THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Fermentation of lignocellulosic material:

A study on bacterial contamination and yeast physiology

EMMA JOHANSSON

Department of Chemical and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
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Systems Biology

Department of Chemical and Biological Engineering

Chalmers University of Technology

SE-412 96 Göteborg

Sweden

Telephone: +46 (0) 31 772 1000

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ABSTRACT

Progressive depletion of oil and conventional fossil fuels, an increased energy demand and an increased struggle for national energy security has led to the development of alternative biobased fuels, bioethanol being one of them. Bioethanol can be made from many different raw materials, and based on this are classified as 1st generation and 2nd generation. This research work dealt with 2nd generation bioethanol using lignocellulosics as raw material. Lignocellulosic material is an abundant, non-edible raw material that can be converted to ethanol using the yeast *Saccharomyces cerevisiae*. Due to the recalcitrant nature of lignocellulosics, it is a harsh substrate to ferment. Despite this, bacterial contaminations do occur. This research work aimed to identify potential methods to combat bacterial contamination in industrial fermentations and to identify physiological reactions of the yeast *S. cerevisiae* upon fermentation of lignocellulosic material. The raw material used was spruce processed in a commercial biorefinery and a demonstration plant; spent sulphite liquor and high-gravity dilute-acid spruce hydrolysate, respectively. Two potential methods to combat bacterial contaminations were identified. The cultivation procedure of a pitching agent was proven to influence bacterial concentration and is suggested as a potential antimicrobial activity. Treatment with sodium chloride and ethanol also turned out to selectively support the viability of yeast and reduce the number of bacterial cells. As lignocellulosics are rich in metabolic inhibitors, additional antimicrobial activities may be deleterious for the ethanol production potential of the yeast. By comparing physiological effects and the level of energy of two strains of *S. cerevisiae,* in the two substrates mentioned above, it was found that a commercial-available strain developed for industrial fuel ethanol production performed poorly in fermentations of spent sulphite liquor while a strain originally harvested from a spent sulphite liquor-based fermentation performed well both short-term and long-term. Using high-gravity dilute-acid spruce hydrolysate changed the scenario demonstrating the importance of strain selection.

Keywords: Lignocellulosic bioethanol, bacterial contamination, yeast physiology, energy metabolism

LIST OF PUBLICATIONS

This thesis is based on the following research papers, referred to as Paper I-IV in the text:

Paper I

Johansson, Emma; Brandberg, Tomas; Larsson, Christer (2011)

Influence on cultivation procedure for *Saccharomyces cerevisiae* used as pitching agent in industrial spent sulfite liquor fermentations

Journal of Industrial Biotechnology 38:1787-1792.

Paper II

Albers, Eva; Johansson, Emma; Franzén, Carl-Johan; Larsson Christer (2011)

Selective suppression of bacterial contaminants by process conditions during lignocellulose based yeast fermentations

Biotechnology for Biofuels 4:59-67

Paper III

Johansson, Emma; Xiros, Charilaos; Larsson, Christer (2013)

Fermentation performance and physiology of two strains of *Saccharomyces cerevisiae* during growth in high gravity spruce hydrolysate and spent sulfite liquor (Submitted)

Paper IV

Johansson, Emma; Westerlund, Monika; Larsson, Christer (2013)

The effect on yeast physiology and performance during sequential batch cultivations in spent sulfite liquor medium. (Manuscript)

CONTRIBUTION SUMMARY

Paper I

Performed the wet-lab experiments and took part in the design of experiments, analysis of data and writing of the manuscript.

Paper II

Performed parts of the wet-lab experiments and took part in the design of experiments, analysis of data and writing of the manuscript.

Paper III

Performed the wet-lab experiments and took part in the design of experiments, analysis of data and writing of the manuscript.

Paper IV

Performed the wet-lab experiments and took part in the design of experiments, analysis of data and writing of the manuscript.

PREFACE

This dissertation partly fulfills the requirements for a PhD degree at the Department of Chemical and Biological Engineering at Chalmers University of Technology, Sweden. The industrial PhD project was initiated in September 2008 in collaboration with SP Processum (formerly Processum Biorefinary Initiative AB), SEKAB and Domsjö Fabriker, Aditya Birla. The research was carried out under the supervision of Professor Christer Larsson with additional help and supervision by PhD Tomas Brandberg, PhD Roland Agnemo and PhLic. Monika Westerlund.

The research work concerns bacterial contamination in industrial fermentations of lignocellulosic material and the influence of lignocellulosic material on the physiology of *Saccharomyces cerevisiae*.

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Emma Johansson
October 2013

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1. INTRODUCTION

Lignocellulosic media is a harsh substrate to ferment, partly due to the lignin degradation products as well as the presence of acids (Palmqvist and Hanh-Hägerdal, 2000b). Lignocellulosics are a diverse group of substrates and the amount of inhibitors is highly dependent on the origin of the material as well as the pre-treatment and hydrolysis method used (Olsson and Hanh-Hägerdal, 1996). Despite the harsh environment bacterial contaminations do occur and decrease the ethanol yield in commercial lignocellulose-based fermentation plants. Many investigations of bacterial contamination during industrial fermentations have been conducted, lbut few have dealt with lignocellulosic material as the fermentation substrate. The complex structure of lignocellulosics also contributes to high stress levels which hamper ethanol production and biomass formation in the fermenting organism.

The organism in focus in this research work was *Saccharomyces cerevisiae*, more commonly known as "baker's yeast." It has been shown that *S. cerevisiae* have a greater potential to produce ethanol if adapted to the fermentation media prior to fermentation (Alkasrawi et al., 2006; Kadar et al., 2007; Paper I). In order to further study this physiological behavior the levels of adenosine phosphate were studied.

S. cerevisiae consumes sugars and produces biomass and ethanol, along with CO_2, in order to reproduce. In these reactions adenosine phosphates are formed and consumed. The ATP production and consumption can therefore be coupled to the need for maintenance or growth of the cell and therefore also ethanol production. If the need for maintenance is high a minor part of ATP goes to biomass formation, which may, under non-inhibitory conditions, be beneficial for ethanol production. But under more severe conditions this can be detrimental for a continuous ethanol production as it is dependent on a vital cell culture. As lignocellulosics are complex substrates it is of interest to study any difference in ATP levels of the cell in relation to nutrient supplementation in order to maintain a high ethanol production in the plant.

1.1 Aim

With respect to the above, the aim of this research work has been to investigate different methods for combating bacterial contaminations in lignocellulosic fermentations and to understand how lignocellulosics affect the energy metabolism in the yeast *Saccharomyces cerevisiae.*

2. BACKGROUND

2.1 Composition of lignocellulosic material

Lignocellulosic material or woody material mainly consists of plant cell walls. The plant cell walls are composed of energy rich polymers such as cellulose, hemicellulose and the polyphenol lignin (Sjöström, 1981; Somerville, 2004). Lignocellulosics are a heterogeneous group in terms of the relative amounts of the polymers. Plant cell walls have evolved to be recalcitrant to internal and external degradation (Ralph et al., 2004). They give tensile strength to the cells and the entire plant, function as the water transportation system and act as a guard against pathogens. Due to the cell wall's many different functions, it possesses immense structural variation between species but also among different cell types within the same species (Pauly and Keegstra, 2008). This complicates the use of lignocellulosic material as a feedstock in the production of biofuels and chemicals. In this research work softwood has been used as raw material.

2.1.1 Cellulose

Cellulose is the main structure in wood and Kräsig (1993) describes the large abundance and the poor use of the polymer in his book *Cellulose – Structure, Accessibility and Reactivity*. It is a high molecular weight linear homopolysaccharide composed of several D-glucose molecules linked by β(1→4)-glycosidic bonds. The degree of polymerization, measured as the number of glucose units, in one cellulose molecule is on average 10 000 to 15 000 (O´Sullivan, 1997; Rowell et al., 2005). The cellulose fibers are packed into microfibrils linked with hydrogen bonds contributing to the crystalline structure and the rigidity and strength of the cell wall (Brett and Waldron, 1996).

Cellulose is hydrolyzed to glucose which in a harsh environment is degraded to 5-hydroxymethylfurfural (HMF). HMF can be further degraded to levulinic acid and formic acid (Forss, 1961; Ulbricht et al., 1984) (Fig. 1). It has been reported that carbohydrates also can be degraded to phenolic compounds (Popoff and Theander, 1976).

2.1.2 Hemicellulose

Hemicellulose is a heteropolysaccharide cross-linking the cellulose in wood. In contrast to cellulose hemicellulose is composed of both hexoses and pentoses, *e.g.* D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose and L-rhamnose. The composition is strongly dependent on the plant species. The polysaccharide can be highly branched and the degree of polymerization is on average 100-200 molecules (Sjöström, 1981; Rowell et al., 2005).

Hydrolysis of hemicellulose can, along with monosaccharides, produce acetic acid (Fig. 1). Acetic acid is cleaved off and released during hydrolysis of wood. The pentoses can be further degraded to furfural (Forss, 1961).

2.1.3 Lignin

Lignin is an aromatic polymer that gives rigidity to the wood (Adler, 1977). Its complex network is composed of phenyl propane units (i.e. *p*-coumaryl, coniferyl and sinapyl alcohols), which can differ greatly between plant species. Softwood lignins mainly consist of coniferyl alcohols. Paracoumaryl alcohol (*p*-coumaryl) is also present in smaller amounts (Klinke et al., 2004).

Lignin is a complex structure that during hydrolysis partly is decomposed to high and low molecular weight phenolic compounds of which the low molecular weight compounds have been suggested to be the most inhibitory to *S. cerevisiae* (Larsson et al., 1999; Palmquist and Hahn-Hägerdal, 2000; Klinke et al., 2004).

2.1.4 Extractives and non-extractives

Wood extractives protect the wood from biological damage and constitute a supply of reserve food. The amount and type of extractives vary between tree species. They also vary within different parts of the same tree (Torssell, 1997). Extractives can be divided into three groups: aliphatic compounds, terpenes and phenolic compounds (present mainly in hartwood and bark). Non-extractives consist of mainly inorganic ash components such as silica and alkali salts but also some pectin, proteins and starch. In wood the concentration of non-extractives is low (<1%) (Umezawa and Higuchi, 1991; Bierman, 1996).

