sugar contents and the toxicity of ethanol and C₈ and C₁₀ saturated carboxylic acids. Because these effects are well known, the juice is usually supplemented with DAP and thiamine. More difficult to counter are the inhibitory polysaccharides produced by *B. cinerea* (Ribéreau-Gayon et al., 1979), and the disruptive action of acetic acid released by acetic acid bacteria. Up-take of acetic acid can decrease the pH of yeast cytoplasm from about 7.2 to below 6. This disrupts protein function, notably the glycolytic enzyme enolase (Pampulha and Loureiro-Dias, 1990). The presence of 10⁵–10⁶ acetic acid bacteria/mL can be lethal to *S. cerevisiae* (Grossman and Becker, 1984). Some of this effect may be caused by activation of a prion (GAR⁺) in some strain of *S. cerevisiae* (Walker et al., 2016). Activation reduced fermentative capacity and occurred especially in the absence of sulfur dioxide.

If the juice is insufficiently protected by sulfur dioxide, and its pH is sufficiently high, indigenous lactobacilli may produce enough acetic acid to retard or inhibit fermentation. The best confirmed inhibitor is *Lactobacillus kunkeei* (Huang et al., 1996). Adding sulfur dioxide is the best-known means of controlling this relatively rare spoilage bacterium (Edwards et al., 1999). Conversely, excessive addition of sulfur dioxide can retard the growth of inoculated yeasts. Thus, as so often, sulfur dioxide should be employed judiciously, based on need not habit.

Another potential cause of stuck fermentation involves killer yeasts. Killer yeasts produce a protein typically toxic to similar yeasts that do not produce the protein. This feature is associated with joint infection by a mycovirus and a satellite dsRNA. The mycovirus is considered a helper, regulating replication and encapsulation of the satellite dsRNA. The latter synthesizes a toxic protein. Although most killer proteins act only on the same species, forms are known that are active against other yeasts as well as filamentous fungi and bacteria (see Magliani et al., 1997). These do not appear to be important in wine fermentations.

Under worst-case scenarios (low inocula with highly sensitive strains or continuous fermentation), killer yeasts can replace inoculated strains, even when the initial concentration of the killer strain is as low as 0.1% (Jacobs and van Vuuren, 1991). Killer *S. cerevisiae* strains may cause sluggish or stuck fermentations as well as donate undesirable sensory attributes (Maqueda et al., 2012). Even more serious are situations where potential spoilage yeasts, notably *Kloeckera apiculata* or *Zygosaccharomyces bailii*, possess killer factors that can inactivate *S. cerevisiae*.

Most killer yeast problems can largely be countered by inoculation with commercial strains, constructed to possess both common killer satellite dsRNAs strains (K1 and K2) (Boone et al., 1990; Sulo et al., 1992). Possession of the killer factor protects the producing cell from the effects of the toxin. Addition of sulfur dioxide may also suppress killer yeasts in the indigenous flora, if combined with inoculation with a resistant wine yeast strain. Temperature control and the addition of bentonite can also reduce the activity of killer proteins.

Killer properties have been isolated from naturally occurring *S. cerevisiae* strains in wine as well as other yeast genera (e.g., *Hansenula*, *Pichia*, *Torulopsis*, *Candida*, and *Kluyveromyces*). Although relatively uncommon, yeast strains resistant to killer toxins have been found that do not possess the killer factor. These are termed neutral strains. Typically, yeasts immune to a particular killer protein possess the gene that produces it.

Expression of the killer factor can vary among various yeasts. In *S. cerevisiae*, both K1 and K2 strains are associated with a helper virus of the Totiviridae group, whereas in *Kluyveromyces lactis*, linear dsDNA plasmids control the property. In some genera, chromosomal genes may be involved. In all cases, the toxic principle is associated with the production and release of a protein or glycoprotein.

With K1 and K2 toxins, toxicity involves the attachment of the protein to β -1,6-D-glucans of the cell wall. Subsequently, the toxin binds with a component of the cell membrane. It induces the formation of a pore, permitting unregulated ion movement across the membrane. The consequence is cell death. The nuclear genes that encode the killer proteins KHR and KHS are less well known, but also result in an increase in ion permeability of sensitive cells. In contrast, the K28 toxin attaches to α -1,3-mannose of cell-wall mannoproteins. It inhibits DNA synthesis and further cell division of the affected cell.

The killer proteins produced by *S. cerevisiae*, notably K1, act optimally at a pH above that normally found in wine. Consequently, K2 is of greater significance to wine production. Cells appear to be most sensitive during the exponential growth phase, when the growing tip of buds provide β -1,6-D-glucan receptors in close proximity to the cell membrane. Thus, the significance of killer toxins depends on juice pH, the addition of protein-binding substances (such as bentonite or yeast hulls), the ability of killer strains to ferment effectively, and the number of division cycles wine yeasts undergo during fermentation.

In general, the incidence of stuck fermentation may also be reduced by: limiting prefermentative clarification (restricting nutrient depletion); moderate aeration (~ 5 mg O₂/liter) at the end of the exponential growth phase; the addition of ergosterol or long-chain

unsaturated fatty acids (i.e., oleic, linoleic, or linolenic acids); the addition of yeast ghosts or other absorptive materials, such as bentonite (associated with agitation to assure adequate mixing); or the addition of ammonium salts periodically or halfway through fermentation. The addition of absorptive substances, such as yeast hulls, appears to have optimal effects when applied midway or near the end of exponential growth.

The need for such treatments can be partially predicted by discovering the root cause(s) of past instances of stuck or sluggish fermentations. This is greatly aided by close scrutiny, and recording, of the conditions and dynamics of fermentation. These details provide a basis for diagnosing the cause(s), the commencement of early corrective measures, or preventive actions in the future. Yeast analysis may also provide additional clues. For example, specific genes are activated under low-nitrogen conditions (Mendes-Ferreira et al., 2007).

Bisson and Butzke (2000) have divided problem fermentations into four categories: slow initiation (eventually becoming normal); continuously sluggish; typical initiation, but becoming sluggish; and normal initiation but abrupt termination. Their studies indicate that comparing sugar consumption, temperature, nutrient profiles, and records of procedures used in previous fermentations often provides early indications of potential problems and their possible quick resolution. Once fermentation has stopped, reinitiation is more complicated.

When stuck fermentation occurs, successful reinitiation usually requires incremental reinoculation with special yeast strains, following racking off from the settled lees (see Bisson and Butzke, 2000). The special strains usually possess high ethanol tolerance as well as greater ability to utilize fructose (the sugar whose proportion can increase markedly during fermentation). The latter property appears to depend on the mutated activity of one of the multiple sugar transport proteins located in the plasma membrane—HXT3 (Guillaume et al., 2007). The inoculum appears to be more successful if it is derived from colonies harvested in their stationary phase and exposed to ethanol (Santos et al., 2008). The addition of nutrients (if deficient), yeast hulls (to remove toxic fatty acids), must aeration, and adjustment of the fermentation temperature (if necessary) usually achieves successful refermentation.

Ideally, treatment should be based on the cause. However, this usually requires access to rapid laboratory analysis. Although available in large wineries, this may not be an option for small wineries. In this instance, use of one of the many commercial preparations for stuck fermentation is about the only solution once fermentation has ceased.

Typically, vintners do their best to avoid stuck fermentation. However, in the production of certain

specialty sweet wines, premature termination can be intentional—usually by chilling and clarification to remove the yeasts. It achieves the low alcohol, high residual sweetness desired. Because such wines are particularly sensitive to microbial spoilage, stringent measures must be employed to counteract spoilage.

Malolactic fermentation

After years of intensive investigation, the desirability of malolactic fermentation is finally becoming less controversial. The contention related to its seemingly capricious nature—at times improving quality, at others degrading. How the “second” fermentation was perceived has also varied historically. Pliny (*Historia Naturalis* 14.25) considered it undesirable, as did Jullien (1817), (page 125). However Chaptal et al., 1801, (page 140) considered it beneficial. Such divergence probably relates to their referring to different events, some probably caused by spoilage organisms. In addition, wines in which a second fermentation (if malolactic) had beneficial effects occurred erratically, whereas conditions that favored its frequent occurrence could often have undesirable consequences.

The principal effects of malolactic fermentation are a rise in pH and a reduction in perceived acidity. This involves the decarboxylation of a dicarboxylic acid (malic acid) to a monocarboxylic acid (lactic acid). This replaces the harsher taste of malic acid with the less aggressive sensation of lactic acid. The malic acid content usually is reduced to less than 300 mg/L.

Winemakers in most cool wine-producing regions (where high acidity is usually associated with residual malic acid) tend to view malolactic fermentation positively, especially for red wines. Nonetheless, raising the pH can result in a loss in color depth. Conversely, wines produced in warm regions may be low in acidity, high in pH, or both. Thus, malolactic fermentation can

aggravate an already difficult situation, potentially leaving the wine tasting “flat,” with undesired flavors, and microbially unstable.

Originally, deacidification was considered the paramount benefit of malolactic fermentation. More recently, many winemakers have begun to view it as a means of adjusting and improving wine flavor. For example, it is thought to reduce the incidence of vegetal notes and accentuate fruit flavors (Laurent et al., 1994). Nonetheless, the reverse may occur (see below). Malolactic fermentation is most commonly promoted in red wines but is also being encouraged with some white wines. Where desired, and for wines marginally high in pH, tartaric acid may be added prior to inducing malolactic fermentation.

Lactic acid bacteria

Lactic acid bacteria are characterized by several unique properties. As their name implies, a major by-product of their metabolism is lactic acid. On this regard, they are typically classified as to whether the ferment sugars to lactic acid or a combination of lactic acid, ethanol, and carbon dioxide. The former mechanism is termed homofermentation, whereas the latter is called heterofermentation. Homofermentation potentially yields two ATPs per glucose (similar to yeast fermentation), whereas heterofermentation yields but one ATP. Species possessing either type of fermentation can grow in wine.

The most beneficial member of the group, from an enologic perspective, is a heterofermentative species—*Oenococcus oeni* (formerly *Leuconostoc oenos*). Recently, its genome has been deciphered (Mills et al., 2005). It contains 1701 ORFs (open reading frames). Of these, 75% have been classified as related to functional genes. This information is facilitating the study of its physiology and genetic diversity.

Not only is *O. oeni* the species most frequently found in wine, it is also the only species inducing malolactic fermentation in wines at a pH ≤ 3.5 . It is also more tolerant to ethanol, low temperatures, and sulfur dioxide than most lactic acid bacteria. Nonetheless, interest is being shown in the use of *Lactobacillus plantarum*, alone or in combination with *O. oeni*, especially with wines with a pH ≥ 3.5 . *L. plantarum* also produces a β -D-glucosidase that can release glycosidically bound aromatic compounds.

Usually, though, most *Lactobacillus* and *Pediococcus* spp. are viewed as spoilage organisms (see the section on “Lactic acid bacteria” in Chapter 8). *Lactobacillus* contains both homo- and heterofermentative

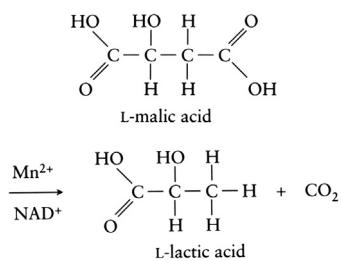


ILLUSTRATION 7.3 The deacidification of wine involving the decarboxylation of malic acid to lactic acid.

members, whereas *Pediococcus* spp. are strictly homofermentative.

Although lactic acid bacteria are categorized on the basis of sugar fermentation, the extent to which sugar metabolism is important to their growth in wine is unclear. Even dry wines, possessing primarily trace amounts of pentose sugars, can support considerable bacterial growth. Intriguingly, the concentration of hexose sugars (glucose and fructose) may increase marginally during malolactic fermentation (Davis et al., 1986a). This increase, however, is apparently unrelated to malolactic fermentation, as it can occur in its absence.

Lactic acid bacteria are further distinguished as a group by their limited biosynthetic abilities. They require a complex set of nutrients including B vitamins, purine and pyrimidine bases, and several amino acids. Indicative of their limited synthetic capabilities is their inability to synthesize heme proteins. As a consequence, they produce neither cytochromes nor catalase (both heme proteins). Without cytochromes, lactic acid bacteria cannot respire. Consequently, their energy metabolism is strictly fermentative.

