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Volatile phenols in wine: Control measures of *Brettanomyces/Dekkera* yeasts

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ABSTRACT

This review focuses on the considerable amount of research regarding volatile phenols production by *Brettanomyces* and on microbiological and technological parameters that influence development of these compounds during all stages of grape processing and winemaking. Also, volatile phenols impact on wine aroma and quality and prevention methods were discussed. The yeast genus *Brettanomyces* is the major microorganism that has the ability to convert hydroxycinnamic acids into significant concentration of phenolic compounds, especially of 4-ethylphenol and 4-ethylguaiacol, in red wine. When volatile phenols reach concentrations above the sensory threshold in wine, it is then characterized as wine with fault. In order to control the growth of *Brettanomyces* and preclude volatile phenols production, it is helpful to keep good quality of grape, winery sanitation, control of oxygen and sulphite level, as well as orderly check physiochemical composition of wine.

Key words: wine, volatile phenols, *Brettanomyces*, growth, factors, prevention

IZVLEČEK

HLAPNI FENOLI V VINU: KONTROLNI UKREPI ZA KVASOVKE *Brettanomyces/Dekkera*

Ta pregled se osredotoča na znatno število raziskav, ki preučujejo tvorbo hlapnih fenolov s kvasovkami rodu *Brettanomyces*, ter na mikrobiološke in tehnološke parametre, ki vplivajo na sintezo tovrstnih fenolov v vseh fazah predelave grozdja in predelave vina. Prav tako je obravnavan tudi vpliv hlapnih fenolov na aromo in kakovosti vina ter preventivni ukrepi. Kvasovke rodu *Brettanomyces* so glavni mikroorganizmi, ki imajo sposobnost pretvorbe hidroksicimetnih kislin v značilne vsebnosti prisotnih hlapnih fenolov v rdečem vinu, predvsem 4-etilfenola in 4-etilgvajakola. Ko hlapni fenoli dosežejo koncentracije nad senzoričnim pragom zaznave za vino, je potem le-to spoznano kot vino z napako. Za nadzor rasti kvasovk rodu *Brettanomyces* in preprečevanje nastajanja hlapnih fenolov je koristno upoštevati kontrolo kakovosti grozdja ob trgatvi, higienske razmere v vinski kleti, kontrolo količine kisika in sulfita, ter redno kontrolo fizikalno-kemijske sestave vina.

Ključne besede: vino, hlapni fenoli, *Brettanomyces*, rast, dejavniki, preprečevanje

1 INTRODUCTION

Wine is a complex mixture of hundreds of compounds and most of them contribute to sensory characteristics of wine such as the colour, mouth-feel and aroma. There is a huge interest in wine aroma and numerous components are identified as playing a role in specific sensory notes. Flavour and aroma of wine are determined by many factors including grapevine variety, viticultural and winemaking practices, wine maturation and aging conditions. Besides, a wide range of microorganisms influence wine aroma. These microorganisms come into contact with wine

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during grape processing and wine production and their metabolic activities, synthetic and degrading enzymes impact wine aroma. Volatile phenols are important group of compounds that can be formed in wine and their elevated concentrations are associated with unpleasant smelling aroma often described as "phenolic", "leather", "horse sweat", "stable" or "varnish", etc. (Chatonnet et al., 1993; Chatonnet et al., 1992; Rodrigues et al., 2001). Within this group of compounds the most widely represented are 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol. The most unpleasant odoured are 4-vinylphenol (reminiscent of pharmaceuticals, gouache paint and 'Band Aids') and 4-ethylphenol (stables and sweaty saddles). Also, 4-vinylguaiacol (carnations) and 4-vinylguaiacol, (smoky, spicy aromas) are much less unpleasant, but however they are always associated with 4-vinylphenol and 4-ethylphenol, respectively. The olfactory impact of the two vinylphenols or ethyl-phenols should therefore be considered together, in the proportions in which they are present in the wine (Ribéreau-Gayon et al., 2000).

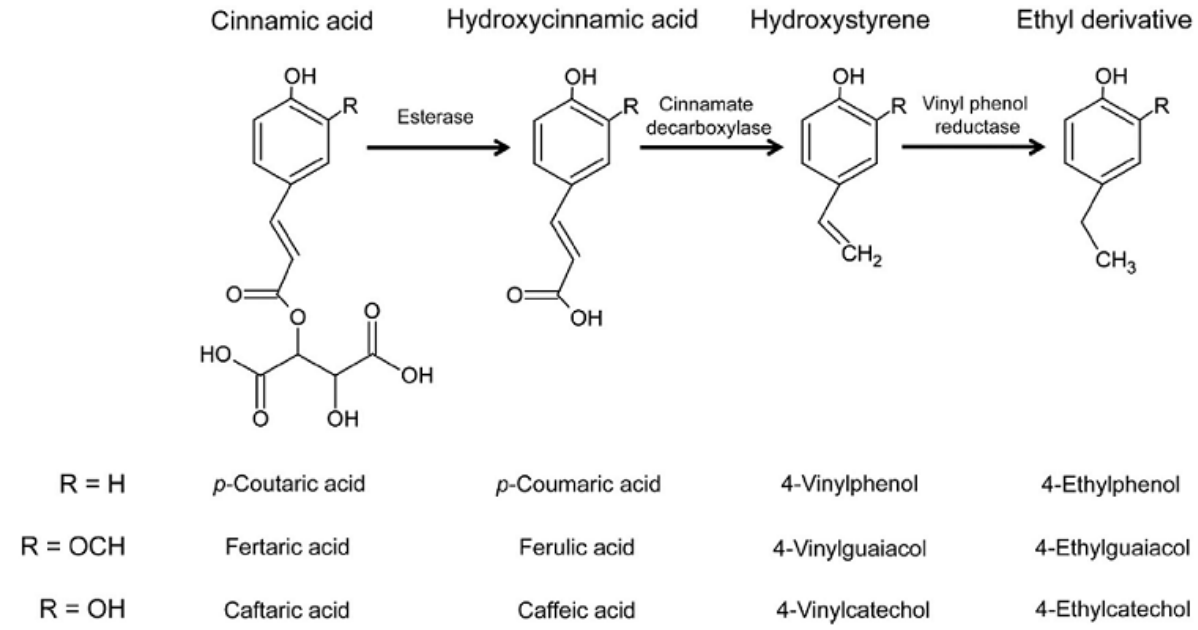
The origin of ethylphenols in wine aroma is due to different sources, but the most usual ways of formation are enzymatic processes of wine yeast and aging. Volatile phenols formation involves the sequential action of two enzymes on a hydroxycinnamic acid (ferulic, *p*-coumaric or caffeic acid) substrate. Hydroxycinnamate decarboxylase first turns these hydroxycinnamic acids into hydroxystyrenes (vinylphenols), which are then reduced to ethyl derivatives by vinylphenol reductase (Edlin et al., 1998; Dias et al., 2003). The enzyme that facilitates decarboxylation is present in a large number of bacteria, fungi, and yeasts, but it is shown that the reduction step is only performed by the species *Brettanomyces bruxellensis* Kufferath & von Laer (*Dekkera bruxellensis* van der Walt), *Dekkera anomala* Smith et van Grinsven, *Pichia guillermondii* Wickerham, *Candida versatilis* (Etchells & T.A. Bell) S.A. Mey. & Yarrow, *Candida halophila* Yarrow & S.A. Mey and *Candida mannitofaciens* (Onishi & Tom. Suzuki) S.A. Mey. & Yarrow (Chatonnet et al., 1995; Edlin et al., 1995; Chatonnet et al., 1997; Dias et al., 2003). Lactic acid bacteria may produce significate amounts of vinylphenols but produce only traces of ethylphenols under wine conditions (Chatonnet et al., 1995, 1997).The fermenting yeast *Saccharomyces cerevisiae* Meyen ex E.C. Hansen and other wine contaminants (e.g. *Pichia* sp., *Torulaspora* sp., *Zygosaccharomyces* sp.), may also produce 4-vinylphenol but are incapable of producing 4-ethylphenol (Chatonnet et al., 1993, 1995; Rodrigues et al., 2001). In *D. bruxellensis* the enzymes cinnamate decarboxylase and vinylphenol reductase are active under wine conditions and so these yeasts should be regarded as the off-flavour producers (Chatonnet et al., 1995, 1997). It is turned out that, 4-ethylguaiacol and 4-ethylphenol are formed in very small concentrations during malolactic fermentation by *Lactobacillus* able to transform phenolic acids in ethyl phenols (Baumes et al., 1986; Dubois, 1983). It has also been observed that ethyl phenols increase in wine during the aging and high levels were found in wine aged in used barrels (Chatonnet et al., 1992).

