Significance of *Brettanomyces* and *Dekkera* during Winemaking: A Synoptic Review

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Wine comprises a complex microbial ecology of opportunistic microorganisms, some of which could potentially induce spoilage and result in consequent economic losses under uncontrolled conditions. Yeasts of the genus *Brettanomyces,* or its teleomorph *Dekkera,* have been indicated to affect the chemical composition of the must and wine by producing various metabolites that are detrimental to the organoleptic properties of the final product. These yeasts can persist throughout the harsh winemaking process and have in recent years become a major oenological concern worldwide. This literature review summarises the main research focus areas on yeasts of the genera *Brettanomyces* and *Dekkera* in wine. Specific attention is given to the spoilage compounds produced, the methods of detection and isolation from the winemaking environment and the factors for controlling and managing *Brettanomyces* spoilage.

Winemaking comprises a diverse set of factors that play a crucial role during the transformation of grapes to wine. The most important factors generally considered by winemakers include vineyard management, grape quality, winemaking practices and commercial yeast selection. However, the microbiology behind the wine should also be considered because this is one of the parameters often neglected as a quality control constraint. Wine microbiology entails a complex interaction of a variety of microorganisms that play an essential role on the outcome of the final product and, if the microbiology of wine is disregarded, there will simply be no wine.

The involvement of microorganisms in the fermentation of alcoholic beverages has been a subject of interest for centuries. As early as the mid-1800s, Louis Pasteur observed the conversion of grape juice into wine by the action of yeast and noticed the presence of bacteria that were capable of causing wine spoilage (Drysdale & Fleet, 1988). Since then the microbiology of wine has been the topic of many investigations and a large diversity of microorganisms that are present during the winemaking process have been identified (Fugelsang, 1997; Loureiro, 2000).

Apart from the principal wine yeast *Saccharomyces cerevisiae,* different genera and species of bacteria and non-*Saccharomyces* yeasts have been identified. These microorganisms form a natural part of the active biomass involved in the winemaking process as they are found on grapes, in the must and wine and can therefore contribute to the organoleptic properties of the final product (Heard & Fleet, 1988; Fleet & Heard, 1993; Lambrechts & Pretorius, 2000). The contributions are, however, not always positive with regard to the wine's flavour because microbial activity often results in wine spoilage.

Wine spoilage is a serious problem for the wine industry because it renders the products unacceptable and can lead to large economic losses. For this purpose, research is targeted towards the microorganisms that are responsible for spoilage during the winemaking process. The typical focus areas include: methods of detection, identification and characterisation of spoilage microorganisms; the monitoring and control of spoilage compounds; and fundamental investigations to gain more knowledge on the metabolism and activities of spoilage microorganisms.

One of the controversial yeasts that has gained increasing attention in recent years, specifically as it is associated with wine spoilage, belongs to the genera *Brettanomyces* and *Dekkera* well-known for the production of ethyl phenols. This review presents a summary of some of the above-mentioned major scientific focus areas about the yeasts *Brettanomyces* and *Dekkera* during winemaking.

BRETTANOMYCES AND *DEKKERA* DURING WINEMAKING

History of *Brettanomyces*

The first reference to the genus *Brettanomyces* dates back to 1904 when N.H. Claussen isolated a yeast from a slow secondary fermentation of an old English stock beer (Gilliland, 1961). The flavours produced by this yeast became characteristic of the British beers of that time and so the name '*Brettanomyces*' was derived from 'British brewing fungus'. It was not until the 1940s, when M.T.J. Custers performed the first systematic study on *Brettanomyces* yeast, that *Brettanomyces* was associated with wine (Custers, 1940). Although this study included 17 strains, of which most were isolated from beer, one strain originated from a French wine (Krumbholz & Tauschanoff, 1933).

Different species in wine

The taxonomy of the genus *Brettanomyces* has seen numerous reclassifications over the years from the handful of species that were initially identified. Originally, these species included *Brettanomyces bruxellensis*, *Brettanomyces lambicus, Brettanomyces clausenii, Brettanomyces anomalus* and *Brettanomyces intermedius,* which reproduced asexually by means of budding (Custers, 1940;

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Van der Walt & Van Kerken, 1958). The genus *Dekkera* was introduced to the taxonomy in 1964 after the production of ascospores (sporulating-form) was observed (Van der Walt, 1984). Currently, the five species jointly belonging to the genera *Brettanomyces* and *Dekkera* are: *Brettanomyces custersianus, Brettanomyces naardenensis, Brettanomyces nanus, B. anomalus* and *B. bruxellensis* (Kurtzman & Fell, 2000; Cocolin *et al*., 2004). Teleomorphs (perfect state) are known for the last two species, *Dekkera anomala* and *Dekkera bruxellensis*, respectively (Kurtzman & Fell, 2000). The details regarding the morphological, biochemical and physiological characteristics of these species are well described in recent classification manuals (Barnett *et al*., 2000; Boekhout *et al*., 2002; Kurtzman & Fell, 2000). From the five species currently known, the species primarily associated with winemaking is *B. bruxellensis* (*D. bruxellensis*) (Egli & Henick-Kling, 2001; Stender *et al.,* 2001; Cocolin *et al.,* 2004), although *B. anomalus* (*D. anomala*) and *B. custersianus* isolations from must fermentations have been reported in two instances (Querol *et al*., 1990; Esteve-Zarzoso *et al*., 2001). With advances in DNA-based methods, recent wine-related investigations often include *D. anomala* along with the predominant species *D. bruxellensis* as conventional methods had showed difficulty in differentiating between these two species (Loureiro & Malfeito-Ferreira, 2006). Although current taxonomical classifications suggest that *Dekkera* should be used in reference with the species *bruxellensis* and *anomala* (Boekhout *et al*., 1994)*,* many discrepancies exist and some authors frequently prefer using the technically incorrect naming of *B. bruxellensis* and *B. anomalus* when referring to these yeasts in a winemaking context. This is largely attributed to the fact that the sexual or sporulating form, *Dekkera,* is yet to be found in wine.

Some authors have made the point that the separation of *Brettanomyces* and *Dekkera* in the context of wine is meaningless because current molecular DNA techniques reveal no distinction between the anamorph and teleomorph forms (Loureiro & Malfeito-Ferreira, 2006). This might explain why it is not uncommon to see the use of '*Brettanomyces/Dekkera* spp.' in wine research. In this review, the same context will be used in which the original authors used the naming in their publications. This can either be *B. bruxellensis* or *D. bruxellensis*.

Occurrence and distribution during winemaking

Brettanomyces/Dekkera spp. are ubiquitously distributed in nature and their occurrence and spoilage activities have been well summarised by Loureiro and Malfeito-Ferreira (2006). The majority of reports associate *Brettanomyces/Dekkera* spp. with fermented food products ranging from cheeses and fermented milk to various alcoholic beverages including wine, beer, cider, kombucha (fungus-tea) and tequila (Davenport, 1976; Kumara & Verachtert, 1991; Lachance, 1995; Kosse *et al*., 1997; Licker *et al.*, 1998; Gadaga *et al*., 2002; Teoh *et al*., 2004; Loureiro & Malfeito-Ferreira, 2006). Less frequent reports of their isolations from other sources (bees, fruit-flies, olives and carbonated drinks) are also available (Van der Walt & Van Kerken, 1958; Phaff *et al*., 1978; Deák & Beuchat, 1995; Kotzekidou *et al.*, 1997). *Brettanomyces/Dekkera* spp. have been and still are isolated from wines and wineries all around the world, predominantly from red wines. These yeasts are less frequently isolated from white wines (Licker *et al.*, 1998; Dias *et al.*, 2003b) although their loss of viability and the consequent non-existence of ethylphenol levels in white wines is largely ascribed to the efficiency of sulfur dioxide (SO_2) at lower pH conditions (Loureiro & Malfeito-Ferreira, 2006). Hence, the focus of the research on these yeasts has primarily fallen on their occurrence in red wine.

The winemaking process hosts multiple sources where *Brettanomyces/Dekkera* spp. can survive and numerous debates about the initial source and dispersion of these yeasts have occurred (Licker *et al.*, 1998). The vineyard provides many sources, including the soil, rootlets, bark, leaves and grapes. Davenport (1976) investigated all of these but could not isolate any *Brettanomyces* spp. In 1987, Guerzoni and Marchetti reported their isolation from grapes damaged by sour rot. This agrees with recent knowledge suggesting a connection between *Brettanomyces/Dekkera* and damaged grapes (*Botrytis*-affected) (Taillandier, 2007). Surprisingly, only one investigation has been successful in recovering *Brettanomyces/Dekkera* spp. from grapes (Renouf & Lonvaud-Funel, 2006) despite the fact that they have been isolated many times from fermenting musts during earlier research (Licker *et al*., 1998; Pretorius, 2000; Jolly *et al*., 2003; Prakitchaiwattana *et al*., 2004; Van de Water, 2004). The poor detection of *Brettanomyces/Dekkera* spp. on grapes has been speculated to be the result of their low cell numbers amid a diverse microbial ecosystem where other wild yeast and bacterial species dominate. Renouf and Lonvaud-Funel (2006), however, overcame this problem by developing an enrichment medium that enabled them to detect *B. bruxellensis* on grape berries. They were subsequently able to detect this yeast from several vineyards and at different stages of grape berry development.