Most bacteria incapable of synthesizing heme molecules are strict anaerobes—that is, they are unable to grow in the presence of oxygen. Oxygen reacts with certain cytoplasmic components, notably flavoproteins, producing toxic oxygen radicals (superoxide and peroxide). In aerobic organisms, superoxide dismutase and catalase rapidly inactivate these toxic radicals. Anaerobic bacteria produce neither enzyme. Lactic acid bacteria escape the fate of most anaerobic bacteria in the presence of oxygen (death) by accumulating large quantities of Mn²⁺ ions and producing peroxidase. Manganese detoxifies superoxide by converting it back to oxygen. The rapid action of manganese also limits the synthesis of hydrogen peroxide from superoxide. Peroxidase rapidly detoxifies any hydrogen peroxide that does form by oxidizing it to water in the presence of organic compounds. Lactic acid bacteria are the only prokaryotes that are both strictly fermentative and able to grow in the presence of oxygen.

Although fermentative metabolism is an inefficient means of generating biologically useful energy, the production of large amounts of acidic wastes quickly lowers the pH of most substrates. As a consequence, the growth of most potentially competitive bacteria is arrested. Lactic acid bacteria are one of the few bacterial groups capable of growing below pH 5. Although lactic acid bacteria grow under acidic conditions, growth is still comparatively poor at the low pH values typical of must and wine. For example, species of *Lactobacillus* and *Pediococcus* commonly cease growth below pH 3.5.

Even *Oenococcus oeni*, the primary malolactic bacterium, is inhibited by pH values below 3.0–2.9. *O. oeni* grows optimally within a pH range of 4.5–5.5. Thus, a major benefit of malolactic fermentation for the bacterium (other than generating ATP) is surprisingly acid reduction (producing conditions more suitable to its growth). By metabolizing malic to lactic acid, the number of carboxyl groups is halved, acidity is reduced, and the pH raised. The degradation of arginine, one of the major amino acids in must and wine, releases ammonia, which may also help raise the pH.

Another distinctive feature of lactic acid bacteria is the malolactic enzyme. Unlike other enzymes converting malic to lactic acid, the reaction decarboxylates L-malic acid to L-lactic acid, without free intermediates. The enzyme functions in a two-step process. First, malic acid is decarboxylated to pyruvic acid (which remains bound to the enzyme). Then, pyruvic acid is reduced to lactic acid. ATP is generated through the joint export of lactic acid and hydrogen ions (protons) from the cell (Henick-Kling, 1995). The hydrogen ions that accumulate outside the cell are sufficient to activate the phosphorylation of ADP to ATP in the cell membrane, as protons move back into the cytoplasm. Alternatively, lactic acid may be generated either by decarboxylating malic acid to pyruvic acid (and its subsequent reduction to lactic acid) or via its dehydrogenation to oxaloacetate (followed by decarboxylation to pyruvate and reduction to lactic acid).

Another energy-yielding mechanism is associated with the metabolism of citric acid. Its catabolism appears to assist glucose utilization, increasing growth (Ramos and Santos, 1996). Small amounts of reducing energy (NADH) may also result from the action of a minor alternative oxidative pathway.

Although the decarboxylation of malic acid to lactic acid is central to the enologic importance of malolactic fermentation, malic acid is not the primary energy source for lactic acid bacteria. Currently, the primary energy source for bacterial growth in wine remains uncertain. This may be because the source changes throughout growth—initially involving small amounts of sugars (exponential phase), followed by malic acid (early stationary phase), and subsequently by citric acid (late stationary phase) (Krieger et al., 2000). However, data from Henick-Kling (1995) supports the view that much of the ATP required for growth/maintenance during the log and stationary phases of malolactic fermentation comes from malic acid catabolism (Henick-Kling, 1995). Nonetheless, the situation is complicated by the pronounced influence of pH on the ability of bacteria to ferment sugars, and the marked variability among

strains. *Oenococcus oeni* shows little ability to ferment sugars, at least below pH 3.5 (Davis et al., 1986a; Firme et al., 1994). However, data from Liu et al. (1995) suggest that sugars may be the primary carbon and energy sources for the bacterium's slow-growth phase. Growth may be increased by the joint metabolism of several compounds, such as glucose with fructose or citrate. This improves their redox balance and increases ATP production.

The metabolism of citric acid also has sensory significance. It is the prime source of the diacetyl, acetoin (Shimazu et al., 1985), and acetic acid generated during malolactic fermentation. Citric acid fermentation is enhanced by the presence of phenolic compounds (Rozès et al., 2003) as well as ethanol (Olguín et al., 2009).

Amino acids, notably arginine, may also act as energy sources (Liu and Pilone, 1998). Thus, the degradation of yeast and grape proteins may be important, both in terms of energy generation and providing essential growth factors (Manca de Nadra et al., 1999).

All lactic acid bacteria assimilate acetaldehyde and other carbonyl compounds. However, their metabolism may retard malolactic fermentation. It may accrue from inhibitory amounts of sulfur dioxide being liberated by the catabolic degradation of carbonyl sulfonates (Wells and Osborne, 2011)—a microbial trojan horse.

Anaerobic bacterial metabolism, as with its yeast counterpart, can result in the generation of an excess

of NAD(P)H. To maintain an acceptable redox balance, lactic acid bacteria must regenerate NAD(P)⁺. How lactic acid bacteria accomplish this in wine is unclear. Some species reduce fructose to mannitol, presumably for this purpose (Nielsen and Richelieu, 1999; Richter et al., 2003). This may explain the common occurrence of mannitol in wine associated with malolactic fermentation. Some strains also regenerate NAD(P)⁺ with flavoproteins and oxygen. This reaction probably accounts for the reported improvement in malolactic fermentation in the presence of trace amounts of oxygen. However, oxygen also inactivates the pathway reducing pyruvate to ethanol—one of the means by which *O. oeni* regenerates NAD(P)⁺ for the metabolism of glucose.

In addition to important physiological differences, morphological features help to distinguish the various genera (Fig. 7.50). *Oenococcus* usually consists of spherical to lens-shaped cells (Plate 7.7). They commonly occur in pairs or chains but occasionally singly. *Leuconostoc mesenteroides*, closely related to *Oenococcus*, is similarly shaped and may also be isolated from wine. *Pediococcus* species usually occur as packets of four spherical cells. *Lactobacillus* produces long, slender, occasionally bent, rod-shaped cells commonly occurring in chains. Some of the more common lactic acid bacteria that may occur in wine are noted in Table 7.4.

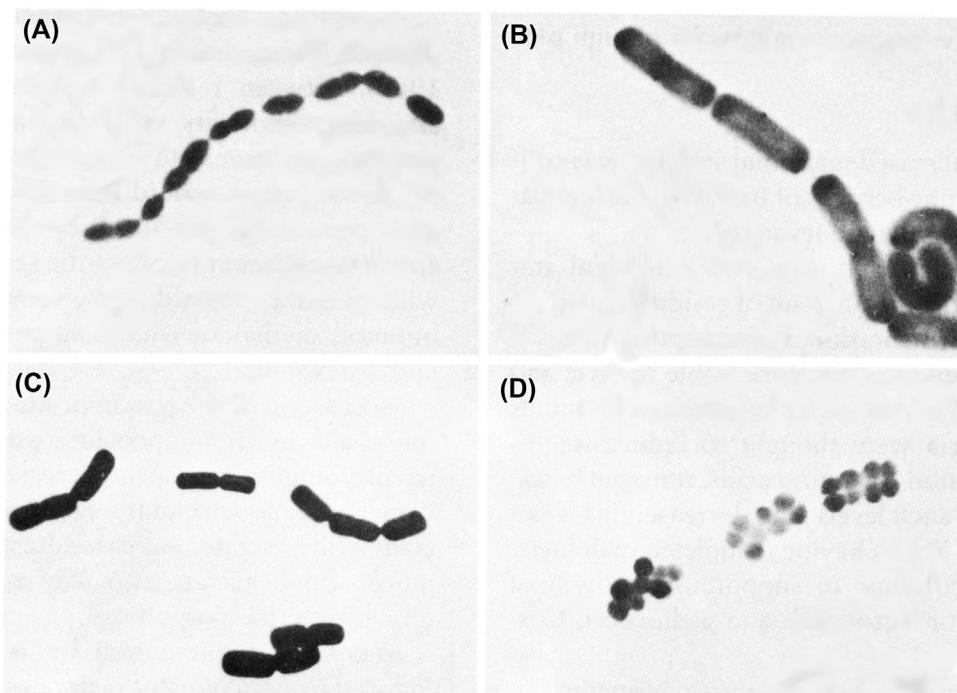


FIGURE 7.50 Micrographs of important members of the Lactobacillaceae found in wine: (A) *Oenococcus oeni* ($\times 6000$); (B) *Lactobacillus casei* ($\times 8500$); (C) *Lactobacillus brevis* ($\times 5500$); (D) *Pediococcus cerevisiae* ($\times 5000$). Cell shape and grouping may depend on the medium in which the bacteria grew. From Radler, F., 1972. Problematik des bakteriellen Säureabbaus. Weinberg Keller 19, 357–370, reproduced by permission of Dr Radler.

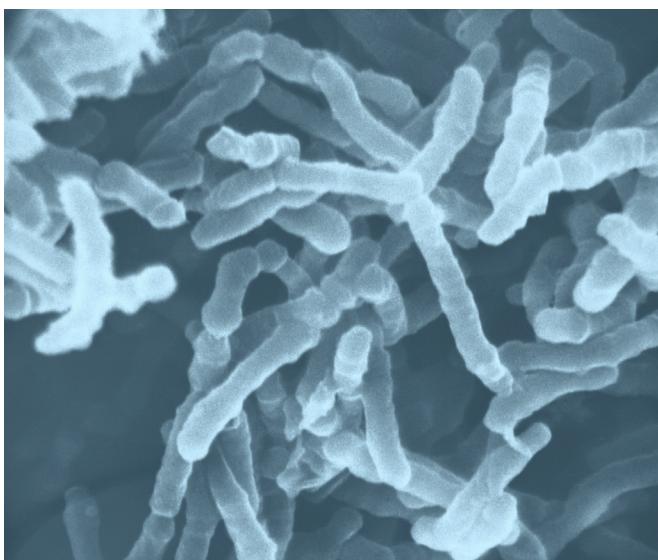


PLATE 7.7 Scanning electron micrograph of *Oenococcus oeni*. Photo courtesy Lallemand.

Effects of malolactic fermentation

Bacterial malolactic fermentation has three distinct, but interrelated, effects on wine quality. It reduces acidity, influences microbial stability, and affects the wine's sensory attributes.

Acidity

Deacidification and a rise in pH are the most consistent effects of malolactic fermentation. This adjustment usually does not commence until the bacterial population has reached a threshold of about 10^8 cells per mL. A reduction in acidity increases the smoothness and drinkability of red wines but can generate a flat taste in wines marginally low in acidity. The desirability of deacidification depends primarily on the initial pH and acidity of the grapes. Typically, the higher the acidity and the lower the pH, the greater the benefit; conversely, the lower the acidity and higher the pH, the greater the likelihood of undesirable consequences. In addition, the higher the relative proportion of

tartaric acid in the juice, the less likely malolactic fermentation will significantly affect the acidity and pH of the wine.

As the pH of wine changes, so too do the relative concentrations of the various colored and uncolored forms of anthocyanins. The metabolism of carbonyl compounds (notably acetaldehyde) by lactic acid bacteria, and the accompanying release of SO₂ bound to them, can result in pigment bleaching. In general, color loss associated with malolactic fermentation is sensorially significant only in pale-colored wines or those with an initially high pH.

Microbial stability

Formerly, increased microbial stability was considered one of the prime benefits of malolactic fermentation. This view still holds, but its relative significance is now in some doubt and its mechanism uncertain.

Improved microbial stability was thought to result from the metabolism of residual nutrients left after alcoholic fermentation. It removed the more readily metabolized malic and citric acids, leaving the more microbially stable tartaric and lactic acids. In addition, the complex nutrient demands of lactic acid bacteria were thought to reduce the concentration of amino acids, nitrogen bases, and vitamins. Although their levels do tend to decrease, wines that have completed malolactic fermentation may still support the growth of *Oenococcus oeni* or lactobacilli and pediococci (Costello et al., 1983) as well as other spoilage microorganisms.

