Thesis for the degree of Doctor of Philosophy

Towards More Robust *Saccharomyces cerevisiae* Strains for Lignocellulosic Bioethanol Production

Lessons from process concepts and physiological investigations

MAGNUS ASK

Industrial Biotechnology
Department of Chemical and Biological Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

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Schematic representation of the workflow followed in the thesis, by Magnus Ask.

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ABSTRACT

Dwindling oil reserves and the negative impacts of fossil fuels on the environment call for more sustainable energy sources. First-generation bioethanol produced from sugar cane and corn has met some of these needs, but it competes with the food supply for raw materials. Lignocellulosic biomass is an abundant non-edible raw material that can be converted to ethanol using the yeast *Saccharomyces cerevisiae*. However, due to the inherent recalcitrance to degradation of lignocellulosic raw materials, harsh pretreatment methods must be used to liberate fermentable sugars, resulting in the release of compounds such as acetic acid, furan aldehydes and phenolics, that inhibit yeast metabolism. This thesis research aimed to identify bottlenecks in terms of inhibitory compounds related to ethanol production from two lignocellulosic raw materials, *Arundo donax* and spruce, and furthermore to harness the physiological responses to these inhibitors to engineer more robust yeast strains. A comparative study of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) revealed that acetic acid limits xylose utilization in pretreated *Arundo donax*, whereas the furan aldehydes furfural and 5-hydroxymethyl-2-furaldehyde (HMF) were hypothesized to be key inhibitors in pretreated spruce. The impacts of furfural and HMF on the redox and energy metabolism of *S. cerevisiae* were studied in detail in chemostat and batch cultivations. After adding the inhibitors to the feed medium of chemostat cultivations, the intracellular levels of NADH, NADPH, and ATP were found to decrease by 40, 75, and 19%, respectively, suggesting that furan aldehydes drain the cells of reducing power. A strong effect on redox metabolism was also observed after pulsing furfural and HMF in the xylose consumption phase in batch cultures. The drainage of reducing power was also observed in a genome-wide study of transcription that found that genes related to NADPH-requiring processes, such as nitrogen and sulphur assimilation, were significantly induced. The redox metabolism was engineered by overproducing the protective metabolite and antioxidant glutathione. Strains with an increased intracellular level of reduced glutathione were found to sustain ethanol production for longer duration in SSF of pretreated spruce, yielding 70% more ethanol than did the wild type strain.

Keywords: Lignocellulosic bioethanol, SHF, SSF, *Arundo donax,* spruce, furfural, HMF, redox metabolism, transcriptome, glutathione

LIST OF PUBLICATIONS

This thesis is based on the following research papers, referred to as **Papers I – IV** in the text:

Paper I: Magnus Ask, Kim Olofsson, Tommaso Di Felice, Laura Ruohonen, Merja Penttilä, Gunnar Lidén, and Lisbeth Olsson (2012)
Challenges in enzymatic hydrolysis and fermentation of pretreated *Arundo donax* revealed by a comparison between SHF and SSF.
Process Biochemistry 47:1452-1459.

Paper II: Magnus Ask, Maurizio Bettiga, Valeria Mapelli, and Lisbeth Olsson (2013)
The influence of HMF and furfural on redox-balance and energy-state of xylose-utilizing *Saccharomyces cerevisiae*.
Biotechnology for Biofuels 6:22.

Paper III: Magnus Ask, Maurizio Bettiga, Varuni Raju Duraiswamy, and Lisbeth Olsson (2013)
Pulsed addition of HMF and furfural to batch-grown xylose-utilizing *Saccharomyces cerevisiae* results in different physiological responses in glucose and xylose consumption phase.
Submitted for publication.

Paper IV: Magnus Ask, Valeria Mapelli, Heidi Höck, Lisbeth Olsson, and Maurizio Bettiga (2013)
Engineering glutathione biosynthesis of *Saccharomyces cerevisiae* increases robustness to inhibitors in pretreated lignocellulosic materials.
Submitted for publication.

CONTRIBUTION SUMMARY

My contributions to each of the above publications are as follows:

Paper I: Designed and performed wet-lab experiments, analysed the data, and wrote the manuscript.

Paper II: Designed and performed wet-lab experiments, analysed the data, and wrote the manuscript.

Paper III: Designed and performed most wet-lab experiments, analysed the data, and wrote the manuscript.

Paper IV: Designed and performed wet-lab experiments, analysed the data, and wrote the manuscript.

PREFACE

This PhD dissertation partially fulfils the requirements for a PhD degree at the Department of Chemical and Biological Engineering, Chalmers University of Technology, Sweden. The PhD project was initiated in May 2009 as part of NEMO, a collaborative research project funded by the European Union. The research was carried out under the supervision of Professor Lisbeth Olsson, Assistant Professor Maurizio Bettiga and Professor Gunnar Lidén.

The main part of the work concerns the impacts of lignocellulose-derived inhibitors on the metabolism of *Saccharomyces cerevisiae* in the context of second-generation bioethanol production.

My PhD project was mainly funded by grant 222699 from the European Union. Personal travel grants were obtained from Ångpanneföreningen Research Foundation, Adlerbert Research Foundation and the Nils Pihlblad Foundation.

Magnus Ask
August 2013

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INTRODUCTION

Since the start of the industrial revolution, the carbon dioxide content of the atmosphere has increased steadily, a change attributed mainly to the increased consumption of petroleum-based fuels. The utilization of such fuels has indeed contributed to the increased wealth of the industrialized nations, but since petroleum constitutes a finite resource and since net emissions of carbon dioxide contribute to the greenhouse effect, renewable energy sources with smaller or negligible greenhouse gas emissions are needed. Moreover, the production of renewable fuels would not only reduce our dependence on fossil fuel resources, but also increase our energy security (Hahn-Hägerdal et al., 2006; Lynd, 2010). These issues have been recognized by world-leading economies such as those of the USA and EU, prompting the investments of considerable resources into transforming a fossil fuel-based economy into a biobased economy (European Commission, 2012; US Department of Energy, 2012).

Bioethanol is the fuel dominating the renewable biofuel market today, and most new gasoline engines can use fuel blends containing up to 20% ethanol, whereas flexi-fuel vehicles can run on fuel blends containing up to 100% ethanol (E100). The main ethanol producers in the world are the USA and Brazil, which according to the Renewable Fuels Association, produced 40.1 and 24.9 billion litres, respectively, in 2009 from the first-generation feedstocks corn and sugar cane. Bioethanol is produced by microbial fermentation of the constituent sugars of the raw materials, and *Saccharomyces cerevisiae*, also known as baker's or brewer's yeast, is the most commonly used cell factory for this application. As first-generation feedstocks compete with food production, voices have called for changing the raw material basis to a non-edible one. Lignocellulose, the most abundant biopolymer on Earth, cannot be digested by humans and thus fulfils this criterion (Tilman et al., 2009).

Lignocellulose is generally referred to as a second-generation feedstock. It is a complex heteropolymer of cellulose, hemicellulose, and lignin and is the main component of plant biomass. Not only is lignocellulose available in vast amounts, but it has also outperformed corn-based first-generation bioethanol production in life-cycle assessments in terms of lower greenhouse gas and air pollutant emissions (Spatari et al., 2010; Williams et al., 2009). However, the industrial production of second-generation bioethanol has been hampered by its elevated production cost compared with that of ethanol from first-generation feedstocks (Galbe et al., 2007; Stephen et al., 2012).

Although lignocellulosic bioethanol hold great promise for sustainable fuel production, some challenges remain. One of the main obstacles to industrially viable lignocellulosic bioethanol production originates from the recalcitrant nature of the raw material. For this reason, harsh pretreatment methods have to be used to increase the susceptibility of the raw material to enzymatic hydrolysis, which is used to release the fermentable sugars. The pretreatment process has the negative consequence of producing byproducts that inhibit hydrolytic enzymes and microorganisms, reducing sugar and ethanol yields and productivities. Moreover, as highly concentrated hydrolysates are needed to achieve ethanol concentrations up to 40–50 g L^{-1} to minimize the energy expenditure in the final separation step after fermentation, the inhibitor concentration is increased accordingly. Consequently, robust microorganisms are needed that can tolerate high concentrations of inhibitors; to obtain such organisms by rational engineering, the inhibitory compounds have to be identified and the molecular mechanisms of inhibition revealed.

In my thesis research, I have identified challenges related to bioethanol production from two lignocellulosic feedstocks of considerable interest in Europe: the energy crop *Arundo donax* and the softwood spruce. I then studied the inhibitory mechanisms of different inhibitor classes such as furan aldehydes and weak acids on *S. cerevisiae*, focusing mainly on the redox and energy metabolism. My work has not only provided clues as to the molecular mechanisms of action of these inhibitors, but has also resulted in metabolic engineering strategies to improve microbial robustness under industrially relevant conditions. At present, two papers presented in the thesis (**Papers I** and **II**) have been published in peer-reviewed journals, whereas **Papers III** and **IV** have been submitted for publication.

In **Paper I**, I identified challenges in the enzymatic hydrolysis and fermentation of the lignocellulosic feedstock *A. donax* using the two principal process concepts separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). **Paper II** assess the long-term influence of two frequently occurring lignocellulosic inhibitors, furfural and HMF, on the redox and energy state of *S. cerevisiae* in chemostat cultivations by adding the inhibitors to the feed medium. To investigate the dynamic physiological responses of *S. cerevisiae* to furfural and HMF, batch cultivations were performed in which the inhibitors were pulsed in either the glucose consumption phase or the xylose consumption phase (**Paper III**). In **Paper IV**, the results obtained in **Papers II** and **III** were harnessed to engineer *S. cerevisiae* with improved redox buffering capacity and

consequently increased robustness to inhibitors present in pretreated spruce in an SSF process.

The thesis is divided into two main parts. In the first part, I give a general overview of second-generation bioethanol production and the challenges connected to various aspects of the production process, highlighting the significant findings of my work in relation to what has been reported in the literature. The second part contains the research papers on which this thesis is based (**Papers I-IV**).

The first part is divided into four chapters. **Chapter 1** provides a general overview of the lignocellulosic feedstocks and process steps involved in second-generation bioethanol production. In **Chapter 2**, I elaborate on challenges connected to converting pretreated *A. donax* and spruce to bioethanol, identifying furfural and HMF as important inhibitors in the case of spruce. **Chapter 3** describes in greater depth the influence of furfural and HMF on the redox and energy state of *S. cerevisiae*. This is discussed in relation to the two experimental approaches used: chemostat and batch cultivation. In **chapter 4**, I present strategies to improve the robustness of *S. cerevisiae* to lignocellulose-derived inhibitors, including the engineering strategy for the redox metabolism that was developed as part of this thesis research.

CHAPTER 1

ETHANOL PRODUCTION FROM LIGNOCELLULOSIC BIOMASS: AN OVERVIEW

In the present chapter, the general structure of lignocellulosic raw materials will be described and the basic process steps in the production of bioethanol from lignocellulosic raw materials will be covered (Figure 1). As there are several feasible process configurations, a discussion will be given on which parameters that govern the choice of process mode.

