Brettanomyces bruxellensis yeasts: impact on wine and winemaking

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Abstract

Yeasts belonging to the *Brettanomyces/Dekkera* genus are non-conventional yeasts, which affect winemaking by causing wine spoilage all over the world. This mini-review focuses on recent results concerning the presence of *Brettanomyces bruxellensis* throughout the wine processing chain. Here, culture-dependent and independent methods to detect this yeast on grapes and at the very early stage of wine production are encompassed. Chemical, physical and biological tools, devised for the prevention and control of such a detrimental species during winemaking are also presented. Finally, the mini-review identifies future research areas relevant to the improvement of wine safety and sensory profiles.

Introduction: *Brettanomyces/Dekkera* yeasts and their occurrence in the wine processing chain

The term *Brettanomyces* was first introduced in 1904 by Claussen to describe a yeast used in the production of English beer. *Brettanomyces* became a recognized genus in 1920 when it was isolated from lambic beers in Belgium (Henschke et al. 2007) and first detected in wine in 1930 (Krumbolz et al. 1933).

Brettanomyces refers to the anamorphic (asexual) form, while the genus name *Dekkera* was proposed for the teleomorphic (sexual) form (Van Der Walt 1964). Currently the genus *Brettanomyces/Dekkera,* belonging to the *Pichiaceae* family, includes five species: *B. custersianus*, *B. naardenesis*, *B. nanus*, *B. anomalus* and *B. bruxellensis*. The species *B. intermedius* and *B. lambicus* are considered synonyms of *B. bruxellensis*. Teleomorphic forms have only been found in *B. anomalus* and *B. bruxellensis*, denominated *Dekkera anomala* and *Dekkera bruxellensis*, respectively (Kurtzman et al. 2011). *Dekkera* asci are formed directly from diploid vegetative cells, and contain from one to four hat-shaped or spheroidal ascospores, which tend to agglutinate when released (van der Walt 1964). *Brettanomyces* species multiply by multilateral budding, or, more rarely, by bipolar budding. The cell shape appears polymorphic - ellipsoidal, ogival or cylindrical - with dimensions ranging from 2 to 7 μm, which often form pseudomyceliums (Kurtzman et al. 2011). Cells may become smaller under stress, thus filtration through 0.45 membranes is sometimes ineffective (Millet and Lonvaud-Funel 2000).

Under aerobic conditions *Brettanomyces/Dekkera* species produce high amounts of acetic acid and ethanol. The presence of oxygen stimulates growth, which subsequently stops for the inhibitory effect of acetic acid. *Brettanomyces/Dekkera* species, except for *B. naardenesis,* could also grow anaerobically and they can be classified as facultative anaerobic and Crabtree-positive yeast, as *Saccharomyces cerevisiae* (Rozpędowska et al., 2011). In 1940, Custer showed that oxygen stimulates the fermentation of *Brettanomyces claussenii*, a phenomenon called "negative Pasteur

effect". Subsequently, Scheffers and Wikén (1969) introduced the concept of the Custer effect, defined as the inhibition of alcoholic fermentation during the transition to anaerobic conditions and underlined that this effect was common to all species of *Brettanomyces*, suggesting it as a taxonomic criterion of the genus. Ciani and Ferraro (1997) reported that after 7-8 hours without oxygen, the culture adapted to the anaerobic conditions and growth resumed, although slowly, and with a lower production of ethanol.

To date, *Brettanomyces/Dekkera* yeasts have been found in grape berries, wine, wine-making equipment, beer, sherries, dairy products, sourdough, cider, kombucha, olives (Curtin et al., 2015), tequila (Lachance 1995), tamarind (Nassereddin and Yamani 2005), ogi, mawè, gowé, and tchoukoutou (Greppi et al. 2013).

Dekkera anomala may spoil beer, cider and soft drinks (Gray et al. 2011), but is not common in wine (Loureiro and Malfeito-Ferreira 2006), where *B. bruxellensis* is more prevalent. In the first phases of vinification, it is usually present at lower concentrations than other yeasts responsible for alcoholic fermentation. Successively and during malolactic fermentation (MLF), *B. bruxellensis* may increase (Renouf et al. 2006) and become the dominant yeast, therefore seriously affecting the sensorial traits of wine. The ability to tolerate environmental stresses such as high ethanol concentrations (up to 14,5-15%), low pH and oxygen, low sugar (smaller than 300 mg/l) and fermentable nitrogen concentrations suggests that *B. bruxellensis* adapted to this peculiar niche (Curtin et al. 2015).

