Yeast Primer

The wine and beer yeast *Dekkera bruxellensis*

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Abstract

Recently, the non-conventional yeast *Dekkera bruxellensis* has been gaining more and more attention in the food industry and academic research. This yeast species is a distant relative of *Saccharomyces cerevisiae* and is especially known for two important characteristics: on the one hand, it is considered to be one of the main spoilage organisms in the wine and bioethanol industry; on the other hand, it is 'indispensable' as a contributor to the flavour profile of Belgium lambic and gueuze beers. Additionally, it adds to the characteristic aromatic properties of some red wines. Recently this yeast has also become a model for the study of yeast evolution. In this review we focus on the recently developed molecular and genetic tools, such as complete genome sequencing and transformation, to study and manipulate this yeast. We also focus on the areas that are particularly well explored in this yeast, such as the synthesis of off-flavours, yeast detection methods, carbon metabolism and evolutionary history. © 2014 The Authors. *Yeast* published by John Wiley & Sons, Ltd.

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Introduction to basic characteristics

Dekkera bruxellensis is considered to be a major cause of wine spoilage worldwide (Boulton *et al.*, 1996; Fugelsang, 1996; Delfini and Formica, 2001; Loureiro and Malfeito-Ferreira, 2003). Infected wines develop dinstinctive, unpleasant aromas due to volatile phenols produced by this species (Woolfit *et al.*, 2007), also called 'Brett' taints (Chatonnet *et al.*, 1995) and normally associated with aromas of barnyard, burnt plastic, wet animal and horse-sweat (Licker *et al.*, 1998). However, this species is also known for its positive contribution of acetic acid flavour to Belgian lambic beers (Dequin *et al.*, 2003; Dufour *et al.*, 2003) and to the fermented and sweetened tea Kombucha (Mayser *et al.*, 1995; Teoh *et al.*, 2004). The occurence of the species in feta cheese (Fadda *et al.*, 2001) and sour dough (Meroth *et al.*, 2003) has also been reported. This yeast also provides the characteristic aroma profile in some wines, such as the French *Château de Beaucastel*

wines. *D. bruxellensis* is also often associated with high-ethanol biotechnological habitats (de Souza Liberal *et al.*, 2007; Passoth *et al.*, 2007). It has previously been isolated from Belgian stout, lambic beer and grape must as well as sparkling wine, sherry and porter. Its presence on the surface of grape berries has been shown by Renouf *et al.* (2007). This yeast is now also becoming a model for the deduction of yeast evolution processes (Rozpedowska *et al.*, 2011).

It is still common in the current literature to use both *Dekkera* and *Brettanomyces* as the genus name. The anamorphs *Brettanomyces/Dekkera anomala*, *B./D. bruxellensis*, *B. custersianus*, *B. naardenensis* and *B. nanus* build this genus. Teleomorphs have been reported for two out of these five species, *D. anomala* and *D. bruxellensis*. The first reference to the genus *Brettanomyces* dates back to 1904, when Hjelte Claussen first isolated the yeast from British beers. The flavours produced by this yeast became characteristic of certain British beers of that time and the name

Brettanomyces was derived from 'British brewing fungus' (greek: 'brettano' means British brewer, 'myces' means fungus). First, the genus *Brettanomyces* was shown to occur in wine (Custers, 1940). The yeast *Mycotorulum intermedia*, which was isolated from French wine by Krumholz and Tauschanoff in 1933, was named as a *Brettanomyces* species and later renamed as *Brettanomyces intermedius* (van der Walt and van Kerken, 1959); in 1960 the latter authors reported ascospore formation in *B. bruxellensis*, which led to reclassification of the genus *Brettanomyces* (van der Walt and van Kerken, 1960). The new genus *Dekkera* was proposed to accomodate the ascosporogenous forms – the name was chosen in honour of Nellie Margaretha Stelling-Dekker, for her contribution to the taxonomy of the ascosporogenous yeasts (van der Walt, 1964). After the first description of spore formation (van der Walt and van Kerken, 1960), spores have, to our knowledge, never been reported again.

