Dekkera bruxellensis – a Competitive Yeast for Ethanol Production from Conventional and Non-conventional Substrates

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Cover: To the left: YPD agar + $CaCO_3$. To the right: The same plate with *D. bruxellensis*. Due to acetic acid production the plate became transparent when *D. bruxellensis* grew. Under the plates an enlarged microscopic photo of *D. bruxellensis* is shown.
(photo: J. Blomqvist)

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Abstract

In the ethanol industry and research community, *Saccharomyces cerevisiae* is regarded as the most competitive fermentation yeast and therefore industrial yeast populations are rarely studied. This thesis investigates yeast and lactic acid bacteria (LAB) populations in a Swedish ethanol plant. Interestingly, the yeast population was dominated by *Dekkera bruxellensis* and not by the inoculated *S. cerevisiae*. High numbers of lactic acid bacteria (LAB) were also found, with *Lactobacillus vini* dominating. Since there was no indication of a reduction in productivity, we regarded *D. bruxellensis* together with *L. vini* as a production consortium.

In test fermentations, the industrial *D. bruxellensis* strains had similar or higher ethanol yield than two industrial *S. cerevisiae* strains. Glycerol yield was lower and biomass yield higher, indicating more energy-efficient metabolism of *D. bruxellensis*.

To test the ability of our isolates to produce ethanol from lignocellulosic substrate, we cultured *D. bruxellensis* in aspen sawdust hydrolysate. *D. bruxellensis* was slightly more sensitive to the hydrolysate than *S. cerevisiae* in batch culture, but was able to adapt to the inhibitors, achieving an ethanol production comparable to *S. cerevisiae*.

Five years after the first isolation, the ethanol plant made substantial process changes. Renewed investigations showed that *D. bruxellensis* and *L. vini* were still the dominant microbial population.

In continuous competition experiments, *D. bruxellensis* was only able to outcompete *S. cerevisiae* during glucose limitation. This suggests that in glucose-limited conditions, *D. bruxellensis* is more competitive due to its more energy-efficient metabolism, probably together with a higher affinity for glucose uptake in such conditions. Although *D. bruxellensis* is indeed facultatively anaerobic, it has a higher nutritional demand under anaerobic conditions than *S. cerevisiae.* This is probably due to a redox imbalance caused by low glycerol production.

The results show that *D. bruxellensis* is a competitive yeast owing to its energy-efficient metabolism in glucose-limited conditions. It can act as production yeast for ethanol production from both first- and second-generation substrates under conditions of optimal aeration and nutrient supply.

Keywords: *Dekkera bruxellensis*, *Saccharomyces cerevisiae*, fermentation, lignocellulosic hydrolysate, continuous cultivation, competition, anaerobic growth,

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“Pain is inevitable. Suffering is optional.”

Haruki Murakami (What I talk about when I talk about running)

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Passoth V, Blomqvist J and Schnürer J (2007). *Dekkera bruxellensis* and *Lactobacillus vini* form a stable ethanol-producing consortium in a commercial alcohol production process. *Appl Environ Microbiol* 73, 4354-4356.

II Blomqvist J, Eberhard T, Schnürer J and Passoth V (2010). Fermentation characteristics of *Dekkera bruxellensis* strains. *Appl Microbiol Biotechnol* 87, 1487-1497.

III Blomqvist J, South E, Tiukova I, Momeni MH, Hansson H, Ståhlberg J, Horn SH, Schnürer J and Passoth V (2011). Fermentation of lignocellulosic hydrolysates by the alternative industrial ethanol yeast *Dekkera bruxellensis*. *Lett Appl Microbiol* 53, 73-78.

IV Blomqvist J, Nordstedt P and Passoth V. Yeast-yeast and yeast-lactic acid bacteria interactions in industrial ethanol fermentation (manuscript).

V Blomqvist J, Sànchez-Nogué V, Gorwa-Grauslund M and Passoth V. Physiological requirements for growth and competitiveness of *Dekkera bruxellensis* under oxygen limited or anaerobic conditions (submitted manuscript).

The contribution of Johanna Blomqvist to the papers included in this thesis was as follows:

I Took part in planning the project. Performed all laboratory work. Minor part in writing the manuscript.

II Took part in planning the project. Performed all laboratory work. Main writer of the manuscript.

III Took part in planning the project. Supervisor of Master's student (ES). Performed a minor part of laboratory work. Main writer of manuscript.

IV Took part in planning the project. Involved in the supervision of PN. Performed the competition experiment. Main writer of manuscript.

V Took part in planning the project. Performed all laboratory work. Main writer of manuscript.

1 Introduction

Yeasts are eukaryotic microorganisms that are found in many different habitats and can be both beneficial and detrimental for society. They are best known as the active 'ingredient' in leavening of bread and as the production organism in the production of alcoholic beverages (for example wine and beer) and in the ethanol industry. Humans have used yeasts as production organisms for several thousand years and there is evidence that alcoholic beverages fermented by yeast were used as early as 8500-4000 BC in China, Iran and Egypt (Kurtzman *et al.*, 2011).

Yeasts are also used in the fermentation of foods and in feed production, as well as in the biotech industry, for instance for the expression of heterologous proteins. The residues from ethanol industry can be used for either feed production (Olstorpe *et al.*, 2010), or as substrate for the biogas process (Dererie *et al.*, 2011). Yeasts can also act as spoilage organisms, disturbing industrial processes or contributing to deterioration of conserved food and feed (Deak, 2004). In addition, some yeast species can be pathogenic to humans and animals (Kurtzman *et al.*, 2011).

In the beginning of the 19th century, fermentation was thought to be a pure chemical process. During 1837-1838, three independent researchers (Cagniar-Latourd, Kützing and Schwann) concluded that the process was due to the activity of an organism that fermented sugar to ethanol (Barnett, 1998). Schwann, together with a mycologist, Meyen, gave the yeast its generic name *Saccharomyces*, which stands for sugar fungus (Barnett, 1998). However, it was not until Pasteur's work on alcoholic fermentation that it became fully accepted that a microorganism caused the conversion of sugar to ethanol (Barnett, 2000; Pasteur, 1857).

The most well-known yeast is *Saccharomyces cerevisiae*, also known as baker's yeast. It is considered the main fermentation yeast and is used as production yeast in the beer, wine and ethanol industry and for heterologous

protein production. Other examples of production yeasts are *S. pastorianus* for the production of lager beer (Tamai *et al.*, 2000) and the methylotrophic yeast *Komagatella pastoris* (formerly *Pichia pastoris*) for the production of heterologous proteins (Eckart & Bussineau, 1996).

1.1 Yeast and lactic acid bacteria (LAB) populations in industrial ethanol production plants

Since it have almost become a dogma that *S. cerevisiae* is the fermentation yeast in ethanol production, there has been little interest in studying the production yeasts in such systems. In one study, the yeast population dynamics of *S. cerevisiae* strains were investigated in six different distilleries using PCR-fingerprinting (da Silva *et al.*, 2005). The aim was to find strains adapted to the harsh conditions of the industrial fermentations for future use as starter cultures. For two groups of distilleries, running either on sugar cane or molasses, there were common amplification patterns (strains), but each group had a different distribution of the strains. At the end of the season, both groups of distilleries had one dominant specific strain (da Silva *et al.*, 2005). That research group also developed a method for detection of contaminant yeasts by amplification of the ITS1-5.8S-ITS2 rDNA. Different yeast species could then be distinguished by the length of the PCR product (Liberal *et al.*, 2005). Later, a fast mtDNA isolation method was developed, followed by mtDNA-RFLP analysis to keep track of genetic changes in the industrial yeast during industrial fermentation (Petrova *et al.*, 2009).

For wine processes there are several published studies on production yeast population dynamics during wine fermentation (Mercado *et al.*, 2007; Pretorius *et al.*, 1999; Martinez *et al.*, 1997). For ethanol processes, if yeast populations have been studied at all, the focus has mainly been on contaminating yeasts and LAB (Lucena *et al.*, 2010; Basillo *et al.*, 2008; Liberal *et al.*, 2007; Skinner & Leathers, 2004).

1.2 Aim

The limited knowledge available about the microbial ecosystems of industrial ethanol processes led us to investigate the yeast and LAB population in an industrial ethanol plant in Sweden (**I**). The specific process runs in continuous mode with recirculation of the yeast cells. To start the process, one tonne of vacuum-dried baker's yeast was added and during the first three weeks the fermentation was unstable. When it had stabilised, the process personnel noticed a change in the cell shape of the fermenting yeast. The change was

regarded as either a physiological adaptation or as a selected genetic variant of the inoculated *S. cerevisiae*. The aim of the study described in (**I**) was to investigate the reason for the change in cell shape and to analyse the occurrence of potentially contaminating LAB. Surprisingly, the yeast population was found to be dominated by *D. bruxellensis*. No *S. cerevisiae* was detected in the process, either by cultivation-based techniques or with qPCR on DNA isolated directly from the fermentation broth. This means that in spite of massive inoculation with *S. cerevisiae*, the process had been taken over by a different yeast species. The LAB population was almost completely dominated by the species *Lactobacillus vini*. Since the ethanol productivity of the process was economically satisfactory, *D. bruxellensis* and *L. vini* represent the production consortium for this specific ethanol process.

The results of study (**I**) raised several questions:

- What impact has this outcompetition by *D. bruxellensis* on the process? What about its ethanol-producing abilities and by-product formation? (**II**).
- Can this competitive yeast ferment substrates derived from lignocellulosic material? (**III**).
- What is the mechanism of outcompetition by *D. bruxellensis* and under which conditions outcompetition can occur? (**IV**, **V**).
- How stable is *D. bruxellensis* over a long time period? (**IV**)
- Finally, we investigated the competitiveness of *D. bruxellensis* in defined continuous fermentations and its ability to grow under anaerobic conditions (**V**).