Following the initial stages of winemaking, *Brettanomyces/Dekkera* spp. have been more consistently associated with wine and cellar equipment (Fugelsang, 1998). As their populations are usually minor in the presence of numerous other rapidly fermenting yeasts, their increase in numbers only occurs during more nutritionally favourable conditions that suit their slow-growing characteristics (Fugelsang *et al*., 1993). These conditions are created once alcoholic fermentation is completed and traces of residual sugars allow them to proliferate more easily. Malolactic fermentation (MLF) and ageing in used barrels have therefore been recognised as the most critical stages of wine production for *Brettanomyces/Dekkera* contamination (Chatonnet *et al*., 1992, 1995; Fugelsang *et al*., 1993; Licker *et al*., 1998; Renouf *et al*., 2006b; Suárez *et al*., 2007). During MLF, *Brettanomyces/Dekkera* spp. is presented with conditions of low free sulfur dioxide, low residual sugar concentrations and yeast autolysis with the release of nutrients occurring along with modest microbial competition. The main characteristics of oak barrels (new and old) that are beneficial to *Brettanomyces/Dekkera* growth are the porous microstructure, which allows the influx of small amounts of oxygen (Swaffield & Scott, 1995; Loureiro & Malfeito-Ferreira, 2006) and the presence of cellobiose that can serve as sugar resource (Boulton *et al*., 1996). In addition, difficulty of sanitation (old barrels) is favourable to established *Brettanomyces/Dekkera* populations and promotes contamination of wine (Pollnitz *et al*., 2000; Yap *et al*., 2007). When MLF is performed in barrels these characteristics can aid the growth of *Brettanomyces/Dekkera*. That these yeasts have also been recovered from wines in concrete or stainless steel tanks is more likely due to other reasons of survival

than those pertaining in barrels (Chatonnet *et al*., 1992; Rodrigues *et al*., 2001). Furthermore, numerous finished and bottled wines have also been known to host *Brettanomyces/Dekkera* populations. These wines have been linked to prior conditions of long periods of barrel ageing, lower SO_2 concentrations and less filtration prior to bottling (Herezstyn, 1986a; Arvik *et al*., 2002).

Wineries and equipment that have been investigated revealed the presence of *Brettanomyces/Dekkera* yeasts in winery air samples and on cellar walls, drains, pumps, transfer lines and other pieces of equipment that are difficult to sterilise (Van der Walt, 1984; Alguacil *et al*.,1998; Fugelsang, 1998; Connel *et al*., 2002). It is therefore not surprising that wineries are often considered as the primary source of *Brettanomyces/Dekkera* contamination, as opposed to grapes. However, as its occurrence is often inconsistent, each winery can present a unique situation that requires the determination of the specific origin and route of contamination.

DETECTION AND IDENTIFICATION METHODS

Isolation media

The isolation of *Brettanomyces/Dekkera* spp. from winemaking environments is not easy as they are slow growing and have low occurrence (Fugelsang, 1997). Additionally, it has been described that *Brettanomyces/Dekkera* yeasts are difficult to recover from materials heavily contaminated with other microorganisms (Van der Walt & Van Kerken, 1960). For this purpose, several authors have investigated different possibilities of selective media by altering the main constituents and carbon sources (Heard & Fleet, 1986). The earlier media for *Brettanomyces* isolation included maltose and sucrose as carbon sources and it was reported that the use of sorbate, ethanol and cycloheximide as antimicrobials was not satisfactory (Van der Walt & Van Kerken, 1960; Wright & Parle, 1974). More recent studies included glycerol and trehalose with sucrose as carbon sources with a wider range of antimicrobial agents (gentamicin, oxytetracycline, cycloheximide and sorbic acid) to suppress the growth of unwanted yeasts and bacteria (Chatonnet *et al*., 1992; Fugelsang *et al*., 1997; Alguacil *et al*., 1998). Furthermore, vitamins such as thiamine and biotin have also been suggested as these can be beneficial to the growth of *Brettanomyces/Dekkera*, although some authors do not regard this as necessary (Fugelsang *et al*., 1997; Loureiro & Malfeito-Ferreira, 2006). The development of a selective or differential medium specifically for the isolation of *Brettanomyces/Dekkera* spp. was presented by Rodrigues *et al*. (2001). This medium, named DBDM (*Dekkera/Brettanomyces* Differential Medium), was reported as being able to recover less than 1% of the target yeasts from a total microbial population in combination with the Most Probable Number (MPN) technique. Along with yeast nitrogen base (YNB) this medium contained two antimicrobial agents (ethanol and cycloheximide), a pH indicator (bromocresol green) to indicate media acidification and a substrate (*p*-coumaric acid). The latter compound was included as its degradation results in a distinct phenolic off-odour that can be indicative of *Brettanomyces/Dekkera* activity. For a more comprehensive list of media that have been tested for the detection of *Brettanomyces/Dekkera* spp. refer to Rodrigues *et al*. (2001).

The development of a selective liquid medium that enabled the detection of *Brettanomyces/Dekkera* spp. followed the works of Rodrigues *et al*. (2001). This WLN (Wallerstein Laboratory Nutrient)-based medium was aimed at the development of a simple detection system for *Brettanomyces/Dekkera* yeast that could be used on a routine basis in the wine industry (Couto *et al*., 2005a). Liquid media have been described as having a resuscitation function that could be beneficial for the recovery of some microorganisms (e.g. yeast) while reducing mould growth (Loureiro & Malfeito-Ferreira, 2006).

As mentioned before, the prevalence of *Brettanomyces/Dekkera* spp. on grapes has been remarkably low and the lack of recoveries has been ascribed to the short-comings of optimal isolation media and poor detection limits. Renouf and Lonvaud-Funel (2006) proposed the use of an enrichment medium to overcome this problem and obtained good success with the detection of *D. bruxellensis* on the surface of grape berries. The use of enrichment steps has previously proven very useful for the detection of scarcely represented *S. cerevisiae* and *S. paradoxus* on grapes (Van der Westhuizen *et al*., 2000; Redzepovic *et al*., 2002) and should definitely be considered at times when the presence of *Brettanomyces/Dekkera* is uncertain. It is also necessary to emphasize the importance of incubation time while performing detection and isolation of *Brettanomyces/Dekkera* spp. from the winemaking environment. Their low growth rate and fastidious nutritional requirements demand incubation times of up to two weeks (Rodrigues *et al*., 2001) making the general incubation periods (three to six days at 25-30°C) used for other yeasts inadequate for routine microbiological screenings.

Direct methods of enumeration by plating on selective growth media can be inaccurate resulting from the possible viable but non-culturable (VBNC) state of microorganisms. Cells in the VBNC state are metabolically active but unable to undergo cellular division for growth in liquid or on agar and are therefore non-culturable (Oliver, 1993). Moreover, evolution to a VBNC state is related to the intensity of the stress (Oliver *et al.*, 1995) and there are hypotheses currently about whether SO_2 and ethanol could induce this state amongst *Brettanomyces/Dekkera* spp. populations in wine (Millet & Lonvaud-Funel, 2000; Arvik *et al*., 2005; Du Toit *et al*., 2005). This is especially important for *Brettanomyces/Dekkera* spp. as we have found instances where wines contained objectionable levels of ethyl phenols but yielded no culturable cells.

DNA-based identification techniques

As discussed in the previous section, the conventional identification methods for *Brettanomyces/Dekkera* spp. are insufficient, especially during the winemaking process where a period of a week is crucial. The low relative occurrence, prolonged incubation times and variable identification results often obtained due to their mixed morphological features (Smith, 2002) prompted development of more rapid and reliable identification techniques of these spoilage yeasts. Therefore recent years have seen the development of several molecular DNA-based techniques (Loureiro & Querol, 1999).

Stender *et al*. (2001) developed a technique that does not require DNA extraction and utilises microscopic visualisation of fluorescent *Brettanomyces/Dekkera* cells after *in situ* hybridisation of species-specific PNA (peptide nucleic acid) probes to the 26S ribosomal RNA (RNA-FISH hybridisation). The authors have assigned a high specificity to this method which uses pelleted *D. bruxellensis* cells from a centrifuged wine. Considering the difficulties encountered with microscopic identification of cell

morphologies this method can be very useful. A similar study described the use of FISH probes on sequence regions beyond the D1/D2 domains of the 26S rRNA gene that can successfully detect all of the five currently known *Brettanomyces/Dekkera* species *(D. bruxellensis, D. anomala, B. custersianus, B. nanus* and *B. naardenensis*) (Röder *et al*., 2007).

