

# *Dekkera bruxellensis*, a Non-conventional Ethanol Production Yeast

Studies on Physiology, Transcriptomics and Interactions  
with Industrial Microbial Isolates

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Doctoral Thesis  
Swedish University of Agricultural Sciences  
Uppsala 2014

Acta Universitatis agriculturae Sueciae

2014:40

ISSN 1652-6880

ISBN (print version) 978-91-576-8028-0

ISBN (electronic version) 978-91-576-8029-7

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Print: SLU Service/Repro, Uppsala 2014

# *Dekkera bruxellensis*, a Non-conventional Ethanol Production Yeast. Studies on Physiology, Transcriptomics and Interactions with Industrial Microbial Isolates

## Abstract

*Dekkera bruxellensis* has been shown to outcompete an initial inoculum of *Saccharomyces cerevisiae* in several ethanol production plants, which nevertheless had a high efficiency in one of the monitored processes. Co-occurrence of *D. bruxellensis* with lactic acid bacteria (LAB) *Lactobacillus vini* has been observed. The aim of this thesis was to broaden the knowledge on *D. bruxellensis* physiology in respect to its high competitiveness.

Global gene expression analysis of *D. bruxellensis* under conditions similar to those in which it outcompeted *S. cerevisiae* was performed by whole transcriptome sequencing. Low expression of genes involved in glycerol biosynthesis, and expression of NADH-ubiquinone reductase (complex I) are probably the basis for an efficient energy metabolism. Genes of putative high affinity glucose transporters might be involved in the efficient glucose transport of *D. bruxellensis*.

*D. bruxellensis* also has a good potential to ferment lignocellulose hydrolysate to ethanol. Adaptation to lignocellulose hydrolysate inhibitors by pre-cultivation was demonstrated. Adapted cells had a shorter lag phase and produced higher amounts of ethanol compared to non-adapted cells.

The role of *L. vini* during co-cultivation with *D. bruxellensis* or *S. cerevisiae* was also investigated. Formation of LAB–yeast cell aggregates consisting of a bacterial core with an outer layer of yeast cells was identified. It was noted that addition of mannose to the aggregates dissolved them, but higher mannose amounts were required to inhibit co-flocculation between *L. vini* and *S. cerevisiae* compared to *L. vini* and *D. bruxellensis*.

Growth and metabolite profiles of *D. bruxellensis* during cultivation on different combinations of carbon and nitrogen sources were studied. Repression of genes involved in nitrate assimilation in *D. bruxellensis* under oxygen-limited conditions in presence of ammonium was shown.

In conclusion, *D. bruxellensis* has a great potential for industrial ethanol production due to a highly efficient energy metabolism, adaptability to lignocellulose hydrolysate, utilisation of an alternative nitrogen source and robustness against bacterial contaminants.

**Keywords:** *Dekkera bruxellensis*, *Saccharomyces cerevisiae*, *Lactobacillus vini*, bioenergy, ethanol production, microbial interaction, transcriptome, gene expression

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## List of Publications

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

- I Tiukova IA, Petterson ME, Tellgren-Roth C, Bunikis I, Eberhard T, Pettersson OV, Passoth V (2013). Transcriptome of the alternative ethanol production strain *Dekkera bruxellensis* CBS 11270 in sugar limited, low oxygen cultivation. *PLoS One* 8: e58455.
- II de Barros Pita W, Tiukova I, Leite FC, Passoth V, Simões DA, de Morais MA Jr (2013). The influence of nitrate on the physiology of the yeast *Dekkera bruxellensis* grown under oxygen limitation. *Yeast* 30: 111-117.
- III Tiukova IA, de Barros Pita W, Sundell D, Haddad Momeni M, Horn SJ, Ståhlberg J, de Morais MA Jr, Passoth V (2014). Adaptation of *Dekkera bruxellensis* to lignocellulose-based substrate. *Biotechnology and Applied Biochemistry* 61: 51-57.
- IV Tiukova I, Eberhard T, Passoth V (2014). Interaction of *Lactobacillus vini* with the ethanol-producing yeasts *Dekkera bruxellensis* and *Saccharomyces cerevisiae*. *Biotechnology and Applied Biochemistry* 61: 40-44.

Papers I-IV are reproduced with the permission of the publishers.

The contribution of Ievgeniia A. Tiukova to the papers included in this thesis was as follows:

- I took an important part in planning the project, performed almost all (wet) laboratory work, took an important part in the bioinformatics work and a major part in writing the manuscript.
- II took part in planning the project, was running parts of the experimental work and participated in evaluation of results and writing the manuscript.
- III took a major part in planning the project, performed most of the laboratory work and writing the manuscript.
- IV took a major part in planning the project, performed almost all the laboratory work and took a major part in writing the manuscript.

In addition to the papers I-IV, the author contributed to the following papers within the timeframe of the project:

Blomqvist J, South E, Tiukova I, Momeni MH, Hansson H, Ståhlberg J, Horn SJ, Schnürer J, Passoth V (2011) Fermentation of lignocellulosic hydrolysate by the alternative industrial ethanol yeast *Dekkera bruxellensis*. *Letters in Applied Microbiology* 53:73-78.

Passoth V, Tabassum MR, Nair HA, Olstorpe M, Tiukova I, Ståhlberg J (2013) Enhanced ethanol production from wheat straw by integrated storage and pre-treatment (ISP). *Enzyme and Microbial Technology* 52:105-110.

## Abbreviations

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
ADH	Alcohol dehydrogenase
CoA	Coenzyme A
CRP	Cytoplasmic ribosomal proteins
DHOD	Dehydroorotate dehydrogenase
DNA	Deoxyribonucleic acid
FAME	Fatty acid methyl esters
HMF	Hydroxymethyl furfural
LM	Lignocellulosic material
MRP	Mitochondrial ribosomal proteins
mRNA	Messenger RNA
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
SHAM	Salicyl hydroxamic acid
TCA	Tricarboxylic acid
tRNA	Transfer ribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
ROS	Reactive oxygen species



# 1 Introduction

Fermentation of sugar into ethanol is one of the oldest microorganism-mediated reactions mankind has mastered (Barnett, 1998). Archeological findings have demonstrated that Neolithic people used fermentation techniques to make alcoholic beverages 9000 years ago (McGovern et al., 2004). Historical evidence of ancient brewing has also been found in Iran and Egypt (Kurtzman et al., 2011). Brewing traditions in Europe were spread by Germanic and Celtic tribes 3000 BC (Barnett, 1998). Alcoholic fermentation was performed on domestic scale until the industrial revolution in the 19<sup>th</sup> century, when it was transformed into an industrial manufacturing process. The conversion of glucose into ethanol was for a long time believed to be a chemical reaction, until the work of Cagniar-Latour, Kützing, Schwann and Pasteur from the 30<sup>th</sup> to the 50<sup>th</sup> of the 19<sup>th</sup> century demonstrated that alcoholic fermentation is performed by microorganisms (Pasteur, 1857, Barnett, 2000). In 1897, Buchner showed that an extract of ground yeast cells catalysed alcoholic fermentation (Buchner, 1897). This was one of the landmark experiments of enzymology (Lagerkvist, 2005).

The development of industrial ethanol production was associated with an expanded range of ethanol utilisation, extending from food ingredient, drug, and disinfectant in medicine to a transportation fuel (Vallee, 1998). Intensive development of land transport in the 19<sup>th</sup> century initiated the gradual shift from animal-powered to motorised private vehicles. The invention of the internal combustion engine subsequently led to the development of the ethanol-powered engine. However, the invention of kerosene distillation from petroleum (1847) enabled the use of gasoline as a major motor fuel, and slowed down the development of alternative approaches. In the first half of 20<sup>th</sup> century, gasoline became the most popular fuel in many parts of the world. Contrary to this, in Brazil, a strategy for the development of ethanol production from sugarcane for motor fuels was implemented (Carlos Basso et al., 2011). The world energy crisis in 1970 highlighted the dangers of dependency on

fossil fuel. The search for alternative energy sources has awoken interest in ethanol as a renewable biofuel (Cheng and Timilsina, 2011, Tilman et al., 2009). Today, ethanol remains one of the major biotechnological products on the global market. 68% of produced ethanol is used as fuel, which represents 2% of the current global transport fuel consumption (Berg, 2004, Caspeta et al., 2013). In the frame of alternative energy research, investigations on the optimisation of ethanol production have intensified (Gnansounou, 2010, Solomon et al., 2007).

### 1.1 Role of *Dekkera bruxellensis* in various ethanol production systems

Although fermentation has been widely exploited by humanity for thousands of years, the microbial population in ethanol production-related habitats remains poorly investigated (Beckner et al., 2011). The yeast *Saccharomyces cerevisiae* was believed to be the major industrial ethanol production organism until researchers in both Sweden and Brazil independently discovered the yeast *Dekkera bruxellensis* as dominant ethanol-producing microbe in industrial ethanol plants, where it had sometimes completely replaced the initial inoculum of *S. cerevisiae* (Liberal et al., 2007, Passoth et al., 2007). In the majority of documented cases, *D. bruxellensis* also co-occurred with the lactic acid bacterium (LAB) *Lactobacillus vini* (Passoth et al., 2007, Lucena et al., 2010).

It has been estimated that *S. cerevisiae* and *D. bruxellensis* diverged from a common ancestor some 200 million years ago (Rozpedowska et al., 2011). Yet these two phylogenetically distant yeasts have evolved features beneficial for ethanol production organism independently of each other. Both yeasts display high ethanol tolerance, the capacity to produce ethanol under aerobic conditions, the ability to grow under oxygen-limited conditions and to survive without mitochondria. Parallel evolution of both yeasts resulted in their high fitness in niches with glucose access. The fermentative lifestyle obviously conferred efficient adaptation to the environment, allowing more competitive substrate utilisation with production of ethanol that inhibits growth of other microbes (Piskur et al., 2006, Rozpedowska et al., 2011)

*D. bruxellensis* is often isolated from various fermented beverage production systems. In these habitats, *D. bruxellensis* was classified as a spoilage organism, due to the production of volatile by-products (Silva et al., 2004). Isolation of *D. bruxellensis* from distilled alcohol production systems, where traces of volatile metabolites are not essential for final product quality,

triggered reconsideration of its role, from spoilage organism to production yeast (Blomqvist et al., 2010).

*D. bruxellensis*' domination has been reported exclusively during secondary fermentation of alcoholic beverages, when only minute amounts of sugar are available (Uden, 1967). This is reflected by the designated status of *D. bruxellensis* as a "spoilage yeast of second fermentation" (Silva et al., 2004). The slowly growing *D. bruxellensis* does not dominate in batch systems with glucose excess when co-cultivated with *S. cerevisiae* (Abbott et al., 2005).

In contrast, continuous cultivation with cell recirculation favoured domination of *D. bruxellensis* over *S. cerevisiae*, because the maximum specific growth rates of both yeasts were above the dilution rate and, thus, not a crucial competition factor. Glucose concentration has been shown to play an essential role in the outcome of *D. bruxellensis*/*S. cerevisiae* competition in continuous cultivation, as *S. cerevisiae* dominated over *D. bruxellensis* under glucose excess, but *D. bruxellensis* outcompeted *S. cerevisiae* under glucose limitation. This indicates that more efficient substrate utilisation by *D. bruxellensis* is involved in its high competitiveness in glucose-limited continuous systems (Blomqvist et al., 2012). The molecular basis of this feature in particular, and physiology of *D. bruxellensis* in general, remain poorly investigated.

## 1.2 Aim

Although spoilage capacity is the major subject of investigations on *D. bruxellensis*, this thesis project aimed to address *D. bruxellensis* physiology from the perspective of its ethanol production properties. The specific objective was to characterise different aspects of *D. bruxellensis* competitiveness, recently reconsidered as essential trait for a production strain (I). Global transcriptome analysis aimed to identify genes involved in efficient glucose uptake and metabolism, the presumable basis of *D. bruxellensis* competitiveness.