D. bruxellensis has adapted to harsh and limiting environmental conditions, such as very high ethanol concentrations, low pH values (Fugelsang, 1996; Rozpedowska *et al.*, 2011) and 'poor' nitrogen sources. For example, *D. bruxellensis* preferentially uses ammonium ions but can also use nitrate (de Barros *et al.*, 2011; Galafassi *et al.*, 2013). Renouf *et al.* (2006) have shown a higher adaption rate of *D. bruxellensis*, in comparison to other wild yeasts, to survive in must and during alcoholic fermentation. *D. bruxellensis* yeast grows between 19°C and 35°C, shows variable growth between 37°C and 42°C and cannot grow at 45°C (www.cbs.knaw.nl, May 2013; and Figure 1). Van der
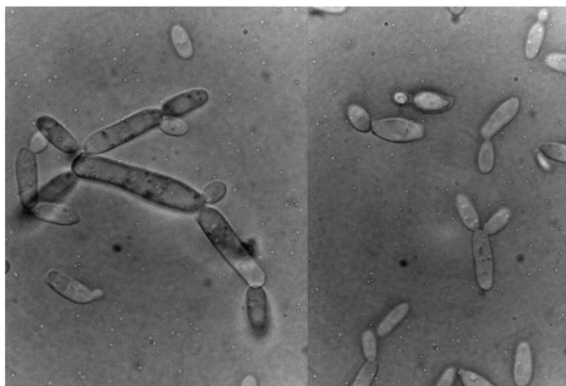
Walt (1964) has also characterized the colony colours, ranging from cream to light brown and usually shiny and smooth.

The first attempt to determine the *D. bruxellensis* genome sequence was in 2007 (Woolfit *et al.*, 2007) and provided almost half of the open reading frames. In 2012, the whole genome of two different *Dekkera bruxellensis* strains were determined and are now publicly available (http://genome.jgi.doe.gov/Dekbr2/Dekbr2.home.html by Piškur *et al.*, 2012; and GenBank: AHIQ01000137.1 by Curtin *et al.*, 2012). Piškur *et al.* (2012) have determined the whole genome sequence of the strain Y879 (CBS2499) and used it to deduce several 'food-relevant' properties of this yeast. To date there are 5636 predicted genes based on the sequenced strain CBS2499 (Y879) (DOE Joint Genome Institute), and so far the UniProt database comprises 4929 protein sequences. Phylogenetic analyses, based on 3930 individual gene trees in the context of 21 closely related fungal species, placed *D. bruxellensis* as a sister group to *Pichia (Komagataella) pastoris*. The genus *Komatagaella* and its closest relatives are known as aerobic poor ethanol-producer yeasts (de Schutter *et al.*, 2009), which is just opposite to *D. bruxellensis* and *Saccharomyces cerevisiae*. Analyses of growth parameters and carbon metabolism of several *D. bruxellensis* isolates have demonstrated that this yeast produces ethanol under aerobiosis and has the ability to grow without oxygen, similar to *S. cerevisiae* (Nissen *et al.*, 2000) and its closest relatives. *D. bruxellensis* can thus be described as a Crabtree-positive and facultative anaerobic yeast (Rozpedowska *et al.*, 2011), which can particularly dominate in harsh environments.

Figure 1. *Dekkera bruxellensis* Y879 (CBS 2499) in minimal medium. Courtesy of Concetta Compagno, Milan, Italy

Genetic and molecular tools

Although spores have been observed previously (van der Walt and van Kerken, 1960), neither mating types nor mating events, nor crosses of two haploid strains, have been observed so far. Thus, it could be that this yeast is asexual. There is little known about variation within the whole genus *Dekkera* at genomic level, but preliminary investigations suggest that differences may be large and that the *D. bruxellensis* clade could consist of several sister species (Hellborg and Piškur, 2009; Galafassi *et al.*, 2011).

