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Brettanomyces yeasts — From spoilage organisms to valuable contributors to industrial fermentations

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ABSTRACT

Ever since the introduction of controlled fermentation processes, alcoholic fermentations and *Saccharomyces cerevisiae* starter cultures proved to be a match made in heaven. The ability of *S. cerevisiae* to produce and withstand high ethanol concentrations, its pleasant flavour profile and the absence of health-threatening toxin production are only a few of the features that make it the ideal alcoholic fermentation organism. However, in certain conditions or for certain specific fermentation processes, the physiological boundaries of this species limit its applicability. Therefore, there is currently a strong interest in non-*Saccharomyces* (or non-conventional) yeasts with peculiar features able to replace or accompany *S. cerevisiae* in specific industrial fermentations. *Brettanomyces* (teleomorph: *Dekkera*), with *Brettanomyces bruxellensis* as the most commonly encountered representative, is such a yeast. Whilst currently mainly considered a spoilage organism responsible for off-flavour production in wine, cider or dairy products, an increasing number of authors report that in some cases, these yeasts can add beneficial (or at least interesting) aromas that increase the flavour complexity of fermented beverages, such as specialty beers. Moreover, its intriguing physiology, with its exceptional stress tolerance and peculiar carbon- and nitrogen metabolism, holds great potential for the production of bioethanol in continuous fermentors. This review summarizes the most notable metabolic features of *Brettanomyces*, briefly highlights recent insights in its genetic and genomic characteristics and discusses its applications in industrial fermentation processes, such as the production of beer, wine and bioethanol.

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1. Introduction

Since early Neolithic times, humans relied on fermentation processes to introduce desirable flavours and increase the shelf life and safety of foods and beverages (Chambers and Pretorius, 2010; Sicard and Legras, 2011). Whilst initially conducted spontaneously, most modern fermentation processes are initiated and managed by a well-defined, single-strain starter cultures (Barnett and Lichtenthaler, 2001; Steensels and Verstrepen, 2014). In alcoholic fermentations, these starter cultures most commonly consist of a strain belonging to *Saccharomyces cerevisiae*, or one of its close relatives.

However, pure culture fermentations also have disadvantages; selecting one single strain with all beneficial characteristics necessary for an efficient and high-quality fermentation might prove difficult, since the diversity of wild, contaminating yeasts and bacterial species creates a distinct fermentation and flavour profile and a lot of the complexity or subtle aromatic notes might be eliminated in pure culture fermentations. Despite that several strain improvement methods are described to enrich the aromatic profile or fermentation efficiency of *S. cerevisiae* strains (Steensels et al., 2014a,b), these techniques also have their limitations, and non-*Saccharomyces* (or non-conventional) yeasts are becoming increasingly popular in the fermentation industry (Ciani and Comitini, 2011; Cordero-Bueso et al., 2013; Gonzalez et al., 2013; Johnson, 2013). Whilst many of these yeasts are still stigmatized as unwanted spoilage organisms, some of them can have a beneficial role by increasing the fermentation efficiency, lowering the spoilage risk or changing the flavour profile of the end product (Steensels and Verstrepen, 2014). One of such microbes is *Brettanomyces*. This yeast was originally described in 1904 by Niels Hjelte Claussen, a younger colleague of the famous Emile Christian Hansen, at the Carlsberg brewery. Claussen isolated this peculiar yeast from beer, where it was held responsible for performing the secondary fermentation and development of characteristic flavours of the finest English stock ales (Claussen, 1904). Interestingly, this initial isolation of *Brettanomyces* resulted in the first patented microorganism in history (UK patent GB190328184). In the claims of the patent, Claussen targets "the employment in the manufacture of English beers such as ale, stout and porter, of cultures of the new species of micro-organisms hereinbefore called *Brettanomyces* (which do not form endospores and thus differ from the Saccharomycetes) in order to produce the flavour and condition peculiar to such beers".

One century later however, the role of *Brettanomyces* in the food industry is confounded and ambiguous. For example, *Brettanomyces* yeasts are considered to be some of the worst spoilage microbes in wine (Wedral et al., 2010), whilst their presence is imperative in spontaneously fermented beers (Verachtert, 1992). Nevertheless, the potential of *Brettanomyces* as a starter culture in industrial fermentation processes is increasingly recognised. Its unique flavour profile and amylase activity make them very well suited for the production of novel alcoholic beverages (Daenen et al., 2009), whilst their tolerance to low pH, their nutrient-efficient metabolism and their ability to produce high concentrations of ethanol caught the eye of the bioethanol industry (Passoth et al., 2007). Hence, whilst *Brettanomyces* spp. are still not used frequently as starter cultures in food fermentations and their eradication is still a popular topic in wine research, their biotechnological potential becomes more and more apparent, and an increasing number of studies target the peculiar *Brettanomyces* genome, transcriptome, metabolome, proteome and phenome.

In this review, we provide a comprehensive overview of the currently available information on *Brettanomyces* yeasts. In the first section, the biological and genetic diversity and the taxonomy of the *Brettanomyces* genus are addressed. In a second part, the genetic features of *B. bruxellensis*, and how evolution shaped the genome of this species, are reviewed. Next, we review the phenotypic characteristics, such as production of flavour-active compounds, growth patterns in industrially relevant conditions and aspects of microbial safety. In the last part, we discuss the role and potential of *Brettanomyces* in three fermentation processes, namely the production of spontaneously fermented beers, wines and bioethanol, and we discuss future perspectives. However interesting, other aspects regarding *Brettanomyces* fall outside the scope of this review and are only mentioned briefly, but are reviewed in detail elsewhere. These topics include *Brettanomyces* detection, identification and enumeration (Barata et al., 2012; Fugelsang and Edwards, 2007; Wedral et al., 2010), genomic evolution (Curtin and Pretorius, 2014) and genetic modification methods (Schifferdecker et al., 2014).

2. Taxonomy of *Brettanomyces*

The etymological origin of the *Brettanomyces* yeast genus lies in Great Britain, where it was first isolated by Claussen in 1904 (see also above). Whilst Claussen did call his isolate "*Brettanomyces*" (deducted from the Greek words Brettano [British brewer] and Myces [fungus]), he initially classified it as a *Torula* species. However, in 1921, Kufferath and Van Laer isolated a yeast strain from Belgian lambic beers with the same characteristics described by Claussen and classified it as *Brettanomyces bruxellensis* (Custers, 1940). The first systematic investigation of *Brettanomyces* yeasts was conducted and reported by Mathieu Custers in 1940, who characterized 17 different strains, isolated from English and Belgian beers.

Since its first description, the taxonomy of *Brettanomyces* has been the subject of debate and there have been many reclassifications over the years. Initially, the classification was solely based on a few, asexually reproducing (anamorphic) variants (Custers, 1940). However, a few decades later (in 1960), the formation of ascospores was observed in some strains and the genus *Dekkera* was introduced in the taxonomy as the teleomorphic (sexual) counterpart of *Brettanomyces* (Van der Walt, 1984). Thus, in current classifications, yeasts belonging to the genus *Brettanomyces* are non-spore forming (anamorph), whilst the genus name *Dekkera* describes the spore forming (teleomorph) variants of the yeast. However, they are currently often used as synonyms. Interestingly, the distinction between *Dekkera* and *Brettanomyces* is still somewhat unclear, especially since current molecular DNA detection techniques were not able to detect systematic differences between the anamorph and teleomorph states (Oelofse et al., 2008). Moreover, according to the new International Code of Nomenclature for algae, fungi, and plants (the Melbourne Code), fungal species should be assigned only a single valid name. Since the name *Brettanomyces* is well-known and used more commonly in the food and beverage industries, it will likely be prioritized over *Dekkera*. However, since both genera were used frequently in the past decades, the name *Brettanomyces*/*Dekkera* or *B/D* will be used in this review.

In the first edition of their manual on yeast characteristics and identification, Barnett and co-workers described the following 9 *B/D* species: *Brettanomyces abstinens*, *Brettanomyces anomalus*, *Brettanomyces claussenii*, *Brettanomyces custersianus*, *Brettanomyces custersii*, *Brettanomyces lambicus*, *Brettanomyces naardenensis*, *Dekkera bruxellensis* and *Dekkera intermedia* (Barnett et al., 1983). Currently,

five species of *B/D* are described, based on molecular analysis of the genera: the anamorphs *B. bruxellensis*, *B. anomalus*, *B. custersianus*, *B. naardenensis*, and *Brettanomyces nanus*, with teleomorphs existing for the first two species, *D. bruxellensis* and *Dekkera anomala*. Details regarding the taxonomical rearrangements and cross references from the original classification to the current version are depicted in Table 1.

3. The *Brettanomyces* genome and phenome

Most currently known ecological niches colonized by *B/D* spp. are spontaneous alcoholic fermentation processes, as well as soft drinks, dairy products, kombucha tea, sourdough and olives (Table 1). These niches are characterized by varying combinations of environmental stresses: high ethanol concentrations, low pH, the absence of readily fermentable nitrogen and carbon sources, low oxygen, etc. This section will give a concise overview on *B/D* genomics and how evolutionary processes shaped its genome and consequently its phenome (i.e. the combination of phenotypes or "traits"), allowing it to adapt to these harsh environments and outcompete other microbes. We also discuss the role of environmental factors on the growth and flavour production. Since *B/D bruxellensis* is the most encountered and best-studied *B/D* representative, this section will largely focus on this species.

