

Brettanomyces bruxellensis yeasts: impact on wine and winemaking

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14

15 **Abstract**

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

24

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

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

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

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

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

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

59 *Dekkera anomala* may spoil beer, cider and soft drinks (Gray et al. 2011), but is not common in wine
60 (Loureiro and Malfeito-Ferreira 2006), where *B. bruxellensis* is more prevalent. In the first phases of
61 vinification, it is usually present at lower concentrations than other yeasts responsible for alcoholic
62 fermentation. Successively and during malolactic fermentation (MLF), *B. bruxellensis* may increase
63 (Renouf et al. 2006) and become the dominant yeast, therefore seriously affecting the sensorial traits
64 of wine. The ability to tolerate environmental stresses such as high ethanol concentrations (up to
65 14,5-15%), low pH and oxygen, low sugar (smaller than 300 mg/l) and fermentable nitrogen
66 concentrations suggests that *B. bruxellensis* adapted to this peculiar niche (Curtin et al. 2015).

67 *B. bruxellensis* may produce 4-vinylphenol and 4-ethylphenol from p-coumaric acid, and 4-
68 vinylguaiacol and 4-ethylguaiacol from ferulic acid. At low concentrations, these volatile compounds
69 can contribute to wine aroma complexity (cider, pepper, clove). However, at concentrations higher
70 than their perception threshold, they negatively impact the sensory profile of wine, conferring off-
71 flavours, such as animal odours, barnyard, horse sweat, medicine and animal leather (Chatonnet et al.
72 1992). Moreover, this yeast is able to produce biogenic amines. This trait was first described by

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

77 During the last twenty years the molecular and functional diversity of *B. bruxellensis* isolates
78 collected worldwide has been extensively studied (see Oelofse et al. 2008, Curtin et al. 2015).
79 Different strains of *B. bruxellensis* can produce variable yields of volatile phenols not always
80 correlated with its growth (Silva et al. 2004; Conterno et al. 2006; Renouf et al. 2006; Curtin et al.
81 2007; Vigentini et al. 2008; Romano et al. 2008; Joseph et al. 2013). Barata et al. (2008), studying the
82 effect of sugar concentration and temperature on cellular viability and 4-ethylphenol production,
83 found that the levels of ethylphenols were intrinsically linked to *B. bruxellensis* growth. Agnolucci et
84 al. (2009) reported that seven *B. bruxellensis* strains showed a relationship between growth rate and
85 production kinetics of volatile phenols, while Curtin et al. (2013) found that under oxygen-limiting
86 conditions, three predominant Australian *B. bruxellensis* strains did not differ in their capacity to
87 produce ethylphenols in a chemically-defined wine medium.

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

92

93 **Methods to detect *B. bruxellensis* in winemaking**

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

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

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

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

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

114 Quantitative PCR (qPCR) has been subsequently used for detecting and quantifying *B. bruxellensis*.
115 The first qPCR protocol was developed by Phister and Mills in 2003, followed by other experimental
116 studies, which confirmed the advantages of this method (Delaherche et al. 2004; Tessoniere et al.
117 2009).

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

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

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

128 In addition to PCR and qPCR methods, other approaches targeted *B. bruxellensis* in wine samples,
129 such as fluorescence in situ hybridization (Stender et al. 2001; Röder et al. 2007), dot blot
130 hybridization (Cecchini et al. 2013) and the use of biosensors (Cecchini et al. 2012; Manzano et al.
131 2016). Finally, several spectroscopy methods, such as Raman (Rodriguez et al. 2013) and Fourier
132 transform mid-infrared (FTMIR) (Oelofse et al. 2010), in combination with chemometrics, have
133 been devised to identify *B. bruxellensis*, although no applications in wine have been carried out so
134 far.

135

136 **Preventing and controlling *B. bruxellensis* in winemaking**

137 Culturable *B. bruxellensis* cells close to 10^6 can heavily spoil wine in few weeks by the production of
138 4-ethylphenol (4EP) and 4-ethylguaiacol (4EG) (Agnolucci et al. 2014). The production of 4-
139 vinylphenol (4VF) and 4-vinylguaiacol (4VG) can be carried out also by VBNC *B. bruxellensis*, even
140 shortly after their death (Agnolucci et al. 2010; Laforgue and Lonvaud-Funel 2012). The ability of
141 such a spoilage yeast to affect wine sensory traits even in the VBNC state, can encompass serious
142 economic and safety consequences, and should be further and more in-depth investigated. Although
143 vinyl compounds have negative off-flavour properties, their role in red wine is of minor importance,
144 compared with 4EP and 4EG, in particular in anthocyanins rich wines, as 4VP and 4VG easily bind
145 anthocyanins, forming unvolatile pyranoanthocyanin derivatives (Pozo-Bayon et al. 2004).