B. bruxellensis may produce 4-vinylphenol and 4-ethylphenol from p-coumaric acid, and 4-vinylguaiacol and 4-ethylguaiacol from ferulic acid. At low concentrations, these volatile compounds can contribute to wine aroma complexity (cider, pepper, clove). However, at concentrations higher than their perception threshold, they negatively impact the sensory profile of wine, conferring off-flavours, such as animal odours, barnyard, horse sweat, medicine and animal leather (Chatonnet et al. 1992). Moreover, this yeast is able to produce biogenic amines. This trait was first described by

Caruso et al. (2002), who detected the production of phenylethylamine in wine by five strains. Vigentini et al. (2008) and Agnolucci et al. (2009) then showed that some strains of *B. bruxellensis* produce cadaverine, hexylamine, phenylethylamine, putrescine and spermidine, under wine-model conditions.

During the last twenty years the molecular and functional diversity of *B. bruxellensis* isolates collected worldwide has been extensively studied (see Oelofse et al. 2008, Curtin et al. 2015). Different strains of *B. bruxellensis* can produce variable yields of volatile phenols not always correlated with its growth (Silva et al. 2004; Conterno et al. 2006; Renouf et al. 2006; Curtin et al. 2007; Vigentini et al. 2008; Romano et al. 2008; Joseph et al. 2013). Barata et al. (2008), studying the effect of sugar concentration and temperature on cellular viability and 4-ethylphenol production, found that the levels of ethylphenols were intrinsically linked to *B. bruxellensis* growth. Agnolucci et al. (2009) reported that seven *B. bruxellensis* strains showed a relationship between growth rate and production kinetics of volatile phenols, while Curtin et al. (2013) found that under oxygen-limiting conditions, three predominant Australian *B. bruxellensis* strains did not differ in their capacity to produce ethylphenols in a chemically-defined wine medium.

Overall, the functional traits related to spoilage activity, such as growth rate, volatile phenol production and sulphite tolerance, were greatly affected by physico-chemical factors (*i.e.* sugar and nitrate source and concentration, temperature, oxygenation, ethanol content, pH) and by different wines and synthetic wine solutions (Curtin et al. 2015).

Methods to detect *B. bruxellensis* in winemaking

B. bruxellensis has been the focus of decades of investigations, most of which aimed at developing methods to detect its presence in the wine chain, from the vineyard to the bottle. The need for more efficient tools to detect its occurrence and biological activity is related to several aspects of its physiology, which makes its cultivation very difficult in traditional microbiological media, due to its

very slow growth rate. In addition, it can enter in a viable-but-not-culturable (VBNC) state (Du Toit et al. 2005; Agnolucci et al. 2010; Serpaggi et al. 2012), remaining viable and potentially able to provoke spoilage.

Selective media have been developed for the isolation of *B. bruxellensis*, such as the Differential Brettanomyces Dekkera Medium, DBDM, (Couto et al. 2005a). Moreover, the formulation of novel enrichment media, allowed its isolation from grape surfaces (Renouf and Lonvaud-Funel 2007).

In the last 20 years new detection methods have been devised, targeted on nucleic acids, RNA and DNA. It is important to note that, as DNA is a molecule that remains stable long after cell death, it can be detected even if the microorganism is no longer alive. In the specific context, this aspect is extremely important as only viable cells can initiate spoilage.

Polymerase chain reaction (PCR)-based approaches have been the most frequent option for detecting *B. bruxellensis*. Species-specific PCR protocols were developed first (Egli and Henick-Kling 2001), followed by further studies in which PCR was coupled with other molecular methods in order to differentiate the species within the genus (Cocolin et al. 2004). Furthermore, the use of the loop-mediated isothermal amplification method has also been used to detect *Brettanomyces* species in wine and beer (Hayashi et al. 2007).

