

# Organic Acid Metabolism and the Impact of Fermentation Practices on Wine Acidity - A Review

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**The conversion of grape sugar to ethanol and carbon dioxide is the primary biochemical reaction in alcoholic wine fermentation, but microbial interactions, as well as complex secondary metabolic reactions, are equally relevant in terms of the composition of the final wine produced. The chemical composition of a wine determines the taste, flavour and aroma of the product, and is determined by many factors such as grape variety, geographical and viticultural conditions, microbial ecology of the grapes and of the fermentation processes, and the winemaking practices. Through the years, major advances have been made in understanding the biochemistry, ecology, physiology and molecular biology of the various yeast strains involved in wine production, and how these yeasts affect wine chemistry and wine sensory properties. However, many important aspects of the impact of yeast on specific wine-relevant sensory parameters remain little understood. One of these areas of limited knowledge is the contribution of individual wine yeast strains to the total organic acid profile of wine. Wine quality is indeed very directly linked to what wine tasters frequently refer to as the sugar–acid balance. The total acidity of a wine is therefore of prime sensory importance, and acidity adjustments are a frequent and legal practice in many wineries. However, the total acidity is the result of the sum of all the individual organic acids that are present in wine. Importantly, each of these acids has its own sensory attributes, with descriptors ranging from fresh to sour to metallic. It is therefore important not only to consider total acidity, but also the contribution of each individual acid to the overall acid profile of the wine. This review will summarise the current knowledge about the origin, synthesis and analysis of organic acids in wine, as well as on the management of wine acidity.**

## INTRODUCTION

Organic acids and total acidity play a pivotal role in wine sensory perception, and directly influence the overall organoleptic character of wines. It is generally acknowledged that too much acidity will taste excessively sour and sharp, while wines with too little acidity will taste flabby and flat and present a less-defined flavour profile (Mato *et al.*, 2005). Desirable acidity is also a function of wine sweetness, which is mostly, but not uniquely, derived from residual grape sugars. Sweeter wines usually require higher levels of acidity to be considered of good sensory quality (Schmit *et al.*, 2013). Organic acid concentrations in grape musts are primarily a function of grape maturity and variety (Conde *et al.*, 2007). Alcoholic fermentation, however, change the concentration and content of wine acidity, and may result in a higher or lower total acidity of the wines (Volschenk *et al.*, 2006; Yabaya *et al.*, 2016). Importantly, different organic acids have different organoleptic properties, and the impact of organic acids is therefore not only linked to total acidity

and pH, but to the specific concentration of each acid in the wine.

In general, malic, citric and tartaric acids are the primary acids in wine grapes, and these acids also contribute the highest proportion of acidity (known as titratable acidity) to the final wine (Defilippi *et al.*, 2009). However, during alcoholic fermentation, several other important organic acids, such as succinic, pyruvic, lactic and acetic acid, are produced by yeast and bacteria and are mainly associated with the fresh, tart, sour and sometimes metallic taste of wines (Usseglio-Tomasset, 1995; Margalit, 1997; Bely *et al.*, 2003). These acids have also been found to contribute to the stability of wines, especially white wines (Tita *et al.*, 2006; Kučerová & Široký, 2014). Moreover, depending on the requirements for acid balance and maintenance, as well as the winemaking practices of some wines, acids such as ascorbic, sorbic and sulphurous acids are also used during winemaking.

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As grapes ripen, their sugar concentrations generally increase while their acidity declines. It has been shown that grapes from cooler wine regions generally have higher levels of acidity, which is attributed to slower grape ripening compared to grapes from warmer climate areas (Schmit *et al.*, 2013). It has also been reported that lower acidity levels in white wine are often the cause of the polymerisation of phenolic compounds, resulting in brown deposits and therefore causing darkening of white wine (Darias-Martín *et al.*, 2000). On the other side of the acidity spectrum, general concerns about undesirably high levels of acidity are common in oenology, and in some cases winemakers can resort to malolactic fermentation as a way to reduce wine acidity (López *et al.*, 2008). Although malolactic fermentation is considered the most natural method for the adjustment of wine acidity, microbial stability and organoleptic complexity, there are a number of concerns, such as spoilage (especially in warm viticultural regions with grapes containing less malic acid) and undesirable changes in wine flavour associated with the metabolic activity of lactic acid bacteria, which makes this technique inappropriate for certain types of wine (Bauer & Dicks, 2005).

Acidity is a primary driver of important management decisions related to contamination risks and sensorial properties (Akin *et al.*, 2008). In terms of contamination risks, it is well known that lower acidity and higher pH generally support the growth of microorganisms, including several unwanted or spoilage species (Bisson & Walker, 2015). Wines with a high pH therefore usually require more careful microbiological management, including the use of larger amounts of SO<sub>2</sub>. Acidity and pH are also central features of the sensorial properties of wine, although pH and acid taste are not always directly correlated. For this reason, the adjustment of acid in grape must is a critical part of winemaking. Under conditions of normal alcoholic fermentation, the titratable acidity (TA) of wine increases by 1 to 2 g/L from the start to the finish of alcoholic fermentation as a result of the evolution of acids such as succinic, acetic, lactic, malic and pyruvic acids (Volschenk *et al.*, 2006). While it is essential to monitor pH and acidity throughout fermentation, acid management includes the addition of acids, mostly tartaric acid, to grape must with a high pH (Petrie & Sadras, 2007). This practice is of particular importance in warm viticultural regions, where tartaric acid is most commonly added at the start of alcoholic fermentation in order to prevent the proliferation of spoilage LAB and other bacteria during alcoholic fermentation (Volschenk *et al.*, 2006). Several methods have been established for identifying and quantifying organic acids in grape juice and wine. Such methods include non-enzymatic and enzymatic spectrophotometric, chromatographic and electrophoretic methods (Mato *et al.*, 2005). However, high performance liquid chromatography (Castellari *et al.*, 2000; Pereira *et al.*, 2010; Park *et al.*, 2017), ion chromatography (Kupina *et al.*, 1991), gas chromatography (West & Mauer, 2011; Pinu & Villas-Boas, 2017) and capillary zone electrophoresis (Kandl & Kupina, 1999; Cortacero *et al.*, 2005; Mato *et al.*, 2006) are the preferred methods for organic acid analysis.

Acid control and regulation in wine is therefore regarded as a key process for winemakers to control wine character and

quality, combining controlled pH adjustments and informed yeast selection and management. However, the impact of many other environmental and nutritional management practices that may modulate yeast organic acid metabolism during the winemaking process, and thus final wine acidity, have not yet been fully elucidated.

### Wine organic acids

Organic acids in wine derive either directly from the grape, or are the result of microbiological activities that take place before, during or after alcoholic and malolactic fermentation. While the most commonly measured feature of wine acidity is the total acidity (TA) and pH, some organic acids are important markers for fermentation management and wine flavour and aroma. Malic acid is monitored to measure the progress of malolactic fermentation, acetic acid is monitored as an indicator of fermentation problems or of spoilage, and citric acid may be added to adjust acidity and chelate metal ions to prevent nutrients from precipitation resulting from the interaction of nutrients with metal ions, such as iron precipitating with phosphorus (Fowles, 1992).