Multiple *D. bruxellensis* strains have been screened for auxotrophic mutants after mutagenesis by UV or ethane methyl sulphonate (EMS) treatment. The frequency of auxotrophs was 0.1% (Šiurkus, 2004). A *ura3*-deficient mutant has been isolated, which promotes the use of the *URA3* gene as a selection marker (Šiurkus, 2004). For subsequent transformation experiments, the *URA3* gene has been subcloned from the genomic DNA (originating from the strain CBS2499) into the pUC57 vector, resulting in plasmid P892. This plasmid has been used to develop the first transformation protocol. A lithium acetate electrotransformation procedure (based on Becker and Guarente, 1991; Boretsky *et al.*, 2007) has been modified to obtain transformants and the plasmids integrated at random sites into the genome (Hagström, 2008; Ishchuk *et al.*, in preparation). Genomic libraries from two different strains (Y879 and Y881) have been constructed to find putative autonomous replication elements. Three types of replicating loci have been deduced; *CIGO 1, 2* and *3* and have been used for the construction of autonomously replicating plasmids (Ishchuk *et al.*, in preparation). A reporter system based on the *Kluyveromyces lactis LAC4* gene, under the control of the *D. bruxellensis* promoter from *YPR100W* (*MRLP51*) and the sub-cloned *CIGO1* locus (Figure 2), have been used to determine the plasmid copy number, which is approximately 10–15. This reporter system can also be used for the screening of different promoter elements. Apart from the *URA3* gene, the *Sh ble* gene can also be used as a dominant selection marker (Figure 3). This marker and *URA3* have also been used for targeted deletions. The length of the homologous end sequences, which promoted homologous recombination and integration, varied in the range 350–600 bp (unpublished data).

D. bruxellensis shows much greater diversity among strains in chromosome number and ploidy

Figure 3. A scheme of the development of molecular and genetic tools for *D. bruxellensis*, from auxotrophic mutants to targeted deletions, based on Šiurkus (2004), Hagström (2008) and Ishchuk *et al.* (in preparation)

than does *S. cerevisiae*. The proposed genome size is in the range < 20– > 30 Mb (Hellborg and Piškur, 2009). Analysis of 30 *D. bruxellensis* isolates showed a range in chromosome sizes from < 1 Mb to > 6 Mb, the different strains contained between four and nine chromosomes, suggesting that the genome has been rearranged very fast upon the separation of single lineages. Almost all strains had polymorphic sites at the analysed loci, suggesting a > 1 ploidy status (Hellborg and Piškur, 2009). Curtin *et al*. (2012) have suggested that the sequenced AWRI 1499 strain has a triploid genome and the strain speciation has occurred through interspecific hybridization. Native *D. bruxellensis* strains indeed exhibit a large diversity of genotypes and phenotypes. Fugelsang and Zoecklein (2003) have shown that not all *D. bruxellensis* wine isolates are able to grow in Pinot Noir wine. Analyses of 244 wine isolates obtained from wineries located in 31 winemaking regions of Australia by using the multilocus AFLP fingerprinting method revealed large diversity and the presence of three major genotypes in Australian wine. Differences in the

