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# Non-*Saccharomyces* Yeasts: Biotechnological Role for Wine Production

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Additional information is available at the end of the chapter

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## Abstract

Non-*Saccharomyces* yeasts play a substantial role in the early stages of wine fermentation. With the increase in alcohol concentration, indigenous or commercial strains of *Saccharomyces cerevisiae* take over and complete the transformation of the grape must sugars into ethanol, CO<sub>2</sub>, and other secondary metabolites. The presence of non-*Saccharomyces* during the fermentation has an impact on the wine composition, and consequently, their contribution during the fermentation process cannot be ignored. The new challenges to enhance the appeal and value of wine elaborated by traditional technology are being achieved by selecting and using autochthonous non-*Saccharomyces* and *Saccharomyces* strains that may enhance regional identity of wines. Greater understanding of yeast biochemistry and physiology is enabling the selection and development of yeast strains that have defined specific influences on process efficiency and wine quality. The aim of this chapter was to show the different aspects of non-*Saccharomyces* species that may play a positive incidence in the biotechnological process to conduct to wine elaboration.

**Keywords:** non-*Saccharomyces* yeast, wine production, aroma, enzymes, mixed culture

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## 1. Introduction

Since 1866 when Louis Pasteur first elucidated the bioconversion of grape juice into wine, this complex biochemical process and the role of the yeast therein have been studied continuously. *Saccharomyces cerevisiae* has often been the wine yeast that has received the most attention in the wine elaboration. This yeast is not only related with the conversion of grape sugar into

alcohol and CO<sub>2</sub> in the fermentation process which also plays an important role in the generation of secondary metabolites, just as the production of varietal wine aromas from grape aroma precursors [1]. The spontaneous fermentation of the wine is produced by a mixture of yeast species simultaneously present in the grape must [2]. Although the control of *S. cerevisiae* (inoculated or native) in the fermentation is predicted and desired, the presence of indigenous non-*Saccharomyces* yeasts in the must leads to a competitive situation for the nutrients contained in the must. These non-*Saccharomyces* yeasts are perfectly adapted to the specific environment and frequently are in greater number of *S. cerevisiae* [3].

Yeast and bacterial microbial dynamics have been studied since the 1970s [4]. Using classical methods, microbial count and diversity is determined by employing synthetic culture media containing agar. The biodiversity in the complex ecosystems is impossible to characterize with precision using classical microbiological culture-dependent methods. When using enrichment methods and growth on culture medium, the microbiota naturally present in the sample are open to undergo important changes because of the ability of the certain species take control of the system and overcome other microbial components [5]. Due to this, populations less numerous or sensitive to the stress conditions are hard to find again and identify. Therefore, the use of culture-dependant methods could cause a misidentification of the microbial ecology of complex ecosystems [6].

Since the end of the 1990s, molecular techniques have helped provide a good overview of microbial ecology. These methods, generally named culture-independent methods, are used for the identification of microorganisms directly in the system through the study of their DNA and RNA without the need for isolation and cultivation. There are several advantages of the direct characterization of wine microbial DNA against to culture-dependent methods. Firstly, not all microbial populations are able to grow in enrichment media due to injury, lack of appropriate nutrients, or presence of viable but not culturable states. Secondly, the direct analyses allow saving time in comparison with the enrichment methods. This advantage could enable winemakers to use microbial detection data during the fermentation process, being able to anticipate possible spoilage problems in the wine. Furthermore, the DNA-based studies allow processing a larger numbers of samples than plating methods [7].

Nevertheless, in the past few years, successful culture-independent methods such as denaturing gradient gel electrophoresis (DGGE), real-time PCR, fluorescence in situ hybridization (FISH), or Fourier transform infrared spectroscopy (FT-IR) have been described. The PCR-DGGE method was first developed for the study of the microbial ecology in the environmental samples but soon found application in food microbiology [8]. This method is based on the separation of same length DNA fragments, but of different sequences. PCR-DGGE method has reported detection limits between 10<sup>2</sup> CFU/mL in pure cultures and 10<sup>4</sup> CFU/mL in wine or must samples [9].

In recent years, scientists have used real-time quantitative PCR (QPCR) to detect and quantify microorganisms in different alimentary environments [10]. The advantages of QPCR are the low detection level, often as low as one cell per mL, the speed by which assays are performed, and the ability to quantify yeasts present following alcoholic fermentation.

Fluorescence in situ hybridization (FISH) is a very promising technique for wine ecology studies for its simplicity and rapidity as well as the ability to observe the cell morphology by a microscope and the high sensitivity obtained using a flow cytometer [11].

Fourier transform infrared (FT-IR) spectroscopy is used to identify isolates according to the different components of the cell [12]. The relative success of this method is directly dependent on the complexity within a reference spectral library, identification results on genus and species level. Due to high automation and cost efficiency, this high-throughput method gives much deeper insights into functional yeast diversity during wine production.