### Wine organic acids derived from grapes

#### Tartaric acid

Unlike most other fruits, grapes contain significant amounts of tartaric acid. It is regarded as the main contributor to wine acidity, and presents a tart taste in wine (Volschenk *et al.*, 2006). Tartaric acid is not metabolised by grape berry cells via respiration in the same manner as malic acid, and the level of tartaric acid in the grapes remains relatively consistent throughout the ripening process. The concentration of tartaric acid in grapes depends largely on the grape variety and soil composition of the vineyard. Levels usually range from 4.5 to 10 g/L at the end of the grape's vegetative growth phase (Ribéreau-Gayon *et al.*, 2006). In cold climates, concentrations of above 6 g/L are commonly reached, while low levels of 2 to 4 g/L are more commonly observed in warm climates (Apichai *et al.*, 2007). Because of its stability, and the fact that yeast and other microorganisms are unable to metabolise tartaric acid, it is the acid that is most commonly employed for pH adjustment in the wine industry (Volschenk *et al.*, 2006).

#### Malic acid

L-malic acid is commonly found in many fruits, including green apples and grapes (Krueger, 2012). Mature grapes contain between 2 and 6.5 g/L of L-malic acid (Ribéreau-Gayon *et al.*, 2000). Excessive amounts of malic acid (15 to 16 g/L) may be present in grapes harvested from exceptionally cool climatic regions (Gallander, 1977). The highest concentration of malic acid attained depends on the grape variety, with some, such as Barbera, Carignan and Sylvaner, being naturally prone to higher malic acid levels. Before the change in colour of grapes at véraison, the malic acid content can reach up to 25 g/L, before declining to 2 to 6.5 g/L by maturation (Ribéreau-Gayon *et al.*, 2000). When malic acid levels are too high, wines may taste sour and may require the use of lactic acid bacteria to convert malic acid to the less harsh and softer lactic acid. The induction of malolactic fermentation is beneficial to some wines,

but in white wines, such as Chenin blanc, it may result in the production of off-flavours such as diacetyl (Bartowsky & Henschke, 2004). Nevertheless, the use of alternative technologies to develop malolactic fermentation in wine is now a priority (Maicas, 2001; Betteridge *et al.*, 2015; Lerena *et al.*, 2016; Lucio *et al.*, 2017).

### **Citric acid**

Citric acid is an intermediate of the TCA cycle and is widespread in nature (e.g. in lemons). It plays a critical role in the biochemical processes of grape berry cells, bacteria and yeast. High citric acid levels during fermentation could lead to a slower yeast growth rate (Nielsen & Arneborg, 2007). However, concentrations of citric acid in must and wine prior to malolactic fermentation are usually relatively low, between 0.5 and 1 g/L (Kalathenos *et al.*, 1995). Citric acid addition during fermentation influences the acidity and flavour of wines by promoting the perception of “freshness”, while, at the same time, promoting microbial instability and the growth of unwanted microorganisms.

## **Organic acids derived from fermentation**

### **Succinic acid**

Succinic acid occurs widely in nature in both plants and animals. Succinic acid levels vary between grape varieties, as concentrations are usually very low in white cultivars but slightly higher in red grapes. Succinic acid is one of the most important acids that develop during fermentation due to yeast metabolism, with concentrations averaging approximately 0.5 to 1.5 g/L in wine. It is a dicarboxylic acid produced mainly as an intermediate of the tricarboxylic acid (TCA) cycle during aerobic respiration, but is also one of the fermentation end products of anaerobic metabolism. Songa and Lee (2006) reported that the organic acid responsible for the largest part of the increase in titratable acidity during fermentation is succinic acid. Bertolini *et al.* (1996) previously reported the same observations, with succinic acid accounting for 50% (1.23 g/L) of the observed increase in wine acidity. In general, it is expected that, during fermentation, the formation of non-volatile organic acids ranges from 1 to 4 g/L, but such ranges vary significantly with different fermentation conditions (Lamkanra, 1997). The organoleptic character of succinic acid has been described as sour with a salty, bitter taste, and its threshold concentration is approximately 35 mg/L (Benito *et al.*, 1999). Because of its bitter-salty flavour, winemakers pay particular attention to succinic acid levels in wine. Although succinic acid is relatively resistant to microbial utilisation under fermentative conditions, it cannot be used as an acidulating agent due to this bitter-salty taste attribute (Ribéreau-Gayon *et al.*, 2006).

### **Lactic acid**

Lactic acid is an organic acid that also contributes to the overall acidity of wine. The reason why it is attractive to winemakers is that it is much softer on the palate than malic acid (Robinson, 2006). Lactic acid concentrations normally average from 1 to 3 g/L in wines (Boulton *et al.*, 1996), but can be higher in wines that have undergone malolactic fermentation, whereby malic acid is decarboxylated to lactic

acid (Volschenk *et al.*, 2006). Unlike malic and tartaric acid, lactic acid is a softer and milder acid that contributes to a creamier mouthfeel of the wine. During winemaking, lactic acid production is usually controlled by sulphur dioxide addition, which suppresses the metabolic activities of lactic acid bacteria such as those belonging to the *Oenococcus* and *Lactobacillus* genera (Osborne *et al.*, 2000). However, small amounts of lactic acid can also be synthesised through cellar practices such as maceration and cold stabilisation (Jackson & Schuster, 1997). While high lactic acid levels present no major problems in wine, lactic acid bacteria are capable of changing the sensorial characteristics of certain wines through the degradation of terpenes and other flavour molecules produced during alcoholic fermentation, as well as producing potentially undesirable aromatic compounds such as diacetyl (Lonvaud-Funel, 1999).

### **Acetic acid**

Acetic acid is a two-carbon volatile organic acid produced during wine fermentation and is mostly responsible for the sour and vinegary smell and taste in wines. Alcoholic fermentation of grapes usually results in the production of acetic acid. This process occurs mainly at the beginning of alcoholic fermentation, and again towards the end (Bartowsky *et al.*, 2003). Apart from yeast metabolic activity, the involvement of aerobic acetic acid bacteria during fermentation can also produce acetic acid by oxidising ethanol (Pronk *et al.*, 1996; Ghosh *et al.*, 2012).

In *S. cerevisiae*, a direct relationship has been established between glycerol and acetic acid production during fermentation (Remize *et al.*, 1999, Erasmus *et al.*, 2004; Goold *et al.* 2017). *S. cerevisiae* continuously has to equilibrate redox imbalances, which are a feature of alcoholic fermentation. Indeed, anabolic reactions related to biomass formation divert glycolytic intermediates away from ethanol production, requiring other pathways for the regeneration of NAD<sup>+</sup>, which is required to maintain flux through glycolysis. NAD<sup>+</sup> is therefore regenerated through glycerol biosynthesis. However, excess production of NAD<sup>+</sup> may occur, which is balanced through the production of acetic acid from acetaldehyde, a reaction that works as a redox sink to convert NAD<sup>+</sup> to NADH (Michnick *et al.*, 1997; Remize *et al.*, 1999). Wine yeasts therefore also produce acetic acid in response to hyperosmotic stress conditions. The primary response to such conditions is indeed the production of glycerol to act as a compatible compound (Hohmann, 2002). As a consequence, the redox balance is disturbed, since NADH is oxidised to NAD, leading to acetic acid production to regenerate NADH. Such hyperosmotic conditions tend to prevail at yeast inoculation at the start of alcoholic fermentation due to the high initial sugar concentrations (Erasmus *et al.*, 2004).