2 *Dekkera bruxellensis*

2.1 The organism

Dekkera bruxellensis (anamorph *Brettanomyces bruxellensis* Kufferath et v. Laer) was described by van der Walt (1964) and the type strain was isolated from Belgian Lambic beer by Custers in. 1940. The first isolation of 'Brettanomyces' yeast was reported in 1904, when a yeast species was isolated from different kinds of British beers (Claussen, 1904). However, the use of the word Brettanomyces was not initially intended as a taxonomic name, but just to indicate the origins of the yeast (derived from 'British brewing fungus' (Oelofse, *et al.* 2008)). *Brettanomyces* was proposed as the generic name for two new anamorphic species, *B. bruxellensis* and *B. lambicus*, isolated from the Belgian beer Lambic in 1921 (Kufferath & Laer, 1921).

According to Smith *et al.* (1990), there are six different species in the genus *Dekkera/Brettanomyces*: two teleomorphic, *D. anomala* and *D. bruxellensis*, and four anamorphic, *B. anomalus, B. bruxellensis*, *B. custersianus* and *B. naardensis*. However, *D. bruxellensis* and *B. bruxellensis* can be regarded as synonyms (Smith, 2011). The synonyms of *D. bruxellensis* are shown in Table 1.

D. bruxellensis and *S. cerevisiae* separated from each other about 200 million years ago (Woolfit *et al.*, 2007). They have some common characteristics, such as ethanol production under aerobic conditions and the ability to grow under anaerobic conditions (see Chapter 6) and to be petite positive, which have evolved independently in the two yeast species (Rozpędowska *et al.*, 2011; Prochazka *et al.*, 2010; Hellborg & Piskur, 2009).

Table 1. Synonyms of *D. bruxellensis* (*B. bruxellensis*) according to Smith (2011), Boekhout et al. (1994) and Smith et al. (1990)

Synonym
D. intermedia
B. abstinens
B. custersii
B. intermedius
B. lambicus
Other synonyms (less frequently used)
B. bruxellensis var. *non-membranifaciens*
B. bruxellensis var. *vini*
B. patavinus
B. schanderlii
B. vini
D. abstinens
D. lambica
Mycotorula intermedia

D. bruxellensis cells are ellipsoidal to spherical, often ogival, but also cylindrical to elongated. Pseudomycelium is often formed (Figure 1; van der Walt, 1964). The growth is slow in malt extract and on malt agar and the cultures are generally short-lived. Under aerobic conditions acetic acid is formed in large amounts from glucose (van der Walt, 1964). The metabolism can be either oxidative or fermentative. One of the best-known characteristics of *Dekkera/Brettanomyces* yeasts is Custer's effect, which is temporary inhibition of fermentation under anaerobic conditions (Wijsman *et al.*, 1984; Scheffers, 1979). *D. bruxellensis* cannot grow in vitamin-free medium and requires biotin and/or thiamine for growth (Barnett *et al.*, 2000).

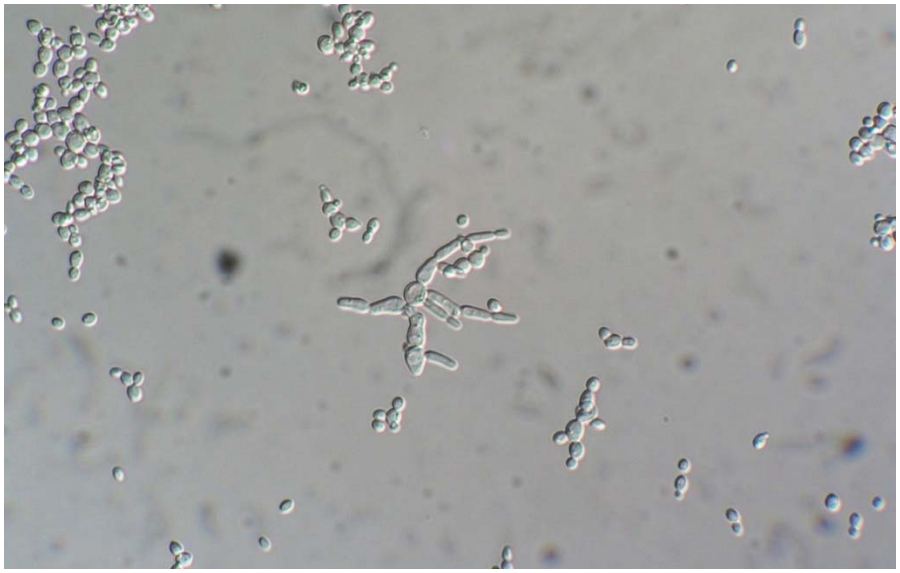

Figure 1. An example of pseudomycelium formed by *D. bruxellensis* CBS 11269.

2.2 Contaminating yeast

D. bruxellensis has until quite recently mainly been considered a contamination yeast in both the wine industry (Oelofse *et al.*, 2008; Renouf *et al.*, 2007; Cocolin *et al.*, 2004) and the ethanol industry (Basillo *et al.*, 2008; Liberal *et al.*, 2007).

2.2.1 Contaminant of wine processes

Contamination with *D. bruxellensis* occurs more frequently in red wine than in white wine, probably due to the low pH and the inhibitory efficiency of SO_2 in white wine processes (Loureiro & Malfeito-Ferreira, 2006). In wine, *D. bruxellensis* produces off-flavours that have been described as wet horse, stable, mouse-like, band-aid odours, etc. (Mitrakul *et al.*, 1999; Chatonnet *et al.*, 1997; Heresztyn, 1986). These off-flavours are caused by ethylphenols produced by *D. bruxellensis* from precursors present in wine (Andrade *et al.*, 2005). One of the precursors is hydroxycinnamic acid, which is decarboxylated into 4-ethylphenol via vinylphenol derivatives by different enzymes in *Dekkera*/*Brettanomyces* yeasts (Harris *et al.*, 2009; Chatonnet *et al.*, 1992). Other precursors for off-flavours in wine include ρ-coumaric acid and ferrulic acid (Dias *et al.*, 2003; Chatonnet *et al.*, 1995).

The main sugars in grape must are glucose and fructose and *S. cerevisiae* prefers to ferment glucose to ethanol. Under conditions of high ethanol content and low sugar concentration (as in wine at the end of alcoholic fermentation), it

has been shown that *D. bruxellensis* consumes fructose as carbon source (Vigentini *et al.*, 2008). *Dekkera*/*Brettanomyces* contamination is therefore most prominent at the end of alcoholic fermentation, during malolactic fermentation and during ageing of the wine in used barrels (Oelofse *et al.*, 2008; Renouf *et al.*, 2007; Chatonnet *et al.*, 1992). During malolactic fermentation LAB, most commonly *Oenococcus oeni*, transform malic acid into lactic acid, which decreases the acidity and enhances the aroma of wine by the production of other metabolites (Renouf *et al.*, 2005). *B. bruxellensis* has been found on grapes (Renouf & Lonvaud-Funel, 2007), which may explain the origin of wine contamination by *B. bruxellensis*. The microorganisms on the surface of oak barrels have been studied and *D. bruxellensis* was one of the most common species on those surfaces (Renouf *et al.*, 2007). Since *D. bruxellensis* is found on the surfaces of oak barrels and is known to produce biofilms (Joseph *et al.*, 2007), it is very difficult to remove once inside the system.

2.2.2 Contaminant of ethanol processes

Since it is not feasible to run large-scale industrial ethanol fermentation under sterile conditions, contamination by unwanted yeasts and bacteria (especially lactic acid bacteria) is a very common phenomenon (Schell *et al.*, 2004). This contamination often leads to lower productivity of the process, meaning that less ethanol is produced, which is detrimental for the industry. Investigations have identified the major contaminating yeasts in the bio-ethanol industry to be *Candida tropicalis*, *Pichia galeiformis* and *D. bruxellensis* (Basillo *et al.*, 2008; Liberal *et al.*, 2007). The latter yeast is regarded as the most common contaminant in industrial ethanol fermentation (Liberal *et al.*, 2007).

The yeast population in an industrial ethanol fermentation process running on crude sugar cane juice with re-circulation of the yeast cells was investigated by Liberal *et al.* (2007). As the number of *D. bruxellensis* cells increased at the expense of the number of *S. cerevisiae* cells, the ethanol productivity decreased. When the number of *D. bruxellensis* cells reached 50% of the total population, the whole yeast biomass was replaced with fresh *S. cerevisiae* cells. However, already after 30 days *D. bruxellensis* had taken over the fermentation again and these oscillations continued during the whole season.

In an unpublished study, I investigated the yeast population in another industrial ethanol plant with continuous cultivation and recirculation of the yeast cells, where process problems had occurred. The yeast colonies were slow-growing on agar plates, which is one of the characteristics of *D. bruxellensis* (see above). Sequencing the D1/D2 region of the 26S rRNA of a number of the slow-growing isolates confirmed that they were all

D. bruxellensis. The substrate in that fermentor was starch-based, so the occurrence of *D. bruxellensis* is most likely not coupled to a specific type of substrate.

2.3 Production yeast

D. bruxellensis can also act as a production organism, perhaps most well known as one of the wild yeasts in the production of Lambic beer. It is also one of the yeasts that have been isolated from the production process of the Brazilian alcoholic beverage 'cachaça' and can in principle generate a beverage that fits the product characteristics of cachaça (Dato *et al.*, 2005). The yeast has also been found in certain types of sour dough (Meroth *et al.*, 2003). In the present work, *D. bruxellensis* was found to be a production organism in the ethanol industry (**I**).