1.1 From raw material to ethanol: a schematic overview of the process

Although the nature of lignocellulosic raw materials varies considerably, certain process steps are common regardless of the type and source of feedstock:

1) Collection
2) Milling/Chipping
3) Pretreatment/Hydrolysis
4) Fermentation
5) Downstream processing (e.g. distillation)

All these steps contribute to the cost of the final product, making it important to minimize the energy input in each step (Chandra et al., 2007). Figure 1 depicts the material flow in a typical biorefinery with ethanol production as the main product. After the feedstock has been collected, the material is usually milled or chipped into smaller pieces to increase the specific surface area (Hendriks and Zeeman, 2009). The processed raw material is subsequently hydrolysed either completely or partially using physical, chemical, or biological methods, or combinations thereof, to liberate fermentable sugars from the raw material (Olsson et al., 2005). The released monomeric sugars are then fermented to ethanol by microbes. In the final step, the produced ethanol is stripped from the fermentation broth by distillation, other process streams being either recirculated or further refined depending on the plant configuration. The various process steps will be described in more detail in the present chapter.

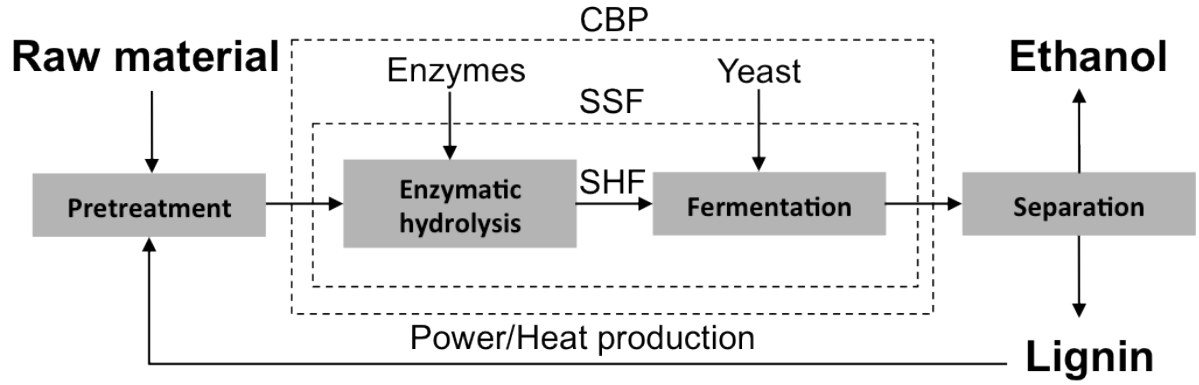

Figure 1. Principal steps involved in converting lignocellulosic biomass to ethanol. The raw material is first pretreated, after which either SHF, SSF or CBP is used to convert the constituent sugars of the raw material to ethanol. At the end of the fermentation, the ethanol is separed from the fermentation broth by distillation. The lignin can be burned to produce heat for the pretreatment step. SHF: separate hydrolysis and fermentation, SSF: simultaneous saccharification and fermentation, CBP: consolidated bioprocessing.

1.2 The raw material

Through photosynthesis, plants utilize energy from sunlight to assimilate carbon dioxide in the atmosphere into complex polymeric sugars. Lignocellulose is the generic name of the main components that make up the major part of plant cell walls. Although lignocellulosic feedstocks are quite heterogeneous in terms of the relative amounts of their constituent components, all are complex biopolymers of cellulose and hemicellulose intertwined in a matrix of lignin (Figure 2). A simplistic view often referred to is that cellulose confers rigidity to the structure, while hemicellulose provides flexibility, lignin being said to function as glue between the other two components. Apart from cellulose, hemicellulose, and lignin, lignocellulosic raw materials also contain varying amounts of inorganic ash components and extractives such as resins and terpenes (Klinke et al., 2004). Suggested feedstocks for second-generation bioethanol production include forestry residues from softwood and hardwood, agricultural and municipal wastes, and dedicated energy crops. The ultimate choice of feedstock will likely depend on factors such as geographical location and agricultural tradition (Lynd, 1996; Somerville et al., 2010). The biomass composition depends not only on the species, but also on the growth conditions and plant part. Nevertheless, lignocellulose typically comprises 40-45% cellulose, 20-30% hemicellulose, and 15-25% lignin (Olsson et al., 2005). The composition in terms of constituent sugars and lignin of several classes of feedstocks is shown in Table 1.

The largest differences between the classes of raw materials reside in the varying amounts of glucan, mannan, xylan, and lignin (Table 1). Softwoods have generally been considered more

recalcitrant to decomposition due to their high lignin content. Softwoods are also characterized by mannan-rich hemicellulose fractions, whereas xylan-containing hemicellulose is lower than that of other feedstock classes (see 1.2.2). In contrast, hardwoods, agricultural residues, and energy crops are characterized by high amounts of xylan in their hemicellulose fractions. In this thesis research, I evaluated two raw materials, *Arundo donax* and spruce, which are feedstocks of considerable interest for lignocellulosic bioethanol production in Europe. As can be seen in Table 1, spruce represents a hexose- and lignin-rich material, whereas *A. donax* contains a high fraction of xylose, thereby requiring an efficient xylose-utilizing yeast strain for economically viable conversion of both hexoses and pentoses to ethanol.

Table 1. Compositions of various lignocellulosic raw materials (% of dry matter).

	Glucan	Mannan	Galactan	Xylan	Arabinan	Lignin
Softwoods						
Spruce[a]	49.9	12.3	2.3	5.3	1.7	28.7
Pine[b]	44.8	11.4	1.4	6.0	2.0	29.3
Hardwoods						
Poplar[c]	42.2	2.0	0.6	13.4	0.6	25.6
Birch[b]	42.6	1.8	0.6	26.4	0.5	18.9
Agricultural residues						
Corn stover[d]	36.8	0.3	0.7	21.7	2.6	17.2
Sugarcane bagasse[e]	43.0	-	0.4	26.0	1.5	24.6
Energy crops						
Arundo donax[f]	35.2	-	-	18.2	0.8	23.0
Switch grass[g]	37.8	0.4	1.1	24.9	3.4	21.4

[a](Soderstrom et al., 2003)
[b](Timell, 1967)
[c](Sannigrahi et al., 2010)
[d](Zhu et al., 2007)
[e](Rudolf et al., 2008)
[f](Caparrós et al., 2007)
[g](Wiselogel et al., 1996)

1.2.1 Cellulose

Cellulose is a linear polysaccharide and the dominant structural carbohydrate in plants (Figure 2). The basic repeating unit of cellulose is cellobiose, which consists of two 1,4-β-linked D-glucopyranose units linked together. The degree of polymerization has been found to range from 10,000 to 15,000 (O'Sullivan, 1997), and the long linear cellulose chains are stabilized by hydrogen bonding in the direction of the molecule. As the aliphatic hydrogen

groups and polar hydroxyl groups are in axial and equatorial positions, respectively, the tops and bottoms of the cellulose chains are hydrophobic. This contributes to the packing of cellulose fibres into crystalline structures, so-called microfibrils, which typically consist of 30–200 independent chains (Wyman et al., 2005). Microfibrils are relatively inert and confer rigidity and strength to the cell wall (Brett and Waldron, 1996).

1.2.2 Hemicellulose

In contrast to the linear structure of cellulose, hemicellulose is more heterogeneous and can be highly branched (Figure 2). Hemicellulose has a lower degree of polymerization than does cellulose and is connected to cellulose chains by hydrogen bonds, thereby stabilizing the structure of the cell wall (Wyman et al., 2005). It comprises hexoses such as glucose, mannose, and galactose as well as pentoses such as xylose and arabinose. Hemicellulose is usually classified according to the main sugar type in the backbone of the polymer, xylans and mannans being the most common. Depending on the plant species, the hemicellulose composition can vary considerably (Table 1). For example, the major hemicellulose in softwoods is glucomannan, while hardwoods and agricultural residues contains glucoronoxylans and arabinoxylan, respectively. The backbone can be substituted with arabinose, galactose, glucuronic, acetic, and/or ferulic acid groups (Saha, 2003).

1.2.3 Lignin

Lignin is the major non-polysaccharide polymer in lignocellulose and comprises a complex network of phenyl propane units (i.e. p-coumaryl, coniferyl and sinapyl alcohols) with a degree of polymerization of 450–500 (Wayman and Parekh, 1990) (Figure 2). The terms *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) are commonly used to denote the three kinds of aromatic rings in the monomers, and the respective amounts of H, G, and S can differ considerably between feedstocks (Klinke et al., 2004). Lignin can form covalent bonds with hemicellulose, creating a matrix in which hemicellulose and cellulose are embedded (Jorgensen et al., 2007). In general, lignin is more abundant in softwood and hardwood than in agricultural residues (Table 1). In summary, cellulose, hemicellulose and lignin create a complex biocomposite recalcitrant to challenges encountered in the natural habitats of plants (Ralph et al., 2004). Hence, it is not surprising that lignocellulosic biomass has been found to be difficult to break down into its building blocks.

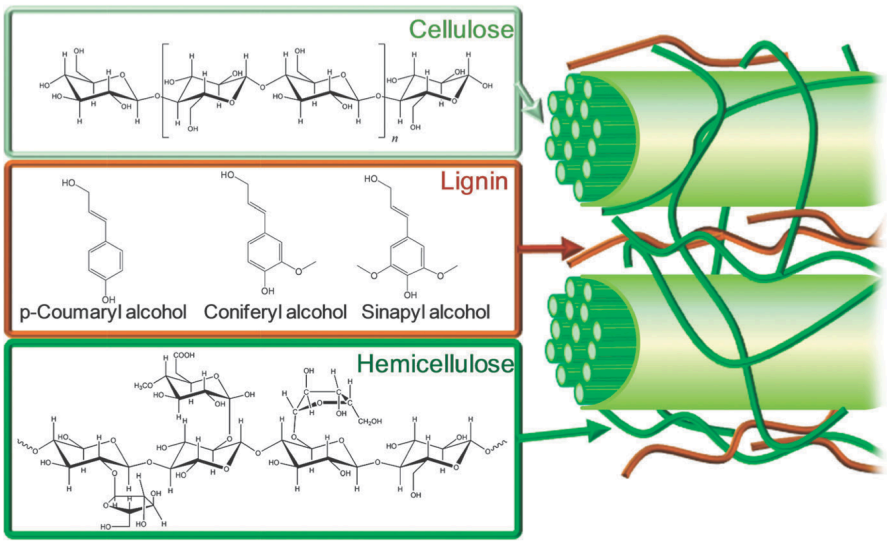

Figure 2. Schematic on the structure of lignocellulose (Alonso et al., 2012). Reproduced by permission from the Royal Society of Chemistry.

1.3 Pretreatment

Concentrated and dilute acid hydrolysis have both been used frequently in the past as methods for liberating the constituent sugars from lignocellulosic feedstocks. Although they have shown some promise, disadvantages connected to corrosion of reactor parts, limited possibility of cost-effective acid recovery, and sugar degradation have been identified as major obstacles (see review by Mosier et al., 2005). Degradation of lignocellulose solely by biological means by white-rot and brown-rot fungi has also been an area of active research (Perez et al., 2002). Nevertheless, due to the inherent recalcitrant structure of the raw material, with lignin and hemicellulose shielding the cellulose fibres from the hydrolytic enzymes, biological pretreatment has been found to be both slow and inefficient (Chandra et al., 2007). Hence, recent research has focused on methods for altering the native structure of the raw material, to make it more amenable to hydrolytic attack by cellulolytic enzymes (Hendriks and Zeeman, 2009). An ideal pretreatment method should have the following characteristics (in no particular order) (Chandra et al., 2007):

1) Applicability to a wide range of feedstocks.
2) Requirement for minimal energy-consuming preprocessing steps.
3) Removal of shielding structures such as hemicellulose and lignin from cellulose fibres.

4) Generation of activated cellulose fibres with decreased crystallinity, degree of polymerization, and particle size that are amenable to enzymatic hydrolysis.
5) Recovery of all sugars, preferably in monomeric form (**Paper I**), minimizing the amount of inhibitory degradation products.
6) Low capital and operating costs.