Other direct molecular techniques that are fast, sensitive and accurate involve polymerase chain reaction (PCR). Specific sequences spanning the 5.8S ribosomal RNA genes and their flanking internal transcribed spacer (ITS1 and 2) regions can be targeted for species identification of *Brettanomyces/Dekkera* (Esteve-Zarzoso, 1999; Egli & Henick-Kling, 2001). The 5.8S rRNA and ITS regions have been documented in many studies for yeast identification (White *et al*., 1990; Guillamon *et al*., 1998; Esteve-Zarzoso, 1999; Granchi *et al*., 1999) and can include restriction fragment length polymorphisms (PCR-RFLP) for interspecies discrimination of *Brettanomyces/Dekkera* yeasts (Esteve-Zarzoso, 1999; Nisiotou & Gibson, 2005). A nested PCR method comprising two primer sets has been developed for the direct detection of *Brettanomyces/Dekkera* strains in sherry (Ibeas *et al*., 1996). This approach is very efficient for identification of *D. bruxellensis* strains from intact yeast cells. Another highly specific PCR (targeting the D1-D2 loop of the 26S rRNA) was developed by Cocolin *et al*. (2004) that form amplification products only with the species *B. bruxellensis* and *B. anomalus*. Differentiation between these two species could be achieved after restriction enzyme analysis (*Dde*I) of the amplified products. The use of denaturing gradient gel electrophoresis (PCR-DGGE) for the characterisation of yeast diversity within wine fermentations has also been indicated to detect *Brettanomyces* yeasts (Cocolin *et al*., 2004; Renouf *et al*., 2006a).

One of the concerns about direct PCR methods is that the sensitivity can depend on the level of contamination (Loureiro & Malfeito-Ferreira, 2006) and that only a high detection limit ($\geq 1x10^4$ cfu/mL) may provide a positive result. Several authors have reported that wines could be tainted with a phenolic off-flavour character by *Brettanomyces* counts below this value (Ibeas *et al*., 1996; Phister & Mills, 2003; Cocolin *et al*., 2004) and therefore PCR detection limits of less than 10^4 cfu/mL are required. Phister and Mills (2003) employed real-time PCR and showed detection of *D. bruxellensis* in wine at levels as low as one to 10 cells/mL, depending on the dilution factor of the sample. In contrast however, Delaherche *et al*. (2004) obtained a detection limit of 10^4 cfu/mL with real-time PCR and this currently questions the routine use of this technique. A change of the DNA extraction method has greatly improved the detection limit to 10 cfu/mL by the same authors (personal communication, 2007). Another recent suggestion for achieving detection levels of about 10 cfu/mL for *Brettanomyces/Dekkera* yeasts has included the use of a loop-mediated isothermal amplification (LAMP) method (Hayashi *et al*., 2007).

Genetic diversity and techniques for strain discrimination

The identification of *D. bruxellensis* as the primary spoilage species during winemaking was soon followed by investigations that focused on determining the genetic diversity amongst this species. Intraspecies identification of *Brettanomyces/Dekkera* yeasts has not been frequently reported and some of the first techniques that have been described used random amplified polymorphic DNA (RAPD-PCR) and amplified fragment length polymorphisms (AFLPs) (de Barros Lopes *et al*., 1999; Mitrakul *et al*., 1999). Genetically different strains of *D. bruxellensis* wine isolates were revealed from different vintages and exhibited different chromosomes (three or four) and consequently different chromosomal fingerprints (Mitrakul *et al*., 1999). It was also shown that the wine strains of *D. bruxellensis* were genetically different from reference strains. Several studies have since been performed that allow for strain identification and they included techniques such as, AFLPs (Bellon *et al*., 2003; Curtin *et al*., 2007); PCR fingerprinting with microsatellite primers; intron splice site - PCR (de Barros Lopes *et al*., 1998); sequencing a portion of the 26S rDNA gene (Conterno *et al*., 2006); restriction enzyme analysis of mitochondrial DNA and RAPD-PCR with OPA-primers (Martorell *et al*., 2006); restriction enzyme analysis – pulsed field gel electrophoresis (REA-PFGE) (Miot-Sertier & Lonvaud-Funel, 2007); and PCR-DGGE (Renouf *et al*., 2006c). Genetic characterisation studies have relevance in the wine industry because they connect different *D. bruxellensis* strains with geographic origin, vintage year and wine variety (Conterno *et al*., 2006). Renouf *et al*. (2006c) found three different chromosomal patterns (after digestion with restriction enzymes) for *D. bruxellensis* isolates from different French wineries, but concluded that the same strains were predominant throughout the winemaking process at the specific wineries. Conterno *et al*. (2006) found that a total of 47 wine isolates of *B. bruxellensis* could be grouped into six clusters. The same authors also found that physiological traits were highly variable and did not correlate with the groupings from the DNA analysis. Therefore, the genetic diversity that exists among species with related genomes should be further explored to obtain correlations between phenotype (visible and biochemical properties) and genetic composition (Bellon *et al*., 2003).

In a large study using AFLP analysis for the characterisation of *D. bruxellensis* isolates from Australian wineries, eight genotypes have been found (Curtin *et al*., 2007). These eight strain groupings originated from a total of 244 *D. bruxellensis* isolates from 31 red winemaking regions, in which some strains regularly prevailed. It was also found that the wine strains were highly divergent from the *D. bruxellensis* type strain (Bellon *et al*., 2003; Curtin *et al*., 2007).

Future studies on strain identification might give clearer information on the origin of these species during the vinification process. By tracing the routes of *Brettanomyces/Dekkera* contamination genetically, it might be possible to gather beneficial information for the winemakers which could be considered along with preventative measures (Miot-Sertier & Lonvaud-Funel, 2007).

WINE SPOILAGE BY *BRETTANOMYCES* AND *DEKKERA* SPP.

Brettanomyces/Dekkera yeasts obtained their significance in wine due to the formation of various spoilage compounds that are detrimental to wine quality. For this reason, most of the investigations performed on these yeast species focussed on their wine spoilage capabilities.

This section will summarise the current knowledge of the main compounds and describe their impact on the organoleptic properties of wine.

Production of volatile phenols

The production of phenolic off-flavours (POF), specifically volatile phenols, defines the importance of *Brettanomyces/Dekkera* yeasts during winemaking and has been well documented (Heresztyn, 1986a; Chatonnet *et al*., 1992, 1995, 1997; Edlin *et al*., 1995;

Licker *et al*., 1998; Suárez *et al*., 2007). Volatile phenols represent a large family of aromatic compounds of which the vinyl- and ethylphenols are implicated with *Brettanomyces* spoilage (Chatonnet *et al*., 1992). These volatile phenols, especially the ethylphenols, are responsible for off-odours that have been described as 'animal', 'medicinal', 'Elastoplast', 'sweaty leather', 'barnyard', 'spicy' and 'clove-like' and are detrimental to the aroma profile of wines at high concentrations (Chatonnet *et al*., 1992; 1995; Suárez *et al*., 2007).

The formation of volatile phenols by *Brettanomyces/Dekkera* yeast has been shown to be the result of enzymatic transformation of phenolic (hydroxycinnamic) acids present during winemaking (Heresztyn, 1986a; Chatonnet *et al*., 1992). Hydroxycinnamic acids are naturally present in grape juice and wine and originate from the grapes, where they are generally esterified with tartaric acid or anthocyanin esters (Dugelay *et al*., 1993). The action of enzymes with cinnamoyl-esterase activity releases these weak acids to their free forms (Gerbaux *et al*., 2002), in which they can be inhibitory towards the growth of many microorganisms (Stead, 1995; Zaldivar & Ingram, 1999; Barthelmebs *et al*., 2001). However, *Brettanomyces/Dekkera* spp. overcome the toxicity problem by converting these acids into volatile phenols. The formation of volatile phenols by *Brettanomyces/Dekkera* spp. is shown in Figure 1. The free hydroxycinnamic acid precursors (*p*-coumaric, ferulic and caffeic acid) are decarboxylated into hydroxystyrenes (4-vinylphenol, 4-vinylguaiacol and 4-vinylcatechol, respectively), and then reduced into their corresponding ethyl-derivative forms (4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol, respectively) (Heresztyn, 1986a; Chatonnet *et al*., 1992; Hesford *et al*., 2004). It is believed that the free available hydroxycinnamic acids can be released by the action of fungal enzymes or by grape juice heating (Gerbaux *et al*., 2002), although other mechanisms may exist. It is speculated that *Brettanomyces/Dekkera* spp. might be able to hydrolyse bound phenolic acids, but there is no scientific proof for this. The bound or the free hydroxycinnamic forms are however not the sole requirement for these yeasts to produce the ethyl-derivatives as *B. bruxellensis* has been shown to produce 4-ethylphenol directly from 4-vinylphenol as substrate (Dias *et al*., 2003b).

The two enzymes that facilitate the biotransformation of phenolic acids involve a phenolic (cinnamic) acid decarboxylase (PAD) for the formation of the vinyl derivatives and a vinyl phenol reductase (VPR) for the formation of the ethyl derivatives thereafter. The decarboxylation step has been linked to the *POF1* (phenolic off-flavour) or *PAD1* (phenylacrylic acid decarboxylase) gene of *S. cerevisiae* (Clausen *et al*., 1994). Similar decarboxylase activities exist in numerous bacteria, fungi and yeast species, of which some are present during the winemaking process (Heresztyn, 1986a; Chatonnet *et al*., 1992; Cavin *et al*., 1993; Degrassi *et al*., 1995; Edlin *et al*., 1995; Cavin *et al.*, 1997; Edlin *et al*., 1998; Shinohara *et al.*, 2000; Van Beek & Priest, 2000; Barata *et al*., 2006; Couto *et al*., 2006). The reduction step and ethylphenol formation occurs less frequently in microorganisms (Chatonnet *et al*., 1995; Barthelmebs *et al*., 2001), but is particularly effective in wine by the species *D. bruxellensis* and *D. anomala* (Edlin *et al*., 1995; Chatonnet *et al*., 1997; Dias *et al*., 2003a). Furthermore, *S. cerevisiae* are not able to produce ethylphenols (Chatonnet *et al*., 1993), and LAB, predominantly *Lactobacillus* spp., are only capable of producing low amounts under oenological conditions (Chatonnet *et al*., 1995; Couto *et al*., 2006). Recently, strains of *Pichia guilliermondii* have also been reported as producing considerable quantities of ethylphenols in grape must, to an extent similar to *D. bruxellensis* strains (Dias *et al*., 2003a). As *P. guilliermondii* have been recovered from grapes, grape juice and grape juice-related environments such as winery equipment, they have great significance for wine spoilage through the production of volatile phenols. However, these species are not capable of producing high levels of 4-ethylphenol in wine (Barata *et al*., 2006).