The critical acetic acid detection threshold in wine is estimated to be approximately 600 mg/L. However, the normal desirable acetic acid level in wines is about 100 to 300 mg/L (Ribéreau-Gayon *et al.*, 2006). High volatile acidity in wine presents a major problem, with most wineries recommending the use of lower initial sugar-containing must to reduce acetic acid formation during fermentation. However, acetic acid concentrations can reach above 1 g/L,

depending on environmental factors and the nutritional composition of the must, as well as the influence of spoilage yeasts and bacteria (Bely *et al.*, 2003). Since the aroma threshold for acetic acid varies depending on the wine variety and style, its maximum acceptable limit for most wines is 1.2 g/L (OIV, 2010). However, the volatile acidity of ice wines and botrytised wines can reach a maximum acetic acid concentration of 2.1 g/L (OIV, 2010).

### Pyruvic acid

Pyruvic acid is generally present in wine as a secondary product of alcoholic fermentation, and the amount of pyruvic acid in wine varies considerably. Concentrations of pyruvic acid average from anywhere between 10 to 500 mg/L in dry wines (Usseglio-Tomasset, 1995). In terms of its sensory attributes, this acid imparts a slightly sour taste. It is formed at the onset of fermentation and decreases towards the end of fermentation (Usseglio-Tomasset, 1995). It also plays an indirect role in wine quality due to its ability to bind sulphur dioxide. SO<sub>2</sub> is widely used in winemaking, and its germicidal effect is hugely dependent on the levels of free sulphur dioxide. Free SO<sub>2</sub> is indeed the most antimicrobial form of SO<sub>2</sub>, and bound SO<sub>2</sub> has much weaker antimicrobial properties (Fugelsang & Edwards, 2007; Pezley, 2015). Binding of SO<sub>2</sub> by pyruvic acid thus enables the growth of bacteria such as those involved in malolactic fermentation (Wells & Osborne, 2012). Any compound which binds sulphur dioxide reduces its effectiveness, and pyruvic acid is second only to acetaldehyde in this regard.

## Yeast metabolism

### Yeast central carbon metabolism

Most yeast species have similar central carbon metabolic pathways, but differences in nutrient uptake and utilisation, as well as in the regulation of fermentation and respiration, have been noted (Flores *et al.*, 2000; Conrad *et al.*, 2014). Few yeast species are capable of growing under close-to-anaerobic conditions as successfully as *S. cerevisiae* (Visser *et al.*, 1990; Moller *et al.*, 2001). Therefore, the physiology of this organism during fermentative, respiratory and respiro-fermentative conditions has attracted considerable research interest. This interest is mainly driven by the industrial significance of this species, and is linked to its ability to produce ethanol, proteins, cell biomass and other commercially relevant products (Khan & Dwivedi, 2013). The metabolism of yeast, as of all living cells, is interconnected through the coupling of anabolic and catabolic pathways. As summarised in Fig. 1, ATP is provided by the oxidation of organic carbon sources, yielding energy, ethanol, carbon dioxide and various intermediate metabolites such as organic acids (Rodrigues *et al.*, 2006).

### Glycolysis

The principal source of energy production in *S. cerevisiae* is hexoses, primarily glucose, and the conversion of such hexoses to pyruvate is achieved via the glycolytic pathway (Fernie *et al.*, 2004). Glycolysis provides the yeast with energy, together with essential glycolytic intermediates under both aerobic and anaerobic conditions. Under aerobic conditions, the pyruvate formed by glycolysis enters the TCA

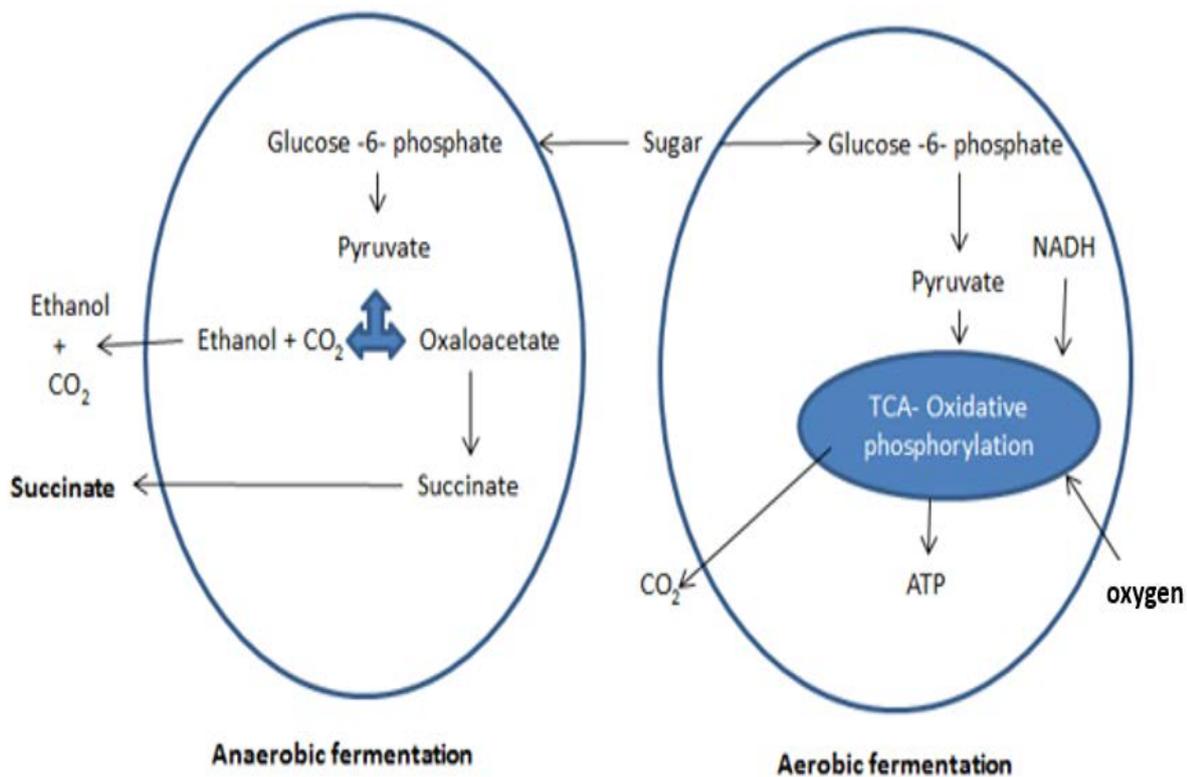


FIGURE 1

Summary of major sugar catabolic pathways in *S. cerevisiae* under aerobic versus anaerobic conditions.

cycle and energy is subsequently generated by substrate-level phosphorylation in the presence of oxygen.

However, sugar dissimilation during the anaerobic growth of yeast occurs via alcoholic fermentation that enables the re-oxidation of the NADH formed during glycolysis. Moreover, the reduction of the glycolytic dihydroxyacetone phosphate to glycerol-3-phosphate during glycolysis (in the production of glycerol) is also essential to re-oxidise the NADH formed by sugar catabolism under anaerobic conditions. Re-oxidation of NADH provides  $\text{NAD}^+$ , which enables the continuation of glycolysis in the absence of oxygen (and thus without a final electron acceptor). The redox balance is thus maintained by both ethanol and glycerol formation (Rigoulet *et al.*, 2004; Navarrete *et al.*, 2014). The glycolytic pathway is also responsible for pyruvate production. Pyruvate is a key metabolite not only in energy generation, but also as an intermediate in many other yeast metabolic pathways, including anabolic pathways involved in biomass formation (Zhu *et al.*, 2008). Besides its role in cellular metabolism, it is also an important organic acid, which contributes to the overall acid balance and organoleptic properties of wine.