Nitrate has been shown to be an important component of fermentation broth in Brazilian ethanol production plants. The ability to assimilate nitrate as a source of nitrogen can confer a competitive advantage of *D. bruxellensis* when other preferred nitrogen sources are depleted. Investigating the effect of nitrate on the physiology of *D. bruxellensis* can therefore provide essential information on the relationship between nitrogen source and ethanol production (II).

The use of lignocellulose-based substrates for ethanol production is currently under intensive study. It was therefore highly relevant to investigate

adaptation of the non-conventional yeast *D. bruxellensis* during fermentation of lignocellulose hydrolysate (III).

An ethanol production consortium composed of *D. bruxellensis* and *L. vini* had first been discovered in a Swedish ethanol production plant. The co-occurrence of *D. bruxellensis* together with *L. vini* has since then also been reported in other distilleries. However, the nature of the interaction between *D. bruxellensis* and *L. vini* as well as the role of this bacterium in the fermentation process is still poorly understood (IV).

The thesis work aimed to gain insight into the physiology of *D. bruxellensis* under different conditions that are relevant for industrial ethanol production: i) simulated conditions of industrial fermentation in which *D. bruxellensis* outcompeted *S. cerevisiae*; ii) combination of different carbon (glucose, fructose, sucrose, maltose) and nitrogen (ammonium, nitrate, mixed) sources iii) fermentation of lignocelluloses-based medium; iv) co-cultivation with the LAB *L. vini*.

The investigation of *D. bruxellensis* physiology under the conditions mentioned above was expected to broaden our understanding of: i) transcriptional activity in yeast cells in general and global gene expression patterns of *D. bruxellensis* in particular; the molecular physiology of *D. bruxellensis* competitiveness; ii) how yeast carbon metabolism is regulated by nitrogen source; iii) adaptation of the yeast to lignocellulose hydrolysate inhibitors; iv) ecology and microbial interactions in industrial ethanol production habitats.

In order to achieve these research aims a combination of established and novel methods were used. For instance, the next generation sequencing technique SOLiD allowed the analysis of genome-wide expression in the non-conventional yeast *D. bruxellensis*.

In summary the specific aims of this thesis were:

- to determine the global gene expression profile of *D. bruxellensis* when cultivated under glucose- and oxygen-limited conditions (I)
- to study *D. bruxellensis* physiology during the growth in different combinations of carbon and nitrogen sources under nitrogen limitation (II)
- to investigate adaptation of *D. bruxellensis* to lignocellulosic substrate and to verify the stability of the phenotype of lignocellulose hydrolysate- adapted cells (III)
- to characterise the interaction of the yeasts *D. bruxellensis* and *S. cerevisiae* with the LAB *L. vini* (IV)

## 2 Global development of biobased technology

### 2.1 Renewable energy

Global energy demand is currently increasing rapidly as a direct result of a growing world population in combination with an increase in living standards. The eventual depletion of fossil fuel reserves and the threat of climate change, and the question of how to supply the future global population with energy in an environmentally sustainable way presents a great political, economical and technological challenge. The rise of industrial production in Asia is one the main factors in the increase of global energy consumption (Peters et al., 2007). Contrary to this trend, Sweden experienced a decline in total energy use from 1970 to 2009, mainly due to increased energy efficiency within the residential sector. However, within the transport sector, energy consumption has increased by 70% since 1970 (Swedish energy agency, 2010).

The current interest in alternative energy sources is motivated by issues of energy security (in terms of supply and price stability) and environmental impact (Cheng and Timilsina, 2011). A possible solution can be found in the development of renewable technologies for fuel and goods production (Faaij, 2007). There are various alternative energy forms available to replace fossil fuel: hydro and wind power, bio, solar and geothermal energy. These forms of energy have different properties and, correspondingly, are appropriate for specific applications. Hydro and wind power can be used for electricity generation. Biomass, solar and geothermal energy are used for heat production. Biofuels can replace transportation fossil fuels.

Numerous efforts have been undertaken by developed countries to start reorganising the energy system towards minimising fossil fuel use and implementing alternative sources (Vanholme et al., 2013). Renewable energy now supplies 16% of the world's total energy demands. Low-grade processed

biomass currently leads the renewable energy applications, meeting 10% of the world's energy use. Hydropower supplies 3.4% of the world's energy. Wind power, solar and geothermal energy and motor biofuels currently represent only 2.8% of global energy use, although there is a strong trend for further development (REN21, 2011).

In Sweden, renewable energy comprised 34% of total energy consumption in 2009. Most of this was used for industrial electricity production (from biomass, hydro and wind power), followed by residential heating. The transport sector consumes only a very small part of renewable energy in the form of biogas, ethanol and fatty acid methyl esters (FAME). The renewable energy applied in Sweden is derived from: black liquors, hydro power, heat absorbed by heat pumps, organic waste, biobased motor fuels and wind power (Swedish energy agency, 2010).

There is political support for the development of an environmentally sustainable economy (Gnansounou, 2010). For example, the USA Department of Energy has set goals to replace 30% of fossil fuel and 25% of industrial organic chemicals with biomass-derived products by 2025 (Ragauskas et al., 2006). In Sweden, the renewable energy proportion is planned to reach 50%, with 10% in the transport sector, by 2020. The long-term aim is to liberate the transport sector from fossil fuel dependency by 2030 (Swedish energy agency, 2010). The main challenge in renewable energy implementation is the need for global investments for reorganisation of existing infrastructure (Vanholme et al., 2013).

## 2.2 Biofuels: risks and benefits

Biomass can be considered a cheap, renewable and widely accessible source of alternative energy (Perlack et al., 2005). There is a great variety of biomass-derived products that can potentially be used to replace fossil fuels: wood fuels, black liquors, energy grass and straw, peat and combustible waste (Swedish energy agency, 2010).

Technologies for biomass conversion into highly refined biofuels have been developed, resulting in FAME, biogas, bioethanol and biodiesel production (Wu et al., 2010). The diversity of biofuels implies that the multidimensional problem of energy generation cannot be solved with the help of only one universal technology.

Biofuel production faces two major difficulties: a limited supply of arable land and environmental aspects (Goldemberg et al., 2008, Tilman et al., 2009). The limited area of arable land may cause competition between biofuels and food production (Dale et al., 2011). The so called "first generation liquid

biofuels”, such as ethanol is produced from plants which alternatively could be used as food. Implementation of “second generation liquid biofuels” that are produced from inedible lignocellulosic substrates can partly ease the tension with food security (Caspeta et al., 2013, III). However, this technology is still under development. There are ongoing active debates concerning the choice of sources for lignocellulose production, between forestry (Jonsell, 2007, Skogforsk, 2011) and agriculture (Wright, 2006, Weih et al., 2008).

Negative environmental impacts of biofuel production in terms of declines in biodiversity, a reduction in soil fertility, and even an increase in greenhouse gas (GHG) emissions from conversion of forests to crop lands have been reported in ecological studies (Fargione et al., 2009). On the other hand, efficient land management such as use of abandoned agricultural land (Campbell et al., 2008), application of fast-growing plants, agroforestry, crop rotation and gentle harvesting techniques may ameliorate the negative effect of biofuel production (Mirck et al., 2005).

It has been estimated that biofuels can meet approximately 25% of the world’s total fuel demands, contributing to food security and environmental protection (Koonin, 2006, International Energy Agency, 2010). Implementation of biofuels can contribute to security of energy supply (Borras Jr. et al., 2010), infrastructure development, creation of new job opportunities (Sills, 2009), and potentially decreased GHG emissions (Searchinger et al., 2009).

## 2.3 Bioethanol industry

Bioethanol is considered to be a promising alternative transportation fuel (Farrell, 2006). The antiseptic, alimentary and energetic properties of ethanol determine its widespread occurrence in different spheres of human life (Vallee, 1998, McGovern et al., 2004). Around 12% of the ethanol produced is used for beverages, 20% in the chemical industry and 68% for fuel (Berg, 2004).

Nowadays, the increased demand for energy in transport is partly supplied by development of renewable motor fuels, among which ethanol is one of the major biofuels. Ethanol represents nearly 2% of current world transport fuel, with total world production of 84.6 billion litres in 2011 (Caspeta et al., 2013). Globally, ethanol is produced by USA (50.4%), Brazil (39.2%), European countries of Organisation for Economic Co-operation and Development (5%), and other countries (5.4%) (Gnansounou, 2010). 52.7 and 21.1 billion litres of ethanol are produced per year in the USA and Brazil, respectively (Caspeta et al., 2013).

In Sweden, ethanol consumption as transportation fuel was 420 million liters in 2011. Ethanol and raw materials for its production are partly imported from different countries. Today, ethanol comprises less than 5% of energy used for transport in Sweden (Swedish energy agency, 2012).

Although ethanol has a lower energetic content compared with other longer chain alcohols, the long-term experience of ethanol production has facilitated its extended application from a food additive to a transport fuel (Lynd, 1996). Currently, the feedstock for ethanol production consists of various crops with a high starch content, e.g. maize and wheat, as well as sucrose-based sugarcane and sugar beets (Berg, 2004, **II**).

Research is also underway to introduce new lignocellulose-based feedstock, e.g. waste straw and sawdust, coppiced willow and poplar, reed canary grass, cord grass, miscanthus and sorghum (Hess et al., 2007, Passoth et al., 2013, **III**). Apart from a number of pilot and demonstration lignocellulose-derived ethanol production plants, there are examples of commercial platforms in Italy, Norway, Russia, and USA (Passoth, 2014).

An important disadvantage of ethanol is its lower fuel quality characteristics. Ethanol has a lower energy value per litre than conventional petrol (Roayaei and Taheri, 2009). In addition, ethanol has corrosive and hygroscopic properties (Topgul et al., 2006, Turner et al., 2011). However, ethanol as an additive to petrol provides several positive effects, for instance, lowering the freezing temperature of fuel. In addition, the higher octane number of ethanol provides more complete combustion of petrol, and thus a reduction in GHG emissions (Yucesu et al., 2007). Bioethanol non-GHG emissions are equivalent to those of petrol in terms of environmental pollution and harm to human health (Beer et al., 2011, Costa and Sodre, 2010). Formaldehyde, acetaldehyde and aromatic aldehydes are the most abundant carbonyls in bioethanol exhaust fumes (Magnusson et al., 2002).

The main concern regarding the industrial ethanol production is verification of the technology's sustainability. Results of studies on calculation of the input of non-renewable energy (mainly for biomass processing and distillation) in ethanol production considerably vary (Fu et al., 2003, Goldemberg et al., 2008).

The future domination of the fuel market by certain biofuels will be largely steered by present day investment, especially regarding engine specialisation towards particular types of fuels (Dufie, 2006). Availability of different biofuel production infrastructures can contribute to the prevention of market monopolisation and provide opportunities to involve different substrates in the production process, creating integrated wasteless, closed biorefinery systems (Earley and McKeown, 2009).

## 3 Ethanol production overview

### 3.1 Industrial ethanol production

Industrial ethanol production consists of several stages: (1) biomass processing; (2) fermentation; and (3) ethanol concentration. Processing of starch-based biomass includes milling (disintegration to smaller particles) and cooking (heat-catalysed sugar release, material sterilisation) (Kelsall and Lyons, 2003a). Sucrose extraction from sucrose-rich materials occurs in few main stages: biomass milling; chemical treatment; and evaporation (Pennington and Baker, 1990). Ethanol concentration can be achieved by distillation and subsequent dehydration.

Fermentation is the crucial stage in the ethanol production process, determining the level of residual sugars and the final ethanol concentration (Amorim et al., 2011). Fermentation can be performed either in batch or continuous systems. The main characteristic of batch cultivation is that all components of the medium are present from the start of fermentation. Continuous fermentation is performed with constant supply of new nutrients and removal of metabolites. Continuous fermentation can be performed in vessels connected together in a cascade mode. Industrial batch fermentors usually have a volume of 100 to 3,000 m<sup>3</sup>, whereas the fermentors for continuous cultivation can be much smaller (Kelsall and Lyons, 2003b).

Steady state in a continuous fermentation is defined by constant cell biomass, substrate and products concentrations (Postma et al., 1989, **D**). The growth rate in a continuous system is equal to the dilution rate in steady state, as long the dilution rate is lower than the maximum specific growth rate of the cultivated organism.