In this review we examine important group of compounds that influence wine aroma – volatile phenols, their origin in wine, emphasizing parameters that influence growth and activities of *Brettanomyces/Dekkera* yeast, as well as, the measures of prevention its development and volatile phenols production.

2 VOLATILE PHENOLS IN WINE

The accumulation of volatile phenols in wine has been a cause of great concern in modern oenology being now a key point in the control of wine quality. The quality of wine is considered to be mainly affected by the accumulation of 4-ethylphenol and 4-ethylguaiacol, whose presence is commonly described as responsible for sensorial notes reminiscence of leather, horse sweat, animal, and medicinal. Actually there are six compounds responsible for the phenolic flavour: 4-ethylguaiacol, 4-ethylphenol, 4-ethylcatechol and their precursors 4-vinylguiacol, 4-vinylphenol and 4-vinylcatechol (Fig. 1).Volatile phenols found in wines are microbial derived product formed from

hydroxynnamic acids naturally present in grapes (Boulton et al., 1996). Vinylphenols (4-vinylphenol and 4-vinylguaiacol) and ethylphenols (4-ethylphenol and 4-ehtylguaiacol) may be produced in wine, in a sequential pathway, due to microbial activity, imparting undesirable odours and flavours. Mainly, they are formed by metabolism of hydroxycinnamic acid (ferulic, *p*-coumaric or caffeic acid) substrate by *Brettanomyces/Dekkera* yeast which involves the sequential action of two enzymes. Hydroxycinnamate decarboxylase first turns these hydroxycinnamic acids into hydroxystyrenes (vinylphenols), which are then reduced to ethyl derivatives by vinylphenol reductase (Figure 1). The decarboxylation step is present in a large number of bacteria, fungi and yeast species (Degrassi et al., 1995; Edlin et al., 1995; Suezawa et al., 1995). The reduction step is much less frequent and has been reported as particularly effective in the species *Dekkera bruxellensis* (Chatonnet et al., 1995, 1997), *D. anomala* (Edlin et al., 1995), *Pichia guilliermondii* (Dias et al., 2003), *Candida versatilis, C. halophila* and *C. mannitofaciens* (Suezawa, 1995).



R = H	*p*-Coutaric acid	*p*-Coumaric acid	4-Vinylphenol	4-Ethylphenol
R = OCH	Fertaric acid	Ferulic acid	4-Vinylguaiacol	4-Ethylguaiacol
R = OH	Caftaric acid	Caffeic acid	4-Vinylcatechol	4-Ethylcatechol

Figure 1: The formation of volatile phenols from their hydroxycinnamic acids precursors
Slika 1: Tvorba hlapnih fenolov iz prekurzorjev hidroksicimetnih kislin

The presence of this kind of aroma character can be considered either negative or positive depending on the concentration and expectation of a particular wine. At low concentration, these compounds can contribute to aroma complexity, but in concentration above threshold can create an unpleasant experiences (Chatonnet et al., 1990). Also, the judgment of brett wines is controversial and depends on individual and cultural preferences (Wedral et al., 2010).

Based on the literature, there are a lot of reports on presence of *Brettanomyces* metabolites in wine indicating a worldwide issue. Although only trace amounts are present in must, wine contains volatile phenols at concentrations between a few tens and several hundreds of µg l^{-1} (Dubois, 1983; Chatonnet et al., 1988). The perception threshold of an odoriferous compound is conventionally considered to be the minimum concentration at which its presence in a model dilute alcohol solution is detectable by 50 % of trained tasters. The recognition threshold of an odoriferous compound corresponds to its perception threshold in wine. The preference threshold of a compound is the concentration above which the overall aroma of a wine is affected. In the case of vinyl- and ethylphenols, the preference thresholds have been estimated at 720 µg l^{-1} for 4-vinylphenol and 4-vinylguaiacol in white wines, and at 420 µg l^{-1} for 4-ethylphenol and 4-ethylguaiacol in red wines. It has been reported (Licker et al., 1999) that wines

with high, medium and no brett character have an average 4-ethylphenol concentrations of 3.00, 1.74 and 0.68 mg l^{-1}, respectively.

Volatile phenols concentration in red wine and its sensory descriptors are presented in Table 1 (Steensels et al., 2015; Curtin et al., 2005). White wines contain variable quantities of vinyl-phenols but comparing to red wines almost no of ethylphenols. On the contrary, reds contain only small quantities of vinyl-phenols and have variable concentrations of ethyl-phenols (Table 2). The volatile phenol composition of rose wines is between those of red and white wines (Chatonnet et al., 1992b, 1993b). The variety of grapevine used also affects the sensorial perception of ethylphenols. Phister and Mills (2004) indicated the detection thresholds to be high in mono-varietal Cabernet Sauvignon wines, and lower in Tempranillo wines. Pollnitz et al. (2000) analysed 61 bottles of different commercially available varietal Australian red wine, where a 4-ethylphenol was detected in all analysed wines. The concentrations found in the wines varied between 2 µg l^{-1} in a Merlot and 2660 µg l^{-1} in a Shiraz, with a mean concentration of 795 µg l^{-1}. 4-Ethylguaiacol was also found in every red wine analysed, varying in concentration from 1 µg l^{-1} in a Pinot Noir up to 437 µg L^{-1} in a Merlot with a mean concentration of 99 µg l^{-1}. An average ratio of 4-ethylphenol and 4-ethylguaiacol was approximately 10:1 for Cabernet Sauvignon, 9:1 for Shiraz, 8:1 for Merlot and 3.5:1 for Pinot Noir, what is in accordance with reports by Chatonnet et al. (1992; 1995).