### Glyoxylate pathway

Another pathway responsible for the replenishment of TCA intermediates such as oxaloacetate and  $\alpha$ -ketoglutarate is referred to as the glyoxylate cycle (Fig. 2), which is most active when yeast oxidises acetate (Lee *et al.*, 2011). This pathway is essential for the continuous flow of carbon through the TCA cycle (Servi, 1990), because when intermediates of the TCA cycle are withdrawn for anabolic reactions, the cycle is replenished by the glyoxylate cycle (Wendisch *et al.*, 2006). The enzymes of the TCA cycle

and the glyoxylate cycle are physically segregated, with the glyoxylate cycle enzymes of yeast and fungi localised in a specialised organelle called the glyoxysome/peroxisome (Donnelly *et al.*, 1998). Glyoxysomes import fatty acids and aspartate, which present acetyl-CoA to the shunt. During this process, aspartate transaminase converts aspartate into oxaloacetate, permitting the incorporation of acetyl CoA into citrate via citrate synthase (Pronk *et al.*, 1996). However, the maintenance of the glyoxylate pathway is mostly controlled by the oxidation of succinate to oxaloacetate, which can be converted back to aspartate by aspartate transaminase (Popov *et al.*, 2005). When the glyoxylate pathway is active, it bypasses some reactions of the TCA cycle in which  $\text{CO}_2$  is released, thus conserving four-carbon compounds responsible for the further biosynthesis of other metabolites, such as organic acids. (Songa & Lee, 2006). While this pathway is fully active primarily under respiratory conditions, parts of it play important roles during fermentation and act as a source of organic acids, such as succinic acid (Heerde & Radler, 1978).

### TCA cycle

The tricarboxylic acid (TCA) cycle is directly involved in the formation of most intermediate carboxylic acids, including succinic acid. Under aerobic conditions, the main function of the TCA cycle is the reduction of the coenzymes that are necessary for the full operation of the respiratory electron transport chain (Ferne *et al.*, 2004). Its role in anaerobic conditions was understated in the past, but proof of the TCA cycle's importance in anaerobic fermentation was provided by showing that all of its enzymes are present within anaerobically grown yeast cells (Kuyper *et al.*, 2004). Under

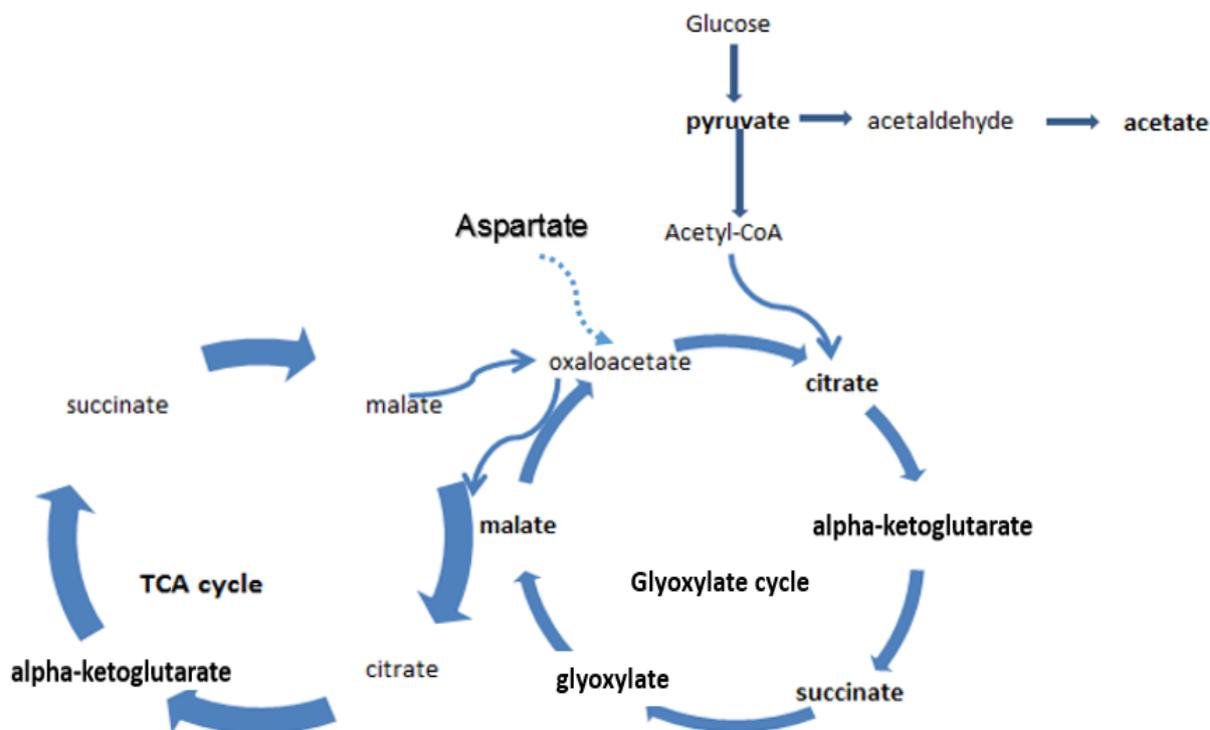


FIGURE 2

A simplified pathway diagram showing yeast-derived acids and their connection to the TCA and glyoxylate cycles.

anaerobic conditions, however, the TCA pathway more frequently operates in a branched manner, with a reductive arm working in the reverse direction of the normal cycle and leading to the formation of succinate, and an oxidative arm leading to the formation of  $\alpha$ -ketoglutarate (Tu *et al.*, 2005).

The TCA cycle is in large part responsible for citrate, malate and succinate production (Heerde & Radler 1978; Albers *et al.*, 1996). While citric acid and malic acid depend mostly on TCA cycle reactions, succinic acid can be formed in yeast via four main pathways, including amino acid catabolism, depending on the growth conditions and the availability of nitrogen sources in the culture media (Cartledge, 1987; Finley *et al.*, 2012). Under fermentative conditions, the TCA cycle operates in a branched manner, with a reductive branch leading to succinate formation and the oxidative branch leading to  $\alpha$ -ketoglutarate. However, the flux through these pathways depends on nitrogen availability, since  $\alpha$ -ketoglutarate is the primary ammonium-fixing compound. The reductive branch of the TCA cycle is the principle metabolic pathway for succinate formation under anaerobic conditions, particularly in the absence of glutamate. In contrast, glutamate is responsible for the production of succinate via the oxidative arm of the TCA cycle. Pyruvate and aspartate also play an important role in the formation of succinic acid *via* the reductive branch of the cycle, or from pyruvate *via* the oxidative branch.

#### Nitrogen metabolism as another source of organic acids

Nitrogen availability has a noteworthy (although indirect) impact on organic acids formed *via* the TCA cycle. For instance, glutamate (a preferred amino acid) can lead to the production of succinic acid *via* the deamination of glutamate to  $\alpha$ -ketoglutarate (Sanborn *et al.*, 1979) (Fig. 3). In a study by Alberts *et al.* (1994), the assimilation of 3-<sup>13</sup>C glutamate led to significant succinate concentrations that were labelled at the second and third carbon positions. The results prove that 3-<sup>13</sup>C glutamate was deaminated to 3-<sup>13</sup>C  $\alpha$ -ketoglutarate, which was then oxidatively decarboxylated to succinyl CoA and succinate. In their observations, Alberts *et al.* (1994) found that 17% of the carbon from glutamate was converted to succinate, which further supports succinate synthesis from glutamate. When glutamate is used as a nitrogen source, the reduced synthesis of 2-oxoglutarate from glucose causes fewer reducing equivalents to be formed, which often reduces the production of glycerol and ethanol (Camarasa *et al.*, 2003). Consequently, a metabolic flux (shift) towards organic acid synthesis might occur.