Fortunately, DNA-based approaches have largely helped to clarify modern taxonomy. DNA sequence analysis is now widely used in the identification and classification of yeasts and other fungi therefore helping to reassign species within genus level because of the new species that are now discovered [13]. In food and beverage industry, these name changes influence our ability to notify the identity of spoilage microorganisms and to be in accordance with the regulations governing the presence of certain microorganisms in this industry. Current taxonomies recognize 149 yeast genera comprising nearly 1500 species [14]. Of these, more than 40 species have been isolated from grape must [15].

## 2. Use of non-*Saccharomyces* yeasts in the wine production

Non-*Saccharomyces* yeasts were originally seen as responsible for microbial spoilage in wine production due to their isolation from spoiled wines [16]. Traditionally, the use of non-*Saccharomyces* in the wine elaboration has not been usual due to preceding investigations showed that several species produce high levels of undesirable compounds that affect the wine quality such as acetoin, ethyl acetate, acetic acid, and acetaldehyde [17]. Unfortunately, this exclusion of non-*Saccharomyces* yeasts from the fermentation process may result in a loss of complexity and wines lacking distinctive characteristics.

The initial belief that all non-*Saccharomyces* yeasts died soon after the commencement of an alcoholic fermentation due to the rising ethanol concentration and added SO<sub>2</sub> has not been sustained by later research [18]. Currently, the cellar technology and hygiene in modern cellars have improved greatly; as a consequence of this, the use of SO<sub>2</sub> has been significantly reduced. For these reasons, the survival of a higher number and diversity of non-*Saccharomyces* yeasts has increased. The great number of non-*Saccharomyces* yeasts reported in recent literature has also been influenced for the use of modern laboratory techniques that have made the detection of non-*Saccharomyces* yeasts easier [19].

During fermentation, and more evident in spontaneous fermentations, which lack the initial high-density inoculum of *S. cerevisiae*, there is a sequential succession of yeasts. Initially, species of *Hanseniaspora* (*Kloeckera*), *Rhodotorula*, *Issatchenkia*, *Pichia*, *Debaryomyces*, *Zygosaccharomyces*, *Torulaspota*, *Schizosaccharomyces*, *Candida*, *Metschnikowia*, and *Cryptococcus* are found at low levels in fresh must [20]. Of these, the most common yeast present in the highest numbers is *Hanseniaspora uvarum*, followed by different *Candida* spp. This is normally more

evident in red must than white, probably as a result of the higher pH in red wine. However, *Hanseniaspora* may sometimes be absent or present at low levels [15].

Despite the sustained presence of certain non-*Saccharomyces* yeasts, the majority are not possible to recovery on plates during the early stages of a vigorous fermentation. This might be due to their slow growth and inhibition by the combined effects of SO<sub>2</sub>, pH, increase in ethanol and oxygen deficiency [21]. This is consistent with their oxidative or weak fermentative metabolism. Nutrient limitation and size or dominance of *S. cerevisiae* inoculum can also have a suppressive effect, sometimes separate from temperature or ethanol concentration [22]. It has been reported that *Torulaspota delbrueckii* and *Lachancea thermotolerans* are less tolerant to low oxygen levels, and this, rather than ethanol toxicity, affects their growth and leads to their death during fermentation [23]. It was also shown that a cell–cell contact mechanism in the presence of high concentrations of viable *S. cerevisiae* yeasts played a role in the inhibition of these two non-*Saccharomyces* species [24]. But these mechanisms are not corroborated by Pérez-Nevado et al. [25] and Wang et al. [26]. Both authors were able to show that some metabolites produced by *S. cerevisiae* may be the responsible of the inhibitory effect on the growth of non-*Saccharomyces* wine yeasts. Recently, Branco et al. [27] showed that one derived peptide of the GAPDH could be the responsible of this inhibitory effect.

Species	Metabolites and/or physical properties	References
<i>Candida stellata</i> or <i>Starmerella bacillaris</i> (formerly <i>Candida zemplinina</i> )	Acetic acid	[9, 35, 42, 43]
	Dodecanoic acid	
	Ethyl octanoate	
	Glycerol	
	Higher alcohols	
	2-Methyl propanoic acid	
	Succinic acid	
	Terpenoids	
<i>Schwanniomyces vanriijiae</i> (formerly <i>Debaryomyces vanriiji</i> )	Esters	[44]
	Medium-chain fatty acids	
	Terpenoids	
<i>Hanseniaspora uvarum</i>	Acetate and ethyl esters	[9, 47]
	Sulfur compounds	
	Higher alcohols	
	Medium-chain fatty acids	
<i>Hanseniaspora vineae</i>	2-Phenylethyl acetate	[48]
<i>Hanseniaspora guilliermondii</i>	Acetate and ethyl esters	[47]
	Acetone	
	Heavy sulfur compounds	
<i>Hanseniaspora osmophila</i>	Ethyl acetate	[49, 50]