Contrary to common belief, malolactic fermentation can occasionally decrease microbial stability. This can occur when the wine is marginally too high in pH. The resulting increase in pH can favor the subsequent growth of spoilage lactic acid bacteria. Spoilage organisms generally do not grow in wines at a pH below 3.5, but their ability to grow increases rapidly as the pH rises from 3.5 to 4.0 and above.

The stabilizing effect associated with malolactic fermentation may arise more indirectly, from the preservation practices applied after its completion. The onset and completion of malolactic fermentation permits the application of procedures, such as the addition of sulfur dioxide, storage at cool temperatures, and clarification, that are definitively preservative. Delayed onset, without the application of standard preservation techniques, exposes the wine to potential infection by acetic acid bacteria, spoilage lactic acid bacteria, and spoilage yeasts (notably *Brettanomyces/Dekkera* spp.). In the case of *Brettanomyces*, however, there is evidence that malolactic fermentation also reduces the presence of ethyl phenols, their principal spoilage by-product (Gerbaux et al., 2009). In addition, the early completion of

TABLE 7.4 Lactic acid bacteria occurring in wine.

Genus	Species
<i>Oenococcus</i>	<i>O. oenos</i>
<i>Pediococcus</i>	<i>P. pentosaceus</i> , <i>P. dammosus</i> (<i>P. cerevisiae</i>), <i>P. parvulus</i>
<i>Lactobacillus</i>	<i>L. plantarum</i> , <i>L. brevis</i> , <i>L. cellobiosis</i> , <i>L. buchneri</i> , <i>L. casei</i> , <i>L. hilgardii</i> , <i>L. trichodes</i> , <i>L. mesenteroides</i>

malolactic fermentation reduces its possible, and inappropriate (sediment generating) occurrence in bottled wine.

Flavor modification

The greatest controversy concerning the relative merits or demerits of malolactic fermentation relates to flavor modification. The diversity of opinion probably reflects both the biologic and physicochemical conditions which exist during malolactic fermentation. For example, malolactic fermentation in barrels may enhance attributes characteristic of oak maturation, but diminish the wine's varietal distinctiveness (Fig. 7.51). Varietal attributes, such as the apple and grapefruit-orange essences often detected in Chardonnay and the strawberry-raspberry attributes of Pinot noir wines, may be replaced by hazelnut, fresh bread, and dried fruit aromas, and animal and vegetable notes, respectively (Sauvageot and Vivier, 1997). In contrast, malolactic fermentation has been associated with increases in the fruity aspects of Cabernet Sauvignon wines (Bartowsky et al., 2011). Long-term studies on these sensory influences are needed.

Considerable variation in effects also depends on the strain and species involved (Malherbe et al., 2013).

Although potentially undesirable, strain selection does permit winemakers to modify the sensory characteristics of their wines. These influences, however, are affected by the wine's temperature, pH, and varietal origin. Thus, it is not surprising that the relative sensory merits of malolactic fermentation are often contested. Table 7.5 lists some of the substrates metabolized and by-products produced by lactic acid bacteria. Fig. 7.52 illustrates not only the influence of different bacterial strains, but also the response variability of two sets of panelists.

Prominent among the many malolactic by-products is diacetyl. At a concentration between 1 and 4 mg/L, diacetyl may contribute positively to a wine's fragrance (the threshold varying with the type of wine). It is often referred to as having a buttery, nutty, or toasty character. However, at concentrations above 5–7 mg/L, its buttery character can become pronounced and undesirable. Mild aeration during malolactic fermentation dramatically increases diacetyl synthesis. Maximum accumulation correlates with the completion of malic and citric acid metabolism (Nielsen and Richelieu, 1999). Surprisingly, sensory differences among wines, with and without malolactic fermentation, often do not correlate with their diacetyl content (Martineau and Henick-Kling, 1995). In addition, presence of a buttery aspect does not necessarily correlate well with diacetyl contents (Bartowsky et al., 2002). Thus, even with a single compound, the sensory influence of malolactic fermentation is complex.

Other flavorants occasionally synthesized in amounts sufficient to affect a wine's sensory character include acetaldehyde, acetic acid, acetoin, 2-butanol, diethyl succinate, ethyl acetate, ethyl lactate, and 1-hexanol. Much of the acetic acid associated with malolactic fermentation appears to be derived from citric acid metabolism. If the strain is a significant producer of acetic acid, it can leave the wine unacceptably high in volatile acidity.

Most lactic acid bacteria produce esterases. Although this could result in important losses in the fruity character of young wines, such decreases generally are negligible. Their enzymes work poorly at wine pH values (Matthews et al., 2007). Conversely, nonenzymatic synthesis of esters tends to increase during malolactic fermentation, especially ethyl acetate. Ethyl acetate may also accumulate via direct bacterial synthesis. Many strains of lactic acid bacteria also produce β -glucosidases. If released in sufficient quantities, and not inhibited by unfavorable temperature and ethanol conditions (Spano et al., 2005), these enzymes could hydrolyze odorless glycosidic complexes, enhancing the development of a varietal character (D'Incecco et al., 2004). Enzymic activities may also modify oak aromatics (if malolactic fermentation occurs in wood cooperage)

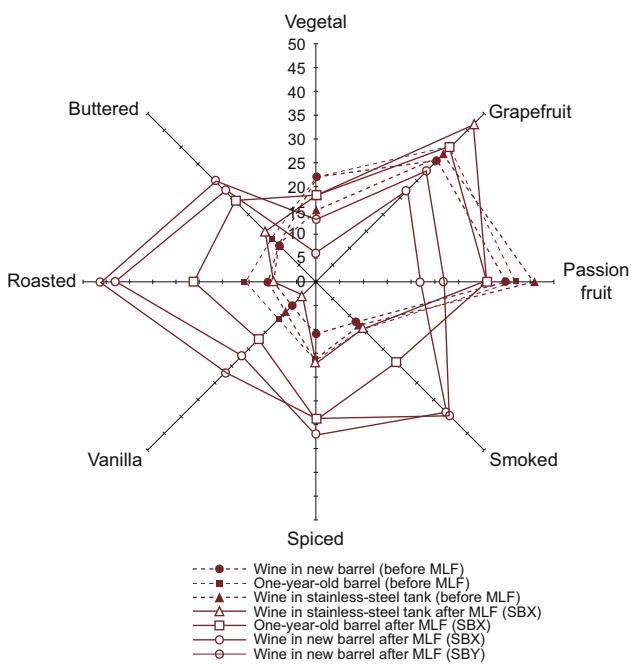


FIGURE 7.51 Presentation of eight descriptors (average of the note of 11 tasters) made it possible to differentiate wines before and after malolactic fermentation (MLF) in different tanks (stainless steel or oak barrels) with two starter-culture preparations (SBX, SBY). From de Revel, G., Martin, N., Pripis-Nicolau, L., Lonvaud-Funel, A., Bertrand, A., 1999. Contribution to the knowledge of malolactic fermentation influence on wine aroma. *J. Agric. Food Chem.* 47, 4003–4008. Copyright 1999, American Chemical Society, reproduced by permission.

TABLE 7.5 Substrates and fermentation products of lactic acid bacteria.

Substrate	Products
<i>Acids</i>	
L-Malate	L-Lactate, CO ₂ , succinate, acetate
Citrate; pyruvate	Lactate, acetate, CO ₂ , acetoin, diacetyl
Gluconate	Lactate, acetate, CO ₂
2-Oxoglutarate	4-Hydroxybutyrate, CO ₂ , succinate
Tartrate	Lactate, acetate, CO ₂ , succinate
Sorbate	2,4-Hexadien-l-ol (sorbic alcohol)
Chlorogenate	Ethylcatechol, dehydroshikimate
<i>Sugars</i>	
Glucose	Lactate, ethanol, acetate, CO ₂
Fructose	Lactate, ethanol, acetate, CO ₂ , mannitol
Arabinose, xylose, or ribose	Lactate, acetate
<i>Polyols</i>	
Mannitol	(Probably from glucose)
2,3-Butanediol	2-Butanol
Glycerol	1,3-Propanediol
<i>Amino acids</i>	
Arginine	Ornithine, CO ₂ , NH ₄
Histidine	Histamine, CO ₂
Phenylalanine	2-Phenylethylamine, CO ₂
Tyrosine	Tyramine, CO ₂
Ornithine	Putrescine, CO ₂
Lysine	Cadaverine, CO ₂
Serine	Ethanolamine, CO ₂
Glutamine	Aminobutyrate, CO ₂
<i>Unknown substrates (probably sugars)</i>	
	Propanol, isopropanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, ethyl acetate, acetaldehyde, <i>n</i> -hexanol, <i>n</i> -octanol, glycerol, 2,3-butanediol, erythritol, arabitol, dextran, diacetyl

After Radler, F., 1986. Microbial biochemistry. *Experientia* 42, 884–893, reproduced by permission.

and change the wine's soluble polysaccharide fraction (Dols-Lafargue et al., 2007). *Oenococcus oeni* may also contribute directly to the polysaccharide content of wine (separate from the occasional undesirable

production of β-1,3-glucan chains (ropiness) (Cieza et al., 2010).

The metabolism of arginine, which has a bitter, musty taste, could influence the taste perception of wines high in residual arginine. Occasionally, residual arginine values can reach the g/liter range. Incomplete arginine metabolism can, however, liberate citrulline. Its reaction with ethanol can produce the carcinogen, ethyl carbamate. Thankfully, this appears more likely with other lactic acid bacteria than *Oenococcus oeni* (Mira de Orduna et al., 2001) and tends to occur preferentially within a pH range higher than that typical of wine.

Malolactic fermentation has traditionally been encouraged more in red wines than white wines. This preference may relate to the tendency of lactic acid bacteria to metabolize compounds responsible for an excessively vegetative, grassy aspect, for example the herbaceous aspect of Cabernet Franc wines (Gerland and Gerbaux, 1999). *O. oeni* also appears to increase the presence of C₄ to C₈ fatty acid ethyl esters and 3-methylbutyl acetate (Ugliano and Moio, 2005). In addition, red wines typically possess more flavor than white wines. Thus, they are less likely to be overpowered by malolactic flavors.

O. oeni can modify hydroxycinnamic acid derivatives to vinylphenols, but is not known to decarboxylate them to ethyl phenols. Regrettably, other lactic acid bacteria can (Cavin et al., 1993). For example, *Lactobacillus brevis*, *L. plantarum*, and *Pediococcus* spp. can convert ferulic and *p*-coumaric acids to 4-ethylguaiacol and 4-ethylphenol. The production of these compounds can donate distinct phenolic or stable-like off-odors. At sub-threshold levels, however, they may contribute to a wine's overall aromatic complexity. Because people differ markedly in their detection thresholds, this may help to explain why there is such diversity in opinion relative to the flavor influences of malolactic fermentation.

Also relating to phenolics, but in a clearly positive vein, *O. oeni* has the potential to enhance the concentration of several aromatic compounds, notably oak lactones, vanillins, and phenolic aldehydes. This is associated with in-barrel malolactic fermentation (de Revel et al., 2005).

Malolactic fermentation occurring below pH 3.5 (typically by *O. oeni*) rarely generates undesirable off-odors. Undesirable buttery, cheesy, or milky odors are usually confined to malolactic fermentation induced by pediococci or lactobacilli, at or above pH 3.5. Cold soak and sluggish fermentation conditions may also favor the production of malolactic off-odors.