146 Sulfur dioxide is the traditional antimicrobial compound used in winemaking for an effective
147 counteraction of *B. bruxellensis* in grape must and wine. The ability of *B. bruxellensis* to grow in the
148 presence of sulfur dioxide is comparable to that of *Saccharomyces cerevisiae* (Agnolucci et al. 2010;
149 Usseglio-Tomasset 1992). Though, doses as high as 1 mg/L of molecular SO₂ (mSO₂) have been
150 found not effective against *B. bruxellensis*, which continued growing and producing volatile phenols
151 in wine, or synthetic wine solution, with differences among strains and experimental conditions
152 (Agnolucci et al. 2010; Curtin et al. 2012; Zuehlke et al. 2013; Vigentini et al. 2013). Moreover,
153 whether the authors considered the effect of ethanol content on SO₂ dissociation is not always clear,
154 though it can affect the mSO₂ concentration values up to about 70% (Usseglio-Tomasset 1992).
155 Indeed, the mSO₂ concentrations needed for either killing or preventing the growth of *B. bruxellensis*
156 in red wine may be higher than in model wine solutions, as most of the SO₂ combined with
157 anthocyanins is quantified as free form when official analytical methods are applied (Usseglio-
158 Tomasset et al. 1982). However, mSO₂ concentrations as high as 1.4 mg/L were not able to induce *B.*
159 *bruxellensis* VBNC status in wine, whereas only SO₂ concentrations exceeding 2.1 mg/L were
160 needed to kill *B. bruxellensis* (Agnolucci et al. 2014). Such high concentrations can have detrimental
161 effects on vinification, by increasing the overall sulfite concentration beyond the legal limits,
162 hindering the malolactic process, slowing down the phenolic evolution in ageing red wine, interfering
163 with the olfactory properties of wine, and producing harmful effects on human health. The increased
164 antimicrobial effect of sulfites in ethanol solutions (Sturm et al. 2014; Chandra et al. 2015) is likely
165 due also to the thinning of the cell membrane due to ethanol, which affects cell permeability
166 (Vanegas et al. 2010).

167 Several preservatives other than SO₂ but safer to humans, though not as much effective, have been
168 proposed (Curtin et al. 2015), including killer toxins (Ciani and Comitini 2011; Oro et al. 2014a;

169 2014b, Villalba et al. 2016). The stability killer toxins and their selectivity against specific yeasts
170 make this tool very interesting for practical use in both grape must and wine.

171 Physical approaches aimed at preventing *B. bruxellensis* contamination have been also
172 proposed. Most of them show either limited effectiveness or important drawbacks. Ultra-high
173 pressure is hard to apply in industrial winemaking because the high pressure/time conditions are
174 unsuitable for bulk treatments (Gonzalez-Arenzana et al. 2016). Low electric currents proved as
175 effective as SO₂ in killing *B. bruxellensis* with in-barrel ageing of wine (Lustrato et al. 2015),
176 although the effects of applying an electric current through a sensitive redox system, such as wine,
177 are far from being understood. An effective microbial destruction under continuous treatment can be
178 obtained in grape must by exposure to UV-C light. Unfortunately the shielding effect exerted by the
179 anthocyanins prevents effective results in red wine (Rizzotti et al. 2015).

180 The eradication of *B. bruxellensis* is even more important when wine is intended for wood barrel
181 ageing. The residual yeast cells in wine can penetrate the wood pores and crevices and give rise to
182 privileged ecological niches located as deep as 8 mm into the wood, contaminating and spoiling the
183 aging wine (Malfeito-Ferreira et al. 2004). The upper inner surfaces of the barrel show the highest
184 contamination of *B. bruxellensis* cells (Leaute and Giboulot 2013) probably because of the highest
185 concentration of dissolved oxygen and the resulting lower SO₂ content. *B. bruxellensis* can multiply
186 in wood barrels owing to its ability to exploit the wide number of pentoses, hexoses and
187 disaccharides, including cellobiose, released from wood owing to the bending and toasting process of
188 the staves (Crauwels et al. 2015).

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

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

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

203

204 **Concluding remarks**

205 The occurrence of *B. bruxellensis* in the wine processing chain has been widely investigated and
206 worldwide established as part of grape and cellar microbial diversity. Scientists and winemakers
207 should join collaborative efforts to integrate the new findings from experimental studies and wine
208 industry into a coherent body of knowledge, aimed at understanding the physiology of *B. bruxellensis*
209 in order to prevent and control its detrimental effects on wine industry and consumers safety. Further
210 studies are needed to answer questions as to whether different strains may differentially affect wine
211 spoilage and at what extent; whether environmental variables and winemaking cellar conditions may
212 differentially modulate the production of off-flavours and biogenic amines; whether *B. bruxellensis*
213 different strains are inhibited by the differential concentrations of SO₂ both during normal vegetative
214 growth and in their VBNC status; whether the physical, chemical and biological tools utilized for the
215 control of *B. bruxellensis* show a differential efficiency against diverse strains.

216

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219

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221

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