The ratio of 4-ethylphenol to 4-ethylguaiacol also varied from wine to wine with reports varying from 3:1 to over 40:1 (Gawel et al., 2004; Steensels et al., 2015). The reason for these differences in wine are still not fully understood, even though they are likely caused by the combined effect of differing ratios between wines *p*-coumaric and ferulic acids (the precursors of 4-ethylphenol and 4- ethylguaiacol, resp.) and of different strains of *Brettanomyces/Dekkera* with some being more effective in producing one compound relative to the other (Buron et al., 2012; Gawel, 2004; Vigentini et al., 2008). Fariña et al. (2007) analysed six Tannat wines from Uruguay and results indicated that in three of the six analysed wines the 4-ethylphenol was found, and quantified in two of them with concentrations of 1120 and 170 g l^{-1}. 4-ethylguaiacol was quantified only in one of the six wine samples, in concentrations of 120 g l^{-1}. Recently Baša-Česnik et al. (2016) determined 4-ethylphenol, 4-vinylphenol, 4- ethylguaiacol and 4-vinylguaiacol in Teran PTP wines that were produced in the Kras winegrowing district (Slovenia). During the 2011-2013 periods, these authors found average concentrations: 153±193 µg l^{-1} for 4-ethylphenol, 1265±682 µg l^{-1} for 4-vinylphenol, 69±94 µg l^{-1} for 4-ethylguaiacol and 128±106 µg l^{-1} for 4-vinylguaiacol. Earlier from the same region the concentration of 4-ethylphenol in bottle-aged Teran PTP wines was reported to be 1016, 678 and 616 µg l^{-1} for the 2007, 2008 and 2009 vintages, respectively (Čuš et al., 2011).

Volatile phenols are usually analysed by gas chromatography, after their extraction from the sample. Traditionally, liquid–liquid extraction methods were employed (Monje et al., 2002; Chatonnet, 1988), but now simpler and more selective extraction methods are applied, such as solid-phase extraction (SPE) (López et al., 2002; Domínguez et al., 2002) solid-phase microextraction (SPME) (Monje et al., 2002; Martorell et al., 2002) or stir bar sorptive extraction (SBSA) (Díez et al., 2004).

Table 1: Volatile phenols in wine (Steensels et al., 2015; Curtin et al., 2005)
Preglednica 1: Hlapni fenoloi v vinu (Steensels in sod., 2015; Curtin in sod., 2005)

	Concentration in red wine (ppb)	Sensory descriptor
4-vinylphenol	8.8 – 43	Phenolic, medicinal
4-vinylguaiacol	0.2 – 15	Clove-like
4-ethylphenol	118 - 3696	Medicinal, horsy
4-ethylguaiacol	1 – 432	Spicy, clove-like
4-ethylcatechol	27 – 427	Phenolic, medicinal

Table 2: Ethyl- and vinyl-phenol concentrations in different wines (μg l^{-1}) (Chatonnet et al., 1992, 1993)
Preglednica 2: Vsebnosti etil- in vinilfenolov v različnih vinih (μg l^{-1}) (Chatonnet in sod., 1992, 1993)

Volatile phenols	White wines *n* = 54	Rose wines *n* = 12	Red wines *n* = 83
Vinyl-4-phenol			
Minimum	73	3	0
Maximum	1150	215	111
Mean	301	71	35
Standard deviation (%)	79	99	75
Vinyl-4-guaiacol			
Minimum	15	4	0
Maximum	496	75	57
Mean	212	17.5	12
Standard deviation (%)	44	113	79
Ethyl-4-phenol			
Minimum	0	0	1
Maximum	28	75	6047
Mean	3	20	440
Standard deviation (%)	229	122	179
Ethyl-4-guaiacol			
Minimum	0	0	0
Maximum	7	15	1561
Mean	0.8	3	82
Standard deviation (%)	225	159	230

Coulter et al. (2004) concluded that the extent to which the sensory properties of a wine may be affected by 4-ethylphenol depends on the style and structure of the wine, i.e. the concentration and intensity of other wine compounds that could mask (e.g. volatile oak compounds) or accentuate (e.g. 4-ethylguaiacol) the aroma of 4-ethylphenol. For example, in a light-bodied red wine with little oak influence, the sensory perception threshold of 4-ethylphenol may be as low as concentration of 350 μg l^{-1}, compared with 1000 μg l^{-1} in a full-bodied red wine with intense fruit and considerable oak influence.

3 THE PRODUCTION OF VOLATILE PHENOLS BY *BRETTANOMYCES/DEKKERA*

Brettanomyces bruxellensis is yeast found on surfaces of grapes as well as in barrels, but the greatest concern is its presence in wine. The yeasts of the genus *Brettanomyces*, or its teleomorph *Dekkera*, were first described by Claussen in 1903, in beer production (Gilliland, 1961). *Brettanomyces*/*Dekkera* yeasts exist in two forms: *Brettanomyces*, the asexual, non-sporulating form, and *Dekkera*, the sexual, sporulating form. These genera are particularly known as spoiling agents in beer, wine, cider and soft drinks industries (Deak and Beuchat, 1996). The current taxonomy includes five species within the genera of *Dekkera*/*Brettanomyces*. Those are the anamorphs *Brettanomyces bruxellensis*, *Brettanomyces anomalus* Custers, *Brettanomyces custersianus*van der Walt, *Brettanomyces naardenensis* Kolfsch. & Yarrow, and *Brettanomyces nanus* (M.T. Sm., Bat. Vegte & Scheffers) M.T. Sm., Boekhout, Kurtzman & O'Donnell, with teleomorphs existing for the first two species, *Dekkera bruxellensis* and *Dekkera anomala* (Cocolin et al. 2004; Oelofse et al., 2008). Different strains of *Brettanomyces* can show great differences in their production of volatile phenols (Joseph and Bisson, 2004). In wines, the metabolic products responsible for spoilage by *Brettanomyces/Dekkera* sp. are mainly volatile phenols, isovaleric (3-methylbutyric) acid, tetrahydropyridines and acetic acid (Heresztyn, 1986; Licker et al., 1999; Larue et al., 1991; Ciani and Ferraro, 1997). Coulter et al. (2004) found that 4-ethylphenol and 4-ethylguaiacol (both compounds associated with 'Band-aid', 'medicinal', 'barnyard' and 'stable' aroma characters and 'metallic' taste attributes) were the main compounds derived from *Brettanomyces* that were associated with off-odours in red wines. They found that the concentration of isovaleric acid (associated with 'sweaty', 'cheesy' and 'rancid' characters) was independent of the concentrations of these two key spoiler compounds. It therefore appears that isovaleric acid may be involved in additional sensory effects with other *Brettanomyces*-derived compounds, thereby enhancing the apparent aroma intensity of those other compounds.