Amine production

Lactic acid bacteria, notably the pediococci, produce amines via amino acid decarboxylation. Nonetheless, the prevalence of *Oenococcus oeni* suggests that it is the

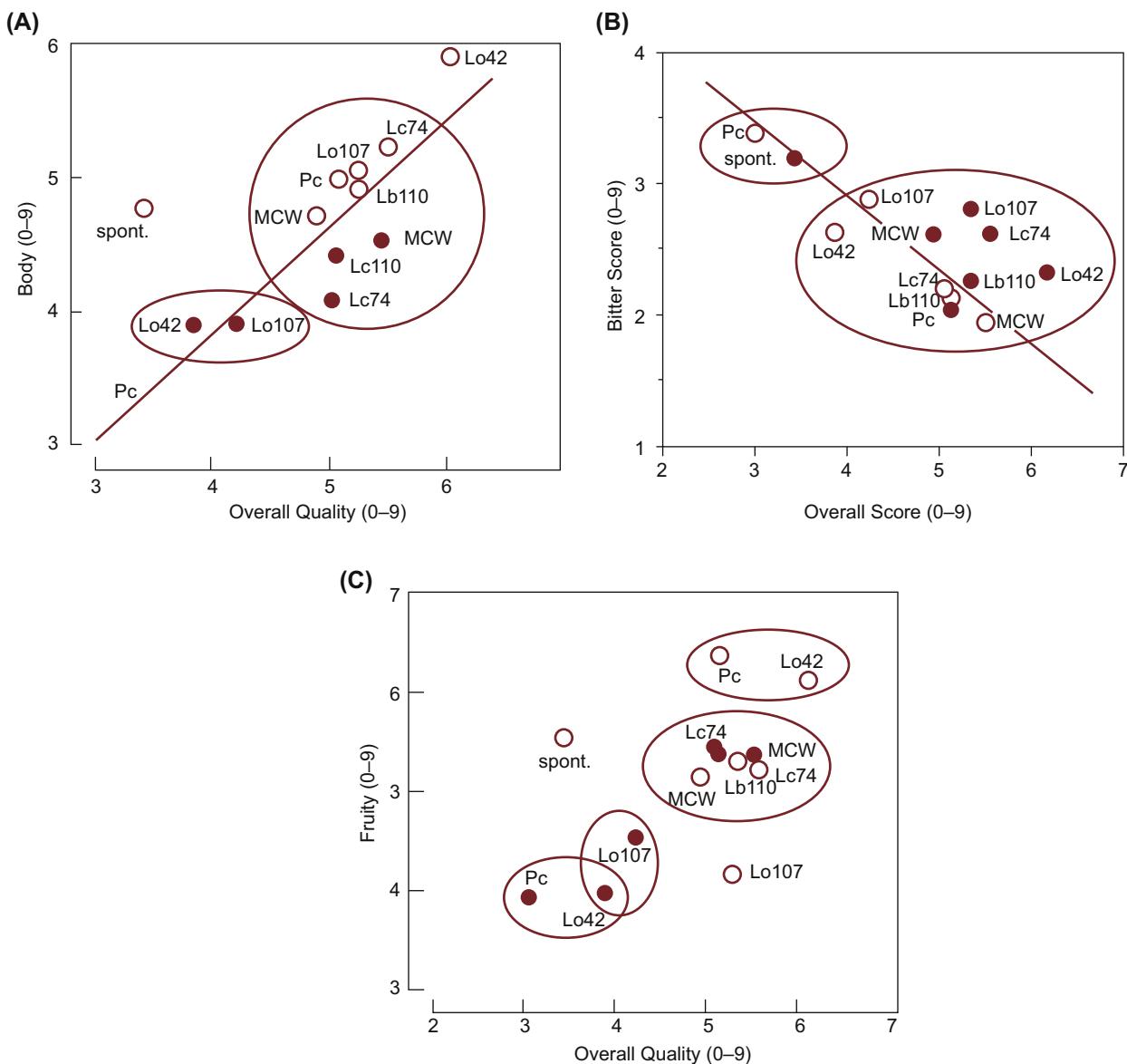


FIGURE 7.52 Relationship of body (A), bitterness (B), and fruitiness (C) to overall quality of Cabernet Sauvignon wine fermented with various malolactic cultures, as evaluated by two taste panels, composed of winemakers (●) and a wine research group (○). From Henick-Kling, T., Acree, T., Gavitt, B.K., Kreiger, S.A., Laurent, M.H., 1993. Sensory aspects of malolactic fermentation. In: Stockley, C.S. (Ed.), Proc. 8th Aust. Wine Ind. Tech. Conf. Winetitles, Adelaide, Australia, pp. 148–152, reproduced by permission.

primary source of histamine production in wine. Surprisingly, this can occur even when the bacteria are in a dormant, unculturable state (Coton et al., 1998). The potential for metabolic activity in unculturable microbes suggests that aspects of their activity in wine may be unsuspected (Piao et al., 2015).

Thankfully, biogenic amine synthesis appears to be significant only in wines of high pH. In addition, amine production appears to be less with inoculated than spontaneous malolactic fermentations (Smit and du Toit, 2013). Although some biogenic amines can induce blood-vessel constriction, headaches, and other associated effects, their contents in wine have not been demonstrated to be adequate to induce these physiological effects in humans (Radler and Fäth, 1991).

Origin and growth of lactic acid bacteria

The ancestral habitat of *Oenococcus oeni* is unknown. Although rarely isolated from grapevines (Bae et al., 2006), the bacterium has no known habitat other than wine. Its isolation from winery walls and equipment probably relates to dispersion with wine residues. From the relatively small variation in its genetic makeup, *O. oeni* appears to have evolved from a few individuals that have specialized to grow in wine (Zavaleta et al., 1997). These appear to have subsequently spread worldwide.

The only other member of the genus is *O. kitaharae*. Its endemic habitat appears to be compost. Although closely related to *O. oeni*, it does not produce the malolactic

enzyme, prefers growth at higher pH values, and expresses other distinctive physiologic attributes.

As noted, grape surfaces rarely possess colonies of *O. oeni*, nor are they a typical habitat for other lactic acid bacteria. Nonetheless, species of *Pediococcus*, *Lactobacillus*, and *Leuconostoc* may occur in the range of 10^3 – 10^4 cells/mL shortly after crushing (Costello et al., 1983). The population size depends largely on the maturity and health of the fruit—higher numbers occurring on mature, wounded, or infected fruit.

Although malolactic fermentation could originate from bacteria derived from grape surfaces, spontaneous fermentations usually appear to arise from winery equipment. Stemmers, crushers, presses, and fermentors may harbor populations of lactic acid bacteria. The relative importance of grape versus winery sources in spontaneous malolactic fermentation has yet to be clearly established.

The current trend is for winemakers to inoculate their wines with commercial strains when malolactic fermentation is desired. Of particular concern is avoiding slow, delayed, or partial fermentation, the production of off-flavors and high histamine levels, and the partial metabolism of arginine to citrulline. Citrulline and its breakdown product, carbamoyl-P, can generate ethyl carbamate (a suspected carcinogen).

Unlike yeast growth during alcoholic fermentation, no consistent bacterial growth sequence develops in the must or wine during malolactic fermentation. A pattern occasionally found in spontaneous malolactic fermentations is shown in Fig. 7.53. Significant variations occur due to factors such as pH, total acidity, malic acid content, temperature, duration of skin contact, and grape cultivar (wine matrix).

Several strains of *Oenococcus oeni* may be present at the beginning of alcoholic fermentation. In spontaneous malolactic fermentations, the most common strains frequently disappear by the end of alcoholic fermentation. Malolactic fermentation is usually conducted by one or more of the strains that are initially uncommon (Reguant and Bordons, 2003). When malolactic fermentation is induced by inoculation, it is typical for the inoculated strain to dominate deacidification or, at least, be a common member of the population inducing malolactic fermentation (Bartowsky et al., 2003).

In most spontaneous fermentations, preexisting bacteria rapidly lyse as alcoholic fermentation commences. This reduces the bacterial population from about 1×10^3 to about 1 cell/mL. Most species of lactic acid bacteria initially found die out during alcoholic fermentation. Wines with pH values higher than 3.5 may show the temporary growth of species such as *Lactobacillus plantarum*. Although capable of producing ethyl phenols, the species may reduce oxidative browning by delaying the formation of xanthylum pigments

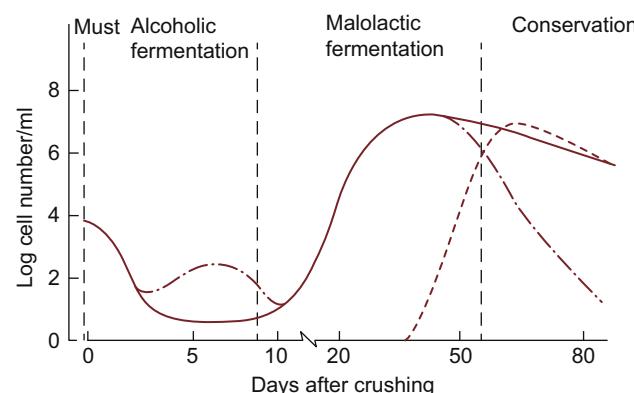


FIGURE 7.53 Diagram of the growth of indigenous lactic acid bacteria during the vinification of a red wine. Population development of *Oenococcus oeni* below (solid line) and above (dot-dashed line) pH 3.5, before and during malolactic fermentation. Near the end of fermentation and during maturation, the growth of other species of lactic acid bacteria (dashed line) may result in an accelerated decline in the *O. oeni* population (dot-dashed line). From Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H., Lee, T.H., 1985. Occurrence and growth of lactic acid bacteria in wine: a review. *Am. J. Enol. Vitic.* 36, 302–313, reproduced by permission.

from flavan-3-ols (Cureil et al., 2010). Occasionally, when sulfiting is low and the pH is above 3.5, *Oenococcus oeni* may induce malolactic fermentation coincident with alcoholic fermentation.

The usual initial population decline has been variously ascribed to sulfur dioxide toxicity, acidity, the synthesis of ethanol and toxic carboxylic acids, or the increasingly nutrient-poor status of fermenting must or wine. All these factors may be involved to some degree. At the end of alcoholic fermentation, a lag period generally ensues before the bacterial population begins to rise. This phase may be of short duration or may last several months. Once growth initiates, the bacterial population may rise to 10^6 – 10^8 cells/mL. In most wines of low pH, only *O. oeni* grows. However, there can be variations in the proportion of different *O. oeni* strains throughout malolactic fermentation. At high pH values, species of both *Lactobacillus*, and especially *Pediococcus*, may predominate. Depending on the strain or species involved, malic acid decarboxylation may occur simultaneously with bacterial multiplication or only after cell multiplication has ceased.

At the end of the exponential growth phase, the bacterial population enters a prolonged decline phase. The slope of the decline can be dramatically changed by cellar practices. For example, storage at above 20–25°C or the addition of sulfur dioxide can result in a rapid dying off of *O. oeni*. If the pH is above 3.5, and other conditions are favorable, previously inactive strains of *Lactobacillus* and *Pediococcus* may begin to multiply. Their growth often produces a corresponding decline in the population of *O. oeni*. The nature of this apparently competitive antibiosis is unknown.

Factors affecting malolactic fermentation

Growth conditions in wine are harsh for any bacterium—cool, acidic, alcoholic, anaerobic, nutrient poor, and possessing toxic fatty acids. Thus, *Oenococcus oeni* is exposed to multiple stresses. Its relative tolerance to these factors appears to be under the control of a master regulator (CtsR) (Grandvalet et al., 2005). It controls the synthesis of a variety of stress response proteins, such as HSPs and Clp ATP-dependent proteases. The first group facilitates maintenance of proper protein folding, while the second degrades improperly folded proteins. In addition, the bacterium produces membrane-bound ATPases, thought to maintain cytoplasmic electrolytic balance. Many of these proteins are synthesized at specific phases in colony growth. The influence of these stress factors on bacterial growth and physiology are outlined below.

Physicochemical factors

pH

The initial pH of juice and wine strongly influences not only if and when malolactic fermentation occurs, but also how and what species will conduct the process (Fig. 7.54). Low pH not only slows the rate, but also delays its initiation. Below a pH of 3.5, *Oenococcus oeni* is the predominant species inducing malolactic fermentation, whereas above pH 3.5 *Pediococcus* and *Lactobacillus* spp. become increasingly prevalent (Costello et al., 1983). Some of the inhibitory effects observed at low pH are probably indirect, acting through increased cell-membrane sensitivity to ethanol and the enhanced proportion of molecular sulfur dioxide. The pH also significantly affects the composition and degree of cell-membrane fatty acid unsaturation, similar to ethanol (Draci-Cachon et al., 1996).

Through these indirect influences, the pH modifies the metabolic activity of lactic acid bacteria. For example, sugar fermentation is much more effective at higher values. Similarly, the synthesis of acetic acid increases, whereas diacetyl production decreases in relation to increased pH (see Wibowo et al., 1985). The metabolism of malic and tartaric acid is also affected, with decarboxylation of malic acid being favored at low pH values, whereas the potential for tartaric acid degradation increases above pH 3.5. This results primarily from the preferential growth of *Lactobacillus brevis* and *L. plantarum* at higher pH values (Radler and Yannissis, 1972). This can lead to dramatic increases in wine pH, if both malic and tartaric acids (strong acids) are metabolized to lactic acid (a weak acid).

