Chapter 14
The Fermentative and Aromatic Ability of *Kloeckera* and *Hanseniaspora* Yeasts

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Contents

Abstract Spontaneous alcoholic fermentation from grape, agave and others musts into an alcoholic beverage is usually characterized by the presence of several non-*Saccharomyces* yeasts. These genera yeasts are dominant in the early stages of the alcoholic fermentation. However the genera *Hanseniaspora* and *Kloeckera* may survive at a significant level during fermentation and can influence the chemical

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composition of the beverage. Several strains belonging to the species *Kloeckera apiculata* and *Hanseniaspora guilliermondii* have been extensively studied in relation to the formation of some metabolic compounds affecting the bouquet of the final product. Indeed some apiculate yeast showed positive oenological properties and their use in the alcoholic fermentations has been suggested to enhance the aroma and flavor profiles. The non-*Saccharomyces* yeasts have the capability to produce and secrete enzymes in the medium, such as β-glucosidases, which release monoterpenes derived from their glycosylated form. These compounds contribute to the higher fruit-like characteristic of final product. This chapter reviews metabolic activity of *Kloeckera* and *Hanseniaspora* yeasts in several aspects: fermentative capability, aromatic compounds production and transformation of aromatic precursor present in the must, also covers the molecular methods for identifying of the yeast.

Keywords Fermentation, bouquet, apiculate yeast, aroma and flavor, grape must

14.1 Introduction

Alcoholic beverage production in today's world is a complex process. There are infinite parameters that can be altered to produce alcoholic beverage with different flavor profiles. Spontaneous alcoholic fermentation occurs mainly by a succession of different yeast and bacteria population, which are affected by environmental factor. In fact yeasts, medium composition and culture conditions impact the alcoholic fermentation process and aromatic quality of final beverage.

Several authors have reported that the first fermentation stage is dominated by *non-Saccharomyces* apiculate yeast activity, mainly the *Kloeckera* and *Hanseniaspora* strains genus. The high substrate concentration tolerance (>200 g l^{-1}) of the *Kloeckera* and *Hanseniaspora* strains explains their dominance at the initial stages of fermentation. The growth of these yeasts is limited to the first days of fermentation. The progressive disappearance of the *non-Saccharomyces* strains is attributed to lower capability adaptation to gradual increase of ethanol concentration. This fact makes possible the growth of more tolerant *Saccharomyces* strains. Considerable physiological characteristics found in the *Saccharomyces* strains allow them to dominate alcoholic fermentation, the main one being high alcohol concentration tolerance.

The aroma-developing properties of *Kloeckera* and *Hanseniaspora* and their contribution to beverage bouquet determined by the survival period in fermentation make this species a very important object of study. Persistence of the *non-Saccharomyces* species depends on several factors, such as the temperature, pH, nutrient availability, *Saccharomyces* inoculum concentration, kind and concentration of antimicrobial compound, toxin killer sensibility, indigenous microorganism concentration present in fermentation juice and process technology.

This chapter focuses mainly on *Kloeckeras* and *Hanseniaspora* yeast strains studied in wine process. Metabolic, nutritional and aromatic aspects are reviewed.

In addition, fermentative and aromatic abilities are compared to other non-*Saccharomyces* and *Saccharomyces cerevisiae*, the universal yeast used in alcoholic fermentation.

14.2 Biodiversity and Ecology of the Yeasts Used in Fermented Alcoholic Beverages

Yeasts are found throughout nature all over the World. To this day, more than 700 yeast species have been classified in 100 genera (Kurtzman and Fell, 1998). However this number is only a tiny fraction of biodiversity. Hawksworth and Monchacca (1994) estimated that 62,000 genera and 669, 000 yeast species are yet to be described. Yeasts are not capable of moving and depend on vectors, such as wind, insects or man. Fermentative yeasts are found in 2 different habitats: raw material and factories where they processed. In wine-production, grape microflora varies according to the grape variety, climatic influences, viniculture practices etc. (Pretorius, 2000). *Kloeckera* and *Hanseniaspora* are the predominant species on the surface of the grape and represents 50–75% of the total yeast population (Fleet, 1993). The other yeast genera present are: *Candida, Brettanomyces, Cryptococcus, Kluyveromyces, Metschnikowia, Pichia* and *Rhodotorula.* In contrast, *S. cerevisiae* is scarce in vineyards, but abundant in grape juice and must-coated surfaces of winery equipment (Fleet and Heard, 1993). *S. cerevisiae* is preferred for initiating wine alcoholic fermentation due to their most efficient fermentative catabolism. However in the future, some winemakers might prefer using a mixture of indigenous yeast species and strains as starter cultures to increase aroma production.

14.2.1 Spontaneous Fermentation

The natural fermentation of grape must is usually started by low-alcohol-tolerant apiculate yeasts (*Kloeckera/Hanseniaspora*) dominating the initial stages of alcoholic fermentation in spontaneous and inoculated fermentation. These yeasts die off when ethanol concentration increases and are replaced by the strongly fermentative *S. cerevisiae* yeast (Heard and Fleet, 1986; Satora and Tuszynski, 2005). In non-inoculated fermentation, *Kloeckera* yeast is present up to 10^6 CFU ml^{-1}, being the dominant species and representing 50 to 75% of the total population. The main yeast, *S. cerevisiae*, is present, generally with very low population, less than 50 CFU ml^{-1} (Fleet and Heard, 1993). It has been reported that the growth and the survival of *K. apiculata* is not suppressed by the inoculated *S. cerevisiae* strain (Heard and Fleet, 1986). Nevertheless, another author suggests that *S. cerevisiae* produces compounds that are toxic to apiculate yeasts, other than ethanol and killer toxins (Perez-Nevado et al., 2006).

In addition, during various fermentation stages, it is possible to isolate other cultures belonging to other yeast genera, such as *Candida, Torulaspora, Kluyveromyces* and *Metschnikowia* (Fleet et al., 1984; Heard and Fleet, 1986; Pardo et al., 1989).

According to Pretorius et al. (1999), the intervention of only 15 genera of yeasts has been shown during the wine-making process: *Brettanomyces* (and their sexual Dekkera), *Candida, Cryptococcus, Debaryomyces, Hanseniaspora* and their equivalent asexual *Kloeckera, Kluyveromyces, Metschnikowia, Pichia, Rhodotorula, Saccharomyces, Saccharomycodes, Schizosaccharomyces* and *Zygosaccharomyces*. In the specific case of tequila, only one paper has been published, Lachance (1995), about yeast characterization in the manufacturing process. The author identified 10 genera: *Brettanomyces, Candida, Hanseniaspora, Kluyveromyces, Pichia, Saccharomyces, Saccharomycodes, Zygosaccharomyces, Torulaspora* and *Iwatchenkia,* eight of which are present in the wine production process.

14.2.2 Inoculated Fermentation

The introduction of a pure strain allows fermentation to be regulated and accelerated, thereby avoiding the action of certain indigenous populations and, consequently, the production of a specific aromatic compound. In winemaking, inoculation with a starter culture allows a high population of selected *S. cerevisiae* strain to assure its dominance.