2.3.1 Production yeast in the beer industry

Beer production based on wild yeast is mainly carried out in Belgium, but there are also examples of British beers produced from wild yeasts (Martens *et al.*, 1997; Kumara & Verachtert, 1991). For Lambic and Geuze beers, produced in the region around Brussels in Belgium, *D. bruxellensis* is one of the production yeasts (Verachtert & Dawoud, 1990). Lambic beer is produced from spontaneously fermented wort, while to produce Geuze the wort is transferred to bottles, where the fermentation is continued (Verachtert and Dawoud, 1990). In both Geuze and Lambic beers, *Dekkera*/*Brettanomyces* yeasts occur in the later stages of fermentation of the wort (Van Oevelen *et al.*, 1977). The esterase activity of *Brettanomyces* yeasts gives Lambic and Geuze beers their characteristic flavour (van Nedervelde & Debourg, 1995). The ester concentration in Lambic beer follows the growth of *Brettanomyces*, with the highest amounts of esters and cells being found in the later stages of the fermentation (Verachtert & Dawoud, 1990). The esters present are (in order of abundance): isoamyl acetate, ethyl acetate, ethyl lactate and ethyl caprate (van Nedervelde & Debourg, 1995).

2.3.2 Production yeast in the ethanol industry

As described in the introduction, *D. bruxellensis* was isolated from an industrial ethanol plant in Sweden where it was the actual production yeast. To investigate how *D. bruxellensis* entered the process, samples were withdrawn from the saccharification line and the process water, as well as from commercial baker's yeast. No *D. bruxellensis*, but several other yeasts, were found in those samples (**I**) (Table 2). Thus the source of *D. bruxellensis*

remained unknown. According to the process personnel, the change in yeast cell shape happened several times after start-up of the process, so *D. bruxellensis* has probably been a production yeast in that plant for several years (**I**). There are strains of *B. bruxellensis* that are efficient biofilm producers (Joseph *et al.*, 2007). This could explain why *D. bruxellensis* is recurring in the ethanol plant and why it is a major problem for the wine industry. When yeasts and bacteria produce biofilms it is difficult to remove them from the surface to which they are attached. Such biofilms are most frequently occurring on places that are difficult to clean, *i.e.* corners, joints and transfer lines (Kumar & Anand, 1998). However, even if *D. bruxellensis* produces biofilms in the ethanol plant investigated, this still does not explain its origin in the process.

In February 2011, five years after the first sample was taken from the fermentor in Sweden, samples were withdrawn to investigate the yeast population again (**IV**). The reason for taking these samples was that after several years running the process without disturbances and reconstruction (to a larger fermentor), a decrease in productivity was noticed. Unsaccharified cereals had accidentally been added to the fermentor and to give the yeast population the opportunity to recover it was run at lower ethanol concentrations. The dominant yeast was as before *D. bruxellensis*. Surprisingly, *Wickerhamomyces anomalus* (synomyms *Hansenula anomala*, *Pichia anomala*) was also found in the fermentor broth. This was most likely due to the use of unsaccharified cereals as substrate, since *W. anomalus* has frequently been identified within the storage flora of cereals (Olstorpe & Passoth, 2011). Following our recommendations, the process was re-started and run at higher ethanol concentrations. About four weeks after that re-start, a sample was withdrawn and only *D. bruxellensis* and no *W. anomalus* was found and the productivity of the process was improved. This is the first report showing that the non-conventional production yeast *D. bruxellensis*, usually regarded as a contaminating yeast, is in competition with another contaminating yeast (**IV**).

Table 2. Yeasts and lactic acid bacteria found in and around the fermentation process in an industrial ethanol plant in Sweden in 2006 (**I**).

	Baker's yeast	**Saccharification line**	**Process water**	**Fermentor**
Yeast	*S. cerevisiae*	*Candida* cf. *sorbosivorans*, *C. magnoliae*	*S. cerevisiae*, *Kluyveromyces marxianus*, *Pichia galeiformis*	*D. bruxellensis*
LAB	*Lactococcus lactis*, *Leuconostoc pseudomesentetoides*, *Pediococcus* sp.	*Lactobacillus parabuchneri*, *L. casei*	*L. fermentum*, *L. delbruecki* subsp. *bulgaricus*, *Weisella confusa*, *L. salivarius*	*L. vini* (*L. fermentum*, *L. panis*)

”Ju mer man tänker, desto mer inser man att det inte finns något enkelt svar.”

Nalle Puh, A. A Milne

3 LAB in the ethanol industry

LAB are usually present in industrial ethanol fermentations, but the number should be kept to a minimum since LAB compete with the yeast for the substrate and generate organic acids that can inhibit the yeast, causing stuck fermentation (Skinner & Leathers, 2004).

The LAB population in an ethanol process with recirculation of yeast cells was investigated and was found to be almost completely dominated by *Lactobacillus vini* (**I**). Samples were withdrawn from the same fermentor one month, six months and 18 months after the first sample and the yeast and lactic acid bacteria populations were found to be dominated by *D. bruxellensis* and *L. vini* on all occasions. In the fermentor the number of LAB was high, sometimes even higher than the number of yeast cells, without affecting the ethanol production. Therefore, one might regard *D. bruxellensis* and *L. vini* as a production consortium (**I**).

Among the 60 LAB strains isolated at three different time points, 58 belonged to the species *L. vini* and the two other belonged to *L. fermentum* and *L. panis* (**I**). *L. vini* was first isolated from fermenting grape must and is able to produce lactic acid from pentoses (Rodas *et al.*, 2006). *Lactobacillus fermentum* has been isolated from malt whisky production (van Beek & Priest, 2002) and *L. panis* has been isolated from wheat distillers grain (Pedersen *et al.*, 2004) and thus may be a typical microorganism in an ethanol production process.

A consortium of *D. bruxellensis* and *L. vini* has recently also been discovered in an industrial ethanol fermentor in Brazil with recirculation of yeasts, but running on a different substrate, sugar cane juice. However, in this case the yeast population was a mixture of *D. bruxellensis* and *S. cerevisiae* (Lucena *et al.*, 2010). The composition of LAB species was more diverse at the beginning of the fermentation, while at the end the majority of the LAB species belonged to *L. vini* and *L. fermentum* (Lucena *et al.*, 2010). Thus the yeast

population was not completely dominated by *D. bruxellensis* and *L. vini* as in (**I**). *L. fermentum* has also been shown to be one of the dominant LAB species in a starch-based industrial ethanol plant (Bischoff *et al.*, 2009; Chang *et al.*, 1995).

When sampling five years after the first study (**I**), the LAB population was investigated again and *L. vini* was still found in the fermentor broth (**IV**). In the first sampling of that study the only LAB other than *L. vini* was *Lactobacillus hamsteri*, which comprised 10% of the isolates investigated. *L. hamsteri* was first isolated from the intestinal flora of hamster (Mitsuoka & Fujisawa, 1987). In the samples taken after the second re-start, only *L. vini* could be detected (**IV**).

3.1 Yeast and LAB interactions

There are a number of yeast/LAB interactions that contribute positively to the end-product quality of the fermented food, for instance in the production of kefir (Simova *et al.*, 2002), in sourdoughs (De Vuyst *et al.*, 2009) and in wine making during malolactic fermentation (Renouf *et al.*, 2005). In ethanol production, LAB are always present, but should preferably be kept to a minimum. In contrast, the number of LAB in the ethanol process described in (**I**) was high, sometimes even higher than the number of yeasts, without affecting the process negatively. In a study on laboratory-scale continuous cultivation with recirculation, the number of LAB was found to be high and it was concluded that the set-up was the reason for the high number of LAB (Chang *et al.*, 1995).

With regard to the *D. bruxellensis* and *L. vini* interaction in the industrial ethanol plant, we can only speculate at present (there is an ongoing investigation in our laboratory about the interaction between these microorganisms; I. Tiukova, pers. comm. 2011). It is possible that the two organisms can live side by side without affecting each other (neutralism), *i.e.* both have created their own niche in the fermentation tank. For example, *L. vini* is able to ferment pentoses and might survive on those compounds present in the substrate. However, the possibility of competition between the two organisms cannot be excluded, as up to now no comprehensive study of ethanol yield and productivity in *D. bruxellensis*/*L. vini* co-cultures has been performed.

4 Mechanisms of outcompetition – physiological background

Studies of the contamination of wine by *D. bruxellensis* (Renouf *et al.*, 2005) and the microbial composition in the production of Lambic beer (Verachtert and Dawoud, 1990) have found that the number of *D. bruxellensis* cells increases after malolactic fermentation in wine processes and at the later stages of fermentation of the wort for Lambic beer production. The common denominator is that the sugar level is low when *D. bruxellensis* levels are increasing. All reports describing outcompetition of *S. cerevisiae* by *D. bruxellensis* in the ethanol industry originate from distilleries run in continuous mode, especially from those plants that re-circulate the yeast cells, *i.e.* where the capacity for rapid growth is of minor importance (**I;** de Barros Pita *et al.*, 2011; Liberal *et al.*, 2007).

Therefore our starting hypothesis was that *D. bruxellensis* is more competitive during sugar limitation and when the growth rate has a minor influence. However, other possible explanations, such as killer activity of *D. bruxellensis* and production of inhibitory metabolites, cannot be excluded.

4.1 Fermentation characteristics of *D. bruxellensis*

Due to the unexpected finding that *D. bruxellensis* had outcompeted *S. cerevisiae* (**I**), the first objective was to investigate the ethanol-producing abilities of the yeast in order to determine the impact this outcompetition could have on the process.