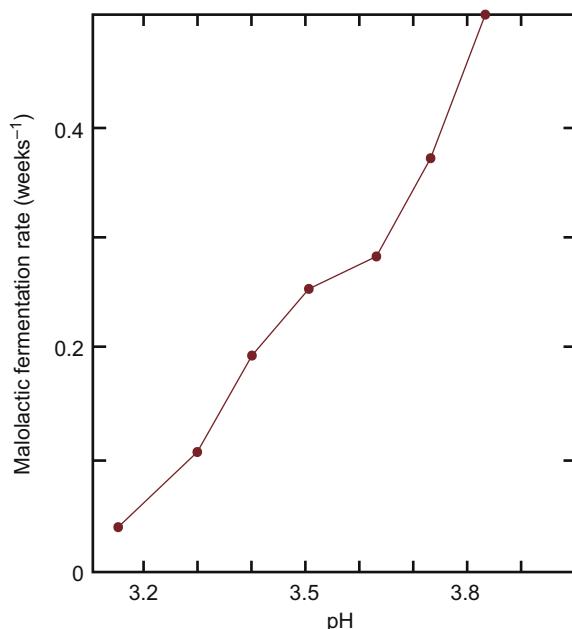


FIGURE 7.54 Connection between initial pH and the rate of malolactic fermentation. From Bousbouras, G.E., Kunkee, R.E., 1971. Effect of pH on malo-lactic fermentation in wine. Am. J. Enol. Vitic. 22, 121–126, reproduced by permission.

Temperature

The pronounced effect of temperature on malolactic fermentation has long been realized—the process often occurring when cellars began to warm in the spring. To speed its initiation, cellars may be heated to maintain the wine's temperature above 20°C. Although temperature directly affects bacterial growth rate, it most significantly influences the rate of malic acid decarboxylation. Maximal decarboxylation occurs at between 20 and 25°C, whereas growth is roughly similar within a range of 20–35°C (Ribéreau-Gayon et al., 1975). Outside this range, decarboxylation slows dramatically. At temperatures below 10°C, decarboxylation essentially ceases. In addition, most strains of *Oenococcus oeni* grow very slowly or not at all below 15°C. They do maintain cell viability, though. Thus, wines cooled after malolactic fermentation commonly retain a high viable population for months. Temperatures around 25°C favor a rapid decline in the *O. oeni* population (Lafon-Lafourcade et al., 1983), but can encourage the growth of pediococci and lactobacilli.

Cellar practices

Many cellar practices can affect when, and if, spontaneous malolactic fermentation occurs. Maceration commonly increases the likelihood and early onset of malolactic fermentation (Guilloux-Benatier et al., 1989).

The precise factors involved are unknown, but they may entail the action of phenols as electron acceptors in the oxidation of sugars (Whiting, 1975). This would help to explain why malolactic fermentation has occurred more commonly in red wines than white wines. The higher pH of most red wines is undoubtedly involved as well.

Clarification can directly reduce the population of lactic acid bacteria by favoring their removal, along with yeasts and grape solids. Racking, fining, centrifugation, and other similar practices also remove nutrients or limit their uptake into wine as a consequence of yeast autolysis.

Chemical factors

Carbohydrates and polyols

The chemical composition of must and wine has a profound influence on the outcome of malolactic fermentation. Carbohydrates and polyols constitute the most significant group of fermentable compounds (Davis et al., 1986a). Most dry wines contain between 1 and 3 g/L residual hexoses and pentoses. There are also variable amounts of di- and trisaccharides, sugar alcohols, glycosides, glycerol, and other polyols.

There is considerable heterogeneity among strains and species in their use of these nutrients. Ethanol and pH also influence their ability to ferment carbohydrates. Consequently, few generalizations about carbohydrate use appear possible. The major exception may be the poor use of most polyols. Few lactobacilli metabolize glycerol, the most prevalent polyol in wine. Although uncommon, glycerol metabolism can produce a bitter-tasting compound, acrolein (Meyrath and Lüthi, 1969).

Skin contact, before or during fermentation, promotes bacterial growth and malolactic fermentation. This is associated with an increased release of mannoproteins during fermentation, and reduced production of toxic mid-chain fatty (carboxylic) acids. A similar boost is also associated with *sur lies* maturation. Regrettably, it increases the likelihood of higher biogenic amine production (Smit and du Toit, 2013). Must clarification before fermentation appears to enhance the subsequent release of yeast polysaccharides, but also reduces the natural population of lactic acid bacteria (an important factor if early spontaneous malolactic fermentation is desired). Synthesis of α - and β -glucosidases by lactic acid bacteria increases in the presence of yeast polysaccharides, suggesting that polysaccharide hydrolysis may be a carbon source for cell growth (Guilloux-Benatier et al., 1993).

Occasionally, increases in the concentration of glucose and fructose have been noted following malolactic fermentation. These increases appear to be coincidental. The sugars may arise from the nonenzymatic

breakdown of various complex sugars, such as trehalose, by release from glycosides or from the liberation of sugars following the pyrolytic hydrolysis of oak hemicelluloses.

Organic acids

Although the decarboxylation of malic acid is the principal reason for promoting malolactic fermentation, other acids are also metabolized. Of particular importance is the oxidation of citric acid. It is associated with the synthesis of acetic acid and diacetyl (Shimazu et al., 1985). Few bacteria in wine, other than *O. oeni*, appear to metabolize citric acid. Where the flavor of diacetyl is undesired, a commercial strain of *O. oeni* (CiNi) is available that does not metabolize citrate. Gluconic acid, characteristically found in botrytized wines, is also metabolized by lactic acid bacteria (excepting the pediococci). Some lactic acid bacteria, notably the lactobacilli, have been reported to degrade tartaric acid (Radler and Yannissi, 1972). This has been associated with a wine fault called *tourne*.

The relative significance of organic acid metabolism to the energy budget of lactic acid bacteria is unclear. Both malic and citric acids are fermented after the major growth phase, when bacteria enter the stationary phase. The decarboxylation of malic acid, and the subsequent release of lactic acid from the cell, activates H⁺ uptake via membrane-bound ATPase. This is associated with the generation of ATP. A small proportion of malic acid is also metabolized directly to lactic acid. This may generate reducing power (NADH).

Fumaric acid addition was once proposed as an inhibitor of malolactic fermentation. However, its activity decreases dramatically at pH values above 3.5 (Pilone et al., 1974). Thus, it becomes progressively ineffective under the precise conditions where protection becomes increasingly needed.

The rise in pH associated with malic acid decarboxylation was particularly a problem when sorbic acid was added to control spoilage yeasts in sweet wines. Metabolism of sorbic acid by lactic acid bacteria resulted in the formation of 2-ethoxyhexa-3,4-diene, a compound possessing a strong geranium-like off-odor.

The ability of lactic acid bacteria to metabolize or tolerate fatty acids is largely unknown. However, some fatty acids are toxic to lactic acid bacteria, notably octanoic, decanoic, and dodecanoic acids produced by yeasts. These are more inhibitory in their acidic forms, but more cytotoxic in their esterified forms (Guilloux-Benatier et al., 1998). Tolerance to their toxicity is one of the multiple features for which current strain selection and/or breeding is being conducted. Adsorption of these acids onto added yeast hulls helps diminish their toxicity.

Nitrogen-containing compounds

Lactic acid bacteria are noted for their complex nitrogen growth requirements. Nonetheless, few generalizations about the nitrogen composition of wines appear evident (Remize et al., 2006). For example, the concentration of individual amino acids may increase, decrease, or remain stable during malolactic fermentation. Simple interpretation of the data is confounded by the release of bacterial proteases. These hydrolyze soluble proteins into smaller peptides and amino acids. Of these, transporting peptides is more energy-efficient than the uptake of individual amino acids. The latter have higher energy requirements. Besides grapes, an additional important source of nitrogenous compounds is yeast autolysis.

Of amino acids, only the concentration of arginine appears to change consistently during malolactic fermentation. This involves its bioconversion to ornithine. The conversion is associated with the generation of ATP. Arginine uptake, along with fructose, also activates stress-responsive genes that enhance survival (Bourdinneaud, 2006).

Where reduction in amino acid content is evident, it is probably associated with their uptake and incorporation into proteins. The direct uptake and utilization of ammonia apparently does not occur.

Ethanol

At low concentrations (1.5%), ethanol appears to favor bacterial growth (King and Beelman, 1986). At higher concentrations, it progressively retards growth, and even more effectively inhibits malolactic fermentation (Fig. 7.55). Of lactic acid bacteria, *Lactobacillus* is the most ethanol-tolerant. For example, *L. trichodes* can grow in wines at up to 20% ethanol (Vaughn, 1955). A few strains of *Oenococcus oeni* grow in culture media at up to 15% alcohol (Guzzon et al., 2009). This trait is becoming more important as the production of table wines with high alcohol contents increases. Modified inoculation procedures appear also to have some benefit in acclimating *O. oeni* to higher alcohol levels (Zapparoli et al., 2009). Alcohol tolerance appears to decline, both with increasing temperature and decreasing pH values.

The source of ethanol's inhibitory action is unknown, but likely involves changes in the semifluid nature of the plasma membrane and protein aggregation. These changes can induce enhanced passive H⁺ ion influx and loss of cellular constituents (Da Silveira et al., 2003). A small HSP (Lo18) helps limit both effects (Maitre et al., 2014). A reduction in the concentration of neutral lipids and an increase in the proportion of glycolipids have been correlated with high alcohol concentrations (Desens and Lonvaud-Funel, 1988). Ethanol tolerance has also been associated with augmented

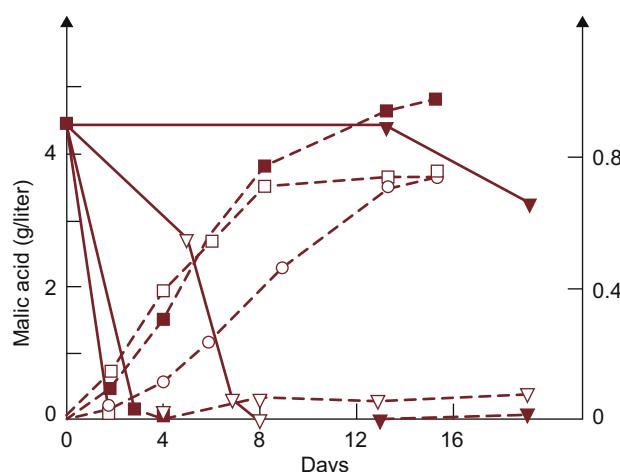


FIGURE 7.55 Influence of ethanol on the growth and malolactic activity of *Oenococcus oeni* malic acid (g/L); —, bacterial growth (OD_{620 nm}), - - -; □, 0%; ■, 5%; ○, 8%; ▽, 11%; ▼, 13% ethanol. From Guilloux-Benatier, M., 1987. Les souches de bactéries lactiques et les divers essais d'ensemencement de la fermentation malolactique en France. Bull. O.I.V. 60, 624–642, reproduced by permission.

synthesis of certain phospholipids (phosphoethanolamine and sphingomyelin), and the incorporation of lactobacillic acid in the plasmalemma (Teixeira et al., 2002).

Alcohol-induced membrane malfunction probably explains the growth disruption, reduced viability, and poor malolactic fermentation at high alcohol contents. Up to an 80% reduction in the rate of malic acid decarboxylation has been reported with an increase in alcohol content from 11% to 13% (Lafon-Lafourcade, 1975).

Other organic compounds

During malolactic fermentation, lactic acid bacteria assimilate a wide range of compounds. Occasionally, this can affect the progress of malolactic fermentation. The most well-known example involves the metabolism of carbonyl sulfonates. This has the potential to liberate sufficient SO₂ to slow or terminate malolactic fermentation.

Many lactic acid bacteria produce esterases. Despite this, the concentration of most esters, such as 2-phenethyl acetate and ethyl hexanoate, changes little during malolactic fermentation. Any reductions that occur appear to be insufficient to cause sensory impact. Other esters, such as ethyl acetate, ethyl lactate, and diethyl succinate, may increase. Although the last two are unlikely to have a sensory impact, due to their low volatility, increased ethyl acetate content could donate an off-odor, resembling nail-polish remover (acetone).