The results are quick alcohol production, short fermentation, more predictable aromas and a decrease in the growth of non-*Saccharomyces* present in the must. The foregoing prevents the risk of stuck fermentation and the production of undesirable aromas. However, simpler and less interesting aroma production has been detected when fermentation is dominated by only one yeast type (Romano et al., 1992; Romano et al., 1997; Ciani and Maccarelli, 1998). Ciani et al. (2006), reported that the use of multistarter with apiculate yeast and *Saccharomyces*, showed analytical profiles of wines comparable to or better than those exhibited by pure culture of *S. cerevisiae*. Highest means for acceptability were also obtained by Pinot Noir musts fermented by *P. membranaefaciens* and Chardomay, fermented by *Kloeckera apiculata* (Mamede et al., 2005). This strategy revalues the role of non-*Saccharomyces* yeast and can increase the interest of starter cultures alone or cultures mixed with *S. cerevisiae.* However, our knowledge of the metabolic interactions between *S. cerevisiae* and non-*Saccharomyces* wine yeasts under winemaking conditions needs to be improved.

There are a few studies reporting the kinetic parameter of wine yeast in pure and mixed cultures. Moreira et al. (2005) reported that the specific growth rates in pure culture of *S. cerevisiae, H. uvarum* and *H. guilliermondii* are similar to those in mixed culture of, *H. guilliermondii-S. cerevisiae* and *H. uvarum-S. cerevisiae*. Nevertheless, in mixed cultures of the three yeasts, the specific growth rates of *S. cerevisiae* and *H. uvarum* decreased significantly, while the *H. guilliermondii* rate was not affected. Charoenchai et al. (1998); Ciani and Picciotti (1995) reported variations of specific growth rate of *S. cerevisiae* and *K. apiculata* cultivated on chemically defined grape juice and modified grape juice respectively. During

tequila production, the specific growth rates in pure culture of *Saccharomyces and Kloeckera* strains shown significant differences between genera: 0.373 ± 0.073 h^{-1} for *Saccharomyces* and 0.22 ± 0.1 h^{-1} for *Kloeckera* (Díaz-Montaño, 2004). Additionally, *Saccharomyces* specific growth rates in agave juice fermentation were similar to those reported in grape juice fermentation.

14.3 Molecular Techniques for the Analysis and the Identification of Yeasts

Traditionally, yeast identification has been made by conventional physiological techniques that are labor-intensive and can give ambiguous results (Kurtzman and Fell, 1998). Another disadvantage is their inability to discriminate among strains belonging to the same species. To avoid doubtful identification or misidentification, molecular techniques have been used by numerous authors to discriminate different wine yeast *Saccharomyces* and non-*Saccharomyces* (Capece et al., 2003, 2005). The sequence of the large subunit (26S) rDNA, especially region D1/D2, has been applied to study the phylogeny of different yeast groups and is an important tool in yeast identification (Balerias Couto et al., 2005). Recently, multigene sequence analysis was used, regarding their usefulness for reconstruction of phylogenetic relationships in the *Hanseniaspora–Kloeckera* species group (Cadez et al., 2006); Schültz and Gafner (1993) characterized strains of *Metschnikowia pulcherrima* and *Hanseniaspora uvarum* on the basis of their electrophoretic karyotypes. Esteve-Zarzoso et al. (1999) evaluated the use of restriction fragment length polymorphism (RFLP) of rDNA, amplified by polymerase chain reaction (PCR) to generate a database of restriction patterns for the routine identification of yeast species most frequently isolated from food. The same methods have been used to identify wine yeast species (Granchi et al., 1999; Torija et al., 2001). The molecular techniques employed to differentiate strains at intraspecific level include mtDNA restriction analysis (Comi et al., 2000), comparison of chromosomal DNA profiles (Cardinali et al., 1995), and analysis of random amplified polymorphic DNA by PCR (RAPD-PCR) (Quesada and Cenis, 1995; Cadez et al., 2003; Walczak et al.,2007). Recently (Flores et al., 2005) determined the variability and compared the genetic diversity obtained using amplified fragment length polymorphism (AFLP) markers in analyses of wine, tequila, mezcal, sotol and raicilla yeasts. This is the first report of molecular characterization of yeasts isolated from different traditional Mexican agave-distilled beverages, which shows high genetic differences with respect to wine strains.

14.4 Alcoholic Fermentative Process

Alcoholic fermentation consists of three main stages: transporting sugars to the interior of the cell, transforming sugars into pyruvate by means of glycolysis, and finally converting of acetaldehyde to ethanol.

14.4.1 Carbohydrate Transport

Transporting sugar is a key stage in alcoholic fermentation, as the internal concentration of sugars always remains low compared to the external concentration, (Gancedo and Serrano, 1989). Several authors (Mauricio and Salmon, 1992; Salmon et al.,1993) demonstrated in studies on winemaking that inhibiting sugar transport is the main factor restraining fermentative metabolism of wine yeast (particularly *S. cerevisiae*). Limiting fermentative metabolism in an industrial process can interrupt fermentation produce spontaneously, even when fermentable sugars are present in the must.

In *S. cerevisiae*, glucose and fructose are transported mainly by facilitated diffusion rather than active transport (Kruckeberg, 1996). The hexose transporter family (Hxt) consists of more than 20 proteins of high and low affinity (Bisson et al., 1993). Hexose transport through the plasmatic membrane in *S. cerevisiae* is known to be a control point in the metabolism of carbon compounds during fermentation (Elbing et al., 2004). However, for some non-conventional yeast, transporter kind can be different (Flores et al., 2000). *Candida utilis* transports sugar for proton symport, when the organism is grown to a low sugar concentration (van den Broek et al., 1997). Hofer and Nassar (1987) identified the hexose transporters in *Schizosaccharomyces pombe*, as H^+-symport. Scarce information exists in literature with regard to the transport mechanisms of monosaccharide through the membrane and its regulation in apiculate yeasts. With regard to the selectivity of sugar transport, it has been considered that the *S. cerevisiae* yeast is mainly glucosophilic. The residual fructose in wine is the result of the low capacity of fructose transport presented by *S. cerevisiae* yeast (Schültz and Gafner, 1995). The consumption of glucose and fructose in several strains of wine apiculate yeasts of the *Hanseniaspora guilliermondii*, *Hanseniaspora uvarum* (Ciani and Fatichenti, 1999) and *H. osmophila* genera (Granchi et al., 2002), were evaluated. The apiculate strains showed a wide viability in their preference with regard to the type of sugar. Most were glucosophilic (*H. osmophila/K. cortices* strains), although various were fructosophilic. Other yeasts consumed the two carbohydrates at the same speed (*Hansenispora uvarum/Kloeckera apiculata* and *H. guilliermondii*). These results show that the selective consumption of fructose is widely distributed along apiculate yeasts.

14.4.2 Carbohydrate Assimilation and Their Regulation

After the glucose is transported to the interior of the cell, it is phosphoriled (hexoquinases), to enter into the glycolysis cycle. The glycolysis final product is pyruvate that can be incorporated in two different metabolic ways: respiratory and fermentative. Both ways are regulated by the concentration of the substrate and dissolved oxygen in the medium, showing several phenomenons that are illustrated in Table 14.1.