Batch fermentors are commonly used in beverage production, whereas many bioethanol fuel plants use continuous systems. The advantages of continuous fermentation include: higher productivity per unit time (>2.4 to 3.3-

fold more than corresponding batch fermentation (Kosaric et al., 1987); and reduction of maintenance cost (Cysewski and Wilkie, 1978). One of the disadvantages of batch fermentation is connected with the lag phase of yeast growth that can increase the risk for contamination (Kelsall and Lyons, 2003b).

Cell recirculation in continuous system may allow to increase the proportion of sugar available for ethanol production, due to the reduction of cell biomass increment (Caylak and Sukan, 1998). A disadvantage of these systems is the additional cost for cell separators.

Fermentation under industrial conditions is connected with a variety of stresses for production organism, e.g. high temperature, low pH and competition with other microbes (**I**, **IV**). Glucose catabolism to ethanol is exothermic, and the release of heat can locally change the fermentation temperature, affecting fermentation efficiency (Abdel-Banat et al., 2010). The fermentation product, ethanol, is itself very toxic and therefore represents another limiting factor for the production yield (Hallsworth, 1998). Fungal contamination of grain may also inhibit yeast metabolism. In addition, the grain component phytic acid has a high chelating capacity (ability to bind metals), thus reducing the nutritional properties of the fermenting broth (Kelsall and Lyons, 2003b). Biopreservation of cereal grain by *Wickerhamomyces anomalus* prevents fungal contamination and introduces phytase activity into the system (Passoth et al., 2009, Olstorpe et al., 2009).

## 3.2 Biology of alcoholic fermentation

### 3.2.1 Ethanol production in microorganisms

*S. cerevisiae* is the most studied ethanol producing yeast with a long history of use in industry. There are more than 2000 other described yeast species, which are commonly referred to as “non-conventional yeasts”, since they are less well studied. Examples of non-conventional yeasts capable of ethanol production include *Cyberlindnera jadinii*, *D. bruxellensis*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Ogataea polymorpha*, *Pachysolen tannophilus*, *Scheffersomyces stipitis*, *Scheffersomyces shehatae* and *Scheffersomyces segobiensis* (Spencer et al., 2002).

Non-conventional yeasts possess a range of useful traits for improvement of ethanol production, such as broad substrate range and high tolerance to various stresses in industrial habitats (temperature, lignocellulose inhibitors, osmotic stress). These yeasts are a source of traits for genetic engineering of ethanol production strains to improve production performance. For example, *K. lactis* and *K. marxianus* are known for their ability to produce ethanol from lactose. Xylose-fermenting yeasts, such as *S. stipitis*, *P. tannophilus*, *S. shehatae* and *S.*

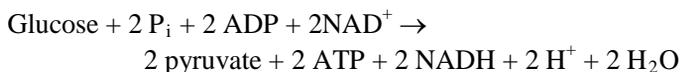
*segobiensis* are of substantial interest for research in biotechnology (Hahn-Hagerdal et al., 2007). Another yeast *O. polymorpha* is of high importance due to its ability to ferment xylose under high temperatures up to 50 °C (Ryabova et al., 2003). *C. jadinii* has been shown to tolerate high concentrations of inhibitors during fermentation of lignocellulose hydrolysate (Villas-Boas et al., 2002). Yeast species isolated as contaminants during industrial ethanol production can be carriers of various stress-resistance phenotypes (osmotolerance, competitiveness to other microbes; see section 7.3).

Some bacteria are also known to be able to ferment glucose to ethanol, and some of these even have industrial applications. For example, the bacterium *Zymomonas mobilis* has been used to produce the traditional Mexican alcoholic beverage *pulque*, which is made from agave juice. Bacteria have lower ethanol tolerance compared with yeasts (Jeffries, 2005). The specific ethanol productivity of *Z. mobilis* is higher than that of yeasts because of the involvement of the Entner-Doudoroff pathway in conversion of glucose into pyruvate, which is less energy efficient than glycolysis with only one molecule of ATP produced per molecule of glucose.

Due to the lower ATP yield, a smaller proportion of sugar is used to produce biomass, which is an attractive property in ethanol production. However, yeasts are the preferred choice in ethanol production due to their resistance to bacteriophage infection, higher tolerance to ethanol and simplicity of separation.

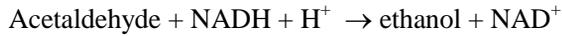
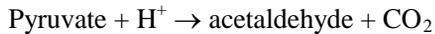
### 3.2.2 Biochemistry of alcoholic fermentation

ATP generation during glycolysis requires the redox carrier nicotinamide adenine dinucleotide in its oxidized form (NAD<sup>+</sup>):



In order to maintain flux through the glycolytic pathway, the intracellular pool of NAD<sup>+</sup> must be replenished through re-oxidation of NADH. If oxygen is available, NADH is re-oxidized through cellular respiration and the end product of glycolysis, pyruvate, is converted into acetyl coenzyme A (CoA), enters the tricarboxylic acid (TCA) cycle, and is oxidised into CO<sub>2</sub> and H<sub>2</sub>O. During oxygen limitation, the respiratory electron transfer chain is no longer functional and NADH is instead re-oxidized through a fermentative pathway. In alcoholic fermentation, the pyruvate is first decarboxylated in a non-oxidative reaction by pyruvate decarboxylase (PDC, 4.1.1.1) to form

acetaldehyde, which is subsequently reduced to ethanol by alcohol dehydrogenase (ADH, 1.1.1.1) (Thomson et al., 2005):



The fermentation pathway generates only 2 molecules of ATP per molecule of glucose compared to respiration, which has been calculated to produce 30 molecules of ATP per molecule of glucose (Berg et al., 2002). Yet some microorganisms ferment sugars into ethanol even when oxygen is available. This is known as the Crabtree effect. Microorganisms that display this behaviour are referred to as Crabtree-positive, whereas the others are termed Crabtree-negative. *S. cerevisiae* is the best known example of a Crabtree-positive yeast.

The seemingly wasteful use of sugar to produce ethanol in Crabtree-positive yeasts may confer certain competitive advantages. Aerobic fermentation by Crabtree-positive yeasts enables them to utilise substrate effectively by first converting glucose to ethanol that is toxic for growth of other microbes, and then, after inactivation of potential competitors, catabolising ethanol to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . This has been called the “make-accumulate-consume” strategy (Piskur et al., 2006).

The ability of *S. cerevisiae* to produce ethanol even under aerobic conditions evolved as the result of a global promoter rewiring event. Unlike Crabtree-negative yeasts, *S. cerevisiae* has lost a characteristic regulatory element referred to as the rapid growth motif (5' AATTTT 3') in the promoters of mitochondrial ribosomal proteins (MRP), which dampens respiratory metabolism in this yeast under aerobic conditions before reaching the diauxic shift.

Consequently the expression of MRP genes is instead coordinated with expression of stress genes induced during growth on  $\text{C}_2$  and  $\text{C}_3$  substrates (acetate, ethanol, glycerol), but not with the ribosomal RNA (rRNA) genes and genes encoding for the cytoplasmic ribosomal proteins (CRPs). This confers active respiratory function of mitochondria during growth on the  $\text{C}_2$  and  $\text{C}_3$  substrates (Rozpedowska et al., 2011).

The Crabtree effect in *D. bruxellensis* evolved in parallel to *S. cerevisiae* as the result of global promoter rewiring (Rozpedowska et al., 2011). The global gene expression profile under fermentative conditions in the Crabtree-positive yeasts *D. bruxellensis* was investigated (I).

### 3.2.3 Fermentation physiology

Several phenomena of metabolic behaviour have been described in response to oxygen and sugar levels: Crabtree, Pasteur and Custer effects. Fermentation under aerobic conditions is known as the Crabtree effect. Activation of glycolysis under anaerobic conditions is referred to as the Pasteur effect. The Custer effect is defined as an inhibition of fermentation under anaerobic conditions.

In glucose-limited aerobic continuous culture, ethanol production by Crabtree-positive yeasts occurs only above a certain dilution rate, which is strain-dependent. In *D. bruxellensis* with increasing dilution rates, three step-wise versions of glucose catabolism under aerobic conditions can be distinguished: respiration with production of CO<sub>2</sub>; acetic acid formation; and ethanol production (van Dijken and Scheffers, 1986).

If glucose is pulsed into an aerobic culture of *S. cerevisiae* running at a low dilution rates, previously respiring cells will start fermenting. This is recognized as the short-term Crabtree effect. The short-term Crabtree effect is connected with glucose overflow from the respiratory to the fermentative pathway (De Deken, 1966, Kappeli, 1986, van Dijken and Scheffers, 1986). If glucose is pulsed into an aerobic culture of *D. bruxellensis* running at low dilution rates, a metabolic profile shift can be observed: at first ethanol and acetate are produced, then ethanol is oxidised to acetate and finally, acetic acid is consumed (van Dijken and Scheffers, 1986).

The long-term Crabtree effect is observed when aerobic batch culture is subjected to glucose excess, or in aerobic continuous cultures at high dilution rates. In contrast to the short-term effect, the long-term Crabtree effect is associated with a shift in gene expression: genes involved in fermentation are induced and genes involved in respiration are repressed.

In contrast to a sudden increase in dilution rate, a slow step-wise increase can result in the establishment of a steady state culture, which performs alcoholic fermentation simultaneously with stable oxygen consumption. Under these conditions, respiratory oscillations can occur, whereby a 40-minute respiration period switches to fermentation synchronously by the whole culture of *S. cerevisiae* (Patnaik, 2003).

Another fermentation-related phenomenon is the Pasteur effect, which is connected with higher rates of glucose uptake under anaerobic conditions compared with aerobic, due to glycolysis inhibition in the presence of oxygen.

The opposite of the Pasteur effect is the Custer effect, which is typical for yeasts belonging to the genera *Brettanomyces/Dekkera* (Scheffers, 1966). The Custer effect can be defined as the inhibition of alcoholic fermentation under anaerobic conditions. This effect manifests itself as a long lag phase followed

by alcoholic fermentation, when aerobic chemostat cultures of *D. bruxellensis* are suddenly subjected to anaerobic conditions. However, the addition of electron acceptors, such as acetoin eliminates this lag period (van Dijken and Scheffers, 1986).

Acetic acid production by *D. bruxellensis* under aerobic conditions leads to overproduction of NADH. This redox imbalance cannot be compensated by *D. bruxellensis* due to its low capacity to produce reduced products such as glycerol. The observed higher expression of genes involved in NADH-producing reactions in the central carbon metabolism compared to that of genes involved in NAD<sup>+</sup>-producing reactions might be the cause of the observed NADH imbalance and resulting Custer effect in *D. bruxellensis* (I). Another hypothesis on the mechanism behind the Custer effect in *D. bruxellensis* is the involvement of alternative respiration (Woolfit et al., 2007). The expression of a gene coding for the salicylhydroxamic acid (SHAM)-sensitive alternative oxidase (AOX) has been reported in *D. bruxellensis* under conditions of oxygen limitation (I).

## 4 Characterisation of the alternative ethanol production yeast *Dekkera bruxellensis*

### 4.1 Genetics

The important role of *D. bruxellensis* in alcohol production habitats has prompted research on *D. bruxellensis* genomics (Borneman et al., 2014). Analysis of 30 isolates of *D. bruxellensis* showed that their karyotypes varied considerably. Aneuploidy of strains has been suggested. The genome of *D. bruxellensis* consists of between 4 and 9 chromosomes depending on the strain. The sizes of the individual chromosomes vary from 1 and 6 Mb (Hellborg and Piskur, 2009) and the total genome size ranges from under 20 to 30 Mb (Woolfit et al., 2007). A high degree of karyotype variability indicates genome rearrangement events in the evolution of *D. bruxellensis*.

Genomes of four *D. bruxellensis* strains have been sequenced to date: AWRI1499 (Curtin et al., 2012), CBS2499 (Piskur et al., 2012), AWRI1608 and AWRI1613 (Borneman et al., 2014).