Species	Metabolites and/or physical properties	References
	Ethyl lactate	
<i>Wickerhamomyces anomalus</i> (formerly <i>Pichia anomala</i> )	Acetate and ethyl esters Higher alcohols	[37, 50, 51]
<i>Issatchenkia orientalis</i>	Color	[41]
	Methanol	
<i>Lachancea thermotolerans</i> (formerly <i>Kluyveromyces thermotolerans</i> )	Glycerol Lactic acid 2-Phenylethanol	[17, 38]
<i>Metschnikowia pulcherrima</i>	Esters	[35, 46]
<i>Meyerozyma guilliermondii</i> (formerly <i>Pichia guilliermondii</i> )	Color	[53]
<i>Pichia kluyveri</i>	Thiols	[52]
<i>Pichia fermentans</i>	Acetaldehyde 2,3-Butanediol Ethyl esters Higher alcohols Polysaccharides	[29, 49]
<i>Schizosaccharomyces pombe</i>	Maloalcoholic deacidification Pyruvic acid Color Propanol	[39, 40]
<i>Torulaspora delbrueckii</i>	Succinic acid Linalool Polysaccharides	[35–37, 66]
<i>Zygosaccharomyces bailii</i>	Polysaccharides	[49]
<i>Zygosaccharomyces florentina</i> (formerly <i>Zygosaccharomyces florentinus</i> )	Polysaccharides	[50]

**Table 1.** Metabolites produced and/or physical properties enhanced in wines fermented with non-conventional yeasts as single or co-fermentations compared with pure fermentation with *Saccharomyces cerevisiae*.

The non-*Saccharomyces* yeasts have an important influence on wine flavor depending of species and strain specific involved in the fermentation process. These yeasts present particular metabolic features that affect to the organoleptic characteristics in the wine including the capacity to secrete enzymes and metabolites related with the primary and secondary aroma of wines, low volatile acidity, release of mannoproteins, or increase in wine color stability [28]. Should be note their potential positive contributions to wine quality, while keeping fermentation kinetics and consistency under control, many researchers have postulated co-inoculation or sequential inoculation of *S. cerevisiae* or closely related species with one or more non-*Saccharomyces* strains (Table 1).

### 3. Influence of non-*Saccharomyces* yeasts in mixed fermentations

In recent years, re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking has resulted in several studies that have looked at the use of controlled fermentations using *Saccharomyces* along with non-*Saccharomyces* yeast species from the winemaking [29, 30]. The mixed fermentations are used as a biotechnological tool in order to enhance especial and specific characteristics of a wine and thus improve their complexity. Indeed, the application of mixed and controlled fermentations in the wine elaboration has changed the standardized way to make wine and can improve the quality of the final product. These fermentations consist in controlled inoculations of *S. cerevisiae* starter culture and non-*Saccharomyces* strains. The non-*Saccharomyces* application could enhance the analytical composition of wine by taking advantages of diverse metabolic pathways of these yeasts.

The use of controlled mixed fermentations of non-*Saccharomyces* yeasts together with *S. cerevisiae* can be suggested as an useful tool for wine production which allows reproduce microbiological and technical aspects that really occur in the spontaneous fermentation, as well as an increase in the wine aroma complexity owing to a more complex synthesis of aromatic compounds [31]. This practice has also been reported as being able to increase some desirable metabolites, such as some acetate esters [32] and glycerol [33]. Moreover, some non-*Saccharomyces* yeasts have been reported as being able to release more polysaccharides than *S. cerevisiae* strains [34].

The influence of multistarter fermentation practices on final wine composition and on growth and death rates of the *S. cerevisiae* and non-*Saccharomyces* strains have been investigated. *Torulaspota delbrueckii*, one of the few non-*Saccharomyces* yeast species currently commercialized, is reported to have a positive effect on the taste and aroma of alcoholic beverages [35] and exhibits low production of acetaldehyde, acetoin, acetate, and ethyl acetate [36].

Recent studies using strains of *T. delbrueckii* and *L. thermotolerans* as starter cultures together with *S. cerevisiae* (specifically in mixed and sequential fermentations) have generated important changes in the wine composition. *Torulaspota delbrueckii* produced a reduction in acetic acid content and *L. thermotolerans* produced a reduction in acetaldehyde concentration and increase in titratable acidity [37, 38].

Other studies have been carried out with the aim of de-acidifying the grape must or wine through malic acid degradation using mixed fermentations of *Schizosaccharomyces pombe* and *S. cerevisiae*; some species of *S. pombe* had been recognized to improve some of sensory parameters of the wine, especially those related to wine color stability due to the correlation between the amount of pyruvic acid released into the medium and the formation of vitisin A (a pyranoanthocyanin, natural polyphenol, found in grapes) [39, 40]. *Issatchenkia orientalis* is one of the indigenous yeasts present in the wine. The strain KMBL 5774 of *I. orientalis* isolated from Korean wine pomace can degrade malic acid thus could be important in decreasing malic acid content in the wine and be useful the wine industry by this attribute [41].