3.1. Genome of Brettanomyces

The sequencing of the complete genome of the baker's yeast *S. cerevisiae* in the 1990s, a multimillion dollar project supported by a worldwide consortium of 74 research groups, was a crucial scientific accomplishment that propelled many new biological discoveries (Goffeau et al., 1996). Now, next-generation sequencing technologies make whole genome sequencing an almost trivial and much cheaper undertaking, and as a result, the genomes of over 40 different yeast species, including several *B/D bruxellensis* strains (Table 2), have been published (Dujon, 2010; Martin et al., 2011). However interesting, a full evaluation of the current knowledge of *B/D bruxellensis* genetics and genome evolution falls outside the scope of this review, but is thoroughly discussed elsewhere (Curtin and Pretorius, 2014).

In 2007, Woolfit and coworkers published the first exploratory genome survey of the French wine spoilage strain CBS 2499, providing a first glimpse of the peculiar nature of the *B/D bruxellensis* genome. In

Table 1
Overview of old and new taxonomical classifications of *Brettanomyces* and *Dekkera* species. Original source of isolation is indicated for each species. *B* = *Brettanomyces*, *D* = *Dekkera*, *C* = *Candida*, NA = not available.

Old classification	Substrate of isolation	New classification
B. sphaericus	Cucumber brine (Etchells and Bell, 1950)	*C. etchellsii*
B. petrophilum	NA (Takeda et al., 1972)	*C. parapsilosis*
B. italicus (*var. membranifaciens*)	Wine (Verona and Florenzano, 1947)	*C. stellata*
B. versatilis	Cucumber brine (Etchells and Bell, 1950)	*C. versatilis*
D. custersiana	Beer (Lee and Jong, 1986)	*B. custersianus*
B. custersianus	Beer (Van der Walt, 1961; Verachtert, 1992), Olives (CBS 8347), Carbonated beverage (Kolfschoten and Yarrow, 1970), Wine (Querol et al., 1990)	
D. naardenensis	Carbonated beverage (Jong and Lee, 1986)	*B. naardenensis*
B. naardenensis	Carbonated beverage (Deak and Beuchat, 1995; Kolfschoten and Yarrow, 1970), Beer (Verachtert, 1992)	
B. nanus	Beer (Scheffers, 1966; Smith et al., 1981; Yamada et al., 1995)	*B. nanus*
D. nana	Beer (Scheffers, 1966; Smith et al., 1981; Yamada et al., 1995)	
E. nana	Beer (Smith et al., 1981)	
B. nonanus	Beer (Verachtert, 1992)	
B. anomalus	Beer (Custers, 1940; Verachtert, 1992), Cider (Morrissey et al., 2004), Sherry wine (Ibeas et al., 1996), Tequila (Lachance, 1995)	*D. anomala*
B. cidri	Cider (Legakis, 1961),	
B/D claussenii (var. *claussenii/sablieri*)	Cider (Legakis, 1961), Beer (Custers, 1940; Lee and Jong, 1985), Sherry wine (Ibeas et al., 1996)	
B. dublin(i)ensis	Beer (Gilliland, 1962)	
Candida beijingensis	NA (Yue and Pna, 1984)	
Torulopsis cylindrica	Beer (Walters, 1943)	
Monilia vini	Wine (Osterwalder, 1912)	
Mycotorula claussenii	NA (Krasil'nikov, 1954)	
Oospora vini	Wine (Janke, 1924)	
D. anomala	Carbonated beverage (Gray et al., 2011; Smith and Van Grinsven, 1984), Kefir (Gadaga et al., 2000; Miguel et al., 2013), Beer (Custers, 1940), Sherry wine (Esteve-Zarzoso et al., 2001; Ibeas et al., 1996), Cider (Coton et al., 2006; Gray et al., 2011)	
B/D abstitens	Carbonated beverage (Yarrow and Ahearn, 1971), Beer (Verachtert, 1992)	*D. bruxellensis*
B. bruxellensis var. vini/bruxellensis/lentus/non-membranifaciens	Beer (Custers, 1940; Kufferath, 1925)	
B. custersii	Beer (Verachtert, 1992), Wine (Florenzano, 1950), Sourdough (Hammes et al., 2005)	
B. intermedius	Carbonated beverage (Put et al., 1976), Beer (Verachtert, 1992), Wine (Van der Walt and Van Kerken, 1959; Wright and Parle, 1974)	
B. lambicus	Beer (Kufferath and Van Laer, 1921; Verachtert, 1992)	
B. patavinus	Wine (Florenzano, 1951)	
B. schanderlii	Beer (Gilliland, 1961), Wine (Peynaud and Domercq, 1956; Van der Walt and Van Kerken, 1959)	
B. vini	Wine (Peynaud and Domercq, 1956)	
D. intermedia	Tea beer (Van der Walt, 1964)	
D. lambica	Beer (Lee and Young, 1986)	
Mycotorula intermedia	Wine (Krumbholz and Tauschanoff, 1933)	
B/D bruxellensis	Kefir (Laureys and De Vuyst, 2014), Sherry wine (Esteve-Zarzoso et al., 2001), Kombucha (Mayser et al., 1995; Teoh et al., 2004),Cider (Morrissey et al., 2004), Wine (Cocolin et al., 2004; Curtin et al., 2007), Bioethanol (Passoth et al., 2007), Sourdough (Meroth et al., 2003), Yoghurt (Kosse et al., 1997), Black olives (Kotzekidou, 1997), Carbonated beverage (Deak and Beuchat, 1995)	

Table 2
Details of the six currently sequenced *Brettanomyces/Dekkera* (*B/D*) genomes. ND = not described.

	Curtin et al. (2012b)	Piskur et al. (2012)	Crauwels et al. (2014)	Borneman et al. (2014)	Borneman et al. (2014)	Valdes et al. (2014)
Species	*B/D bruxellensis*	*B/D bruxellensis*	*B/D bruxellensis*	*B/D bruxellensis*	*B/D bruxellensis*	*B/D bruxellensis*
Strain code	AWRI1499	CBS2499	ST05.12/22	AWRI 1608	AWRI 1613	LAMAP 2480
Ecological niche	Wine	Wine	Beer	Wine	Wine	Wine
Country of origin	Australia	France	Belgium	Australia	Australia	Chile
Genome size (Mbp)	12.7	13.4	13.0	ND	ND	ND
Average coverage	26×	128×	100–110×	61×	68×	26×
Ploidy	Triploid	Diploid	Diploid	Triploid	Diploid	ND

two follow-up studies, the genome of CBS2499 was reanalyzed and subsequently resequenced to a higher coverage (Hellborg and Piskur, 2009; Piskur et al., 2012), enabling the identification of 5600 genes, from which 75% was functionally annotated. Around the same time, the sequence of a second *B/D bruxellensis* wine spoiler (AWRI1499) was published by the Australian Wine Research Institute (Curtin et al., 2012b). More recently, the first genome of a beer-originating *B/D bruxellensis* strain (ST05.12/22) (Crauwels et al., 2014) and the draft genome of a Chilean wine spoiler (LAMAP2480) (Valdes et al., 2014) were published and an in-depth comparison on the genomic structure of four wine isolates, including the previously sequenced AWRI1499 and CBS2499 combined with newly (re)sequenced AWRI1608 and AWRI1613, was performed (Borneman et al., 2014). These studies revealed that although most *B/D bruxellensis* strains share similar general characteristics, the genetic content (ploidy, karyotype, …) can vary significantly.

Comparative analysis of the *B/D bruxellensis* sequences revealed some interesting genomic properties that could be linked to their behaviour and ecological niches. For example, it was shown that many *B/D bruxellensis* strains are equipped with a gene cluster, containing of a nitrate transporter, nitrate reductase, nitrite reductase and two $Zn(II)_2\ Cys_6$ type transcription factors, that enables the utilization of nitrate as a sole nitrogen source (cf. 3.2.1.22.) (Borneman et al., 2014; Crauwels et al., 2014; Woolfit et al., 2007). This gene cluster might confer an important fitness advantage over other species, such as *S. cerevisiae*, in low-nitrogen environments like molasses. In addition, gene content analysis revealed a relative enrichment in genes linked to the cell membrane, membrane-associated transporters, metabolism of alternative carbon sources (such as chitin, N-acetylglucosamine, galactose, mannose and lactose) and oxidoreductase enzymes (Curtin et al., 2012b). Interestingly, many of the enriched membrane-related genes (e.g. *FIG. 2*, *FLO1*, *FLO5*, *FLO9*, *HKR1*, *MUC1*, …) might be advantageous for survival in wine or beer conserved in oak barrels, where they could mediate the adhesion of the cells to the internal wall of the barrel and protect them from washing out during high pressure cleaning (Christiaens et al., 2012; Joseph et al., 2007; Verstrepen et al., 2003b; Verstrepen and Klis, 2006). Further, the enrichment in transporters might be instrumental for the adaptation to relatively low nutrient environments. The (lineage-specific) duplication of oxidoreductase genes may reflect a strategy evolved to enable survival in anaerobic conditions where the species has impaired capacity to regenerate $NAD(P)^+$ and might explain its capacity to produce acetate under aerobic conditions, a trait related to the so-called "Custers" effect (cf. Section 3.2.1.2.1), and the production of some key aromatic compounds (such as isovaleric acid) (Curtin et al., 2012b; Piskur et al., 2012).