Sampling of 1.2% of open reading frames from the genome sequence of AWRI1499 strain showed that more than half of the tested genes are represented by three distinct sequence types. Two sequence types were closely related (~99% identity) and the third was divergent (~95%). This suggests that the genome of this strain is composed of three haplotypes. Allele frequency analysis revealed that genomes of sequenced strains consist of two (CBS2499, AWRI1613) or three (AWRI1499, AWRI1608) haplotypes (Borneman et al., 2014).

*S. cerevisiae* is known to have undergone whole genome duplication (Hagman et al., 2013). Analysis of the *D. bruxellensis* CBS2499 genome revealed the duplication of only certain genomic segments, which indicates that *D. bruxellensis* is not a simple haploid. The number of duplicated regions in

the *D. bruxellensis* genome is lower compared to that of *S. cerevisiae*. This, together with a low number of *Dekkera*-specific duplications, implies that a whole-genome duplication event did not occur in *D. bruxellensis* (Piskur et al., 2012).

The phylogenetic history of *D. bruxellensis* remains unclear. Identification of divergent sequences in the genome of *D. bruxellensis* suggested hybridisation events between two closely related species that resulted in the origin of some *D. bruxellensis* strains. Several meiotic genes have been found in the *D. bruxellensis* genome (*FUS3*, *SGF29*, *NAT1*), indicating the presence of a sexual process in the life cycle of its progenitor. Ascus formation by *D. bruxellensis* has previously been reported, however spores were never successfully mated (Hellborg and Piskur, 2009, Kurtzman et al., 2011). Occurrence of pseudogenes and accumulation of mutations in these sequences suggests that the aneuploid genome of *D. bruxellensis* is in the process of degeneration (Woolfit et al., 2007).

*D. bruxellensis* genes that do not have any homologs in *S. cerevisiae* include genes coding for  $\beta$ -galactosidase,  $\beta$ -glucosidase and L-xylulose reductase (responsible for lactose, cellobiose and L-arabinose catabolism respectively), genes involved in lipid metabolism and peroxisome function, genes coding for several sub-units of the respiratory chain complex I, a salicylhydroxamic acid (SHAM)-sensitive alternative oxidase (AOX), and genes involved in nitrate assimilation (Woolfit et al., 2007, **I**, **II**).

*Dekkera bruxellensis* is a petite-positive yeast (Prochazka et al., 2010). The mitochondrial DNA (mtDNA) of CBS2499 strain is a circular molecule with a size of 76453 bp and a high AT content. Both strands of the mtDNA are coding, in contrast to species belonging to genus *Saccharomyces*.

## 4.2 Physiological characterisation

The species *Brettanomyces bruxellensis* was described by N. Hjelte Claussen at the Carlsberg brewery in 1904, during an investigation on the cause of English ale spoilage (Claussen, 1904). The name *Brettanomyces* is derived from 'British brewing fungus', due to the flavour produced by this yeast, which was typical for the British beer of that time. In 1921 *B. bruxellensis* was also isolated from the Belgian Lambic beer (Kufferath, 1921). Up to the present day, this yeast plays a key role in the distinct style of Lambic beer. In 1940, the type strain of *B. bruxellensis* was isolated from the same source and the first systematic study on this yeast was performed (Custeurs, 1940). *B. bruxellensis* is the anamorph (asexual reproductive stage) of *D. bruxellensis*.

The teleomorph (sexual reproductive stage), *D. bruxellensis*, was classified in 1964 by van der Walt, after sporulation of this microorganism was observed. Vegetative cells of *D. bruxellensis* are capable of ascus formation, generating 1-4 spores per ascus without preceding mating (Kurtzman et al., 2011). Spores of *D. bruxellensis* have never been successfully mated (Hellborg and Piskur, 2009). *D. bruxellensis* and *B. bruxellensis* are conspecific and *D. bruxellensis* will probably soon be renamed *B. bruxellensis* (H.M. Daniel, personal communication).

As already mentioned, *D. bruxellensis* has been isolated from distilleries (Passoth et al., 2007). *D. bruxellensis* is also the most common yeast contaminant during wine making. According to some studies, *D. bruxellensis* is responsible for 90% of the spoilage problems in premium red wines (Silva et al., 2004). However, it is also used as a production organism in some wineries (<http://www.beaucastel.com/en/>, 2014); for Belgian Lambic beer (Vanoevelen et al., 1977, Bokulich et al., 2012); and other alcoholic beverages: *kombucha* (fungus-tea) (Teoh et al., 2004) or tequila (Lachance, 1995). *D. bruxellensis* has also been isolated from Zimbabwean traditional fermented milk (Gadaga et al., 2002).

The source of *D. bruxellensis* contamination in the wine industry remained unclear for a long time, owing to difficulties in formulating an appropriate enrichment medium for this yeast (Oelofse et al., 2009). However, the occurrence of *D. bruxellensis* was later demonstrated in grapes damaged by sour rot, as well as on the surface of fermentation vessels, pumps, transfer lines and oak barrels, which, due to their porous structure, are supplied with oxygen and traces of cellobiose. *D. bruxellensis* can assimilate cellobiose. In nature, *D. bruxellensis* has been isolated from bees, fruit-flies and fruit skin (Oelofse A., 2008).

A distinct feature of *D. bruxellensis* culture is its slow growth and auxotrophy for biotin and thiamine (Blomqvist et al., 2010, Kurtzman et al., 2011). The shape of *D. bruxellensis* cells varies from spheroidal to ellipsoidal. Pseudomycelium formation has also been observed (van der Walt, 1964). Pseudomycelium structure of *D. bruxellensis* cells was shown to determine the shape of flocs when aggregating with contaminant *L. vini* (IV).

Compared to *S. cerevisiae*, the CBS2499 strain of *D. bruxellensis* has a broader spectrum of consumable sugars; apart from maltose, galactose, sucrose and trehalose, it is capable of cellobiose fermentation. Genes encoding  $\beta$ -galactosidase and L-xylulose reductase have been identified in the genome of this strain, although it cannot catabolise lactose and L-arabinose (Woolfit et al., 2007). Interestingly, expression of  $\beta$ -galactosidase and  $\beta$ -glucosidase genes was observed in CBS11270 *D. bruxellensis* (I).

Unlike *S. cerevisiae*, *D. bruxellensis* is able to utilise nitrate as a nitrogen source. Growth and metabolite profiles of *D. bruxellensis* during oxygen-limited cultivation in different combinations of carbon (glucose, fructose, sucrose, maltose) and nitrogen (ammonium, nitrate, mixed) sources were investigated (II). Growth on nitrate as the sole nitrogen source resulted in decreased growth and ethanol production by *D. bruxellensis* for all tested carbon sources. When cultivated in mixed nitrate/ammonium media, most of growth parameters were similar to those observed during growth on ammonium-only media with the exception of ethanol production (II). This indicates that nitrate can influence metabolism of *D. bruxellensis* even in the presence of ammonium under oxygen limitation.

*D. bruxellensis* is reported to have higher biomass and ethanol yields than *S. cerevisiae* in batch oxygen-limited cultivation (Blomqvist et al., 2010). A possible explanation for the higher fermentation rate of *S. cerevisiae* as compared to *D. bruxellensis* is a higher dosage of glycolytic genes, because of the whole genome duplication event in *S. cerevisiae* (Piskur et al., 2012).

*D. bruxellensis* produces high amounts of acetic acid under aerobic conditions. The reason is thought to be the insufficient activity of the acetyl-CoA synthetase responsible for the conversion of acetate to acetyl-CoA (Silva et al., 2004, Wijsman et al., 1984).

*D. bruxellensis* produces very low amounts of glycerol under either aerobic or anaerobic conditions, whereas *S. cerevisiae* produces significant amounts of glycerol (Blomqvist et al., 2010). This is thought to be connected to the Custer effect in *D. bruxellensis*, i.e. inability to ferment under anaerobic conditions (Scheffers, 1966). Genes involved in glycerol biosynthesis in *D. bruxellensis* are expressed at low levels under oxygen-limited conditions (I).

*D. bruxellensis* is reported to have similar tolerance to ethanol as *S. cerevisiae* (Rozpedowska et al., 2011). *S. cerevisiae* exhibits higher multifactorial stress resistance than *D. bruxellensis* in lignocellulose medium, although the reasons for this have yet to be determined (Blomqvist et al., 2011). On other hand, the growth parameters of *D. bruxellensis* in lignocellulose hydrolysate could be improved after corresponding precultivation (III).

### 4.3 Biochemical particularities

For non-fermentative yeasts, activation of an alternative redox sink under aerobic conditions is known, i.e. alternative respiration. Electrons from the ubiquinone pool can be passed to a SHAM-sensitive AOX, which then reduces oxygen to water. In contrast to other Crabtree-positive yeasts, *D. bruxellensis*

is probably capable of utilising alternative respiration (Blondin et al., 1984). *D. bruxellensis* is the first known Crabtree-positive yeast to express respiratory chain complex I and AOX (Woolfit et al., 2007, I).

Gene expression analysis suggests that in *D. bruxellensis*, one *ADH* gene is involved in both ethanol production and consumption in *D. bruxellensis* (III), which is similar to the situation in *S. stipitis* (Passoth et al., 1998) and *W. anomalus* (Fredlund et al., 2006). The *ADH1* gene of *D. bruxellensis* was determined to encode an ADH most probably localised in the cytoplasm due to the missing signal sequence, whereas another identified *ADH* gene, *ADH2*, had a mitochondrial signal sequence (III).

In yeasts, there are two genes known to encode for dihydroorotate dehydrogenase (DHOD 1.3.3.1), involved in uracil synthesis: *URA1* and *URA9*. The *URA1* gene encodes for oxygen-independent cytoplasmic DHOD. The *URA9* gene encodes for an oxygen-dependent mitochondrial DHOD. The cytoplasmic *URA1* gene resulted from a horizontal gene transfer that occurred 100-150 million years ago between yeasts and *Lactococcus lactis* (Gojkovic et al., 2004). Presence of the *URA1* gene allows *S. cerevisiae* to perform uracil synthesis even under anaerobic conditions, whereas the *URA9* gene has been lost in this yeast.

No data on presence of the *URA1* gene in the *D. bruxellensis* genome have been reported; however, the presence and expression of a gene homolog of *URA9* was shown (Woolfit et al., 2007, I). It remains unclear which alternative mechanism *D. bruxellensis* could employ to compensate for uracil auxotrophy under anaerobic conditions. Requirements of *D. bruxellensis* for amino acid-but not uracil supplementation under anaerobic conditions have been reported (Blomqvist et al., 2012). In contrast, *S. cerevisiae* is capable of anaerobic growth in minimal medium supplemented with only non-saturated fatty acids and ergosterol.

Unlike *S. cerevisiae*, *D. bruxellensis* is capable of nitrate assimilation (de Barros Pita et al., 2011). Nitrate is transported into the cell by the nitrate transporter, encoded by *YNT1*. The *YNR1* gene encodes nitrate reductase, which catalyses nitrate conversion to nitrite. Nitrite is reduced to ammonia by nitrite reductase, encoded by the *YNI1* gene. Nitrate assimilation requires a substantial input of energy with four molecules of NAD(P)H required for each molecule of ammonium formed from nitrate.

It has previously been shown that ammonium represses assimilation of nitrate in yeast species, such as *O. polymorpha* and *W. anomalus* (Avila et al., 1998, Garcia-Lugo et al., 2000, Siverio, 2002). However, expression analysis of nitrate assimilation genes in *D. bruxellensis* reported only moderate repression of these genes by ammonium under aerobic conditions (de Barros Pita et al.,

2011). Work in this thesis further demonstrated that the nitrate assimilation genes in *D. bruxellensis* were downregulated in the presence of ammonium under conditions of oxygen limitation (II). Repression of genes involved in assimilation of alternative nitrogen sources in the presence of ammonium is designated as nitrogen metabolite repression (Siverio, 2002).