Various pretreatment methods for lignocellulosic feedstocks are described in the literature, but due to the limited scope if this thesis, just a few are briefly described here. Steam-explosion and ammonia fibre expansion (AFEX) are two well-investigated pretreatment methods that have been applied to several feedstocks with varying degrees of success (Bals et al., 2010; Lau and Dale, 2009; Soderstrom et al., 2003; Varga et al., 2004). Depending on the particular method used, differences in the subsequent structure of the pretreated raw material have been found (Chandra et al., 2007). In steam-explosion, which can be performed either with or without an acid catalyst, the raw material is treated with high-pressure steam at 160–240°C for seconds up to several minutes. When the pressure is released, the material undergoes an explosive decompression, resulting in structural rearrangement. When no catalyst is used, the process is self-catalysed by acetic acid liberated from the raw material (Galbe and Zacchi, 2007). The process has been demonstrated to be more efficient when the raw material is impregnated with SO_2 before the steam-explosion (Clark et al., 1989). Steam-explosion solubilizes the hemicellulose fraction and reduces the crystallinity of the raw material but leaves significant amounts of lignin in the solid phase (Chandra et al., 2007; Galbe and Zacchi, 2007). Moreover, significant amounts of degradation products that inhibit enzymatic hydrolysis and fermentation are formed at high severities (Palmqvist and Hahn-Hägerdal, 2000) **(**see **Chapter 2)**. In the AFEX process, the raw material is treated with 5–15% ammonia at 160–180°C for up to 14 minutes. In contrast to steam-explosion, AFEX alters the cellulose crystallinity, but does not solubilize the hemicellulose fraction (Mosier et al., 2005). Although AFEX produces only a minor amount of inhibitors, the high cost of ammonia and of ammonia recovery are major drawbacks of the AFEX pretreatment method (Mosier et al., 2005; Olsson et al., 2005). Due to the flexibility and relative cost-effectiveness of steam-explosion, this method has been regarded as very promising for industrial bioethanol production (Galbe and Zacchi, 2002; Stephen et al., 2012). The raw materials considered in this thesis*, A. donax* and spruce, were pretreated by non acid-catalysed and acid-catalysed steam-explosion, respectively (**Paper I**).

1.4 Enzymatic hydrolysis

After the raw material has been pretreated, two streams are commonly obtained consisting of a solid fraction comprising mainly cellulose and lignin and a liquid fraction containing monomeric and/or oligomeric sugars. If the pretreatment severity has been low, a considerable amount of hemicellulose is also present in the solid fraction (Galbe and Zacchi, 2007). Thus, the polymeric and oligomeric sugars have to be further degraded to monomers by hydrolytic enzymes before the fermentation can be initiated. Depending on the composition of the solid fraction, a specific set of enzymatic activities is required to degrade the lignocellulosic material (Wyman et al., 2005). A wide range of organisms in nature can degrade cellulose and hemicellulose in lignocellulosic raw materials (Wood, 1985). Collectively, the enzymes produced by these organisms are referred to as cellulolytic and hemicellulolytic enzymes, respectively, and as will be described in the following section, several enzymatic activities are classified under these terms.

1.4.1 Cellulolytic enzymes

The cellulolytic enzyme system from the fungus *Trichoderma reesie* has been used as model system for cellulose hydrolysis since the 1950s (Mandels and Reese, 1964; Wyman et al., 2005). According to the classical model of cellulose degradation, three main classes of enzymes are needed to break down cellulose to glucose: 1) exo-1,4-β-D-glucanases also referred to as cellobiohydrolases (CBH), cleave off cellobiose units from both the reducing and non-reducing ends of cellulose fibres; 2) endo-1,4-β-D-glucanases (EG) act by randomly cleaving β-1,4-glycosidic bonds in cellulose chains; and 3) 1,4-β-glucosidases (BG) cleave cellobiose into two molecules of glucose (Wood, 1985). More recent research has highlighted the importance of monooxygenase enzymes of the GH61 family in the breakdown of crystalline cellulose. These enzymes are thought to attack the dense packing of the crystalline regions, thereby increasing accessibility for the hydrolytic enzymes (see review by Horn et al., 2012). CBH and EG are generally organized into two domains consisting of a catalytic site and a cellulose-binding module (CBM), respectively. CBM is thought to function by directing the catalytic domains of the enzymes into the right configuration so efficient cleavage can occur (Linder and Teeri, 1997). The end-products of the enzymatic hydrolysis (i.e. glucose and cellobiose) can act as inhibitors of the cellulolytic enzymes (Xiao et al., 2004), which can be a problem in an SHF process in which high monomeric sugar concentrations are obtained. In fact, the inhibition constants (K_i) of a crude *T. reesie* cellulase

preparation were found to be 69 g L^{-1} (383 mM) and 3.75 g L^{-1} (11 mM), for glucose and cellobiose, respectively (Ryu and Lee, 1986; Tolan and Foody, 1999). Moreover, partial degradation of xylan to oligomers in the pretreatment has also been demonstrated to have negative effects on cellulolytic enzyme performance, possibly by competitive binding of the oligomers to the cellulolytic enzymes (Qing et al., 2010; Zhang and Viikari, 2012). In **Paper I**, I found that washing of pretreated *A. donax* resulted in a 26% increase in the glucose yield, which was hypothesized to be due to removal of xylooligomers.

1.4.2 Hemicellulolytic enzymes

Hemicellulolytic enzymes constitute a diverse group of enzymes whose activities are required for degrading the hemicellulose fraction of lignocellulosic raw materials. For example, the degradation of xylan into monomers requires enzymatic activities such as endo-xylanase, exo-xylanase, and β-xylosidase. Furthermore, depending on the nature of the substitutions of the main chain, accessory enzymatic activities such as glucuronosidase, acetylxylan esterase, arabinofuranosidase, and feruloyl esterase may also be required (Saha, 2003). As stated above, pretreatment is often designed to degrade the hemicellulose fraction to monomeric sugars. Nevertheless, since this entails harsh pretreatment conditions that generate inhibitory compounds, milder conditions in combination with supplementation of hemicellulolytic enzymes during enzymatic hydrolysis can be advantageous. Moreover, synergistic effects have been observed when cellulolytic and hemicellulolytic enzymes are used together, which further favours the use of these enzymes in the decomposition of lignocellulosic biomass (Gao et al., 2011; Zhang et al., 2011; **Paper I**).

1.5 Fermentation

Fermentation is the key step in bioethanol production, in which the sugars obtained from the raw material are converted to ethanol by microorganisms. Though a range of microbes have been used for this purpose, the yeast *S. cerevisiae*, also known as baker's yeast, has received the most attention due to its GRAS status and traditional use in alcoholic beverage production and bread making. The ideal cell factory for industrial bioethanol production should have a broad substrate range, produce ethanol at a high yield and productivity, and possess high inhibitor tolerance (Zaldivar et al., 2001). The long use of *S. cerevisiae* in commodity production has applied selection pressure for certain useful properties, such as ethanol tolerance, acid tolerance, and osmotolerance (Attfield, 1997). In addition, *S. cerevisiae* can

consume a range of sugars present in lignocellulosic feedstocks, such as glucose, mannose and galactose, though it cannot utilize pentoses such as xylose and arabinose. This section briefly introduces the metabolism of *S. cerevisiae*. More detailed explanations of the redox and energy metabolism are presented as part of Boxes 1 and 2 in **Chapter 3**. Research on conversion of lignocellulosic biomass to ethanol is also being performed with other microorganisms such as *Escherichia coli*, *Zymomonas mobilis*, *Scheffersomyces stipitis* and *Kluyveromyces marxianus* (reviewed by Geddes et al. 2011). However, a more thorough description of these microorganisms is out of the scope of this thesis.

S. cerevisiae is a facultative anaerobe, which means that it can sustain growth in both the presence and absence of oxygen, although supplementation of ergosterol and unsaturated fatty acids are needed to maintain growth under anaerobic conditions (Andreasen and Stier, 1953, 1954). To drive energy-requiring biosynthetic and maintenance reactions in the cell, free energy in the form of ATP has to be generated. Possibly as a result of its natural habitat on fruits, evolution has favoured glucose as the preferred carbon and energy source of *S. cerevisiae*. Yeast can harness some of the energy in glucose by substrate-level phosphorylation in the process known as glycolysis (Figure 3), in which 1 mol of glucose is converted to 2 mol of pyruvate with the concomitant production of 2 mol of ATP and 2 mol of NADH.

If molecular oxygen is present as the terminal electron acceptor, i.e., under aerobic conditions, the pyruvate and NADH formed in glycolysis can be further oxidized to CO_2 and H_2O in mitochondria in the tricarboxylic acid (TCA) cycle and respiration chain, with the concomitant production of additional ATP by oxidative phosphorylation. The amount of additional ATP produced in respiration is dependent on the P/O ratio, i.e., the number of ADP molecules phosphorylated per electron pair transferred to oxygen. A P/O ratio of ~1 has been reported for *S. cerevisiae*, which equates to a total of 16 mol of ATP produced in the complete respiratory dissimilation of glucose (Verduyn et al., 1991). Under anaerobic conditions, yeast ferments pyruvate to ethanol via acetaldehyde in a two-step process catalysed by pyruvate decarboxylase (Pdc1p) and alcohol dehydrogenase (Adh1p), respectively. Ethanol production from acetaldehyde regenerates NAD^+ from NADH, which makes the entire pathway from glucose to ethanol redox neutral. Production of free energy in the form of ATP by fermentation yields 2 mol of ATP per mol of glucose, i.e., eight times less than in respiration. This difference is reflected in the approximately five-fold higher

biomass yield (~0.5 g g^{-1}) during fully respiratory metabolism, versus when no oxygen is present (~0.1 g g^{-1}) (Verduyn, 1991).

S. cerevisiae is known as a Crabtree-positive yeast, which means that it is also able to produce ethanol in the presence of oxygen through respiro-fermentative metabolism. This phenomenon occurs when the glucose concentration is above a critical level (>1 mM) and at high glucose consumption rates even under fully aerobic conditions (Postma et al., 1989; Verduyn et al., 1984). The *Crabtree effect* was first discovered in cancer cells (Crabtree, 1928), but was later also identified in *S. cerevisiae* (De Deken, 1966). The classical explanation attributes the Crabtree effect to the limited respiration capacity of *S. cerevisiae* during high glucose fluxes, leading to an overflow metabolism at the pyruvate node, i.e., the branch point between fermentation and respiration (Postma et al., 1989).

Although glucose is the preferred carbon and energy source of *S. cerevisiae*, other hexoses present in lignocellulosic raw materials, such as mannose and galactose can also be catabolized. Mannose is first converted to mannose-6-phosphate and subsequently isomerized to glucose-6-phosphate, which enters the glycolysis pathway (Herman, 1971). Galactose, on the other hand, is converted to glucose-6-phosphate in five steps in the Leloir pathway (Holden et al., 2003). Whereas mannose can be consumed simultaneously with glucose, galactose metabolism is repressed in the presence of glucose, resulting in a sequential consumption of glucose and galactose, as reviewed by Sellick et al., (2008).