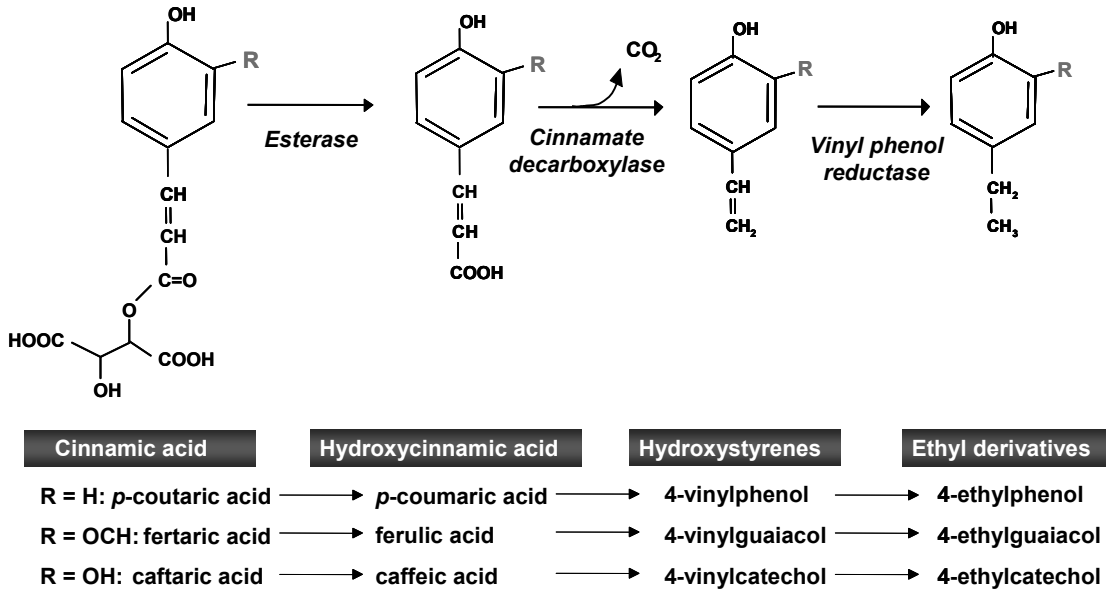

FIGURE 1

Formation of volatile phenols via the decarboxylation of hydroxycinnamic acids.

TABLE 1
Aroma threshold values of volatile phenols in wine (Curtin *et al*., 2005).

Compound	Concentration in red wine (µg/L)	Aroma threshold (µg/L)	Aroma descriptor
4-Vinylphenol	8.8–4.3	440*/600**	Phenol Medicinal
4-Vinylguaiacol	0.2–15	33*/110**	Clove-like
4-Ethylphenol	118-3696	30-60**	Horsy
4-Ethylguaiacol	1–432	20***	Spicy, clove
4-Ethylcatechol	27–427	10*	Phenol Band-Aid® Medicinal Banyard

*model wine, **red wine, ***water

Numerous studies have been performed to elucidate the role of volatile phenolic compounds and the formation of '*Brettanomyces* character' in wine (Heresztyn, 1986a Chatonnet *et al*., 1992; 1995; 1997; Edlin *et al*., 1995; Licker *et al*., 1998; Dias *et al*., 2003b; Coulter *et al*., 2004; Hesford & Schneider, 2004; Francis & Newton, 2005). It has been found that the threshold concentrations of these compounds (Table 1), especially the ethyl derivatives, vary substantially and the perception of the individual aromas is greatly influenced by the wine style, cultivar and the consumer's perceptive abilities.

For more detailed overviews of wine spoilage by volatile phenols and the relevance of *Brettanomyces/Dekkera* yeasts also refer to Loureiro and Malfeito-Ferreira (2006) and Suárez *et al*. (2007).

Other spoilage faults

Brettanomyces/Dekkera yeasts have also been responsible for turbidity or haziness in wine (Van der Walt & Van Kerken, 1958; Van Zyl, 1962) along with the production of several other metabolites that can contribute to wine spoilage. However, the conditions under which some of these are produced in wine and the exact mechanisms involved are not fully understood.

Volatile acidity (VA) and other volatile fatty acids

Brettanomyces/Dekkera spp. have long been known for their ability to affect wine quality negatively through the formation of acetic acid, which constitutes more than 90% of wine's volatile acidity (VA) (Van der Walt & Van Kerken, 1958). Elevated levels of acetic acid can be detrimental to wine quality as it imparts a vinegary/acetone-like aroma (Eglinton & Henschke, 1999) and has also been associated with sluggish/stuck fermentations (Bisson, 1999). *Brettanomyces/Dekkera* yeasts have a particular metabolism that enables them to produce acetic acid (Licker *et al*. 1998; Loureiro & Malfeito-Ferreira, 2006). M.T.J. Custers was the first to describe that the presence of oxygen stimulated glucose fermentation and that this led to the introduction of a biochemical characteristic known as the "negative Pasteur effect" (or 'Custers' effect') (Scheffers & Wiken, 1969; Wijsman *et al*., 1984; Licker *et al*., 1998). Custers also determined that several strains of *Brettanomyces* were capable of producing considerable quantities of acetic acid under conditions of aerobiosis and found that anaerobic conditions inhibited glucose fermentation (Licker *et al*. 1998).

Recently, studies have shown that the availability of oxygen presents a favourable scenario for the development of *Brettanomyces/Dekkera* yeasts during winemaking as it supports their growth and survival and also acetic acid production (Ciani *et al*., 1997; Freer *et al*., 2003; Aguilar-Uscanga *et al*., 2003). In contrast, anaerobosis during alcoholic fermentation may well impede *Brettanomyces/Dekkera* growth, but would not necessarily prevent their development (Ciani *et al*., 1997). Therefore, the risk involved with VA formation by *Brettanomyces/Dekkera* spp. can be reduced by minimising the wine's exposure to oxygen (during racking and transfers) in combination with effective SO_2 usage (Du Toit *et al*. 2005).

Other important volatile fatty acids produced by these yeasts that can have an impact on wine quality include: isovaleric acid (3-methylbutanoic acid), 2-methylbutyric and isobutyric acid (Olsen, 1994; Fugelsang, 1997; Licker *et al*., 1998). However, the focus of this review will fall on isovaleric acid as it can have a major sensory impact on wine aroma. Isovaleric acid has previously been found to be the dominant odorant in wines that were classified as containing a high degree of '*Brettanomyces* character' (Licker *et al*., 1998). The aroma character of isovaleric acid has been described as 'rancid' following Gas Chromatography-Olfactometry (GC-O) analysis, although sensory panels often refer to 'sweaty' and 'cheesy' aromas when describing this compound (Coulter *et al*., 2004). Furthermore, although high concentrations of iso-valeric acid do not correlate with high levels of ethylphenols, it is believed that its presence may enhance the overall perception or intensity of other *Brettanomyces*-derived characters (Coulter *et al*., 2004). The exact cause and the conditions under which isovaleric acid is produced in wine are yet to be determined, but it is known that the amino acid degradation of L-leucine, L-isoleucine and L-valine are involved in the formation of isovaleric acid, 2-methylbutyric and isobutyric acid, respectively. The metabolic pathway of each of these volatile fatty acids can be seen in Figure 2.

Mousiness

Another microbiologically produced off-flavour that can render the aroma and taste of wines unacceptable is 'mousiness'. Mousy off-flavour in wine was first reported by Heresztyn (1986b) who isolated and characterised the compounds responsible for this unpleasant aroma from wines contaminated with species of *Lactobacillus* and *Brettanomyces*. In light of the recent in-depth review on mousy off-flavour by Snowdon (2006) only the main aspects entailing *Brettanomyces/Dekkera* spp. will be mentioned.