Interestingly, not only the DNA sequence itself, but also the general shape of the genome shows several remarkable characteristics. Even though *B/D* species did not undergo a whole-genome duplication event, they do show many instances copy number variation due to local duplications, for example in strain CBS2499. Similar to previous reports in *S. cerevisiae* (Brown et al., 2010; Voordeckers et al., 2013), such copy number variations are frequent in the subtelomeres and often include genes involved in sugar metabolism, indicating that these duplications might aid in the efficient utilization of specific carbon source (Borneman et al., 2014). Apart from copy number variation, gross chromosomal rearrangements were also observed: comparison of the karyotype of 30 different strains revealed remarkable intraspecific differences (Hellborg and Piskur, 2009). Whilst the general chromosome configuration is usually well preserved amongst fungal populations belonging to the same species (e.g. different strains of *S. cerevisiae* are collinear and consist of 16 chromosomes), the chromosome configuration of *B/D bruxellensis* strains was found to be much more variable. *B/D bruxellensis* strains can contain between 4 and 9 chromosomes, and the size of these chromosomes can range from 1 to 6 Mbp. This suggests that *B/D* species might employ frequent variations in chromosome structure to increase their genome variability and competitiveness. Although genomic mutability is beneficial for the adaptability of the species, it can impede sexual reproduction and drive speciation (Fischer et al., 2000).

Haplotype sampling of AWRI1499 revealed that the genome comprises a moderately heterozygous diploid genome, combined with a divergent haploid genome, a phenomenon later also described in AWRI1608 (but absent in CBS2499, AWRI1613 and ST05.12/22) (Curtin et al., 2012b). This suggests a hybridization event of two species or distinct subspecies of *B/D bruxellensis*, one diploid and one haploid. It was suggested that this extra haploid genome might confer a selective advantage in a winery environment, since this triploid genomic structure was detected in 92% of all Australian wine isolates (Borneman et al., 2014). Interestingly, it was shown that the haploid genome fractions of AWRI1608 and AWRI1499 were phylogenetically distant, hinting towards two independent hybridization events (Borneman et al., 2014). Interspecies hybridization events are not rare in fungi and are also observed in the *Saccharomyces* sensu stricto clade. For example, the lager beer yeast *Saccharomyces pastorianus* is an interspecies hybrid of *S. cerevisiae* and *S. eubayanus* (Libkind et al., 2011). Since newly formed hybrid genomes tend to be very unstable [as often shown for *Saccharomyces* hybrids (Antunovics et al., 2005)], mechanisms that drive genome stabilization could explain the extreme karyotype variability observed in *B/D bruxellensis*.

3.2. Phenome of Brettanomyces

Because of its major role in industrial fermentation processes, key phenotypic characteristics of *B/D*, especially *B/D bruxellensis*, have been intensively studied. Despite that many *B/D* strains occupy similar niches as *S. cerevisiae*, their general physiology and phenotypic traits show interesting differences. Below, the main phenotypic characteristics, including growth and fermentation patterns and flavour production, are discussed.

3.2.1. Brettanomyces growth and fermentation

3.2.1.1. Brettanomyces as a well-adapted fermentation specialist. Even though the lineages of *B/D bruxellensis* and *S. cerevisiae* separated approximately 200 million years ago, they both share several peculiar and rather uncommon traits, such as high resistance to osmotic and ethanol stress, and growth in oxygen-limited environments and in low pH, that enable them to thrive in many alcoholic fermentation environments. Despite that some of these traits are widespread in all

yeast genera, these factors are rarely combined in one species (Piskur et al., 2006).

One of the most important phenotypes that determine the habitat of *B/D bruxellensis* is its tendency to ferment sugars to ethanol, even in aerobic conditions where respiration is in principle also possible. This characteristic, called the Crabtree effect, only occurs when sugar concentrations are high and supports the "make–accumulate–consume" strategy, where yeasts first produce ethanol to prevent the growth of competing microbes and then respire the ethanol when glucose is depleted (De Deken, 1966). Another factor that may contribute to the presence of the Crabtree effect is the higher carbon flux and higher ATP production rate through fermentation compared to respiration, even though the ATP yield for respiration is at least an order of magnitude higher (which explains why in aerobic low-sugar conditions, cells generally prefer respiration over fermentation, a trait called "Pasteur effect"). Recently, a study performed by Rozpedowska et al. (2011), deciphered how *B/D bruxellensis* has evolved this phenotype similarly to, but independently of, *Saccharomyces* yeasts. Both lineages used the same strategy relying on global promoter rewiring to change the expression pattern of respiration-associated genes.

Interestingly, *B/D bruxellensis* seems to have an additional strategy to outcompete other microbes. Besides ethanol, they are also capable of producing, accumulating and later consuming high concentrations of acetic acid in aerobic conditions (cf. Section 3.2.2.5), and withstand the resulting low-pH environment. It is important, however, to note that not all *B/D* species share this phenotype. For example, *B. naardenensis*, which separated approx. 100 Mya from *B/D bruxellensis*, is unable to grow in the absence of oxygen (Rozpedowska et al., 2011). Additionally, when grown in oxygen, their metabolism is completely respiratory (and thus no ethanol or acetic acid is formed), indicating that they are Crabtree negative.

3.2.1.2. Role of environmental factors. The growth and fermentation pattern of *B/D bruxellensis* is heavily affected by environmental factors. Below, the effects of oxygen concentration, temperature, nitrogen source, carbon source, sulfur dioxide and ethanol stress are discussed in more detail.

3.2.1.2.1. Oxygen and the Custers effect. The availability of oxygen strongly influences the behaviour of *B/D*. Similar to *S. cerevisiae*, the most commonly studied *B/D* species (*B/D bruxellensis* and *B/D anomala*) are facultative anaerobes. Interestingly, in contrast to the Pasteur effect that poses that yeasts generally prefer respiration over fermentation if oxygen is available and which is observed in *Saccharomyces* spp. when sugar concentrations are low, the carbohydrate metabolism of *B/D bruxellensis* is subjected to a "negative Pasteur effect", meaning that the fermentation of glucose to ethanol is blocked in complete anaerobiosis and is stimulated in the presence of oxygen (Barnett and Entian, 2005; Wijsman et al., 1984; Wikén et al., 1961). The negative Pasteur effect was first described by Mathieu Custers, a student in Albert Kluyver's lab in Delft (Custers, 1940). The results were later confirmed and further analysed by Scheffers and colleagues, who renamed the phenomenon the "Custers effect" (Scheffers, 1961) (Fig. 1).

The exact mechanisms underlying the Custers effect are not completely clear. Several factors appear to be involved, all relating to the inability of *B/D bruxellensis* to restore or maintain their internal redox balance when introduced to anaerobiosis (a schematic representation of the main carbon flux, and other main factors influencing the redox balance in *B/D bruxellensis*, is given in Fig. 1a). As described earlier, *B/D bruxellensis* produces high amounts of acetic acid in aerobic conditions via an NAD^+–aldehyde dehydrogenase (cf. Section 3.2.2.5). This irreversible oxidative conversion from acetaldehyde to acetic acid produces NADH. When oxygen or another external electron receptor is present, this NADH can readily be converted back to NAD^+ (Scheffers, 1961). However, when transferred from an aerobic to anaerobic environment, the lack of NAD^+ generated by the conversion of acetaldehyde to acetic acid quickly results in a blockage of glycolysis (Wijsman et al., 1984). Whilst several yeasts such as *S. cerevisiae* can restore their redox balance in anaerobic conditions by producing secondary metabolites like glycerol, *B/D bruxellensis* is unable to do this, possibly because they lack (or only show very limited) glycerol 3-phosphate phosphatase activity (Tiukova et al., 2013; Wijsman et al., 1984). Despite that this inability to produce glycerol gives *B/D bruxellensis* a competitive advantage over *S. cerevisiae* in nutrient-limiting fermentation environments (since glycerol production is an energy-consuming process), it reduces the growth speed in rich medium in anaerobiosis and causes a considerable lag phase when cells are transferred from an aerobic to an anaerobic environment (Fig. 1b). An additional cause of the Custers effect was revealed by RNA sequencing of *B/D bruxellensis* grown in microaerobic conditions (Tiukova et al., 2013). This study revealed the presence and remarkably high expression of respiratory complex I NADH–ubiquinone reductase in oxygen-limited conditions, which is unusual for Crabtree positive yeasts (Procházka et al., 2010). The activity of this complex of the mitochondrial respiratory chain (which is absent in *S. cerevisiae*) leads to a more efficient metabolism in low-oxygen, nutrient-limiting conditions, since more ATP can be produced through respiration (Leite et al., 2013). This relatively high expression of NADH-generating enzymes (compared to NAD^+-generating enzymes) further supports the primary role of redox imbalance in the Custers effect. Only when other (slow) routes of intracellular re-oxidation of NADH are activated, such as reduction of hydroxystyrenes to their ethyl derivatives (cf. Section 3.2.2.3), or an external electron acceptor, such as acetoin (Scheffers, 1966), is added, *B/D bruxellensis* will be able to escape the aerobic-to-anaerobic lag phase and will start producing ethanol again (without producing acetic acid).