At the level of glucose-6-phosphate, glycolysis branches off into the pentose phosphate pathway (PPP), a network of reactions that functions to supply reducing power in the form of NADPH that is needed in anabolic reactions (Bruinenberg et al., 1983) (Figure 3). Most of the NADPH generated is utilized in the synthesis of amino acids, nucleic acids, and lipids (Bruinenberg et al., 1983). Several PPP intermediates are also important as building blocks for the biosynthesis of nucleic acids and aromatic amino acids. Ribose-5-phosphate is a precursor of nucleotides, tyrosine, and phenylalanine, whereas erythrose-4-phosphate is a building block of phenylalanine, tryptophan, and tyrosine (Braus, 1991). Another important function of PPP is that it serves as the entry point for xylose metabolism. As discussed above, xylose is a pentose sugar abundant in certain types of lignocellulosic biomass. *S. cerevisiae* lacks an active intrinsic pathway for utilizing xylose as a carbon and energy source, whereas several other yeasts have been demonstrated to possess this ability. Evolution has favoured

two approaches for xylose utilization in microorganisms: the xylose reductase/xylitol dehydrogenase (XR/XDH) pathway and the xylose isomerase (XI) pathway (Figure 3). Both these pathways have been successfully cloned into *S. cerevisiae*, thereby generating strains capable of utilizing xylose (Kuyper et al., 2004; Kötter and Ciriacy, 1993). In the research work of this thesis, a strain carrying the XR/XDH pathway was used (**Papers I-III**). A more detailed discussion of xylose metabolism in recombinant *S. cerevisiae* is presented in section 2.1.

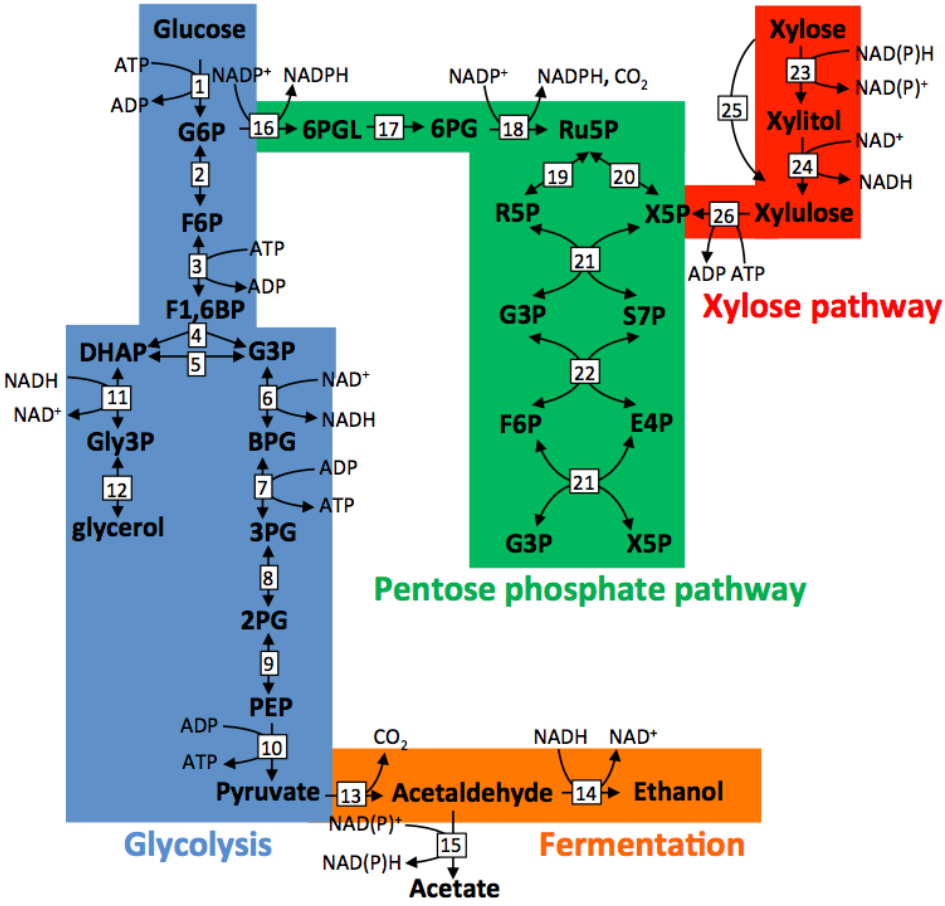

Figure 3. Illustration of glycolysis, fermentation, pentose phosphate pathway, and xylose utilization pathways in yeast. 1: hexokinase, 2: glucose-6-phosphate isomerase, 3: phosphofructokinase, 4: aldolase, 5: triosphosphate isomerase, 6: glyceraldehyde-3-phosphate dehydrogenase, 7: 3-phosphoglycerate kinase, 8: phosphoglycerate mutase, 9: enolase, 10: pyruvate kinase, 11: glycerol-3-phosphate dehydrogenase, 12: glycerol-3-phosphatase, 13: pyruvate decarboxylase, 14: alcohol dehydrogenase, 15: aldehyde dehydrogenase, 16: glucose-6-phosphate dehydrogenase, 17: 6-phosphogluconolactonase, 18: 6-phosphogluconate dehydrogenase, 19: ribose-5-phosphate ketol isomerase, 20: ribulose-5-phosphate 3-epimerase, 21: transketolase, 22: transaldolase, 23: xylose reductase, 24: xylitol dehydrogenase, 25: xylose isomerase, 26: xylulokinase. G6P: glucose-6-phosphate, F6P: fructose-6-phosphate, F1,6BP: fructose-1,6-bisposphate, DHAP: dihydroxyacetone phosphate, G3P: glyceraldehyde-3-phosphate, BPG: bisphosphoglycerate, 3PG: 3-phosphogluconate, 2PG: 2-phosphogluconate, PEP: phosphoenolpyruvate, Gly3P: glycerol-3-phosphate, 6PGL: 6-phosphogluconolactone, 6PG: 6-phosphogluconate, Ru5P: ribulose-5-phosphate, R5P: ribose-5-phosphate, X5P: xylulose-5-phosphate, S7P: sedoheptulose-7-phosphate, E4P: erythrose-4-phosphate.

1.6 Process configurations for lignocellulosic bioethanol production

As indicated in Figure 1, there are three principal process configurations for industrial bioethanol production: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and consolidated bioprocessing (CBP). This section will describe these process concepts and discuss issues involved in choosing the appropriate configuration.

1.6.1 Separate hydrolysis and fermentation (SHF)

In separate hydrolysis and fermentation, the enzymatic hydrolysis and fermentation steps are performed in two distinct processes. One of the main advantages of SHF is that the enzymatic hydrolysis and fermentation can be carried out under their respective optimal conditions, as the cellulolytic enzymes generally require temperatures in the range of 45–50°C for optimal performance, whereas the optimal temperature for growth of *S. cerevisiae* is in the range of 28–32°C (Galbe and Zacchi, 2002). Furthermore, the possibility of recirculating microorganisms benefits the process economy as less yeast has to be added to the process. On the other hand, as high sugar concentrations are reached during the enzymatic hydrolysis step, end-product inhibition of the cellulolytic and hemicellulolytic enzymes by high monomer and oligomer concentrations can be a problem (**Paper I**). Moreover, the capital costs of SHF are higher than those of SSF, as a separate hydrolysis reactor is required.

1.6.2 Simultaneous saccharification and fermentation (SSF)

As the name implies, in simultaneous saccharification and fermentation, the enzymatic hydrolysis occurs concomitantly with the fermentation process in the same reactor. If a xylose-utilizing microorganism is used, the process is often referred to as simultaneous saccharification and co-fermentation (SSCF). The main advantage of running the bioethanol production process in SSF mode is that end-product inhibition of the hydrolytic enzymes can be minimized, as the sugars are directly consumed by the microorganisms after release into the fermentation broth (Olofsson et al., 2008). Another advantage over SHF is the lower capital costs connected to SSF, as the same reactor is used for both enzymatic hydrolysis and fermentation. However, a compromise has to be made between the optimal conditions of the cellulolytic enzymes and microorganisms. Moreover, this process offers no opportunity for microorganism recirculation, as the solid particles in the lignocellulosic feedstock interfere with the separation.

1.6.3 Consolidated bioprocessing (CBP)

Consolidated bioprocessing was not used in this thesis research, but is only mentioned here to give a more complete view of the process configurations developed for lignocellulosic bioethanol production. The goal of CBP is to engineer microorganisms capable of producing ethanol directly from pretreated lignocellulosic raw materials, without the need to add cellulolytic enzymes (van Zyl et al., 2007). This can be achieved either by incorporating genes encoding cellulolytic enzymes into non-cellulolytic microorganisms such as *S. cerevisiae*, or by engineering native cellulolytic microorganisms with improved product-related properties, such as ethanol yield and productivity (la Grange et al., 2010). The major benefit of CBP is the considerable economic impact of incorporating hydrolytic enzyme production into the microorganism, hence circumventing a major cost driver of the bioethanol production process. Nevertheless, problems related to differences in optimal conditions for cellulolytic enzymes and cell growth also apply to CBP, especially if *S. cerevisiae* is used as the production host. As a solution to the problem of the difference in temperature optima between the enzymes and microorganism, the engineering of temperature-tolerant microorganisms such as *K. marxianus* that can grow at temperatures up to 52°C has been proposed. Although CBP is a process configuration with enormous potential, considerable work remains. Promising results have been obtained in co-cultures of metabolically engineered cellulase producers (*Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum*) using the crystalline cellulose model substrate Avicel (Argyros et al., 2011), but as pointed out by Olson et al. (2012), good performance in industrial substrates has yet to be demonstrated.

1.6.4 Choosing the right process configuration

As only SHF and SSF were evaluated as part of this thesis research (**Paper I**), CBP will not be treated in the coming discussion. Numerous comparisons of SHF and SSF have been reported in the literature; in terms of ethanol yield and ethanol productivity, SSF seems to have the upper hand for most raw materials (Table 2).

Table 2. Ethanol yields from a selection of SHF and SSF experiments carried out on different raw materials.

Process configuration	Raw material	WIS content % (w/w)	Mode of operation	Strain	Duration (h)	Ethanol yield (g/g)[f]
SHF						
	Corn stover[a]	8	Batch	*S. cerevisiae* Tembec I	120 + 24	0.23[g]
	Wheat straw[b]	10	Batch	*S. cerevisiae* F12	120[h]	0.24[g]
	A. donax[c]	10	Batch	*S. cerevisiae* VTT C-10880	72 + 96	0.25
	Spruce[d]	10	Batch	*S. cerevisiae* Ethanol Red	72 + 96	0.32
SSF						
	Corn stover[a]	8	Batch	*S. cerevisiae* Tembec I	96	0.28[g]
	Wheat straw[b]	10	Batch	*S. cerevisiae* F12	96	0.29[g]
	A.donax[c]	10	Batch	*S. cerevisiae* VTT C-10880	96	0.21
	Spruce[e]	10	Batch	*S. cerevisiae* Ethanol Red	96	0.35

[a](Öhgren et al., 2007)
[b](Tomás-Pejó et al., 2008)
[c]**Paper I**
[d]Ask et al., Unpublished results
[e]Olofsson et al., Unpublished results
[f]Ethanol yield based on total amount of sugars present in the pretreated raw material.
[g]Estimated from ethanol concentration and material data presented in research article.
[h]120 h refers to the duration of the enzymatic hydrolysis; fermentation time was not reported.