Three chemical compounds have been identified as being responsible for mousy off-flavour in wine: 2-acetyltetrahydro-

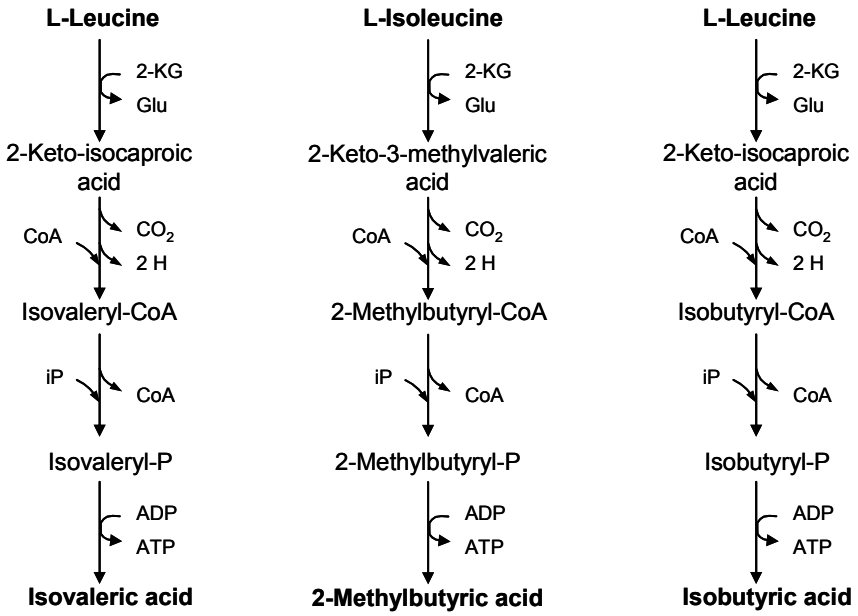

FIGURE 2

Formation of isovaleric acid (3-methylbutanoic), 2-methylbutyric and isobutyric acid (Harwood & Canale-Parola, 1981).

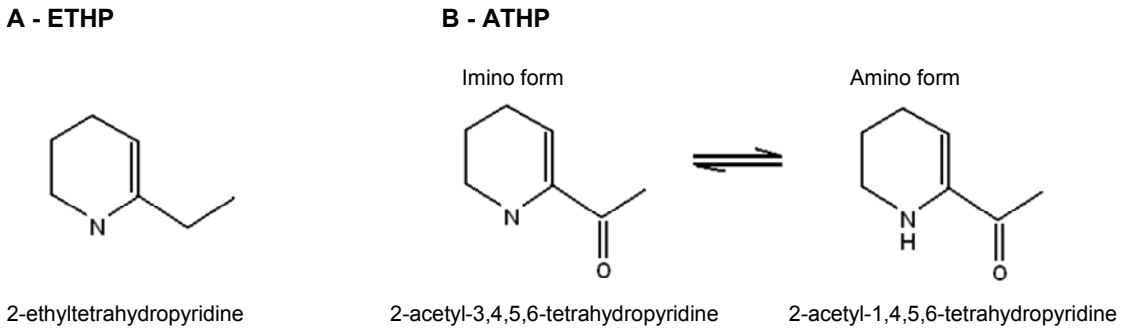

FIGURE 3

Chemical compounds produced by *Brettanomyces/Dekkera* yeasts responsible for mousy off-flavour (Snowdon *et al*., 2006).

pyridine (ATHP), 2-ethyltetrahydropyridine (ETHP) and 2-acetyl-pyrroline (APY) (Heresztyn, 1986b; Grbin *et al*., 1995). Of these, *Brettanomyces/Dekkera* yeasts are capable of producing only the first two (Fig. 3). ATHP appears to be the more prevalent in wine because this compound is normally detected in concentrations (4.8-106 µg/L) (Grbin *et al*., 1995) above its threshold value (1.6 µg/L) (Teranashi *et al*., 1975). ETHP has a much higher threshold value (150 µg/L) (Craig & Heresztyn, 1984) but has only recently been detected in wines in much lower concentrations (Grbin *et al*., 1995).

The amino acid L-Lysine is essential in the formation of these two chemical compounds (Heresztyn, 1986b; Grbin *et al*., 1995; Grbin & Henschke, 2000) and ethanol is a necessary precursor for the flavour to occur in wine (Snowdon *et al*., 2006). Additionally, oxygen has been indicated to have a stimulatory effect on the production of ATHP and ETHP (Grbin, 1998), but this is probably due to higher biomass formation of *Brettanomyces/Dekkera* strains under aerobic conditions. The aroma characters associated with ATHP are reminiscent of 'cracker biscuit' or 'popcorn', however due to the pH of wine, it is more apparent as a bitter, metallic palatable aftertaste. Mousy off-flavours occur infrequently in wine for reasons not fully understood. Chatonnet said: "These compounds are not of major significance when performing sensory screenings of '*Brettanomyces* character' in wine".

Loss of colour

Wines contaminated with *Brettanomyces/Dekkera* strains generally have an undesirable colour. A few reports are available that show glycosidic activity (*b*-glucosidase) amongst *Brettanomyces/Dekkera* strains (Fugelsang *et al*., 1993; McMahon *et al*., 1999; Mansfield *et al*., 2002; Potgieter, 2004; Fia *et al*., 2005). In these cases, these yeasts may produce wines with enhanced aroma and complexity. On the other hand, a large part of the total glycoside concentration of grapes comprises mono-glucosylated anthocyanins, which are considered as the primary red pigments in *Vitis vinifera* (Somers *et al*., 1988). The hydrolysis of glucose usually results in the formation of a corresponding anthocyanin that can be converted to a colourless pseudobase, consequently affecting the

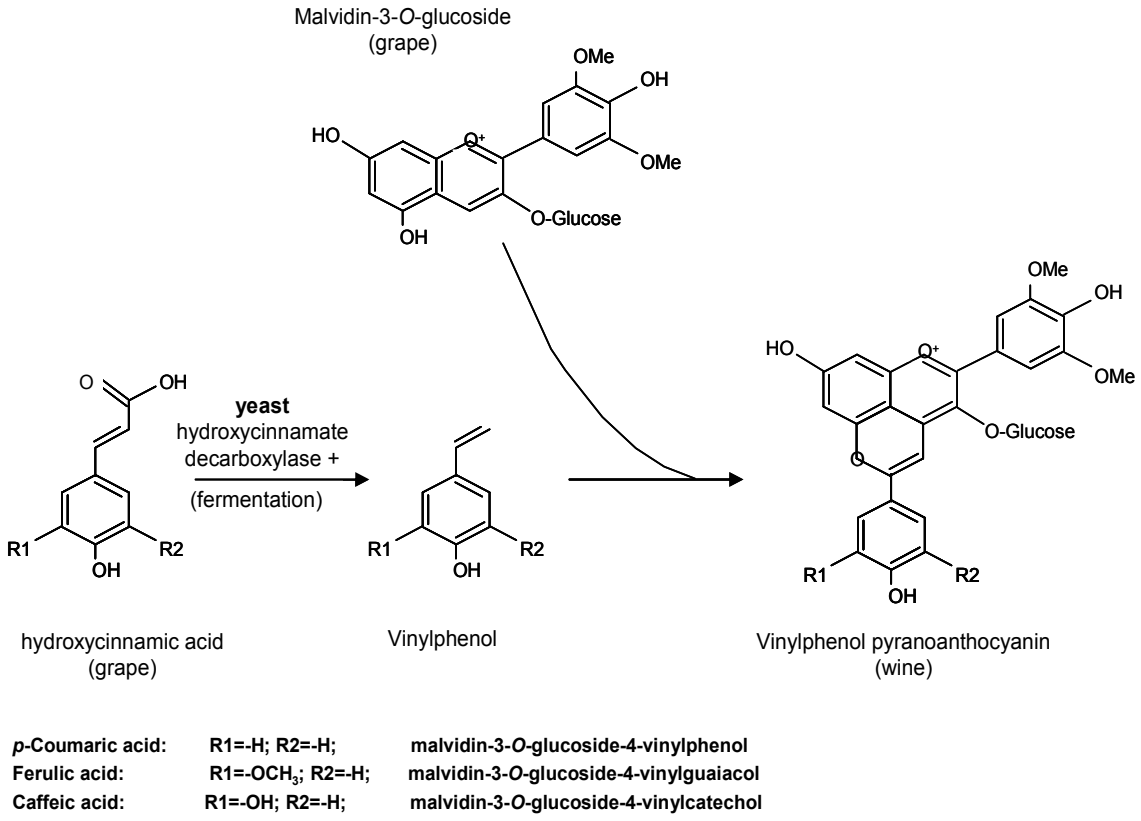

FIGURE 4

Formation of vinylphenolic pyranoanthocyanins during fermentation with yeasts showing hydroxycinnamate decarboxylase activity (Morata *et al*., 2007).

colour negatively (Mansfield *et al*., 2002). This may be a reason why some wines contaminated with *Brettanomyces/Dekkera* spp. have an undesirable colour (Suárez *et al*., 2007).

Another possible theory for loss of colour caused by *Brettanomyces/Dekkera* spp. is presented here. Wine colour is largely determined by various grape-derived anthocyanin pigments. Three types of pyranoanthocyanin pigments, namely vitisins, pyrano-anthocyanin-flavanols and vinylphenolic adducts, have recently been described to increase and stabilise the colour of the wine, particularly during ageing (Morata *et al*., 2007). The vinylphenolic pyranoanthocyanins result from the condensation of vinylphenols with grape anthocyanins, mainly malvidin-3-O-glucoside. Among the group of vinylderivatives, vinylcatechol, 4-vinylguaiacol and vinylphenol have been associated with the formation of vinylphenol malvidin adducts (Fulcrand *et al*., 1996; Francia-Aricha *et al*., 1997; Schwarz *et al*., 2003). The role of yeasts was explained by Morata *et al*. (2007) in that the fermentation by *S. cerevisiae* strains containing hydroxycinnamate decarboxylase activity is favourable for the formation of vinylphenolic adducts. In Figure 4, it can be seen how vinylphenols, formed from the decarboxylation of hydroxycinnamic acids, combine with grape anthocyanins (malvidin-3-O-glucoside) to generate vinylphenolic pyranoanthocyanins.