Figure 2. Linear presentation of the plasmid P1017. The *D. bruxellensis URA3* gene is shown as a grey box, the *K. lactis LAC4* reporter gene as a red box, the *D. bruxellensis* promoter *YPR100W* (*MRLP51*, mitochondrial ribosomal protein large subunit) as a yellow box, the *ClGO1* motif (for autonomous replication) as a black box, and the pUC57 part as a thin line (Ishchuk *et al.*, in preparation)
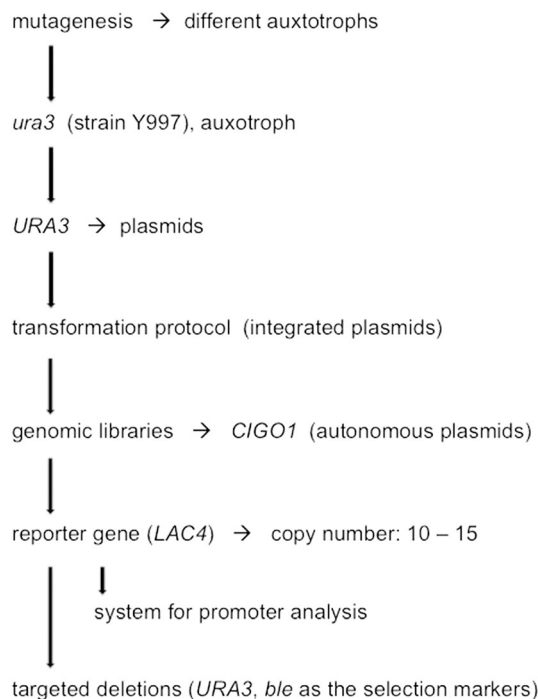
26S rRNA gene sequence imply that some differences between the groups might be highly conserved, showing the existence of isolate-specific genotypes (Curtin *et al.*, 2007).

Although the whole genomes of two *D. bruxellensis* strains were deduced in 2012, commercial microarrays are still not available. Tiukova *et al.* (2013) have introduced an RNAseq approach and detected the expression of 3715 of 4861 annotated genes in the *D. bruxellensis* CBS11270 strain. They have analysed the transcriptome by using the AB SOLiD sequencing technique in conditions of sugar limitation and low oxygen concentrations that are similar to those in industrial fermentations, where *D. bruxellensis* is able to outcompete *S. cerevisiae*. Several genes associated with sugar import as well as glycolysis were highly expressed. Their results also indicate a high frequency of transcription events outside the reading frames (Tiukova *et al.*, 2013).

Methods of detection

For the wine industry it is important to have reliable methods to detect the spoilage yeast *D. bruxellensis*: some of these methods are time consuming (microbiological) and some are less (molecular detection). By using differential media with ethanol as the carbon source, bromocresol green and phenolic precursors, it is possible to distinguish the genus *Dekkera* after a relatively long period of cultivation (Rodrigues *et al.*, 2001). Much faster but also more costly is a method based on fluorescence *in situ* hybridization, using peptide nucleic acid probes, that has been introduced by Stender *et al.* (2001). A PCR method for the species-based identification of *Dekkera*, based on polymorphisms on the ITS regions, has been published by Egli and Henick-Kling (2001). Phister and Mills (2003) have developed a quantitative real-time PCR that targets only species within the genus *Dekkera/Brettanomyces*, while yeast and bacteria common to the winery environment have not been not targeted. Cocolin *et al.* (2004) developed a PCR–restriction enzyme analysis protocol to detect and identify *D. bruxellensis* and *D. anomala* directly in wine samples. This technique allows much faster identification of these species isolated from wine. Molecular detections include a nested PCR method (Ibeas *et al.*, 1996) and amplification of 26S rDNA region,

with further resolving of the PCR product by denaturing gradient gel electrophoresis (Manzano *et al.*, 2004). The spoilage activity is strain-dependent; thus, methods for detection at the strain level are of great oenological importance. Vigentini *et al.* (2012) have developed tools to assess genetic intraspecific variation through the use of introns as molecular targets and designed specific primers annealing to the introns' 5′-splice site sequences (ISSs), where they found a conserved pattern. For interstrain discrimination, restriction enzyme analysis and pulse-field electrophoresis have been introduced by Miot-Sertier and Lonvaud-Funel (2007).