4.1.1 Influence of pH and temperature

In the industrial fermentor from which the strains were isolated, the fermentation was run at an acidic pH (3.6) and at a temperature of 36°C. Studies have shown that when *D. bruxellensis* is grown at a temperature of

35°C, stuck fermentation occurs, *i.e.* the yeast stops growing before all substrate is consumed (Brandam *et al.*, 2008; Bisson & Butzke, 2000). In wine, where *D. bruxellensis* often occurs, fermentation is run at temperatures between 15-35°C (white wine in the lower (15-20°C) and red wine in the higher range (25-35°C)) (Torija *et al.*, 2003). In wine fermentations, *D. bruxellensis* is quite heat-sensitive and above 35°C a 100% loss in viability has been reported (Barata *et al.*, 2008). However, this is most likely due to the high ethanol content and probably also the phenols present in wine (Couto *et al.*, 2005). When the type strain was grown at pH 3.6 and 36°C, it grew more slowly and reached a lower final biomass compared with the industrial isolates. Another observation was that the type strain formed more extensive pseudomycelia under those conditions (Figure 2) than the industrial isolate grown under the same conditions (Figure 3).

Obviously, there are high variations in the temperature optimum of different *D. bruxellensis* strains. The pH was also rather low (3.6) in the industrial fermentation and it was unknown whether this is the optimum for *D. bruxellensis*. Therefore, we investigated the influence of pH and temperature on growth rate and ethanol yield for one industrial strain of *D. bruxellensis* in bioreactor experiments using a full factorial design. No major differences were observed in the parameters investigated within the test ranges (pH 3-5, 25-37°C), leading to the conclusion that *D. bruxellensis* is robust to changes in environmental conditions. This might be one of the explanations why *D. bruxellensis* is competitive in the harsh conditions in industrial fermentations. The pH tolerance of one strain of *D. bruxellensis* was investigated by Rozpędowska *et al.* (2011), who found that this strain could grow at a pH of 2.3. The ability to decrease the pH and to survive at low pH may be two of the reasons why *D. bruxellensis* is competitive (Rozpędowska *et al.*, 2011).

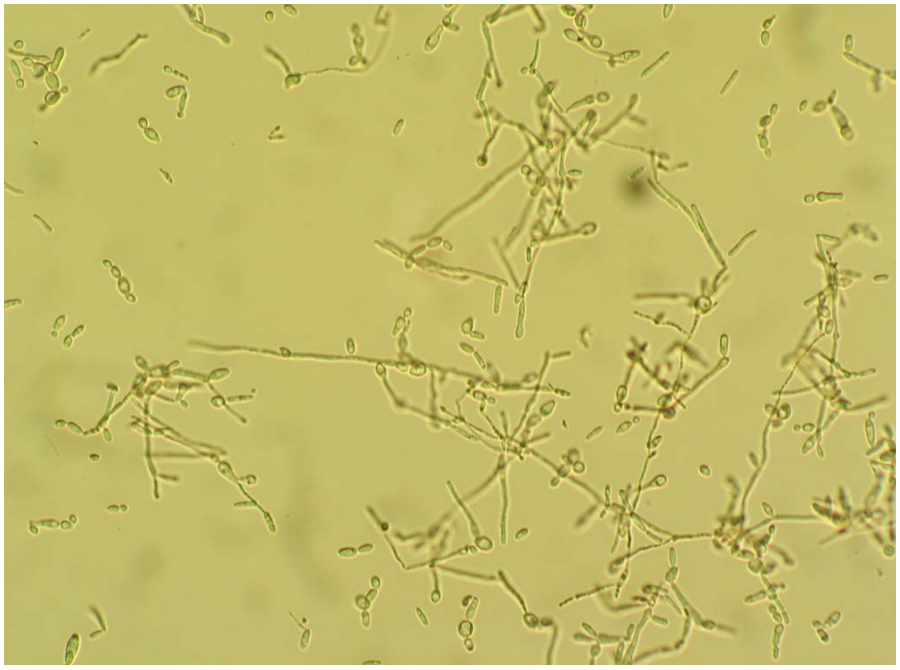

Figure 2. Microscopic image of *D. bruxellensis* CBS 74 grown at pH 3.6 and 36°C.

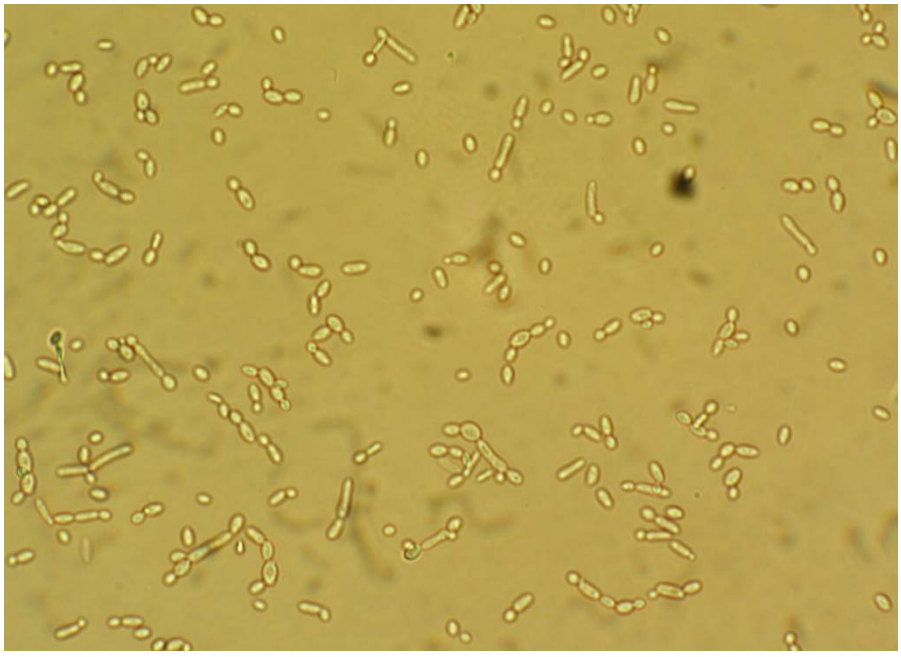

Figure 3. Microscopic image of *D. bruxellensis* CBS 11269 grown at pH 3.6 and 36°C

4.1.2 *D. bruxellensis* vs. *S. cerevisiae*

The growth, ethanol, acetate and glycerol production of different strains of *D. bruxellensis* were compared to those of *S. cerevisiae* under the same pH and temperature as in the industrial fermentor (pH 3.6, 36°C). As found in other studies (Abbott *et al*., 2005), the growth rates of the *D. bruxellensis* strains were much lower (about five-fold) than those of the *S. cerevisiae* strains (**II**). All *D. bruxellensis* strains studied had a similar or even higher ethanol yield than *S. cerevisiae*, higher biomass yield (except the type strain) and a lower glycerol yield (**II**). This is a strong indication that *D. bruxellensis* has a more energy-efficient metabolism than *S. cerevisiae*. Yeasts produce glycerol to restore the imbalance in redox potential when grown under anaerobic or oxygen-limited conditions (Van Dijken & Scheffers, 1986) by re-oxidising the NADH produced from amino acid synthesis and from biomass production. However, glycerol production is also a loss of energy and of carbon, leading to a lower ethanol and biomass yield (Guo *et al.*, 2009). Thus, due to the low glycerol production *D. bruxellensis* can utilise the carbon and energy for ethanol and biomass formation instead. The glycerol production of *D. bruxellensis* is discussed further in Chapter 6.

4.1.3 Acetic acid production

One of the best-known features of *D. bruxellensis* is that it produces acetic acid from glucose under aerobiosis. Some authors actually suggest that *Dekkera/ Brettanomyces* yeasts can be used for acetic acid production (Freer *et al.*, 2003; Freer, 2002).

In yeasts, acetic acid is produced from the oxidation of acetaldehyde, which can be formed as a product of the pyruvate decarboxylase (PDC) reaction during growth on sugars, or as a product of the alcohol dehydrogenase (ADH) reaction during growth on ethanol (Postma *et al.,* 1989; Lutsdorf & Megnet, 1968).

It is under debate whether the acetic acid production by *Dekkera/ Brettanomyces* yeasts is one of the mechanisms behind the outcompetition. De Miniac (1989) considered acetic acid to be the main factor in competition between *D. bruxellensis* and *S. cerevisiae*. However, most studies argue the opposite, since the concentration of acetic acid needed to inhibit *S. cerevisiae* is not obtained by *D. bruxellensis* in industrial ethanol processes (**II**, **V**; Abbott *et al.*, 2005; Phowchinda *et al.*, 1995). In a continuous cultivation experiment where *D. bruxellensis* was able to outcompete *S. cerevisiae*, the culture was sparged with a nitrogen/air mixture that contained approximately 5% oxygen (**V**). Some acetic acid was produced, but not more than 1 g/L, which is unlikely to be the reason for outcompetition. *D. bruxellensis* itself is sensitive to acetic

acid and it has been shown that an acetic acid concentration above 2 g/L negatively affects growth and ethanol production by *B. bruxellensis* (Yahara *et al.*, 2007). The pH value also influences how severely acetic acid affects the yeast, with a pH lower than pKa of acetic acid leading to a higher level of undissociated acid which can enter the yeast cell. When inside, the acid returns to its dissociated form and decreases the intracellular pH (Taherzadeh *et al.*, 1997). The decrease in pH in the cell forces the yeast to pump protons out of the cell by plasma membrane ATPase to maintain the intracellular pH (Taherzadeh *et al.*, 1997; Maiorella *et al.*, 1983). This requires energy (ATP), which in turn decreases biomass production.