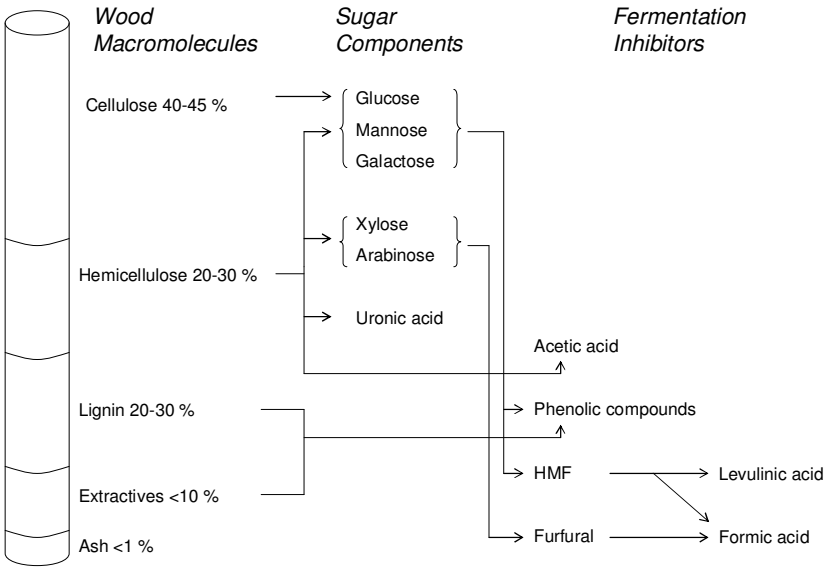

Figure 1 | Wood degradation products. During breakdown of wood both carbohydrates and fermentation inhibitors are formed. The sugars formed are both the hexoses glucose, mannose, and galactose and the pentoses xylose and arabinose. The fermentation inhibitors are both formed directly from the wood components, mainly from lignin but also from hemicellulose, and from the monosaccharides. The relationships vary with tree species and cooking/pre-treatment method.

2.2 Methods for hydrolyzing wood

Hydrolysis of wood has not been performed during this research work but is described here in order to give the whole picture of ethanol production from lignocellulosic material.

Wood is structured so that it is recalcitrant to challenges encountered in its natural habitat (Ralph et al., 2004). It is therefore a challenge to hydrolyze it into its building blocks. Hydrolysis of wood to fermentable sugars can be performed using a variety of methods. Optimal hydrolysis results in a fermentation substrate consisting of high sugar content with low amounts of inhibitors (Fig. 1) (Chandra et al., 2007). A combined severity factor can be calculated using the equation $CS = \log(Ro) - pH$. $\log(Ro) = \log(t \times \exp((T - 100)/14.75)$ where t is the residence time and T is the absolute temperature (Chum et al., 1990; Overend and Chornet, 1987). The most preferable hydrolysis method is still under debate and most probably there will not be one universal method but several, depending on the origin of the

lignocellulosic material and the geographic location in which the process is performed (Kumar et al., 2010; Tomás-Pejó et al., 2011; Binod et al., 2011; Stephen et al., 2013). Hydrolysis of wood can be divided into two steps: pretreatment and chemical/enzymatic hydrolysis (Fig. 3). Depending on the hydrolysis technique of the lignocellulosic material, the amount of degradation products may vary, changing the fermentative severity of the hydrolysate, even though the source is the same (Olsson and Hahn-Hägerdal, 1996; Galbe and Zacchi, 2012). Both pretreatment and chemical/enzymatic hydrolysis have been extensively studied with varying results and many different process procedures exist in the literature. Therefore only a brief overview of the two main components will be mentioned here.

2.2.1 Chemical hydrolysis

Chemical hydrolysis can be used both as a pretreatment step together with a second hydrolysis step (enzymatic or chemical) or as the main hydrolysis step. To be able to produce fermentable sugars from wood it is necessary to break the structure of lignin and cellulose. For the breakage of lignin and opening of the crystalline structure of cellulose, chemical hydrolysis is catalyzed by using inorganic acids (H_2SO_4, SO_2) or an alkaline catalyst (NaOH, NH_3, lime) together with high temperature (121-200°C). Hydrolysis at neutral pH has also been mentioned in literature. Neutral hydrolysis also includes the release of inherent organic acids from the raw material (Galbe and Zacchi, 2012). Treatments with organic solvents (Pan et al., 2005) and ionic liquids (IL) (for review see Brandt et al., 2013) are other forms of chemical hydrolysis mentioned in literature. Chemical hydrolysis can also be combined with physical methods; for review see Galbe and Zacchi (2012).

2.2.2 Enzymatic and fungal hydrolysis

Enzymatic hydrolysis is usually used in combination with a thermal and/or chemical pretreatment, the pretreatment aim to break down lignin structure and open up the crystalline structure of cellulose in order to increase enzyme accessibility (Mosier et al, 2005). A variety of microorganisms in nature can degrade cellulose and hemicellulose in lignocellulosic raw materials (Wood, 1985). These organisms produce cellulolytic and hemicellulolytic enzymes, respectively. The use of fungi for degradation of lignocellulosics is mainly performed using white- and soft-rot fungi (Belkacemi et al., 1998) but other microorganisms have also been considered (Ray et al., 2010; Wan and Li, 2012).

2.2.3 Production of pulp

At present, there are two main strategies to produce chemical pulp (Fellers and Norman, 1998): kraft pulping and sulfite pulping. In this research work material from sulfite pulping has been used (Paper I, II, III, IV). Sulfite pulping is a milder form of dissolving lignin and hemicellulose that leaves high levels of fermentable monosaccharides in the spent sulfite liquor (SSL).

There are several methods to perform sulfite pulping; acid (bi)sulfite, bisulfite, two stage sulfite, three stage sulfite, neutral and alkaline sulfite. The most commonly used method today is probably acid sulfite pulping with Ca^{2+} as "base". Mg^{2+}, NH_4^+ or Na^+ is also used (personal communication with Hans Grundberg, Dominova).

Along with the wood degradation products, *e.g.* hexoses, pentoses, HMF, furfural, phenolic compounds and organic acids, cooking chemicals and sometimes also bleaching chemicals make up the SSL which makes SSL a complex fermentation media and the complexity also varies with tree species and process parameters.

2.3 Fermentation modes

For a fermentation to work properly it might be necessary to use different strategies for substrate addition depending on the composition of the substrate, the organism used and the aim of the study. The basic fermentation modes are displayed here, and based on these three you will have a pallet of different fermentation techniques (Isohla et al., 2013; Matano et al., 2013).

2.3.1 Batch fermentation

In batch fermentations the cultivation media is added from the beginning. Growth in batch mode is characterized by three distinct phases: lag phase (1), exponential phase or log phase (2) and stationary phase (4) (Fig. 2). During lag phase the cells produces enzymes and proteins necessary for growth under the prevailing conditions. When sufficient amount and activity of the desired proteins and enzymes are produced the cell starts to grow and enters the exponential phase. During growth the cells are sensitive to damage from the growth substrate.

If nutrients and vitamins are abundant growth is occurring at maximum specific growth rate defined by Monods kinetics, $\mu = (\mu_{max} \times S)/(K_s + S)$ as long as $S >> K_s$. As growth proceeds metabolic products such as ethanol, glycerol and acetate accumulate and contribute to a constant change throughout the fermentation. In response to a lowered sugar and nutrient concentration growth ceases and cells enter the stationary phase. Growth stops during the stationary phase; this is the most robust state of cell life (Blomberg et al., 1988; Viana et al., 2012). Under aerobic conditions fermentation products secreted during the exponential growth phase can be used as carbon sources. For this to occur, the cells undergo a short secondary lag phase called a diauxic shift (3) during which appropriate enzymes are activated (Fig. 2). Under anaerobic conditions growth on a carbon source other than sugars is not possible.

The culturing conditions prevailing during batch cultivation impose a high stress on the fermenting organism. The concentration of possible inhibitors and sugars are high and the production of secondary metabolites may affect the final yield and titer negatively.

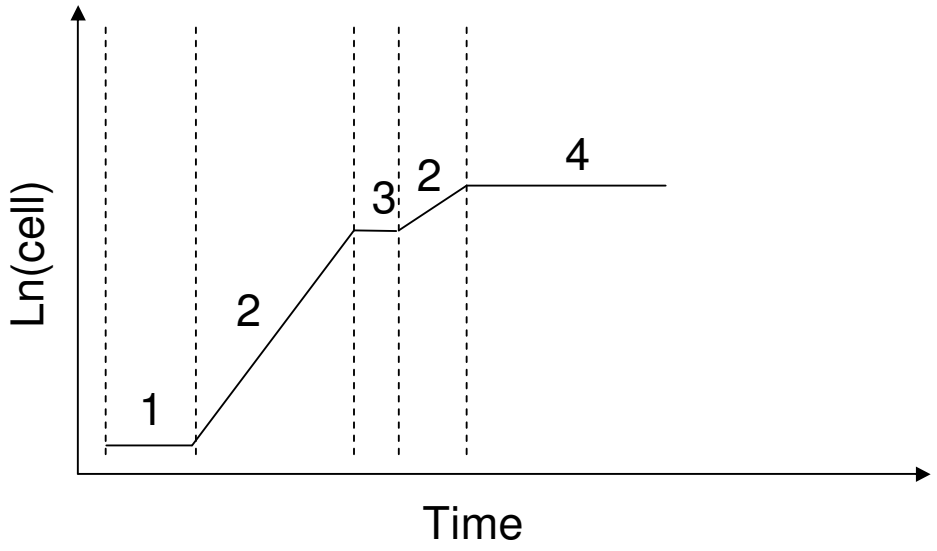

Figure 2 | Schematic overview of cell growth in batch fermentations. 1) lag phase, 2) exponential phase, 3) dioxic shift, 4) stationary phase

2.3.2 Fed-batch fermentation

During fed-batch fermentation the substrate is fed into the system in a well-balanced mode in order to increase productivity of the organism due to low levels of inhibitors. The level of nutrients remains constant and the microorganism remains in log phase until feed is completed, followed by a stationary phase. During fermentations of lignocellulosic material this technique allows for a consumption of the inhibitory furfurals, thus keeping them at a low concentration, not inhibitory to the yeast (Petersson and Lidén, 2007).

2.3.3 Continuous fermentation

A continuous fermentation is constantly fed with substrate (F_{in}) while an equal amount of spent substrate plus microorganisms is constantly withdrawn (F_{out}) which leaves a constant volume (V_r) and dilution rate ($D = F_{in}/V_r$). At steady state the formation of new biomass is in balance with the efflux of cells, and $D = \mu$. With a continuous fermentation a time-independent steady state can be obtained. Different types of continuous fermentations exist but will not be discussed in this thesis.