Carbon metabolism and the ability of anaerobic growth

The yeast *Dekkera/Brettanomyces* can utilize several different sugars (Galafassi *et al.*, 2011). Sugars, like glucose, are broken down into smaller molecules to become a source of energy and building blocks for the synthesis of other molecules. Glycolysis, the major process for sugar degradation, breaks down a glucose molecule into two molecules of pyruvate. Yeasts, depending on conditions, can use pyruvate by fermentation and/or respiration. Since respiration of sugars is energetically more favourable than fermentation, most organisms use fermentation only when respiration is impaired, for example when oxygen availability decreases. However, in several yeast species, such as *S. cerevisiae* and *D. bruxellensis*, the metabolic destiny of pyruvate formed at a high rate is largely switched from respiration to fermentation, even when oxygen is abundant (for review, see Pronk *et al.*, 1996; Rozpedowska *et al.*, 2011). In other words, these two yeasts may also ferment sugars under aerobic conditions, showing the so-called 'Crabtree-positive' phenotype (Figure 4). In contrast, 'Crabtree-negative' yeasts, such as *K. lactis* and *B. naardenensis*, lack fermentative products and, under aerobic conditions, biomass and carbon dioxide are the sole products (Figure 5).

The availability of oxygen varies among different niches. One of the main problems organisms face under anaerobic conditions is the lack of the final electron acceptor in the respiratory chain. The ability of yeasts to grow under oxygen-limited conditions seems to be strictly dependent on the ability to perform alcoholic fermentation. According to the dependence on oxygen during the life
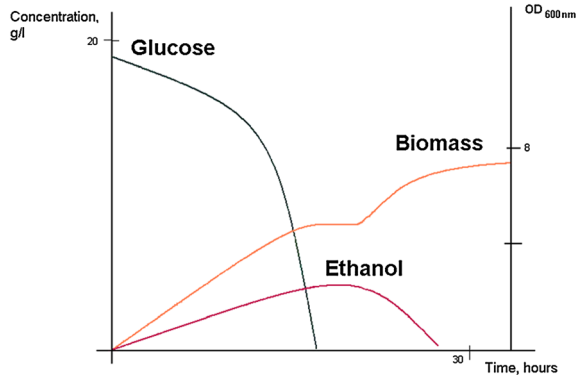
Figure 4. Batch culture of the Crabtree-positive yeast *D. bruxellensis* Y879 (CBS 2499) under aerobic conditions in defined minimal medium. Optical density (OD$_{600nm}$; orange line), glucose (black) and ethanol (violet) concentrations are shown (right-side scale, presented as g/l). Adapted from Rozpedowska *et al.* (2011)

cycle, yeasts are classified as: (a) obligate aerobes displaying exclusively respiratory metabolism; (b) facultative fermentatives (or facultative anaerobes), displaying both respiratory and fermentative metabolism; and (c) obligate fermentatives (or obligate anaerobes) (Merico *et al.*, 2007). *S. cerevisiae* and *D. bruxellensis* are both facultative anaerobes (Rozpedowska *et al.*, 2011), while *K. lactis* and *B. naardenensis* are obligate aerobes (Figure 5).

D. bruxellensis can spontanously generate mitochondrial petite mutants (McArthur and Clark-Walter, 1983). Thus, just like in *S. cerevisiae*, the active respiratory chain is not necessary for survival. However, what is different between these two yeasts

is that the mitochondrial genome of *D. bruxellensis* encodes NADH dehydrogenase (respiratory complex I) (Procházka *et al.*, 2010). The *Dekkera/Brettanomyces* mDNAs exhibit a large size polymorphism (Hoeben and Clark-Walter, 1986; Procházka *et al.*, 2010), and mitochondrial loci represent an efficient tool to 'easily' identify different species of this complex (Hoeben *et al.*, 1993).