During oxygen limitation, almost no acetic acid was produced by the industrial *D. bruxellensis* strains. Acetic acid concentration in the bioreactor experiments was below the detection limit. In shake-flask experiments some acetic acid was produced, mainly in the later stages of fermentation. Interestingly, more acetic acid was produced when *D. bruxellensis* was grown on maltose or cellobiose than on glucose under the same conditions (**II**).

In an experiment (mentioned in **II**) where a *D. bruxellensis* culture was sparged from the beginning with 0.1 L/min of air, acetic acid production started when the glucose was almost zero. When the glucose was consumed and the yeast entered stationary phase, the diauxic shift occurred and *D. bruxellensis* started consuming ethanol (Figure 4). The acetic acid concentration kept increasing, implying that the yeast used ethanol as carbon source for the production of acetic acid. Carrascosa *et al.* (1981) observed that the activity of the NAD^+-linked aldehyde dehydrogenase increased at the end of the exponential phase, where glucose was almost depleted and the acetic acid concentration was highest. The activity of this aldehyde dehydrogenase was also highest in the cultures grown at low glucose concentrations. This suggests that this enzyme is glucose-repressed. Altogether, this would explain why the acetic acid was only produced when the glucose was consumed in the experiment described in Figure 4 and not from the beginning when glucose was present. Freer (2002) investigated the acetic acid production by different *Dekkera/Brettanomyces* strains from glucose or ethanol as carbon source and found that some of the strains produced high amounts of acetic acid from ethanol.

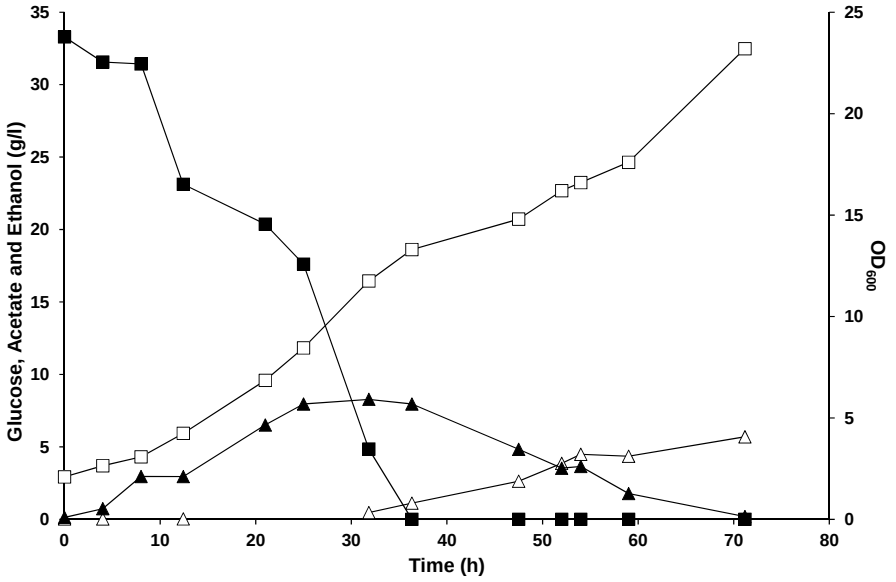

Figure 4. D. bruxellensis CBS 11269 grown aerobically with sparging of 0.1 L/min of air. (Glucose (filled squares) OD (open squares), ethanol (filled triangles) and acetate (open triangles)). The diagram shows the diauxic shift where the yeast started consuming ethanol for growth. Acetic acid production started when glucose was finished and continued as long as ethanol was present.

4.1.4 Pyruvate production

Pyruvic acid (2-oxopropanoic acid, α-ketopropionic acid, acetylformic acid or pyroracemic acid) is used commercially as a starting material for the biosynthesis of certain pharmaceuticals, for example L-tryptophan, L-tyrosine and L-DOPA (L-3,4-dihydroxyphenylalanine) (Enei *et al.*, 1972; Nakazawa *et al.*, 1972). Pyruvic acid is produced for commercial use either chemically by dehydration and decarboxylation of tartaric acid or in biotechnical production by, for example, direct fermentative production using the yeast *Candida glabrata* (syn. *Torulopsis glabrata*) or bacteria (Li *et al.*, 2001a). Pyruvic acid is the last intermediate in the glycolysis before entering the TCA cycle (if yeast growth is respirative) or alcohol formation (fermentative yeast growth). To convert pyruvic acid to acetaldehyde or acetyl-CoA the enzymes PDC (pyruvate decarboxylase) or PDH (pyruvate dehydrogenase) are needed. These enzymes require the active form of thiamine as co-factor, thiamine-diphosphate (Mojzita & Hohmann, 2006). Without thiamine, pyruvic acid accumulates and is excreted to the medium (Li *et al.*, 2001a). The majority of the yeasts that

overproduce pyruvate are auxotrophs for either one or several vitamins or amino acids (Wang *et al.*, 2002). *D. bruxellensis* strains may vary in auxotrophy for thiamine, biotin and several other vitamins (Barnett *et al.*, 2000; Madan & Gulati, 1980).

In a vitamin requirement growth test, *D. bruxellensis* CBS 11269 was pre-grown in medium supplemented with biotin and thiamine and then transferred to a medium without thiamine. *D. bruxellensis* was able to grow for two to three generations but then the growth ceased and a drop in pH was observed, accompanied by pyruvic acid production. The pyruvic acid yield was 0.23 g pyruvic acid/g glucose consumed. However considering only the glucose consumed after the onset of pyruvic acid production (the last 30 h), the yield was as high as 0.53 g pyruvic acid/g glucose consumed (patent pending). This is comparable to strains of *C. glabrata*, which produce 0.50-0.62 g pyruvic acid/g glucose (Li *et al.*, 2001b; Li *et al.*, 2000).

Interestingly, although grown aerobically *D. bruxellensis* produced low amounts of acetic acid, only 1.8 g/L, compared with 6.6 g/L for the control grown in medium containing vitamins. This is an indication that *D. bruxellensis* during pyruvic acid production redirects its carbon metabolism from acetic acid production to pyruvic acid production. *D. bruxellensis* stops growing when thiamine is depleted, so a certain amount of thiamine could be needed in the medium, but no pyruvic acid will be produced at too high concentrations of thiamine. More experiments are needed in order to fully understand the ability of *D. bruxellensis* to produce pyruvic acid and to optimise the cultivation conditions to obtain maximum amounts of pyruvic acid.

4.2 Competition between *D. bruxellensis* and *S. cerevisiae*

From the results obtained in (**II**) and described above, we can state that due to the slow growth of *D. bruxellensis* it cannot outcompete *S. cerevisiae* in conditions where growth rate is critical, *i.e.* in batch fermentations. In a study with batch competition experiments, *S. cerevisiae* was the dominant yeast during the whole experiment, mainly due to its faster growth rate (Nardi *et al.*, 2010). In continuous cultivation the dilution rate is equal to the growth rate of the organism during steady state in a nutrient-limited culture. When the dilution rate is below the μ_{max} of *D. bruxellensis*, the ability for rapid growth may not be decisive for competitiveness.

4.2.1 Glucose limitation is required

The hypothesis was that the competitiveness of *D. bruxellensis* is due to more energy-efficient metabolism, as discussed in (**II**). Considering the slow growth of *D. bruxellensis* and to mimic conditions in the industrial fermentor from where the strains were isolated, competition experiments were run either in small scale in microtitre plates with recirculation of yeast cells (**III**, **IV**)**,** or in glucose-limited continuous cultivation in a bioreactor (**V**).

In 6-well microtitre plates (working volume of 5 mL per well), 2 mL of cell suspension were removed four times per day and samples were taken for OD, viable count and HPLC measurements. The cell suspension was centrifuged and the pellet was resuspended in 2 mL fresh medium and transferred back to the well. At a glucose concentration of 30 g/L in the feed medium, the culture became glucose-limited within 24 h and *D. bruxellensis* numbers remained stable throughout the experiment, while the number of *S. cerevisiae* cells decreased (**IV**).

However, in experiments running at higher glucose concentrations, the cells were not able to consume all the glucose and the numbers of *S. cerevisiae* cells remained stable throughout the experiment (unpublished results). In the control with only *D. bruxellensis* glucose was also detectable, indicating limitation of another essential factor. Adding yeast extract to the medium did not make *D. bruxellensis* able to consume the glucose or outcompete *S. cerevisiae*, so the inability to consume all glucose was not due to nitrogen limitation.

Uscanga *et al.* (2011) investigated the impact of initial glucose concentration on the growth and ethanol production of *B. bruxellensis* and found that at up to 138 g/L, the yeast consumed the glucose completely. Above this concentration there was sugar remaining and the growth rate was severely reduced. They concluded that the reason for this finding was not nutrient limitation but inhibition by metabolites produced by the yeast. They ran the cultivations aerobically and at 165 g/L glucose the acetic acid and ethanol concentrations were 4.5 and 54.5 g/L, respectively. However, in our study only low amounts of acetic acid and ethanol were produced.