The use of *Starmerella bacillaris* (formerly *Candida stellata*) yeast in mixed fermentation with *S. cerevisiae* starter cultures has been widely investigated these last few years, and several studies

have shown an increase in glycerol content in mixed wines. Glycerol is related with the mouthfeel and complexity of wine flavor. The analytical and organoleptic profile of the wine was improved without any negative analytical profile in the fermentation of grape musts with mixed fermentations of *S. bacillaris* and *S. cerevisiae* starter strains. Also, it has been observed that the inoculation of grape must with pure cultures of *S. bacillaris* can result in the production of high concentrations of acetaldehyde and acetoin [42, 43]. In fact, in comparison with *S. cerevisiae* inoculated fermentations, the use of co-fermentations (mixed or sequential inoculations), continuous fermentation, and immobilized cells can contribute the following: (1) complementary consumption of glucose and fructose; (2) enhanced glycerol and succinic acid concentrations; and (3) no increases in acetaldehyde and acetoin contents, due to the presence of the *S. bacillaris* based on the existence of acetaldehyde exchange between the two species without any increment in its levels. The practice of multistarter fermentation can also be used to improve the complexity of organoleptic properties of a wine contributing to the enjoyment of wine. Garcia et al. [44] were able to verify an increase in geraniol production in mixed cultures of *Debaryomyces vanriji* and *S. cerevisiae*. This enhancement was due to the high levels of  $\beta$ -glucosidase activity exhibited by this non-*Saccharomyces* strain.

Another non-*Saccharomyces* yeast with several interesting features from wine industry is *Metschnikowia pulcherrima*. This yeast is generally predominant during the initial stages of alcoholic fermentation and also shown some significant effect in wine composition. In particular, *M. pulcherrima* is a high producer of  $\beta$ -glucosidase [45], and its presence in mixed cultures can provide important improvements in the wine such as decrease in the volatile acidity and increase in the production of medium-chain fatty acids, higher alcohol, esters, terpenoids, and glycerol. Some authors have also reported that *M. pulcherrima* can produce a reduction in the titratable acidity of the final wines. Depending on the initial acidity level of the grape must, this effect could be taken positively or negatively. It has also been reported that *M. pulcherrima* has a higher capacity to release polysaccharides from yeast cell walls during fermentation process compared to *S. cerevisiae*. More recently, sequential fermentations with *M. pulcherrima* and *S. cerevisiae* have shown that a reduction in ethanol concentration is occurring in this type of culture [46].

Yeasts belonging to the *Hanseniaspora* (*Kloeckera*) genus are the non-*Saccharomyces* yeasts found in the highest numbers in grape must. Due to their ability to produce unpleasant compounds, such as acetic acid and ethyl acetate, these apiculate yeasts have long been considered as spoilage yeasts particularly during the early stages of wine fermentation. Mixed fermentation trials in presence of *H. uvarum* and *S. cerevisiae* starter cultures have presented increases in isoamyl acetate [47], while use of *Hanseniaspora osmophila* provides improvements in 2-phenylethyl acetate production [48–50]. According to a report by Kurita [51], mixed inoculations using *Wickerhamomyces anomalus* (formerly *Pichia anomala*) resulted in positive enhancement of isoamyl acetate. Higher concentrations in the varietal thiols have been shown in mixed fermentations with *S. cerevisiae* and another strain belonging to the *Pichia kluyveri* species [52].

Benito et al. [53] have studied the hydroxycinnamate decarboxylase (HCDC) activity of *Meyerozyma guilliermondii* (formerly *Pichia guilliermondii*) in mixed and sequential fermenta-

tions with *S. cerevisiae*. *Meyerozyma guilliermondii* with HCDC activity can be used to decarboxylate hydroxycinnamic acids and form vinylphenols that condense with grape anthocyanins to produce vinylphenolic pyranoanthocyanin adducts—molecules that show great color stability.

## 4. Highlights produced by non-*Saccharomyces* yeasts

### 4.1. Enzymes with oenological interest

Over the last several decades, the utilization of enzymes has become more important in winemaking. Enzymatic treatments of grapes, musts, and wines are nowadays useful for multiple positive aims, reduction of times maceration, clarification and filtration, increase in free and press juice yields, improvements in color and aroma extraction as well as wine stability [54]. The enzymes are proteins usually produced by bacteria or by filamentous fungi [55]. These proteins are very valuable tools for the winemakers; they now strengthen the use of endogenous enzymes over commercial exogenous enzymes. The production of extracellular hydrolytic enzymes by indigenous yeast could be notable and a better understanding to their benefit of wine production is required. Moreover, wine yeast has a decisive role in the production of commercial enzymes to be used in the wine elaboration process [56]. The principal wine yeast, *Saccharomyces cerevisiae*, is not notable as a significant producer of extracellular enzymes, although some strains have been mentioned to degrade polygalacturonate [57].