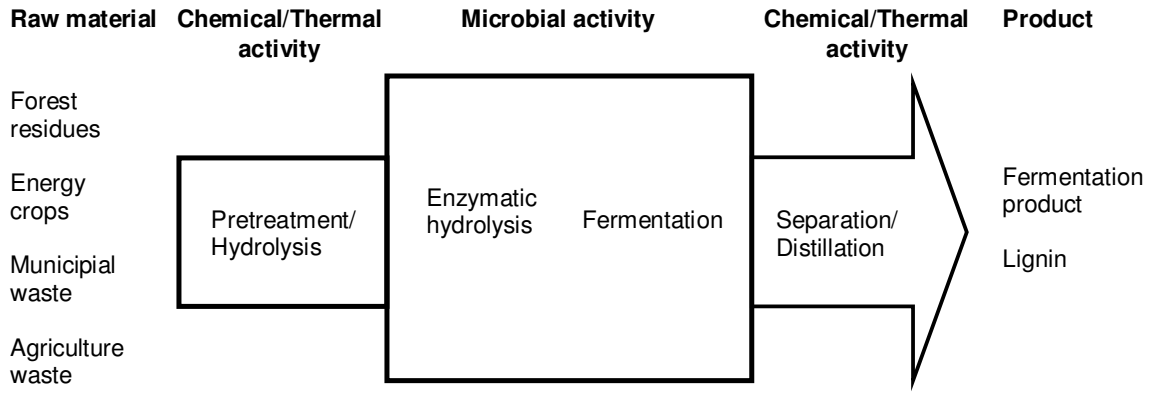

Figure 3 | Schematic process overview of a biomass-based production of biofuels and biochemicals. The components within the box microbial activity can be combined in many different ways, for example consolidated bioprocessing (CBP) in which both degradation and fermentation of the substrate is performed by the same organism; simultanous saccharification and fermentation (SSF) in which the enzymes used for degradation of the substrate and fermenting organism is present at the same time; and separate hydrolysis and fermentation (SHF) in which degradation and fermentation is performed separately.

2.4 Production of liquid biofuels – An overview

Liquid biofuels include bioalcohols such as ethanol, butanol and biodiesel. When manufactured from lignocellulosic material different combinations of the above mentioned hydrolysis and fermentation modes can be used, the most common being SHF (separate hydrolysis and fermentation) and SSF (simultaneous saccharification and fermentation). From these a pallet of different operational modes has evolved, *e.g.* CBP (consolidated bioprocessing) in which the fermenting organism also produces cellulolytic enzymes, among others (Van Zyl et al., 2007).

A schematic view of the production of liquid biofuels is presented in Fig. 3. Depending on the raw material and the product of interest, different obstacles have to be overcome in order to build an efficient production. The composition of the raw material and the hydrolysis of it determine the severity of the fermentation broth, and depending on the choice of fermenting organism and desired product this can be tackled in different ways. Today efforts are made in each step of the process by research focusing on how to modify the raw material to minimize

inhibitory content and increase carbohydrates (for review see Lubieniechi et al., 2012; Lindedam et al., 2012; Saha and Ramachandran, 2013; Yasarla et al., 2013; Bioimprove); how to construct an efficient supply chain of raw material before it reaches the industrial site (Matisons et al., 2012; Mansoornejad et al., 2013); how to perform liquefaction of the raw material including pretreatment, hydrolysis and the development of enzymes (Szijarto et al., 2011; Galbe and Zacchi, 2012); how to mechanically handle the slurry in order to reach optimal conditions (Palmqvist et al., 2011; Wiman et al., 2011); which fermentation technique is to be used (Laluce et al., 2011; Olsson et al., 2012; Erdei et al., 2013; Koppram et al., 2013); and how to separate the desired product (Abels et al., 2013). Research fields spanning over the entire chain are how to determine and perform the required analysis (Lindedam et al., 2010; Hansen et al., 2013; Pribowo et al., 2013) and how to make a process economically feasible (Sassner et al., 2008; Macrelli et al., 2012).

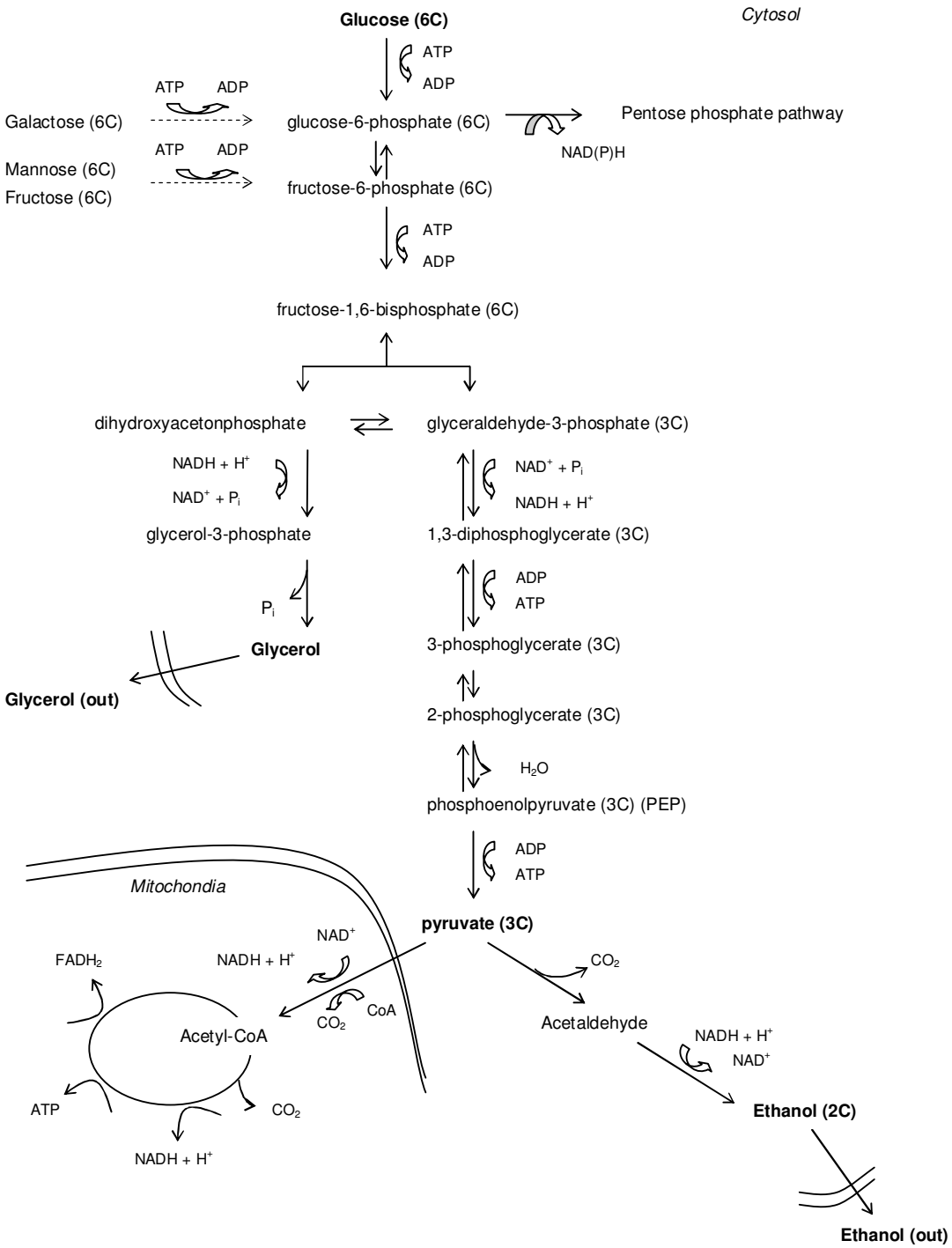

Figure 4 | A schematic view of glycolysis, ethanol and glycerol producing pathway, and TCA cycle in *Saccharomyces cerevisiae.*

2.5 The central carbon metabolism of *Saccharomyces cerevisiae*

Long before the molecular biology of *S. cerevisiae* was known, mankind used this organism to develop alcoholic beverages and other foodstuff. This technique, fermentation of sugars by a culture of *S. cerevisiae* in order to produce ethanol, may be more than 7000 years old (McGovern et al., 1996). The yeast genome was characterized in 1996; it was the first eukaryotic organism to be completely sequenced (Goffeau et al., 1996).

The central carbon metabolism refers to the set of biochemical reactions that serves three important functions for all cellular metabolism. They provide the cell with energy in the form of ATP, reducing power in the form of NAD(P)H, and carbon structures for biosynthesis in the form of a number of different low molecular weight compounds. All pathways in the central carbon metabolism generate precursors for biomass formation and therefore cooperate and are tuned to meet the requirements for building blocks and energy of the cell (Nielsen, 2003). Glycolysis and pentose phosphate pathway (PPP) are reactions included in the central carbon metabolism that occur in the cytosol of the yeast (Fig. 4). When a sugar molecule is taken up by the cell it enters the glycolysis, which refers to a set of reactions in which the sugar is converted to pyruvate while ATP and NADH are generated. Three of the glycolytic reactions are irreversible, the hexokinase and phosphofructokinase reactions and the pyruvate kinase reaction. The glyoxylate cycle is essential to produce carbohydrates during growth on compounds containing two carbon sources, *e.g.* ethanol and acetate, but also during lipid oxidation. The PPP generates the reducing agent NADPH to be used in *e.g* the synthesis of lipids and fatty acids (Lagunas and Gancedo, 1973). It also produces precursor for synthesis of nucleotides and nucleic acids (Berg et al., 2002).

Under aerobic conditions pyruvate from glycolysis is transported into the mitochondria and enters the citric acid cycle through acetyl coenzyme A. The citric acid cycle, also known as the tricarboxylic acid cycle (TCA-cycle) or Krebs cycle, is an amphibolic pathway generating CO_2, NADH and $FADH_2$, GTP or ATP along with biomass precursors. An amphibolic pathway consists both of an anabolic part *e.g.* formations of precursors of biosynthetic reaction, as well as formation of NADH and FADH, GTP or ATP.