Along with the wine yeast *S. cerevisiae* and numerous other wine microorganisms, *Brettanomyces/Dekkera* yeasts also contain hydroxycinnamate decarboxylase activity (Heresztyn, 1986a; Chatonnet *et al*., 1992; Edlin *et al*., 1995) and are capable of producing vinylphenols (Clausen *et al*., 1994; Chatonnet *et al*., 1997; Couto *et al*., 2006). However, *Brettanomyces/Dekkera* yeasts are more likely to reduce the available vinylphenols to ethyl derivatives, consequently influencing the formation of vinylphenolic pyranoanthocyanins. Although this is highly speculative at this stage, it might explain the loss of colour as seen in severe cases of *Brettanomyces/Dekkera* contamination.

Biogenic amines

The metabolic activities of microorganisms may also give rise to the formation of biogenic amines (BA). These amines are produced via the decarboxylation of amino acids; e.g. histidine leads to the formation of histamine (Caruso *et al*., 2002). Most BA research has been undertaken into lactic acid bacteria, especially in fermented foods (Stratton *et al*. 1991; Simon-Sarkadi & Holzapfel, 1994) but also in wine (Lonvaud-Funel, 2001). However, very few studies report on BA production by yeasts during wine-making. Caruso *et al*. (2002) evaluated the production of BA by various wine-related yeasts, including *B. bruxellensis*. They found that *B. bruxellensis* produced the highest concentration of total BA (average of 15 mg/L) in comparison with other yeasts, despite exhibiting the weakest fermentative ability. The biogenic amines that were produced included: ethanolamine, methylamine, tryptamine, putrescine, cadaverine, histamine, agmatine and 2-phenylethylamine. Except for the last two amines, the majority of these were barely detectable. Also, considerable strain variability was seen with agmatine produced by *B. bruxellensis* (Caruso *et al*., 2002). In another study the formation of 2-phenylethylamine

accounted for approximately 50% of the total BA produced by *B. bruxellensis* in a grape must fermentation (Granchi *et al*., 2005). Similar to the findings of Caruso *et al*. (2002), *B. bruxellensis* was the yeast species that produced the highest concentration of BA with an average value of 20 mg/L.

The interest in BA in wine has been triggered as these compounds have been linked with unwanted physiological effects in people who show 'amine intolerance' (Gafner, 2003). The resulting symptoms include: headaches, nausea, diarrhoea and red skin colouration, to name a few. The ability of the human body to degrade BA is drastically affected by alcohol consumption as ethanol inhibits the enzyme diamine oxidase activity (DAO) responsible for converting BA to harmless products (Gafner, 2003). The toxicological importance of BA in wine still needs to be established along with the individual toxic thresholds. Nevertheless, the ability of *Brettanomyces/Dekkera* spp. to produce biogenic amines contributes to their general aptitude for spoiling wine.

INVESTIGATIONS ON *BRETTANOMYCES* MANAGEMENT

Due to the importance of *Brettanomyces/Dekkera* yeasts and their association with wine spoilage, it became evident that strategies for control, monitoring and risk management were needed. The incidences of *Brettanomyces/Dekkera* spoilage in wine have become more prominent during the past decade. Yap *et al*. (2007) attributed this largely to:

(i) recent trends in some winemaking styles – such as wines with higher pH values and residual sugar;

(ii) trends in winemaking practices – decreased use of filtration and SO2;

(iii) general poor cellar hygiene along with improper cleaning and sanitisation of barrels – a critical source of *Brettanomyces/Dekkera* contamination of wine;

(iv) the spread of *Brettanomyces/Dekkera* between wineries and regions due to the use of contaminated barrels which are traded in the second hand barrel market; and

(v) importation of *Brettanomyces/Dekkera*-contaminated wine from other affected wineries.

The past decade has yielded reports that present valuable recommendations and strategies to the wine industry regarding the control and monitoring of *Brettanomyces/Dekkera* spp. This section will focus on and summarise the specific research investigations cited in peer-reviewed journals that focused on these aspects as well as highlighting other more general considerations.

PREVENTATIVE MEASURES TO CONSIDER

As *Brettanomyces/Dekkera* yeasts form a natural part of the winemaking process, they cannot entirely be eliminated. The spoilage caused by this species is inevitable in conditions favouring their proliferation. However, there are a few preventative measures that can be considered, although the issues relating to the complexity of microbiological spoilage in wine are not simply resolved with individual factors, but rather require a holistic approach.

Although there are still conflicts in literature about the origin of *Brettanomyces/Dekkera* spp., the vineyard can definitely play a role and grapes of sound quality can decrease some of the risks leading to the production of phenolic off-flavours (Loureiro & Malfeito-Ferreira, 2006; Taillandier, 2007). In addition, the concentrations of the hydroxycinnamic acid precursors directly responsible for *Brettanomyces/Dekkera* phenolic off-flavours can depend on grape variety, quality (Phister & Mills, 2003) and maceration practices. Gerbaux *et al*. (2002) studied the effect of maceration temperature (heating) and intensity on the release of hydroxycinnamic acids from grape skins as factors influencing the formation of volatile phenols by *Brettanomyces/Dekkera* spp. These factors, along with enzymes used for colour extraction and clarification, such as pectolytic and other enzymes containing cinnamoyl esterase activity, can lead to increased levels of volatile phenol production by *Brettanomyces/Dekkera* spp. (Dugelay *et al*., 1993; Gerbaux *et al*., 2002).

Yeast selection can also play a crucial role. There are several criteria to consider when it comes to selection of commercial *S. cerevisiae* starter cultures (Degré, 1993). These are especially important for the prevention of stuck or sluggish fermentations (Malherbe *et al*., 2007), which create an environment for any form of microbial spoilage to occur. The specific aspect of wine yeasts relating to *Brettanomyces/Dekkera* produced off-flavours revolves around their ability to produce vinylphenols via the decarboxylation of cinnamates (Clausen *et al*., 1994). *S. cerevisiae* strains differ in their ability to produce vinylphenols (Van Wyk & Rogers, 2000; Nelson, 2008) and higher levels of vinyl derivatives can consequently lead to objectionable concentrations of ethylphenols by *D. bruxellensis*. Conversely, vinylphenols are involved in the formation of pyranoanthocyanins and at higher levels these can also be beneficial to the stability and intensity of colour in wine (Morata *et al*., 2007). It remains to be ascertained which aspect of hydroxycinnamic acid decarboxylation by wine yeast is more important.

FACTORS FOR CONTROLLING *BRETTANOMYCES/ DEKKERA*

Sulfur dioxide (SO_2)

The antimicrobial potential of SO_2 makes it ideal for wine preservation, in particular for reducing microbiological instabilities (Romano & Suzzi, 1993). Regarding, SO_2 and its effect on the yeast *D. bruxellensis,* studies have yielded incoherent results as this species is regarded as either sensitive or resistant (Loureiro & Malfeito-Ferreira, 2006). Some authors found this yeast to be sensitive to free SO_2 concentrations exceeding 30 mg/L (Chatonnet *et al*., 1992; Gerbaux *et al*., 2002), explaining why it is frequently isolated from wines with low SO_2 protection (Heresztyn, 1986a). Others observed yeast growth with concentrations of free SO_2 of above 30 mg/L, reflecting the resistance of certain *D. bruxellensis* strains (Van der Walt & Van Kerken, 1961; Froudière & Larue, 1988). This controversy, however, does not lie in the free form of SO_2, but rather in the actual effectiveness of its molecular form (Boulton *et al*., 1996; Margalit, 1997; Ribéreau-Gayon, 2000), which is dependent on many variations in wine composition (pH, ethanol, temperature, anthocyanin levels and nutrient content) (Smith, 1996 in Licker *et al*. 1998). For example, the concentration of molecular SO_2 is pH-dependent and 30 mg/L of free SO_2 can release 0.4 mg/L of molecular SO_2 at pH 3.7, and 0.8 mg/L at pH 3.4 (Margalit, 1997). The use of 0.5 to 0.8 mg/L molecular SO_2 has been recommended to control *Brettanomyces/Dekkera* spp. (Henick-Kling *et al*., 2000). The effectiveness of molecular SO_2 on a strain of *B. bruxellensis* has also been linked with oxygen availability (Du Toit *et al*., 2005). The authors reported that 0.25

mg/L of molecular SO_2 drastically affected the cultureability of the strain, however, the strain remained viable and increased in numbers after exposure to oxygen. This is especially of importance during racking and transfers throughout barrel ageing. Barrels can also reduce SO_2 levels over a period of four to six months of ageing (Chatonnet *et al*., 1993) and therefore SO_2 management is crucial during this time. This agrees with anecdotal evidence that new barrels can absorb up to 15 mg/L of free SO_2 over the same time period (Coulter *et al*., 2004).

A survey done by the Australian Wine Research Institute (AWRI) revealed a tendency among Australian winemakers to use many smaller SO_2 additions, as opposed to larger, less frequent additions during winemaking. This is not advised as it can lead to the unintentional selection or build-up of more resistant yeast species, including *Brettanomyces/Dekkera* spp. (Coulter *et al*., 2004).