3.2.1.2.2. Nitrogen source. One of the most fascinating features of *B/D bruxellensis* is its ability to outcompete *S. cerevisiae*, the fermentation specialist *par excellence*, in certain fermentation conditions, such as in ethanol production plants in Sweden and Brazil (Liberal et al., 2007; Passoth et al., 2007) (cf. Section 4.3). The characteristics and availability of the nitrogen source are often proposed to be a decisive factor in the success rate of indigenous organism in these niches. In keeping with this theory, it was suggested that *B/D bruxellensis* can utilize the available N sources more efficiently compared to *S. cerevisiae* (Conterno et al., 2006; de Barros Pita et al., 2011). In contrast to *S. cerevisiae*, *B/D bruxellensis* is able to utilize nitrate as sole nitrogen source and can also co-consume it together with other N sources (de Barros Pita et al., 2011). Despite that nitrate metabolism requires energy and therefore causes diminished cell growth and ethanol production in oxygen-limited conditions, it can still provide *B/D bruxellensis* with an advantage in bioethanol fermentations where the relative amount of nitrate can be high (de Barros Pita et al., 2013). Indeed, this trait shows a high variability amongst different *B/D bruxellensis* strains (Conterno et al., 2006; Crauwels et al., 2014), possibly explained by the (sometimes disadvantageous) physiological effects of nitrate utilization (Borneman et al., 2014; Galafassi et al., 2013).

Interestingly, in anaerobic conditions, the presence of nitrate in the fermenting medium allows production of acetic acid (normally not encountered in anaerobic conditions) and at the same time abolishes the Custers effect (cf. Section 3.2.1.2.1). Moreover, acetic acid (and not ethanol) is the prime metabolite produced from glucose in aerobic conditions when only nitrate is present as an N source. Both phenomena are probably due to the activity of nitrate and nitrite reductases, which use NAD(P)H as electron donors and function as a redox valve in anaerobic conditions, or compete with alcohol dehydrogenase for NADH in aerobic conditions.

Despite the clear advantages of nitrate utilization in certain niches, such as certain bioethanol fermentations, the cost–benefit balance of nitrate utilization may favour the loss of this trait in certain niches and explain the observed diversity of this characteristic between different strains, even though further research is needed to investigate this hypothesis.

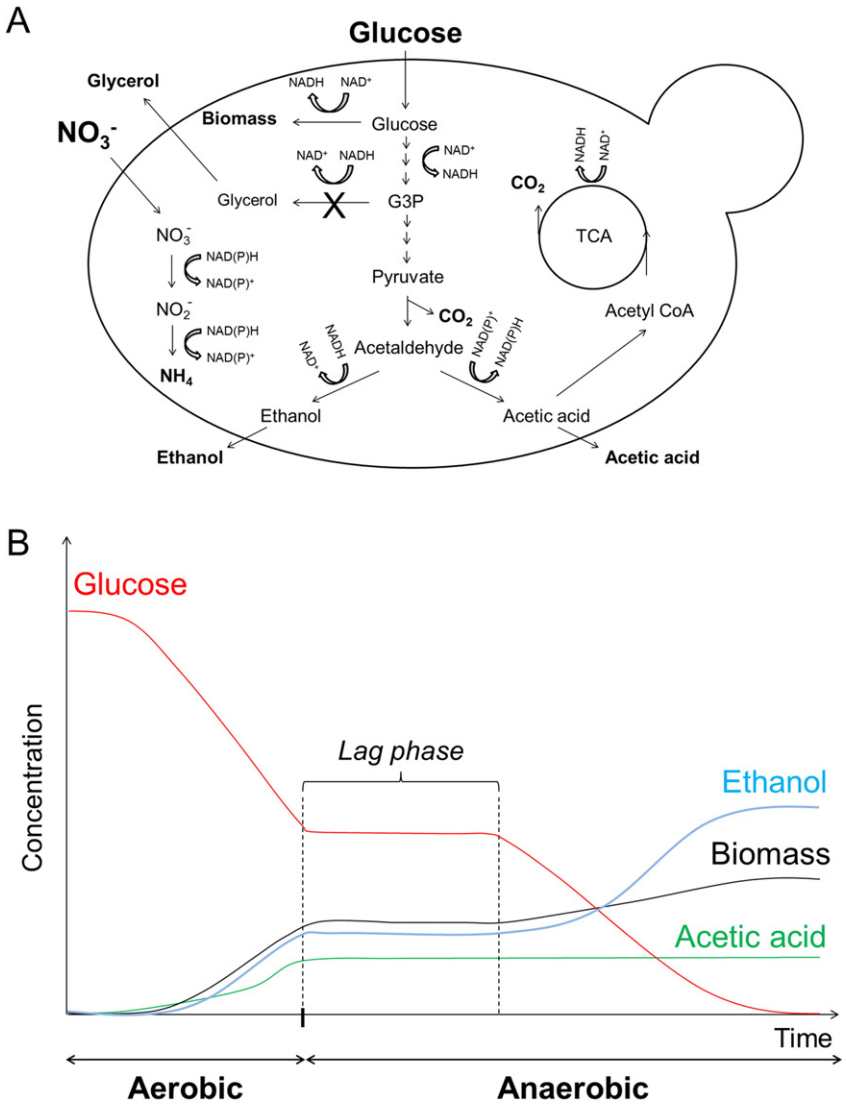

Fig. 1. The Custers effect of *B/D bruxellensis*. (A) Schematic overview of main factors influencing the redox balance and growth pattern. The redox balance is the main responsible for the Custers effect observed in *B/D bruxellensis*, which can ferment glucose to ethanol more rapidly in aerobic than anaerobic conditions. The conversion of glucose-3-phosphate (G3P) to glycerol is typically limited or even absent in *B/D bruxellensis*, due to limited (or absent) glycerol 3-phosphate phosphatase activity (indicated with an X). Nitrate assimilation abolishes the Custers effect by allowing the cell to replenish the NAD(P)H pool through reduction of nitrate to ammonium (cf. Section 3.2.1.2.2). (B) Effect of a shift from aerobic to anaerobic culture conditions on the growth kinetics of *B/D bruxellensis*. The lag phase at the transition from an aerobic to anaerobic environment is caused by the blockage of glycolysis due to a lack of NAD^+. Only when other (slow) routes of intracellular NADH re-oxidation are activated, *B/D bruxellensis* will be able to escape this lag phase and will start producing ethanol again (without producing acetic acid). See text for more details.

3.2.1.2.3. Carbon source. *B/D* is able to ferment a broad range of carbon sources, but they do so at very different rates. For example, it was shown that *B/D bruxellensis* is able to ferment maltose and fructose, albeit at a lower rate compared to glucose (Blomqvist, 2011; de Barros Pita et al., 2013; Leite et al., 2013). Efficient sucrose utilization depends on the expression of a high-efficiency sucrose transporter (for which no homologues exist in *S. cerevisiae*) and might be the key for the high competitiveness of this yeast in sucrose-based fermentations (de Barros Pita et al., 2011; Tiukova et al., 2013). Additionally, it was suggested that a higher affinity for glucose of *B/D bruxellensis* in carbon-limiting conditions (possibly mediated by the orthologous of the *Candida albicans HGT1* gene, encoding a high-affinity H^+-symport glucose transporter) can at least partly explain its success in bioethanol fermentations (Leite et al., 2013). Interestingly, the ability to ferment galactose was shown to vary amongst *B/D bruxellensis* strains (Crauwels et al., 2014). The same variability was encountered in *Saccharomyces kudriavzevii*, with Japanese (but not European) isolates being unable to utilize galactose. It was hypothesized that the fitness cost of having a functional galactose pathway resulted in a selective pressure on the Japanese *S. kudriavzevii* population, leading to the loss of function for all pathway members (Hittinger et al., 2010). However, it is unclear if a similar selective pressure is also at play in *B/D bruxellensis* (Borneman et al., 2014).

Perhaps most importantly, *B/D* is also able to degrade and ferment complex sugars that are not readily utilizable for *Saccharomyces* spp., such as cellobiose and dextrins. Cellobiose, a disaccharide present in second-generation bioethanol substrates [formed by the incomplete hydrolysis of (ligno)cellulose] and wood (e.g. in barrels used in wine or beer fermentations), can be degraded by β-glucosidase, an enzyme often produced by *B/D* (cf. Section 3.2.2.4) (Blondin et al., 1983; Moon et al., 2001). *B/D bruxellensis* strain GDB284, a strain capable of metabolizing cellobiose, was suggested recently as a starter culture for Brazilian bioethanol production (Reis et al., 2014).