Various phenolic acids, such as ferulic, quinic, and shikimic acids as well as their esters are metabolized by some lactic acid bacteria. *Lactobacillus* spp. are notable in this regard. One of the dubious consequences can be

the generation of volatile phenolics, such as ethylguaiacol and ethylphenol. Even at low concentrations, these compounds have spicy, medicinal, creosote-like odors (Whiting, 1975). Because these fragrances do not characterize malolactic fermentations, their synthesis is probably too minimal to be perceptible.

At the concentrations generally found in wine, grape phenolics do not seriously retard malolactic fermentation, even though they have the potential to inhibit growth (Campos et al., 2009). In contrast, anthocyanins and gallic acid appear to favor bacterial viability (Vivas et al., 1997). Nevertheless, leaving stems in the ferment can delay the onset and somewhat slow the rate of malolactic fermentation (Feuillat et al., 1985). The same can occur associated with prolonged maturation in oak cooperage, with ellagitannins suppressing cell viability slightly (Vivas et al., 1995). In this regard, how the oak is treated during barrel assembly appears to be of particular significance. For example, growth of *Oenococcus oeni* was favored when the wood was toasted, but retarded without firing (de Revel et al., 2005).

Fermentors

Spontaneous malolactic fermentation often occurs in the same fermentor as alcoholic fermentation, before racking and maturation. Formerly, malolactic fermentation occurred in oak barrels after transfer from the fermentation tank. This is correlated with increased anthocyanin–tannin polymerization (favoring color stability and intensity) (Vivas et al., 1995). In addition, astringency is reduced, generating a smoother, richer wine, and oak and fruit flavors that are viewed as being more harmonious and balanced. In other studies, though, red wines lose color intensity, associated with reduced polymerization and diminished acetaldehyde and pyruvic acid concentrations (Burns and Osborne, 2015). Lactic acid bacteria may also facilitate the release of flavorants (e.g., vanillin from oak) by hydrolyzing glycoside precursors (Bloem et al., 2008; González-Centeno et al., 2017). In addition, whether malolactic fermentation occurs in barrel or stainless steel appears to affect the wine's sensory characteristics (Izquierdo et al., 2004).

Gases

Sulfur dioxide can markedly inhibit malolactic fermentation. The effect is complex, due to the differing concentrations and toxicities of wine's many sulfur dioxide states. As usual, free forms are more inhibitory than bound forms (Fig. 7.56). Of free forms, molecular SO₂ is inherently the most antimicrobial. Wine pH significantly influences the toxicity of sulfur dioxide by affecting the relative proportions of these forms, as does the concentrations of various wine constituents to which sulfur dioxide can bond. Temperature also dramatically

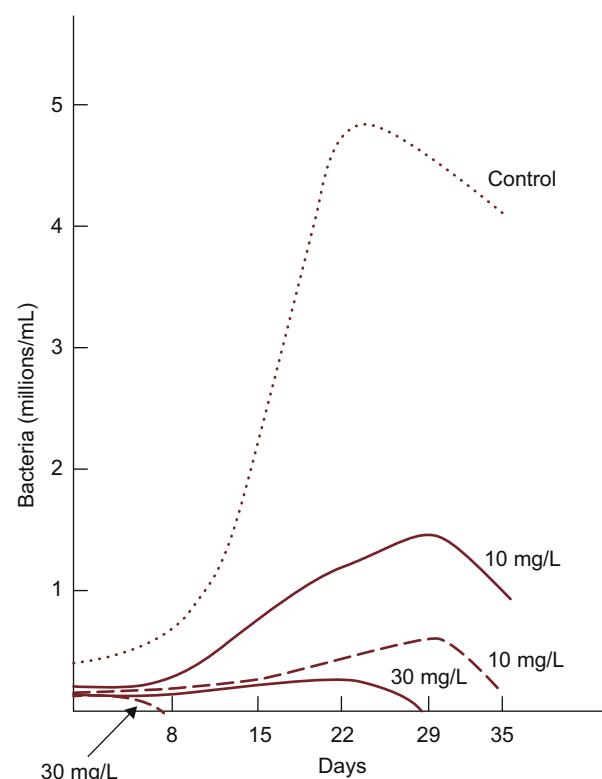


FIGURE 7.56 Action of two levels (10 and 30 mg/L) of free SO₂ and acetaldehyde-bisulfite on the growth of *Oenococcus oeni*. Control,; acetaldehyde bisulfite, ———; free SO₂, - - - - . From Lafon-Lafourcade, S., 1975. Factors of the Malolactic Fermentation of Wines. In: Carr, J.G. (Ed.), *Lactic Acid Bacteria in Food and Beverages* Academic Press, London, pp. 43–53, reproduced by permission.

influences bacterial sensitivity (Lafon-Lafourcade, 1981), as does the concentration of other toxicants (e.g., ethanol and some fatty acids). In all cases, the effect is more bacteriostatic than bactericidal (Delfini and Morsiani, 1992), and partially due to disruption of ATPase activity (Carreté et al., 2002).

Different species and strains vary considerably in their sensitivity to sulfur dioxide. In general, strains of *Oenococcus oeni* are particularly sensitive. Because of the greater tolerance of pediococci and lactobacilli to SO₂, they may be unintentionally selected by sulfur dioxide addition, especially in high pH wines. Thus, where sulfur dioxide is added prior to malolactic fermentation, it is judicious to inoculate the must or wine with a known sulfur dioxide-resistant strain of *O. oeni*. This is of particular concern with white wines, where less sulfur dioxide tends to become bound in inactive forms.

Although lactic acid bacteria are strictly fermentative, small amounts of oxygen can favor malolactic fermentation. As the bacteria neither respire nor require sterols or unsaturated fatty acids for growth, oxygen may act by improving the redox balance via reaction with flavoproteins.

The influence of carbon dioxide on malolactic fermentation is somewhat controversial. One potential mechanism for involvement may be the enhanced production of oxaloacetate, via the carboxylation of pyruvate. This could both help maintain a desirable redox balance and favor amino acid biosynthesis.

Pesticides

Little is known about the possible effects of pesticide residues on the action of lactic acid bacteria. Vinclozolin and iprodione have been reported to suppress malolactic fermentation, but enhance the growth of acetic acid bacteria (San Romáo and Coste Belchior, 1982). Cymoxanil (Curzate) and dichlofluanid (Euparen) have also been reported to inhibit malolactic fermentation (Haag et al., 1988). Copper equally has an inhibitory effect, with the delay in malolactic fermentation being accentuated at higher ethanol and sulfur dioxide concentrations, and lower pH values (Vidal et al., 2001). In contrast, benalaxyl, carbendazim, and triadimefon were found to have no effect on lactic acid bacteria (Cabras et al., 1994). Of seven fungicides and three insecticides tested by Ruediger et al. (2005), only the insecticide dicofol was found to have an inhibitory effect on malolactic fermentation.

Biological factors

Biological interactions in malolactic fermentation are as intricate as the interplay of physical and chemical factors already noted. Although mutually beneficial effects may occur, most interactions appear to be negative.

Yeast interactions

Occasionally, alcoholic and malolactic fermentation occur simultaneously. In this instance, lactic acid bacteria occasionally exert an inhibitory effect on yeast growth, causing stuck fermentations. More commonly, though, yeasts inhibit bacterial growth (Edwards et al., 1990). Cryotolerant yeasts, which are more commonly used with white wines, appear to be more inhibitory than other strains (Caridi and Corte, 1997). There is also marked differences in sensitivity among *O. oeni* strains. Laboratory tests (Costello et al., 2003; Arnink and Henick-Kling, 2005) may help winemakers determine whether, and to what degree, particular yeast and bacterial strain combinations are compatible. This could avoid the often-prolonged delay between the completion of alcoholic fermentation and the onset of malolactic fermentation. Nonetheless, cessation of bacterial growth does not necessarily inhibit malolactic fermentation (if the population is already at the point where malolactic fermentation is possible). Spoilage yeasts, such as *Pichia*, *Candida*, and *Saccharomyces*, can also retard, if not inhibit, malolactic fermentation.

Various explanations have been offered for the inhibitory action of yeasts. Suppression by *Saccharomyces bayanus* (Nygaard and Prahl, 1996) has been associated with its production of SO₂ (Larsen et al., 2003), and the high alcohol tolerance of this yeast species (resulting in a slow loss in its viability, the onset of autolysis, and associated release of nutrients). The depletion of arginine and other amino acids during the early phases of alcoholic fermentation is another possibility. Certain yeast strains coprecipitate with bacteria, removing them from the wine and thus delaying malolactic fermentation. The increasing ethanol content and the accumulation of toxic carboxylic acids (notably octanoic and decanoic acid) are also likely involved (Lafon-Lafourcade et al., 1984). Furthermore, proteins with antibacterial activities (e.g., lysozyme) have been isolated from some strains of *S. cerevisiae* (Dick et al., 1992). Whether this is related to the selectively toxic, proteinaceous agent identified by Comitini et al. (2005) is unknown. Osborne and Edwards (2007) have also isolated a peptide from *S. cerevisiae* that inhibits malolactic fermentation.

After alcoholic fermentation, yeast cells die and begin to undergo autolysis. The associated release of nutrients may explain the initiation (or reinitiation) of bacterial growth after the completion of alcoholic fermentation. Thus, leaving wines in contact with the lees for several weeks tends to encourage malolactic fermentation. Although nutrient release is undoubtedly involved, the maintenance of a high dissolved CO₂ concentration in the lees (Mayer, 1974), and the release of mannoproteins (Guilloux-Benatier et al., 1995) also appear to be involved (Fig. 7.57). Mannoproteins not only inactivate toxic fatty acids, but also provide nutrients when decomposed by bacterial β-glucosidases and proteases.

Surprisingly, malolactic fermentation may be stimulated in botrytized juice (San Romáo et al., 1985). This occurs in spite of the well-known reduction in available nitrogen content. Growth promotion may result from the metabolism of acetic acid (found in higher concentrations in botrytized grapes) or the removal of toxic carboxylic acids (adsorbed by *Botrytis*-synthesized polysaccharides). Regrettably, the increased glycerol content can favor the development of mannitic fermentation, if acidity is too low.

Bacterial interactions

The activity of acetic acid bacteria often favors malolactic fermentation during alcoholic fermentation. This may result indirectly from their suppression of yeast growth (resulting in higher nutrient levels and lower concentrations of alcohol and toxic carboxylic acids) or directly by growth enhancement through the use of acetic acid as a nutrient.

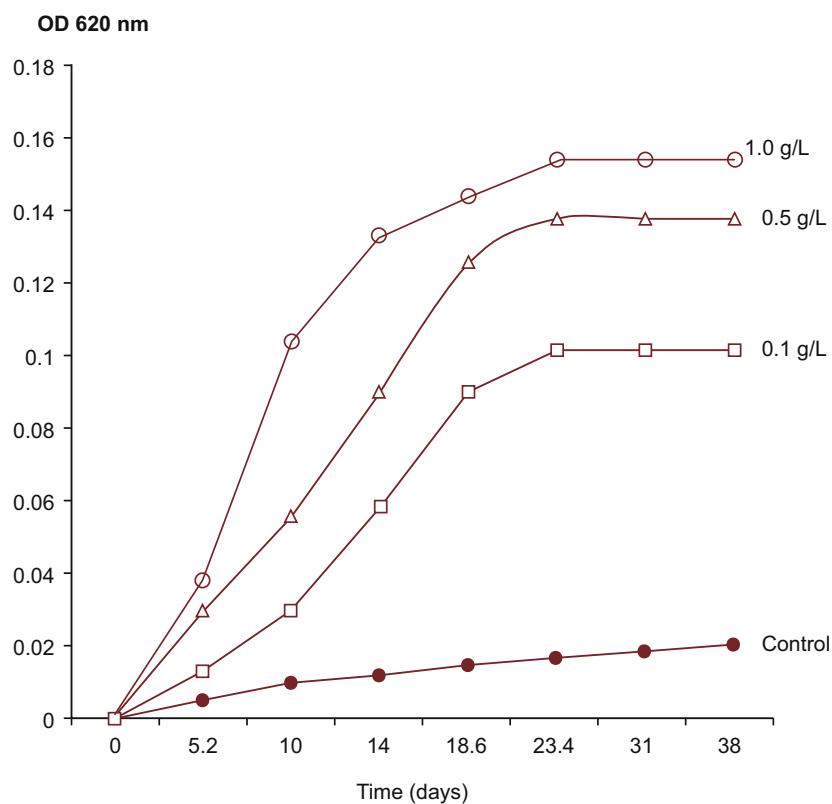


FIGURE 7.57 Influence of yeast mannoproteins on the growth of *Oenococcus oeni*. From Charpentier, C., 2000. Yeast autolysis and yeast macromolecules? Their contribution to wine flavor and stability IPProc. ASEV 50th Anniversary Annual Meeting, Seattle, Washington June 19–23, 2000. American Society for Enology and Viticulture, Davis, CA, pp. 271–277, reproduced by permission.