S. cerevisiae is a facultative anaerobe meaning that it can grow aerobically and anaerobically. In presence of oxygen *S. cerevisiae* can exhibit both respiratory and respiro-fermentative metabolism, called the Crabtree effect. Aerobic growth is therefore regulated by the surrounding conditions such as level and type of carbon source and the presence of oxygen (De Deken, 1966).

During respiratory metabolism the reducing agents produced in the TCA-cycle, *i.e.* NADH and FADH, are oxidized in the electron transport chain, where O_2 is the final electron acceptor, forming water and pumping protons over the mitochondrial inner membrane generating a proton gradient (oxidative phosphorylation). The energy derived from the proton gradient drives the membrane-bound protein complex, ATPase (Berg et al., 2002), resulting in the generation of maximum, 28 mol of ATP per mol of hexose. The amount of ATP produced can be regulated by adjustment of the P/O ratio, *i.e.* the number of ADP molecules being phosphorylated per e^--pair transferred to O_2 (Verduyn et al., 1991). In *S. cerevisiae* the cytosolic NADH is either oxidized by the external NADH dehydrogenase (Luttik et al., 1998; Small and McAlister, 1998). Alternatively, the e^- are fed into the respiratory chain via action of the G3P-shuttle (Larsson et al., 1998).

Under anaerobic conditions, on the other hand, ATP can only be formed via substrate-level phosphorylation generating ethanol and CO_2, and only 2 mol of ATP are formed per mol of glucose consumed. Alcoholic fermentation is a redox neutral process meaning that there is no net production or consumption of NADH. The NADH formed when glycaldehyde-3-phosphate is oxidized to 1,3-diphosphoglycerate during glycolysis is reoxidized by alcohol dehydrogenase (ADH) forming ethanol (Bakker et al., 2001) (Fig. 4). The yeast compensates the lower energy production by increasing the glycolytic flux, increasing sugar consumption (Larsson et al., 1997). Since some of the intermediates in glycolysis (3-phosphoglycerate and pyruvate) are used for the synthesis of amino acids, the redox neutral pathway generating ethanol becomes insufficient for the reoxidation of NADH (Albers et al., 1998). This is compensated for by the production of glycerol, regenerating NAD^+ in the conversion of dihydroxyacetone-phosphate to glycerol-3-phosphate. Glycerol is excreted out of the cell. This pathway may channel up to 8-10% of the carbon source (Nordström, 1968; Oura, 1977).

Glycerol, along with trehalose, is also a major compatible solute in *S. cerevisiae* meaning that in rising external osmolarity these compounds are formed in order to prevent water loss

(Nordström, 1968; Blomberg and Adler, 1992). In industrial production of ethanol glycerol formation is undesirable due to the conversion of sugars to glycerol instead of to ethanol. If glycerol is not formed due to osmolarity its production can be regulated by applying balanced microaerobic conditions since respiratory activities account for some of the regeneration of NAD^+ (Franzén, 2003), but despite this some glycerol will most probably always be formed depending on the composition of the substrate (Albers et al., 1996).

In industrial cultivation of *S. cerevisiae* the conditions are normally respiro-fermentative meaning that parts of the ATP are produced via oxidative phosphorylation and parts via substrate-level phosphorylation. Respiration and fermentations are dependent both on the level of available oxygen but also on the level of sugars present in the cultivation vessel. It is suggested that a level of 0.5-0.8 mM of sugars is the threshold value for respiro-fermentative metabolism, even at high levels of dissolved oxygen (Verduyn et al., 1992). In industrial cultivations the respiro-fermentative conditions may result from the fermentation mode used, uneven mixing or uneven dispersion of the added oxygen.

As mentioned above the pathways of carbon metabolism are tightly linked and regulated. Despite this tight regulation yeast show metabolic uncoupling. Metabolic uncoupling refers to, in a broad sense, an imbalance between anabolic requirements and catabolic production of ATP. It is suggested that the catabolic activity can be higher than required for growth, for instance during limitations of nitrogen (Larsson et al., 1995).

The rate of glycolysis can be controlled both by allosteric control of the involved enzymes and by changes in the amount of the glycolytic enzymes (Sierkstra et al., 1992; Daran-Lapujade et al., 2007). The regulation of glycolytic flux also involves adenine nucleotides that function both as allosteric regulators and as important players in their role as substrate and products in various reactions in the glycolysis (Beauvoit et al., 1993; Larsson et al., 2000).

2.6 Bacterial contaminations

Microbial contamination of industrial fermentations of sugar-based feedstocks is a frequent problem and can occur at any stage of the process (Schell et al., 2007; Bischoff et al., 2009). Microbial contamination comprises both wild yeast (Basilio et al., 2008) and bacteria. Bacterial contaminations cause both a decrease in carbohydrate and nutrient level (Bayrock and Ingledew, 2004) and expose the fermenting organism to stress by the production of organic acids (Narendranath et al., 2001). Common bacteria found in industrial ethanol fermentations are listed in Table 1.

Table 1 | A summary of some bacterial and yeast contaminants found in industrial fuel ethanol fermentations

Bacteria	**Feedstock**	**Reference**
Lactobacillus		
pantheris	Wood	Albers and Larsson
buchneri	Wood, Corn	Albers and Larsson; Skinner and Leathers, 2004; Schell et al., 2007; Bischoff et al., 2007
plantarum	Wood, Corn	Albers and Larsson; Schell et al., 2007
fermentum	Wood, Corn	Albers and Larsson; Bischoff et al., 2007
paracasei	Wood, Corn	Albers and Larsson; Schell et al., 2007
rossiae	Wood	Albers and Larsson
acidophilus	Corn	Skinner and Leathers, 2004
amylovorus	Corn	Skinner and Leathers, 2004; Bischoff et al., 2007
brevis	Corn	Skinner and Leathers, 2004; Schell et al., 2007; Bischoff et al., 2007
casei	Corn	Skinner and Leathers, 2004
crispatus	Corn	Skinner and Leathers, 2004; Bischoff et al., 2007
Lactococcus lactis	Corn	Skinner and Leathers, 2004
Leuconostoc carnosum	Corn	Skinner and Leathers, 2004
Acetobacter		
tropicalis	Wood	Albers and Larsson
syzygii	Wood	Albers and Larsson
peroxydans	Wood	Albers and Larsson
Pedicoccus acidilactic	Corn	Skinner and Leathers, 2004; Schell et al., 2007
Clostridium sp.	Corn	Skinner and Leathers, 2004

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Yeast	Feedstock	Reference
Candida sp.	Sugar cane	Basilio et al., 2008
Dekkera bruxelensis	Sugar cane	Basilio et al., 2008
Exophiala dermatitides	Sugar cane	Basilio et al., 2008
Pichia sp.	Sugar cane	Basilio et al., 2008
Hanseniaspora guilliermondii	Sugar cane	Basilio et al., 2008
Zygosaccharomyces fermenti	Sugar cane	Basilio et al., 2008

2.5.1 Antifungal activity by lactic and acetic acid bacteria

The most commonly encountered bacterial contaminants in industrial ethanol production plants are lactic and acetic acid bacteria (Skinner et al., 2004; Bischoff et al., 2007; Schell et al., 2007). The main inhibitory products formed by lactic acid bacteria (LAB) are lactic and acetic acid. These acids lower the pH in any fermentation contributing to a non-optimal pH range for *Saccharomyces cerevisiae,* but the true inhibitory effect of these acids is seen in their undissociated form and their ability to diffuse into the cell. The level of dissociation is dependent on the acids' pKa and pH. Once inside the cell they can dissociate and release H^+. In order to maintain the cytosolic pH, the H^+ are pumped out of the cell by an ATP-driven transport system. Besides lactic and acetic acid, LAB produces a number of other products with antifungal activity, of which a summary can be found in Fig. 5 (Lindgren and Dobrogosz, 1990; Claisse and Lonvaud-Funnel, 2000; Magnusson and Schnürer, 2001; Sjögren et al., 2003; Schnürer and Magnusson, 2005). The species of the genera lactic acid bacteria determine what antifungal product will be formed.

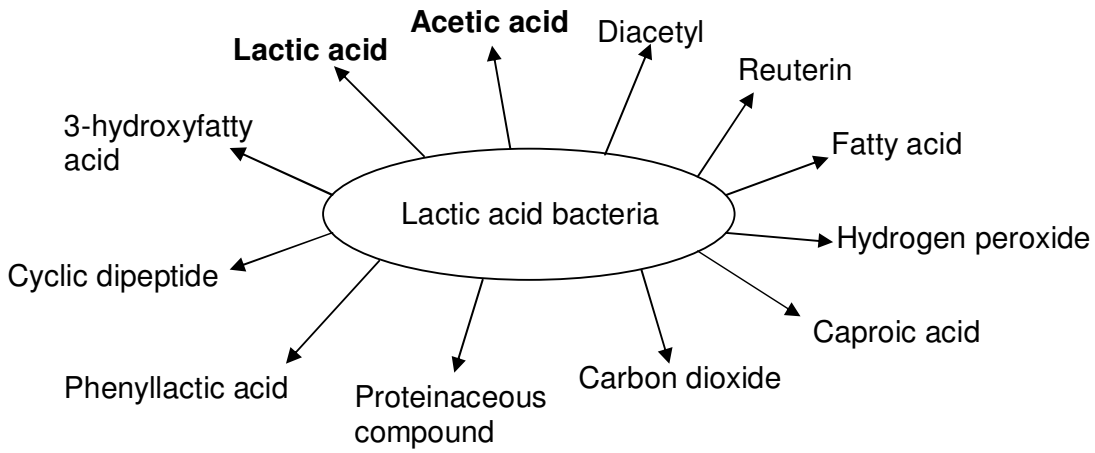

Figure 5 | A schematic view of antifungal products formed by lactic acid bacteria. Lactic and acetic acid are considered the most troublesome in ethanol fermentations by *Saccharomyces cerevisiae* (Modified from Beckner et al., 2011)

3. RESULTS AND DISCUSSIONS

3.1 Bacterial contamination during ethanol fermentation of lignocellulosic material

Ethanol production by *S. cerevisiae* can be negatively affected in numerous ways, one being the presence of bacteria, namely lactic acid bacteria and acetic acid bacteria. Bacterial infections are a fact of life as total sterilization is not practical in this type of industrial production. *S. cerevisiae* is a hexose-consuming yeast leaving the present pentoses untouched and available for other potential organisms to thrive on.