#### Acetic acid metabolism

The yeast biochemical pathways, as well as the individual enzymatic reactions, involved in acetic acid formation during fermentation are reasonably well characterised (Jost & Piendl, 1975). They include (1) the reversible formation from acetyl Co-A and acetyl adenylate through acetyl Co-A synthetase; (2) the cleavage of citrate by citrate lyase; (3) the production of pyruvate by pyruvate dehydrogenase (PDH), yielding acetyl Co-A, which can be hydrolysed into acetate through acetyl Co-A hydrolase; (4) the reversible formation of acetyl-phosphate by acetyl kinase; (5) and the oxidation of acetaldehyde by aldehyde dehydrogenase (Boulton *et al.*,

1996; Ribéreau-Gayon *et al.*, 2006). In wine, acetic acid production is mainly due to the latter enzymatic oxidation of acetaldehyde to acetate by acetaldehyde dehydrogenases (Remize *et al.*, 2000).

Under fermentative conditions, acetic acid production is also linked to glycerol formation via redox balancing (Remize *et al.*, 1999; Eglinton *et al.*, 2002; Goold *et al.* 2017). Glycerol is formed in order to reoxidise the NADH formed during glycolysis (Jackson, 2008), and *S. cerevisiae* may convert NAD<sup>+</sup> back to NADH through acetic acid production. Alterations in glycerol metabolism, such as increased glycerol production, are generally accompanied by an increase in acetic acid production to compensate for changes in the cellular redox balance (De Barros Lopes *et al.*, 2000; Prior *et al.*, 2000; Goold *et al.*, 2017).

#### Factors affecting organic acid production in wine

The sensor systems of the yeast cell act to identify variations in environmental conditions (osmolarity, temperature, pH, nitrogen and carbon starvation, chemical and physical agents, etc.). The responses to the sensing of changes in conditions are set in motion by a series of signal transduction pathways, which result in changes to gene expression networks, synthesis of protective molecules and/or modulation of protein activity by post-translational modifications or sub-cellular localisation (Estruch, 2000). The downstream effects of the regulatory responses to these physicochemical factors will, in most cases, have an impact on the primary and secondary metabolism of the yeast, thus affecting the quality and organoleptic characters of the wines produced. These environmental and compositional factors are thus important focus areas in wine research due to their impact on the attributes and perceived quality of the final product. Several influential wine-relevant environmental and nutritional factors that require extensive investigation, as indicated in the current review, include temperature, aeration, pH, nitrogen levels, osmotic stress and vitamins. Furthermore, the effect of individual strains with different genetic backgrounds on organic acid production is also an important area to consider.

#### Effect of pH on organic acid production by yeast

The large majority of wines have pH values between 3 and 4. As with sugars, the pH level will vary according to the ripeness of the grapes, with increasing ripeness leading to lower acidity and increased pH. For white wines, winemakers often recommend pH levels between 3.1 and 3.2, and if the pH is too high (> pH 3.4), it may be a sign that the grapes are overripe. It is generally known that the activity of enzymes involved in central carbon metabolism is pH sensitive, which could account for the pH-related changes in the production levels of several organic acids in wine (Agarwal *et al.*, 2007). For this reason, the mono-factorial impacts of pH on organic acid production have been studied, and several authors have presented significant variation effects of pH on yeast and bacterial strains in terms of organic acid profiles. The impact of pH on organic acid productivity is often related to the specific strain employed, which may account for the different trends observed in different studies. For example, high succinic acid levels have been linked to an increased

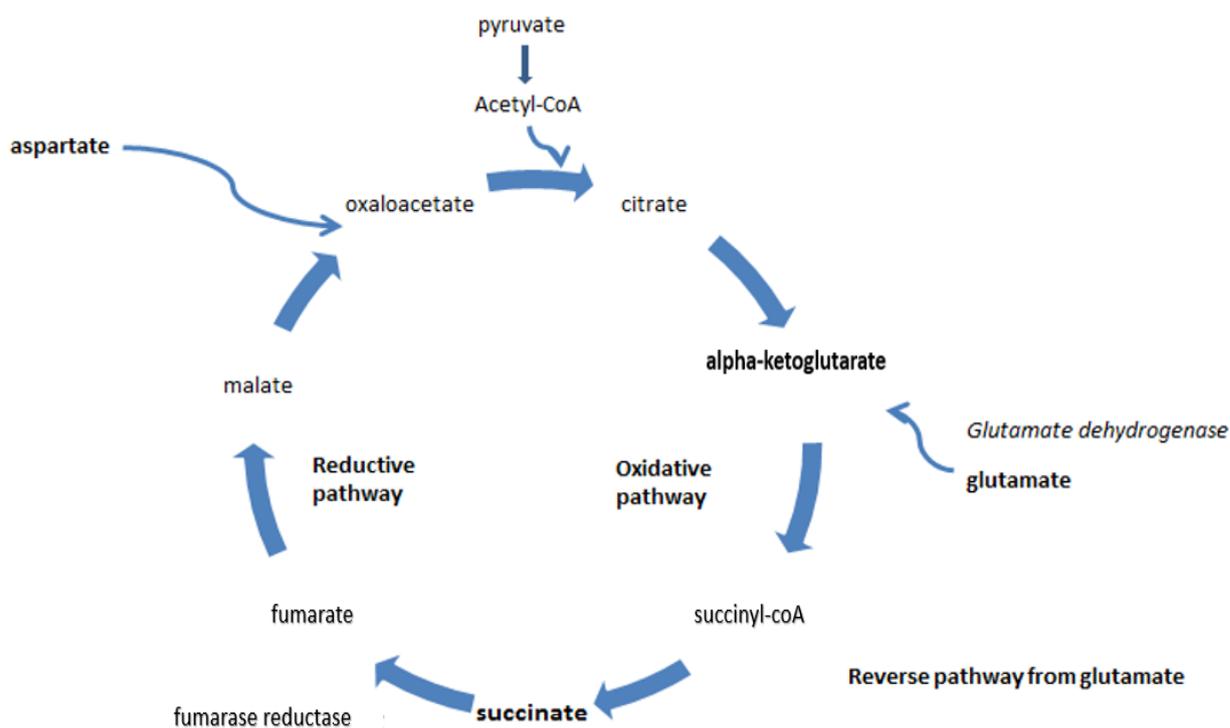


FIGURE 3

Summary of the main pathways involved in succinic acid production/utilisation during anaerobic fermentation.

initial fermentation pH (Thoukiss *et al.*, 1965). The authors observed a slight increase in succinic acid by *S. cerevisiae* when the grape must was adjusted to pH values ranging from 3.0 to 3.8, but more when it was raised from 3.8 to 4.8. The study also indicated a strong direct relationship between glycerol and succinic acid formation at a higher initial pH of the must. Recently, Liu *et al.* (2015) reported that lower initial pH (2.75) showed properties of prolonged yeast lag phase, increased acetic acid levels, and decreased final content of succinic acid when three *S. cerevisiae* strains were tested under winemaking conditions. Several authors have also noted a significant increase in pyruvic acid levels when fermentation pH was increased (Graham *et al.*, 1979). Rankine (1967) also showed that yeast strain variability and pH appeared to be the two most influential factors affecting the pyruvic acid content of wines. In this study, however, pyruvic acid production only showed pH-dependent trends for some of the strains evaluated.