Dextrins, such as maltotetraose and maltopentaose, are present as residual sugars after the main fermentation of beer. *B/D* produces α-glucosidase, enabling them to hydrolyze these complex sugars into glucose units (Kumara et al., 1993; Kumara and Verachtert, 1991), yielding "superattenuated" (over-fermented) beers with slightly higher ethanol

levels and lower concentrations of residual sugars (and thus lower caloric contents).

3.2.1.2.4. Sulfur dioxide resistance and viable but non culturable (VBNC) state. The influence of SO_2 on the metabolism of *B/D* is of special importance to the wine industry, where there is no boiling step to disinfect the fermentation medium (like e.g. in beer brewing) and the use of preservatives like SO_2 is the most common way to control microbial contamination. Therefore, several teams have investigated the sensitivity of *B/D*, especially *B/D bruxellensis*, to SO_2 (Agnolucci et al., 2010; Barata et al., 2008; Curtin et al., 2012a; Duckitt, 2012).

It has been suggested that during wine fermentations, various yeast species may enter in a so-called *viable but non culturable* (VBNC) state following sulfite stress (Salma et al., 2013; Serpaggi et al., 2012). VBNC has been described as a physiological state where cells display low levels of metabolic activity but cannot grow or multiply on nonselective media. This state is frequently described in bacteria, but is more rare (or at least less frequently reported) in yeasts. However, SO_2 was shown to induce loss of culturability but maintenance of viability in wine-related yeast species such as *S. cerevisiae* (Salma et al., 2013) and *B/D bruxellensis* (Agnolucci et al., 2010; du Toit et al., 2005; Serpaggi et al., 2012; Zuehlke and Edwards, 2013). Moreover, it was shown that this state was reversible, since removal of the stress by increasing the pH of the medium (to decrease the concentration of toxic SO_2) allowed the VBNC yeast cells to resuscitate (Salma et al., 2013; Serpaggi et al., 2012). However, reports on the effect of SO_2 on *B/D bruxellensis* inactivation are often contradictory (Barata et al., 2008; Chatonnet et al., 1992; Gerbeaux et al., 2002). Indeed, the strain-dependent nature of this trait is highlighted by Curtin et al. (2012a), who found that the maximal sulfite tolerance for the 41 tested *D. bruxellensis* isolates varied over a five-fold range. Recently, a similar test was performed by Vigentini et al. (2013), who tested 108 *B/D bruxellensis* strains for SO_2 resistance. They confirmed the remarkable intraspecies variability and identified two strains that could tolerate up to 0.6 mg L^{-1} of molecular SO_2 (Vigentini et al., 2013). Aromatic characterization of infected wines revealed that VBNC *B/D* cells often maintain their spoilage capacity and continue to produce (low concentrations of) volatile phenols (Agnolucci et al., 2010; Serpaggi et al., 2012), although results are sometimes contradictory (Zuehlke and Edwards, 2013).

Several recent studies investigated the physiological changes of *B/D bruxellensis* upon SO_2 stress and VBNC entry. The cytotoxicity of sulfite in plants and other eukaryotes is mediated by free radicals (such as $OH^{\bullet}$ and H_2O_2) formed when SO_2 is converted from HSO_3^- or SO_3^{2-} to SO_4^{2-} and could therefore generate oxidative stress and thus an increased NADPH demand (Vigentini et al., 2013). Studies targeting the metabolome (Vigentini et al., 2013) and proteome (Serpaggi et al., 2012) of SO_2-stressed *B/D bruxellensis* cells indeed seem to support this hypothesis. Serpaggi et al. (2012) showed that there is a reduced glycolytic flux coupled to changes in redox cell homeostasis and protection mechanisms in VBNC cells (hinting towards a conservation of some large-scale molecular mechanisms between yeast and bacteria when entering a VBNC state). Vigentini et al. (2013) identified several metabolic changes in response to SO_2 stress, most of which pointed towards an increased NAD(P)H demand. More specifically, they observed a decrease in cytoplasmic levels of polyols, and a change in metabolites involved in the glycerolphospholipid pathway (glycerol-3-phosphate and myo-inositol) when cells were exposed to sublethal concentrations of SO_2. The authors did not detect an alteration in the pentose phosphate pathway, which led to the conclusion that NADPH usage could be diverted to other pathways (Vigentini et al., 2013). Additionally, they observed an increase concentration of some amino acids (alanine, glutamic acid, glycine, proline, 5-oxoproline, serine and valine), which could be due to general repression of protein synthesis, increased glycolytic or tricarboxyilic acid pathways or detoxification mechanism (Vigentini et al., 2013).

3.2.1.2.5. Temperature. Optimal growth rates of *B/D* generally lie between 25 °C and 28 °C (Fugelsang and Edwards, 2007; Zuehlke and Edwards, 2013). Several studies indicated that ethanol yield, productivity and growth in general are only marginally influenced by temperature (Blomqvist et al., 2010; Brandam et al., 2008; Yakobson, 2009). A study by Blomqvist et al. (2010) demonstrated that the productivity of *B/D bruxellensis* CBS11269 was only very slightly dependent on temperature between 25 °C and 37 °C, even at varying pH values. This flexibility might further explain the robustness of *B/D* in quickly changing environments such as fermentation processes.

3.2.1.2.6. Ethanol. Most *B/D* strains show a high resistance towards ethanol, a trait crucial for survival in a fermentation environment. However, in general, *B/D* is slightly more sensitive compared to most *S. cerevisiae* strains (Barata et al., 2008). In *B/D bruxellensis*, experiments in synthetic media indicate that 14.5–15.0% (v v^{-1}) of ethanol is likely to be the upper limit that allows *B/D* growth in wines. However, this trait depends on the strain and on environmental factors, such as pH and free sulfite concentration (Sturm et al., 2014). Importantly, different levels in ethanol stress affect the flavour production of *B/D*, with a positive correlation between ethanol stress and production of several ethyl esters, phenyl ethanol and 4-ethylguaiacol (Conterno et al., 2013).

3.2.2. Brettanomyces flavour production

As indicated previously, *B/D* can strongly affect the aroma of fermentation products. Many different terms, including clove, spicy, mousy, barnyard, smoky, plastic, phenolic, medical, "band-aid", metallic, humid leather, cracker biscuit, sweaty, goat-like, apple, floral, tropical fruit, citrus and/or spicy, are used to describe the (often pungent) aroma profile of *B/D* ferments (Heresztyn, 1986; Licker et al., 1999), but these are more conveniently summarized as "*Brett* flavour". Apart from heavily influencing the sensorial characteristics of various foodstuffs, it was recently hypothesized that some of these compounds (more specifically the ethyl phenols) play a crucial role in the dispersal through insect vectors (Dweck et al., 2015). An overview of the most important and industrially relevant aroma-active compounds produced by *B/D*, both positive and negative, is given below.

3.2.2.1. Mousy off-flavours. Mousy off-flavours are regularly encountered in wines infected with lactic acid bacteria (LAB) or *B/D*. There are three known compounds involved this off-flavour: 2-ethyltetrahydropyridine (ETHP), 2-acetyltetrahydropyridine (ATHP), and 2-acetylpyrroline (APY) (Snowdon et al., 2006). Two of these substituted tetrahydropyridines, ETHP and ATHP, are produced by *B/D*, although the absolute concentrations vary between different species and strains (Romano et al., 2008).

The aromas associated with mousy off-flavours are sometimes similar to cracker biscuit aromas, but under low pH conditions they can be perceived as metallic or bitter (Oelofse, 2008). They are usually only perceived after swallowing (or expectoration) and the flavour can persist for more than 10 min (Snowdon et al., 2006). Notwithstanding the huge impact on the quality of beverages, the metabolic pathways leading to the production of ETHP and ATHP in *B/D* are not yet known, but pathways leading to these N-heterocycles in LAB are already described (Costello and Henschke, 2002). ATHP and APY synthesis starts from ethanol, a fermentable sugar (either fructose or glucose) and an amino acid (L-lysine and L-ornithine for ATHP and APY, resp.) with oxygen stimulating their production (Heresztyn, 1986). ETHP on the other hand is likely to be the product of ATHP reduction (Romano et al., 2008).