Works on *D. bruxellensis* have mainly focused on its early detection to reduce economic losses (Wedral et al., 2010). Also, studies have been carried out to understand the mechanisms of 4-vinylphenol and 4-ethylphenol production (Dias et al., 2003; Godoy et al., 2008; Harris et al., 2008; Harris et al., 2009). Barata et al. (2008) concluded that the production of 4-ethylphenol in red wines is related to the presence of growing populations of *D. bruxellensis*, demonstrating that the primary management objective should not be their complete elimination but their maintenance at constant levels. If the numbers of *D. bruxellensis* increase, removal or inactivation procedures must be performed. Coulon et al. (2009) highlighted a relationship between the physiological state of *B. bruxellensis*, and its capacity to produce volatile phenols. Cultivable populations seem indeed able to synthesize higher amounts of ethyl-phenols than viable but non-cultivable cells. They also stated that sequential ethyl-phenol production could systematically be correlated to the *B. bruxellensis* physiological state and found that maximum vinyl-phenol concentrations were found with fast-multiplying cells. They then decreased, indicating that the yeast metabolism is centred on ethyl-phenol production. This was generally accompanied by a population regression or/and a non-cultivable state shift. Other authors had already noted that ethyl-phenols synthesis occurred during the late exponential phase or stationary growth phase in synthetic media (Dias et al. 2003; Harris et al. 2008) or wine (Romano et al. 2008). This indicates that the overall information about the growth physiology of *D. bruxellensis* and metabolite production appears to be sometimes contradictory. Some authors concluded that production of ethylphenols was intrinsically related to *D. bruxellensis* growth (Barata et al., 2008b; Dias et al., 2003; Vigentini et al., 2008), whereas other studies suggest the existence of a sulphite-induced viable but non-culturable subpopulation, which is able to produce vinylphenols and ethylphenols (Agnolucci et al., 2010; Laforgue and Lonvaud-Funel, 2012; Serpaggi et al., 2012).

*Brettanomyce*s spoilage of wine usually occurs when they are fermented or aged in oak barrels (Rayne et al., 2008). The yeast grows slowly so it usually imparts flavours only when the wine is aged. Since *Brettanomyces* is present at low

numbers early in the fermentation, it is outnumbered by other indigenous yeast and may go undetected (Wedral et al., 2010). Garde-Cerdán et al. (2002) investigated the behaviour of the barrels at the completion of their cycle of use (5–6 years old) on the volatile composition of a red wine. In all the wines high concentrations of 4-ethylphenol and 4-ethylguaiacol were found, and these have a negative impact on the quality of the product, probably due to the presence of contaminating microflora as the barrels were old (Garde-Cerdán et al., 2002).

The results of Silva et al. (2011) suggest that the conversion of 4VP into 4EP, catalysed by the vinylphenol reductase, may lead to the re-oxidation of NADH. An analogous statement was made by Fugelsang and Edwards (2007) for *Brettanomyces/Dekkera*: since a reduced cofactor is generally required for the enzymatic reduction activity, it is likely that the production of volatile phenols, specifically the reduction of 4-vinylphenol into 4-ethylphenol, is a source of NAD^+ during growth of this organism by maintaining the redox balance of its cells in red wines.

However, red wines are more susceptible to *Brettanomyces bruxellensis* due to their lower acidity, higher polyphenol concentration and barrel aging. Therefore *Vitis vinifera* red varieties with higher polyphenol concentration are the most susceptible to the *Brett* (Wedral et al., 2010). The loss of viability in white wines is largely due to the efficacy of sulphur dioxide at low pH (Loureiro et al., 2006) and due to absence of precursor compounds (Chatonnet et al., 1992).

3.1 Factors affecting growth of *Brettanomyces* and production of volatile phenols

3.1.1. Carbon and energy sources

Bioconversion of ethylphenols precursors, like *p*-coumaric, by *B. bruxellensis* is highly dependent on the growth media (Dias et al., 2003) and cinnamic acid ratios (Romano et al., 2008). Dias et al. (2003) showed that reduction step was dependent on the carbon and energy source, i.e. high conversion rates of *p*-coumaric acid to 4-ethylphenol only occurred when glucose or ethanol were substrate. Regarding to trehalose sugar, Chatonnet et al. (1995) suggested that this residual sugar may allow synthesis of 4-ethylphenol. These results are not in agreement with Dias et al. (2003) who demonstrated that contribution of this sugar to the overall production of 4-EP is not relevant in wine.

D. bruxellensis grown in the presence of glucose showed relatively low growth rate compared to other wine related yeast such as *S. cerevisiae* and *Z. bailii* (Lindner) Barnet et al. (Rodrigues et al., 2001a). Vigentini et al. (2008) found that *D. bruxellensis* used fructose as a preferred carbon source in a synthetic medium with a high concentration of ethanol. Also, the amount of less than 2 g l^{-1} fermentable sugar in "dry" wines is still not limitation to the production of 4-EP by *D. bruxellensis.* The production of 4-ethylphenol was detected at sugar concentrations over 0.2 g l^{-1} and increased under higher sugar concentrations (Barata et al., 2008). In line with this, Sturm et al. (2015) showed that 210 mg l^{-1} of sugars (glucose, fructose and trehalose) was enough to allow *B. bruxellensis* growth. Sugars were completely consumed during the first growth phase, whilst consumption of glucose and fructose was faster than trehalose.

The growth of *Brettanomyces/Dekkera* in synthetic media containing autolysed *Saccharomyces cerevisiae* has also been studied (Guilloux-Benatier et al., 2001). Under these conditions it was observed that these contaminating yeasts grew easily, even in glucose concentrations of less than 150 mg l^{-1}. However the quantity of ethylphenols formed was smaller than expected, probably because of the adsorption of the phenolic compounds by the cell wall fragments. More recent studies have reported the high capacity of yeast cell walls to adsorb phenolic compounds (Morata et al., 2005; Morata et al., 2003).

The inhibition of growth and 4-ethylphenol production by 13 % (v/v) of ethanol is observed in wines with high concentration of ethanol and these wines did not show high concentrations of this phenol (Rodrigues et al., 2001; Dias et al., 2003). These authors showed that 4-ethylphenol concentrations in wines were not correlated with acetic acid concentrations expect in media with high sugar concentration, as also observed by Gerós et al. (2000) in *D. anomala.* This observation indicates that these two *D. bruxellensis*

spoiling features (acetic acid and 4-ethylphenol production) are independent. However, acetic acid production is variable among strains of the genera *Dekkera/Brettanomyces* cultivated under the same conditions (Freer, 2002).

Barata et al. (2008) demonstrated that even in the presence of a carbon and energy source, the levels of volatile phenols do not increase in commercial red wines when *D. bruxellensis* is not growing. This conclusion has direct implications in the management of the phenolic taint, because it shows that the primary objective should be the prevention of actively growing populations and not the reduction of *D. bruxellensis* to the lowest possible level, as suggested by Renouf et al. (2007). In fact, complete absence of viable cells of *D. bruxellensis* is not easy to achieve under winery conditions, especially when oak aging is used due to the porous nature of the wood (as discussed by Loureiro and Malfeito-Ferreira, 2006).

Coulon et al. (2009) confirmed as it has previously been observed that *B. bruxellensis* is not very demanding from a nutritional point of view and that it can grow with other energy sources than glucose and fructose (Alguilar Uscanga et al., 2000; Conterno et al., 2006).