On the contrary than *Saccharomyces* species, the non-*Saccharomyces* yeasts produce and secrete several enzymes as well as esterases, glycosidases, lipases,  $\beta$ -glucosidases, proteases, cellulases, etc., to the periplasmic space and the medium, where they have the capacity to bind with grape precursors compounds to produce aroma active compounds and thus play an important role in varietal aroma and flavor profiles [58].

Terpenoids, fatty acid esters, higher alcohols, glycerol, acetaldehyde, acetic acid, and succinic acid are some metabolic products generated from non-*Saccharomyces* growth. There is a distinction in types of flavors according to their origin, the primary flavor of wine comes naturally from grapes, each grape variety offers a unique set of aromas and flavors, and the fermentation process creates a group of bouquets that are commonly referred to secondary flavor [59]. Several flavor and aroma compounds are present in grapes as glycosidic precursors without sensory properties. The  $\beta$ -glucosidase enzyme might hydrolyze these compounds to form free volatiles increasing the flavor and aroma of wine and contributing to the higher fruit-like characteristic of final product; this enzyme is not encoded by the *S. cerevisiae* genome [60]. Instead, certain non-*Saccharomyces* genera as *Debaryomyces*, *Hansenula*, *Candida*, *Pichia*, and *Hanseniaspora* (*Kloeckera*) have different degrees of  $\beta$ -glucosidase activity which can have on the sensory character of wines [61].

Proteolytic and pectinolytic (polygalacturonase) are other extracellular enzymatic activities produced by non-*Saccharomyces* yeasts which may also be beneficial to winemaking. For

example, proteolytic activity of some non-*Saccharomyces* yeast reduces the protein concentration of the grape juice by approximately one-third with accompanying increase in protein stability of the final product. The protein haze reduction is one of most significant changes for alcoholic beverages manufacturers. Protein precipitation in bottled wines especially in whites and red with low amounts of polyphenols causes protein haze where a coagulation of proteins occurs in alcoholic beverage with unfavorable storage conditions. These denatured proteins can either flocculate into a hazy suspension or form sediments in bottle [62]. Species found to produce the greatest number of extracellular enzymes are *C. stellata*, *H. uvarum*, and *M. pulcherrima* [56].

Non-*Saccharomyces* yeasts have also been reported to affect the concentration of polysaccharides in wine [49]. An enzymatic degradation happens in the dead yeast cells; the cells components such as proteins, nucleic acids, and lipids are broken down into smaller compounds as amino acids, peptides, fatty acids and nucleotides and also occur in releasing soluble polysaccharides (mannoproteins) from the cell wall. Most of these products have flavor impact or flavor-enhancing potential [63], but their specific contributions to wine character require more focused research and will depend on the extent to which the wine is exposed to the lees [64]. Moreover, there is evidence that peptides released during yeast autolysis could have antioxidant and other bioactive properties. Polysaccharides improve the sensory properties in wines, as they can positively influence mouthfeel (texture) by increasing its viscosity and mouth-filling [65]. Some non-*Saccharomyces* yeasts die in the early stages of fermentation process and they can also be a nutrient source that *S. cerevisiae* used to ferment optimally. Charoenchai et al. [58] reported the effect of nitrogen sources on the production of extracellular proteases by non-*Saccharomyces* wine yeasts. From 26 yeast strains, protease activity was observed in strains of *M. pulcherrima*, *Kloeckera apiculata*, and *W. anomalus*. Also, *T. delbrueckii* has reported as releasing higher amount of polysaccharides, Gonzalez-Royo et al. [66] have found that the high content of polysaccharides obtained by sequential culture with *T. delbrueckii* has a positive effect on the foam properties in sparkling wines.

The role of pectinases in winemaking has been evaluated by Canal-Llaubères [67]. Some of the applications in mash treatment are to improve juice extraction, clarification process, filterability, and also color extraction. The use of pectolytic enzymes for maceration may also accelerate the extraction of phenolic compounds, reducing the maceration time needed for high quality of wine [68]. The addition of fungal pectinase preparations is a normal practice in wine industry even though pectin esterase and polygalacturonase enzymatic activities increase during grape ripening and are produced by non-*Saccharomyces* yeasts present in must.