Saccharomyces yeasts are Crabtree-positive, which means that glycolytic activity increases in the presence of high glucose concentration, diminishing the breathing

Table 14.1 Metabolic regulation: combined effect of sugar concentration and oxygen

Glucose (g l^{-1})	1–5	5–150	>150
Aerobiosis	Respiratory metabolism Pasteur Effect	Fermentative metabolism Crabtree Effect	Inhibition by substrate at respiratory and fermentative pathways
Anaerobiosis	Fermentative metabolism	Slow fermentative metabolism	

capacity in presence of oxygen, leading to an increment of the intracellular pyruvate concentration and ethanol formation (Käppeli, 1986). This phenomenon is due to the repressive action of glucose on the breathing enzymes. Albergaria et al. (2003) found that *Hanseniaspora guilliermondii* yeast was Crabtree-positive, in contrast to *Hanseniaspora uvarum*, that was Crabtree-negative, because aerobic alcoholic fermentation did not develop in batch cultures (Venturin et al., 1994) and chemostat cultures (Venturin et al., 1995a, b) in the presence of high concentrations of sugar. The different responses between Crabtree-positive and Crabtree-negative yeasts have been explained in terms of sugar consumption, glycolytic efficiency, anabolic limitation and enzymatic levels of pyruvate descarboxylase, alcohol dehydrogenase, pyruvate dehydrogenase, acetaldehyde dehydrogenase and acetyl CoA synthetase (**v**an Urk et al., 1988; Postma et al., 1989). Numerous studies have been performed on the balance of oxidative and fermentative metabolism in *S. cerevisiae* and *Candida utilis* (Verduyn, 1991, Verduyn et al., 1992; Käppeli, 1986). In Crabtree-positive yeasts (*S. cerevisiae*), the response to a pulse of glucose induces transcription mainly of the genes of the fermentative enzymes: pyruvate descarboxylase and alcohol dehydrogenase. On the other hand, Crabtree-negative yeasts (*C. utilis*) present low levels of fermentative enzymes and high activities of oxidative enzymes: pyruvate dehydrogenase, acetaldehyde dehydrogenase and the acetyl CoA synthetase, allowing mainly the generation of high concentrations of biomass, CO_2 and small quantities of ethanol. However, *H. uvarum* presents low acetyl CoA synthetase activity (0.05 U mg^{-1}) (Venturin et al., 1995a, b) with regard to other Crabtree-negative yeasts: *C. utilis* (0.50 U mg^{-1}) and *Kluyveromyces marxianus* (0.37 U mg^{-1}) (**v**an Urk et al., 1990).

The deficiency of acetyl CoA synthetase allows the accumulation of acetate and afterwards of ethanol in *H. uvarum*. Also, *H. uvarum* produces glycerol during the oxidative metabolism probably allowing the reoxidation of NADH generated during glycolysis (Venturin et al., 1995a). Likewise, the *H. guilliermondii* yeast (Crabtree-positive) presented yields similar to Crabtree-negative yeasts (Yx/s = 0.49 g g^{-1}) in a purely oxidative metabolism. Also under the same aerobic conditions, *H. guilliermondii* synthesized biomass, CO_2 and glycerol and in biosynthesized respiro-fermentative conditions, mainly ethanol, acetic acid, glycerol, CO_2 and malic acid. Venturin et al. (1995b) analyzed pyruvate descarboxylase activity in Crabtree-positive and Crabtree-negative yeasts in glucose limiting chemostat at different decreased flows. In the case of *S. cerevisiae* (Crabtree-positive), high levels

of pyruvate descarboxylase (0.67 U mg^{-1}) were present even in a low aerobic glucose-limited condition (Pronk et al., 1994). These activities were increased only two-fold under respiro-fermentative conditions (Weusthuis et al., 1994), in contrast to *H. uvarum*, in which single low activities of pyruvate descarboxylase could be detected in aerobic glucose limited cultures (0.20 U mg^{-1}). Likewise, *C. utilis* showed similar behavior (0.30 U mg^{-1}) (Weusthuis et al., 1994). These activities were increased in *H uvarum* (x 3.5) and in *C. utilis* (x7) when grown under oxygen limitation. This information suggests that the metabolism regulation of the Crabtree-negative yeasts *H. uvarum* and *C. utilis* can be controlled by the levels of oxygen (Weusthuis et al., 1994). Alcoholic fermentation occurs out only if oxygen is the limiting factor (Venturin, 1995b). On the other hand, (Steel et al., 2001) compared glucose catabolism for the pentose phosphate pathway in *S. cerevisiae* and *Kloeckera apiculata* yeasts, showing that *K. apiculata* catabolize smaller quantities of glucose through the pentose phosphate pathway than *S. cerevisiae*. The pentose phosphate or hexose monophosphate pathway is considered an alternative pathway in the degradation of glucose. This pathway allows the formation of two important products: NADPH and pentose phosphate. NADPH is used as a reducer in numerous reactions, mainly in lipid biosynthesis as well as other compounds whereas pentose phosphate (ribose 5-phosphate) is a precursor of nucleotides and nucleic acids. Likewise, it provides erythrose 4-phosphate for the synthesis of aromatic amino acids. Todd et al. (1995) reported considerable differences in the ribonucleic acid content between both strains, evidencing the different glucose catabolism requirements of pentose phosphate.

14.5 Factors Affecting Fermentation

Several factors impact fermentation rates and drive sluggish and stuck fermentation, but the important ones are: nutrient limitation, ethanol toxicity, toxicity for fatty and organic acids, the presence of killer factors, cation imbalance, temperatures carried to an extreme, pesticide and fungicide residues, microbial competition (Bisson, 1999).

14.5.1 Nutrients Limitation

The most studied conditions driving stuck and sluggish fermentation is the nutrient limitation (Bisson, 1999). Low fermentative capacities have been observed in *H. guilliermondii* (Albergaria et al., 2003) and *K. africana* (Díaz-Montaño, 2004) which are possibly due to a nutrient limitation.

The two macronutrients were frequently implied in the causes of stuck fermentation when present in small quantities are nitrogen and phosphate (Alexandre and Charpentier, 1998; Henschke and Jiranek, 1993). Micronutrients lacking vitamins

and minerals have been shown to limit fermentation speed (Bisson, 1999). The exhaustion of the thiamine leads to slow fermentation (Bataillon et al., 1996). High ethanol concentrations inhibit the translocation of amino acids and other nitrogen sources, so nitrogen must be available in the first stages of fermentation and stored inside the vacuole for later use (Boulton et al., 1996). Also, the addition of certain amino acids can increase the ability for quickly synthesizing degraded proteins as glucose transporters (Manginot et al., 1997). The phosphate limitation has been shown to impact biomass growth and yield. Phosphate is necessary to maintain cellular pools of Pi, ADP and ATP to drive glycolysis. Furthermore, mineral and cation deficiencies have been shown to impact fermentation rates (Blackwell et al., 1997). The minerals serve as cofactors in glycolysis. Limitations of some minerals such as Zn and Mg affect glucose catabolism while calcium limitation increases ethanol sensibility (Nabais et al., 1988).