Traditionally, treatments with chemicals, antibiotics and natural compounds including plant-derived compounds and extractives as well as bacteriocins have served as methods for suppressing bacterial contamination in breweries and ethanol fermentation plants. Today bacterial infections are controlled mostly by the use of antibiotics (Jessen, 2012) but concerns are raised about the overuse of antibiotics in industrial applications contributing to the emergence of multi-resistant bacteria harmful to animals and humans. Non-antibiotic methods for controlling bacterial contaminations are *e.g.* a lower pH (pH 2-3) (Gibson et al., 2007), different products derived from natural compounds *e.g.* hop bitter acids (Moir, 2000; BetaTec Hopfenproducte GmbH, Germany) and a variety of different chemicals *e.g.* chlorine dioxide (DuPont). However, these products do not always function as expected and there is still a need to find additional ways to prevent bacteria from flourishing in yeast-based fermentation processes.

This project investigated two possible strategies for eliminating bacterial contaminations in fermentation of lignocellulosic material: 1) usage of new fresh yeast called a pitching agent, and 2) treatment with a certain amount of sodium chloride and ethanol.

3.1.1 Combating bacterial contaminations by use of a pitching agent

A pitching agent often refers to new, fresh yeast added to a brew or wine fermentation in order to boost the fermentation, increasing the alcohol concentration and enhance the taste of the final product. The term “pitching agent” here refers to new, fresh yeast, cultivated via any of several available methods, in order to maintain a high viability of the yeast culture in the fermentation.

In the study of cell cultures a number of cultivation methods may be applied depending on the purpose of the study. This study is based on a fed batch methodology during cultivation of the pitching agent and a batch methodology during fermentation. By using a fed batch methodology during cultivation it was possible to adapt the yeast cells to the different cultivation media in order to study a potential difference in their fermentation fitness in the subsequent fermentation (see Box 1 for a description of adaptation).

Box 1 | Adaptation

Adaptation of a microorganism refers to the movement of a population towards a phenotype that best fits the present environment (Orr, 2005), but an exact definition of the term does not exist (Lewontin, 1957; Reeve and Sherman 1993). Adaptation processes are performed by organisms spontaneously in order to survive but can also be used for industrial purposes, for example by the development of certain desired traits such as increased tolerance towards inhibitors and a broadened consumption spectrum of substrate utilisation. The former is more common. An example of adaptive evolution in a *S. cerevisiae* wine yeast strain is the tolerance towards sulfite that is suggested to be due to a gross chromosomal rearrangement of the sulfite efflux gene *SSU1* and *EMC34* genes which leads to an overexpression of the *SSU1* gene (for review see Querol et al., 2003).

S. cerevisiae is considered a robust microorganism partly due to its possibility to adapt to a variety of stress factors related to a changing environment. Many industrial strains of *S. cerevisisae* are diploid, aneuploid or polyploid which has been suggested to be one of the reasons for its high adaptive performance (for review see Querol et al., 2003).

It is well known that adaptation towards one stress factor may in many cases also induce tolerance towards other stress factors (Simpson and Ashe, 2012). This phenomenon is considered to be due to the action of the so-called general stress response (GSR) which is activated by *e.g.* oxidative, pH, heat and osmotic stresses as well as nitrogen starvation. The action of the GSR has been reported to be due to activation of a stress response element consisting of a CCCCT sequence within the promoter region of the activated genes (for review see Gibson et al., 2007). Gasch et al. (2000) found that the stress response genes were triggered upon transition to non-optimal conditions but not in the opposite direction suggesting that the cell respond in a broad manner when subjected to non-optimal conditions. Sánchez et al. (1992) and Sales et al. (2000) have suggested that certain heat shock proteins (Hsp104p and Hsp12) are involved in the tolerance towards several other stress conditions also.

One way of using adaptation to harsh conditions offered by lignocellulosic media is to gradually increase its proportion during cultivation by *e.g.* fed-batch culture, pre-culture(s) with increasing concentrations of lignocellulose etc (Paper I).

3.1.2 Potential effects of a pitching agent and the role of cultivation

It was found that lactic acid bacteria concentrations decreased when a pitching agent grown in nutrient-supplemented SSL was used (Paper I). This pitching agent also gave the highest ethanol concentration and the highest number of viable yeast cells after 12 hours of fermentation. If the fresh yeast is cultivated in a mix of rich nutrients (YD) and SSL it is suggested that the yeast will be able to produce a biomass with proper levels of *e.g.* nutrients, proteins, and energy to sustain a high performance and viability in the harsh, nutrient-poor environment offered by SSL. This may decrease the lag phase and result in a higher productivity as well as a longer life span, which would be of importance in *e.g.* continuous fermentations where the yeast is exposed to the challenging conditions over an extended time period.

The use of pitching agents grown only in nutrient-rich substrate without the fermentation media (YD) or only in the fermentation media resulted in lower ethanol concentrations, lower numbers of viable yeast cells and an increased number of viable bacterial cells compared to pitching agents grown in a combination of YD and SSL. This suggests that both the production of specific enzymes and proteins necessary for growth in lignocellulosic material and an increased viability offered by the addition of nutrients is necessary for the suppression of bacteria and increased ethanol production (Paper I).

The use of dry yeast as a pitching agent did not have any effect at all on the fermentative performance of the culture (Paper I).

3.1.3 Combating bacterial infections by the help of chemicals

The use of chemicals for combating bacterial infections in ethanol production plants is today mainly focused on antibiotics, *e.g.* virginiamycin (Skinner and Leathers, 2004), penicillin G, streptomycin or tetracycline (Aquarone, 1960; Day et al., 1954; Bayrock et al., 2003) but a number of other chemicals such as ammonia (Broda and Grajek, 2009) and hydrogen peroxide (Muthaijan and Ricke, 2010) have also been considered as potential antimicrobial agents (Table 2). In addition to antimicrobial agents against bacteria, there are also a number of agents used to control the level of wild yeast in fermentation. The issue of contamination by wild yeast in bioethanol production will not be discussed here, but a summary of antimicrobial agents can be found in Table 2.

Table 2 | A summary of some antimicrobial agents

Antimicrobial agent	**Microbial target**	**Reference**
Antibiotics	Bacteria	Day et al., 1954; Lushia and Heist, 2005
Hydrogen peroxide	Bacteria	Review: Muthaijan and Ricke, 2010
SO_2	Fungi/Bacteria	Loureiro, 2003; Du Toit et al., 2005
Ammonia	Fungi/Bacteria	Broda and Grajek, 2009
Chitosan	Bacteria	Escudero-Abarca et al., 2004
Hydroxycinnamates	Fungi	Neves et al., 1994
Organic acids	Bacteria	Neves et al., 1994; Simpson and Hammond, 1989
Nisin	Bacteria	Review: Muthaijan and Ricke, 2010
Hop	Bacteria	Moir, 2000
TCC	Bacteria	Oliva-Neto and Yokoya, 1998
PMB	Bacteria	Gibbson and Westby, 1986
Sulfite	Bacteria	Chang et al., 1997
Sulphuric acid	Bacteria	Cunningham and Stewart, 1998
Phosphoric acid	Bacteria	Tang et al., 2010
Bacteriophage lytic enzymes	Bacteria	Roach et al., 2013
NaCl/EtOH	Bacteria	Paper II

3.1.4 The effect of sodium chloride and ethanol on bacterial contaminations

In Paper II different process conditions tested were the addition of NaCl, sugar, ethanol and low pH, which are all known antimicrobial agents, but no single parameter was effective against bacterial contamination in lignocellulosic fermentations. Instead a combination of the above mentioned conditions were tested; a combination of NaCl and ethanol best prevented bacterial contamination without affecting yeast growth in fermentations of lignocellulosic material. This was tested both with a combination of selected bacteria and with a complete natural industrial microbial community.

Investigations with selected bacteria indicated a clear correlation between addition of sodium chloride plus ethanol and a decreased bacterial viability without affecting yeast growth (Paper II). Addition of sodium chloride increases the osmotic pressure and is known to induce cell shrinkage, up-regulate starvation genes and inhibit cell growth by decreasing DNA replication (Csonka, 1989; Csonka and Hanson, 1991; Wood, 2011; Pilizota and Shaevitz, 2012). It has also been suggested that osmotic stress affects membrane fatty acid composition (Guillot et al., 2000). Ethanol affects the bacterial cell by increasing the permeability of the plasma membrane, inducing an uncontrolled leakage (Dombeck and Ingram, 1984). The ethanol would in this case act as a barrier breaker, decreasing the ability of the cell to maintain its intracellular ionic composition, pH and metabolites levels (Csonka et al., 1991); in combination with sodium chloride, this leads to cell deterioration. It is also suggested that sodium salt has a specific negative effect apart from the osmotic stress effect.

Investigations with a complete industrial microbial community confirmed this observation but intensive fine-tuning of the amount of NaCl and ethanol added was required depending on the substrate used. This underscores the importance of process-specific treatment (Paper II).

Regarding combined strategies for dealing with infections, sodium chloride has been suggested by other authors as an effective antimicrobial agent in fermentations of non-cellulosic media, in combinations with low pH (Abu-Ghazaleh, 2010) and nisin (Chollet et al., 2008).

3.2 Findings regarding the energy metabolism of *Saccharomyces cerevisiae* during fermentation of lignocellulosic material

In order to control an industrial fermentation, for ethanol production or any other fermentation product, it is of interest to know how the energy metabolism of the fermenting organism is affected by the prevailing conditions.

Energy metabolism is essential for cell growth and therefore also for product formation. Fermentations of lignocellulosic material impose a high stress on the fermenting organism even though this can be mitigated by the choice of hydrolysis and fermentation method.

It has been shown that *S. cerevisiae* have a greater potential to produce ethanol if adapted to the fermentation media prior to fermentation (Paper I, Falla et al., 2013). In order to further understand this physiological behavior the energy metabolism and the fermentation capacity were studied.