3.1.2. Precursors

The biosynthesis of volatile phenols is related to the sequential activity of two enzymes which decarboxylate hydroxycinnamic acids (ferulic, *p*–coumaric and caffeic acids) spontaneously present in grapes into vinylphenols, which are then reduced to ethylphenols (Steinke et al., 1964). The formation of volatile phenols in wine depends on the presence of precursors and is proportional to the size of the *Brettanomyces/Dekkera* population (Gerbeaux et al., 2000, Suárez et al., 2007). It has previously been suggested that microorganism's decarboxylate HCAs in order to produce less toxic compounds (Goody et al., 1982). Vinyl-phenol reductase and cinnamate decarboxylase, the two enzymes involved in ethyl-phenol production in *B. bruxellensis* are precursor inducible, but these precursors also have an inhibitory effect on *B. bruxellensis* growth (Harris et al., 2008). Variations in wines can be noted because of the instability, esterification and cell-adsorption of *p*-coumaric acid (Salameh et al., 2007).

Grapevine varieties differ in the quantity of phenolic acids present in the berries (Rodrigues et al., 2001; Morel-Salmi et al., 2006; Morata et al., 2007; Rentzsch et al., 2007). The presence of the three hydroxycinnamic acids (caffeic acid, ferulic acid and *p*-coumaric acid) in grapes originate from their bound form with tartaric acid known as caftaric acid, fertaric acid and coutaric acid, respectively. Goldberg et al. (1998) measured the concentration of *p*-coumaric acid, the precursor to 4-ethylphenol, in single variety red wines from various countries and found that Pinot Noir had a lowest concentration of *p*-coumaric acid, among all studied varieties and countries. General concentrations for hydroxycinnamic acids present in *Vitis vinifera* L. juice (oxidative and hydrolytic losses prevented) are about 150 mg l^{-1} of caftaric acid, 20 mg l^{-1} of coutaric acid and 1.0 mg l^{-1} of fertaric acid (Boulton et al., 1996). Other grapevine varieties have been identified to have higher amounts of hydroxycinnamic acids for example wines from Grenache variety can contain between 270-460 mg l^{-1} of caftaric acid (Morel-Salmi et al., 2006). Other wine varieties mean values for caftaric acid range from 50 to 60 mg l^{-1} (Rentzsch et al., 2007). Dias et al. (2003) studied the capacity of a number of yeasts present in wine microbiota to produce 4-ethylphenol from *p*-coumaric in model media. Molar conversions of 90 % were reported for *D. bruxellensis, D. anomala* and *P. guillermondii*; other fermentative yeasts were incapable of producing 4-ethylphenol at these rates of conversion.

Recently, Cabrita et al. (2012) showed that phenolic acids concentration decreases while volatile phenols concentration increases and the proportion of caffeic acid taken up by *Dekkera bruxellensis* is lower than that for *p*-coumaric or ferulic acid, i.e. less 4-ethylcatechol is formed. Before, the presence of 4-ethylcatechol, has been reported only once, by Hesford et al. (2004), until Carrillo and Tena (2007) reported the presence of 4-ethylcatechol in some wines affected by *Brettanomyces*. Study of Cabrita et al. (2011) has shown that 4-ethylcatechol is the last one to appear in wine, and it is the least significant. In this study conversion rates greater than 90 % were obtained for the conversion of *p*-coumaric acid into 4-ethylphenol and from ferulic acid into 4-ethylguaiacol, but a rate smaller than 20 % was

ethylcathecol. These results may justify why only the presence of 4-ethylcatechol in wines was reported (Hesford et al., 2004). Although caffeic acid is present in wines in considerable amounts and has a structure similar to the other phenolic acids, these results seem to indicate that *p*-coumaric and ferulic acids are easily used by yeast metabolism.

In the study of Sturm et al. (2015), all five examined *D. bruxellensis* strains were able to simultaneously metabolise *p*-coumaric and ferulic acid with production of their respective volatile phenols, being the conversion rate of ferulic acid lower than *p*-coumaric acid. In contrast, Oelofse et al. (2009) suggested that the conversion pathway of ferulic acid as precursor was preferred to *p*-coumaric acid by different *D. bruxellensis* strains grown in wine spiked with similar amounts of both compounds (100 mg l^{-1}). Besides, the ratio of *p*-coumaric and ferulic acid was 8:1 (Sturm et al., 2015) and it has been proven that ferulic acid is slightly more toxic to the yeast than *p*-coumaric acid (Harris et al., 2008).

Recently, Lentz et al. (2015) showed that caffeic acid was the weakest inhibitor of the HCAs tested. This observation is in general agreement with published data for other strains that show weak or no inhibition of growth by this compound compared to other cinnamic acids (Harris et al., 2009). When a strain showed variation for inhibition by ferulic acid and *p*-coumaric acid, it's turned out that ferulic acid was always a more potent inhibitor (Lentz et al., 2015). This data supports results from similar experiments using different strains of *B. bruxellensis* and *B. anomalus* (Harris et al., 2009). It appears that *Brettanomyces* in general are only weakly inhibited by caffeic acid, and are slightly more susceptible to ferulic than *p*-coumaric acid.

Kosel et al. (2014) examined the impact of ethanol, and hidroxycinnamic and vinylphenol precursors on the production of volatile phenols in fermentations of mixed and pure cultures of yeasts *Saccharomyces cerevisiae* and *Dekkera bruxellensis*. Results showed that in mixed culture fermentations less vinylphenols and more ethylphenols were produced in comparison with *D. bruxellensis* pure culture fermentations. Vinylphenol precursors significantly inhibited the growth of *S. cerevisiae* and the production of ethylphenols. It was found that *D. bruxellensis* genes encoding for enzymes coumaric acid decarboxylase (CAD) and vinylphenol reductase (VPR) are more responsive to vinylphenol precursors in comparison with hidroxycinnamic acids. Consequently, higher concentrations of vinylphenols in the cell were found to be more cytotoxic than hidroxycinnamic acids. Also, these authors showed that 10 % of ethanol strongly reduced the growth and volatile phenol production of yeasts *D. bruxellensis* and *S. cerevisiae*.

3.1.3. Temperature, ethanol concentration, pH

The impact of different chemical factors and different temperatures on volatile phenol production has been well studied. Different strains of *B. bruxellensis* vary in their capacity to produce volatile phenols and it is always greater at lower alcohol concentrations (more are made at 12 % v/v than at 14 % v/v) and at higher temperatures (e.g., more is produced at 18 °C than at 13 °C) (Gerbeaux et al., 2000). Recently, study of Kosel et al. (2014) confirmed that low ethanol concentrations induced higher production of volatile phenols by *S. cerevisiae* and *D. bruxellensis.* Little significance is attributed to the pH of the wine or the presence of residual sugars in this respect. The intensity and temperature of maceration and the use of pectolytic enzymes have been studied as possible factors conditioning the formation of volatile phenols by *Brettanomyces* and *Dekkera* from hydroxycinnamic acids released from the grape skins (Gerbeaux et al., 2002). Godoy et al. (2008) demonstrated that both enzymatic activities were stable at pH 3.4, but in the presence of ethanol the coumarate decarboxylase activity decreased drastically while the vinyl reductase activity was more stable.