14.5.2 Antimicrobial Compounds

Nutritional requirements of yeasts during the fermentation of grape juice can be influenced by the inhibitory substances present in the media. These compounds include killer toxins, chemical preservatives (especially sulfite) and agrochemicals containing heavy metals. Chemical preservatives can affect microbial activity causing an increment in the latency phase (Bisson, 1999). This behavior has been observed in pesticides containing copper (Tromp and De Klerk, 1988). Several pesticides have shown high antiseptic activity even with yeasts (Cabras et al., 1987). Recently, Cabras et al. (1999) studied the influence of six fungicides (azoxystrobin, cyprodinil, fludioxonil, mepanipyrim, pyrimethanil and tetraconazole), on *S. cerevisiae* and *K. apiculata* fermentative activity. The most of these pesticides improved the alcoholic production; this fact was especially observed with *K. apiculata*, which increased the alcoholic production from two- to three folds. Sulfur dioxide is used widely to suppress the growth of spoilage microorganisms in grape juice. The sulfite transport in wine yeasts is for simple diffusion (Walker, 1998), causing a decrease of intracellular pH. Even though *S. cerevisiae* is more tolerant to high concentrations of SO_2 than the non-*Saccharomyces* yeasts and bacteria, excessive doses of SO_2 can cause sluggish or stuck fermentation (Boulton et al.,1996). The susceptibility to SO_2 in non-*Saccharomyces* yeasts varies. For example, *K. apiculata* has been found to be susceptible to less than 5 mg l^{-1} free SO_2, but *Candida guilliermondii* and *Zygosaccharomyces* spp. were resistant to at least 10 times that concentration (Romano and Suzzi, 1993). Likewise, significant differences in resistance to sulphur dioxide was found in non-*Saccharomyces* grape and agave strains (Fiore et al., 2005).

Recently, the production by *S. cerevisiae* of fermentative metabolites potentially toxic for some non-*Saccharomyces* has been reported and not yet identified (Pérez-Nevado et al., 2006).

14.5.3 Toxin Killer

The killer activity was first reported in *S. cerevisiae* strains (Bevan and Makower, 1963). Since then, the characteristic killer has been detected in other yeast genera such as *Pichia* (Sawant et al., 1988), *Hansenula* (Polonelli et al., 1983), *Williopsis* (Walker et al., 1995) and *Kluyveromyces* (Young and Yagiu, 1978). Killer yeast strains produce an extracellular protein or glycoprotein (killer factor) that kills other sensitive yeasts. Neutral type yeasts are resistant to the killer factor but do not produce it. Killer-sensitive strains have also been discovered, these strains are immune to their own toxins but may be sensitive to other strains of toxins. The *Saccharomyces* yeasts produce species of protein killers: K_1, K_2, K_3 and K_{28}. The non-*Saccharomyces* yeasts generate species of protein killers: K_4 to K_{11}. The most of *Kloeckera* yeasts are neutral type and they are resistant to killer factor (Rodriguez et al., 2004; Sangorrín et al., 2001), an exception of toxin Kpkt of *K. phaffi* (Ciani and Fatichenti, 2001).

14.5.4 Temperature, pH, Oxygen and Culture Media Effects

The growth and the permanency of the non-*Saccharomyces* yeasts depend on fermentation conditions such as: temperature (Fleet and Heard, 1993), ethanol concentration (Kunkee, 1984), substrate concentration and pH (Charoenchai et al., 1998). Low temperature at the beginning of the fermentations results in prolonged survival of the non-Saccharomyces yeast. In contrast, *Saccharomyces* yeast populations dominated throughout the fermentation when the temperature of the cellar was maintained at a constant 16–18°C (Domizio et al., 2007). Several studies performed in wine (Erten et al., 2002) and cider (Bilbao et al., 1997) suggest that the growth of *K. apiculata* in the presence of *S. cerevisiae* is favored during fermentations performed below 20°C, allowing *K. apiculata* to prevail together with *S. cerevisiae* during fermentation (Bilbao et al., 1997). This situation can alter the chemical composition of wines, since the aromatic compounds depend mainly on the yeast (Mateo et al., 1991; Gil et al., 1996; Antonelli et al., 1999) and on fermentation temperature (Aragon et al., 1998).

Variation of medium pH between 3.0 and 4.0 did not significantly affect the growth rate or cell biomass of the non-*Saccharomyces* and *S. cerevisiae* strains (Charoenchai et al., 1998).

Culture media kind affects growth and fermentative capability of yeast. Recently, Arrizon et al. (2006) assessed different non-*Saccharomyces* and *S. cerevisiae* strains isolated of different origins cultivated on agave and grape must. Non-*Saccharomyces* grape strains did not ferment agave must in any conditions, whereas than non-*Saccharomyces* agave strains showed a moderate fermentative activity both in low sugar and high sugar concentration. On the contrary, non-*Saccharomyces* grape and agave strains were able to consume sugar and to produce ethanol in YPD medium, although to a lesser extent than *S. cerevisiae*.

During alcoholic fermentation, oxygen is a limiting factor for yeast growth. Visser et al. (1990) showed that *S. cerevisiae* is capable of rapid growth under strictly anaerobic conditions, whereas other yeasts, including the wine-related genera *Hanseniaspora, Kloeckera* and *Torulaspora,* grow poorly under the same conditions. The effect of oxygen on the survival of non-*Saccharomyces* yeasts during mixed culture fermentations of grape juice with *S. cerevisiae* has been reported. Oxygen clearly increased the survival time and decreased the death rate of *T. delbrueckii* and *K. thermotolerans* in mixed cultures, whereas it did not affect the growth and survival of *S. cerevisiae* (Hansen et al., 2001). It has also been shown that oxygen increases the time during which *Hanseniaspora valbyensis* coexists with *S. cerevisiae*, diminishing the mortality rate of *Hanseniaspora valbyensis* (Panon, 1997).

14.5.5 Ethanol Tolerance

Several studies have reported the role of the plasmatic membrane in the ethanol tolerance of *S. cerevisiae* (Beavan et al., 1982; D'Amore et al., 1990). High tolerance to ethanol thus correlates markedly with the level of fatty acid saturation and the fluidity of the membrane (Beavan et al., 1982; Alexandre et al., 1994). Sterols and unsaturated fatty acids also named survival factors cannot be synthesized under anaerobic conditions (Mauricio et al., 1997; Morrisey et al., 1999). Several authors have reported that the addition of fatty acids and ergosterol to the culture medium increases the ethanol yield and the ethanol tolerance of *S. cerevisiae* without requiring oxygen in the culture (Mauricio et al., 1997; Mishra and Prasad, 1989). *Hanseniaspora/Kloeckera* yeast is sensitive to ethanol concentrations of 5–6% v/v (Kunkee, 1984). Low temperatures increase ethanol tolerance (Fleet et al., 1989).