Regarding process performance, there are several parameters to consider, and a final decision on process configuration is likely a function of the lignocellulosic feedstock involved and must be based on a complete techno-economic evaluation. Not only is the final ethanol yield important, but factors such as ethanol productivity, potential for process stream recirculation, and capital costs must also be taken into account. In my thesis research, pretreated *A. donax* was evaluated as a feedstock for bioethanol production in both SHF and SSF. Interestingly, SHF resulted in a higher final ethanol yield (0.25 g g^{-1}) based on the total amount of sugars in the pretreated raw material, than did SSF (0.21 g g^{-1}) (**Paper I**). This was likely a result of the small amount of monomeric sugars initially present in the liquid fraction of the pretreated raw material, hence minimizing the initial end-product inhibition of the cellulolytic enzymes

that otherwise can be a problem for steam-pretreated raw materials in an SHF setup. As the end-product inhibition was relatively weak, the advantages of SSF in this respect did not outweigh the benefits of running the enzymatic hydrolysis under optimal conditions, as in SHF. On the other hand, it is important to keep in mind that the duration of the SHF process was 72 + 96 hours, whereas SSF needed only 96 hours for completion. The process time greatly influences the ethanol productivity and hence the process economy. Nevertheless, the final ethanol concentrations achieved in the SHF and SSF of pretreated *A. donax* in the present study (18.5 and 17.0 g L^{-1}, respectively) are far below 40–50 g L^{-1}, which is considered the lower limit to make the distillation step sufficiently energy efficient in an industrial process (Galbe et al., 2007).

In contrast to the results obtained with pretreated *A. donax* (in **Paper I)**, SSF of pretreated spruce resulted in a higher final ethanol yield than in SHF (Table 2) (Ask et al., Unpublished data; Olofsson et al., Unpublished data). As the pretreated spruce contained a high concentration of monomeric glucose prior to the enzymatic hydrolysis (see **Paper IV** for composition of the pretreated spruce), the cellulolytic enzymes were probably subjected to a higher degree of end-product inhibition in SHF than in SSF, hence explaining the higher total ethanol yield achieved in SSF. A likely explanation for the significant difference in ethanol yields between *A. donax* and spruce could be the higher [hexose]:[pentose] ratio of spruce (Table 1) and the fact that hexoses such as glucose and mannose can be consumed by *S. cerevisiae* at a considerably higher rate than pentoses.

In **Paper I**, I concluded that the major reason for the low ethanol yields was the low sugar yields obtained in enzymatic hydrolysis. The low hydrolysis yield resulted partly from the high concentrations of oligomeric xylose, demonstrated to be inhibitory for cellulolytic enzymes (Qing et al., 2010; Zhang and Viikari, 2012). Hence, either the pretreatment should be designed to degrade the xylan to monomers, or a hydrolysis step using for example acids could be applied after the pretreatment. It is worth mentioning that the enzymes used during the enzymatic hydrolysis reported in **Paper I** (Celluclast and Novozyme 188) are considered out-dated compared with new enzyme systems such as Cellic CTec2 and CTec3, so another obvious way to increase the hydrolysis yield is of course to use the latest high-performing cellulolytic enzymes.

CHAPTER 2

MICROBIAL CHALLENGES IN LIGNOCELLULOSIC BIOETHANOL PRODUCTION

Although lignocellulosic bioethanol research has made substantial progress in recent years, several challenges still impede viable industrial production. Identifying these challenges is critical to further progress. Of course, all unit operations involved in the production process can be improved (Figure 1), but only challenges related to the microbial production hosts and, in particular, to *S. cerevisiae* will be discussed in this chapter. Some of these challenges ultimately originate in the pretreatment of the lignocellulosic feedstock, as significant amounts of inhibitory degradation compounds are formed or released if the conditions are sufficiently harsh. Furthermore, the raw material composition in terms of the relative contents of hexoses and pentoses in the hemicellulose fraction is important since wild-type *S. cerevisiae* lacks an active enzymatic system for utilizing pentose sugars such as xylose and arabinose. Moreover, the degree of acetylation of the hemicellulose chains can affect the process performance, since these groups are cleaved off during pretreatment, potentially resulting in high concentrations of acetic acid that can negatively affect yeast metabolism. In this chapter, I will discuss challenges related to broadening the substrate range and the effects of inhibitors on yeast metabolism. As the major research work for this thesis was performed using the softwood *Picea abies* (spruce) and the herbaceous energy crop *A. donax* (giant reed), these raw materials will be discussed as case studies.

2.1 Xylose utilization in *S. cerevisiae*

As can be seen in Table 1, xylose accounts for a considerable fraction of the total sugars in several kinds of lignocellulosic materials, whereas arabinose occurs in smaller amounts. Consequently, a microorganism capable of utilizing pentoses and, in particular xylose, is needed for economically viable conversion of lignocellulosic feedstocks to ethanol. Most of the literature on xylose utilization in *S. cerevisiae* simply states that this microorganism lacks the necessary enzymes to metabolize this sugar. However, as has been demonstrated by several research groups, some *S. cerevisiae* strains in fact have an inherent capacity to utilize xylose for growth, although at a very low rate compared with glucose (Attfield and Bell,

2006; van Zyl et al., 1989; Wenger et al., 2010). Despite the existence of native xylose-consuming *S. cerevisiae* strains, most research efforts have focused on expressing heterologous enzymes in order to engineer *S. cerevisiae* strains with xylose utilization capability.

As briefly mentioned in **Chapter 1**, two principal pathways have been exploited in the metabolic engineering of *S. cerevisiae* for xylose utilization: the xylitol reductase/xylitol dehydrogenase (XR/XDH) pathway and the xylose isomerase (XI) pathway (Figure 3). From xylose, both these pathways generate xylulose, which can be metabolized by *S. cerevisiae* after entry into PPP. The first attempts to engineer *S. cerevisiae* strains with the ability to utilize xylose were undertaken by expressing the *S. stipitis* genes *XYL1* and *XYL2* encoding xylose reductase and xylitol dehydrogenase, respectively (Kötter et al., 1990; Kötter and Ciriacy, 1993). It was later discovered that overexpression of the endogenous *XKS1* gene encoding xylulokinase catalysing the third step in xylose utilization by XR/XDH was necessary to achieve reasonable xylose consumption rates (Ho et al., 1998). It was found early on that *S. cerevisiae* strains overexpressing *XYL1* and *XYL2* produced significant amounts of xylitol, which was excreted into the fermentation broth resulting in substantial carbon loss (Kötter and Ciriacy, 1993). This phenomenon has been attributed mainly to the different cofactor preferences of XR and XDH, as XR can use both NADPH and NADH with higher affinity for NADPH, while XDH exclusively uses NAD^+ (Kötter et al., 1990; Rizzi et al., 1988). Thus, the first two steps in xylose utilization result in a redox imbalance, as the NADH formed in the oxidation of xylitol to xylulose cannot be reoxidized in the XR/XDH pathway. This results in a lack of NAD^+, which is apparent particularly under anaerobic conditions, as the formed NADH cannot be reoxidized in the respiratory chain (Kötter and Ciriacy, 1993).

Several metabolic engineering approaches have been applied to improve xylose utilization in recombinant *S. cerevisiae.* The cofactor preference of XR has been engineered to obtain increased affinity for NADH over NADPH (Jeppsson et al., 2006; Petschacher and Nidetzky, 2008). Moreover, it has been demonstrated that overexpression of genes such as *RPE1, RKI1, TKL1,* and *TAL1* in the non-oxidative part of PPP results in improved growth on xylulose, but not on xylose (Johansson and Hahn-Hagerdal, 2002). The intracellular concentration and demand for NADPH and thereby the secretion of xylitol have been modified by either deleting *ZWF1* encoding the NADPH-generating enzyme glucose-6-phosphate

dehydrogenase in the oxidative branch of PPP or deleting *GDH1* encoding NADPH-dependent glutamate dehydrogenase involved in ammonia assimilation (Jeppsson et al., 2002; Roca et al., 2003). It is doubtful whether deletion of *ZWF1* is a viable strategy for lignocellulosic bioethanol production, as NADPH has an important role in the detoxification of inhibitors such as furan aldehydes (Gorsich et al., 2006) (section 2.2.1, **Chapter 3**). In addition, xylose transport, which usually occurs through hexose transporters, has been improved in *S. cerevisiae* by overexpressing the glucose/xylose facilitator Gfx1 from *Candida intermedia* (Runquist et al., 2009).

Xylose isomerase converts xylose directly to xylulose, circumventing the problems associated with the carbon loss resulting from xylitol accumulation (although some xylitol can still be produced by an endogenous aldose reductase encoded by *GRE3* (Kuhn et al., 1995). The first functionally expressed XI in *S. cerevisiae* originated from the thermophilic bacterium *Thermus thermophilus*, which resulted in low enzyme activity probably because the optimal activity of this enzyme was found to occur at 85°C (Walfridsson et al., 1996). More successful results have been achieved by overexpressing XI from the anaerobic rumen fungus *Piromyces* sp. E2 and thereafter selecting for clones with increased xylose utilization capacity by evolutionary engineering (Kuyper et al., 2003; Kuyper et al., 2004). Recently, it was also demonstrated that heterologous expression of *XylA* encoding XI from *Clostridium phytofermentans* in *S. cerevisiae* resulted in the ability to use xylose as the sole carbon and energy source and that the kinetic properties of XI from *C. phytofermentans* were comparable to those of XI from *Piromyces* sp. (Brat et al., 2009). *S. cerevisiae* engineered with XI also displayed improvement upon overexpression of genes in the non-oxidative part of PPP (Kuyper et al., 2005). In addition, deletion of *GRE3,* which encodes an endogenous aldose reductase, benefits xylose utilization by XI strains, as xylitol is an inhibitor of XI (Träff et al., 2001). Similar to *ZWF1*, there are indications that expression of *GRE3* is important for inhibitor tolerance (Ma and Liu, 2010a). Thus, although *GRE3* and *ZWF1* deletion improves xylose utilization, it is imperative to demonstrate that improvements also are achieved in a pretreated lignocellulosic raw material to investigate potential trade-offs in inhibitor tolerance.

2.2 Inhibitors in pretreated lignocellulosic raw materials

As mentioned previously, the recalcitrant nature of lignocellulosic feedstocks requires that

the raw materials are pretreated before fermentation to facilitate enzymatic hydrolysis. Depending on the conditions during pretreatment (i.e. temperature, pH, and residence time), greater or lesser amounts of degradation products originating from the raw material constituents will be formed or released. The harshness of the pretreatment can be estimated from a severity factor calculated from: $\log(Ro) = \log(t \times \exp((T - 100)/14.75))$, where t is the residence time and T is the absolute temperature (Overend and Chornet, 1987). As pH influences the formation of degradation products, a combined severity factor (CS) has been developed by modifying the original equation as follows: $CS = \log(Ro) - pH$ (Chum et al., 1990). From these equations, it is clear that at high temperatures, low pH, and long residence times, a high severity factor is obtained, which in turn results in significant carbon loss and large amounts of inhibitory degradation products.

Lignocellulose-derived inhibitors have traditionally been divided into three general classes: furan aldehydes, weak acids, and phenolics (Palmqvist and Hahn-Hägerdal, 2000), although some researchers have argued that a classification based on the functional group(s) of the compound in question is more suitable (Liu, 2011). The concentrations of several of these compounds in various pretreated raw materials are shown in Table 3. It is important to keep in mind that the concentrations of inhibitors are highly dependent on the pretreatment method and raw material used (Klinke et al., 2004). Thus, process parameters such as ethanol productivity can vary considerably between materials, as illustrated in Figure 4, where the volumetric ethanol production rate differs significantly between SHF of *A. donax* and spruce (**Paper 1**; Ask et al., Unpublished results). In this section, the origin and the effects of these inhibitory substances on yeast metabolism will be discussed.