Dias et al. (2003) described that a 5 % ethanol concentration is adequate to obtain volatile phenols in the culture medium, since an increase in ethanol concentration is detrimental to the yeast population and consequently leads to a decrease in volatile phenols. Garde-Cerdán et al. (2008) showed that alcohol concentration was the oenological parameter that had the greatest impact on the accumulation of volatile compounds in wines and according to same author accumulation of ethylphenols in the wines diminished as the

alcohol concentration of the wines increased from 12.5 % to 13.5 %.

Results obtained by Ganga et al. (2011) are similar to that obtained by Dias et al. (2003) and Garde-Cerdán et al. (2008), where although *D. bruxellensis* presents basal coumarate decarboxylase activity, it is necessary to add ethanol to the culture medium to increase its production. Ganga et al. (2011) investigated the influence of the interaction between the concentration of *p*-coumaric acid, ferulic acid and ethanol as well as growth temperature on the production of CD activity and the expression of a putative gene that codes for this enzymatic activity. These authors concluded that the interaction of cinnamic acids with growth temperature, and growth temperature with ethanol concentration, as well as ethanol concentration, are highly important variables in the production of CD activity. Analysing the assayed growth temperatures (16 ºC to 28 ºC) shows that the increase of this parameter brings about a decrease of coumarate decarboxylase activity. This affirmation is not in accordance with reports by Benito et al. (2009), who indicated that at a temperature between 20 and 30 ºC the yeast consumes the greatest quantity of *p*-coumaric acid, which is indirectly associated to the presence of higher coumarate decarboxylase activity . At 22 ºC with 10 vol. % alcohol, the yeast on average only metabolizes 38 % of the *p*-coumaric acid in the culture medium, while with 3 % ethanol, 74 % of the initial *p*-coumaric acid was metabolized by the yeast. This result was also obtained at 16 ºC. Salameh et al. (2008) indicated that *p*-coumaric acid can react with the ethanol in the medium or be absorbed through the yeast wall, which leads to a decrease in the acid concentration in the culture medium. A slow metabolization of *p*-coumaric acid in the culture medium is closely related to the growth rate of the yeast, with yeast growth slower at 10 % than at 3 %.

3.1.4. Conditions during aging and storage of wine

The use of old wooden casks can increase the presence of *Brettanomyces* and *Dekkera* species in wine due to difficult cleaning and impossibility of their sterilization. *Brettanomyces/Dekkera* has been found at 8 mm down within the wood of barrel staves (Malfeito-Ferreira, 2006). These yeasts survive treatments where contact with SO_2 is limited, e.g., around bung holes, in the oak structure, and in yeast sediments (lees). Besides, *Brettanomyces custersii* and *Dekkera* intermedia metabolise cellobiose, a disaccharide, forming the basic repeating unit of cellulose (a structural polysaccharide of wood) (Freer, 1991; Park et al., 1999; Park et al., 2000).

The age of the barrel greatly influences the growth of *Brettanomyces* populations during the long aging of red wines. Old barrels favour *Brettanomyces* contamination – oak wood is extremely porous and yeasts deep in the barrel staves are difficult to eliminate – and any ethylphenols in the mass of the wood are released (Chatonnet et al., 1999). However, some authors (Lonvaud-Funel and Renauf, 2005) report that, due to their higher oxygen and sugar contributions, new barrels are even more likely to favour the maintenance of large *Brettanomyces* populations. It is probable that the wood pores become blocked as the barrels are used, so the oxygen arriving via them decreases. The frequent re-use of casks and the use of the micro-oxygenation technique to accelerate wine maturation, facilitates the polymerisation of wine pigments and the modification of the wine volatile profile (frequently associated with the use of oak chips or barrel aging), resulting in the proliferation of *Brettanomyces/Dekkera* (Aguilar-Uscanga et al., 2003; Ciani and Ferraro, 1997; Ciani et al., 2003).

Pollnitz et al. (2000) determined 4-ethylphenol and 4-ehtylguaiacol concentration in red wine aged in new and used French and American oak barrels of different ages. Wine stored in shaved and renovated with fireing oak barrels contained up to 85 % less 4-ethylphenol and 4-ethylguaiacol than wine stored in usual barrels of the same age that were not shaved. Oak barrels that become contaminated with *B. bruxellensis* cannot be effectively sterilized. Neither careful washing followed by rinsing with sulphited water, nor shaving and firing, nor ozone treatment achieves sterilization (Pollnitz et al., 2000) – a result of the large internal volume and porous nature of oak barrels. The sanitation of barrel wood requires at least 7 g of SO_2 gas per barrel. Filled wine barrels should receive 20–25 mg l^{-1} of free SO_2 (30–35 mg l^{-1} during hot summers) (Henick-Kling et al., 2000). Malfeito-Ferreira (2005) tested four barrel sanitation procedures: (I) cold water rinse followed

by three hot water rinses (70 °C); (II) the same as the previous plus filling with an aqueous solution of SO_2 (200 mg l-1, pH 3) and storing for one month; (III) cold water rinse, followed by filling the barrel with hot water (90 °C for 10 min); (IV) cold water rinse, followed by a hot water rinse (70 °C) and low pressure steam (10 min). The last one appeared the most effective and author recommended isolation of brett infectioned barrels to reduce the contamination of others during disinfection and wine pumping.

Garde-Cerdán et al. (2010) analysed 510 wines, from four different Spanish geographic zones, and aged in different oak barrels types for at least 6, 12 and 18 months. Theyconcluded that accumulation of volatile oak compounds and ethylphenols was affected mainly by the storage time of the wines in the oak barrels, while the oenological parameters, the geographic origin and the oak barrel type had smaller influences on the accumulation of these volatile compounds in the wines. The total average of the ratio 4-ethylphenol and 4-ethylguaiacol of the aged-6 wines was below that of the aged-12 and aged-18 wines. A ratio for the aged-12 and aged-18 wines was within the range found by Pollnitz et al. (2000), i.e. between 3.5 and 10.1.

However, young red wines in stainless-steel vessels or bottled wines are also prone to this type of spoilage (Rodrigues et al., 2001; Renouf et al., 2007). At the end of barrel aging, before bottling, residual population of *B. bruxellensis* can often be detected (Nisiotou and Gibson, 2005; Renouf et al., 2006; Curtin et al., 2007). Although populations are usually too low at this point to synthesize ethyl-phenols, they could further develop and spoil the wine during bottle storage, when the winemaker can no longer intervene (Coulon et al., 2010).

Recently, results obtained by Rubio et al. (2015) indicated the spoilage risk exists when *Brettanomyces* cells are present, even at a low level, in wines subjected to aging, both in the cask and the bottle. *Brettanomyces* presence and ethylphenol production during aging, is affected more by the aging conditions (aerobic/anaerobic and sulphiting) than by the origin of the oak. They had shown that aging only under aerobic conditions with racking and sulphur dioxide addition, showed lower *Brettanomyces* levels than the combined aerobic and anaerobic maturation with racking but without sulphur dioxide addition. Also, wines aged in Chinese oak revealed different behaviour to the other three in terms of the level of *Brettanomyces* and in the ethylphenol concentration, probably due to the fact that this oak has the highest porosity.