Expression analysis of genes involved in i) the pentose phosphate pathway, ii) the TCA cycle and iii) ATP synthesis demonstrated that i) *TKL1* (encoding for transketolase), ii) *MDH1* (encoding for malate dehydrogenase) and iii) *ATP1* (encoding for the  $\alpha$ -subunit of the mitochondrial F1F0) were upregulated, while i) *GND1* (encoding for phosphogluconate dehydrogenase) and ii) *ACO1* (encoding for aconitase) remained unaltered in the presence of nitrate as sole nitrogen source (II).

The presence of nitrate led to a downregulation of *ADH1*, which is supported by diminished ethanol production under these conditions. This work demonstrated that under oxygen limitation nitrate had an inhibitory effect on ethanol production (II).

A notable biochemical property of some strains of *D. bruxellensis* is their vinyl phenol reductase activity, which is responsible for the formation of off-flavours (ethyl hydroxysterenes) in wine (Oelofse A., 2008). *D. bruxellensis* has been shown to produce volatile compounds, which are products of L-leucine, L-lysine, L-isoleucine and L-valine catabolism (Oelofse A., 2008, Grbin, 1998). During wine making, enhanced production of biogenic amines by *D. bruxellensis* compared with other yeasts has been observed (Caruso et al., 2002).

#### 4.4 Hypotheses about the physiological basis of *D. bruxellensis* competitiveness

It has previously been shown that *D. bruxellensis* can only outcompete *S. cerevisiae* under glucose-limited conditions in continuous culture (Blomqvist et al., 2012).

Two hypotheses have been proposed to explain the physiological basis of *D. bruxellensis*' competitiveness over *S. cerevisiae* under glucose limitation. The first hypothesis is that *D. bruxellensis* possesses a more efficient energy metabolism than *S. cerevisiae*, whereas the second hypothesis is that *D. bruxellensis* has a higher affinity to sugar than *S. cerevisiae*. These mechanisms are not mutually exclusive and could act in combination. Low glycerol production may be the molecular basis for high efficiency of *D. bruxellensis* metabolism under glucose limitation, since glycerol production is

energetically demanding and also removes carbon from the ATP-generating pathways.

Low expression of genes involved in glycerol production was observed (I). Another aspect of energy metabolism of *D. bruxellensis* is the possibly higher efficiency of the respiratory chain. Documented expression of NADH-ubiquinone reductase (complex I) in *D. bruxellensis* indicates that it may be active under oxygen limitation (I). This suggests a high affinity of *D. bruxellensis* to oxygen, and higher ATP gain per NADH oxidation in the respiratory chain of *D. bruxellensis* as compared to *S. cerevisiae* (which, as Crabtree-positive yeast, is deficient for complex I).

High expression of sugar transporter genes would support the hypothesis of a more efficient sugar uptake by *D. bruxellensis* under glucose-limited conditions (I). Highly expressed sugar transporter genes in *D. bruxellensis* were homologous to *S. cerevisiae* and *Komagataea pastoris* high-affinity glucose transporters. Higher affinity of *D. bruxellensis* for the limiting substrate would enable it to become dominant during continuous fermentations when only minute amounts of the sugar are available.

Several other factors such as the presence of nitrate or *L. vini* in the outcompetition of *S. cerevisiae* by *D. bruxellensis* have been discussed (II, IV). A relationship between nitrate assimilation by *D. bruxellensis* and its competitiveness has been suggested (de Barros Pita et al., 2011). On the other hand, outcompetition of *S. cerevisiae* by *D. bruxellensis* in glucose-limited culture has been demonstrated also in the absence of nitrate (Blomqvist, 2011, Blomqvist et al., 2012). Regardless of the decrease in *D. bruxellensis* growth parameters when nitrate is the sole nitrogen source (III), the capability for nitrate assimilation can confer a physiological advantage to *D. bruxellensis* when ammonium is depleted from sugar cane juice and growth of *S. cerevisiae* is restricted.

*L. vini* has been repeatedly co-isolated with *D. bruxellensis* from industrial ethanol production plants in Sweden and Brazil. Almost nothing is known about the reasons for its frequent co-occurrence with *D. bruxellensis*. Investigation of co-cultures of *L. vini* with *D. bruxellensis* and *S. cerevisiae* revealed formation of structured LAB-yeast cell aggregates. Involvement of mannose residues in flocculation between yeast and bacterial cells was demonstrated. Interestingly, a higher mannose concentration was required to induce deflocculation of *S. cerevisiae* compared to *D. bruxellensis* (IV). The observed phenomenon is most probably due to the higher mannose content in the cell wall of *S. cerevisiae* as compared to *D. bruxellensis* (Prillinger et al., 1990). Stronger binding of *S. cerevisiae* cells to flocs, as compared to *D. bruxellensis* cells could lead to faster removal of *S. cerevisiae* from the culture

due to sedimentation. It was shown that outcompetition of *S. cerevisiae* by *D. bruxellensis* can occur without involvement of *L. vini* (Blomqvist et al., 2012), however this does not preclude the potential for additional effects in the presence of this bacterium.

## 5 Transcriptomics in yeasts

### 5.1 Introduction to transcriptomics

Development of a platform for genome-wide gene expression analysis is often defined as one of the main breakthroughs in functional genomics (Bro et al., 2003). The transcriptome is the set of all RNA molecules transcribed under certain conditions. In contrast to the genome sequence, which compiles the total gene content of the organism, transcriptome analysis determines only genes that are transcribed (Goffeau et al., 1996). Identification of open reading frames from the bulk of non-coding regions in a genome sequence is complicated compared with the highly refined transcripts produced by mRNA sequencing (Grabherr et al., 2011).

Initially, when sequencing runs were expensive, determination of the total gene content was of high priority, and genome rather than transcriptome sequencing was preferred. At the time of high cost sequencing runs, gene expression analysis was accomplished through the application of arrays (Malone and Oliver, 2011).

Development of second generation sequencing technologies reduced the cost of sequencing, and extended its application from genomes to transcriptome analyses under various conditions (Agarwal et al., 2010). This facilitated the analysis of coding regions and the discovery of new genes (Shah et al., 2006).

Compared to microarrays, transcriptome sequencing provides higher accuracy in gene expression analysis and enables the discovery of new genes that were not predicted computationally. There are currently several next generation sequencing technologies available, such as 454, Illumina, SOLiD, LifeTech and PacBio (Wall et al., 2009, Mardis, 2011).

Many non-conventional yeasts lack established tools for genetic manipulation, which limits the use of targeted mutagenesis for the

determination of gene function. Gene sequence and expression analysis can shed light on gene function. Sequencing a whole transcriptome can provide unbiased information on gene expression levels when the number of sequenced reads for the specific transcripts can be determined (I).

Massive gene expression analysis under various conditions represents an efficient tool for molecular characterisation of certain physiological phenomena. Comparative analysis of total transcriptomes expressed under different conditions allows detection of co-regulated gene groups and reconstruction of the cellular gene expression regulation network (Yassour et al., 2009). The increasing number of non-conventional yeast genomes will simplify transcriptome analysis (Shah et al., 2006).

## 5.2 ABI SOLiD sequencing for transcriptome analysis

The later upgrades of ABI SOLiD (5500xl and 5500 xl-Wildfire) sequencing machine can produce both colour space and base space data. However, the transcriptome of *D. bruxellensis* presented in this thesis was generated at a time when only the older version SOLiD 4 was available, producing solely colour space data.

SOLiD-generated data are different from those derived from any other sequencing technology. Reads produced by SOLiD are only 75 bp long. The colour space coding principle means that sequences of two nucleotides are coded by a certain colour, which is read as a number (from 0 to 3) (Wall et al., 2009). One colour corresponds to four different dinucleotide sequences, which increases the potential for misinterpretation during decoding of the colour space sequence. Thus, if the first base is decoded incorrectly, it shifts the decoding frame in all reads. However, due to the colour-coding, a correct combination of two colours is required to correctly call a base, thus the error rate of SOLiD technology is generally lower than any other existing technology on the sequencing market. This feature is especially suitable for detection of single nucleotide polymorphism sites in resequenced genomes.

Another disadvantage of colour space SOLiD-sequence data is the current shortage of available software that can analyse this type of data. *De novo* assembly of colour space SOLiD-derived data is complicated because it requires conversion of the colour space into base space format before it can be analysed with the commonly used assembly programs (Grabherr et al., 2011).

The initial aim of the transcriptomics study on *D. bruxellensis* presented in this thesis was to perform *de novo* assembly of the transcriptome based on SOLiD colour space data. However, the short read length in combination with the limitations of colour space coding restricted the ability to complete the

stated research aim. Once the genome of *D. bruxellensis* became available, colour space data could be used to determine global gene expression levels.

Software that can handle SOLiD-derived data for gene expression analysis, but not for *de novo* transcriptome assembly, has been developed by Life Technologies. Mapping SOLiD -sequenced reads to a reference genome can be accomplished using Life Scope software (I). The read count numbers are used to calculate Reads Per Kilobase per Million mapped reads (RPKM) values for each gene, which is the normalised measure of expression level (Mortazavi et al., 2008). Alternatively, programs such as BWA or Novoalign can be used to align colour space reads to reference genomes, and expression levels can be calculated with read counting software such as Htseq (Li and Durbin, 2009, Anders and Huber, 2010, Ruffalo et al., 2012).

### 5.3 Complexity of yeast transcriptome

The transcriptome of an actively dividing yeast cell consists of rRNA (~80%), tRNA (~15%) and mRNA (~5%). 50% of mRNA is derived from ribosomal protein genes (Warner, 1999). RNA polymerase II is responsible for the transcription of mRNA. However, 90% of transcription events by RNA polymerase II were estimated to be transcriptional noise. Recent studies have revealed pervasive transcription in yeast cells, which means initiation of transcription in intra- or intergenic regions from both DNA strands: in upstream common transcription starting sites (TSS); within ORFs; inside intergenic regions; or in termination sites (antisense RNAs). A total of 10 000 unique transcription units were estimated to exist within the *S. cerevisiae* transcriptome (Ito et al., 2008).

The function of these non-coding RNAs is poorly understood; however, a role in transcriptional interference or histone modification has been shown for some of them. Several types of stable non-coding RNAs with putative regulatory function were identified: small nuclear RNAs (snRNAs); small nucleolar RNAs (snoRNAs); small interfering RNAs (siRNAs); and microRNAs (Jacquier, 2009).

A large repertoire of non-coding RNAs, which will be degraded, seems to be wasteful. Non-coding RNAs, however, might convey flexibility for yeast metabolism. First, different regulation mechanisms may act dependently on structure of TSS. Second, loose process control may represent potential for rapid evolution. The ability to generate a versatile pool of RNAs from a limited number of genes by varying TSS may have contributed to the evolutionary success of eukaryotes (Jacquier, 2009).

A similar degree of complexity in the *D. bruxellensis* transcriptome was observed in the course of this thesis. The fact that the majority of data produced in this study (81.17% of rRNA-depleted transcriptome reads) did not map to regions identified by gene models points to the high frequency of transcription events outside coding regions (I).

Several studies have shown that there is only weak positive correlation between transcript and protein levels in *S. cerevisiae*, which demonstrated limitations of gene expression analysis studies for the understanding of cell metabolism. For example, after introduction of anaerobic conditions, glycolytic proteins levels increased 2- to 10-times in *S. cerevisiae*, however transcript levels remained the same (de Groot et al., 2007). Comparative analysis of the transcriptome and corresponding proteome has demonstrated post-transcriptional regulation of cellular processes. Post-transcriptional regulation can be accomplished by mRNA decay and protein degradation, capping, splicing, mRNA export, translational initiation, etc. mRNA decay can be induced in the case of large proteins (under stress conditions) or by the presence of a conserved motif in the 3' UTR of mRNA (Olivares-Hernandez et al., 2010).

Work in this thesis showed that low expression of acetyl-CoA synthetase genes in combination with high expression of aldehyde dehydrogenase genes did not correlate with acetate production. This indicates that regulation mechanisms other than transcription prevent acetate formation under low oxygen conditions (I). However, determining the fraction of transcribed genes facilitates framing a set of putative key players in metabolism, by omitting untranscribed genes. Competition of enzymes for substrate and co-factors represent another level of regulation. Transcriptomics studies appears as more affective for interpretation of induced genes, rather than altered expressions of constitutive genes.