In **V**, the competition experiments were run in continuous cultivation, one without sparging and the other with sparging of a nitrogen/air mixture (approximately 5% oxygen in the inflow). In the former, *D. bruxellensis* was not able to consume the glucose completely before spiking with *S. cerevisiae*, but in the latter the glucose level was zero at the time of spiking. The reason for this is discussed in Chapter 6. Following the same pattern as observed with the microtitre plates when the *D. bruxellensis* culture was not glucose-limited, *D. bruxellensis* was not able to outcompete *S. cerevisiae*, but instead the yeasts co-existed in the fermentor. When the culture was glucose-limited,

S. cerevisiae decreased throughout the experiment, while *D. bruxellensis* remained stable. This shows that for *D. bruxellensis* to be able to outcompete *S. cerevisiae*, the culture needs to be glucose-limited. This could have something to do with *D. bruxellensis* having a more energy-efficient metabolism than *S. cerevisiae*. In addition, *D. bruxellensis* may have a higher affinity for glucose than *S. cerevisiae* during glucose limitation, while when glucose is present *S. cerevisiae* is more efficient in glucose uptake. A number of studies have measured the Km for glucose and all show that the *Dekkera*/*Brettanomyces* yeast studied had a high affinity transport system, with a Km value 30-60 times lower than for the high affinity system of *S. cerevisiae* (Walsh *et al.*, 1994; Van Urk *et al.*, 1989; Bisson & Fraenkel, 1983). The low affinity transporter of *S. cerevisiae* had a Km of around 20. If our strains of *D. bruxellensis* have a high affinity transporter with a Km in the same range as other investigated strains, this would explain why *D. bruxellensis* is more competitive at low glucose conditions and not so when glucose is present. When glucose is present, the low affinity transporter of *S. cerevisiae* will be active, while the high-affinity transporter is repressed. During glucose limitation *D. bruxellensis*, due to a lower Km for glucose for its high affinity transporter, will be more efficient in glucose uptake compared with *S. cerevisiae*. However, the Km for glucose for the strains of *D. bruxellensis* remains to be determined experimentally.

4.2.2 The role of nitrate in the outcompetition

D. bruxellensis is one of few yeast species that is able to assimilate nitrate as nitrogen source. Other examples are *W. anomalus*, *C. nitratophila*, and *Rodotorula glutinis*. In contrast, *S. cerevisiae* is not able to assimilate nitrate (Kurtzman *et al.*, 2011).

de Barros Pita *et al.* (2011) suggested that one of the explanations for the competitiveness of *D. bruxellensis* is its ability for nitrate assimilation. Growth and ethanol production of *D. bruxellensis* are affected positively by the presence of nitrate. In competition experiments between *D. bruxellensis* and *S. cerevisiae*, *D. bruxellensis* was only able to outcompete *S. cerevisiae* in medium with high nitrate and low ammonium concentrations. Sugar cane juice, one of the major substrates for the ethanol industry in Brazil, has a high nitrate content (Liberal *et al.*, 2007). In samples from an industrial fermentor using sugar cane as substrate, the nitrate concentration of the substrate was correlated to the number of *D. bruxellensis* cells such that when the concentration of nitrate was high the number of *D. bruxellensis* was at its highest level and *S. cerevisiae* at its lowest and *vice versa* (de Barros Pita *et al.*, 2011).

However, not all *D. bruxellensis* strains have the ability to assimilate nitrate (Barnett *et al.*, 2000). Furthermore, in the competition experiments described above and in (**IV**) and (**V**), no nitrate was added to the medium. The substrate in the industrial fermentor from which the strains were isolated was investigated for the presence of nitrate (**IV**). Using nitrate sticks that give either a positive or negative indication for nitrate with a detection limit of 0.5 mg/L showed that no nitrate was present in the substrate. Thus, *D. bruxellensis* can be competitive without the presence of nitrate, but this does not exclude the possibility that nitrate increases the competitiveness of *D. bruxellensis*. The ability of our strains to assimilate nitrate still needs to be experimentally determined.

4.2.3 Killer activity

Certain yeasts can produce killer proteins that inhibit the growth of other yeasts (Santos *et al.*, 2011), and bacteria (Meneghin *et al.*, 2010). Killer proteins are often glycoproteins and can have molecular weights ranging from 5000-100 000 kDa. Killer activity has been found in more than 90 yeast species (Buzzini *et al.*, 2007).

The competitiveness of *D. bruxellensis* in wine and ethanol processes could be due to possible production of killer proteins by *D. bruxellensis*. However, our continuous competition experiments with *D. bruxellensis* and *S. cerevisiae* (**IV** and **V**) indicated that production of killer toxin by *D. bruxellensis* does not occur. In cases where *D. bruxellensis* outcompeted *S. cerevisiae*, the decrease in *S. cerevisiae* was too slow to be caused by killer toxin. In the study by Santos *et al.* (2011) where the killer yeast *Ustilago maydis* was co-cultivated with *B. bruxellensis*, the viable count of *B. bruxellensis* decreased by 90% in just 8 h, while a 90% decrease in *S. cerevisiae* cells in the continuous cultivation experiment took several days (**V**).

W. anomalus is another yeast that produces killer proteins (Fredlund *et al.*, 2002). Since *W. anomalus* was isolated from the industrial fermentor at a time when the fermentation was not running properly, one might suspect killer activity in those strains. Therefore, a killer assay was performed testing the isolated strains of *W. anomalus* toward *D. bruxellensis* and *S. cerevisiae*, but no killer activity was seen (unpublished results).

5 Lignocellulosic application

The transportation and chemical industries both depend heavily on the diminishing resource of mineral oil. The transport sector is increasing world-wide, leading to an increase in greenhouse gas (GHG) emissions, so there is a urgent need for alternative fuels. To cover the demand for alternative fuels in the future, bio-ethanol from the substrates used today, so-called first-generation biomass (cereals, maize or sugar-cane), will soon reach its limit and may also compete with food production. Using first-generation biomass can also result in intensification of agriculture, which will not result in a reduction in GHG emissions. Lignocellulosic substrates (second-generation biomass) are currently under-utilised in biofuel production. In order to improve the raw material supply and efficiently decrease GHG emissions, the utilisation of lignocelluloses for biofuel production is required (Gnansounou & Dauriat, 2010).

5.1 Lignocellulosic biomass and pre-treatment

Lignocellulosic biomass consists of cellulose (poly β-1,4 glucose), hemicelluloses (mainly poly β-1,4-xylose) and lignin. It is a rigid structure that is not fermentable by yeasts. To release the sugar monomers, pretreatment of the material is required. This usually includes combined thermochemical and enzymatic degradation. Thermochemical pretreatment, for example steam explosion, acid pretreatment or ammonia fibre explosion, breaks up the recalcitrant structure of lignocelluloses and increases the availability of the polysaccharides to the enzymes (Alvira *et al.*, 2010). Cellulases are used for enzymatic pre-treatment. The cellulases are divided into three subgroups according to their activity. Endoglucanases randomly cut the inner β-1,4 glycosidic bonds of cellulose, reducing the degree of polymerisation. Exoglucanases bind to the glucan ends, releasing cellobiose units, while

β-glucosidases cleave the cellobiose into two glucose molecules (Olofsson *et al.*, 2008). The pretreatment may also release compounds that are toxic to the fermentation yeasts. Examples of these toxic compounds are furfural, hydroxymethyl furfural (HMF), acetic acid and phenolic compounds. Acetic acid is produced during steam explosion from the acetyl groups on hemicellulose, furfural is formed by the degradation of pentoses, HMF from glucose and phenolic compounds from the degradation of lignin (Alvira *et al.*, 2010; Palmqvist & Hahn-Hägerdal, 2000).

In thermochemical pretreatment sulphuric acid is frequently used. When considering ethanol production as a partial process in a biorefinery approach, the use of sulphate-containing compounds is problematic as sulphate can disturb subsequent biogas processes (Dererie *et al.*, 2011; Pender *et al.*, 2004).

Preatreatment of the lignocellulosic biomass releases not only hexoses but also pentoses, which are not fermentable by the majority of yeast species (Kurtzman *et al.*, 2011). Those pentose sugars create a niche for contaminating bacteria that cause problems for the fermentation process (Schell *et al.*, 2004). Since *D. bruxellensis* tolerates high numbers of LAB (**I**), is robust to environmental changes such as pH and temperature and can ferment cellobiose (**II**), it is an interesting candidate for lignocellulosic fermentation.

5.2 Assimilation/fermentation of sugars other than glucose

Glucose is the preferred carbon source for the majority of yeasts, but they can also use other sugars as carbon source (Kurtzman *et al.*, 2011). Maltose and cellobiose were tested as fermentation substrates for *D. bruxellensis* (**II**). For both sugars, the lag phase was longer than for growth on glucose and the ethanol yields were lower. This is probably due to more steps being required for the yeasts to convert the sugars before they can enter glycolysis.

Cellobiose is a disaccharide that is especially interesting for future lignocellulosic applications, since it may be one of the sugars released from the enzymatic hydrolysis of lignocelluloses. The description of the species *D. bruxellensis* by van der Walt (1964) states that *D. bruxellensis* is not able to ferment or assimilate cellobiose. However, strains isolated later have been shown to be able to ferment this sugar (**II**; Barnett *et al.*, 2000; Spindler *et al.*, 1992).

5.3 Fermentation of aspen sawdust in batch cultures

The ability of *D. bruxellensis* to ferment steam-exploded enzymatically hydrolysed aspen sawdust was investigated and compared with *S. cerevisiae* (**III**). The steam explosion of aspen sawdust was performed in a unit constructed by Cambi A/S, Norway. In small-scale batch fermentation, both yeasts were unable to grow in undiluted or 1:2 diluted hydrolysate, while *S. cerevisiae* grew in 1:5 diluted. *D. bruxellensis* was only able to grow in 1:10 diluted hydrolysate and needed external vitamins (yeast extract or YNB) to grow, while *S. cerevisiae* considerably improved its growth in the presence of an external vitamin source (**III**).