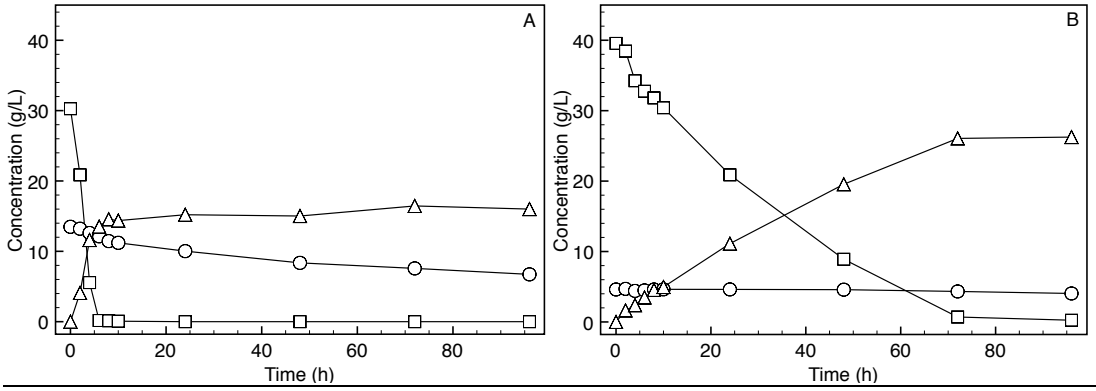

Figure 4. Fermentation of pretreated *A. donax* (A) and spruce (B) hydrolysates in SHF with VTT C-10883 (**Paper I**) at an inoculum concentration of 4 g L^{-1}. Squares: glucose, circles: xylose, triangles: ethanol. Note the large difference in volumetric ethanol production rate between the two fermentations, which is a result of the more inhibitory spruce hydrolysate.

Table 3. Concentrations of furan aldehydes and weak acids in a selection of pretreated lignocellulosic raw materials.

Raw materials	Pretreatment	Solid consistency (%) (w/w)	Furfural (g L^{-1})	HMF (g L^{-1})	Acetic acid (g L^{-1})	Levulinic acid (g L^{-1})	Formic acid (g L^{-1})
Softwood							
Spruce[a]	Steam, 4% H_2SO_4, 195°C, 15 min	10% dry matter (DM)[g]	1.2	3.2	4.7	0.2	0.6
Spruce[b]	Steam, 0.5% H_2SO_4, 222°C, 7 min	n.a.	1.0	5.9	2.4	2.6	1.6
Pine[c]	Steam explosion, H_2SO_4, 198°C, 7 min	33% DM[h]	1.3	5.7	3.1	n.a.	n.a.
Hardwood							
Aspen[c]	Steam explosion, H_2SO_4, 198°C, 7 min	33% DM[h]	2.8	1.3	10.1	n.a.	n.a.
Birch[c]	Steam explosion, H_2SO_4, 198°C, 7 min	25% DM[h]	1.8	0.8	9.2	n.a.	n.a.
Energy crops							
A. donax[d]	Steam explosion, 207°C, 4.5 min	10% water insoluble solids (WIS)[g]	0.4	0.1	5.7	n.a.	0.2
Agricultural waste							
Wheat straw[e]	Steam explosion, 210°C, 5 min	13.5% WIS[g]	1.4	0.1	5.1	n.a.	1.3
Sugar cane bagasse[a]	Steam, 2.5% SO_2, 203°C, 5 min	10 % DM[g]	1.0	3.0	3.2	0.2	0.6
Corn stover[f]	Steam, 2% SO_2, 200°C, 5 min	13.7 % WIS[g]	1.5	0.2	2.2	n.a.	n.a.

n.a.: not available
[a](Alriksson et al., 2011)
[b](Larsson et al., 1999)
[c](Taherzadeh et al., 1997b)
[d]**Paper I**
[e](Tomás-Pejó et al., 2008)
[f](Öhgren et al., 2006)
[g]Concentration after pretreatment
[h]Concentration before pretreatment

2.2.1 Furan aldehydes

In the context of this thesis, furan aldehydes refer to two compounds, 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF), frequently encountered in considerable amounts in lignocellulosic hydrolysates. Furfural and HMF are formed in dehydration reactions at high temperatures and low pH from pentoses and hexoses, respectively (Dunlop, 1948;

Ulbricht et al., 1984). Furfural is produced industrially in volumes of several hundred thousand tons per year, and its main uses are as a solvent and for producing furfuryl alcohol used in furan resin production (Moreau et al., 2004). Furfural is also used as flavour enhancer in the food industry (Adams et al., 1997). HMF has not yet attracted the same industrial attention, although utilization of 2,5-furandicarboxylic acid obtained by oxidizing HMF has been suggested as a starting point for producing polyesters and polyamides (Moreau et al., 2004). As HMF and furfural are produced at elevated temperatures from sugars, both compounds have been found in heat-treated food (Adams et al., 1997; Ramirez-Jimenez et al., 2000).

Despite the presence of furfural and HMF in food, these compounds have been demonstrated to have numerous adverse effects on microbial metabolism, such as reducing the fermentation rate, increasing the lag phase of growth, reducing viability, inhibiting several enzymes in glycolysis and fermentation, and inducing reactive oxygen species (ROS) resulting in DNA and membrane damage (Allen et al., 2010; Banerjee et al., 1981; Heer and Sauer, 2008; Modig et al., 2002; Palmqvist et al., 1999a; Taherzadeh et al., 1999). Inhibitory effects have been demonstrated not only in *S. cerevisiae*, but also in other eukaryotes such as *K. marxianus, S. stipitis,* and *T. reesei* as well as in prokaryotes such as *Escherichia coli* and *Zymomonas mobilis* (Delgenes et al., 1996; Oliva et al., 2003; Szengyel and Zacchi, 2000; Zaldivar et al., 1999). The reactivity of the molecules has been attributed to the aldehyde group of the compounds, and the toxicity of inhibitors generally decreases in the following order: aldehydes > ketones > acids > alcohols (Almeida et al., 2007).

Under anaerobic conditions, furfural and HMF can be converted *in situ* to their less toxic corresponding alcohols, furfuryl alcohol and furan-2,5-dimethanol, whereas under fully respiratory conditions, furfural is oxidized to furoic acid (Liu et al., 2004; Morimoto and Murakami, 1967; Sárvári Horváth et al., 2003; Taherzadeh et al., 2000). The *in situ* reduction of furan aldehydes to alcohols is thought to be mediated by NAD(P)H-dependent oxidoreductases. If furfural and HMF are initially present in a cultivation, the lag phase lasts until sufficient furan aldehydes have been converted to reach a strain-specific threshold concentration, after which growth resumes (Almeida et al., 2007; Heer and Sauer, 2008). The fact that furfural and HMF can act as electron acceptors in the cell benefits xylose-consuming *S. cerevisiae* strains engineered with the XR/XDH pathway, since the NADH produced during the oxidation of xylitol to xylulose can be reoxidized by furan aldehydes at non-

inhibitory concentrations, thereby reducing xylitol accumulation (Wahlbom and Hahn-Hägerdal, 2002) (**Paper III**). Nevertheless, the additional NADPH demand imposed by the reductive detoxification of HMF and furfural can have other consequences for the cells, as will be discussed in **Chapter 3** (**Papers II-III)**.

Furan aldehydes and, in particular, furfural have been identified as key inhibitors in pretreated lignocellulosic materials (Heer and Sauer, 2008; Taherzadeh et al., 1999). Thus, detailed investigation of the molecular mechanisms of action of these compounds is needed in order to develop novel yeast strains with improved inhibitor tolerance. As the conversion of furfural and HMF to less toxic alcohols is thought to involve redox reactions mediated by NAD(P)H and $NAD(P)^+$ and as stress responses may require free energy in the form of ATP, I investigated the influence of furan aldehydes on these parameters as part of this thesis research (**Papers II-III**). Indirect evidence for perturbations in redox metabolism caused by furan aldehydes has been inferred from the redistribution of fluxes of extracellular metabolites such as glycerol, acetate and xylitol (Horvath et al., 2001; Palmqvist et al., 1999a; Wahlbom and Hahn-Hägerdal, 2002), but data are currently unavailable on how these inhibitors affect the distribution of intracellular metabolites and, in particular, the redox cofactors. As will be further elaborated on in **Chapter 3**, redox metabolism plays a central role in the overall yeast metabolism, as NAD(P)H and $NAD(P)^+$ function as cofactors in numerous intracellular reactions. Hence, much is at stake when perturbing the intracellular levels of NAD(P)H.

2.2.2 Weak acids

The growth-inhibitory effects of weak acids on microorganisms have long been known, and have been exploited in food preservation, as reviewed by Mollapour et al. (2008). Weak acids in the context of pretreated lignocellulosic materials generally refer to acetic acid, formic acid, and levulinic acid, but several weak acids are also part of the phenolics group (Klinke et al., 2004). Acetic acid is released during pretreatment as a result of the deacetylation of hemicellulose chains, whereas formic acid and levulinic acid are degradation products of furfural and HMF (Dunlop, 1948; Ulbricht et al., 1984). Acetic acid occurs in considerable amounts in some xylan-rich lignocellulosic raw materials such as hardwoods and in herbaceous energy crops such as *A. donax*. At low concentrations (~3 g L^{-1}), acetic acid has stimulatory effects on ethanol yield at the expense of biomass yield due to the uncoupling of ATP utilization from growth (Taherzadeh et al., 1997a; Verduyn et al., 1990a). The decrease

in biomass yield results in a concomitant decrease in glycerol yield (see box 2, **Chapter 3**), which consequently results in an increased carbon-flux towards ethanol. At higher acetic acid concentrations, negative effects on specific growth rate and lag phase have been observed (Taherzadeh et al., 1997a).

The inhibitory effects of weak acids are strongly correlated with pH and have been attributed to two factors: intracellular acidification and intracellular accumulation of anions (Russell, 1992; Warth, 1989). Intracellular acidification results from the passive influx of weak acids in their undissociated form from the extracellular medium (usually pH 5) over the plasma membrane into the cytosol (pH ~7). This phenomenon is particularly apparent when the pH of the growth medium is near or lower than the pK_a of the weak acid in question. The pK_a values of the acids relevant in the context of the fermentation of lignocellulosic feedstocks (i.e. formic, levulinic and acetic acids) are in the range of 3.75 – 4.75, which means that nearly half of the acetic acid is present in its undissociated form at pH 5. To counteract the decrease in pH caused by the influx of protons, *S. cerevisiae* can pump out the protons through the plasma membrane H^+-ATPase encoded by *PMA1* (Serrano et al., 1986). When the proton efflux capacity of the cells is exhausted, intracellular acidification occurs, ultimately leading to cell death (Pampulha, 1989). An alternative theory of weak acid toxicity, which probably should be considered a complementary theory, is based on the fact that anions accumulate in the cytoplasm after the diffusion of weak acids into the cell (Russell, 1992). High intracellular anion concentrations have been correlated with lower glycolytic activity (Pampulha and Loureiro-Dias, 1990). In addition, anion efflux has been demonstrated to occur through Pdr12p, which is another transporter with ATPase activity (Piper et al., 1998).

As the xylose utilization rate and consequently, the ATP production rate, is lower than the glucose consumption rate in recombinant xylose-utilizing *S. cerevisiae*, acetic acid stress is a major challenge to efficient xylose consumption in strains carrying the XR/XDH pathway as well as the XI pathway (Bellissimi et al., 2009; Casey et al., 2010; Helle et al., 2003). In my thesis research, I corroborated the impact of acetic acid on xylose utilization using industrial substrates, where pretreated *A. donax* was converted to ethanol in SHF and SSF (**Paper I**). Figure 5 shows the time courses of glucose, xylose, and ethanol concentrations from fermentation experiments using two different batches of pretreated *A. donax* (batches 1 and 2); batch 1 contained 7.0 g L^{-1} of acetic acid, whereas some of the acetic acid in batch 2 was

removed, resulting in a material with 2.8 g L^{-1} of acetic acid. No major differences were observed in terms of glucose utilization, which reached completion after 6 hours in both pretreated materials. However, 50% of the xylose initially available in batch 1 was still present after 96 hours of fermentation, whereas 96% of the xylose present in batch 2 was consumed.