3.2.1 The effect of lignocellulosic material on the energy metabolism of *Saccharomyces cerevisiae*

Lignocellulosic materials are a diverse group of substrates; depending on how the hydrolysis is performed, different stress factors may be present (Klinke et al., 2004). In batch cultures the production of ethanol is highest during exponential growth of yeast, and during this stage the ATP consumption and production is high. Ethanol production can sometimes continue after a decrease of cell growth, for several reasons. One reason may be an uncoupling between the anabolic energy requirements and the catabolic energy production. If, for example, the biomass formation is hampered due to limited amounts of a nutrient, the glucose consumption will remain high, with a flux towards ethanol production, generating ATP through a respiro-fermentative pathway (Larsson et al., 1997). The sugar degradation products present in lignocellulosics, HMF and furfural are in low concentrations consumed by the yeast, leading to an increased consumption of ATP (Petersson and Lidén, 2007). The organic acids present lower intracellular pH, which also leads to an increased demand for ATP as excess protons are pumped out of the cell (Taherzadeh et al., 1997). In a non-inhibitory media an increased consumption of ATP can lower biomass formation and increase the ethanol production rate

through an increased glycolytic flux, but in an environment containing many stress factors an increased demand for ATP can lead to a dysfunctional glycolysis resulting in decreased cell growth and ethanol productivity.

Box 2 | A review of earlier work on nutrient supplementation to lignocellulosic material

It is generally recognised that fermentation performance can be improved by the presence of nutrients (Jones and Ingledew, 1994; Casey et al., 1984). The lack of nutrients during ethanol production diminishes the catabolic capacity, probably due to several factors. It is known that glucose transport capacity, the level of storage carbohydrates, and allosteric effectors along with the concentration of glycolytic enzymes play a crucial role in tolerance to nutrient starvation (Larsson et al., 1997; Larsson et al., 2000; van Hoek et al., 2000; Thomsson et al., 2003; Thomsson et al., 2005). The prevailing conditions when depletion of nutrients occurs influence the entire protein and molecular setup of the cell, causing it to respond to nutrient starvation and subsequent nutrient addition in different ways. The act of nutrient supplementation therefore needs to be considered in relation to the process in question.

Commonly-employed chemical compounds for studies on nutrient supplementation of lignocellulosic-based fermentations are urea, sulphates and phosphates, which work as nitrogen and/or mineral sources (Asgher et al., 2010) but others are also considered. Brandberg et al. (2005) investigated the effect of yeast extract supplementation to dilute-acid spruce hydrolysate but found this insufficient to reach steady state. Jorgensen (2009) performed an SHF on high-gravity wheat straw hydrolysate and found that supplementation of yeast extract to the fermentation improved fermentation performance significantly. Tomás-Pejó et al. (2011) found a positive effect on ethanol production from pre-treated wheat straw using an inoculum grown in the presence of urea and $(NH_4)_2HPO_4$: $(NH_4)SO_4$. The studies of nutrient supplementations to lignocellulosic fermentations are all performed differently with respect to raw material, strain and mode of operation, which makes comparison between them difficult.

In this research work physiological effects including nucleotide levels in the cells were studied using *Saccharomyces cerevisiae* Thermosacc, and *Saccharomyces cerevisiae* CCUG 53310 in undiluted dilute-acid spruce hydrolysate (SH) and spent sulfite liquor (SSL), with and without yeast extract, respectively. The experiments were performed in batch mode during 22 hours (Paper III), and sequential batch mode continued for six batches (Paper IV).

The chemical composition of the two substrates is listed in Table 3. The inhibitory compounds that can be observed are the phenolic compounds, HMF, furfural and organic acids that reach a total concentration of 5, 2, 2 and 8.4 g/l, respectively in SH while SSL only contains 1.0 g/l phenolic compounds, 0.3, 0.2 and 6.5 g/l of HMF, furfural and organics acids, respectively (see section 2.1.3 and 2.1.4 for a description of phenolic compounds).

Table 3 | An example of the difference in composition between two different lignocellulosic materials depending on the hydrolysis method used. The spruce hydrolysate is treated with dilute acid in a demonstration plant with a capacity of 1000 kg day^{-1} (SEKAB E-Technology, Örnsköldsvik, Sweden). The spent sulfite liquor is derived from a commercial biorefinary production plant based on a sulfite process (Domsjö Fabriker, Aditya Birla, Örnsköldsvik, Sweden).

Chemical compound	Spruce hydrolysate	Spent sulfite liquor
Sulfite	1.1	3.8
Glucose	34.0	9.0
Mannose	33.0	24.0
Galactose	7.0	4.0
Furfural	2.0	0.2
HMF	2.0	0.3
Acetic acid	7.0	5.0
Formic acid	0.4	1.2
Levulinic acid	1.0	0.3
Phenolic compounds	5.0	1.0

Fermentation using spruce hydrolysate as media and CCUG as fermenting organism revealed an increased fermentative performance when supplemented with yeast extract. This increase in hexose consumption and ethanol production (Fig. 6) indicates an increased metabolic activity towards ethanol production. This increased metabolic activity upon nutrient addition also reflects the severity imposed by the substrate, showing a decreased ATP concentration

and an increased AMP concentration in the absence of nutrients. The energy charge (see Box 3) in this system was therefore also low, 0.5, due to an increased concentration of AMP and a decreased level of ATP indicating low viability of the population probably due to high maintenance requirements. Despite the increase in ethanol production upon addition of nutrients, the ethanol titer remained low for CCUG fermenting SH (Paper III).

Box 3 | Energy charge

The importance of the adenine nucleotides as major regulatory factors in controlling metabolic processes within a cell is well established. The energy charge is a numeric value of the total concentration of metabolically available energy stored in the adenylate pool, in ATP, ADP and AMP. It was first defined by Atkinson in 1968 using the following equation:

$$EC = ([ATP] + \tfrac{1}{2}[ADP]) / ([ATP] + [ADP] + [AMP])$$

The energy charge in almost all well-functioning cells lies in the range between 0.8 and 0.9.

As the response mechanisms to the combination of nutrient starvation and lignocellulosic inhibitors in *S. cerevisiae* are still somewhat unclear, the control of the metabolic pathways upon nutrient addition to a fermentation using non-detoxified lignocellulosic substrate is complex. What is suggested is that HMF and furfural, when present in sufficient amounts, redirect energy to repair cellular damage imposed by these compounds (for review see Almeida et al., 2007). It is also suggested that they affect the redox balance negatively while leaving the energy metabolism unaffected (Ask et al., 2013; Bajwa et al., 2013). Other negative effects have also been reported (Modig et al., 2002). The weak acids, on the other hand, may contribute to intracellular anion accumulation and affect the sugar uptake system and energy metabolism through uncoupling (for review see Almeida et al., 2007; Abbott et al., 2007; Bajwa et al., 2013). The inhibition mechanisms of the phenolic compounds are yet not known, partly due to the heterogeneity of the group but it has been suggested that these compounds contribute more to decreased ethanol productivity than to a decreased final ethanol yield (Almeida et al., 2007). Some studies are done on, for example, vanillin, which is

considered one of the most troublesome phenolic compounds mainly due to the low concentrations of it needed for inhibition (Klinke et al., 2004). It is suggested that this compound is converted to vanillic acid and vanillyl alcohol in yeast (Larsson S et al., 2000; Fitzgerald et al., 2003). The minimum inhibitory concentration of vanillin in non-lignocellulosic media has earlier been shown to be 3 g/l for *S. cerevisiae* (Fitzgerald et al., 2003). As lignocellulosic media consist of many inhibitors, both inherent and formed, this concentration can only serve as an indication of the tolerance of *S. cerevisiae* to vanillin. Ayako et al. (2008) showed a decreased tolerance towards vanillin if subjected to additional stress factors. Almeida et al. (2007) listed common phenolic compounds present in spruce hydrolysates produced with dilute acid, and summarized potential metabolic effects of phenolics. An explanation of the inhibitory effects of phenolics was suggested to be an increased permeability of cellular membranes and destruction of the electrochemical gradient by transportation of the protons back into the mitochondrial matrix. Addition of nutrients, especially a complex nutrient source such as yeast extract, may provide the cells with additional molecules and compounds that help the cells to cope with the inhibitors present in lignocellulosic material, thereafter showing the increased hexose consumption and ethanol production revealed (Paper III and IV).

With spruce hydrolysate as fermentation media, nutrient addition did not help CCUG to maintain a high ATP level, although it did not decrease as much as in the absence of nutrients (Paper III). The ATP level in Thermosacc fermenting spruce hydrolysate was high during the whole fermentation, indicating a higher tolerance to the stress factors imposed by this substrate in comparison to CCUG. The energy charge for Thermosacc was high, irrespective of nutrient addition. The fact that Thermosacc maintained a low level of AMP and a reasonably high ATP level still suggests a low impact of spruce hydrolysate on this strain (Paper III).

When SSL was used as a fermentation media, a somewhat different scenario unfolded (Paper III). The CCUG strain could, in this substrate, maintain a high ATP level and low AMP level throughout the fermentation, with or without nutrient addition. Thermosacc, on the other hand, noticeably decreased ATP levels, but no increase in AMP was detected (Paper III). The decreased ATP level expressed by Thermosacc together with the fact that both CCUG and Thermosacc had high hexose consumption and final ethanol titer in this condition suggests that Thermosacc is more severely affected by SSL and may have problems maintaining a high

ethanol production in fermentations over a longer period of time, while CCUG is less affected and will perform well. The ability of sustained physiological activity was tested in sequential batch fermentations revealing a high ethanol titer for both strains but a lower biomass formation for Thermosacc. No difference in the level of adenine nucleotides between the two strains could be detected (Paper IV).