Internal competition/antibiosis between *Oenococcus oeni* strains is suggested by changes in the relative proportions of different strains throughout malolactic fermentation. More importantly, there may be antagonism between different species of lactic acid bacteria. Such antagonism is rare below pH 3.5, at which typically only *O. oeni* grows. However, above pH 3.5, pediococci and lactobacilli progressively have a selective advantage over *O. oeni*. Antagonism seems clearest when a decline of *O. oeni* is mirrored by an equivalent rise in the number of lactobacilli and pediococci. The mechanism(s) of this apparent antagonism are unknown.

Viral interactions

Bacterial viruses (bacteriophages) can severely disrupt malolactic fermentation by attacking *Oenococcus oeni*. Virally infected *O. oeni* may also exist in an inactive (prophage) state (Cavin et al., 1991). Because the prophage state is more unstable at higher pH values, the lytic phase may reestablish itself as the pH rises during malolactic fermentation. Under such conditions, malolactic fermentation may continue, but less predictably, under the action of *Lactobacillus* and/or *Pediococcus*.

O. oeni is most sensitive to phage infection during its exponential growth phase. The virus attaches to the bacterium and injects its DNA into the cell (Fig. 7.58). In the

cytoplasm, viral DNA begins to replicate and establish control over bacterial functions. After multiple copies of both the viral DNA and its protein coat have been produced, these components self-assemble into virus particles. At this point, the bacterium bursts open (lyses), releasing the virus particles. These may subsequently initiate additional infection/lytic cycles.

The main defense against viral disruption involves massive inoculation with *Oenococcus*. A large population reduces the number of cell divisions and thereby the duration of bacterial infection-sensitivity. The use of a mixed culture, containing several resistant strains, is also protective. Mixed cultures minimize the likelihood that all strains will be sensitive to any phage race present. Thus, infection may delay, but unlikely inhibit, malolactic fermentation. Besides delaying or inhibiting malolactic fermentation, phage infection can leave the wine open to the undesirable development of pediococci and lactobacilli. Specificity among phage strains means that those attacking *O. oeni* are unlikely to attack Pediococci or lactobacilli or vice versa.

Oenococcus oeni strains may possess one or more of at least three types of plasmids (Prévost et al., 1995). The significance of their presence is uncertain, but may favor adaptation to vine conditions (Favier et al., 2011). Plasmids are also being investigated as vectors for the incorporation of properties in the breeding of new strains of *O. oeni*. Traditional breeding is complicated by the

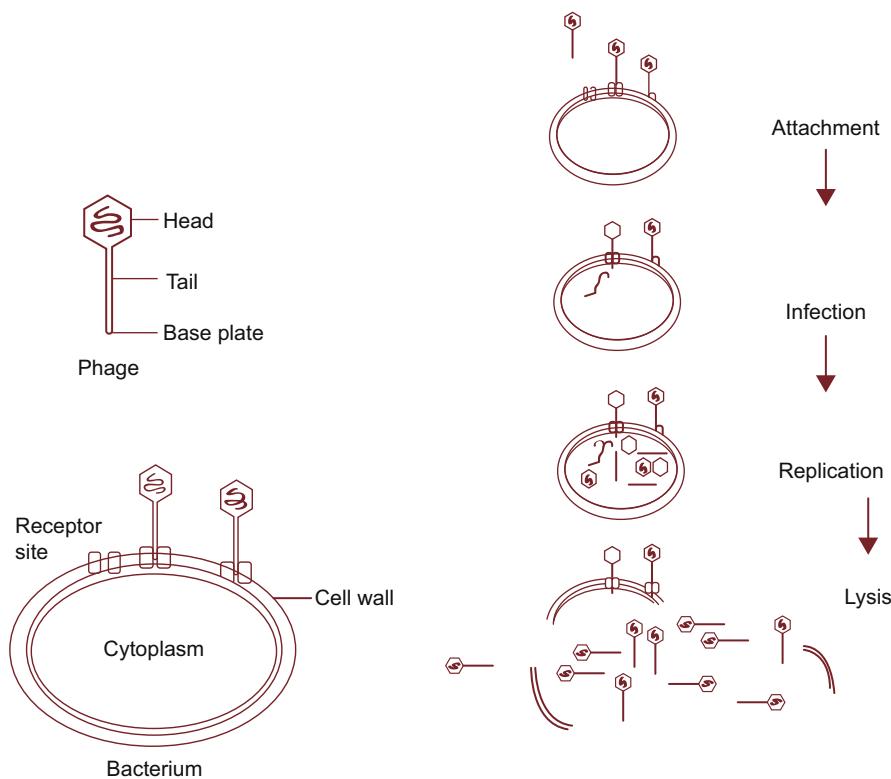


FIGURE 7.58 Attachment and infection cycle of phage viruses on bacteria. From Henick-Kling, T., 1985. Phage can block malolactic. *Wines Vines* 69 (6), 55–59, reproduced by permission.

absence of a well-developed sexual reproductive system in bacteria.

Control

Spontaneous malolactic fermentation has been notoriously difficult to manage. Malolactic fermentation may take months to begin, even under conditions chosen to favor its development. It can also occur when least desired. The following procedures are used to either encourage or inhibit malolactic fermentation.

Inoculation

To encourage malolactic fermentation, winemakers commonly inoculate with one or more strains of *Oenococcus oeni*. Addition is concomitant with the development of conditions favorable to its activity: warm temperatures ($\sim 18\text{--}22^\circ\text{C}$), minimal or no sulfiting, and delayed racking. Their combination usually induces the rapid onset and completion of malolactic fermentation. Without inoculation, it may take weeks or months for the indigenous bacterial population to achieve a size sufficient to initiate malic acid decarboxylation.

Inoculation often involves lyophilized or frozen concentrates. With most preparations, proper reactivation

is critical to maintaining malolactic ability (Hayman and Monk, 1982). Reactivation commonly takes place in nonsulfited, diluted, grape juice (1:1 with water), adjusted to a pH of 3.6 or above. Additives, usually yeast extract, may be added at a concentration of about 0.05% w/v. Reactivation generally requires 24 h, after which the organisms are cultured to achieve the desired population. Treating cells to various types of shock, for example 42°C for 1 h, appears to increase resistance to subsequent inoculation (Guzzo et al., 1994). This appears to involve the induction of several HSPs. However, with several preparations, the bacteria can be directly added to wine without prior reactivation or multiplication.

Once malolactic fermentation has occurred in one batch of wine, it may be used to inoculate other batches. However, this is generally not recommended, as it risks unwittingly transferring unsuspected contaminants that the same time. Details on how to restart stuck malolactic fermentation are given in Krieger-Weber (2009).

Inoculation customarily aims to achieve a population of about $10^6\text{--}10^7$ viable cells/mL. This usually assures the quick onset of malolactic fermentation and the dominance of the inoculated strain(s). However, laboratory studies suggest that higher populations ($\geq 10^8$ cells/mL) may be preferable under highly acidic conditions

(Maicas et al., 2000). At this population size, the cells have reached a physiological state where malic acid decarboxylation can occur without cell division. Once malic acid decarboxylation commences, it usually takes from 1 to 3 weeks to complete. High inoculation levels also diminish the synthesis of secondary metabolites, such as diacetyl and acetoin.

Although inoculation tends to be followed by the rapid onset of malolactic fermentation, it is not assured. Multiple factors could result in the inoculated strain becoming inactive or being superseded by endemic strains or species. Thus, the outcome of malolactic fermentation is less predictable than equivalent inoculations with yeast strains in alcoholic fermentation. Various mousy, bitter, acetic flavors may develop, and viscous textures may result if spoilage lactobacilli or pediococci come to dominate. Typically, though, these undesirable consequences do not occur if the inoculated strain initiates early malolactic fermentation and the wine possesses an initial pH below 3.6.

Where initiation has frequently been unacceptably slow, addition of a commercial activator 24 h before reinoculation may eliminate this problem. The activator often contains a combination of bacterial nutrients and components that absorb toxic fatty acids from the wine (Gindreau and Dumeau, 2005).

When simultaneous alcoholic and malolactic fermentation is desired, the activation medium is inoculated with both yeasts and bacteria. Occasionally, however, this has been reported to result in the production of high concentrations of acetic acid. To avoid this, Prahl et al. (1988) suggest the use of *Lactobacillus plantarum*. It is insensitive to low pH, rapidly decarboxylates malic acid, does not generate acetic acid, and rapidly dies out as the alcohol content rises. Nonetheless, the ability of many strains of *L. plantarum* to produce volatile phenols is a concern (Silva et al., 2011).

There is considerable variation in opinion as to when to inoculate. The Bordeaux school recommends inoculation after the completion of alcoholic fermentation, to avoid the risk of off-odor production (Lafon-Lafourcade, 1980) and the potential for stuck fermentation. Delayed inoculation avoids much of the toxicity of C₈ and C₁₀ carboxylic acids (their concentration declines after alcoholic fermentation), but exposes the cells to the highest levels of ethanol.

Several researchers have not confirmed French findings linking the development of high concentrations of acetic acid, yeast antagonism, or stuck fermentation with earlier inoculation (Jussier et al., 2006). They recommend concurrent alcoholic and malolactic fermentations. This option avoids any shortage of nutrients, exposure to toxic alcohol concentrations, and results in shorter overall fermentation times (Abrahamse and Bartowsky, 2012). This permits earlier racking, cooling,

and sulfiting of the wine. The sensory benefit of joint malolactic and alcoholic fermentation may be the production of less buttery, more fruity wines—albeit not consistently (Antalick et al., 2013). This may result from interactions between their metabolic by-products (Rossouw et al., 2012).

An alternative method for achieving joint malolactic and alcoholic fermentation involves the use of a yeast strain transformed with the malolactic enzyme and a malic acid transport protein (Husnik et al., 2007). It is a means of achieving the desired deacidification without any of the potential flavor changes associated with bacterial malolactic fermentation.

Another suggested method involves inoculation midway through alcoholic fermentation. Theoretically, this should avoid the toxicity of high concentrations of ethanol and suppression by sulfur dioxide. Any sulfur dioxide added during the grape crush is largely inactivated by binding to carbonyl and other compounds in the juice. However, the most intense level of yeast-induced antagonism by its metabolites, such as decanoic acid, may be encountered with this option.

Reasons for such a diversity of opinion remain unclear. Possible sources may involve differences in bacterial and yeast strains as well as grape nutrient status and vinicultural procedures. Because of this uncertainty, winemakers ideally should conduct their own trials to establish what works best for them.

Periodically, interest has been shown in the application of industrial approaches to malolactic fermentation. This has involved both the use of immobilized bacterial cells and enzymes. Such techniques offer the possibility of faster decarboxylation of malic acid, better control over the degree of the conversion (especially useful in wines with pH values above 3.5), and a reduction in the production of undesirable flavor compounds. These advantages also come with the disadvantages of cost and complexity.

Immobilization involves coating a dense population of active cells with a gel, such as pectinate, alginate, carrageenan, or polyacrylamide (Genisheva et al., 2013). Extrusion of the gel-cell mixture forms small beads, about 2–3 mm in diameter. The gel helps prevent cell division and infiltration into the wine. In many industrial uses, division of the entrapped cells is unnecessary. This is equally valid with malolactic fermentation, in which stationary phase cells actively decarboxylate malic acid. A support system (reactor) holds the beads in a rigid framework, through which the wine is slowly passed. This can achieve rapid malic acid decarboxylation (Simó et al., 2017). Other benefits of encapsulation include reduced bacterial sensitivity to pH, ethanol, and sulfur dioxide (Kosseva and Kennedy, 2004). Recent improvements in the useful life of the reactor may improve the procedure's economics. An alternate approach uses

a cellulose sponge (given a basic charge), to which individual bacterial cells adhere (Maicas et al., 2001). This avoids the development of inactive central regions, potentially found in gel-cell aggregates.