The reason for the lack of growth in undiluted and 1:2 diluted hydrolysate was most probably the low pH of the hydrolysate (3.6), in combination with high amounts of acetic acid (10-11 g/L) and fairly high amounts of HMF and furfural (at least 1.65 g/L and 1.5 g/L, respectively). The severity of the inhibition by acetic acid largely depends on the pH (see above). The pH of the hydrolysate was lower than the pKa of acetic acid and it has been shown that in the presence of 10 g/L acetic acid, the lowest pH at which *S. cerevisiae* can grow is 4.5 (Taherzadeh *et al.*, 1997). The furfural concentration in the hydrolysate was also higher than the concentration reported to be causing problems for an indigenous yeast species (Wikandari *et al.*, 2010). In that study, furfural concentrations above 1 g/L negatively influenced growth and ethanol production.

The level of furfural was high and the level of xylose in the hydrolysate was low (1.8 g/L), which means that most xylose had been degraded to furfural during steam explosion (Horn & Eijsink, 2010; Pienkos & Zhang, 2009).

5.4 Fermentation of aspen sawdust in continuous cultivation with recirculation of yeast cells

In small-scale, high cell density continuous cultivations with cell recirculation and step-wise increasing concentration of hydrolysate, *D. bruxellensis* was able to grow in and ferment 1:5 diluted hydrolysate, unlike the batch cultures described in (**III**). This is an indication that *D. bruxellensis* can adapt to the rough conditions of the hydrolysate. To run the fermentations in a more controlled environment, the continuous experiment was repeated in a bioreactor. Here *D. bruxellensis* was able to grow and ferment 1:2 diluted hydrolysate. However, the pH of the bioreactor experiment was controlled at 5. This was higher than the pKa of acetic acid, so the effect of acetic acid was probably not as high as in the microtitre plates and in the batch fermentations, where the pH was around 3.6-3.7. However, the effect of other inhibitors in the

hydrolysate should not be influenced by the pH and thus this result indicates that *D. bruxellensis* can adapt to the inhibitors present in the hydrolysate. Furthermore, *S. cerevisiae* also adapted to the hydrolysate, since in the bioreactor experiments it was able to grow and ferment 1:2 diluted hydrolysate, while in batch it could not.

Comparing the two yeasts in the small-scale microtitre plates and in the bioreactor, *S. cerevisiae* had in most cases a slightly higher ethanol yield (**III**). However, the ethanol yields of *D. bruxellensis* were comparable and the ability for adaptation and the competitiveness with regard to contaminating bacteria (**I**) make it an interesting candidate for lignocellulose fermentation.

6 Physiological requirements of *Dekkera bruxellensis* in anaerobic environments

There are only a small number of yeasts that can grow under strict anaerobic conditions (Visser *et al.*, 1990), among them *S. cerevisiae* and its close relatives. In order to grow without molecular oxygen the yeasts need sterol and unsaturated fatty acids (Andreasen & Stier, 1954; Andreasen & Stier, 1953).

The growth of *D. bruxellensis* under anaerobic conditions has only been investigated in a limited number of studies. Rozpędowska *et al.* (2011) compared the ethanol, biomass, acetate and glycerol production of *D. bruxellensis* in aerobic and anaerobic batch cultures. Uscanga *et al*. (2003) and Ciani and Ferraro (1997) both investigated the role of oxygen on acetic acid production, which was not produced during anaerobic condition. However, in Uscanga *et al.* (2003) the cultivation was not performed under strict anaerobic conditions, since no sparging of nitrogen was done before or during the cultivation. Even if the oxygen present at the beginning of the cultivation was consumed rapidly by the yeast, the culture should still be regarded as a oxygen-limited culture, not an anaerobic culture. In the experiments reported in (**II**), (**III**) and parts of (**V**), the cultivations were performed without N_2 sparging, thus in oxygen-limited fermentations. However, the growth rates obtained in (**II**) and (**V**) during oxygen limitation (0.05-0.07 h^{-1}) were very similar to those under anaerobic conditions (0.07-0.075 h^{-1}) (Rozpędowska *et al.*, 2011). This suggests that the conditions in (**II**) were strongly oxygen-limited. Alternatively, strain variations may exist and the isolates in (**II**) and (**V**) had slower growth compared with the strains in Rozpędowska *et al.* (2011).

6.1 Behaviour of *D.* bruxellensis during oxygen limitation

Growing *D. bruxellensis* under oxygen limitation caused unexpected problems during the continuous cultivations. The feed pumps were started when *D. bruxellensis* had consumed all glucose in the batch phase, but before the glucose was depleted the growth slowed down and continued at a much slower growth rate (**V**). This bi-phasic growth behaviour can be seen in earlier published growth curves, although it was not discussed by the authors (**II**; Galafassi *et al.*, 2011; Rozpędowska *et al.*, 2011). This change in growth rate was not observed for *S. cerevisiae* (**II**). The decreased growth rate during the second growth phase could explain why it was not possible to run the *D. bruxellensis* culture at a dilution rate of 0.03, which is much lower than the maximum growth rate at the exponential phase.

The aim was to run the continuous cultivation under glucose limitation. However, during the continuous cultivation, the glucose concentration increased almost immediately and never returned to zero, indicating another type of limitation than for glucose. Sparging with a mixture of air and nitrogen (approximately 5% O_2) stimulated growth of *D. bruxellensis* and the remaining glucose was then consumed within 24 hours and the culture remained glucose-limited during the experiment (**V**). This strongly indicates that the culture was oxygen-limited. Comparing the product formation during oxygen limitation with that of glucose limitation during sparging, the ethanol yield was slightly lower in the latter, glycerol yield approximately the same and acetate and biomass yields were higher (**V**). This agrees with the results from other studies run under oxygen limitation/anaerobic environment (Galafassi *et al.*, 2011; Rozpędowska *et al.*, 2011).

6.2 Custer's effect and glycerol production

Dekkera/*Brettanomyces* yeasts are best known for Custer's effect, meaning that the fermentation is inhibited in the absence of oxygen. However, the effect is transient and fermentation re-starts after a certain lag phase (Wijsman *et al.*, 1984). Custer's effect was initially called the 'negative Pasteur effect', since *Dekkera*/*Brettanomyces* yeast ferments glucose at a faster rate in aerobic conditions than in anaerobic conditions. In addition, large amounts of acetic acid are produced under aerobic conditions (Barnett & Entian, 2005). The acetic acid production leads to the reduction of NAD^+ to NADH. The NADH generated from the production of acetic acid, from the assimilation of sugars into biomass and in the amino acid synthesis is re-oxidised by the production of glycerol (Van Dijken & Scheffers, 1986). In theory, the production of 1 mol glycerol reoxidises 1 mol of NADH (Nielsen *et al.*, 2000). On the other hand,

glycerol production is also a loss of carbon and energy but its production is essential under anaerobic conditions since no other way exists to re-oxidise NADH. However, *D. bruxellensis* only produces glycerol in small amounts, which can result in a redox imbalance. In theory, for each mole of acetic acid produced, two moles of glycerol must be produced in order to keep the redox balance (Van Dijken & Scheffers, 1986). Some studies claim that *D. bruxellensis* is unable to produce glycerol (Geros *et al.*, 2000; Wijsman *et al.*, 1984) and that this is the reason for Custer's effect. However, other studies, including (**II**), (**IV**) and (**V**), have shown that *D. bruxellensis* produces glycerol, though not in large amounts (Rozpędowska *et al.*, 2011; Liberal *et al.*, 2007; Uscanga *et al.*, 2003). The amount of glycerol produced is obviously not enough to restore the redox imbalance during anaerobic conditions. Galafassi *et al*. (2011) measured the enzyme activities of *D. bruxellensis* during growth and found that the activity of glycerol 3-phosphate dehydrogenase was only detected in oxygen-limited conditions.

6.3 The role of amino acids in anaerobic growth of *D. bruxellensis*

In preliminary experiments *D. bruxellensis* CBS 11270 was grown under strict anaerobic conditions with sparging of 0.2 L/min of nitrogen in a mineral medium without amino acids (Verduyn *et al.*, 1992). However, *D. bruxellensis* did not grow. When the sparging was stopped, *D. bruxellensis* started to grow after a lag phase (Figure 5), although slowly. There are indications that yeasts need some CO_2 to start growing (Garcia Sanchez, 2010). To test this hypothesis, we sparged the medium before inoculation with nitrogen but not during the cultivation, to avoid removal of the CO_2 formed by the fermentation. Under these conditions *D. bruxellensis* grew, but too slowly to initiate any continuous culture (**V**).

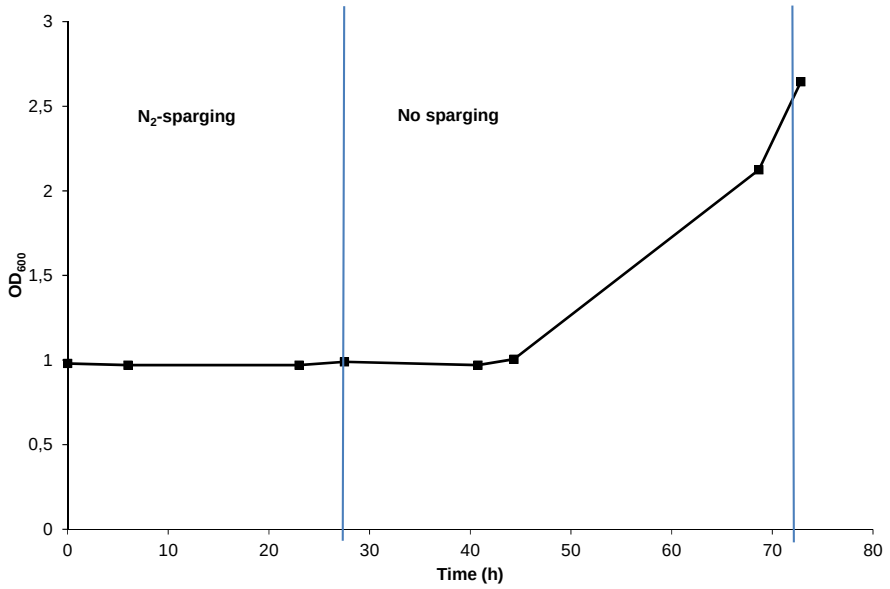

Figure 5. D. bruxellensis grown in anaerobic batch culture at 36°C and in mineral medium without amino acids. The culture was first sparged with 0.2 L/min of ultra-pure nitrogen and no growth was seen. When the sparging was turned off, *D. bruxellensis* started growing, but only slowly and after a lag phase.