Pina et al. (2004) studied the kinetics of cell inactivation at high ethanol concentrations (>22.5% v/v) in different oxygen conditions with the addition of survival factors. These authors reported that the most abundant compounds found in the *S. cerevisiae*, *H. uvarum* and *H. guilliermondii* were: palmitic acid, oleic acid and ergosterol. However, the ergosterol/fatty acids ratio differs according to culture conditions and the yeast genus (Pina et al., 2004; Alexandre et al., 1994). *S. cerevisiae* aerobe cultures contain nearly 80% of unsaturated fatty acids, whereas in anaerobic ones, the phospholipids are typically enriched with the saturated fatty acids (Steels et al., 1994). *H. guilliermondii*, cultivated under aerobic conditions, produced high ergosterol and oleic acid contents, whereas anaerobiosis showed mainly palmitic acid and low ergoesterol concentration (Pina et al., 2004). The ergosterol and Tween 80 addition as sources of oleic acid in anaerobe cultures cultivated on high ethanol concentrations (> 22.5% v/v) has allowed an increase of cellular viability in *S. cerevisiae* and *H. guilliermondii* yeasts (Pina et al., 2004). Oleic acid and ergosterol play an equivalent role when they modulate ethanol tolerance in *H. guilliermondii*, although this response is not observed to all non-*Saccharomyces* yeasts. In the case of the most sensitive yeasts, *H. uvarum* and *T. delbrueckii*, the

presence of survival factors in anaerobic does not increase ethanol tolerance, even though the lipids have been incorporated into the membrane.

14.6 Aromatic Compounds

Alcoholic fermentation by yeast is associated with the production of a wide variety of fermentation products contributing to the flavor of drinks, as aromatic compounds or their precursors. There are nearly 400 volatile constituents, classified as: higher alcohols, fatty acid esters, bencenic compounds, lactones, terpens and certain particular metabolites (Cordonnier and Bayonove, 1986). Nevertheless, only the portion of these substances reaching the receiving organs is important from an aromatic point of view. The concentrations of most aromatic compounds in fermented alcoholic drinks are small, in the order of 10–50 ppm or fewer (Belitz and Grosch, 1988). To study them, it is necessary to perform extraction and concentration processes that allow their identification and later quantification (Mamede and Pastore, 2006).

14.6.1 Higher Alcohols

Higher alcohols are secondary metabolites of yeasts in alcoholic fermentation and they constitute the largest group of aromatic compounds in alcoholic drinks. Higher alcohols, also called fusel alcohol, have a strong pungent smell and taste of alcoholic drink (Rapp and Mandery, 1986). They are found in variable concentrations in wines, between 80 and 540 mg l^{-1}. When they are present in concentrations under 300 mg l^{-1}, they contribute to a desired complexity, but if they are above 400 mg l^{-1}, these compounds have a negative effect on the aroma. Higher alcohols are divided into two categories: aliphatic and aromatic. Aliphatic alcohols are the most significant in this group (Bertrand, 1986) and include propanol, isoamyl alcohol, isobutanol and isoamylic and amylic alcohols. Aromatic alcohol consists of 2-phenylethyl alcohol and tyrosol. It has been observed in wine that several factors influence the final concentration of higher alcohols in alcoholic beverages: vinicultural conditions and the use of different yeast strains during fermentation (Giudici et al.,1990). Also, the amino acid concentration in the medium affects the production of higher alcohols (Hernández-Orte et al., 2002). Likewise, ethanol concentration, fermentation temperature, must pH, aeration levels, solids levels, grape variety, maturity and skin contact time also affect the higher-alcohol concentration (Fleet and Heard, 1993).

Most aromatic compounds can be formed by yeast starting with exogenous amino acids for degradation, or starting with the assimilable sugars for biosynthesis of ketonic acids. Pyruvate decarboxylase converts the resulting keto acid into the corresponding branched-chain aldehyde, and the alcohol dehydrogenase catalyzes

the NADH-dependent reduction of this aldehyde to the corresponding fusel alcohol (Derrick and Large, 1993). Likewise, higher alcohols can be generated by the degradation of branched-chain amino acids following the Ehrlich pathway: leucine (isoamyl alcohol), valine (isobutanol), 2-amino-butyric acid (n-propanol), isoleucine (amyl alcohol) and phenylalanine (phenethyl alcohol) (Henschke and Jiranek, 1993). The yeast uses at least three aminotransferases, five descarboxylases and six deshydrogenases. Branched-chain amino acid uptake in *S. cerevisiae* is mediated by at least three transport proteins: the general amino acid permease Gap1p, the branched-chain amino acid permease Bap2p, and one or more unknown permeases (Didion et al., 1996).

14.6.1.1 n-Propanol

n-Propanol concentrations in wine are in the range of 14 to 17 ppm. The yeast genus (*S. cerevisiae, H. uvarum/Kloeckera* and *H. guilliermondii*) in pure and mixed culture (Rojas et al., 2003; Gil et al., 1996; Moreira et al., 2005) and the temperature (Erten, 2002) do not influence the concentration of this metabolite. However, differences have been observed in tequila; *Saccharomyces* yeasts generated greater quantity than *Kloeckeras* yeasts: 23 ± 9 ppm and 17.8 ± 5 ppm respectively (Díaz-Montaño, 2004). Similar results have been observed in wines (Romano et al., 2003).

14.6.1.2 Phenethyl Alcohol

The production of phenethyl alcohol using *Saccharomyces* yeasts is influenced by temperature. High concentrations of this metabolite have been detected at low temperatures (13°C). The production of phenethyl alcohol is also influenced by the yeast genus. Phenethyl alcohol is present in wine and tequila production in concentrations of 82.09 ± 0.97 (Rojas et al., 2003) and 22.4 ± 4.9 ppm (Díaz-Montaño, 2004) respectively. Other authors have reported a higher production in *H. guilliermondii* in mixed culture with *H. uvarum* (Moreira et al., 2005). Nevertheless, Gil et al. (1996) did not observe significant differences among the concentrations of this metabolite in pure and mixed cultures with *K. apiculata*, *H. uvarum* and several species of *Saccharomyces spp*.

14.6.1.3 Isobutanol

Isobutanol synthesis is strongly affected by the yeast genus. *S. cerevisiae* biosynthesizes isobutanol (Romano et al., 2003; Moreira et al., 2005; Aragon et al., 1998) in the range of 34.4–64.3 ppm in wine (Gil et al., 1996) and 20.9 ± 7.5 ppm in tequila (Díaz-Montaño, 2004). However, Rojas et al. (2003) observed that *H. guilliermondii* presented higher isobutanol production (57.61 ± 9.35 ppm)

than *S. cerevisiae* (16.43 ± 1.56 ppm), with a production of 32.34 ± 1.89 ppm in mixed culture. Other yeast genus, such as *H. uvarum and K. apiculate,* showed a low production (5–29 ppm) of this compound (Gil et al., 1996). On the another hand, *Kloeckera* yeast showed very low concentrations of isobutanol in the order of 7.7 ± 1.3 ppm in a tequila production process (Díaz-Montaño et al., 2004). Pinal et al. (1997) found that the isobutanol production by *S. cerevisiae* in agave fermentation is influenced mainly by yeast type and the carbon-nitrogen relationship.