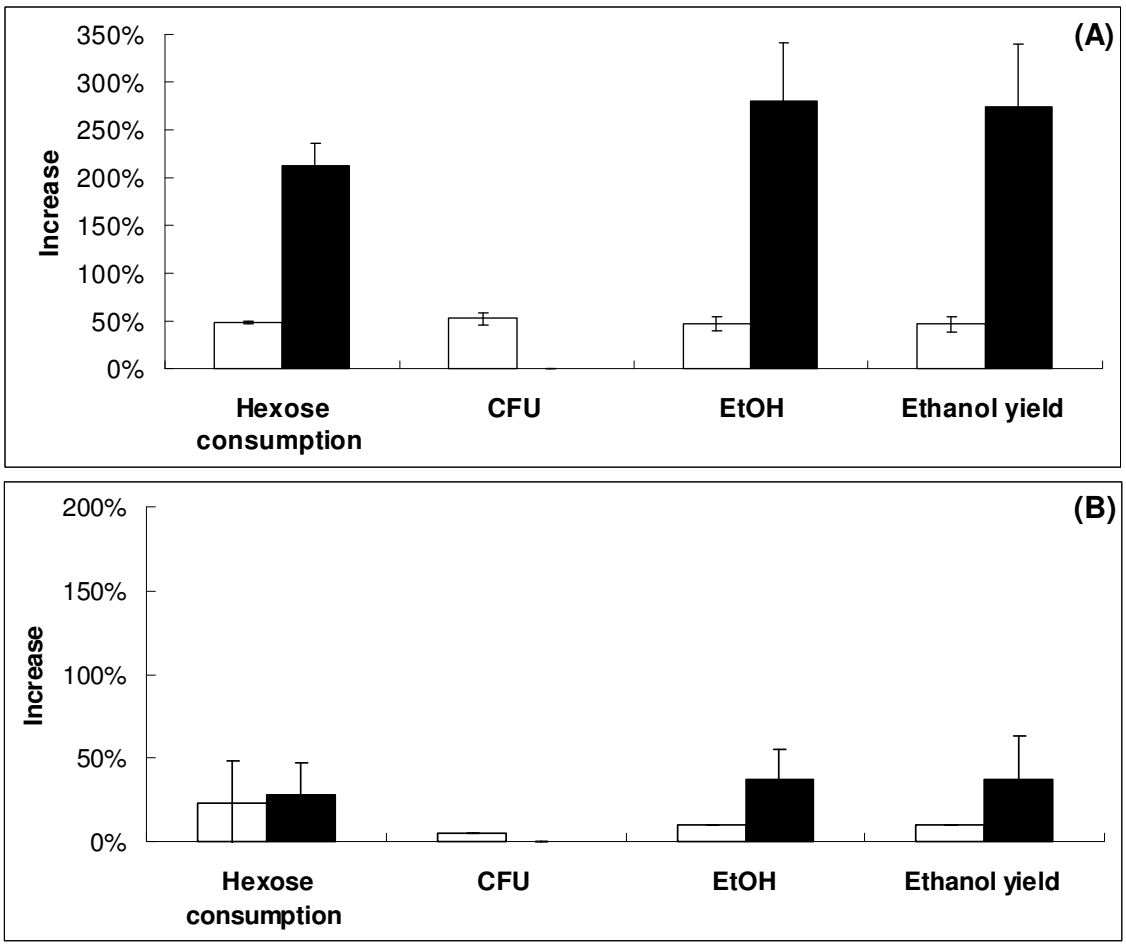

Figure 6 | Increase in fermentation performance upon supplementation with yeast extract. (A) The fermentation substrate is spruce hydrolysate. (B) The fermentation substrate is SSL. Black bars indicate values for Thermosacc, gray bars indicate values for CCUG. Error bars indicate max/min values from two separate fermentations.

As mentioned earlier, the level of adenine nucleotides may reveal the tolerance of an organism to a certain fermentation medium, but the reactions involved in energy metabolism and their response to complex substrates, such as lignocellulosics, are complex. The many pathways and reactions are both energy-requiring, and energy-producing, and are also

regulated by the absolute amount of adenine nucleotides which makes the overall picture very complicated. Other methods are therefore needed to support or reject data received from measurements of adenine nucleotides.

Fermentation capacity may be used as such additional tool to investigate the fitness of an organism to the substrate in question. The definition of fermentation capacity varies in the literature but the definition used during this research work is the following:

> The ability of the yeast to regain its fermentative performance when exposed to non-inhibitory nutrient-rich media after cultivation in inhibitory media.

The ability of a cell to regain a high fermentative performance after cultivation in inhibitory media is a measurement of how severely the inhibitory media has affected the fermenting organism. The different challenges offered by SH and SSL are reflected in the fermentation capacity of the two strains along with the detected differences in adenine nucleotide levels. The fermentation capacity of CCUG harvested from an SH fermentation is quickly decreased without regaining any fermentative capacity during the 22-hour fermentation unless nutrients are added (Fig. 7).The fermentation capacity of Thermosacc in the same environment was considerably higher but still somewhat decreasing suggesting that Thermosacc is not unaffected by SH but can still withstand the challenges imposed by this substrate in a much better way than CCUG (Paper III).

Supplementing the SH fermentation in question with yeast extract increased fermentation capacity for both strains (Fig. 7). The effect of nutrient addition to CCUG was seen first after 22-hour fermentation. The increased fermentation capacity was accompanied by increased sugar consumption and ethanol production in the original SH-based fermentation for both strains. For CCUG the level of residual hexoses was still rather high, but for Thermosacc addition of yeast extract resulted in a complete consumption of hexoses. This suggests that the fermentation capacity is positively affected by nutrient addition. The positive effect of nutrient addition may be explained by a nutrient limitation in the lignocellulosic material but also by an increased demand of nutrients upon the presence of inhibitory compounds.

SSL-based fermentations, on the other hand, affected both strains more negatively, revealing a fermentation capacity below 2 mmol $(g_{CDW} \cdot h)^{-1}$, which is in the same range as for CCUG

fermenting SH without nutrients (Paper III and IV). Addition of yeast extract increased the fermentation capacity for CCUG to the same level as Thermosacc fermenting SH, while it had no effect on the fermentation capacity for Thermosacc. The level of fermentation capacity was also again unaffected by carbon and energy limitation prevailing in the SSL fermentation. The inability of Thermosacc to regain its fermentative performance suggests that excess of nutrients does not help to increase the fermentation capacity for this strain after cultivation in SSL.

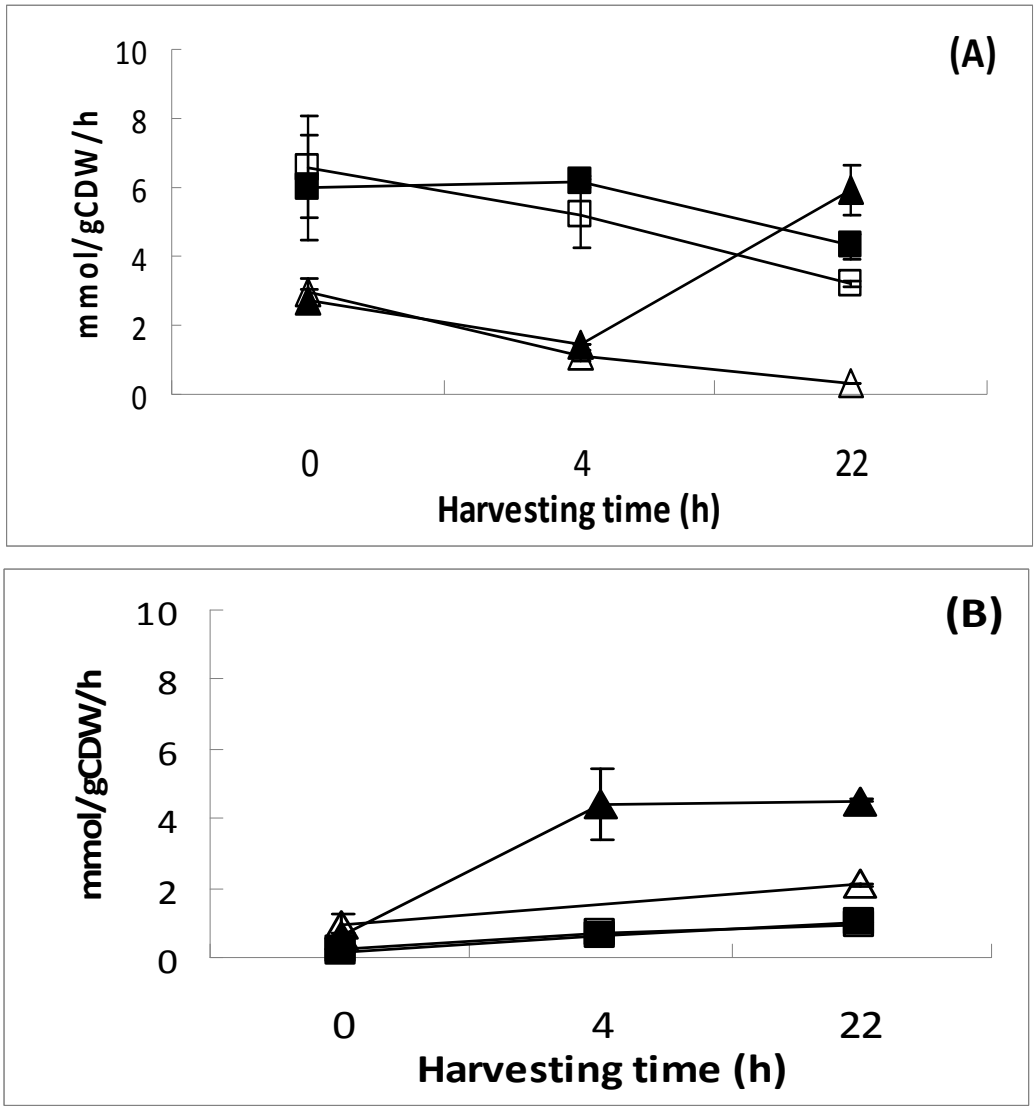

Figure 7 | Fermentation capacity upon reinoculation into YPD media. ▲represents CCUG and ■ represents Thermosacc. Open symbols represent cells harvested from fermentations without nutrient supplementation. Closed symbols represent cells harvested from fermentations with nutrient supplementation. (A) Fermentation capacity for cells harvested from SH. (B) Fermentation capacity for cells harvested from SSL. Error bars indicate max/min values from two separate fermentations (Paper III).

3.2.1.1 Sequential batches

Industrial fermentations of ligncoellulosics often deteriorate periodically. To study the recovery potential of yeast cells, a combination of oxygen and nutrients were applied after sequential batch fermentation, using SSL as fermentation media along with the two *S. cerevisiae* strains previously described (Paper IV). For an overview of the effects of oxygen on yeast physiology see Box 4. In this study supplementation of oxygen did not have any effect on fermentative performance or biomass formation, irrespective of the background of the fermenting organism. Supplementation of nutrients on the other hand made it possible to maintain the fermentative capacity for both strains (Fig. 8), suggesting that yeast extract may be added periodically to eliminate a decreased ethanol production.

The difference in fermentation capacity between the two strains seen in single-batch fermentations (Paper III) was maintained during sequential-batch fermentations. Thermosacc could hardly manage to uphold a fermentative capacity while CCUG showed an increased fermentation capacity during the first three batches, suggesting a more adapted metabolism of this strain to SSL (Fig. 8) (Paper IV).