Due to the unexpectedly low ability of *D. bruxellensis* to grow under anaerobic conditions, anaerobic growth tests were performed in serum flasks using different nitrogen sources (**V**). *D. bruxellensis* grew only in media containing yeast extract or glutamic acid as nitrogen source, although with retarded growth in the latter. This leads to the hypothesis that *D. bruxellensis* needs amino acids to grow under strict anaerobic conditions.

The common denominator for previous studies on anaerobic growth of *D. bruxellensis* is that the medium used either contained yeast extract or casamino acids (Rozpędowska *et al.*, 2011; Uscanga *et al.*, 2003; Ciani & Ferraro, 1997). In mineral medium, Visser *et al.* (1990) were unable to grow *D. bruxellensis* anaerobically, but even in medium containing yeast extract that particular strain of *D. bruxellensis* was not able to grow under anaerobic conditions.

An amino acid test for anaerobic growth was performed with almost all proteinogenic amino acids (**V**). Lysine, histidine, arginine, asparagine, aspartic acid, glutamic acid and alanine all promoted growth, while cysteine, tryptophan and proline did not stimulate growth under anaerobic conditions (Table 3). During the synthesis of amino acids NADH is released (Albers *et*

al., 1996). The synthesis of certain amino acid releases more NADH than that of others. The NADH that is produced from the synthesis of amino acids is reoxidised in *S. cerevisiae* by the production of glycerol (Verduyn *et al.*, 1990). However, since *D. bruxellensis* has limited glycerol production and thereby a low ability to correct the imbalance, the hypothesis was that adding amino acids to the medium should stimulate anaerobic growth of *D. bruxellensis*. Indeed, arginine, lysine and glutamic acid can generate high amounts of NADH during their synthesis (Albers *et al.*, 1996). However, histidine, asparagine and alanine also stimulated growth of *D. bruxellensis*, although during their synthesis only 2, 1 and 1 mol NADH/mol amino acid, respectively, are released.

Factors other than NADH release during synthesis may be involved in the growth promotion of amino acids in anaerobic conditions. For example, amino acids can have different uptake rates; for *S. cerevisiae* the preferred amino acids that also stimulated the growth best for *D. bruxellensis* were: aspartic acid, glutamic acid, threonine and lysine. Proline was only slightly consumed in the anaerobic growth of *S. cerevisiae* (Perpete *et al.*, 2005) and did not stimulate growth under anaerobic conditions for *D. bruxellensis* (Table 3). Many of the amino acids listed at the bottom of Table 3, *i.e.* those that do not stimulate anaerobic growth of *D. bruxellensis* to any great extent, are aromatic and/or hydrophobic chain amino acids, for example tryptophan, proline, phenylalanine and isoleucine. This may indicate that the uptake system for those amino acids is insufficient during anaerobic conditions.

It has also been shown that industrial strains of *S. cerevisiae* cannot grow in mineral media without amino acids. Thomas *et al.* (1998) tested 10 industrial strains of *S. cerevisiae* and found that nine of these were not able to grow in anaerobic conditions without supplementation of amino acids. They postulated that the amino acids were involved in some unknown reactions that formed sufficient traces of molecular oxygen to promote the growth of *S. cerevisiae*. However, in the case of *D. bruxellensis*, it seems more likely that the amino acids helped restore the imbalance in redox potential under anaerobic conditions.

A conclusion is that *D. bruxellensis* is a facultative anaerobic yeast, but its nutritional requirements under anaerobic conditions are higher than those of the majority of *S. cerevisiae* strains.

Table 3. Number of duplications of *D. bruxellensis* grown in anaerobic conditions in serum flasks containing mineral medium and one of the following amino acids (modified from Table 2 in **V**)

Amino acid	**Duplications**	**NADH released**[†]
Arginine	3.7	4-5
Lysine	3.5	6-7
Asparagine	3.3	1
Histidine	3.3	2
Alanine	3.1	1
Glutamic acid	3	3-4
Serine	2.7	2
Aspartic acid	2.6	1
Methionine	2.5	1-4
Threonine	2.5	0-1
Glycine	2.4	2
Leucine	2.4	5
Glutamine	2.3	3-4
Phenylalanine	2.3	2
Valine	2.1	2
Isoleucine	2.0	1-2
Tryptophan	1.9	3
Proline	1.9	1-4
Cysteine	1.2	2-5
Yeast extract[‡]	4.2	-
w/o amino acid	1.4	-

[†]Amount of NADH released from the synthesis of that amino acid in *S. cerevisiae* (mol NADH/mol amino acid) (Albers *et al.*, 1996).
[‡]*D. bruxellensis* finished growing within 3 days in yeast extract.

7 Conclusions

The results presented in this thesis show that:

- *Dekkera bruxellensis* outcompetes *Saccharomyces cerevisiae* in certain industrial fermentations.
- In batch fermentation, *D. bruxellensis* has higher biomass and lower glycerol yields, indicating a more energy-efficient metabolism, which is probably the reason for its competitiveness in sugar-limited fermentations.
- Ethanol yields in *D. bruxellensis* were almost constant over a broad range of pH and temperature values, and were similar to or higher than those of *S. cerevisiae* strains. The *D. bruxellensis* yeast could also cope with a high number of lactic acid bacteria in the fermentation. Therefore *D. bruxellensis* can have great potential as an ethanol production organism. However, this is restricted to continuous systems with cell recirculation, as the specific growth and ethanol production rates of *D. bruxellensis* are relatively low.
- Dominance of *D. bruxellensis* in industrial fermentations seems to be connected with the presence of *Lactobacillus vini*.
- Due to its ability to adapt to inhibitors and to ferment cellobiose, *D. bruxellensis* also has potential for use in the fermentation of lignocellulosic biomass to ethanol. Its ability to cope with high numbers of acid-forming bacteria may also be an advantage, because lignocellulose fermentations may be more prone to contamination due to the presence of non-utilised sugars *e.g.* pentoses.
- When growing anaerobically, *D. bruxellensis*, in contrast to *S. cerevisiae*, has a demand for complex organic compounds, especially amino acids. Based on genomic analyses, it has been concluded that the abilities to grow anaerobically / perform aerobic fermentation evolved independently in the *Saccharomyces* and *Dekkera/ Brettanomyces* clades (Rozpędowska *et al.*, 2011). The

nutrient demands identified in *D. bruxellensis* indicate that the regulation and capacity of certain metabolic pathways differ considerably between the yeasts, thus confirming independent evolution.

- The high nutrient demand for *D. bruxellensis* during anaerobic growth and its more energy-efficient metabolism have one common denominator: the low glycerol production by *D. bruxellensis*. Low glycerol production is a problem on one hand, as during anaerobic growth the NADH produced from, say, amino acid synthesis cannot be reoxidised, which causes redox imbalance. Thus, additional nutrients, *e.g.* amino acids, are required in these conditions. On the other hand, low glycerol production also means that more carbon and energy can be directed into ethanol and biomass formation, leading to a more energy-efficient metabolism.

8 Future perspectives

There is a growing interest in research on the yeast *D. bruxellensis*. This interest is driven by several factors. The main factor is still prevention of its growth, since this yeast is a major contaminant in wine and distillery ethanol production. The results of the present study suggest that *D. bruxellensis* can be established as a production organism in continuous ethanol processes. In general, it may also be regarded as a model for a highly competitive yeast, which can help in understanding microbial interactions in industrial ethanol processes. The yeast is also interesting for fundamental research, due to its unique evolutionary position as a non-*Saccharomyces* fermentative yeast. All the above-mentioned issues can be approached using global metabolic analyses, mainly metabolic flux and transcriptome analyses. These analyses will reveal how the substrate is distributed among the different cellular processes. For example, during the course of this project, it emerged that *D. bruxellensis* is very competitive in continuous culture, but has a high demand for organic nutrients during anaerobic and severe oxygen-limited conditions. Identifying the factors involved in both competitiveness and the fermentation metabolism would provide opportunities to improve its performance in ethanol production, but possibly also to prevent contamination by this yeast. One approach to identify those factors could be to analyse the metabolic fluxes and global gene transcription during anaerobic growth, in order to identify the genes and pathways involved in these conditions. Arising from these analyses, targets for metabolic engineering of certain key metabolic steps might be identified. Recently, a transformation system for *D. bruxellensis* has been developed (J. Piskur, personal communication), so at least gene overexpression is possible in *D. bruxellensis*. For instance, overexpression of the genes involved in glycerol production might improve the anaerobic growth of *D. bruxellensis*. Fine-tuning of this pathway may provide both anaerobic growth and energy-efficient metabolism.

Knowledge about microbial ecology in industrial ethanol production is still limited. Strains dominating a fermentation are often different from those that were initially inoculated. Even the well-established process of ethanol production could probably be greatly improved by running it with appropriate

strains from the start and regulating cultivation conditions according to the demands of the production strain. Identifying factors of competitiveness and production efficiency in a non-*Saccharomyces* species will greatly extend the perspective on industrial fermentation ecology and enable a theory-based selection of production strains and conditions

"*Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning.*"

Albert Einstein

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