3.2.2.2. Volatile esters. Since they are responsible for the fruity or flowery character of fermented beverages, volatile esters constitute an important group of aromatic compounds (Verstrepen et al., 2003a). The ester fraction in lambic beers (where *B/D* plays a leading role, cf. Section 4.1) is typically characterized by a very low amount of isoamyl acetate, a high concentration of ethyl caprylate and ethyl lactate and significant amounts of ethyl caprate in comparison to beer produced with traditional *S. cerevisiae* and *S. pastorianus* beer yeasts (Verachtert, 1992). Interestingly, this difference in the concentrations of esters only appears

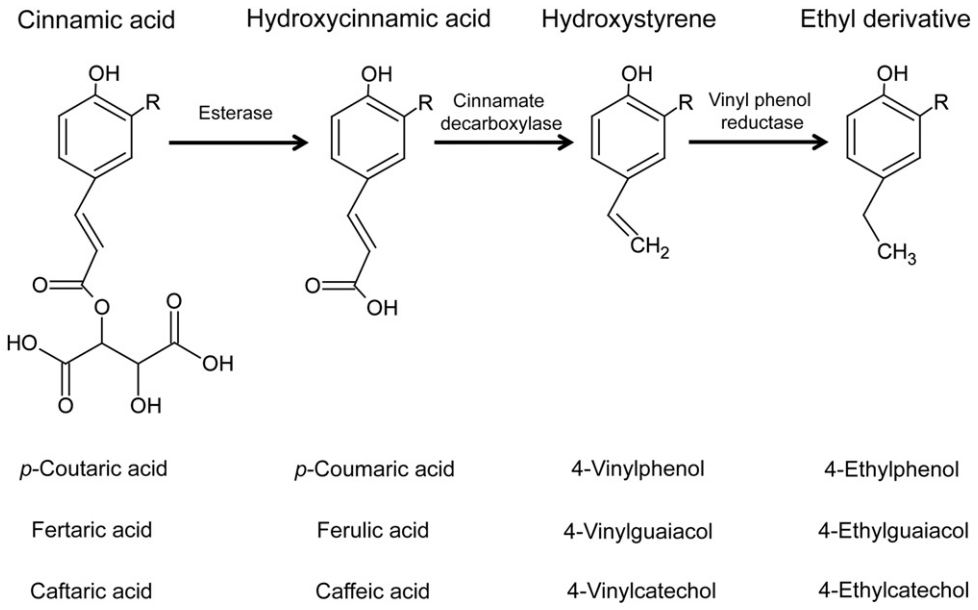

Fig. 2. Formation of volatile phenols by *B/D*. Whilst PAD activity is also commonly encountered in *Saccharomyces* strains, the reductase activity is specific for *B/D*. PAD = phenylacryl acid decarboxylase. VPR = vinylphenol reductase.

with the emergence of *B/D* in the course of the fermentation. Further analysis confirmed that the esterases present in *B/D* spp. are responsible for the formation of ethyl esters, such as ethyl acetate and ethyl lactate, along with the hydrolysis of acetate esters, such as isoamyl acetate and phenethyl acetate (Spaepen et al., 1978; Spaepen and Verachtert, 1982). The imbalance between acetate and ethyl ester concentration is caused by the degradation of acetate esters by the *B/D* esterase, which is much more efficient compared to hydrolysis of non-acetate esters.

3.2.2.3. Volatile phenolic compounds. Volatile phenolic compounds are responsible for some of the most perceptible flavours associated with *B/D* (Chatonnet et al., 1995; Chatonnet et al., 1992; Edlin et al., 1998; Heresztyn, 1986; Licker et al., 1999; Oelofse, 2008). In fact, volatile phenolic (off-)flavours are the main indicators of *B/D* activity in wine. Their production depends on the fermentation medium, since precursor concentration can vary significantly. For example, *B/D* contamination is reported much more frequently in red wines, where the extraction of precursors of volatile phenols from the grape skin is much more intense compared to white wines (Dias et al., 2003; Licker et al., 1999). However, the impact of these compounds on overall wine quality is subjective, with some (rare) reports describing the presence of low concentrations of phenolic compounds as pleasant, since it "adds a distinctive aged character to young red wines", whilst most other tasters find it less desirable due to the "diminished flavour complexity" accompanying low concentration of phenolic compounds (Fugelsang and Edwards, 2007; Malfeito-Ferreira, 2011). There are six compounds responsible for the phenolic flavour: 4-ethylguaiacol (4-EG), 4-ethylphenol (4-EP), 4-ethylcatechol (4-EC) and their precursors 4-vinylguaiacol (4-VG), 4-vinylphenol (4-VP) and 4-vinylcatechol (4-VC) (visualized in Fig. 2). Whilst 4-EG, 4-EP, 4-VG and 4VP have been studied intensively in the last 40 years, the role of 4-EC and 4-VC in the aroma of fermented beverages, mainly cider, was only investigated recently (Buron et al., 2011; Larcher et al., 2008).

The metabolic pathway leading to the synthesis of phenolic off-flavours was systematically investigated in 1986 by Heresztyn (1986). Remarkably, these researchers detected high concentrations of 4-EG and 4-EP in *B/D*-containing fermentations, but only trace amounts of 4-VG and no 4-VP. This is due to the presence of vinylphenol reductase (VPR) in addition to a phenylacrylic acid decarboxylase (PAD), an enzyme also present in *S. cerevisiae* (encoded by the *PAD1* gene) (Dias et al., 2003; Godoy et al., 2014; Godoy et al., 2008; Harris et al., 2009). The reduction of 4-VP leads to 4-EP, reduction of 4-VG to 4-EG and reduction of 4-VC to 4-EC (Buron et al., 2011; Hixson et al., 2012; Vanbeneden et al., 2006; Vanderhaegen et al., 2003) (Fig. 2). The physiological role of these enzymes is not fully understood. However, the decarboxylase gene likely contributes to detoxification processes, since overexpression of *PAD1* in *S. cerevisiae* resulted in an improved growth rate and ethanol productivity in the presence of ferulic acid, cinnamic acid, and in a dilute acid hydrolysate of spruce (Larsson et al., 2001). Additionally, since VPR uses NADH as a cofactor when reducing hydroxystyrenes to their ethyl derivatives, it could be argued that this enzyme might play a role in maintaining the redox balance of the cell. Observations that oxygen-limited conditions enhance VPR activity (Curtin et al., 2013), thereby increasing NAD^+ availability, further supports this theory. Additionally, it was recently shown that ethyl phenols produced by *B/D* can serve as an attractant for insects (Dweck et al., 2015). In this paper, the authors show that the "vinegar fly" (or "fruit fly") *Drosophila melanogaster* detects the presence of hydroxycinnamic acids (HCAs, which are potent dietary antioxidants) via olfactory cues. These flies are not able to smell these acids directly, but are equipped with dedicated olfactory sensory neurons detecting yeast-produced ethylphenols that are exclusively derived from HCAs. Therefore,

Table 3
Volatile phenols in beer and wine (Curtin et al., 2005; Vanbeneden et al., 2006; Witrick, 2012). ND = not detectable.

	Concentration in red wine (ppb)	Concentration in lambic beer (ppb)	Sensory descriptor
4-Vinylphenol	8.8–43	ND–69	Phenolic, medicinal
4-Vinylguaiacol	0.2–15	ND–258	Clove-like
4-Ethylphenol	118–3696	63–1130	Medicinal, horsy
4-Ethylguaiacol	1–432	427–5770	Spicy, clove-like
4-Ethylcatechol	27–427	ND	Phenolic, medicinal

production of ethyl phenols by *B/D* might pose an important life strategy, since it aids in attracting flies that can serve as vectors that promote dispersal of the yeast cells. Similar mechanisms are described in *S. cerevisiae*, where acetate esters are shown to attract flies and promote dispersal (Christiaens et al., 2014).

Interestingly, whilst 4-EP and 4-EG strongly contribute to an undesirable spoilage flavour in wines, the same compounds are considered essential contributors to the flavours of lambic, American Coolship Ale and various Belgian acidic ale beers. The interesting discrepancy between the perceived effect of *B/D* on wines and beers could be caused by the difference in relative concentration of volatile phenols: beer generally contains higher concentrations 4-EG (clove-like, or spicy aroma), whilst wine contains more 4-EP (medicinal, "band-aid" aroma) (Romano et al., 2008; Vanbeneden et al., 2008) (Table 3). The ratio of 4-EP over 4-EG also varies substantially from wine to wine, with reports varying from 3:1 to over 40:1 (Gawel, 2004). The reason for these differences in wine are still not fully understood, even though they are likely caused by the combined effect of differing ratios between wines coumaric and ferulic acids (the precursors of 4-EP and 4-EG, resp.) and of different strains of *B/D* with some being more effective in producing one compound relative to the other (Buron et al., 2012; Gawel, 2004; Vigentini et al., 2008).

Because of its devastating effects on wine flavour, several researchers investigated the factors affecting the production of 4-EP. The results show that *B/D bruxellensis* spp. produce 4-EP compounds under conditions with little residual sugar during the later maturation process, indicating that this process is not subjected to catabolite repression by glucose (Dias et al., 2003; Malfeito-Ferreira, 2011). In addition, pH, temperature and the presence of oxygen and sulfite also seem to influence production of 4-EP (Dias et al., 2003; Zuehlke and Edwards, 2013).

3.2.2.4. Sugar-bound flavour-active compounds. *B/D* spp. are commonly cultured from beer or wine conditioned in oak barrels (Vanderhaegen et al., 2003). Interestingly, some *B/D* species have the ability to hydrolyze cellobiose (a complex sugar present in wood) and further ferment it to ethanol (Moon et al., 2001), which might help to explain how *B/D* can survive for years in wooden casks. This requires a β-glucosidase, which is frequently found in several *B/D* strains (Daenen et al., 2008a; Gonde et al., 1984; Moon et al., 2001).