Wine and beer aroma-associated aspects

Dekkera yeasts often cause wine spoilage. The odours of wines contaminated with *Dekkera* could be described as 'pharmaceutical', 'smoky' or 'wet horse' and are mainly caused by two groups of chemical compounds. 'Mousiness' can be the consequence of carbonyl compounds. Among these nitrogenous compounds are 2-acetyl-3,4,5,6-tetrahydropyridine, 2-acetyl-1,2,5,6-tetrahydropyridine and 2-ethyl-3,4,5,6-tetrahydropyridine. The second group is presented by volatile phenols such as 4-vinylphenol, 4-vinylguiacol, 4-ethylphenol and 4-ethylguiacol (Pretorius, 2000). *Dekkera* is almost unique among other yeast because of its ability to convert hydroxycinnamic acids – antimicrobial non-volatile compounds present in grape must – into ethyl derivatives. Also, the wine yeast *Meyerozyma guilliermondii* can perform similar metabolic activity (Barata *et al.*, 2006). Many other microorganisms with hydroxycinnamate activity would only form vinyl derivatives, with no further conversion to ethyl derivatives (Chatonnet *et al.*, 1992).
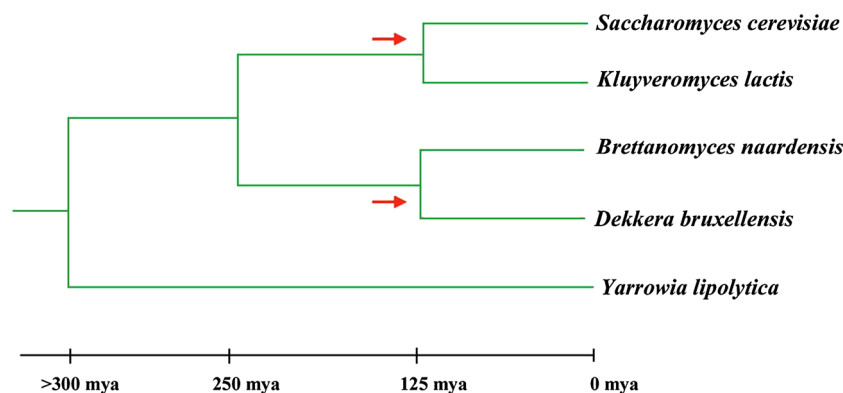

Figure 5. A simplified phylogenetic relationship among the *Saccharomyces/Kluyveromyces* and *Dekkera/Brettanomyces* clades, which separated more than 200 million years ago (mya). The *Saccharomyces* and *Dekkera* lineages have independently evolved (red arrows) the ability to produce ethanol in the presence of oxygen (Crabtree effect) and the ability to propagate under anaerobic conditions

The origin of phenolic metabolites and enzymes involved in this hydroxycinnamic acid conversion pathway has been described only recently. Godoy *et al.* (2009) have purified the corresponding *p*-coumarate decarboxylase (CD) and vinylphenol reductase (VR). It has also been shown that the *PAD* gene is present in the genera *Dekkera* and *Saccharomyces* and encodes the CD activity. However, the sequence of *PAD* in *Dekkera* is much more similar to bacterial phenolic acid decarboxylase than to the *S. cerevisiae PAD1* gene. Among all budding yeasts described (Curtin *et al.*, 2012), only *M. guilliermondii* has the *D. bruxellensis PAD* homologue. On the other hand, VR activity has not been found in *Saccharomyces*. Harris *et al.* (2009) have published the sequence of the *D. anomala* gene with putative decarboxylase activity, and de Souza Liberal *et al.* (2012) have described the existence of two genes in *D. bruxellensis*, which might be paralogues of phenyl pyruvate decarboxylase (*Db ARO10*).

Dekkera species are also important in the sour dough and beer industries. When present at high levels, food spoilage occurs. However, low amounts of *Dekkera* contribute metabolites desirable in bread, lambic beer, ale and kombucha tea. In beer, the concentration of 4-ethylphenol (medicinal aroma) is lower than that of 4-ethylguiacol (clove, spicy smell). In the case of the wine situation, this is the opposite. Thus, the relative concentrations determine different effects in different food products (Curtin *et al.*, 2012). In contrast to *S. cerevisiae*, some *Dekkera* isolates possess *β*-glucosidase activity, which catalyses the release of desirable bonded phenolic compounds from hops in beer (Daenen *et al.*, 2008). The kinetic properties of *α*-glucosidases and their role during beer fermentation have also been described (Shantha Kumara *et al.*, 1993).