The accumulation of esters in wine is known to be a result of the balance between the yeast's ester-synthesizing enzymes and hydrolysis reactions involving esterases (responsible for cleavage and in some cases, formation of ester bonds). The production of extracellular esterases in *Saccharomyces* wine yeasts is well known [69], but the situation for non-*Saccharomyces* needs further investigation. Yeast esterases studied include those of the genus *Brettanomyces* [70] and *Rhodotorula mucilaginosa* [71]. Also, one strain of *Debaryomyces hansenii* has been reported as producing strain of an esterase enzyme [72].

The lipids proceeding from the grape or from autolytic activity of yeasts can be degraded by lipases. After this enzymatic reaction, free fatty acids would be released into the juice or wine, which can lead to changes in wine quality. While properties of lipoxygenase and peroxide-cleaving enzymes from grapes have been well established [73], the knowledge about lipase enzyme production by non-*Saccharomyces* yeasts is not well documented yet. Ratledge and Tan [74] reported data about the production of extracellular lipases by yeasts, only a single species of *Yarrowia lipolytica* (formerly *Candida lipolytica*) and *Saccharomycopsis lipolytica*.

*Hanseniaspora* and *Torulasporea* genera are reported as good producers of enzymes such as  $\beta$ -glucosidases, pectinases, proteases, and those involved in xylan degradation [58, 75]. However, the secretion of each enzyme is not characteristic of a particular genus or species, but depends on the yeast strain analyzed [76].

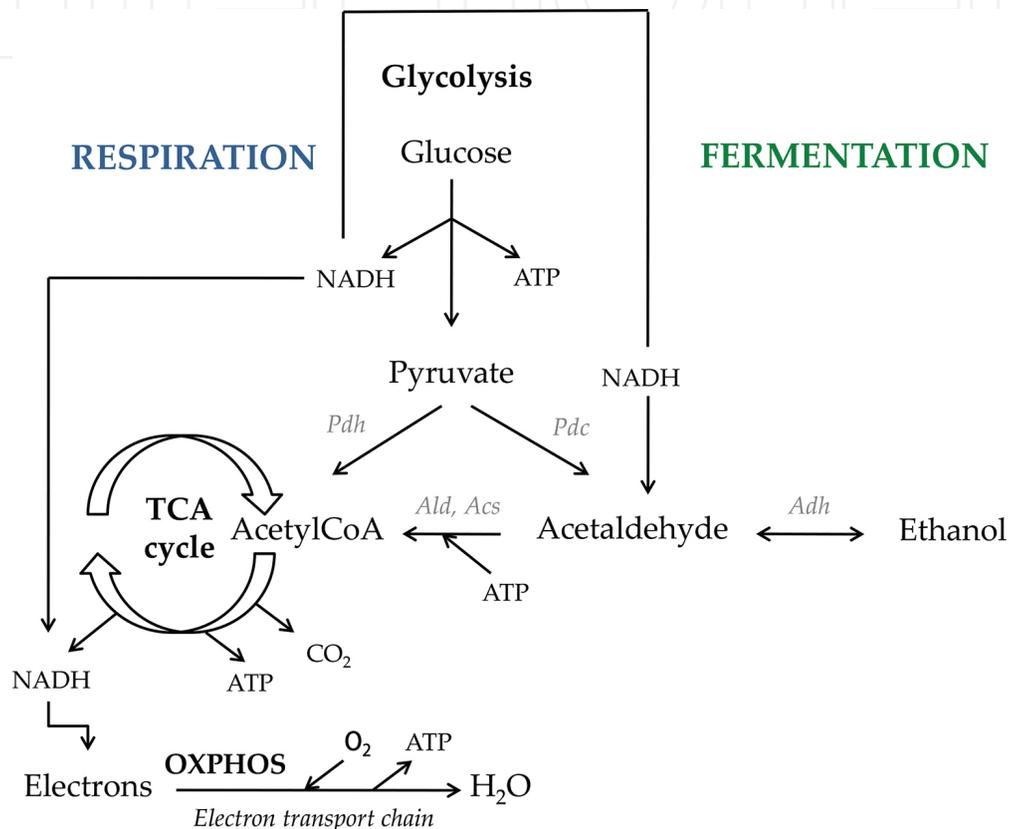
#### 4.2. Use of lower ethanol efficiency yeasts

In recent decades, the increasing alcohol level in wine is one of the most important challenges facing in the enological industry. The problem is related in part to global warming, which results in modifications of fruit maturation patterns, as well as a lack of balance between sugar accumulation and the phenolic ripeness of berries [77]. Fermentations with higher initial sugar content combined with high final ethanol concentration may have impact on microbiological, technological, sensorial, and financial aspects of winemaking. Higher sugar level delivers shifts in alcohol, altering flavors and mouthfeel. Musts with higher sugar concentrations cause a stress response in yeast leading to an increased formation of fermentation co-products, such as acetic acid. Also, this increasing sugar content leads to delay harvest period so as to insure appropriate aromatic and phenolic maturity. On the commercial side, excess ethanol can get worse sensory quality of wine, discourage consumers, because of the health effects associated with the excessive alcohol consumption or become a drawback in the global market, due to regulations and taxes associated with the alcohol content of beverages. All these reasons stimulate the creation of strategies directed to reduce alcohol level in wine.