4 MEASURES FOR THE PREVENTION

Prevention of the growth of *Brettanomyces/Dekkera* in wine involves attention to fruit quality and winery sanitation, control of sulphite and oxygen levels, as well as to the use of uncontaminated barrels (Wedral et al., 2010). *B. bruxellensis* is found on damaged grapes and in winery equipment, so effective general sanitation is the first step in the prevention. Adequate amounts of SO_2 should be added to fermentation vessels and maintained during fermentation in order to inhibit growth of *B. bruxellensis.* After addition of SO_2, *Brettanomyces* enter in to viable but not culturable state, so the yeast may be still present after depletion of free SO_2 (Umiker et al., 2007). As control factors, winemakers should consider using a starter yeast culture, alcohol, acid levels, temperature and oxygen exposure. Using a starter culture can decrease indigenous fermentation, decreasing the opportunity for *Brettanomyces* to grow. Though fermenting wines to a higher alcohol level may often inhibit development of flavour precursors, ethanol tolerance is also a strain dependent character (Vigentini et al., 2008).

Apart from limiting *Dekkera* growth one way of avoiding ethylphenol production is to minimize the concentration of precursors in wine (Gerbaux et al., 2002). A common oenological technique is the addition of enzyme preparations during maceration to aid in the release of phenolic compounds from the grape berries. These preparations have been shown to be relatively effective in releasing free hydroxycinnamic acids from their esterified form, which then leaves these available for conversion into volatile phenols. Therefore it has been

recommended that enzyme preparations possessing cinnamoyl esterase not be used in winemaking as it increases the chance of spoilage by volatile phenols (Gerbaux et al., 2002).

Chatonnet et al. (1993) stated that wines are more susceptible to the phenolic taint in warmer months. In fact, the impact of air temperature on 4-ethylphenol production was related with the production rate, and not with the total amount produced. Thus, keeping wines at low cellar temperatures only delays the process, being an efficient prevention measure if cell growth is fully inhibited. Couto et al. (2005) already presented lethal heat-treatment parameters for *D. bruxellensis*, showing that significant inactivation of *D. bruxellensis* in wine began at 35 °C, stimulated by the ethanol concentration of wine. In addition, other data (Barata et al., 2008) showed that relatively mild temperatures (about 36 °C) overnight are a reasonable technological option when wines are found contaminated by viable *D. bruxellensis* and 4-ethylphenol tends to increase. According to these authors, this mild temperature has no obvious detrimental effects on wine quality and may be achieved using electric devices to heat wine in stainless-steel vessels or simply by heating bottled wine, without disgorging, if contamination could not have been avoided during bottling. Barata et al. (2008) managed to achieve stable levels of 4-ethylphenol (100 µg l^{-1}) with viable but non-growing *D. bruxellensis* populations of 2000 CFU ml^{-1} during barrique storage at low temperatures (6–8 °C). Removal and inactivation of *D. bruxellensis* by strict process operations (e.g. heat treatment, sterile filtration) would only be advisable when there is an increase in the 4-ethylphenol levels.

Decreases of 4-ethylphenol and 4-ethylguaiacol concentrations were found in red wine containing yeast lees compared to the same wine aged without lees (Guilloux-Benatier et al., 2001). Chassagne et al. (2005) shown that yeast lees were effective in removal of 4-ethylguaiacol and 4-ethylphenol. Also the presence of other wine constituents sorbed by yeast influenced the sorption of both volatile phenols, with a greater effect in the case of 4-ethylphenol. Yeast lees provide a cost-effective and efficient approach to remove or to decrease organoleptic defects in wine due to phenols.

However, preventive methodologies have been based on the generation of conditions unfavourable to *Brettanomyces/Dekkera* (Benito et al., 2009). Sturm et al. (2015) also concluded that potential spoilage of wine by *D. bruxellensis* was more related to the ability of the strains to develop in the wine environment than the CD and VR enzymatic activity recorded in laboratory conditions and therefore, the most efficient way to prevent wine spoilage by *D. bruxellensis* should be the control of its development.

Certain additives can inhibit the growth of *Brettanomyces* (Suarez et al., 2007). The most common is sulphur dioxide (SO_2), although it is hard to keep the concentration stable over prolonged aging periods in casks in which the environment is mildly oxidizing. It is known that, in a red wine at pH 3.65, initial doses of free SO_2 of 15, 25, 30 and 35 mg l^{-1} are significantly reduced after four months of aging in barrels to 6, 11, 10 and 15 mg l^{-1}, respectively (Chatonnet, et al., 1993).

The action of SO_2 on *Brettanomyces* seemed to be rapid, with cells having their viability greatly reduced and losing their culturability completely within 330 min of exposure. The uptake of free molecular SO_2 by *Brettanomyces* is fast, as exposure of the cells to molecular SO_2 for only a short period of time showed (Du Toit et al., 2005). Also, the addition of O_2 to wine that contains low concentrations of SO_2 can support the survival and growth of *Brettanomyces* and it usually happen during racking and other transfer or transport. Therefore, when it is suspected that wine has been contaminated by *Brettanomyces*, it should be avoided excessive O_2 exposure and molecular SO_2 concentrations should be checked and regularly adjusted to 25–35 mg l^{-1} of free SO_2. Winemakers should bear in mind that at excessive concentrations, SO_2 might affect the aroma and colour of red wine because the reaction of SO_2 with the red form of anthocyanins leads to the bleaching of red wine colour (Ribéreau-Gayon et al., 2000). The use of between 0.5 and 0.8 mg l^{-1} of molecular SO_2 is recommended and it should be remembered that the molecular SO_2 concentration achieved is pH-dependent; 30 mg l^{-1} of free SO_2 releases 0.4 mg l^{-1} of molecular SO_2 at pH 3.7, and 0.8 at pH 3.4 (Henick-Kling, et al., 2000). The antimicrobial potential of SO_2 against

Brettanomyces, and the effectiveness of physical treatment like racking to remove yeast cells from barrels have been shown also by (Oelofse et al., 2008; Suárez et al., 2007). Besides, the inhibitory effect of these two actions (sulphating and racking) overcame the stimulating effect of oxygen on *Brettanomyces* growth (Kheir et al., 2013; Aguilar-Uscanga et al., 2003). Also, lower *Brettanomyces* levels is achieved when wine is aged only in aerobic conditions (12 months cask) with racking and sulphur dioxide addition, than the combined aerobic and anaerobic maturation (6 months in cask and 6 in bottle) (Rubio et al., 2015).