An industrially important side effect of this enzyme is its capacity to liberate "locked" natural flavours from various substrates. Besides the presence of flavour-active volatile compounds in a free form, fruits, flowers and other plant parts that are often used for food and beverage fermentations also contain volatiles which are glycosidically bound, resulting in water soluble, non-volatile and odourless compounds (Daenen, 2008). The capacity of *B/D* to hydrolyze these non-volatile chemically-bound aroma compounds is interesting in industrial practices because once released, these natural compounds can contribute positively to the aroma profile (Daenen et al., 2009).

β-Glucosidase activity was investigated in *Brettanomyces intermedia* (now *B/D bruxellensis*) (Blondin et al., 1983; McMahon et al., 1999), *B. anomalus* (now *B/D anomala*) (Fia et al., 2005) and *B. custersii* (now *B/D anomala*) (Daenen et al., 2008a). The activity was found to be cell-bound, intracellular and slowly released into the medium. Daenen and coworkers further discovered that *B. custersii* was able to hydrolyze glycosidically bound flavour compounds from hops during the beer maturation phase (Daenen et al., 2008b). Similarly, they showed that *B/D* glycosidase activity likely contributes to the typical flavour development in traditional Kriek (cherry beers) production processes because it liberates flavour-active compounds present in cherries (Daenen, 2008). Also in wines, it was shown that *B/D bruxellensis* and *B/D anomala* display β-glucosidase activity and make the hydrolysis of monoterpene glucosides present in grape juice possible (Fia et al., 2005; Villena et al., 2007).

3.2.2.5. Acetic acid. Acetic acid, often referred to as volatile acidity or vinegar taint, is generally present in fermented beverages in concentrations varying from 0.2–0.6 g/L in wine to 0.4–1.2 g/L in lambic beers. They are generally considered to be negative when concentrations reach 1.2–1.3 g/L. As discussed previously (cf. Section 3.2.1.2.1), *B/D* can produce acetic acid in aerobic conditions, but this trait is strain- and species-dependent (Castro-Martinez et al., 2005; Rozpedowska et al., 2011). Since *B/D bruxellensis* strains have been shown to utilize both glucose and ethanol to produce acetic acid in aerobic conditions, they have been suggested as interesting candidates for industrial acetic acid production (Freer, 2002; Freer et al., 2003).

3.2.2.6. Glycerol. Glycerol is a non-volatile compound that does not have a specific aroma. However, its viscous nature and sweet taste contributes to the quality of fermentation products by providing sweetness, body and mouthfeel (Langstaff and Lewis, 1993; Nurgel and Pickering, 2005; Pretorius, 2000). In most *Saccharomyces* fermentation processes, it is quantitatively the most import product after ethanol and carbon dioxide. *B/D bruxellensis*, however, does not produce large quantities of glycerol, which is most likely due to the absence of glycerol 3-phosphate phosphatase activity (Wijsman et al., 1984). As discussed above, the lack of glycerol production may play an important role in the Custers effect (cf. Section 3.2.1.2.1). Recent studies, however, observed glycerol production under anaerobic conditions by some *B/D bruxellensis* strains, albeit in very low concentrations (Aguilar Uscanga et al., 2003; Blomqvist et al., 2010; Liberal et al., 2007; Rozpedowska et al., 2011).

3.2.2.7. Volatile fatty acids. *B/D* can produce several volatile fatty acids, including isovaleric acid (sensorially described as rancid and/or cheesy). Together with phenolic compounds, this volatile compound is the main contributor to undesirable *B/D* character in wines (Licker et al., 1999). Additionally, it might indirectly affect the aroma by changing the overall perception or intensity of volatile phenolic compounds (Oelofse et al., 2008).

The exact metabolic pathways involved in the production of volatile fatty acids and the conditions influencing their production in *B/D* are yet to be determined, but it was shown that the degradation of the amino acids L-leucine, L-isoleucine and L-valine is involved in the formation of respectively isovaleric acid, 2-methylbutyric and isobutyric acid (Harwood and Canaleparola, 1981; Oelofse, 2008). Isovaleric acid is generally produced by transamination of leucine to α-ketoisocaproatic acid, subsequent decarboxylation to isoamylaldehyde and finally oxidized to isovaleric acid (Harwood and Canaleparola, 1981; Styger et al., 2013). Genes responsible for these processes have not yet been pinpointed in *B/D*, but alcohol and aldehyde dehydrogenase genes, which encode oxidoreductases and which were shown to be duplicated in the *B/D bruxellensis* genome, were raised as potential determinants of volatile fatty acid production potential of *B/D bruxellensis* (Curtin et al., 2012b; Piskur et al., 2012).

3.3. Microbial safety of Brettanomyces

Interestingly, little research has focused on the safety of *B/D* for application in food fermentations. Because the yeasts are commonly found in traditional beverage fermentations, like the Belgian gueuze and lambic beers, which have been produced and consumed for ages, *B/D* is often considered safe. However, there are two factors that do require some attention, namely their potential to produce certain biogenic amines and their resistance to antimicrobial cycloheximide.

3.3.1. Biogenic amine production

Biogenic amines (BAs) are potentially hazardous biological compounds that can have undesirable physiological effects when absorbed in high concentrations. They can provoke hormonal disadjustments, gastric acid secretion, increased heart pulse, migraine, tachycardia,

and higher blood pressure (Shalaby, 1996). According to their chemical structure, BAs can be classified as aliphatic (putrescine, cadaverine, spermine and spermidine), aromatic (tyramine and phenylethylamine) or heterocyclic (histamine and tryptamine) (Spano et al., 2010). The most toxic representative is histamine.

BAs can enter fermented foods in two ways, either directly from raw materials, or from production during the fermentation process. In the fermentation process, they are produced by decarboxylation of amino acids. This reaction occurs in many microbial species, including certain *B/D* strains. Consequently, these can produce significant concentrations of BAs, especially when they are grown on complex media with an enriched concentration of amino acids. The biological function of this reaction is not fully understood, but it was hypothesized that this decarboxylase activity favours growth and survival in acidic media, since it induces an increase in pH (Spano et al., 2010).

In wine, more than 20 different BAs have been identified and their total concentration has been reported to range from a few mg/L to about 50 mg/L (Landete et al., 2005; Lonvaud-Funel, 2001; Spano et al., 2010). The literature published on BA production in wine by *B/D bruxellensis* is not always univocal. In a study executed by Vigentini et al. (2008), putrescine, cadaverine and spermidine were found in wines inoculated with *B/D bruxellensis*, but they were considered to be harmless to human health due to the low concentrations and the absence of the most physiologically active molecules (Vigentini et al., 2008). However, in the work of Caruso et al. (2002), a strain of *B/D bruxellensis* was able to form up to 15 mg L^{-1} of total amines, mainly 2-phenylethylamine (Caruso et al., 2002).

The production of biogenic amines is considered a major factor for the safety of micro-organisms in food fermentation processes: a recently published Scientific Opinion of the Panel on Biological Hazards of the European Food Safety Authority (EFSA) discusses the control of BAs formation in fermented foods (EFSA, 2011). However, the longstanding traditional use of foods produced by *B/D* fermentations and the strong interstrain variability indicates that selection of specific *B/D* strains might reduce the risk of BA contamination in *B/D*-inoculated fermentations.

3.3.2. Cycloheximide resistance

B/D is generally highly resistant to cycloheximide (sometimes referred to as actidione), a common antifungal agent that inhibits protein biosynthesis in many eukaryotic organisms (Leach et al., 1947; Morneau et al., 2011). As a consequence, the antibiotic is often used as one of the main selective agents for the isolation of *B/D* strains. Although resistance to antimycotics is generally not transmissible amongst yeasts, it was recently decided by EFSA that yeasts resistant to antimycotics used for human treatments should be avoided in fermentation processes (EFSA, 2009). Cycloheximide however, is currently not applied as therapeutic antimycotic and should therefore not pose a problem when requesting the Qualified Presumption of Safety (QPS) status for *B/D*.

4. *Brettanomyces* in industrial fermentation processes

B/D spp. are isolated from a number of ecological niches, such as spontaneous alcoholic beverage fermentation processes (beer, wine, cider, tequila, cachaça …), soft drinks, dairy products, kombucha, sourdough and olives (Table 1). However, as mentioned before, the role and perception of *B/D* in these fermentations are often ambiguous. In some industrial processes, such as Belgian lambic or the American Coolship Ale fermentations, the presence of *B/D bruxellensis* and its accompanying aroma profile is considered essential and beneficial, whilst the same aroma compounds are considered as severe off-flavours when encountered in wine (Verachtert, 1992; Wedral et al., 2010). Moreover, whilst the frequent presence of *B/D bruxellensis* in bioethanol plants is mostly unintentional, it was recently shown that some of these strains might actually be superior production strains (Passoth et al., 2008; Reis et al., 2014).

Below, three industrial habitats in which *B/D* are commonly encountered (beer, wine and bioethanol fermentations) and their impact on the fermentation profile are discussed.