Researchers, engineers, and oenologists are working together to develop approaches to limit ethanol content of wines, targeting almost all the steps in the production cycle [78], including among other examples, grapevine clonal selection, vineyard management, winemaking practices adapted to unripe grapes [79], use of lower ethanol efficiency yeast strains [80] or metabolic inhibitors [81] and partial dealcoholization by physical means [82]. While some of these technologies are still in need of fundamental research, others are in several stages of regulatory support and implementation by the industry.

The development of low-alcohol yeasts is a current challenge in wine industry. During the last years, researchers have been investigating *S. cerevisiae* metabolism to reduce the yield ethanol/sugar consumed. Two approaches were used as follows: metabolic engineering strategies diverting sugar metabolism towards products other than ethanol [Genetically Modified Organisms (GMO) strategy] [83] and more recently an adaptive evolution-based strategy [84]. An alternative to these approaches is to select low-ethanol producers in *S. cerevisiae* species by screening wild yeast population or to use breeding strategies.

Given the vast potential for diverse wine relevant phenotypes among non-*Saccharomyces* yeast, it has been proposed that strains able to utilize oxygen grape sugars could be used to decrease ethanol concentration in wine [85]. Unlike *S. cerevisiae*, which favors fermentative metabolism over aerobic respiration when sugar concentration exceeds 10 g/L (due to the Crabtree effect), many non-*Saccharomyces* yeast are able to use oxygen for growth regardless of sugar concentration and thus divert carbon into other metabolites and therefore away from ethanol formation.



**Figure 1.** Yeast energy metabolism.

Respiration and fermentation are two pathways for ATP production from glucose used by yeasts. Both pathways start with glycolysis, the major process for sugar degradation where the breakage of one glucose molecule results in the production of two molecules of pyruvate and ATP. In fermentation, pyruvate is finally transformed into ethanol by pyruvate decarboxylase (*Pdc*) and alcohol dehydrogenase (*Adh*) enzymes. This process does not produce additional ATP but the NADH that is released in glycolysis is recycled by *Adh* into NAD<sup>+</sup>, and thus, alcoholic fermentation can occur in the absence of oxygen. In respiration, pyruvate is transformed into acetyl-coenzyme A by pyruvate dehydrogenase (*Pdh*) which is then oxidized to CO<sub>2</sub> through the TCA cycle and oxidative phosphorylation (OXPHOS), where it yields additional ATP but requires oxygen. At abundant levels of sugars and oxygen, Crabtree-positive yeasts use fermentation and respiration simultaneously. Once glucose has been depleted in the environment, one way to generate ATP is recycling the ethanol accumulated.

This process causes a loss in terms of ATP because of the conversion of ethanol to acetyl-CoA carried out by aldehyde dehydrogenase (*Ald*) and acetyl-CoA synthetase (*Acs*) requires one additional ATP per ethanol recycled (**Figure 1**).

Yeasts can be classified depending on the way they regulate their respiro-fermentative metabolism. Crabtree-positive yeasts could ferment under aerobic conditions only if sugar concentration is above certain thresholds. The prime example of Crabtree-positive species is *S. cerevisiae*, yeast with clear preference towards fermentative metabolism. This Crabtree-positive character has allowed to *S. cerevisiae* adapt in sugar rich environments [86]. In contrast, the extent of fermentative metabolism for Crabtree-negative species would be very limited whenever enough oxygen is available [87]. *Hanseniaspora uvarum* and *Candida utilis* are examples of Crabtree-negative yeasts [88]. In spite of his preference for respiratory metabolism, some Crabtree-negative yeasts, such as *Kluyveromyces lactis* and *Kluyveromyces marxianus*, can grow in the absence of oxygen [89]. Finally, some yeast species are not able to ferment sugars and are obligate aerobes.

It is thought that redox balance in the metabolism of sugars generates the production of metabolic by-products as acetic acid, ethanol, and glycerol. In recent years, it is trying to take the control of metabolic systems in order to redirect carbon flux towards desirable compounds release, for example, glycerol overproduction. An added benefit of this approach is that enhanced glycerol concentrations can have a favorable influence on wine by enhancing its sweetness, smoothness, and overall body [90].

Several yeast strains, including *M. pulcherrima*, *K. lactis*, and *Candida sake* isolates, were found to be good candidates to develop fermentation procedures aiming at reducing alcohol content in wine by respiration. Results of previous work also indicated that, besides the study of yeast ability in sugar respiration metabolism under aerated winemaking conditions, it is necessary to find a compromise between ethanol yield, acetic acid production, and growth performance in grape must. Differences of up to one order of magnitude in acetic acid yield were found among the different yeast strains studied [91].