Fermentation characteristics of *Dekkera*

During the fermentation process, yeasts need to adapt to high osmotic pressure, high sugar concentrations, partially anaerobic conditions, deficiency of nitrogen and the presence of ethanol. It has been previously noted that the genus *Dekkera* shows the Custer effect during fermentation (van Dijken and Scheffers, 1986; Vigentini *et al.*, 2008). When growing partly anaerobically, fermentation is partially inhibited because of acetic acid production and redox imbalance (Vigentini *et al.*, 2008). Blomqvist *et al.* (2010) described the fermentation properties of *D. bruxellensis* using a full factorial design; it was noted that the growth rate and ethanol yield on maltose are lower than that on glucose as a carbon source. When comparing *D. bruxellensis* with industrial *S. cerevisiae* strains, the latter grew five times faster but with lower ethanol yields. *S. cerevisiae* was also the 'winner' in glycerol amounts produced (six-fold higher). After a while, however, *S. cerevisiae* biomass levels reached 72–84% of *D. bruxellensis* biomass (Blomqvist *et al.*, 2010). Nardi *et al.* (2010) demonstrated that *Dekkera* consumes sugar and grow much more slowly than *S. cerevisiae*. The roles are changed at the end of fermentation, when sugar is depleted and only low amounts of nitrogen are available. The expression of *MSN4* (transcription factor involved in activation of heat shock, osmotic stress and high ethanol stress) in *Dekkera* is activated at higher ethanol concentrations than in *S. cerevisiae*. When comparing with the response on sugar, the situation is the opposite. Late activation of *MSN4* might mean a different ability of *Dekkera* to survive on an alternative carbon source under glucose-starvation conditions. For *VPS34*, *ERG6* and *ATP1*, essential in the presence of ethanol, expression patterns in *S. cerevisiae* and *Dekkera* are different. In *Dekkera*, *ERG6* is not repressed in stationary phase and *ATP1* expression level at the start of fermentation is extremely high. Nardi *et al.* (2010) explained this as being due to poorly established anaerobiosis. In general, some stress response genes in *D. bruxellensis* are expressed at the beginning of fermentation, but some of the genes are expressed much later than those in *S. cerevisiae*. It seems that, just like the conventional yeasts, *Dekkera* is well adapted to fermentation conditions through changes in expression of its genome or some morphophysiological features. For example, it has been demonstrated that *D. bruxellensis* is able to form biofilms, which improves attachment on the barrel surface (Joseph *et al.*, 2007).

Rozpedowska *et al.* (2011) have studied several isolates of *D. bruxellensis* under controlled batch cultivation for their growth parameters and carbon metabolism. Under aerobic conditions, *D. bruxellensis* produced substantial amounts of ethanol and, as Aguilar Uscanga *et al.* (2003) have described previously, good aeration stimulated

acetate production. After glucose depletion, both ethanol and acetate were completely consumed. This yeast could also grow under anaerobiosis if the minimal medium was supplemented with Tween 80, ergosterol and amino acids (see also Blomqvist *et al.*, 2012). In contrast, in the presence of oxygen *B. naardenensis* exhibited a completely respiratory metabolism and could not grow under anaerobiosis. The *D. bruxellensis* ethanol yield in aerobiosis and the ability to grow without oxygen are very similar to those reported for *S. cerevisiae* (Nissen *et al.*, 2000) and its sister species. During anaerobic growth glucose is converted into biomass at lower yields than in aerobiosis, due to higher ethanol production.

Biofuel production

Dekkera yeasts are often present during the biofuel production process. While Basilio *et al.* (2008) have considered the presence of these yeasts as spoilers of the production processes, other authors refer to the genus *Dekkera* as an alternative yeast to *S. cerevisiae* for ethanol production (Passoth *et al.*, 2007). There are a few important features that make *Dekkera* suitable for bioethanol production. *D. bruxellensis* is a Crabtree-positive yeast, so it is possible to obtain ethanol from yeast culture when high sugar concentrations are available, even under aerobiosis. It has also been revealed that the Custer effect takes place under increasingly anaerobic conditions. In this case alcoholic fermentation is inhibited due to a redox imbalance. The other special feature is high acetic acid production during aerobic alcoholic fermentation (Leite *et al.*, 2013).