## 6 Lignocellulosic ethanol

### 6.1 Overview

Lignocellulose is a collective term for plant biomass that is inedible to humans, and therefore, it is a promising alternative substrate for biofuel production (Farrell, 2006, **I**). On the other hand, lignocellulosic sugars are not readily available for ethanolic fermentation (Olsson and HahnHagerdal, 1996).

Lignocellulose is composed of three main components: cellulose; hemicellulose; and lignin. Cellulose consists of homopolymers, in which glucose monomers are bound by  $\beta(1-4)$  glycosidic bonds. Hemicelluloses are branched acetylated heteropolymers that include pentoses (L-arabinose, D-xylose), hexoses (D-galactose, D-glucose, D-mannose) and uronic acids. Lignin is a complex poly-phenolic compound, formed by monomers of *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol incorporated into the lignin structure in the form of phenylpropanoids in a haphazard manner.

Softwood contains a higher average amount of lignin (26-36%) and less cellulose (38-52%) and hemicellulose (16-27%) than hardwood (17-30%, 37-57% and 20-37%, respectively) (Foyle et al., 2007). The complex molecular structure of lignocellulose makes it highly resistant to degradation, and harsh pre-processing is required for sugar release (Girio et al., 2010, **III**).

The process of ethanol production from lignocellulosic material (LM) consists of several stages: (1) LM pre-treatment; (2) saccharification of polysaccharides; (3) fermentation of the hydrolysate monosaccharides into ethanol; and (4) ethanol concentration (Lee, 1997, **III**).

The purpose of LM pre-treatment and saccharification is to liberate fermentable sugars from polysaccharides. Various techniques for LM processing have been developed: chemical pre-treatment, such as with acids or alkali, or physical pre-treatment, such as steaming, milling or freezing (Kumar et al., 2009).

Saccharification can be performed by either enzymatic or chemical methods. Enzymatic saccharification provides highly efficient specific hydrolysis (Himmel et al., 2007). Simultaneous saccharification and fermentation was elaborated to optimise efficiency of lignocellulosic substrate conversion to ethanol (Olofsson et al., 2008). The use of ionic liquids has been demonstrated to recover a high proportion of fermentable sugars in the process of biomass saccharification without enzymes (Binder and Raines, 2010).

Nevertheless, thermo-chemical pre-treatment in combination with enzymatic hydrolysis is a commonly used technique, achieving a tolerable level of toxic compounds and high sugar yield during processing of LM (Zhu et al., 2010, **III**).

Pre-treatment of LM can lead to the formation of toxic compounds that inhibit fermentation of the released sugars (Larsson et al., 1999, **III**). The inhibitor composition of the resulting hydrolysate depends on the type of LM and pre-treatment method. Inhibitors fall mainly into one of three groups: (1) weak organic acids; (2) furan compounds; and (3) phenolic compounds. The weak organic acids include acetic acid, which is mainly generated from acetyl side chains of hemicellulose and lignin, as well as formic and levulinic acid, which are products of furan degradation. Furan compounds are heterocyclic aldehydes that arise from sugars during thermo-chemical pre-treatment. The two main furan compounds are furfural and hydroxymethyl furfural (HMF), which are derived from the dehydration of pentoses and hexoses, respectively. Phenolic compounds are produced as a result of lignin decomposition.

Adaptation of the ethanol producing organism to some inhibitors has been observed, and therefore, the yeast cultivation technique can be optimised (Petersson and Liden, 2007). Step-wise increases in the lignocellulose hydrolysate concentration or precultivation in lignocellulose hydrolysate have been shown to facilitate yeast growth on lignocellulosic substrates (Blomqvist et al., 2011, **III**). Additionally, a cultivation mode with cell recirculation can provide high cell density that may be beneficial for the yeast survival in lignocellulose-derived inhibitory medium (Palmqvist et al., 1998, **III**).

## 6.2 Effects of lignocellulose-derived inhibitors

The effects of weak acids, furfurals and phenols on yeast metabolism have mainly been studied in *S. cerevisiae*. Acetic acid is an abundant inhibitor in lignocellulose hydrolysate (Mira et al., 2010). At low extracellular pH, undissociated weak acids are able to diffuse into the cytosol. The higher intracellular pH leads to dissociation of the acids in the cytoplasm, which inhibits growth by the drop in intracellular pH, ATP depletion and intracellular

accumulation of anions (Russel, 1992). ATP is consumed by the plasma membrane ATPase, which neutralises pH by pumping out protons. ATP consumption leads to a decline in synthetic processes and, thereby, biomass production. Decreased intracellular pH results in inhibition of DNA replication (Imai and Ohno, 1995). Acetic acid can also induce generation of reactive oxygen species (ROS) in the cell (Ludovico et al., 2001, Sousa et al., 2012).

Phenolic compounds are thought to derive their inhibitory activity due to their high hydrophobicity (Endo et al., 2008, Ibraheem and Ndimba, 2013), which results in a relatively easy incorporation into the cellular membrane and disruption of its integrity (Jonsson et al., 2013). Phenolic compounds may induce generation of ROS (Mikulasova et al., 1990).

Furfural and HMF can affect enzymatic activity, damage DNA, inhibit RNA and protein synthesis, and induce the generation of ROS (Sanchez and Bautista, 1988, Khan and Hadi, 1994, Modig et al., 2002, Allen et al., 2010). NADH depletion (Palmqvist et al., 1999) in combination with inhibition of glycolytic enzymes (Banerjee et al., 1981) in the presence of furfural might be involved in reported acetaldehyde accumulation in yeast cells.

The cell can reduce furfural and HMF to the less toxic furfuryl alcohol and 2,5-bis-hydroxymethylfurfural, respectively. Detoxification of furfural mainly involves NADH-dependent oxidoreductases, while HMF is predominantly reduced by NADPH-dependent oxidoreductases (Liu, 2006). Variation in cofactor preferences for oxidoreductases, which detoxify HMF has been described (Laadan et al., 2008, Nilsson et al., 2005).

### 6.3 Adaptation to lignocellulose-derived inhibitors

A rapid and efficient response to stress conditions is essential for survival of an organism (Berry and Gasch, 2008). The exposure of yeast cells to lignocellulose-derived inhibitors induces a cellular adaptation response that includes the activation of inhibitor detoxification pathways, stress resistance mechanisms and metabolic regulation (Nicolaou et al., 2010). Detoxification can be achieved through direct enzymatic conversion of inhibitors to less toxic compounds or active export of inhibitors from the cell (Piper et al., 2001, Liu, 2006). Stress resistance mechanisms include immediate general stress response and long-term protection against intracellular damage, such as alteration of biosynthetic pathways to provide compensatory changes in cellular composition (sugars, amino and fatty acids)(Pereira et al., 2011). Regulation of metabolism under stress conditions can compensate for redox and energy loss (Liu, 2011).

Introduction of inhibitors into growth medium, leads to differential transcription of genes involved in adaptation in yeast cells (Ma and Liu, 2010, Li and Yuan, 2010). Many of these responses, such as those permitted by genes of the general stress response system represent secondary unspecific reparative processes, such as responses to DNA, protein or lipid damage etc (Ruis and Schuller, 1995).

Adaptive changes in protein composition of the plasma membrane have been reported in *S. cerevisiae* in response to acetic acid exposure. Incubation in the presence of acetic acid resulted in induction of the specific ATP binding cassette transporter (Pdr12p) in the plasma membrane that actively extrudes acid anions from the yeast cell (Piper et al., 2001). Increase in expression of *PMA1*, encoding for H<sup>+</sup>-ATPase has been shown to be connected with weak acids tolerance mechanisms in *S. cerevisiae* (Holyoak et al., 1996). Decrease or increase in ethanol production by *S. cerevisiae* can occur dependently on the acetic acid concentration and pH (Larsson et al., 1999). Changes in membrane unsaturated and saturated fatty acids composition in response to phenolic compounds and acetic acid exposure has been reported (Heipieper and de Bont, 1994, Lindberg et al., 2013).

Exposure to furan compounds has been shown to increase the expression of genes involved in the reduction of furan compounds (Ma and Li, 2010, Liu, 2011). Detoxification of furfural and HMF requires NADH and NADPH (Gorsich et al., 2006, Liu, 2011). Therefore, a compensatory regulation of metabolism occurs in the presence of these compounds. Furfural has been reported to affect gene expression in *S. cerevisiae*: upregulating genes involved in alcohol production, TCA cycle and downregulating genes involved in the glycerol biosynthesis (Lin et al., 2009). Inhibition of glycerol formation in *S. cerevisiae* culture in the presence of furfural has been shown. Increase or decrease in ethanol production has been shown in response to different furfural concentrations (Palmqvist et al., 1999). Upregulation of genes involved in pentose-phosphate pathway has been shown in the presence of HMF in evolutionarily engineered *S. cerevisiae* resistant to furan compounds (Liu et al., 2009).

It was previously shown that a furfural concentration of 2 g/l induced an increase in ADH activity of 78% after 48 hours (Banerjee et al., 1981). Precultivation in lignocellulose hydrolysate did not alter the expression levels of genes in *D. bruxellensis* homologous to *S. cerevisiae* *ADH1* and *ADH2*. However, this does not preclude that precultivation affected activity of the corresponding enzymes (**III**), or the involvement of other enzyme isoforms. When this study was performed, only the sequences of two *D. bruxellensis* *ADH* genes were known.

Numerous studies have examined the effects of single inhibitors on yeast fermentation performance. Nevertheless, cumulative cellular responses to multifactorial stress induced by lignocellulose hydrolysate remains poorly investigated. The effect of adaptive precultivation in lignocellulose hydrolysate on the fermentation performance by *D. bruxellensis* was investigated as part of this thesis. Adaptation of *D. bruxellensis* cells in lignocellulose hydrolysate resulted in shortening of the lag phase, moderate growth acceleration, and increased ethanol yields (III). However, after exposing adapted cells to non-selective conditions, these properties were partly lost. The stability of phenotypic characteristics of cells adapted in batch and continuous cultivation differed. Low deviation between biological replicates supports a physiological mechanism of *D. bruxellensis* adaptation, rather than selection of randomly-generated better-adapted mutants (III).

Only partial loss of the adapted phenotypic characteristics may point to the complex character of this adaptive phenomenon, arising from a heterogeneous culture composed of distinct subpopulations. Fractional structure of *S. cerevisiae* cultures has been revealed during cultivation in the presence of acetic acid; only a subpopulation of cells resumed growth under stress conditions, whereas other cells were in a viable but non-proliferating state. This phenomenon has been shown to be caused by phenotypic cell-to-cell heterogeneity (Swinnen et al., 2014).

Alternatively to genetic engineering efforts, pre-treatment or long-term cultivation in presence of inhibitors is becoming more frequently used to improve fermentation performance or obtain better-adapted strains (Hasunuma and Kondo, 2012, Wallace-Salinas and Gorwa-Grauslund, 2013, Gu et al., 2014).



## 7 Microbial contaminants in ethanol production

### 7.1 Role of contaminants

Contamination is one of the main problems in industrial large-scale biotechnology operations that can threaten the profitability of production (Skinner and Leathers, 2004). Although bacteria constitute a large group of microbial contaminants during ethanol production, a variety of yeast species have also been isolated from this habitat. Contaminants are capable of causing yield reductions through competition for substrate, and may also directly inhibit the growth of the production organism (Thomas et al., 2001, **IV**). Harsh cultivation conditions are maintained to minimise the risk of contamination during industrial fermentation. Therefore, only organisms that tolerate high ethanol concentrations, osmotic stress, temperatures of 37 °C, low pH and oxygen limitation can survive under these conditions.