#### Effect of aeration on organic acid production by yeast

The fermenting must is easily saturated in oxygen (6 to 8 mg/L) at cellar temperature (Du Toit *et al.*, 2006). Several techniques are available to provide at least limited amounts of oxygen during the fermentation process, including pumping-over and micro-oxygenation. Pumping-over is not recommended in white winemaking because of oxidation concerns; however, oxygen can be introduced during the pressing of whole clusters, transfer from tank to tank, filtration, racking, centrifugation, bottling and barrel ageing (Cheynier, 1993). According to Saa *et al.* (2012), for oxygen solubility in fermentation media at 25°C (*i.e.* about 7 mg/L), 15% oxygen saturation (with air) would be equivalent to

around 1 mg/L of molecular oxygen. Aeration has been shown to play a fundamental role in the production of many secondary metabolites. For example, the level of citric acid production by yeast as well as other fungi was previously shown to be strongly dependent on the oxygen availability in the fermentation vessel (Sakurai *et al.*, 1996). Sufficient oxygen supply is extremely important during citric acid metabolism, since even short interruptions in oxygen supply can result in a complete loss of citric acid formation (Yigitoglu, 1992; Rywińska *et al.*, 2012). Previously, Rehm *et al.* (1980) indicated that the formation rate of citric acid strongly coincides with the ratio between ATP and ADP at various pH values. The authors also indicated that the energy generated by substrate-level phosphorylation is used to promote the excretion of organic acids (in particular citric acid) by the pH-dependent active transport system.

In many cases, aeration during fermentation might increase the production of several secondary metabolites, such as succinic, pyruvic and acetic acid. This behaviour has been correlated with the increase in the levels or activity of the TCA enzymes involved in the biosynthesis of these secondary metabolites during aeration, of which succinic acid is an intermediate (Boulton *et al.*, 1996). The synthesis of succinic acid has also previously been tested in apple and grape juices. For instance, when *Saccharomyces cerevisiae* EC1118 was assessed in white wine fermentations, acetic acid was produced only under conditions of strict anaerobiosis (0.3 C-mmol g/DW/h). On the other hand, a striking and significant increase in the level of succinic acid production occurred between 1.2 and 2.7 µM dissolved oxygen conditions (from 0.02 to 0.27 C-mmol g/DW/h) (Aceituno *et al.*, 2012). In another study (Estela-Escalante

*et al.*, 2012), compounds of sensory importance produced by *Saccharomyces cerevisiae* RIVE V 15-1-416 cultivated in apple juice at 28°C in static and agitated cultivation (200 rpm) were analysed. At the end of fermentation, the authors reported an increase (0.77 g/L under static and 1.32 g/L under agitate cultivation) in succinic acid levels under agitated cultivation, whereas acetic acid levels decreased (51.0 g/L under agitated and 266 g/L under static cultivation). Recently, oxygenation levels in wine were assayed with *M. pulcherrima* CECT12841 and *S. cerevisiae* EC1118 containing 10% or 25% air (maximum dissolved oxygen levels around 0.7 and 1.7 mg/L respectively). A negative correlation was found between air concentration and ethanol yield, while a positive correlation was confirmed between acetic acid yield and oxygenation level (Morales *et al.*, 2015).

### The impact of temperature on organic acid production by yeast

Wine fermentations are generally conducted across a wide range of temperatures, with red wine fermentation being carried out at higher temperatures (18°C to 25°C) and white wine fermentation at lower temperatures (10°C to 15°C). The higher fermentation temperature in red wines is essential for the extraction of anthocyanins and other non-volatile compounds from the grape skins during fermentation, while lower temperatures are recommended for the retention of fruity volatile compounds and the prevention of undesirable volatile flavour compounds in white wines (Lambrechts & Pretorius, 2000; Styger *et al.*, 2011; Fairbairn *et al.*, 2014).

Torija *et al.* (2003) observed temperature effects on the growth of strains of *S. cerevisiae* in grape must and noted a significant increase in succinic and acetic acid as fermentation temperature increased. Aragon *et al.* (1998) investigated the influence of yeast type and fermentation temperature on organic acid content and observed that wines fermented at 18°C showed lower succinic acid content compared to those fermented at 21°C. Significant differences were also observed for acetic acid and citric acid concentrations. Taing and Taing (2007) found that temperature (optimum at 25°C) increased succinic and malic acid levels in high-sugar fermented food. Apart from wine, temperature-dependent production/consumption profiles of lactic, succinic, malic and acetic acid were previously observed in rice wine (Liu *et al.*, 2014).

Data also suggest that the intrinsic ability of yeast strains to grow at different temperatures appears to be another influential element in terms of temperature-dependent succinic acid production during fermentation. Castellari *et al.* (1994) showed that mesophilic strains AWIR 796 and *Endoferm* M2 were average producers of succinic acid, but cryo-tolerant strains of *S. bayanus* produced an additional 0.8 g/L of succinic acid. The study concluded that succinic acid production was strain dependent. The same study also showed variations in acetic acid production among the wine yeast strains tested.

### Effect of vitamins on organic acid production by yeast

According to the United States Department of Agriculture (National Nutrient Database for Standard Reference

Release 28), 100 grams of grapes contain about 5.4 mg of vitamin C, 0.09 mg of vitamin B1, 0.2 mg of vitamin B2, 0.08 mg of vitamin B6, 0.70 mg of vitamin E and 0.2 mg of niacin. Vitamins commonly required by yeast include the following: Biotin (which serves as a cofactor in carboxylase-catalysed reactions), pantothenic acid (the functional group of coenzyme A, which is involved in acetylation reactions), nicotinic acid (in the form of nicotinamide, which is involved in redox reactions) and thiamine (in the form of thiamine pyrophosphate, which is involved in decarboxylation reactions) (Walker, 1998). Organic co-factors, such as vitamins, are required for the enzymatic complexes of several intermediates such as organic acids (Tu *et al.*, 2005). The study of Ribéreau-Gayon *et al.* (1956) demonstrated that the absence of individual vitamins such as thiamine, biotin and pantothenate in synthetic medium significantly reduced succinic acid concentrations in wine, whilst increasing acetic acid and ethanol yields. In addition, a vitamin, nicotinic acid, was the limiting factor for lactic acid production during fermentation with the K1-LDH strain in batch conditions (Colombie & Sablayrolles, 2004).

### Effect of nitrogen on organic acid production by yeast

The range of yeast available nitrogen (YAN, mainly ammonium and amino acids, with the exception of proline) recommended by oenologists varies from 150 mg/l YAN (Weeks & Henschke, 2013) to 400 mg of nitrogen per litre (Ugliano *et al.*, 2007). However, Zoecklein (1998) showed that maximum fermentation rates can be achieved with YAN in the 400 to 500 mg N/L range, although most winemakers do not prefer higher nitrogen concentrations due to the impact that they can have on wine aroma. According to Bisson & Butzke (2000), in order to successfully complete fermentations, optimal nitrogen levels at harvest should be part of viticulture considerations, hence sugar-nitrogen balancing is also an important aspect of fermentation. For this reason, the UC Davis Department of Viticulture and Enology made recommendations to yeast and nutrient manufacturers based on harvest Brix level (a measure of must sugar). They recommended that, at a Brix level of 21 to 27°Brix, about 200 to 350 mg N/L should be made available in order to successfully complete fermentation.