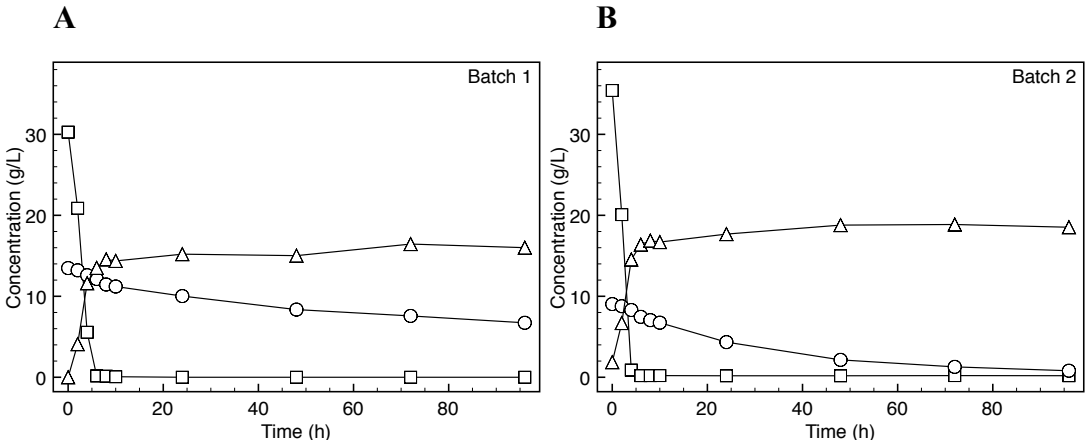

Figure 5. Time courses of glucose (squares), xylose (circles) and ethanol (triangles) concentrations from SHF of two different batches of pretreated *A. donax* fermented with VTT-10883 (**Paper I)** at an inoculum concentration of 4 g L^{-1}. Of the initially present xylose, 50% was consumed in batch 1 (A) and 96% was consumed in batch 2 (B). The main difference between the two batches was that batch 1 contained a significantly higher concentration of acetic acid (7.0 g L^{-1}) compared with batch 2 (2.8 g L^{-1}).

To verify that acetic acid was the reason for the decreased xylose utilization, the low-acetic acid-containing pretreated material (batch 2) was supplemented with additional acetic acid equivalent to the acetic acid content of batch 1. Indeed, this resulted in similarly decreased xylose utilization as in the batch 1 material (Figure 6). To demonstrate the importance of extracellular pH for acetic acid stress during xylose consumption, the pH was increased to 5.5 after 96 hours. By increasing the pH, the relative ratio of the dissociated to undissociated forms of acetic acid can be increased, which is described by the Henderson-Hasselbach equation, i.e., $pH = pK_a + \log_{10}([A^-]/[HA])$. Interestingly, the 0.5 unit increase in pH resulted in a 46% increase in the xylose consumption rate (**Paper I**).

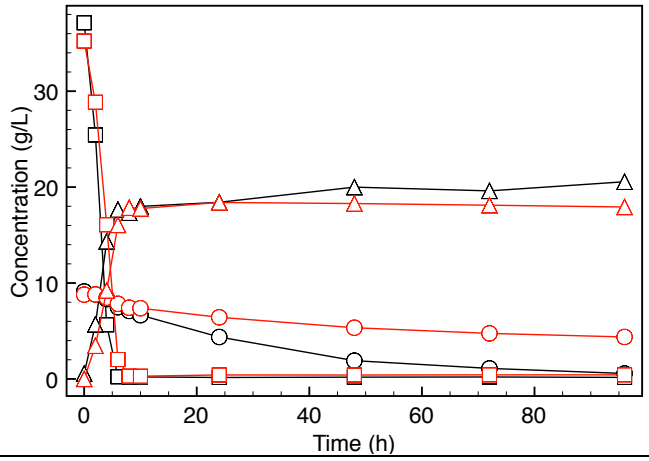

Figure 6. Time courses of glucose (squares), xylose (circles), and ethanol (triangles) concentrations from SHF of batch 2 *A. donax* with (red lines) and without (black lines) supplementation of acetic acid to a final concentration equivalent to that of the batch 1 material. The fermentation was performed with VTT-10883 at an inoculum concentration of 4 g L^{-1} (**Paper I**).

Bellissimi et al. (2009) demonstrated that a limiting glucose feed could alleviate the inhibitory effects of acetic acid on xylose consumption by providing free energy for proton and possibly anion extrusion, thereby corroborating the key role of ATP in acetic acid tolerance. As glucose is continuously released during SSF, a positive effect on xylose utilization similar to that of glucose feeding could be expected. However, SSF of *A. donax* containing a high acetic acid concentration (batch 1) did not result in improved xylose uptake compared with SHF, possibly due to the low glucose release during the later part of the process. The results obtained in **Paper I** highlight the importance of dealing with acetic acid for economically viable conversion of *A. donax* to ethanol. The problems with acetic acid could be dealt with as in **Paper I**, by partly removing it or by increasing the pH. However, both these processes fall short in terms of either the process economics or the increased risk of contamination incurred by increasing the pH. Thus, an approach that increases the inherent capacity of *S. cerevisiae* to tolerate high acetic acid concentrations is suggested. This could be achieved by either evolutionary engineering or rational methods based on, for example, transcriptome data.

2.2.3 Phenolics

The phenolics constitute a heterogeneous group of compounds that originate from the partial degradation of lignin (Ando et al., 1986). The nature and amount of phenolics are dependent on the pretreatment technology and type of feedstock used, since the composition in terms of the lignin building blocks and internal bonding can differ considerably. For example, softwood materials produce almost exclusively guaiacyl-derived phenolics, whereas the

phenolics composition is more variable for hardwood and herbaceous materials (Klinke et al., 2004). Despite the great variability of phenolic compounds, after reviewing the literature on the phenolic compositions of pretreated lignocellulosic raw materials, Klinke et al. (2004) concluded that 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, syringaldehyde, and syringic acid were the most frequently occurring among the reported phenolics.

Most studied phenolic compounds increase the lag phase of growth and reduce the specific growth rate and volumetric ethanol productivity of *S. cerevisiae* (Ando et al., 1986; Clark and Mackie, 1984; Klinke et al., 2003; Larsson et al., 2000b). In a pretreated willow hydrolysate, removing most of the phenolics by enzymatic treatment significantly increased the fermentability, illustrating the significance of these compounds as inhibitors in industrial hydrolysates (Jönsson et al., 1998). Furthermore, the inhibitory effect was demonstrated to increase with decreasing degree of substitution of the aromatic ring (Klinke et al., 2003). In addition, the location of the methoxyl and hydroxyl substituents (i.e., *para, ortho,* and *meta*) reportedly influences the toxicity (Larsson et al., 2000b).

The functional group of the phenolics is important, as phenolic aldehydes and ketones are generally more inhibitory than are phenolic acids and alcohols (Ando et al., 1986; Clark and Mackie, 1984; Klinke et al., 2003). Like the furan aldehydes, the phenolic aldehydes are converted *in situ* to their corresponding alcohols, although no conversion of ketones has been observed (Heer and Sauer, 2008; Klinke et al., 2003; Larsson et al., 2000b). The inhibitory mechanisms of the phenolics remain to be elucidated, although phenolics have been hypothesized to cause loss of membrane integrity, thereby destroying the membrane's capacity to act as a selective barrier (Heipieper et al., 1994). As phenolic aldehydes, like the furan aldehydes, are converted to their corresponding alcohol forms in metabolism, it is tempting to speculate that phenolic and furan aldehydes both act to perturb redox metabolism. This hypothesis was investigated in the case of furan aldehydes in **Papers II-III (Chapter 3)**, but the influence of phenolic aldehydes on redox metabolism remains to be elucidated.

2.2.4 Other inhibitors

A certain amount of ash components present mainly as inorganic salts, is contained in lignocellulosic materials. Moreover, inorganic ions are also added with chemicals used in

pretreatment, during pH adjustment of the pretreated raw material, and are plausibly also leached off from process equipment (Jönsson et al., 2013; Klinke et al., 2004). Despite *S. cerevisiae* being an osmotolerant microorganism, high concentrations of salts have been demonstrated to reduce the volumetric ethanol production rate, with more pronounced effects when xylose was used as the sole carbon source (Casey et al., 2013). In addition, the amplitude of the inhibitory effect is dependent on the nature of the ion, with Ca^{2+} being the most inhibitory (Maiorella et al., 1984).

Ethanol is also a potential inhibitor of *S. cerevisiae,* as it has been reported to reduce the specific growth rate, viability, and specific fermentation rate (D'amore and Stewart, 1987). However, since the concentration of ethanol in lignocellulosic bioethanol processes rarely exceeds 50 g L^{-1}, ethanol probably elicits a minor effect on its own. However, ethanol may act synergistically in combination with other inhibitors.

2.2.5 Combined effects of inhibitors

Synergism means that the combined effect of several inhibitors is greater than the summed effects exerted by individual inhibitors acting in isolation. Few studies report on the combined effects of several inhibitors in a robust experimental design. This is probably due to the great many experiments that would have to be carried out to systematically decipher synergistic effects. Thus, most studies of the effects of lignocellulose-derived inhibitors have been performed by examining the inhibitors in isolation, except for a few studies that will be mentioned here. Palmqvist et al. (1999b) have performed a full 2^3 factorial design to elucidate the potential synergistic effects of acetic acid, furfural, and *p*-hydroxybenzoic acid on *S. cerevisiae*. It was found that furfural and acetic acid interacted synergistically by reducing the specific growth rate, ethanol yield, and biomass yield (Palmqvist et al., 1999b). Furthermore, syringaldehyde and acetovanillone are known to interact synergistically in *S. cerevisiae* by reducing the volumetric ethanol productivity when added to a wheat straw hydrolysate pretreated by wet oxidation (Klinke et al., 2003). Furfural was also found to act synergistically with HMF and aromatic aldehydes such as vanillin, 4-hydroxybenzaldehyde, and syringaldehyde in *E. coli* by reducing the specific growth rate and ethanol yield, whereas all other binary combinations were additive (Zaldivar et al., 1999). In another study of *E. coli*, furfural acted synergistically with its conversion product furfuryl alcohol by reducing the specific growth rate and ethanol yield (Zaldivar et al., 2000). Finally, it was found that

none of the weak acids commonly found in lignocellulosic hydrolysates acted synergistically on the specific growth rate of *E. coli* in binary combinations (Zaldivar and Ingram, 1999).

There is no doubt that inhibitory substances formed or released when pretreating lignocellulosic materials are challenging for microorganisms, acting as impediments to increasing the solids consistency in fermentation processes as the inhibitor concentration would increase accordingly. All inhibitor classes and combinations thereof are obviously important, as described above. However, the furan aldehydes and, in particular, furfural have been identified as key mediators of hydrolysate toxicity in several investigations (Heer and Sauer, 2008; Taherzadeh et al., 1997b). Despite the considerable research into the effects of furfural and HMF, unanswered questions remain relating to the mechanisms of inhibition, which will be discussed in the next chapter.