Immobilizing the malolactic enzyme into an inert permeable medium would be simpler because it would avoid the need for maintaining living cells. Enzyme immobilization also would avoid any flavor modification (if desired)—only malic acid decarboxylation would be induced. However, a considerable obstacle to its successful application is the instability of the crucial enzyme cofactor (NAD^+) at low pH values. This problem has been avoided by separating the enzyme reaction mixture (at a favorable pH) from the wine. A central membrane divides the reactor into two chambers, one each for the wine and enzymic solution (Formisyn et al., 1997).

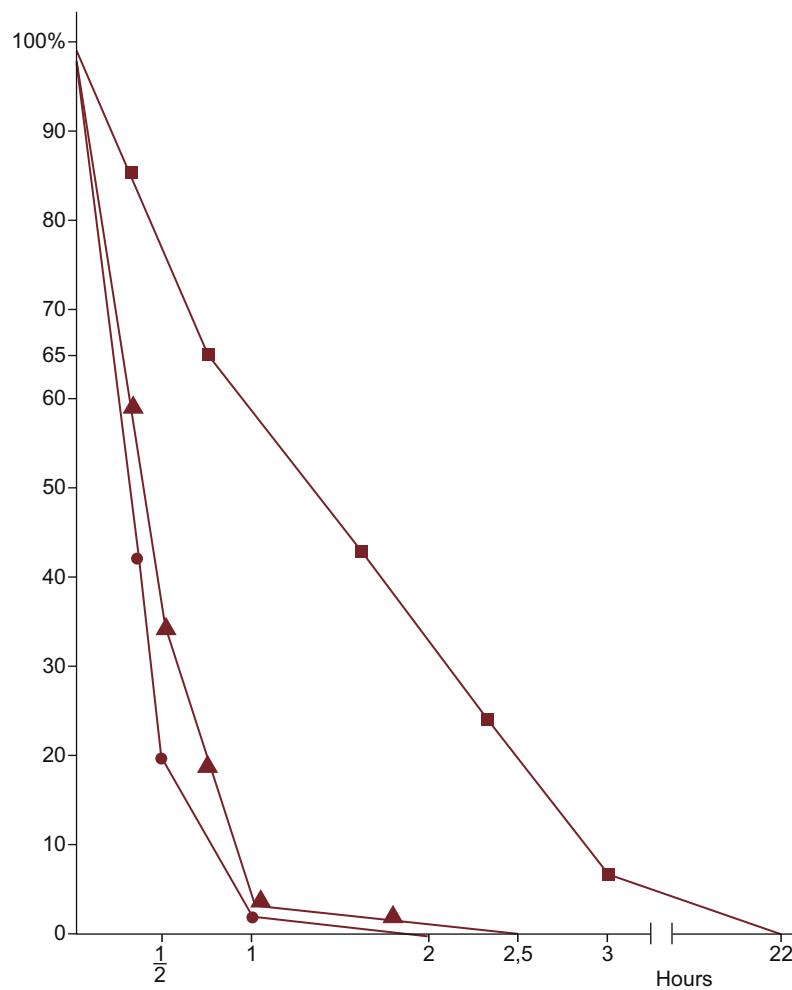
Inhibition

Inhibition of malolactic fermentation involves the reverse of factors that favor its occurrence. This usually involves wine storage at or below 10°C, early and

frequent racking, early clarification (both before and/or after alcoholic fermentation), acidification if the pH is high, minimal maceration, avoidance of *sur lies* maturation, and maintenance of a total sulfur dioxide content above 50 mg/L. Fig. 7.59 gives a representative example of the sensitivity of *Oenococcus oeni* to sulfur dioxide.

Lysozyme (muramidase) is a natural antimicrobial agent. It is particularly active against gram-negative bacteria, due to its ability to degrade the cell wall. It is present in many animal secretions (for example, tears), and is part of the vertebrate innate immune system. Because of its abundance in egg whites, it is the principal source of commercial lysozyme. It has been approved in several countries for the control of lactic acid bacteria. It has the advantage of being more effective at higher pH values (where it is most needed), and being primarily active against dividing cells. However, it is less effective in red wines than white wines (Azzolini et al., 2010), and is inactive against several strains of spoilage lactic acid bacteria (Delfini et al., 2004). Lysozyme appears to have no detectable effect on wine aroma, taste

FIGURE 7.59 Survival of *Oenococcus oeni*, expressed in terms of cellular survival, in the presence of 0.84 (■), 2.58 (▲) and 3.72 (●) mg/L of H_2SO_3 . From Delfini, C., Morsiani, M.G., 1992. Resistance to sulfur dioxide of malolactic strains of *Leuconostoc oenos* and *Lactobacillus sp.* isolated from wines. *Sci. Aliments* 12, 493–511, reproduced by permission.



(Bartowsky et al., 2004a), or effervescence. If added, it should not coincide with the addition of protein fining agents. The latter remove the enzyme along with other soluble proteins. Binding with tannins also limits lysozyme's effectiveness in red wines (Bartowsky et al., 2004a). In addition, it may complicate issues relative to haze development in white wines. This is usually corrected when the wine is fined (Weber et al., 2009). Overuse has the disadvantage of increasing color loss.

Alternative agents are the bacteriocins, nisin, and pediocin (Ruiz-Larrea et al., 2007). These are natural antimicrobial peptides or proteins produced by bacteria. They have the advantages of being colorless, odorless, and food safe (Cleveland et al., 2001). Both appear to show promise in preventing malolactic fermentation.

Procedures identical to those just noted may be employed following malolactic fermentation to limit further metabolic activity by viable *O. oeni* or the activity of other lactic acid bacteria. In this regard, the addition of lysozyme was found especially useful in limiting the production of acetic acid and several histamines after the completion of malolactic fermentation (Gerbaux et al., 1997). Because lysozyme results in cell rupture, amine-generating enzymes in viable, but dormant lactic acid bacteria, are inactivated upon release into the wine.

Depending on the likelihood of postbottling malolactic fermentation, the wine may be given sulfur dioxide, sterile-filtered into sterilized bottles or subjected to other procedures. In-bottle malolactic fermentation is undesirable. It can generate not only clouding and petillance (from the released carbon dioxide trapped in the bottle) but also off-odors.

Appendix 7.1

Partial Synonymy of several important wine yeasts.

Synonyms		
<i>Brettanomyces intermedius</i> (Krumbholz and Tauschanoff)	<i>Mycotorula intermedia</i> Krumbholz and Tauschanoff	<i>Candida lambica</i> (Lindner and Genoud) van Uden and Buckley
van der Walt and van Kerken	<i>Brettanomyces vini</i> Peynaud and Domercq	<i>Pichia membranaefaciens</i> Hansen
Perfect state: <i>Dekkera intermedia</i> van der Walt		Imperfect state: <i>Candida valida</i> (Leberle) van Uden and Buckley
<i>Candida stellata</i> (Kroemer and Krumbholz) Meyer and Yarrow	<i>Brettanomyces italicus</i> Verona and Florenzano	<i>Saccharomyces bayanus</i> Saccardo
	<i>Torulopsis bacillaris</i> (Kroemer and Krumbholz) Lodder	<i>Saccharomyces abuliensis</i> Santa Maria
	<i>Torulopsis stellata</i> (Kroemer and Krumbholz) Lodder	<i>Saccharomyces globosus</i> Osterwalder
		<i>Saccharomyces inusitatus</i> van der Walt

—cont'd

Synonyms	
<i>Saccharomyces cerevisiae</i> Meyen ex Hansen	<i>Saccharomyces aceti</i> Santa María
	<i>Saccharomyces beticus</i> Marcilla ex Santa María
	<i>Saccharomyces capensis</i> van der Walt and Tscheuschner
	<i>Saccharomyces chevalieri</i> Guilliermond
	<i>Saccharomyces coreanus</i> Saito
	<i>Saccharomyces diastaticus</i> Andrews and Gilliland ex van der Walt
	<i>Saccharomyces ellipsoideus</i> Meyen ex Hansen
	<i>Saccharomyces fructuum</i> Lodder and Kreger-van Rij
	<i>Saccharomyces italicus</i> nom. nud. (Castelli)
	<i>Saccharomyces norbensis</i> Santa María
	<i>Saccharomyces oleaceus</i> Santa María
	<i>Saccharomyces oleaginosus</i> Santa María
	<i>Saccharomyces oviformis</i> Osterwalder
	<i>Saccharomyces prostoserdovii</i> Kudriavzev
	<i>Saccharomyces steineri</i> Lodder and Kreger-van Rij
	<i>Saccharomyces vini</i> Meyer ex Kudriavzev
<i>Saccharomyces pastorianus</i> E. C. Hansen	<i>Saccharomyces carlsbergensis</i> E. C. Hansen
	<i>Saccharomyces monacensis</i> E. C. Hansen
<i>Saccharomyces uvarum</i> Beijerinck	
<i>Saccharomyces ludwigii</i> Hansen	<i>Saccharomyces ludwigii</i> Hansen
<i>Torulaspora delbrueckii</i> (Lindner) Lindner	<i>Saccharomyces delbrueckii</i> Linder
Imperfect state: <i>Candida colliculosa</i> (Hartmann) Meyer and Yarrow	<i>Saccharomyces fermentati</i> (Saito) Lodder and Kreger-van Rij
	<i>Saccharomyces rosei</i> (Guilliermond) Lodder and Kreger-van Rij
<i>Zygosaccharomyces bailii</i> (Lindner) Guilliermond	<i>Saccharomyces acidifaciens</i> (Nickerson) Lodder and Kreger-van Rij
	<i>Saccharomyces bailii</i> Linder

—cont'd

Synonyms	
<i>Zygosacch. Bisporus</i> (Naganishi) Lodder and Kreger-van Rij	<i>Saccharomyces bisporus</i> Naganishi
<i>Zygosacch. florentinus</i> Castellli ex Kudriavzev	<i>Saccharomyces florentinus</i> (Castelli ex Kudriavzev) Lodder and Kreger-van Rij
<i>Zygosacch. Rouxii</i> (Boutroux) Yarrow	<i>Saccharomyces rouxii</i> Boutroux
Imperfect state: <i>Candida mogii</i> Vidal-Leiria	<i>Zygosacch. barkeri</i> Saccardo and Sydow

Based primarily on Kurtzman, C.P., Fell, J.W. (Eds.), 1998. *The Yeasts: A Taxonomic Study*, fourth ed. Elsevier, Amsterdam, The Netherlands, Kreger-van Rij, N.J.W. (Ed.), 1984. *The Yeasts: A Taxonomic Study*, third ed. Elsevier, Amsterdam, and Gouliamova, D.E., Hennebert, G.L., 1998. *Phylogenetic relationships in the Saccharomyces cerevisiae complex of species*. Mycotaxon 66, 337–353.

Appendix 7.2

Physiological races of *Saccharomyces cerevisiae* previously given species status.

Fermentation						
	Galactose	Sucrose	Maltose	Raffinose	Melibiose	Starch
<i>S. aceti</i>	—	—	—	—	—	—
<i>S. capensis</i>	—	+	—	+	—	—
<i>S. cerevisiae</i>	+	+	+	+	—	—
<i>S. chevalieri</i>	+	+	—	+	—	—
<i>S. coreanus</i>	+	+	—	+	+	—
<i>S. diastaticus</i>	+	+	+	+	—	+
<i>S. globosus</i>	+	—	—	—	—	—
<i>S. betrogenicus</i>	—	+	+	—	—	—
<i>S. hienpiensis</i>	—	—	+	—	+	—
<i>S. inusitatus</i>	—	+	+	+	+	—
<i>S. norbensis</i>	—	—	—	—	+	—
<i>S. oleaceus</i>	+	—	—	+	+	—
<i>S. oleanginosus</i>	+	—	+	+	+	—
<i>S. prostoserdovii</i>	—	—	+	—	—	—
<i>S. steineri</i>	+	+	+	—	—	—

After van der Walt, J.P., 1970. *The genus Saccharomyces* (Meyer) Reess. In: Lodder, J. (Ed.), *The Yeasts: A Taxonomic Study*, second ed. Amsterdam, The Netherlands, North-Holland, pp. 555–718, reproduced by permission.

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