Other additives

In addition to SO_2 usage, alternative additives have also been investigated as growth inhibitors for *Brettanomyces/Dekkera* yeasts (Loureiro & Malfeito-Ferreira, 2006; Suárez *et al*., 2007). Dimethyl dicarbonate (DMDC), also commercially known as Velcorin®, has been evaluated for *Brettanomyces/Dekkera* yeast inhibition. It was found that DMDC could not completely inhibit the growth of *B. anomalus* with a dosage of 400 mg/L, but 250 mg/L inhibited the fermentation of *B. bruxellensis* (Delfini *et al*., 2002). A very recent study described the effectiveness of DMDC for the prevention of *B. bruxellensis* in wine and evaluated its use during different winemaking stages (Renouf *et al*., 2007). The authors obtained variable results with strains of *B. bruxellensis* showing moderate resistance with 150 mg/L DMDC in grape must and 250 mg/L causing only a transitory inhibition during MLF. The effectiveness of DMDC was, however, dependent on ethanol content (Malfeito-Ferreira *et al*., 2004). It was also found that DMDC did not completely eliminate *B. bruxellensis* populations in the presence of lees at 200 mg/L (Renouf *et al*., 2007). On the other hand, in finished wines the *B. bruxellensis* population declined to less than 100 cfu/mL. Renouf *et al*. (2007) advised against the use of DMDC before the end of MLF as it can act on fermenting species such as *S. cerevisiae* and *Oenococcus oeni* and instead recommended the use of DMDC prior to bottling. Regular additions up to 200 mg/L (maximum permitted level in wine) can help to control *Brettanomyces* growth during barrel maturation (Loureiro & Malfeito-Ferreira, 2006) in countries where its use is allowed.

The effect of DMDC is not directly pH dependent (Threlfall & Morris, 2002) and it yields no residual odours or flavours (Ough, 1983). The use of DMDC requires an approved dosing machine and must be carefully handled.

Weak acids, such as sorbic, benzoic and fumaric acids have also been investigated for use against *Brettanomyces/Dekkera* spp. as they contain antifungal activity. *D. bruxellensis* are regarded as one of the most tolerant species to sorbic acid and dissolved carbon dioxide (Ison & Gutteridge, 1987; Loureiro, 1997). Although these weak acids are included in selective media (Chatonnet *et al*., 1992, Rodriquez *et al.*, 2001), they are not favourable during winemaking and face consumer resistance (Pretorius, 2000; Suárez *et al*., 2007). Weak-acid preservatives have been shown to be more effective at low pH values, and that inhibition depended on the actual intracellular concentration of the individual preservatives (Quintas *et al*., 2005). An indirect measure to prevent ethylphenol formation during ageing involves the use of antioxidants such as, ascorbic and erythorbic acids (Suárez *et al*., 2007). Antioxidants can be used to reduce the presence of oxygen, thereby being indirectly detrimental to the proliferation of *Brettanomyces/Dekkera* yeasts.

Haziness/Fining agents

Protein or microbial instabilities are well known for causing turbidity or haziness in wines. This has also been addressed as an issue relating to wine spoilage by *Brettanomyces/Dekkera* after wineries, which apparently practiced effective SO_2 and pH management, barely showed a reduction of 4-ethylphenol concentrations (Coulter *et al*., 2004). In such instances, the effectiveness of SO_2 was compromised as it is rapidly bound. Suárez *et al*. (2007) summarised numerous investigations of fining agents and their impact on *Brettanomyces/Dekkera* yeasts during winemaking. *Brettanomyces* populations can be reduced by 40 to 2000-fold by treatments with fining proteins (Murat & Dumeau, 2003). *Brettanomyces/Dekkera* yeasts in a red wine have also been decreased from an initial population of 10^4 cfu/mL to 170 cfu/mL after fining with liquid gelatine at a dosage of 0.6 ml/L (Suárez *et al*., 2007).

Filtration

Studies that pertain to wine filtration for the removal of *Brettanomyces/Dekkera* cells have also been performed (Calderón *et al*., 2004). The authors reported effective removal of *Brettanomyces* cells using membranes with a pore size smaller than 0.45 µm. Millet and Lonvaud-Funel (2000) studied the VBNC state of wine microorganisms during storage. It was found that non-culturable cells could pass through the 0.45 µm filtration and it is believed that *Brettanomyces* cells can possibly reduce their cellular size when entering the VBNC state. This is a very interesting phenomenon considering that the average size of *Brettanomyces* spp. is (5-8) x (3-4) µm (Millet & Lonvaud-Funel (2000). This might explain why some wines are still subjected to *Brettanomyces/Dekkera* spoilage after they have been declared sterile by agar plate enumerations. The VBNC state of microorganisms, particularly of *Brettanomyces/Dekkera* spp., is an area that needs to be further explored. Moreover, the importance of molecular DNA-based identification techniques is amplified by the shortcomings of microbiological plating techniques. The use of smaller pore sizes during filtration (e.g. cross-flow with 0.22 µm) is recommended for wines destined to be used for topping-up barrels during ageing (Oelofse & Du Toit, 2006). However, filtration poses similar problems to fining as it can be detrimental to the colloidal structure of wine and can lead to a loss of colour (Suárez *et al*., 2007). It should therefore be carefully considered.

Alternative methods

The use of a polysaccharide derived from chitin, called chitosan, has been reported to exert a selective pressure on the growth of *B. bruxellensis* in a mixed bioethanol fermentation with *S. cerevisiae* (Gómez-Rivas *et al*., 2004). The presence of 3-6 g/L of chitosan drastically decreased the growth of *B. bruxellensis* and *B. intermedius* in this study.

The application of high pressure on wine has also been investigated. Total microbial populations comprising AAB, LAB and yeasts (including *Brettanomyces* spp.) could be reduced by 99% by pressures of 400 or 500 MPa for 5 or 15 min at 4 or 20°C, respectively (Puig *et al*., 2003). Wine pasteurisation using

high hydrostatic pressures have been shown not to cause major modifications to the physiochemical and sensorial properties of wine (Mok *et al*., 2006). However, the application of this treatment in oenology still requires a lot of study for the development of appropriate equipment.

The use of temperature for the inactivation of *Brettanomyces/Dekkera* yeasts in wine has also been investigated. Couto *et al*. (2005b) found that a population of 10^6 cfu/mL could be thermally inactivated with a 37.5°C for 6 min and 41°C for 0.6 min treatment. The concern about this approach is its impact on the aroma and flavour characteristics of wine, if the treatment is not carefully controlled.

An alternative strategy to chemical preservation involves the use of antimicrobial agents as part of biopreservation (Pretorius, 2000). Biological control with various antimicrobial agents, such as zymocins is currently being considered, but their efficiency in wine is yet to be determined (Du Toit & Pretorius, 2000). Recently, a potential application of antimicrobial agents active on *Brettanomyces/Dekkera* yeasts during wine ageing and storage has also been hypothesised. Comitini *et al*. (2004) described the use of two killer toxins produced by *Pichia anomala* (DBVPG 3003) and *Kluyveromyces wickerhamii* (DBVPG 6077) that have fungicidal activity against *D. bruxellensis*. The two toxins named Pikt and Kwkt are stable in wine for at least 10 days and show potential use for the future. However, the purification and use of antimicrobial agents might be expensive.

CURATIVE MEASURES FOR VOLATILE PHENOLS

In addition to the methodologies that have been investigated for controlling the microbiological aspects of *Brettanomyces/Dekkera* spp. contamination, there are also some control strategies pertaining to the specific chemical aromas or phenolic off-flavours produced. The volatile phenols, specifically the ethylphenols, which result from a *Brettanomyces/Dekkera* contamination, can also be reduced.

Ugarte *et al*. (2005) obtained a 77% reduction in the total ethylphenols (4-EG and 4-EP) by using reverse osmosis and adsorption. The three hour process comprised a hydrophobic absorbent resin and a membrane with tangential-flow filtration. However, a reduction in some aromatic compounds, namely ethyl- and methyl vanillate and other esters, was also obtained.

Other absorbents commonly used during winemaking have also been reported to reduce off-flavours and odours. Polyvinylpolypyrrolidone (PVPP) and charcoal are used by some winemakers to lower ethylphenol levels (Suárez *et al*., 2007). The prescribed amounts vary and range from 60-480 mg/L for PVPP and 15-240 mg/L for charcoal, depending on the intensity of the off-flavours. Fining agents such as casein and potassium caseinate also present an absorptive function and have been used to reduce low levels of ethylphenols (Ruiz-Hernández, 2003). The adsorption of volatile phenols by using active dried wine yeast and yeast lees as a biosorbent was also investigated (Chassagne *et al*., 2005). This came after decreases in the contents of 4-EP and 4-EG were found in red wine containing yeast lees compared to the same wine aged without lees (Guilloux-Benatier *et al*., 2001). The authors found that active dried yeast of *S. cerevisiae* removed 33% and 26% of the 4-EP and 4-EG concentrations, respectively, in a model wine solution (Chassagne *et al*., 2005). It was speculated that the rapid adsorption process occurs predominantly by yeast surface binding. The affinity of yeast lees for volatile phenol adsorption was sensitive to the level of yeast autolysis and physicochemical parameters, such as ethanol content, temperature and pH.