The nitrogen content of yeast cells is approximately 10% of cellular dry weight (Walker, 1998). Although yeast is unable to fix molecular nitrogen, simple inorganic nitrogen sources such as ammonium salts (e.g. ammonium sulphate) can be utilised effectively (Matthews & Web, 1991; Walker & Stewart, 2016). Yeast can also utilise nitrate and a variety of organic nitrogen compounds such as amino acids, peptides, purines, pyrimidines and amine as nitrogen sources (Webb & Lee, 1990). Insufficient nitrogen during fermentation is one of the biggest challenges faced by yeast under fermentative conditions. Although grape must contains a broad variety of nitrogen sources, such as amino acids and ammonia, sluggish or stuck fermentations are often observed when these nitrogen sources are limited or depleted before the end of fermentation (Bisson, 1999; Sturgeon *et al.*, 2013).

Must nitrogen content influences organic acid production in wine in many ways *via* mechanisms that have only been partially elucidated. According to Remize *et al.*

(2000), the relationship between the initial nitrogen content (ammonium sulphate) and organic acids such as acetic acid in *Saccharomyces cerevisiae* during fermentation is very complex. The effect of the addition of yeast assimilable nitrogen indeed has shown an inverse relationship with acetic acid production in the initial stages of fermentation, but not in the later stages of fermentation.

The impact of different nitrogen sources on the general metabolism of *S. cerevisiae* has also been evaluated in relative depth. Camarasa *et al.* (2003) showed that glutamic acid has a large influence on the formation and production of  $\alpha$ -ketoglutaric acid, succinic acid and acetic acid. Increased concentrations of these compounds were produced during fermentation when glutamate was used as a nitrogen source as compared to ammonium and other amino acids. In a different study, increased *S. cerevisiae* growth and succinic acid production by *S. cerevisiae* were observed at increased nitrogen levels (up to 500 mg/L). However, increases in nitrogen above the 500 mg/L level had little additional impact on the production of succinic acid and acetic acid (Heerde & Radler, 1978). Vilanova *et al.* (2007) also revealed that ammonium supplementation increases the final concentration of L-malic acid, acetic acid and glycerol in wine. Furthermore, higher amounts of proline in cultivars such as Chardonnay, Riesling and Sauvignon blanc were reportedly responsible for higher succinic acid production in the wine (Batch *et al.*, 2009).

The availability of nitrogen sources is central to the utilisation of aspartate and glutamate *via* the reductive and oxidative arms of the TCA cycle respectively (Camarasa *et al.*, 2003). Several authors have reported an increase in succinic acid production when growth media contained glutamate and aspartate as the major nitrogen sources for yeast (Roustan & Sablayrolles, 2002). Agarwal *et al.* (2007) also investigated the effect of nitrogen sources (among others peptone, urea, tryptone and ammonium sulphate) on enzymes involved in succinic acid production. Supplying tryptone as a nitrogen source resulted in the elevated formation of succinic acid (3.8 g/L), as the activities of enzymes involved in succinic acid production were found to have increased. Among the inorganic nitrogen sources tested, ammonium hydrogen phosphate yielded a maximum of 2.43 g/L of succinic acid.

#### **Effect of sugar on organic acid production by yeast**

Under normal circumstances, viticulturists prefer to pick the grapes when the sugars are in specific ranges, depending on the varietal and the style of wine that is targeted. Most wines are harvested at sugar levels of between 190 and 250 g/L, with the sugar composed of similar amounts of glucose and fructose (Boulton *et al.*, 1995). *Saccharomyces* usually converts 95% of the sugar to ethanol and carbon dioxide, while 1% is converted to cellular material and the remaining 4% is converted to other end products such as organic acids, higher alcohols and esters and, to a lesser extent, aldehydes (Rapp, 1998). However, there are other end products, such as acetaldehyde, acetic acid, ethyl acetate, higher alcohols and diacetyl, which may be regarded as undesirable when present in excess concentrations.

Factors that affect the production of some organic acids, and in particular of acetic acid, have been studied extensively

(Usseglio-Tomasset, 1995; Bisson, 1999; Bely *et al.*, 2005; Ferreira *et al.*, 2006). In very high-sugar Riesling ice wine juices (over 400 g/L), alcoholic fermentation tends to result in very low ethanol yields and high acetate production (Caridi, 2003). Pigeau *et al.* (2007) also noted that increasing ice wine juice sugar concentration from 40 to 46°Brix decreased yeast growth, sugar consumption rate, the total amount of sugar consumed and the total amount of ethanol produced. However, acetic acid levels increased from 0.11 g/L (at 40°Brix) to 0.21 g/L (at 46°Brix). A reasonable hypothesis for these findings could be the increased osmotic stress imposed by higher initial sugar contents, leading to higher initial glycerol formation and thus indirectly to acetic acid formation due to redox balancing (Erasmus *et al.*, 2004). Based on these observations, the influence of sugar levels on organic acid production appears to be significant, although no conclusive trends have been established.

#### **Effect of yeast strain on organic acid production in wine**

Numerous studies have provided insights into the response of individual strains to the grape must of specific cultivars and their impact on the sensory and chemical characteristics of the wines produced.

In the case of *S. cerevisiae*, many papers have reported on the influence of yeast strain on wine composition in general (Delfini *et al.*, 1994, Galletti *et al.*, 1996; Kunicka-Styczyńska & Pogorzelski, 2009, Chidi *et al.*, 2015). Aragon *et al.* (1998) specifically addressed the influence of yeast strain on the organic acid composition of wine. The results showed significant differences in acetic, citric and succinic acid production for different yeast strains. Patel & Shibamoto (2003) investigated a total of 18 *S. cerevisiae* yeast strains for the production of volatile acidity, primarily acetic acid. Of all the strains, the composition of the volatile acidity produced by the *A350/VL1/Fermiblanc* and *T73* yeast strains were significantly different from the other strains and contributed significantly to the final characteristic flavours in the corresponding wines. Previously, Kunicka-Styczyńska & Pogorzelski. (2009) also observed significant variations in pyruvic and acetic acid levels when different *Saccharomyces* species were assessed in apple wines. Although there are many other factors that directly/indirectly affect acid degradation/evolution in wine, strain identity seems to be one of the major drivers. Recently, wine strain identity was also regarded as the main factor controlling the organic acid profiles of red and white wine in South Africa (Chidi *et al.*, 2015).

A diversity of yeast species within the genera *Hanseniaspora*, *Pichia*, *Kluyveromyces*, *Candida* and *Saccharomyces* have long been known to be present in freshly crushed grape juice and in the early stages of fermentation (Bisson, 1993). However, there is very little information on how these yeast species affect organic acid production in wine.

#### **Impact of osmotic stress on major fermentation products of yeast**

The ability of wine yeast to carry out alcoholic fermentation under winemaking conditions is largely influenced by its response to the stress conditions such as osmotic stress

(Carrasco-Portugal *et al.*, 2001). Different mechanisms have been developed by the fermenting strains to triumph over these adverse situations. A clear understanding of these mechanisms is essential to improve the overall fermentation process and thus improve the quality of wines (Ivorra *et al.*, 2000).