14.6.1.4 Isoamyl and Amyl Alcohol

The *Saccharomyces* yeast shows a higher production of Isoamyl and amyl alcohol than the *Kloeckera/Hanseniaspora* yeasts in wines (Rojas et al., 2003; Gil et al., 1996; Romano et al., 1998, 2003) and in tequila (Díaz-Montaño, 2004). However, some authors have reported non significant differences in the production of this metabolite with *H. uvarum, H. guilliermondii* and *S. cerevisiae* in pure and mixed cultures (Moreira et al., 2005). The concentrations detected in wines with *Saccharomyces* are in the range of 164–282 ppm (Rojas et al., 2003; Gil et al., 1996), whereas the concentration of *H. guilliermondii* ranged of about 99.76 ± 8.38 ppm and 26.5 to 50.7 ppm for *K. apiculata* and *H. uvarum* yeasts. The concentrations obtained in tequila are lower in both genera: *Saccharomyces* yeasts show concentrations in the order of 64 ± 20 ppm and the *Kloeckera* yeast 18 ± 7 ppm (Díaz-Montaño, 2004). Isoamylic production in mixed culture of *Saccharomyces* and apiculate yeasts is lower than that of pure culture with *Saccharomyces* (167.53 ± 5.99 ppm) (Rojas et al., 2003); Erten (2002) found significant differences in the quantity of the isoamylic alcohols in the range of temperatures from 10 to 25°C in mixed culture of *Kloeckera* and *Saccharomyces* where concentration increased along with temperature increments. Other authors have reported that the production of this metabolite was not temperature-related (Aragon et al., 1998); Pinal et al. (1997) concluded that the production of isoamyl alcohols is influenced by the yeast, temperature and the carbon-nitrogen relationship.

14.6.2 Glycerol and Succinic Acid

Glycerol, together with ethanol, play a very important role in the fixation of aromas contributing to the viscosity of the wine (Navarre, 1992). Glycerol synthesis is a reaction coupled with glycolysis at the glyceraldehyde-3-phosphate level. Ethanol formation is only possible if the NAD^+ is regenerated. Regeneration of the enzymatic cofactor is occurs mainly in glycerol production. Also, the production of succinic acid and acetic acid reestablishes the NAD^+ to NADH. The *Kloeckera* and *Saccharomyces* yeasts produce glycerol in wine in the order of 1.36 to 4.44 g l^{-1} and 4.8 to 8.3 g l^{-1}, respectively (Ciani and Picciotti, 1995; Comi et al., 2001; Granchi et al., 2002; Brandolini et al., 2002). Likewise, *H. guilliermondii* produces glycerol

in similar ranges in S. cerevisiae (Rojas et al., 2003). With regard to succinic acid, Saccharomyces yeasts present concentrations from 0.45 to 0.71 g l^{-1} and 0.25 to 0.54 g l^{-1} with Kloeckera (Ciani and Picciotti, 1995).

14.6.3 Esters

Esters are formed by yeasts and bacteria during alcoholic fermentation (biological esterification) and very slowly in the course of wine aging (chemical esterification). Esters constitute one of most important groups of compounds that contribute largely to the desirable aromas of fermented beverages (Rapp and Mandery, 1986; Gil et al., 1996). The most significant esters are ethyl acetate (fruity, solvent-like), isoamyl acetate (pear-drops), isobutyl acetate (banana), ethyl caproate (apple) and 2-phenylethyl acetate (honey, fruity, flowery) (Rapp and Mandery, 1986). Volatile esters come from the reaction of saturated fatty acids with alcohol and sometimes with phenol.

Ethyl acetate is the most abundant of all esters, and when it is present in high concentrations, it produces off-flavors. Esters, especially ethyl acetate, are produced by *Kloeckera* yeasts, mainly (Romano, 2003; Rojas et al., 2001; Díaz-Montaño, 2004; Bilbao et al., 1997; Mamede et al., 2005; Zohre and Erten, 2002). Other authors report non significant differences in the production of ethyl acetate between pure and mixed cultures with *H. guilliermondii, H. uvarum* and *S. cerevisiae* (Moreira et al., 2005). However, in mixed cultures, the levels of ethyl acetate produced could contribute to the fruity notes and enhance the general complexity (Gil et al., 1996); Rojas et al. (2003) reported that *S. cerevisiae* increases the concentration of isoamyl acetate in mixed cultures with *H. guilliermondii*. Mamede and Pastore (2006) analyzed the volatile compounds on grape must fermentation by *K. apiculata*; ethyl propionate and propyl acetate were the compounds presents in highest concentration. However, Erten (2002) found significant differences in the quantity of ethyl acetate, ethyl butyrate, isoamyl acetate and ethyl hexanoate in the range of temperatures from 10 to 25°C in mixed cultures of *Kloeckera* and *Saccharomyces*. Low temperatures increase ethyl acetate concentration.

14.6.4 Carbonyl Compounds

The main carbonyl compound in wines is acetaldehyde, with a concentration of about 10–300 mg l^{-1}, and a sensory threshold value of 100 mg l^{-1} (Schreier et al., 1976). Wine yeasts produce this compound in very wide ranges, *S. cerevisiae* 0.5–286 ppm, *K. apiculata* 6–66 ppm and *H. guilliermondii* 10.5–28 ppm. The descriptors used for this compound at low concentrations are: apple-like, citrus-like and nutty. However, high concentrations confer an irritating scent to spicy. Acetaldehyde is one of the major metabolic intermediates, because it is the last precursor before the ethanol is formed. The end-product of glycolysis, pyruvate is converted to acetaldehyde

by pyruvate decarboxylase enzymes. Acetaldehyde is converted into ethanol by alcohol dehydrogenase enzymes (ADHI and ADHII) (Mesias et al., 1983). This step is crucial to maintaining the redox balance in the cell, NADH to NAD^+, required for glycolysis. Fermentation conditions and medium composition affect acetaldehyde production (Liu and Pilone, 2000). The use of sulfur dioxide results in an accumulation of this metabolite, and temperatures at 30°C inhibit the activity of the isoenzymes ADHI and ADHII (Romano et al., 1994). However other investigators report that highest acetaldehyde concentrations are produced in low temperatures (10°C) in mixed cultures of *Kloeckera* and *Saccharomyces* (Erten, 2002).

14.6.5 Volatile acids

Volatile acids constitute a large group of aromatic compounds synthesized by yeast. White wines have between 500–1000 mg l^{-1} of volatile acids, which break down into approximately 90% acetic acid and of about 10% fatty acids (Henschke and Jiranek, 1993). The acetic acid in *S. cerevisiae* is produced as an intermediate of the pyruvate dehydrogenase bypass, a pathway responsible for the conversion of pyruvate into acetyl-CoA through a series of reactions catalyzed by pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase. Acetaldehyde dehydrogenase forms acetate by oxidizing the acetaldehyde produced from pyruvate during fermentation (Pronk et al., 1994). Yeasts producing small quantities of acetic acid are characterized by presenting a high activity of the acetyl-CoA synthetase enzyme (Verduyn et al., 1990). This enzyme generates acetyl-CoA, starting with acetic acid, used in the synthesis of lipids.