There are suggestions that some bacterial contaminants can even have a positive effect on fermentations. An ethanol production consortium composed of *D. bruxellensis* and *L. vini* has been isolated from a Swedish ethanol production plant. It has been assumed that *L. vini* might stabilise microbial dynamics in continuous cultures, or lower susceptibility to further contamination (Passoth et al., 2007). Continuous co-cultivations of *L. vini* with both *S. cerevisiae* or *D. bruxellensis* have not revealed any negative impact of this bacterium on ethanol production levels. Moreover, stimulation of ethanol production in *D. bruxellensis/L. vini* co-culture has been reported (de Souza et al., 2012). Yeast and LAB co-cultivation can have practical applications such as malolactic fermentation during wine making (Renouf et al., 2005).

The main sources of contamination are the feedstock and the fermentation equipment (Bayrock et al., 2003). A number of methods are commonly used to prevent contamination, such as sanitation of the fermentation equipment,

maintaining harsh cultivation conditions (high temperature, low pH, high alcohol concentration) or the use of antibiotics (Hynes et al., 1997). However, the use of antibiotics in industrial fermentations is limited by regulations in many countries, due to the possible generation and release of antibiotic-resistant microorganisms into the environment (Martinez, 2012).

The early detection of microbial contaminants in industrial alcoholic fermentations is of high importance and, therefore, methods for monitoring of contamination occurrence have been developed. Plating of diluted fermentation broth on selective media is commonly used to monitor microbial populations in fermentation vessels. However, this method is restricted to the detection of colony-forming microbial population, and cannot detect viable but unculturable populations.

Culture-independent methods provide a better overview of a microbial community (Bokulich and Mills, 2012, Loureiro and Malfeito-Ferreira, 2003). PCR amplification of rDNA and sequencing was shown to be efficient for identification of contaminants (Liberal et al., 2005). PCR-fingerprinting is another frequently used technique for assessing microbial dynamics during fermentation (Basilio et al., 2008).

The microbial ecology of industrial alcohol production remains poorly understood. The role of frequently isolated bacteria requires further investigation. Work in this thesis elucidated a negative impact of an industrial isolate of *L. vini* on ethanol production in batch culture (IV).

## 7.2 Bacterial contaminants

Production fermentors can be contaminated by the Gram-positive LAB such as *Lactobacillus*, *Leuconostoc* and *Pediococcus*, and the Gram-negative *Zymomonas mobilis*. Gram-negative acetate-producing bacteria of the genera *Gluconobacter* and *Acetobacter* and the Gram-positive bacterium *Micrococcus* can contaminate propagator fermentors with efficient oxygenation (Narendranath, 2003).

Fast growing bacteria contaminate production fermentors mainly at the beginning of cultivation before ethanol has reached high concentrations – the contamination is primarily caused by LAB (Lucena et al., 2010). *Lactobacillus fermentum* and *Lactobacillus brevis* are examples of common LAB contaminants in ethanol production.

The LAB are a large group of aerotolerant anaerobic, catalase-negative, non-sporulating Gram positive bacteria. Lactic acid is the major product of glucose fermentation by LAB, but heterofermentative LAB can also produce acetate and ethanol. Heterofermentative LAB, such as *Lactobacillus paracasei*

and *L. fermentum* were reported to have negative impact on ethanol production in continuous system in contrast to homofermentative LAB *L. vini* and *L. plantarum* (de Souza et al., 2012).

LAB are known to require nutrient-rich growth media that contains vitamins, amino acids, oligopeptides, nucleotides, and a fermentable carbohydrate (Moretro et al., 2000). *L. vini*, which had been isolated from a Swedish ethanol production plant (Passoth et al., 2007) was shown to require peptone for growth (IV).

Some LAB species are known to flocculate. Increase in the size of *L. vini* flocs and proportion of cells bound to flocs was observed in response to elevated ethanol concentrations (IV). An ethanol concentration similar to that in industrial habitats was shown to have bacteriostatic rather than bacteriocidal effect on *L. vini* (own unpublished results). This might explain the absence of bacterial metabolites such as lactic acid in the medium analysed from the ethanol production plant, running in continuous mode (Passoth et al., 2007, de Souza et al., 2012). Metabolites of LAB could mainly be detected during batch cultivation before ethanol reached a high level (Narendranath et al., 1997).

Once the habitat is contaminated with LAB, niche availability for further invasion by other microbes will decrease due to the broad antimicrobial activity of these bacteria. Some LAB have antimicrobial activity and can outcompete other microbes by different mechanisms, e.g. production of acids and other inhibitors (Schnürer and Magnusson, 2005, Rouse et al., 2008, Rouse and van Sinderen, 2008).

The production of organic acids by LAB can inhibit the growth of other microorganisms by lowering pH and affecting metabolism after intracellular penetration of the undissociated form of the acids (Narendranath et al., 2001).

Secretion of peptides with a wide range of antibiotic action has been shown for some lactobacilli, e.g. *L. acidophilus* (Shahani et al., 1977). Another mechanism behind the antimicrobial properties of lactobacilli is related to the toxic compound H<sub>2</sub>O<sub>2</sub>, produced when lactobacilli are exposed to O<sub>2</sub> (Condon, 1987).

Competition for the substrate is one of the main aspects of interaction between bacterial contaminants and ethanol production yeast (Narendranath et al., 1997). Work in this thesis demonstrated the ability of *L. vini* to compete for glucose with *D. bruxellensis* or *S. cerevisiae* during batch co-cultivation (IV). Contrary to this, enhanced ethanol production has been shown during continuous co-cultivation of *L. vini* and *D. bruxellensis*. No effect on ethanol production in presence of *L. vini* in continuous co-culture with *S. cerevisiae* has been observed (de Souza et al., 2012). The difference in concentrations of inhibitory ethanol achieved in batch (IV) and continuous (de Souza et al.,

2012) cultures may partly explain the discrepant role of *L. vini* in these systems.

Physical interactions between yeasts and bacteria can also occur. Co-cultivation of yeasts with lactobacilli can lead to co-flocculation of the two organisms (Golowczyc et al., 2009). Studies on co-cultivation of *L. vini* with either *D. bruxellensis* or *S. cerevisiae* described in this thesis demonstrated the formation of yeast-bacteria aggregates, with the yeast forming an external layer surrounding a core of bacteria. The co-flocculation between yeast and bacterial cells was shown to be mannose-dependent (IV). This indicates that the LAB interact with mannose residues within the yeast cell wall. Floccs of *L. vini*-*S. cerevisiae* associated in bigger structures, while it was not observed for *L. vini*-*D. bruxellensis* floccs due to outsticking yeast cells from pseudomycelium (IV).

### 7.3 Yeast contamination

Many yeasts are commonly associated with the alcoholic beverage and food industries, where they are mainly recognised as contaminants (Beckner et al., 2011). Contaminant yeasts are typically introduced to the production process through biomass material, where certain species are members of the natural microbiota.

The yeast species *Candida intermedia*, *Candida parapsilosis*, *Candida tropicalis*, *Clavispora lusitaniae*, *Cyberlindnera fabianii*, *D. bruxellensis*, *Exophiala dermatitidis*, *Hanseniaspora guilliermondii*, *Kodamaea ohmeri*, *Meyerozyma caribbica*, *Meyerozyma guilliermondii*, *Lachancea fermentati*, *Pichia galeiformes*, *Pseudozyma hubeiensis*, *Saccharomycodes ludwigii*, *Schizosaccharomyces pombe*, *W. anomalus*, *Zygoascus hellenicus* and *Zygosaccharomyces rouxii* have been isolated as contaminants in ethanol production habitats (Loureiro and Malfeito-Ferreira, 2003, da Silva-Filho et al., 2005, Basilio et al., 2008).

Most of these yeast species are only capable of surviving in fermentation vessels and do not decrease ethanol production substantially. In contrast to this, species such as *D. bruxellensis*, *P. galeiformes* and *C. tropicalis* are considered severe contaminants that can comprise more than 30% of the yeast population in a fermentation vessel (Loureiro and Malfeito-Ferreira, 2003).

Competition for substrate is one of the mechanisms behind interaction of contaminant and production yeasts. Some yeast contaminants are also capable of killer toxin production, for instance *W. anomalus* and wild strains of *S. cerevisiae* (Passoth and Schnürer, 2003). DsRNA viruses are the carriers of the killer phenotype in *S. cerevisiae* (Schmitt and Breinig, 2002). Production of cell wall degrading enzymes has been shown for *H. guilliermondii* (Guetsky et

al., 2002). Another mechanism behind the inhibitory effect of yeast contaminants is production of toxic metabolites, such as weak organic acids.

Yeast strains isolated from distilleries are often tolerant to the harsh conditions of industrial habitats (Beckner et al., 2011). Understanding the mechanisms behind the tolerance of these strains is of great theoretical and practical interest. For instance, the high osmo- and thermotolerance of *S. pombe* is a promising characteristic for efficient industrial ethanol production (Ohmiya et al., 1995). The osmo- and barotolerance of *Z. rouxii* has also attracted the interest of researchers (Groleau et al., 1995).

*D. bruxellensis* is a very common yeast contaminant in distilleries. As described previously, outcompetition of *S. cerevisiae* by *D. bruxellensis* has been observed in continuous cultivation under sugar limitation (Liberal et al., 2007, Passoth et al., 2007). Work in this thesis examined the transcriptome of *D. bruxellensis* under conditions in which it outcompetes *S. cerevisiae*. Key findings included the identification of genes involved in energy metabolism and glucose uptake, which presumably confers competitiveness to *D. bruxellensis* (I).



## 8 Conclusions

Currently, ethanol is the most widely used vehicle biofuel. Although ethanol production has been applied for millennia, it still faces a variety of challenges. This includes i) the introduction of advanced fermentation techniques such as continuous fermentation with cell recirculation, which redirects carbon flow from yeast biomass increment to ethanol production, and ii) the introduction of novel lignocelluloses-based substrates. These challenges require a deeper understanding of microbial interactions during fermentation processes and new strains with high productivity and fitness in industrial habitats.

The outcompetition of the initially inoculated *S. cerevisiae* strain by a novel fermentation yeast, *D. bruxellensis*, has been observed. At the same time it has been observed and later confirmed, that in industrial fermentations this yeast is always associated with the LAB *L. vini*. It has been shown that *D. bruxellensis* can assimilate nitrate, which is an important component of fermentation broth in Brazil. Recent researches on *D. bruxellensis* suggest that it is a convenient model organism to investigate the complex conditions of industrial fermentation. *D. bruxellensis* is a non-conventional yeast and the molecular techniques to investigate its physiology are still under development.

This thesis aimed to understand the physiological basis of competitiveness of *D. bruxellensis* in industrial fermentations, to study *D. bruxellensis* physiology during nitrate assimilation, to test the applicability of this yeast to ferment lignocellulose hydrolysate, and to understand its interaction with the LAB *L. vini*. Most of these aims could only partially be reached, but in attempts to address them, new aspects of yeast physiology in relation to industrial fermentations have been presented.

- The first transcriptome of *D. bruxellensis* was determined in this thesis. Since no genome was available at the beginning of this work, the transcriptome was analysed by sequencing rather than hybridisation array-based methods. The rapid improvement of RNA sequencing techniques

coupled with decreasing costs will make this the method of choice in the future.