During osmotic stress, glycerol is produced in response to high sugar levels in the must, resulting in excess NAD<sup>+</sup>. To counterbalance the high levels of NAD<sup>+</sup> production, NADH is regenerated by converting acetaldehyde to acetic acid (Caridi *et al.*, 2003). Glycerol metabolism thus plays an essential role in fermentation, not only as an osmo-protectant, but also by aiding the equilibration of the intracellular redox balance (Romano & Suzzi, 1993; Jain, 2010). Beney & Gervais (2001) also found that the resistance of *S. cerevisiae* to high osmotic stress improved at lower temperatures compared to warmer conditions, but a link to organic acid levels was not established. Galafassi *et al.* (2013) showed that osmotic stress is responsible for the production and accumulation of intracellular glycerol in the wine yeast *Dekkera bruxellensis*. Higher expression of NADH-dependent glycerol-3-phosphate dehydrogenase (GPD) activity was observed as a result. The effect of hyperosmotic stress on acetic acid levels was recently demonstrated in ice wines (high-sugar must) (Yang *et al.*, 2017). The authors showed a direct relationship between hyperosmotic stress and the catalysis of NAD<sup>+</sup>-dependent aldehyde dehydrogenase(s) (Aldp(s)), which eventually leads to higher acetic acid production in wines.

Although the current review focuses much attention on several factors that do or may affect organic acid degradation or evolution in wine, very little literature is available on how multiple changes in two or more parameters may affect wine acidity. This is not surprising, since multifactorial experiments are not easy to interpret. Notwithstanding these obstacles, more work is required to investigate the influences of individual and/or multiple changes in wine yeast strains, fermentation pH, sugar and temperature on the acid profiles of different wines.

### The importance of “omics” tools in wine research

#### **Transcriptomic and proteomic approaches in yeast research**

Transcriptome and proteome profiles for several wine yeast strains have been established previously under winemaking conditions (Rossouw *et al.*, 2008, Gomez-Pastor *et al.*, 2010), and many studies have paid special attention to the intrinsic genetic and regulatory pathways that are actively involved in wine fermentation (Erasmus *et al.*, 2003; Rossignol *et al.*, 2003; Varela *et al.*, 2005; Mendes-Ferreira *et al.*, 2007; Marks *et al.*, 2008; Pizarro *et al.*, 2008; Barbosa *et al.*, 2015). A global analysis of gene expression and protein profiles plays an important role in increasing our understanding of how yeast cells adapt to environmental changes, and how their response to environmental conditions affects cellular metabolism and the production of primary and secondary compounds important to wine quality.

Wine fermentation is a process during which yeast must adapt to the significant changes that occur during vinification (Zuzuarregui *et al.*, 2006). At the end of the biological information-transfer system (from genome, to transcriptome, to proteome), the post-translational modifications at the

protein level and/or protein activity play an essential role in further adjustments of the cell to these changing external conditions. Efforts have been made in recent years to investigate gene expression profiles during vinification. The detailed analysis of subsets of genes, transcriptional regulation and gene expression profiles in yeast under winemaking conditions has been the focus of several studies (Puig & Perez, 2000; Rossignol *et al.*, 2006; Rossouw *et al.*, 2008; Margalef-Català *et al.*, 2016).

Previously, commercial wine strains were screened and selected on the basis of desired physiological traits for optimised fermentation performance, but this was not accompanied by knowledge with regard to the molecular basis for the fermentation phenotypes of these selected strains (Aranda & Del Olmo, 2004). The study of Zuzuarregui *et al.* (2006) offered the first proteomic and transcriptomic comparisons involving two commercial strains (*ICV 16* and *ICV 27*) with different fermentative performances. Since then, several other combinatorial transcriptomic-proteomic studies of wine yeast have been carried out (Rossignol *et al.*, 2003; Gomez-Pastor *et al.*, 2010; Orellana *et al.*, 2014). The use of these global analysis strategies has made it possible to analyse variations in gene expression and protein levels at different time points during vinification, and have greatly enhanced our understanding of yeast metabolism during fermentation (Marks *et al.*, 2003). However, and to our knowledge, such approaches have not yet been applied with regard to the genetic regulation of organic acid production. The potential thus exists to utilise these tools with a specific focus on organic acid metabolism in different yeast strains to gain new insights into the regulation of acid metabolism on a molecular level.

### Metabolomics of yeast

The major goal of systems biology is to acquire an overall quantitative description of systems that occur inside the cell. It is a challenging task, as the components and interactions involved in these cellular systems are both numerous and complex (Cakir *et al.*, 2006). Although transcriptome data supplies an overview of the broad expression patterns and regulation of genes involved in metabolism, understanding functional cellular physiology requires metabolomic data to complete the systems picture (Nielsen, 2003).

The metabolomics of wine have been studied extensively over the past few years (Son *et al.*, 2009; Van Dorsten *et al.*, 2009; Cuadros *et al.*, 2010; Orellana *et al.*, 2014). Cuadros *et al.* (2010) provided data demonstrating that unbiased and objective analytical chemistry, in combination with multivariate statistical methods, allows reproducible classification of wine attributes such as variety, origin, vintage and quality through metabolomics studies. Son *et al.* (2009) investigated the changes in metabolites such as pyruvate, succinate, citrate, malate and tartrate in musts during alcoholic fermentation and during ageing by coupling <sup>1</sup>H NMR spectroscopy with multivariate statistical analysis. Elsewhere, the contribution of individual volatile aroma compounds to the overall volatile composition was also accomplished through the development of automated metabolomics data analysis of GC-MS profiles of wines (Schmidtke *et al.*, 2013). In addition, the analysis of wine

micro-oxygenation has also been attempted by untargeted LC-MS (Arapitsas *et al.*, 2012). Recently, Geana *et al.* (2016) successfully classified red wines using suitable markers coupled with multivariate statistical analysis.

The functional analysis of cellular metabolism and integration of metabolome data with other omics data (e.g. transcriptome data) necessitates the large-scale detection and quantification of metabolites of interest. However, a noteworthy challenge is the shortage of targeted quantitative analyses for metabolomics approaches (Nielsen, 2003). Devantier *et al.* (2005) generated a high-throughput GC-MS method for quantifying metabolites that permits semi-quantitative analysis of several metabolites in *Saccharomyces cerevisiae*. Similar metabolomics techniques could also act as valuable tools that will expand our knowledge regarding organic acid metabolism of yeast in wine.

## CONCLUSIONS

Wine acidity contributes significantly to the consumer perception of wine quality. Winemakers have always been challenged in terms of acid management and the balance between sugar and acidity in their wines (both in warm- and cool-climate viticultural regions). While researchers are currently investigating acid evolution in wine, the key issue that is often overlooked by winemakers is how individual organic acids contribute to the flavour profile and organoleptic characteristics of wines. In recent years, the development of several analytical techniques (i.e. electrophoretic, chromatographic and enzymatic) for metabolite quantification has received considerable attention in wine science. Rapid, sensitive and accurate analytical techniques for the quantification of organic acids that are present in wine provide the platform for detailed analysis of these organic acids across a range of experimental conditions. Intelligent experimental design, combined with suitable analytical techniques, form the foundation of meaningful studies of yeast acid metabolism. This will ultimately provide winemakers with a better understanding of acid development in their wines, and of adjustment or management practices in the cellar to favour desirable acid profiles. Factors that impact significantly on the production of organic acids in wine must be investigated thoroughly (with the help of complementary systems biology approaches) in order to understand the fundamental metabolic regulation underlying the evolution of acidity during fermentation.

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