Some investigators have reported that the production of acetic acid depends mainly on elevated temperatures (Erten, 2002) and yeast genus (mainly apiculate yeasts) (Bilbao et al., 1997; Díaz-Montaño, 2004; Romano et al., 2003). However, Rojas et al. (2003) reported similar concentrations of acetic acid with *H. guilliermondii* and *S. cerevisiae* in mixed and pure cultures, presenting concentrations higher than 900 ppm. Bilbao et al. (1997) observed differences in the concentrations with regard to temperature. The range of acetic acid concentrations in wines is in the order of 0.66–0.77 g l^{-1} with *Kloeckera* and 0.02–0.04 g l^{-1} with *S. cerevisiae* (Bilbao et al., 1997). In tequila production, *Kloeckera* synthesizes acetic acid in the range of 92.3 ± 18 ppm (Díaz-Montaño, 2004). These authors did not report acetic acid production by *S. cerevisiae* under these conditions.

14.6.6 Phenols

Volatile phenols can make a favorable contribution to the aroma of some wines; they can also contribute to off-flavor. Ethyl-phenols are known to produce a barnyard or stable smell if present in high concentrations. Vinylphenols produce a pharmaceutical

odor, particularly in white wines (Swiegers and Pretorius, 2005). These compounds are derived from ρ-hydroxycinnamic and ferulic acids under the action of yeasts and bacteria (du Toit and Pretorius, 2000). These phenolic acids can be decarboxylated into volatile phenols. They are usually decarboxylated into 4-vinyl derivatives and then reduced to 4-ethyl derivatives (Cavin et al., 1993). The *Brettanomyces/Dekkera* strains mostly produce 4-ethylphenol from ρ-coumaric acid (Chatonnet et al., 1992). The enzymes responsible for such decarboxylations are called phenolic acid decarboxylases (POF1 from *S. cerevisiae*) and several bacteria and fungi have been found to contain the genes coding them. The enzymes coded by these genes are not inhibited by other grape phenolics, and thus there is a high transformation of the vinylphenols into ethylphenols (Swiegers and Pretorius, 2005); Shinohara et al. (2000) analyzed the activity of wine yeasts to decarboxylated ferulic and ρ-coumaric acids in 74 strains of wild yeast (*S. cerevisiae*) and 23 strains of non-*Saccharomyces* yeast. The authors found that a majority of these yeasts were phenolic off-flavor producing strains.

The non-*Saccharomyces* yeasts belong to the genera: *Brettanomyces, Candida, Cryptococcus, Hansenula, Rhodotorula* and *Pichia* produced high or moderate phenolic off-flavors.

14.6.7 Monoterpenes

Part of the aromas of the wines is present under the form of heterosides and terpenics. These compounds are scentless and are called aroma precursors since they are susceptible to being transformed into volatile compounds participating in the aromas of alcoholic fermentation (Mateo and Jimenez, 2000; Swiegers and Pretorius, 2005). This transformation is made by hydrolysis through levurian enzymes: 1′ α-L-rhamnopiranosidase, *β*-glucosidase and 1′ α-L-arabinofuranosidase, located between the cellular wall and the plasmatic membrane in *S. cerevisiae* (Mateo and di Stefano, 1997). Enzymatic hydrolysis is carried out in two stages, in the first stage the α-L-rhamnosidase and the α-L-arabinofuranosidase or *β*-D-apiofuranosidase (depending on the structure of the aglycon moiety) cleave the 1,6-glycosidic. In the second stage, the monoterpens release mono-terpenyl *β*-D-glucosides by means of the action of *β*-glucosidase (Günata et al., 1990). These compounds are particularly abundant in the aromatic varieties of grapes such as: Muscat, Riesling and Gewürztraminer (Günata et al., 1990). The aroma of geraniol and nerol is described as rose-like, the linalool aroma as coriander, linalool oxides as camphorous, and nerol oxides as vegetative. Certain strains of *S. cerevisiae* possess *β*-glucosidase activity (Fia et al., 2005). However, their activity toward glycoside precursors seems to be very low (Hernandez et al., 2003), due to the inhibition of this enzyme for high substrate and ethanol concentrations (Mateo and di Stefano, 1997). The need to have microorganisms with *β*-glucosidases activities has stimulated the search in the non-*Saccharomyces* yeast group, such as: *Brettanomyces, Candida, Debaryomyces, Hanseniaspora* and *Pichia* (Fernandez et al., 2000; Garcia

et al., 2002; McMahon et al., 1999; Rodriguez et al., 2004). The results obtained have made evident that non-*Saccharomyces* yeasts produce mainly β-D-glucosidase (Ferreira et al., 2001; Manzanares et al., 2000; Rodriguez et al., 2004). The best β-glucosidase activity producers were all non-*Saccharomyces* yeasts belonging to: *Candida* (Rodriguez et al., 2004; Manzanares et al., 2000), *Hanseniaspora* (Manzanares et al., 2000), *K. apiculata* (Rodriguez et al., 2004; Ferreira et al., 2001), *Pichia anomalous* (Manzanares et al., 2000; Ferreira et al., 2001) and *Meshnikowia pulcherrima* (Ferreira et al., 2001). A significant increase in this enzyme's activity in the presence of oxygen has been observed (Rodriguez et al., 2004). On the contrary, (Strauss et al., 2001) studied the behavior of yeasts from the *Kloeckera, Candida, Debaryomyces, Rhodotorula, Pichia, Zygosaccharomyces, Hanseniaspora* and *Kluyveromyces* genera without detecting activity of β-glucosidase enzymes. Fia et al. (2005) used a new, rapid fluorimetric method to assay β-glucosidase activity. The authors found β-glucosidase activity in three *S. cerevisiae* strains, in one *Hanseniaspora valbyensts* strain and one *Brettanomyces anomalous* strain.

14.6.8 Ketones

Acetoin is formed in fermentation by the microbial activity of yeasts and bacteria (Romano and Suzzi, 1996). This compound is biosynthesized starting with pyruvic acid through the condensation of one molecule of pyruvate and another of active acetaldehyde which combines with thiamine pyrophosphate (acetaldehyde-TPP complex), both molecules form α-acetolactate. The diacetyl comes from the oxidative decarboxylation of α-acetolactate. Acetoin can be formed by the non oxidative decarboxylation of α-acetolactate acid or by the reduction of diacetyl (Romano and Suzzi, 1996). The main factor affecting acetoin production is the yeast type (Romano et al., 2003).

Numerous reports show that the production of this metabolite is characteristic of apiculate yeasts (Romano and Suzzi, 1996; Fleet and Heard, 1993; Romano et al., 2000, 2003; Mamede et al., 2005; Ciani et al., 2006). Romano and Suzzi (1996) studied 96 strains of *K. apiculata* and *Hanseniapora guilliermondii*, achieving that up to 60% of the *Kloeckera* yeasts produced between 100–200 ppm together with 60% of the *H. guilliermondii* yeasts. Romano et al. (1998, 2000) evaluated the stereoisomers of 2,3-butanediol and acetoin to differentiate *S. cerevisiae*, *K. apiculata* and other non-*Saccharomyces* wine strains (*C. stellata, M. pulcherrima* and *B. bruxellensis*). Significant differences were observed in the acetoin and 2,3-butanediol isomer concentration among the five species of yeasts, while no differences among strains of the same species were observed. The *S. cerevisiae* strain produces about 80% (R,R)-2,3-butanediol whereas *K. apiculata* produces 90% of (R,S)-2,3-butanediol, respectively. Other studies show that *S. ludwigii* yeast presents high concentrations of acetoin in the order of 100–200 ppm. Some strains of *S. ludwigii* can produce > 300 ppm.