- Non-coding transcripts from intergenic DNA were more abundant than coding transcripts. Extragenic transcription has only been described in a few eukaryotes so far, and its biological significance is poorly understood. However, it may play an important role in cell physiology, and this phenomenon deserves further research attention.
- Global gene expression analysis under conditions in which *D. bruxellensis* outcompeted *S. cerevisiae* revealed potential mechanisms of outcompetition: (i) A more energy efficient metabolism, documented by expression of genes of the respiratory chain, including NADH-ubiquinone reductase (complex I), which is missing in *S. cerevisiae* and other Crabtree-positive yeasts. This may enable the yeast to obtain energy even with trace oxygen in the fermentation. Moreover, genes belonging to the glycerol biosynthetic pathway were only expressed at a low level. Glycerol production is connected with a loss of ATP, and thus, its inhibition results in a more efficient energy metabolism. (ii) The yeast may have a higher affinity to the limiting substrate glucose. Several sugar transporters, homologs of high-affinity transporters in *S. cerevisiae* and *K. pastoris*, were highly expressed.
- *D. bruxellensis* is known to produce acetic acid under aerobic conditions. Although no acetic acid production under oxygen limitation was detected, the genes involved in acetic acid production were nevertheless expressed at detectable levels. This points to additional metabolic regulation at the post-transcriptional level in *D. bruxellensis*.
- This project included one of the first studies of *D. bruxellensis* growth in lignocellulose hydrolysate. Pre-cultivating the yeast in hydrolysate adapted it to the inhibitors present in the hydrolysate. Adapted cells had shorter lag-phase, higher growth rate, and higher final ethanol concentrations compared to non-adapted cells. After cultivation in non-selective medium, the adapted phenotype was partially lost. However, after a longer lag-phase, the adapted phenotype was partly re-established. The uniform behaviour of the different cultures indicates that adaptation was not due to the selection of resistant mutants. These results are in line with recent findings in *S. cerevisiae*, and may indicate the presence of different sub-populations with varying physiological properties also in *D. bruxellensis* cultures.
- *ADH1* was assumed to be localized to the cytosol since it lacked any discernible targeting sequences, whereas *ADH2* had a mitochondrial signal sequence. High expression of *ADH1* during both ethanol production and

consumption indicates that the Crabtree-positive yeast *D. bruxellensis* uses a single alcohol dehydrogenase for both the production and consumption of ethanol, similar to the respiratory yeasts *S. stipitis* and *W. anomalus*.

- Analysis of co-cultivation of the bacterium *L. vini* and the yeasts *S. cerevisiae* or *D. bruxellensis* in batch systems indicated that *L. vini* has a negative impact on ethanol production in this system due to competition for nutrients between the species. The formation of lactic acid bacteria–yeast cell aggregates consisting of a bacterial core with an outer layer of yeast cells was identified. Differences between yeasts were observed in co-flocculation with bacteria, regarding both the shape and the stability of the flocs. This finding may represent an interesting novel aspect in industrial fermentation ecology.
- Unlike *S. cerevisiae*, *D. bruxellensis* is able to assimilate nitrate as a source of nitrogen. Growth and metabolite profiles of *D. bruxellensis* during cultivation in different combinations of carbon and nitrogen sources were studied. Cells cultivated on nitrate as the sole nitrogen source grew slower than if cultivated on ammonium. The upregulation of genes involved in respiratory metabolism (*MDH1* and *ATP1*) and downregulation of *ADH1* during growth of *D. bruxellensis* on nitrate as sole nitrogen source suggests that nitrate assimilation affects the energy and redox metabolism. The ability to assimilate nitrate may provide a selective advantage when other nitrogen sources are depleted.
- Repression of genes involved in nitrate assimilation in *D. bruxellensis* under oxygen-limited conditions in presence of ammonium was demonstrated. The difference in ethanol production and *ADH1* expression in cells grown in mixed nitrate/ammonium and ammonium-only media indicated that nitrate can regulate carbon metabolism of *D. bruxellensis* even in the presence of ammonium under oxygen limitation.
- In general, the yeast *D. bruxellensis* has the potential to become an alternative organism for ethanol production from both first and second generation substrates. It can also be used as a model organism to understand the dynamics of microbial inter-species competition. Candidate genes responsible for the competitive advantage of *D. bruxellensis* can be used in engineering new, more robust yeast strains for ethanol production. Further research may allow us to exploit the full potential of *D. bruxellensis*.



## 9 Future perspectives

### 9.1 Physiology of *Dekkera bruxellensis* from an “omics” perspective

Work within this thesis indicated that in the future transcriptome analysis is likely to rely on sequencing technologies producing base space rather than colour space data. Despite the increasing number of studies on the genomics and transcriptomics of *D. bruxellensis*, the metabolism of this yeast still remains poorly understood. Due to the post-transcriptional regulation of yeast metabolism, transcription reflects only a part of cellular response. A combination of fluxomics and transcriptomics might be better to elucidate the regulatory mechanism in certain pathways. Metabolomics studies of *D. bruxellensis* with focus on NADH/NADPH redox balance as the main drivers of energy metabolism could clarify molecular physiology of this yeast under various conditions. Fluxomics on *D. bruxellensis* could provide an unbiased estimate of the activity of individual metabolic pathways under different fermentation conditions. Using these approaches one could investigate characteristic physiological properties of *D. bruxellensis*, such as the Custer effect, alternative respiration in a Crabtree-positive yeast, compensatory mechanisms for uracil auxotrophy under anaerobic conditions, and competitiveness in glucose and oxygen limitation in continuous culture. These methods can also be used to study the effects of lignocellulose inhibitors on *D. bruxellensis* metabolism.

### 9.2 Nitrogen-specific regulation of *Dekkera bruxellensis* metabolism

Contradictory data was obtained on the regulation of expression of genes involved in nitrate assimilation in *D. bruxellensis* under different oxygen

conditions (de Barros Pita et al., 2011). Fermentations under controlled conditions are required to establish the role of oxygen in nitrogen metabolite repression. Metabolomics and fluxomics studies are required to elucidate the mechanism of metabolism regulation in *D. bruxellensis* in presence of nitrate both as sole nitrogen source and in combination with others under different oxygen levels. Key information on NADPH or NADH cofactor preference of enzymes involved in nitrate assimilation in *D. bruxellensis* remains unclear. Extension of nitrogen source spectrum can be regarded as possible strategy to improve *S. cerevisiae* strain competitiveness.

### 9.3 Laboratory evolution of *Dekkera bruxellensis* for improved lignocellulose hydrolysate conversion into ethanol

As genetic engineering tools for of this yeast are poorly developed, evolutionary engineering may provide an alternative for generating strains for different applications. Long-term cultivation in lignocellulose hydrolysate could, for instance, generate highly adapted and competitive strains for ethanol production from lignocellulose. Comparisons of genomes and transcriptomes between parental and improved strains could then identify the genes responsible for adaptation to lignocellulose hydrolysate. Knowledge of genes involved in adaptation to lignocellulose could also be used to engineer the traditional fermentation yeast *S. cerevisiae* to improve conversion of lignocellulose to ethanol.

Long-term cultivations in lignocellulose hydrolysates may also provide a convenient system for the investigation of dynamics of distinct subpopulations with different physiological properties when the cells are subjected to selection pressure.

### 9.4 Interaction of bacterial and yeast contaminants in industry

The co-occurrence of *D. bruxellensis* and *L. vini* in industrial habitats has been reported by several independent sources. Although some initial investigations of their co-existence in industrial habitats have been described, this phenomenon still requires further research. Comparative studies on the interactions between *D. bruxellensis* and *L. vini* under continuous co-cultivation experiments in industrial fermentation broth under different conditions need to be performed.

This thesis also describes the formation of flocs when *L. vini* was co-cultivated with either *S. cerevisiae* or *D. bruxellensis*. The flocs formed by the two yeasts together with *L. vini* differed in their structure and amount of mannose required

to disperse the flocs. However, the relevance of this phenomenon for industrial environments still needs to be investigated. Pseudomycelium formation and lower mannose content of the cell wall could be a potential strain optimisation strategy to modify flocculation capacity of production strain and increase their fitness within an industrial environments.

## 9.5 Understanding competitiveness of *Dekkera bruxellensis*

The physiological basis of competitiveness of *D. bruxellensis* has yet to be completely established. Due to a lack of molecular tools and the aneuploid character of the *D. bruxellensis* genome, it is difficult to investigate this issue using standard genetic methods. One possible approach would be to test strains of varying competitiveness in co-cultivation experiments with *S. cerevisiae* and to compare their genomes and transcriptomes. Differentially expressed genes may provide some clues about the competition mechanism.

Determining the affinity of putative transporters of *D. bruxellensis* and comparison to those of *S. cerevisiae* could clarify the role of substrate affinity in *D. bruxellensis* competitiveness. One way would be to introduce individual transporter genes from *D. bruxellensis* into a *S. cerevisiae* strain lacking hexose transporters and to determine the affinity of the expressed transporters in this strain. The identification of high affinity transporters from *D. bruxellensis* that can successfully be expressed in *S. cerevisiae* could enable the construction of more competitive *S. cerevisiae* strains.



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## Acknowledgements

This work was financially supported by research programme *Microdrive* at the Swedish University of Agricultural Sciences, Uppsala and Swedish Energy Agency (Energimyndigheten).

I want to thank the people who were the main actors in this research story:

My supervisor Volkmar Passoth for setting my PhD project within the context of open international research efforts. Thank you for always being enthusiastic to discuss stoichiometry of yeast metabolism, for supporting me and trying to teach me to critically approach the “observation-interpretation-expression” path when exploiting the power of yeast to make the world move faster.

My co-supervisors: Hans Ronne for providing me with books, protocols, helpful comments, seminars-everything I needed so much! Olga Vinnere Pettersson for introducing me to the business of decoding genomic messages. SOLiD, Ionproton.... , quantum theory does not look serious any longer to me. Mats Pettersson, for patience and support when learning me how to make a sense out of: (pattern=".+([()4])([:alnum:]]+)",replacement =" \\2"),”.

Johanna Blomquist for sharing your yeast pet with me and setting a good example of discipline and organisational skills. Thomas Eberhard for nearly unlimited assistance with everything to do with fermentation that got me my first milliliters of ethanol produced by Dekkera. I still remember that one hydrolysis when we finally got the lignocellulose to be saccharified! on the ceiling...

Tomas Linder for providing books, comments, protocols as well as proofreading this thesis. Collaborators from the Department of Molecular Biology: Jerry Ståhlberg, Mats Sandgren, Henrik Hansson and Majid Momeni for their limitless knowledge of everything enzymatic. Thanks to you I now see

things around me (chairs, tables... trees) as material waiting to be turned into ethanol. Anna Schnürer and the other members of Bioenergy research School for putting my research efforts in a global context. My gratitude to Johan Schnürer for giving me the opportunity to participate in Microdrive.

From the Uppsala Genome Center Ignas Bunikis and Christian Tellgren-Roth, for teaching me a lot in sequencing projects. Henrik Lantz for valuable hints on bioinformatics.

My fellow “yeastologists” in the EM group: Mattias, Mikael, Ida, Zhen and Niklas- for sharing your knowledge on how to torture the yeast. Su-Lin Heden for valuable comments on this thesis. Salome Schneider for defeating Endnote.

Former students in the lab: David Sundell, Taras Luzhetskui, Hari Nair, Mohamed Tabassum and Swati Sharma, Afsar Ali for all your creativity in finding new applications of lab items and being proactive.

Our collaborators at the Federal University of Pernambuco in Brazil: Will de Barros Pita, Fernanda Cristina Bezerra Leite, Diogo Ardaillon Simoes and Prof Marcos Antonio de Moraes for a truly intercontinental collaboration on mRNA analysis and insight into Brazilian “ethanolic dream”. Prof Svein J. Horn at the University of Life Science in Norway for supplying us with lignocellulosic material.

I also would like to thank some people who were not directly involved in the project but by only saying few words made me realise new dimensions in my project: Hans Johansson, Stefan Roos, Sara Hallin, Lars Frykberg, Jure Piskur, Pernilla Bjerling, Carl Johan Franzén, Diethard Mattanovich.

I’m very grateful for technical assistance from Maria Hellman (everything), Elizabeth Börjesson (HPLC analysis) and Joakim Bjerketorp (RNA analysis) and for generally not letting me to get lost in lab in beginning of the project. Stina, Anki, Sture, Ingemar, Micke, Bengt and Ingvar for frequent assistance with all things practical and admin-related.

I also would like to thank Kajsa, Åsa, Harald, Maren, Chris, Lea, Tina, Anton, Shokoufeh, Daniel, German, Janis, Li, Karin, Alvaro for nice company. All my past and present co-workers at Microbiology for discussions, seminars and cakes.

And thanks to Terhi and Anders, Ane and Amalie, Louise and Jenny, Anna and Dejan, Johan and Britt-Marie, Ulrika, Oskar and Mark, Thea and Kalle, Samantha and Kent, Tuija and Leo for all the support!

Finally, my gratitude to my family for giving me much more than I can say thank you for.

Tack så mycket...