When scaling up, aeration in bioreactors is becoming a problematic and expensive procedure. Under these conditions, *S. cerevisiae* is the most preferable organism. However, *S. cerevisiae* is able to use only ammonium ions as a source of the nitrogen. In this case, nitrate assimilation by *D. bruxellensis* is a superior advantage in lignocellulose media, which are rich in nitrate. Recently, a strict correlation between acetic acid production and nitrate utilization has been reported (Galafassi *et al.*, 2013). Glucose consumption in media with nitrate has also been improved. It seems that NADPH and NADH, which are available in the cell under anaerobic conditions, could neutralize the redox imbalance. Both co-factors play the role of electron donor for nitrate reductase, which is active under anaerobic conditions.

Evolution aspects

A majority of ascomycotic fungi under aerobic conditions convert sugar-based substrates into CO_2. However, at least three groups, including budding and fission yeasts, have apparently independently evolved the metabolic ability to produce ethanol in the presence of oxygen and excess of glucose (reviewed in Rozpedowska *et al.*, 2011; Rhind *et al.*, 2011). This metabolic 'invention' represents in nature a possible tool to poison/outcompete other microbes. The Crabtree-positive budding yeasts *S. cerevisiae* and *D. bruxellensis* can efficiently catabolize ethanol, and therefore their corresponding life style has called a 'make–accumulate–consume (ethanol)' strategy (Thomson *et al.*, 2005; Piškur *et al.*, 2006; Rozpedowska *et al.*, 2011). On the other hand, the third Crabtree-positive group, including the fission yeast *Schizosaccharomyces pombe*, only poorly metabolizes ethanol.

The phylogenetic analysis of Ascomycetes suggests that the *Saccharomyces/Kluyveromyces* and *Dekkera/Brettanomyces* lineages separated at least 200 million years ago (Rozpedowska *et al.*, 2011). In other words, the divergence took place long before the whole-genome duplication (WGD), promoter rewiring, *URA1* horizontal transfer and *ADH* duplication events that occurred in the *S. cerevisiae* lineage and are thought to be involved in the evolution of the 'make–accumulate–consume' strategy (reviewed in Piškur *et al.*, 2006). The *S. cerevisiae* and *D. bruxellensis* lineages, but not the *K. lactis* and *B. naardenensis* lineages, have apparently independently acquired the ability to accumulate ethanol in the presence of oxygen and resistance to high ethanol concentration, which is a crucial trait to accompany efficient ethanol production and accumulation (Rozpedowska *et al.*, 2011) (Figure 5).

The origin of the 'make–accumulate–consume' strategy coincides with the origin of modern plants with fruits, which > 125 million years ago brought to microbial communities a new, larger and increasingly abundant source of food based on simple sugars (reviewed in Piškur *et al.*, 2006). Ancient yeasts could hardly produce the same amount of new biomass as bacteria during the same time interval, and could therefore be out-competed. One

can speculate that slower growth rate could in principle be counter-acted by the production of compounds such as ethanol and acetate that could inhibit the growth rate of competitors. Therefore, it is not surprising that several similar 'winning' traits, such as the ability to grow without oxygen and the Crabtree effect (or 'make–accumulate–consume' strategy), can be found among not so closely related modern yeasts, such as the *Saccharomyces* and *Dekkera* clades. Surprisingly, both lineages used the same tool, global promoter rewiring, to change the regulation pattern of respiration-associated genes, resulting in ethanol accumulation and consequently in the development of the 'make–accumulate–consume' strategy (Rozpedowska *et al.*, 2011). An interesting aspect is also that both yeasts have independently duplicated their alcohol dehydrogenase-encoding *ADH* genes (Piskur *et al.*, 2012).

In conclusion, *D. bruxellensis* will, in the following years, represent one of the central model organisms to understand the evolution of yeast alcoholic fermentations and will also be in focus from the wine and beer makers' perspective.

Acknowledgements

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