Filtration can reduce the presence of contaminating yeasts, but this poses problems of reducing wine aroma and colour (Suárez et al., 2007).. In order to be effective, membranes with a pore size smaller than 0.45 μm must be used (Calderón et al., 2004) and they causes a deterioration of the wine's colloidal structure and can reduce the intensity of its colour. Dormant, elongated forms of *Brettanomyces* cells may be able to pass through a 0.45 μm filter (Suárez et al., 2007). One of the solutions is fining operation of red wines before introducing them into their barrels and in that way contaminating populations of *Brettanomyces* can be reduced by 40 to 2000-fold by treatment with fining proteins (Murat and Dumeau, 2003). There are differences in effectiveness between fining agents and sometimes fining is rejected by winemakers since it also impacts wine aroma and colour. Anyway, the greater reduction in the initial population is achieved when the more fining agents used; i.e. intense finings can almost entirely remove these yeasts (Suárez et al., 2007). Fining with casein or potassium caseinate can reduce ethylphenol levels if these are not too high (Ruiz-Hernández, 2003).

It is known that certain weak acids, such as sorbic, benzoic and fumaric have antifungal activity and can be used against *Brettanomyces/Dekkera*, but their action is not selective and they are not authorized for use in winemaking. Characteristically, weak-acid preservatives do not slay micro-organisms but rather inhibit their growth, causing extended lag phases. Benito et al., (2009) report that sorbic acid can act as inhibitor in conversion of precursor compound to 4-ethylphenol but *Brettanomyces* appear the more resistant yeast species to sorbic acid. Antioxidants such as ascorbic and erythorbic acids can be used to reduce the presence of oxygen during maturation, preventing ethylphenol formation (Suárez et al., 2007).

Other alternative inhibitors are dimethyl dicarbonate and chitosan. Effectiveness of dimethyl dicarbonate has been proven, but Delfini et al. (2002) showed that a dose of 400 mg l^{-1} cannot completely inhibit the growth of *B. anomalus* and in contrast, other fermentative yeasts are inhibited by dosages of 250–400 mg l^{-1}. Chitosan is a polysaccharide derived from chitin and it has a selective effect on *Brettanomyces*, causing a delay in its latent phase in mixed cultures with *Saccharomyces cerevisiae* (Gómez-Rivas et al., 2004). *B. bruxellensis* and *B. intermedius* cannot grow in the presence of 3–6 g l^{-1} of chitosan, while it does not affect the development of *Saccharomyces cerevisiae*. Chitosan at 0.05–0.1 % is known to delay spoilage by yeasts at 25 °C; in fact it even inactivates some other species (Kiskó et al., 2005).

Puig et al. (2003) showed that application of pressures of 400–500 MPa for 5–15 min at temperatures of 5 to 20 °C can reduce populations of certain yeasts (including *B. bruxellensis*) and lactic acid and acetic acid bacteria by more than 99.99 %, without causing major modifications to the wine's physicochemical properties, enzymatic activity, or sensorial properties. Not only is effective, it reduces the use of SO_2. The concentration of ethylphenols can be reduced by reverse osmosis and adsorption (Ugarte et al., 2005). These authors reduced initial concentration (900 μl l^{-1} of 4-ethylphenol plus 4-ethylguiacol) by 77 % after a 3-hours treatment involving reverse osmosis with an appropriate membrane and tangential-flow filtration equipment and a hydrophobic adsorbent resin. Using this method, no significant reduction in wine colour, tannins, body (glycerol and diols) or ethanol was observed, but it was seen reduction in aromatic compounds, like methyl- and ethyl vanillate and other esters (Suarez et al., 2007).

The use of various antimicrobial agents, bacteriolytic enzymes, zymocines and yeast strains with antimicrobial activity constructed by genetic engineering represent biological control of contaminating yeasts and bacteria. However, in the

wine environment they are not very effective and other techniques are usually required as well (Du Toit and Pretorius, 2000).

Some wineries use polyvinylpolypyrrolidone and charcoal to treat wines containing volatile phenols. The recommended doses are from 0.015 to 0.24 g l^{-1} charcoal for slight off-odours, and from 0.12to 0.96 g l^{-1} for more intense off-odours. polyvinylpolypyrrolidone (0.06-0.48 g l^{-1}) is used to remove ethylphenols (Suarez et al., 2007).

Fulcrand et al. (1996) proposed the formation of the malvidin derivative from 4-vinylphenol in order to decrease the production of ethylphenols and in the same way to increase the formation of highly stable pigments during maturations. Vinyl phenolic derivatives are highly stable due to their aromatic heterocyclic ring. Other pyranoanthocyanic derivatives, such as the vitisins, function in the same way (Mateus, et al., 2001). Besides, since the formation of this ring involves carbon 4 of the anthocyanin, decolouration by SO_2 and reductions in colour intensity caused by high pH are less likely (Bakker and Timberlake, 1997).

Yeasts with hydroxycinnamate decarboxylase activity can also be used to decarboxylate hydroxycinnamic acids, and this forms vinylphenols that condense with grape anthocyanins to produce pyranoanthocyanin vinylphenolic adducts of great colour stability (Morata et al., 2006). This eliminates the hydroxycinnamic acid precursors of ethylphenol from wine, and forms highly stable, long-lasting pyranoanthocyanins during fermentation. During fermentation, vitisins A and B, pyranoanthocyanic molecules structurally similar to vinylphenol derivatives, are produced in significant amounts by selected yeast strains (Morata et al., 2003). These adduct formed via the condensation of vitisin A and vinylphenols are very stable molecules that provide wine a red–blue colour (Mateus et al., 2006). Benito et al. (2009) used *S. cerevisiae* strains with high HCDC in order to reduce the concentration of ethylphenol precursors and it is shown that these strains can minimize possible alterations caused by *Brettanomyces/Dekkera* in red wines. This technique has been proven successful in real musts from the Tempranillo variety and significant differences were obtained in the production of ethylphenols. Such a strategy offers natural protection of this undesirable yeast and volatile phenols concentration below the sensorial threshold can be obtained even after *Brettanomyces* contamination.

5 CONCLUSION

The occurrence of volatile phenols in wines has been extensively studied in last decades and there are a lot of reports on the presence of *Brettanomyces* metabolites in wine indicating that it is a worldwide issue. Volatile phenols are very important in term of wine sensory characteristics, because their elevated concentrations in wine are associated with unpleasant aroma. As wine market became very demanding and a big wine competition is present, it is very important to place on the market wine that is from sensory points of view in good condition, i.e. without any flaws. As yeast species *B. bruxellensis* is the main culprit for volatile phenols production and if their presence in wine is noticed, the primary objective should be its elimination or its maintenance at constant level. However, winemakers should consider all factors that influence growth of *Brettanomyces* and volatile phenols production beginning from viticultural practices in order to keep grape healthy, cellar sanitation, physiochemical composition of wine (energy/carbon and precursor sources, alcohol concentration and pH) and very important conditions during wine aging and its storage. Using a starter culture is a good preventive measure that can decrease indigenous fermentation, decreasing the opportunity for *Brettanomyces* to grow. Also, starter cultures can be used to decarboxylate hydroxycinnamic acid precursors of ethylphenol from wine, and forms highly stable, long-lasting pyranoanthocyanins during fermentation i.e. adducts of great colour stability. Generally, from additives the most effective turned out SO_2 and its concentration should be orderly checked and maintained on necessary level. Besides, there are differences among grape varieties in hydroxycinnamic acid precursors accumulation and therefore in volatile

phenols production. Further research that will consider different grapevine varieties, different starter cultures including control of wine in different phases of winemaking process should be put in a word.

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