Quantitative PCR (qPCR) has been subsequently used for detecting and quantifying *B. bruxellensis*. The first qPCR protocol was developed by Phister and Mills in 2003, followed by other experimental studies, which confirmed the advantages of this method (Delaherche et al. 2004; Tessonniere et al. 2009).

The availability of molecular methods, and more specifically of qPCR protocols, enabled scientists to better understand the involvement of this yeast in the wine chain, showing its occurence in red wines from Spain and Italy (Portugal and Ruiz-Larrea 2013; Campolongo et al. 2010), in conventional and organic wines (Tofalo et al. 2012) and in pressed Sangiovese grapes (Agnolucci et al. 2007). The

results of such investigations have shed light on the prevalence and distribution of *B. bruxellensis* in all the winemaking-processing chain, including its occurrence on grape surfaces.

Also the issue of the presence of live/dead *Brettanomyces/Dekkera* cells has been addressed both by targeting messenger RNA (Willenburg and Divol 2012) and by pretreating the samples with intercalating dyes, able to bind covalently to dead cells DNA, preventing its amplification (Andorrà et al. 2010; Vendrame et al. 2014).

In addition to PCR and qPCR methods, other approaches targeted *B. bruxellensis* in wine samples, such as fluorescence in situ hybridization (Stender et al. 2001; Röder et al. 2007), dot blot hybridization (Cecchini et al. 2013) and the use of biosensors (Cecchini et al. 2012; Manzano et al. 2016). Finally, several spectroscopy methods, such as Raman (Rodriguez et al. 2013) and Fourier transform mid-infrared (FTMIR) (Oelofse et al. 2010), in combination with chemiometrics, have been devised to identify *B. bruxellensis*, although no applications in wine have been carried out so far.

Preventing and controlling *B. bruxellensis* in winemaking

Culturable *B. bruxellensis* cells close to 10^6 can heavily spoil wine in few weeks by the production of 4-ethylphenol (4EP) and 4-ethylguaiacol (4EG) (Agnolucci et al. 2014). The production of 4-vinylphenol (4VF) and 4-vinylguaiacol (4VG) can be carried out also by VBNC *B. bruxellensis*, even shortly after their death (Agnolucci et al. 2010; Laforgue and Lonvaud-Funel 2012). The ability of such a spoilage yeast to affect wine sensory traits even in the VBNC state, can encompass serious economic and safety consequences, and should be further and more in-depth investigated. Although vinyl compounds have negative off-flavour properties, their role in red wine is of minor importance, compared with 4EP and 4EG, in particular in anthocyans rich wines, as 4VP and 4VG easily bind anthocyanins, forming unvolatile pyranoanthocyanin derivatives (Pozo-Bayon et al. 2004).

Sulfur dioxide is the traditional antimicrobial compound used in winemaking for an effective counteraction of *B. bruxellensis* in grape must and wine. The ability of *B. bruxellensis* to grow in the presence of sulfur dioxide is comparable to that of *Saccharomyces cerevisiae* (Agnolucci et al. 2010; Usseglio-Tomasset 1992). Though, doses as high as 1 mg/L of molecular SO_2 (mSO_2) have been found not effective against *B. bruxellensis*, which continued growing and producing volatile phenols in wine, or synthetic wine solution, with differences among strains and experimental conditions (Agnolucci et al. 2010; Curtin et al. 2012; Zuehlke et al. 2013; Vigentini at al. 2013). Moreover, whether the authors considered the effect of ethanol content on SO_2 dissociation is not always clear, though it can affect the mSO_2 concentration values up to about 70% (Usseglio-Tomasset 1992). Indeed, the mSO_2 concentrations needed for either killing or preventing the growth of *B. bruxellensis* in red wine may be higher than in model wine solutions, as most of the SO_2 combined with anthocyans is quantified as free form when official analytical methods are applied (Usseglio-Tomasset et al. 1982). However, mSO_2 concentrations as high as 1.4 mg/L were not able to induce *B. bruxellensis* VBNC status in wine, whereas only SO_2 concentrations exceeding 2.1 mg/L were needed to kill *B. bruxellensis* (Agnolucci et al. 2014). Such high concentrations can have detrimental effects on vinification, by increasing the overall sulfite concentration beyond the legal limits, hindering the malolactic process, slowing down the phenolic evolution in ageing red wine, interfering with the olfactory properties of wine, and producing harmful effects on human health. The increased antimicrobial effect of sulfites in ethanol solutions (Sturm et al. 2014; Chandra et al. 2015) is likely due also to the thinning of the cell membrane due to ethanol, which affects cell permeability (Vanegas et al. 2010).