#### 4.3. Bioprotection by non-*Saccharomyces* yeasts

Vinification process is composed by different and delicate steps as growing, harvesting, fermentation, and aging and storage in the winery. Unsuitable precautions or poor practice during any of these steps can lead to growth of wine spoilage organisms and consequent production losses. The major microorganisms involved in wine spoilage are acetic acid bacteria from genera *Acetobacter*, *Gluconobacter*, and lactic acid bacteria from *Leuconostoc*, *Lactobacilli*, and *Pediococcus* genera [92], whereas the yeasts involved in wine spoilage mainly are from genera *Dekkera/Brettanomyces*, *Pichia*, *Zygosaccharomyces*, and *Candida*, usually isolated from wines with aroma defects [93]. Bacterial wine spoilage imparts mousy taint, bitterness, geranium notes, volatile acidity, oily and slimy-texture, and overt buttery characters to the wine [92], whereas the common spoilage effects due to yeasts are off odors, off-tastes, film formation, cloudiness or haziness, sediments, and gas production in bottled wines [93].

Traditionally, sulfur dioxide (as potassium metabisulphite), sorbic acid, fumaric acid, and dimethyl dicarbonate (DMDC) are used for preservation of different wines in various countries. Due to these drawbacks and growing consumer bias against chemical preservatives, research efforts are directed towards use of different physical methods and exploitation of natural antimicrobial compounds obtained from plants, animals, and microorganisms for wine preservation. Many studies have demonstrated the potential of natural products such as hydroxycinnamates and organic acids [94], chitosan [95], nisin [96], lysozyme [97], antimicrobial peptides [98], killer toxins [99], natamycin [100],  $\beta$ -glucanases [101], bovine lactoferrin-derived peptides [102], carvacrol and thymol [103], and vitamin K5 [104] for the control of wine spoilage yeasts and bacteria.

One of the biological mechanisms for the regulation of population dynamics in several microbial ecosystems is the production of toxins capable of kill or inhibit other microorganisms, taxonomically related or not to the producing strains. The toxins synthesized by yeasts, known as killer factor, are proteins or glycoproteins whose action is mediated by specific receptors in the cell wall of the sensitive microorganism. The killer character, first reported on the decade of the 1960s in a *S. cerevisiae* strain, is well distributed among other yeast genera as *Candida*, *Hansenula*, *Pichia*, *Debaryomyces*, *Ustilago*, *Cryptococcus*, *Metschnikowia*, *Williopsis*, *Kluyveromyces*, and *Zygosaccharomyces* [105].

*Saccharomyces cerevisiae*'s killer toxins and their relevance in winemaking have been thoroughly investigated in the literature. However, these killer toxins exhibit narrow spectra of activity limited to the other strains of *S. cerevisiae* [106] except for the Klus killer toxin and the killer toxin from *S. cerevisiae* strain Y500-4 L that are active against a few non-*Saccharomyces* species and are therefore unsuitable as agents to prevent the development of spoilage yeasts. Therefore, the differences in stationary phase cell concentrations between yeast species in wine fermentations may be due to the fact that the non-*Saccharomyces* yeasts are more sensitive to certain growth-inhibitory compounds than *S. cerevisiae*.

The killer toxins secreted by the yeast species *Pichia membranifaciens*, *Kluyveromyces wickerhamii*, *Metschnikowia pulcherrima*, and *Wickerhamomyces anomalus* (formerly *Pichia anomala*) and the filamentous fungus *Ustilago maydis* have been specifically investigated for their killer activity against *Brettanomyces bruxellensis* [107]. These killer toxins successfully inhibited the growth of *B. bruxellensis* in wine and grape juice. Furthermore, the killing activity of certain non-*Saccharomyces* killer toxins has been demonstrated against the apiculate yeast *Hanseniaspora uvarum* [108] and also against the grapevine pathogen *Botrytis cinerea* [109].

## 5. Concluding remarks

Strain selection is of key importance, as not all strains within a species will necessarily show the same desirable characteristics [110]. The accepted list of desirable characteristics as pertaining to the wine yeast *S. cerevisiae* will not necessarily apply to non-*Saccharomyces* yeasts. These wine yeasts will necessarily have a different list of desired characteristics. Thorough briefings and assistance of wine producers will have to accompany any new non-*Saccharomyces*

technology for wine production. However, the aims submitted by Pretorius et al. [111] and other authors advocate the use of selected non-*Saccharomyces* yeasts as able to consume grape juice sugars, enhance production of desirable volatile esters, enhance liberation of grape terpenoids, and produce glycerol to improve wine flavor and other sensory properties. A modern approach to multispecies inoculations backed by science and rigorous research is essential to help winemakers achieve their primary objective of attaining conversion of grape sugar to alcohol and carbon dioxide, at a controlled rate and without the development of off-flavors.

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