Physical and nutritional factors affect acetoin production. Temperature increases the accumulation of this metabolite in the medium (Garcia et al., 1994) as well as high aeration levels (Cowland et al., 1966). Medium composition is important because more acetoin is generated in synthetic medium than in grape juice (Romano and Suzzi, 1992).

14.6.9 Sulfur Compounds

Sulfur-containing compounds have a profound effect on the flavor of wine, owing to their high volatility, reactivity and potency at very low thresholds. Generally, the aromatic contributions of these compounds are considered detrimental to wine quality (Mestres et al., 2000). This type of substance can impart aromas on cheese, cooked vegetables, onion, rubber, garlic, egg and rotten fish. The formation of sulfur compounds is affected by the organic (cysteine and methionine) and inorganic (SO_{4-}) S-containing substances and pesticides in the must, by the nutrient levels of grape musts and by yeast metabolism during fermentation (Swiegers and Pretorius, 2005). Very few reports are available in the literature about the production of sulfur compounds by non-Saccharomyces yeasts (Moreira et al., 2005). Romano et al. (1997) observed several *K. apiculata* and *H. guilliermondii* that produced less than 10 ppm of sulfur dioxide, and *K. apiculata* that produced higher amounts of hydrogen sulfite than *H. guilliermondii.* Moreira et al. (2005) found that heavy sulfur compounds were influenced by the yeast strain used. In general, pure cultures of *H. uvarum* led to the highest production of heavy sulfur compounds. The highest amounts of methionol, 3-methylthiopropionic acid and 2-methyl-tetrahydrothiophen-3-one were found in apiculate yeasts in pure culture and in mixed culture.

14.7 Analysis

In order to achieve alcoholic fermentation control, the complex microbial reaction in the process must be understood. Numerous researchers have studied the participation of *Saccharomyces* and non-*Saccharomyces* yeasts in several alcoholic drinks. Studies have been made on the genetic, metabolic, nutritional and aromatic aspects for distinguishing the fermentative and aromatic capacities of the yeast involved. Evolution of yeast populations and the individual evolution of the most important yeasts during spontaneous fermentation have also been studied under industrial conditions. In addition, yeast characterization by molecular, and physiological methods allow the identification of species and the polymorphism of this species. Currently, alcoholic fermentation control in the industry is performed by starter cultures of *S. cerevisiae,* although low aromatic compound production in alcoholic beverages has resulted from this strategy. Recently, the interest in using starter cultures with apiculate yeast and other non-*Saccharomyces* alone or in

mixed cultures with *S. cerevisiae* has increased. This method caused a positive effect on the aromatic compound production. It increased flavor composition for greater acceptability. It also improved sugar consumption in fermentation with *S. cerevisiae* (glucosophilic yeast) with selective fructose consumption. In addition, different specific growth rates were obtained in *H. guilliermondii, H. uvarum. S. cerevisiae* yeasts in pure and mixed cultures. This suggests that there are interactions between yeasts and culture medium types.

There is very little information on carbohydrate assimilation and its regulation in apiculate yeasts. Differences between non-*Saccharomyces* yeasts have been observed. *H. guilliermondii* behaves like Crabtree-positive yeast such as *S. cerevisiae*, *H. uvarum* and *C. utilis* are Crabtree-negative yeasts. There are other differences also; *Kloeckera* consumes lower quantities of sugar on the pentose-phosphate pathway than *S. cerevisiae*. The development of alcoholic fermentation by apiculate yeast not only depends on its genetic repertoire, certain external factors such as pH, temperature, the presence of inhibitor compounds, nutrient limitation and substrate concentration are involved, too. For years, the first cause of the lowest fermentative capacities by apiculate yeast has been high alcoholic sensibility, although early studies suggest that several factor are involved in these results, such as: high temperatures, low pH, strict anaerobic regime, toxic compounds other than ethanol (excessive SO_2, some pesticide and fungicide residues, the presence of the killer factor and organic and fatty acids) and nutrient limitation. Some studies have shown long periods of apiculate and *S. cerevisiae* yeasts coexisting at low temperatures. High ethanol production in pure culture of apiculate yeast was also reported. The apiculate yeast is characterized as a neutral type resistant to protein killers of *Saccharomyces* and non-*Saccharomyces* yeasts, with the exception of the Kpkt toxin of *K. phaffi*. In addition, they are not tolerant to high concentrations of SO_2.

In the aromatic area, the genotype influences the aromatic compound production. Most authors agree that *S. cerevisiae* strains produce higher amounts of amyl and isoamylic alcohols, n-propanol, 2-phenyl ethanol, acetaldehyde, isobutanol, diacetyl and phenol, whereas *Kloeckera* strains show higher production of acetic acid, monoterpens, acetoin and esters (mainly ethyl acetate). It also shows a significant variability of aromatic compounds between different yeast strains of the same species. Also, fermentation conditions, mainly temperature, oxygen and the medium culture composition, impact the aromatic compound formation and concentrations.

14.8 Perspectives

The use of multi-starter fermentation with non-*Saccharomyces* and *Saccharomyces* yeast could be an interesting alternative to improve alcoholic beverage quality. However, our knowledge of the metabolic interactions between *S. cerevisiae* and non-*Saccharomyces* needs to be improved.

The metabolic, genetics, nutritional and aromatic aspects of several species of apiculate yeasts need to be researched in order to know their fermentative capacities

and improve their yield with the manipulation of some external factors such as: pH, temperature, substrate concentration, oxygen quantities, carbon/nitrogen ratio, oligo-elements and vitamin concentrations. However, we need to detect and establish the physicochemical and biological factors affecting this genus and which do not allow it to survive until fermentation ends. In addition, studying the ethanol tolerance mechanism in *Hanseniaspora* yeasts is very important, as well as the development of efficient and quickest technologies allowing us to evaluate strain biodiversity in the *Hansenispora* genus.

More specific information is required concerning culture conditions affecting aromatic compound formation. Studies of the above points will contribute to enhancing knowledge of these genera, leading to efficient fermentation development with a high control of aromatic compound production.

14.9 Conclusions

The interest in *Hanseniaspora* and *Kloeckera* yeasts has increased throughout the years, as they begin to appear as starter cultures for world class alcoholic drink production.

Although apiculate yeasts do not display as high a fermentative capacity as *S. cerevisiae*, interest in this genus lies mainly in its ability to biosynthesize and/or release interesting aromatic compounds in cultures, thus enabling improvement of wine quality. Nevertheless, the increased knowledge of the nutrition and kinetic aspects of these strains provide us a basis for making this genus more competitive, from a fermentative standpoint.

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