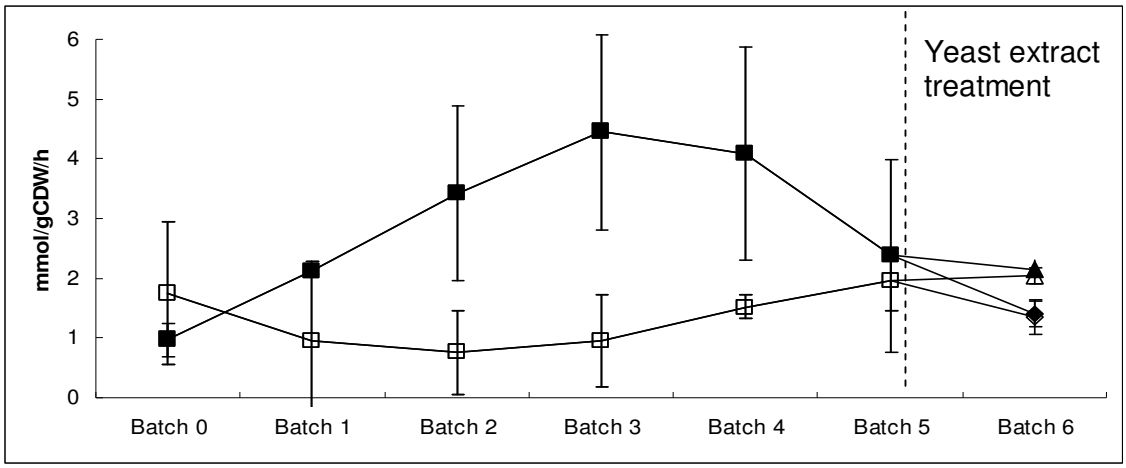

Figure 8 | Fermentation capacity in mmol ethanol $(g_{CDW} \cdot h)^{-1}$ for ■CCUG and □Thermosacc during sequential batch fermentations based on SSL as fermenting substrate. Each batch prolonged for 24 hours. ▲ and Δ indicate yeast extract supplementation. ◊ and ♦ indicate fermentations without yeast extract. Error bars for batches 0-5 indicate values from two separate fermentations. Error bars for batch 6 indicate standard deviation from four fermentations.

Yeast extract is a promising nutrient supplement in lignocellulosic fermentations, but it is also an expensive nutrient and it is therefore of interest to find industrial sustainable methods in the use of yeast extract. Studies have been made on different concentrations of yeast extract in order to decrease the use. This study instead suggests that yeast extract can be added as a pulse treatment after a period of repeated batches (Paper IV). Here a concentration of 10 g l^{-1} yeast extract was used and no elaboration on the concentration was made.

Box 4 | The effect of oxygen on the physiology of *Saccharomyces cerevisiae*

Oxygen is essential for many eukaryotic cells, but as *S. cerevisiae* is a facultative anaerobe, it can grow in the presence or absence of oxygen. However, oxygen has many positive effects on the biomass, *e.g.* increased ethanol tolerance and viability (Hoppe and Hansford, 1984; Ryu et al., 1984). The level of oxygen determines the consumption route of the hexoses. In industrial bioreactors an uneven distribution of oxygen is inevitable due to poor mixing and/or high dry-matter content. This leads to an oxygen gradient with which yeast has to cope. Many studies referring to the physiological effects of oxygen on *S. cerevisiae* are made under fully aerobic or fully anaerobic conditions (Van den Brink et al., 2008), but some have also taken into consideration the industrial obstacle of uneven distribution of the oxygen (Wiebe et al., 2008; Rintala et al., 2008; Rintala et al., 2009; Rintala et al., 2011).

Van den Brink et al. (2008) suggested that the ability of *S. cerevisiae* to perform a rapid regulation of glycolytic flux from respiration to fermentative metabolism is performed in two phases. (i) The initial response is only a regulation in concentrations of low molecular weight substrates, products and effectors; then (ii) a second phase occurs in which the regulation is accomplished by a mix of the concentration of metabolites and the activity of the glycolytic enzymes.

Rintala et al. (2011) have suggested that the transcriptional response of *S. cerevisiae* to sudden anaerobiosis is similar irrespective of the history of the yeast. It was observed that genes related to growth and cell proliferation, mitochondrial translation, protein import and sulfite assimilation were transient down-regulated, while genes related to protein degradation were transient up-regulated. Daran-Lapujade et al. (2007) and de Groot et al. (2007) suggests that glycolytic flux changes are regulated on a post-transcriptional level.

Rintala et al. (2009) reported an up-regulation of genes involved in the respiratory pathway suggesting that in environments with intermediate levels of available oxygen the energy formation efficiency has to be increased. This up-regulation was not seen in either fully anaerobic or fully aerobic conditions. An intermediate oxygen supply also revealed a difference in physiology only on a proteomic level supporting the theory of a post-transcriptional regulation.

3.2.2 Selection of yeast strain

The results retained of the two types of media SH and SSL showed the importance of choosing the right strain for different conditions. Thermosacc showed good fermentable capacity while CCUG was outcompeted in SH (Paper III and IV).

The fact that Thermosacc possessed a high ethanol production performance in SH but had problems maintaining the energy balance in SSL suggests a complexity of the SSL beyond the common inhibitors present in lignocellulosic material, *i.e.* furans, weak acids and phenolics. The intolerance of CCUG to SH may be a response to its increased sensibility to phenolics presented by Westman et al. (2012), pointing to the importance of selecting a microorganism suitable to the process in question.

Microorganisms contribute to the complexity and variety of the biosphere through so-called natural evolution. This contributes to the fitness of the microorganisms in their particular environments (Sauer, 2001) and may therefore be considered the foundation of the development of tolerant microorganisms. Tolerant microorganisms are a prerequisite in the fermentation of lignocellulosic-based substrates due to both the inherent concentration of inhibitors and the increasing demand for high-gravity substrates. Use of high-gravity substrates imposes additional stress on the fermenting organisms, on top of the normal stresses during fermentation. These stresses are mainly of osmotic nature as well as imposed by an increased concentration of inhibitors. Choosing a yeast strain adapted to these conditions is therefore of utmost importance both for direct use and further metabolic engineering.

Numerous studies have pointed to the importance of strain selection in order to achieve high productivity of the desired product (Modig et al., 2008; Berg, 1995). In order to investigate if there exist even better strains we made a screening of 15 strains isolated from different habitats in Ecuador. The comparison was done by performing a pre-culture in which a short adaptation procedure was conducted using 25% SSL in nutrition-rich, non-inhibitory media (YPD) prior to fermentation in 100% SSL. The consumption of glucose and xylose were measured along with the production of ethanol, number of cells and viability. Other metabolites were not analyzed. Little or no growth was detected after 22 hours of fermentation while two species produced approximately 75% ethanol of theoretical yield on

total hexoses which is considered acceptable with respect to the fermentation mode and the short adaptation procedure (Paper IV).

The two most promising strains, both belonging to the genera *Saccharomyces*, were isolated from the cacao fruit and from sap from the tree *Bursera graveolens*.

These two habitats offer distinct challenges and possibilities for growth of microorganisms. *Bursera graveolens* is used in folk medicine in South America for its richness in essential oils and suggested antimicrobial activity (Robles et al., 2005). High amounts of benzofuranoid compounds and monoterpenoids such as limonene, and also triterpenoids has been reported among the volatile metabolites in the stem, bark and resins of *B. graveolens* (Robles et al., 2005; Munoz-Acevedo et al., 2012; Monzote et al., 2012). The terpenes are known for being agents of defense in many plants and trees, including spruce. The cacao fruit pulp is considered a rich media for growth and the peel has, to my knowledge, not been reported to offer any demanding challenges for yeast growth (Schwan and Wheals, 2004).

4. CONCLUDING REMARKS

Microbial contaminations occur at any ethanol fermentation plant. They may at minimum affect the efficiency of fermentations and at worst lead to stuck or sluggish fermentations, requiring shutdown. Both scenarios will lead to unnecessary economic loss and need to be prevented. This research work has identified two possible methods to combat bacterial contamination in fermentations of lignocellulosic material; one concerns the cultivation method of the inoculum in order to prevent a settlement of a bacterial community and one concerns combating an already existing bacterial contamination.

An appropriate cultivation method for combating bacterial infections aims to produce a robust yeast cell culture that can withstand the stress imposed by the substrate and the bacteria existing in most ethanol production plants (Paper I). Other methods primarily minimize the growth of the existing bacterial community, such as treatments with sodium chloride and ethanol investigated during this research work (Paper II). Any method needs to be adjusted to the fermentation plant in consideration in order to work properly.

A robust yeast cell culture is necessary both to withstand a flourishing bacterial contamination and to maintain a high productivity in a production plant. In order to ensure this, a number of parameters and fermentation modes can be varied and an important consideration is the choice of strain. This research work has contributed to the understanding of the importance of strain selection by studying metabolic responses to different lignocellulosic substrates (Paper III and IV).

The energy metabolism of the commercially-available yeast *Saccharomyces cerevisiae* Thermosacc, developed to withstand the high stress factors occurring in fuel ethanol production, proved to be less affected by the dilute-acid spruce hydrolysate than is a strain originally harvested from a commercial ethanol production plant based on spent sulfite liquor, *Saccharomyces cerevisiae* CCUG 53310. The energy metabolism of Thermosacc proved instead to be more negatively affected by the spent sulfite liquor. These results add proof to the importance of strain selection in fermentations of lignocellulosic material.

5. FUTURE PERSPECTIVES

2nd generation bioethanol has undergone intensive research in all aspects of the production process over the last years and is approaching commercial-scale production. There may be a number of reasons way it has not yet reached such a scale, many of which lie out of the scope of this research work. I think the technique, lignocellulosic material to ethanol, is mature enough but the issue lies in the incentives of making it profitable and to find the right combination of industry and regional location.

The research on bioethanol has also encouraged research into other bio-based products involving a variety of microorganisms and products. This is a field of research with immense possibility and I would find it interesting to further study the possibility of using microorganisms in *e.g.* co-cultures to produce value-added products from forest and agricultural residuals. Co-cultures can for example be used as an alternative to engineered organisms to convert all present sugars and carbon sources in order to increase product yield on added sugars and carbon but can also contribute to detoxification of the substrate. To my knowledge this is an area that would benefit from further research.

It would also be interesting to study the interplay between inhibitory compounds, nutritional limitations, nutrient starvation and the choice of strain in order to get a clear picture of various combination and combined effect on fermentation of lignocellulosic material.

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