Several preservatives other than SO_2 but safer to humans, though not as much effective, have been proposed (Curtin et al. 2015), including killer toxins (Ciani and Comitini 2011; Oro et al. 2014a;

2014b, Villalba et al. 2016). The stability killer toxins and their selectivity against specific yeasts make this tool very interesting for practical use in both grape must and wine.

Physical approaches aimed at preventing *B. bruxellensis* contamination have been also proposed. Most of them show either limited effectiveness or important drawbacks. Ultra-high pressure is hard to apply in industrial winemaking because the high pressure/time conditions are unsuitable for bulk treatments (Gonzalez-Arenzana et al. 2016). Low electric currents proved as effective as SO_2 in killing *B. bruxellensis* with in-barrel ageing of wine (Lustrato et al. 2015), although the effects of applying an electric current through a sensitive redox system, such as wine, are far from being understood. An effective microbial destruction under continuous treatment can be obtained in grape must by exposure to UV-C light. Unfortunately the shielding effect exerted by the anthocyans prevents effective results in red wine (Rizzotti et al. 2015).

The eradication of *B. bruxellensis* is even more important when wine is intended for wood barrel ageing. The residual yeast cells in wine can penetrate the wood pores and crevices and give rise to privileged ecological niches located as deep as 8 mm into the wood, contaminating and spoiling the aging wine (Malfeito-Ferreira et al. 2004). The upper inner surfaces of the barrel show the highest contamination of *B. bruxellensis* cells (Leaute and Giboulot 2013) probably because of the highest concentration of dissolved oxygen and the resulting lower SO_2 content. *B. bruxellensis* can multiply in wood barrels owing to its ability to exploit the wide number of pentoses, hexoses and disaccharides, including cellobiose, released from wood owing to the bending and toasting process of the staves (Crauwels et al. 2015).

Given the high cost of wood barrels and the risk of wine spoilage arising from contaminated wood, the effectiveness of approaches aimed at their sanitization has been assessed. Ozone was evaluated both as a gas and water solution, in order to achieve an in-depth removal of *B. bruxellensis*. Although this strong oxidant led to effective results in disinfecting the inner cask surface (Guzzon et al. 2013), its entry inside the staves was hindered by slow diffusion (Palacios et al. 2012). Moreover, the

occurrence in wood of highly oxidizable phenols may decrease the ozone concentration during its entry through the wood pores.

B. bruxellensis can also be removed from wood barrels by different heat treatments. For example, heating conditions such as 60°C for 19 min using hot water led to the effective sanitization of a wood barrel (Fabrizio et al. 2015). However, as the heat sensitivity of *B. bruxellensis* greatly decreases in wine, milder heating conditions proved to be effective (Couto et al. 2005b). Barrel heating can also be achieved by placing a microwave source inside the cask, although the technical tools needed to prevent cellars from being exposed to microwaves may be hard to use. So far, this approach has not given fully effective results (Gonzales-Arenzana et al. 2013).

Concluding remarks

The occurrence of *B. bruxellensis* in the wine processing chain has been widely investigated and worldwide established as part of grape and cellar microbial diversity. Scientists and winemakers should join collaborative efforts to integrate the new findings from experimental studies and wine industry into a coherent body of knowledge, aimed at understanding the physiology of *B. bruxellensis* in order to prevent and control its detrimental effects on wine industry and consumers safety. Further studies are needed to answer questions as to whether different strains may differentially affect wine spoilage and at what extent; whether environmental variables and winemaking cellar conditions may differentially modulate the production of off-flavours and biogenic amines; whether *B. bruxellensis* different strains are inhibited by the differential concentrations of SO_2 both during normal vegetative growth and in their VBNC status; whether the physical, chemical and biological tools utilized for the control of *B. bruxellensis* show a differential efficiency against diverse strains.

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Conflict of interest The authors declare that they have no conflict of interest.

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