4.1. Spontaneously fermented beer

Whilst the vast majority of beers are brewed by pure cultures of *S. cerevisiae* (ale) or *S. pastorianus* (lager) yeasts, several types of specialty beers rely on a natural inoculum (Bokulich and Bamforth, 2013; Martens et al., 1997; Verachtert, 1992). Whilst numerous types of such spontaneously fermented beers are produced, mainly in Africa (e.g. Kayode et al., 2011; Sawadogo-Lingani et al., 2007), the best known spontaneously fermented beer styles are the lambic and gueuze beers produced in the surroundings of Brussels, Belgium. Because of their unique sensorial characteristics, several breweries across the world are mimicking their production process and develop similar beer styles, such as American Coolship Ales (Bokulich et al., 2012).

Lambic-style beers are typified by a very long fermentation time (which can last several years) and a rich, complex flavour with peculiar tones associated with the rich bacterial and fungal flora that thrives during these long fermentations. The microbiome in these fermentations is complex, with several genera of yeast and bacteria coexisting and varying over time (Steensels and Verstrepen, 2014). The ecology of lambic beers was first thoroughly described in 1977 (Van Oevelen et al., 1977) and was recently reinvestigated using modern community profiling techniques based on high-quality sequencing (Bokulich et al., 2012), denaturing gradient gel electrophoresis (DGGE) (Spitaels et al., 2014) and a culture-dependent strategy combined with Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Spitaels et al., 2014). These studies indicate that the microbial population consists mainly of yeasts and LAB (mainly *Lactobacilli* and *Pediococci*). Whilst the major part of the alcoholic fermentation is carried out by *S. cerevisiae*, in later stages, when most short oligosaccharides like maltose and maltotriose are exhausted, *S. cerevisiae* is gradually outcompeted by *B/D*, mainly *B/D bruxellensis*, usually after 4–8 months (Bokulich et al., 2012; Van Oevelen et al., 1977). *B/D* remains the most prevalent yeast genus until the end of the fermentation. During this phase of the fermentation (called the ripening or maturation period), the specific metabolic activity of *B/D*, including esterase, β-glucosidase, α-glucosidase and VPR activity, as well as the metabolism associated with LAB causes drastic changes in the sensory profile of the beverage and results in a strongly attenuated beverage with a unique flavour (Verachtert, 1992).

Together, these studies revealed a core microbial profile that is conserved between consecutive batches. Moreover, the core microbiome is fairly consistent throughout different regions: the main dominant species (such as *S. cerevisiae* and *B/D bruxellensis*) were encountered in all fermentations, whilst the presence of other species, such as the enterobacteria at the early onset of the fermentation, was shown to be region-dependent. These findings suggests the presence of a specific, stable brew house microbiome that is maintained on the machines, kegs, tanks and other surfaces of the brewery.

4.2. Wine

Whilst (inoculated or indigenous) *Saccharomyces* yeasts are the most common drivers of wine fermentations, other organisms, like fungi, bacteria or wild yeasts can infect fermentations, sometimes resulting in aberrant fermentation and flavour profiles. These microbes can be present as contaminants in the starter culture, occur naturally on grape skins, be introduced by insects or have a primary habitat in the winery itself, where they can survive on the winery walls, presses, fermentation tanks, or in the wood of the ageing barrels (Fugelsang and Edwards, 2007). However, to reduce the risk of contamination, specific precautionary practices are often implemented. The most commonly used method is exogenous addition of sulfites (SO_2) (cf.

Section 3.2.1.2.4). However, whilst the combination of wine-related stress conditions (e.g. the high concentrations of ethanol, sulfite and osmolytes) prevents proliferation of most contaminants, some *B/D* species are very tolerant to these conditions and as a consequence, they are still frequently detected in wine fermentations.

In the vast majority of the cases, the presence of *B/D* in wine fermentations is unwanted. Multiple studies have therefore focused on methods to detect *B/D* cells in wine fermentations, aiming to discover contaminations very early in the vinification process (Cecchini et al., 2013; Cocolin et al., 2004; Stender et al., 2001). Nevertheless, some sources report a very slight *B/D* character as being appreciated for certain wine styles (Fugelsang and Edwards, 2007), since it might add positive effects such as sensorial complexity and impart aged characters in some young red wines (Loureiro and Malfeito-Ferreira, 2003). Additionally, many *B/D* strains are able to release favourable glycosidically bound flavour compounds (such as terpenes and norisoprenoids) from naturally present grape glycosides, thereby potentially increasing the natural wine flavour palate (cf. Section 3.2.2.4). However, all in all, winemakers are still at war with *B/D*, and voluntary inoculation of these yeasts in wine fermentations is unlikely to become general practice any time soon.

4.3. Bioethanol

An interesting niche from which *B/D bruxellensis* is frequently isolated is bioethanol production sites (Beckner et al., 2011; Liberal et al., 2007; Passoth et al., 2007). Due to their tolerance to low pH, high ethanol and osmolyte concentrations, nutrient-efficient metabolism and high general stress tolerance, *B/D bruxellensis* is very well adapted to the harsh conditions in bioethanol fermentation tanks (Blomqvist, 2011). Additionally, it has been argued that their presence is at least partly due to their tolerance to LAB (or LAB-related metabolites). LAB are often encountered in these types of fermentations, since they are able to consume the pentose sugars released during the pretreatment of lignocellulosic biomass, and most yeasts are not (Passoth et al., 2007).

Currently, *B/D* are mostly regarded as undesirable spoilage organisms of bioethanol fermentations. However, the recent discovery of a *B/D bruxellensis* strain as the sole production organism active in a starch based continuous industrial alcohol plant suggests that it can in fact be a favourable contributor to, or even the sole driver of, bioethanol fermentations (Passoth et al., 2007; Reis et al., 2014). This discovery suggests that during the high-ethanol fermentation, *B/D bruxellensis* outcompeted the inoculated *S. cerevisiae* strain, without affecting the ethanol yield. Moreover, several interesting features of *B/D bruxellensis*, such as its ability to co-consume nitrate and other nitrogen sources (cf. Section 3.2.1.2.2) and its ability to utilize dextrins and cellobiose as a carbon source (cf. Section 3.2.1.2.3) might broaden the substrate range for industrial bioethanol production. Direct application of this species as a starter culture in bioethanol fermentation sites is still insufficiently studied, but its economical relevance is illustrated by a patent application filed in 2007 (WO 2008072184), that claims protection on the use of "a yeast of the genus *Dekkera* and a lactic acid bacteria for the production of bioethanol" and the recent suggestion of a *B/D bruxellensis* strain for commercial fermentation of lignocellulosic substrates in Brazil (Reis et al., 2014).

5. Concluding remarks and future perspectives

In the past decades, the potential of non-conventional yeasts for novel fermentation processes has received only limited attention. However, the information summarized in this review highlights the potential of *Brettanomyces* (teleomorph: *Dekkera*) for industrial use. Intensive phenotypic characterization, high-quality sequencing of the whole genome and analysis of its metabolome and transcriptome shed light on the peculiar characteristics of this yeast, and provide insight into its remarkable diversity and industrial potential. More specifically, its unique nutrient metabolism and peculiar flavour production hold great potential.

Whereas the lower growth rate of *B/D bruxellensis* compared to *S. cerevisiae* yeasts hampers its competitiveness in short batch fermentations, they are often able to outcompete *S. cerevisiae* in specific conditions that are encountered towards the end of industrial fermentations, where low nutrient availability is combined with low pH and high ethanol levels. Also in the oxygen- and sugar-limited conditions in continuous fermentations with recirculation of yeasts where the dilution rate is below the maximum growth rate of *B/D bruxellensis* (an environment commonly encountered in bioethanol production plants), they often grow more efficiently compared to *S. cerevisiae*. Several research papers highlight this remarkable feature and pose that a more efficient sugar uptake (especially in sugar-limited conditions), energy-efficient energy metabolism (low glycerol, high biomass production), the ability to utilize nitrate and high tolerance to various inhibitors, such as other microbes or chemical inhibitors present in the fermentation medium, might contribute to the competitiveness and potential of *B/D* in these environments. Still, the potential of *B/D* in bioethanol production remains inadequately studied.

B/D is naturally present in spontaneously fermented beverages, such as lambic beer, where they are essential for the typical flavour profile. However, a possible disadvantage of these spontaneous fermentations is the great variability of the microbial population and the dependency on factors such as geographical location and seasonal changes. Moreover, the desired flavours of traditional mixed-culture fermentations containing *B/D* often take several years to develop. Introduction of carefully selected *B/D* strains as a starter culture, either in the main fermentation or the bottle refermentation, may help in obtaining the desired sensorial characteristics without the risk of variability and the need for long fermentation times. Similarly, certain traits of *B/D*, such as the release of glycosidically bound flavours, create an opportunity to increase the sensorial complexity of fermented foods, although production of unfavourable off-flavours might still pose a significant problem that could hamper certain industrial application.

In conclusion, the biotechnological potential of *B/D* has long been overlooked, but new genetic and phenotypic information is bringing this intriguing yeast into the spotlight. Whilst its distinct and pungent aroma profile will likely hamper their commercial application in the wine industry, it is likely that these yeasts will take a more prominent position in several other industrial processes in the future, including the production of specialty beers and (second-generation) bioethanol.

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