CONSEQUENCES OF OAK BARRELS AND CONTROL

Another aspect of winemaking that is increasingly gaining substantial attention, involves the role of oak barrels and their effect on microbial wine spoilage. Wooden barrels are particularly known as an ecological niche where microbial spoilage can occur, especially by yeasts such as *D. bruxellensis* (Swaffield & Scott, 1995; Laureano *et al*., 2005). Due to the difficulty of sanitising barrels several concerns have been raised regarding the control of *Brettanomyces/Dekkera* spp. during barrel ageing and storage of wine.

Brettanomyces/Dekkera yeasts are known to survive in barrels in areas where they are protected against treatments such as SO_2. These include the yeast lees, around bung holes and in the oak structure (Laureano *et al*., 2005). The penetrative capacity of the wine serves as a vector for carrying these yeasts deep into the cracks and crevices of staves (up to 8 mm) (Fugelsang, 1997; Laureano *et al*., 2005). Here, the cells have a large degree of protection against SO_2 gas allowing them to establish themselves (Fugelsang, 1997; Swaffield *et al*., 1997). The survival of established microbial populations becomes a greater sanitary issue with used barrels (Chatonnet *et al*., 1999) as the pores become impregnated or blocked by microbial cells, colour pigments and other colloidal materials. This is further complicated by the presence of fungal growth and the formation of microbial biofilms (Yap *et al*., 2007). The influence of biofilms on wood structure and wine spoilage is not well characterised. However, biofilms are reportedly up to 1000 times more resistant to chemical cleaning agents and sanitisers (Kumar & Anand, 1998; Lewis, 2001). In this regard, Joseph and Bisson (2004) found that 50% from a total of 35 *Brettanomyces/Dekkera* isolates could form biofilms, which adds another dimension to their control.

Barrel cleaning and sanitation is very difficult and there are many inconsistencies in literature as to which methodologies are most effective. Chatonnet *et al*. (1992) advised that the sanitation of barrel wood requires at least 7 g of SO_2 gas per barrel. Henick-Kling *et al*. (2000) recommends that filled barrels should receive approximately 30-35 mg/L free SO_2 during summer months. A recent study by Laureano *et al*. (2005) evaluated different treatments on used French barrels (third fill) that contained red wine with high ethylphenol levels and culturable *D. bruxellensis* cells. These included: (i) cold water rinse followed by three hot water rinses at 70°C and air-drying; (ii) same as above plus filling with an aqueous solution of SO_2 (200 mg/L, pH 3) and storing for one month; (iii) cold water rinse, followed by filling the barrel three-quarters full with hot water at 90°C for 10 min; and (iv) cold water rinse, followed by a 70°C hot water rinse and steaming under low pressure (0.5 kg/cm) for 10 min. It was found that the treatment with steam was the most effective, although none of the approaches was able to significantly reduce the microbial populations as *D. bruxellensis* cells were still recovered from the external surfaces of grooves, side surfaces of staves (2-4 mm) and at the bunghole (4-6 mm).

Studies concerning the use of ozone have also been reported. Cantacuzene *et al*. (2003) evaluated the effect of aqueous ozone and ozone gas on *B. bruxellensis* contaminated oak cubes. The authors found a reduction of the *Brettanomyces* population with

TABLE 2
Summary of investigations on *Brettanomyces/Dekkera* control.

Treatment	Reference
Protein clarification	Murat & Dumeau (2003)
Gelatine	Ruiz-Hernández (2003)
Egg white	
Potassium caseinate	
Caseins	
Filtration	Millet & Lonvaud-Funel (2000)
Membranes (0.45 µm)	Calderón *et al* . (2004)
Ultrafiltration	
Physicochemical variables	Gerbeaux *et al* . (2000)
Low aging temperature	
Low pH	
Reduction of oxygen content	
Avoidance of micro-oxygenation	
High alcohol levels	
Reduction of precursor concentration	Gerbeaux *et al* . (2002)
Low maceration temperature	
Avoidance of pectolytic enzymes and enzymes with cinnamoyl esterase activity	
Additives	Ison & Gutteridge (1987)
SO_2	Delfini *et al* . (2002)
DMDC	Renouf *et al* . (2007)
Chitosan	Gómez-Rivas *et al* . (2004)
Sorbic acid	
Benzoic acid	
Fumaric acid	
Ascorbic acid	
Erythorbic acid	
High pressure processing	Puig *et al* . (2003)
400-500 MPa	
Biological techniques	Du Toit & Pretorius (2000)
Zymocins/killertoxins	Comitini *et al* . (2004)
Bacteriological enzymes	
Genetic egineering	Du Toit & Pretorius (2000)
Transgenic yeasts	
Other alternatives	Guilloux-Benatier (2001)
Ozone	Coggan (2003)
Reverse osmosis	Cantacuzene *et al* . (2003)
Absorbents	Chassagne *et al* . (2005)
PVPP	Yap *et al* . (2007)
Charcoal	
Ultrasonics	

the ozone gas and with a hot water treatment (82°C for 20 min), but not with the aqueous ozone. In contrast, *Brettanomyces* populations were reduced by up to 99% with ozonated water in another investigation (Coggan, 2003). Despite many anecdotal reports, ozone sanitation has been used with good results and is strongly recommended for stainless steel tanks.

Barrel shaving and re-firing have also been investigated by Pollnitz *et al.* (2000) who found that the wine contained up to 85% less 4-EP and 4-EG after being stored in shaved and re-fired barrels in comparison to control barrels (untreated barrels). This was attributed to a reduced microbial population on the inner surface of barrels, which confirmed the prevalence of viable *Brettanomyces/ Dekkera* cells in the wood structure (Pollnitz *et al.,* 2000).

One of the latest additions to barrel cleaning and disinfection techniques use high-power ultrasonics (Yap *et al*., 2007). Laboratory tests have proven that ultrasound or sonification

could effectively kill viable cells of *D. bruxellensis* in synthetic media. More than 97% of the population of 4.4 x 10^6 cfu/mL were destroyed with ultrasound power at 50 watts for 90 to 120 s. Further trials directly in wine are anticipated.

Regarding barrel cleaning, there currently appears to be no substantial scientific evidence on which approach will guarantee complete sterilisation. Barrels are often, if not in most cases, impossible to sterilise and the effectiveness of all the above-mentioned procedures is highly doubted (Boulton *et al*., 1996; Pollnitz *et al*., 2000; Arvik & Henick-Kling, 2002; Malfeito-Ferreira *et al*., 2004). In addition to the uncertainties, there are currently no reliable techniques that allow for the direct detection for *Brettanomyces/Dekkera* spp. in wood. This should be considered for future investigations in order to reveal the true value of the various barrel sanitation techniques.

The possibility that new oak barrels can be beneficial for the growth and survival of the *Brettanomyces/Dekkera* population that is carried by the wine has also been hypothesised (Lonvaud-Funel & Renouf, 2005). This speculation has value as new barrels provide greater sugar resources (higher cellubiose levels) and oxygen contributions than older barrels (Swaffield & Scott, 1995; Boulton *et al*., 1996; Loureiro & Malfeito-Ferreira, 2006; Yap *et al*., 2007). However, new oak is not a source of contamination, since wood is not the natural habitat for the yeast. Moreover, it is expected that the toasting process eliminates new barrels as a source of *Brettanomyces/Dekkera* contamination. Problems related to new barrels are most likely to be caused by poor barrel management at wineries, e.g. pre-rinsing with non-sterile water.

Several recommendations for the control, monitoring and curative procedures of *Brettanomyces/Dekkera* spoilage in wine have been formulated over the years (Godden *et al*., 2004). Combined results from scientific investigations and empirical findings currently indicate that the success relating to the control of *Brettanomyces/Dekkera*-associated spoilage in wine evolves around a holistic approach. More detailed recommendations for the control of *Brettanomyces/Dekkera* yeasts during winemaking can also be found in Coulter *et al.* (2004), Loureiro and Malfeito-Ferreira (2006), Oelofse and Du Toit (2006) and Suárez *et al*. (2007). A summary of all the scientific investigations on the control of *Brettanomyces/Dekkera* yeasts discussed in this overview can be seen in Table 2.

CONCLUSIONS

The microbiology of wine is very complex and this often makes it difficult to pinpoint the exact problem and its origin in a habitat such as wine when spoilage does occur. Nonetheless, large-scale investigations on controlling wine spoilage have been conducted for many years in an attempt to improve wine quality and great progress has been made in the past decade.

With regards to *Brettanomyces/Dekkera* yeasts, only small steps have been taken and there are still many factors to be researched. These yeast species are only a few of the many organisms that exist in the winemaking environment and, because they are living entities, there will be a lot of diversity among their species. Despite their economic importance and the increasing amount of interest by the wine industry, *Brettanomyces/Dekkera* spp. have been largely understudied at genetic level. It is for this reason that a genome sequence project on this wine spoilage yeast has recently been initiated (Woolfit *et al*., 2007). Preliminary results indicate that the proteome of *D. bruxellensis* is rich in transporters and genes involved in lipid and nitrogen metabolism. This may well elucidate their ability to survive in an environment with high ethanol and nutrient limitation. Future studies on the genetic characterisation of this species will resolve their true significance during winemaking.

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