CHAPTER 3

REVEALING PHYSIOLOGICAL RESPONSES TO LIGNOCELLULOSIC INHIBITORS: A CASE STUDY OF FURFURAL AND HMF

Furfural and HMF can be abundant in pretreated lignocellulosic raw materials and, as discussed in the previous chapter, the presence of furfural and HMF can have serious consequences for strain performance, as cellular physiology may be influenced in a number of ways. Detailed physiological studies of the effects of furan aldehydes are imperative if we are to discover rational metabolic engineering strategies that can be applied to improve microbial robustness, as the metabolic responses can provide a key to the mechanisms of inhibition. Most experiments treating the physiological effects of furfural and HMF have been performed in batch cultures, whereas only a few have been carried out in continuous cultivations. As will be discussed in this chapter, the choice of experimental setup is of considerable importance, and should be governed by the research question articulated. During my thesis research, both batch and continuous cultivations were applied in the physiological studies using both glucose and xylose as carbon and energy sources. Using both glucose and xylose is important, since the culture conditions should be as similar as possible to those in a lignocellulosic hydrolysate. This chapter will start with a discussion of the potential advantages and disadvantages of the cultivation methodologies used in my research, i.e., batch and continuous cultivation, in light of the influence of furan aldehydes on gene expression and intracellular metabolites, such as the redox cofactors $NAD(P)^+$ and NAD(P)H, and adenonucleotides.

3.1 Cultivation methodologies

3.1.1 Batch cultivation

Batch cultivation is probably the easiest way to perform a liquid cell culture. This cultivation mode is a closed system (except for gas exchange and possibly pH control) in which all necessary nutrients are supplied in ample amounts at the start of the fermentation. Growth in such a system is characterized by three distinct phases: lag phase, exponential growth phase and stationary phase. In the lag phase, the duration of which is dependent on the history of

the preculture and the inoculum size, the cells are adapting to the new conditions by synthesizing essential constituents needed for growth (Bailey and Ollis, 1986). Since nutrients are available in ample amounts, the cells are growing at their maximum specific growth rate (μ_{max}) in the exponential growth phase, as inferred from Monod kinetics, i.e., $\mu = (\mu_{max} \times S)/(K_s + S)$, as long as $S >> K_s$. As growth proceeds, the cells excrete metabolic byproducts such as CO_2, ethanol, glycerol, and acetate. Hence, the concentrations of biomass and metabolic products in the fermentation broth will change continuously during the course of the cultivation. At the end of the exponential growth phase, cell growth ceases as nutrients start to become limiting and toxic metabolites are accumulating in the fermentation broth. The cells are then entering the stationary phase. Under aerobic conditions, the cells are able to oxidize metabolic products such as ethanol, acetate, glycerol, and pyruvate synthesized and excreted during exponential growth as part of aerobic fermentation due to the Crabtree effect (see section 1.5). Thus, following a short stationary phase called the diauxic shift, growth resumes and continues until all potential carbon and energy sources have been depleted, provided that other nutrients are available. Under anaerobic conditions, which were the conditions applied in this thesis research (**Papers I-IV**), these metabolic products cannot function as carbon and energy sources.

Batch cultivations have been the main cultivation mode whenever the effects of furfural and HMF have been studied in the literature. One of the major reasons for this is probably that batch cultivations are cheap and relatively easy to set up. In addition, batch cultivations can be performed in shake flasks, making it possible to test several compounds and concentrations at once. However, as noted before, the fact that the medium composition changes over time must be considered when batch cultivations are used for physiological studies, by specifying the time intervals over which yields and productivities are calculated. Furthermore, if gene expression studies are performed in batch cultures, it is important to recall that the specific growth rate has been demonstrated to be correlated with the expression of a number of genes (Regenberg et al., 2006). Since both furfural and HMF inhibit growth, this correlation is of particular importance, as gene expression responses specific to the inhibitors may be confounded with changes related to perturbations in the specific growth rate. If shake flasks are used, culture conditions such as pH and degree of anaerobiosis can seldom be controlled. The pH decreases during the course of a batch fermentation due to uptake of ammonium from the growth medium and due to the metabolic production and secretion of acids. As the goal of a physiological study is to find causal relationships specific

to the studied compound, confounding effects due to changes in pH have to be avoided. In most of the work related to this thesis (**Papers I-III**), the cultivations were performed in bioreactors under well-defined conditions with the pH maintained at 5 by automatic addition of base and a continuous flow of nitrogen to sustain anaerobic conditions. Such defined conditions allow better control of the fermentation process, providing the best circumstances for extracting physiological data from batch cultivations. However, it is important to keep in mind that continuously sparging the medium with nitrogen gas causes an increase in ethanol evaporation, which can be reduced by equipping the bioreactor with a condenser.

Several approaches have been tested for studying the effects of furfural and HMF in batch mode. For example, the inhibitors have been added to the growth medium at the start of the fermentation, i.e., before the inoculum (Almeida et al., 2009b; Liu et al., 2004; Palmqvist et al., 1999a). In such studies, a prolonged lag phase is often observed, which is attributed to the cells' need to convert the furan aldehydes to less toxic alcohols before growth can be initiated. As the duration of the lag phase is a common parameter used for measuring yeast tolerance to furan aldehydes, the growth phase from which the inoculum culture is taken (i.e. exponential, post-diauxic, or stationary) is crucial, as an inoculum culture harvested during the stationary or the post-diauxic phase rather than the exponential phase could give rise to different results. In fact, transition from the exponential phase to the diauxic shift resulted in the differential expression of thousands of genes, including the induction of the environmental stress response (ESR), suggesting that cells from different growth phases have different prerequisites for dealing with inhibitors. (DeRisi et al., 1997; Gasch and Werner-Washburne, 2002). Moreover, the preadaptation of inoculum cultures to lignocellulosic hydrolysates by using a hydrolysate feed (Alkasrawi et al., 2006; **Paper I)**, resulted in improved performance in SSF of spruce, possibly from the induction of ESR, further corroborating the importance of precultivation.

Inhibitors can also be pulsed during the exponential phase (Taherzadeh et al., 1999; **Paper III**). In fact, this is the preferred method if batch cultivations are to be used, as the cells are growing at the maximum specific growth rate and the growth is considered balanced, i.e., the rate of biosynthesis of cellular constituents is constant, consequently avoiding the issues connected with the state of the inoculum culture. After inhibitors have been pulsed, the dynamic response of the cell metabolism can be followed on the desired depth (i.e. transcriptome, proteome, and/or metabolome). Such studies provide a wealth of data hinting

about the cellular dynamics involved in responses to inhibitors, data that can advance our understanding of physiological perturbations.

3.1.2 Continuous cultivation

As opposed to batch mode, in continuous cultivations fresh medium is added continuously to the bioreactor over time, while spent medium is removed. Although there are several continuous cultivation operation modes, the vast majority of continuous cultivations reported in the literature have been performed as chemostats (Bull, 2010). In a chemostat, the rates of medium addition and removal are designed so that a constant volume is maintained in the reactor, i.e., the dilution rate (D), defined as F_{in}/V_r, where F_{in} is the flow-rate of medium into the reactor and V_r is the reactor volume, is kept constant. From a mass balance of biomass over the reactor, it can be demonstrated that $D = \mu$, which makes it possible to choose the specific growth rate by setting the desired D. One of the nutrients (often the carbon source) is supplied at a limiting concentration, thereby controlling the biomass concentration in the bioreactor.

One of the major advantages of the chemostat for physiological studies is related to the concept of *steady state.* At steady state, the concentrations of biomass and metabolic products remain constant over time in the bioreactor and equal to the concentration in the outlet. The fact that the specific growth rate and medium conditions are constant has major implications for the conclusions drawn from gene expression data, as the specific growth rate has been found to influence gene expression patterns (Regenberg et al., 2006) and intracellular metabolite concentrations (Boer et al., 2010). Hence, when a growth-inhibiting substance such as furfural and/or HMF is added to a cell culture, it can be difficult to distinguish the effects on gene expression caused by the inhibitor and the effects originating from the change in specific growth rate. By using a chemostat with furfural and HMF present in the feed medium, I could circumvent the influence of the inhibitors on the specific growth rate (**Paper II**). On the other hand, it should be noted that when a chemostat with inhibitors present in the feed medium is used as opposed to pulsed batch cultivations, the longer-term effects of the inhibitors would be the focus of the study, in contrast to the latter case when transient dynamics can be studied (**Paper III**).

The disadvantage or rather limitation of using a chemostat setup to studying the physiological impacts of furfural and HMF on yeast metabolism is that the inhibitor concentrations must be

fine-tuned in order to reach the highest concentration that does not result in wash-out of the culture. Therefore, responses to very high concentrations of inhibitors cannot be studied in this setup; instead, the batch mode would have to be used (**Paper III**). Another risk faced with chemostats in general is contamination due to the prolonged cultivation time compared with that of a batch process; the risk of contamination in chemostats calls for the meticulous application of sterile routines. In addition, it has been demonstrated that prolonged chemostat cultivations can result in cell populations adapted to the conditions prevailing in the bioreactor, manifested in changes in gene expression patterns, morphology, and specific growth rate compared with those of the original strain (Jansen et al., 2005). A suggested rule of thumb is not to cultivate cells for more than 20 generations to avoid adaptation (Ferea et al., 1999). The number of generations in the chemostat experiments in the current work did not exceed 15 (**Paper II**).

3.2 Physiological responses of *S. cerevisiae* to furfural and HMF

As mentioned in **Chapter 2**, although a wealth of data on the physiological responses of *S. cerevisiae* to furfural and HMF exists in the literature, questions remain concerning the influence of furan aldehydes on intracellular metabolites and, in particular, on redox and energy cofactors. As discussed in Boxes 1 and 2, perturbations in redox and energy cofactor levels could result in cell-wide responses. In my thesis research, both batch and chemostat cultivations were used to study the dynamic and long-term effects on the redox and energy metabolism of a xylose-utilizing *S. cerevisiae* strain in the presence of furan aldehydes. In fact, chemostat cultivations can be used for dynamic studies as well by pulsing inhibitors at steady state. However, as I was particularly interested in dissecting the physiological responses to furan aldehydes during both glucose and xylose consumption, batch cultivations had to be used since the strain I used is unable to grow on xylose. The major results of my research and their potential impact will be discussed in the following section.

Box 1. Redox metabolism of *S. cerevisiae*

Nicotinamide adenine dinucleotide (NAD^+) and its phosphorylated counterpart nicotinamide adenine dinucleotidephosphate ($NADP^+$), generally referred to as reducing equivalents or redox cofactors, are two co-enzymes mediating the transfer of electrons in redox reactions in the cell. The reduced forms of the cofactors are denoted NADH and NADPH, respectively. NADH is formed in glycolysis by the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate (Figure 7). In fully respiratory metabolism, NADH formed in the dissimilation of glucose can be reoxidized in the respiration chain with the concomitant production of ATP, thus releasing the energy stored in NADH. In contrast, under anaerobic conditions, which were applied in my thesis research, NAD^+ is regenerated by the conversion of acetaldehyde to ethanol by alcohol dehydrogenase. As such, the conversion of glucose to ethanol is redox neutral, but NADH is also produced during biomass formation in amino acid, nucleotide, and lipid biosynthesis (Bruinenberg et al., 1983). Furthermore, net NADH production also results from the production of various metabolic products such as acetate, acetaldehyde, pyruvate, and succinate. For the cell to maintain the redox balance, the NADH formed in these reactions has to be reoxidized, and this is accomplished by glycerol formation (Figure 7).

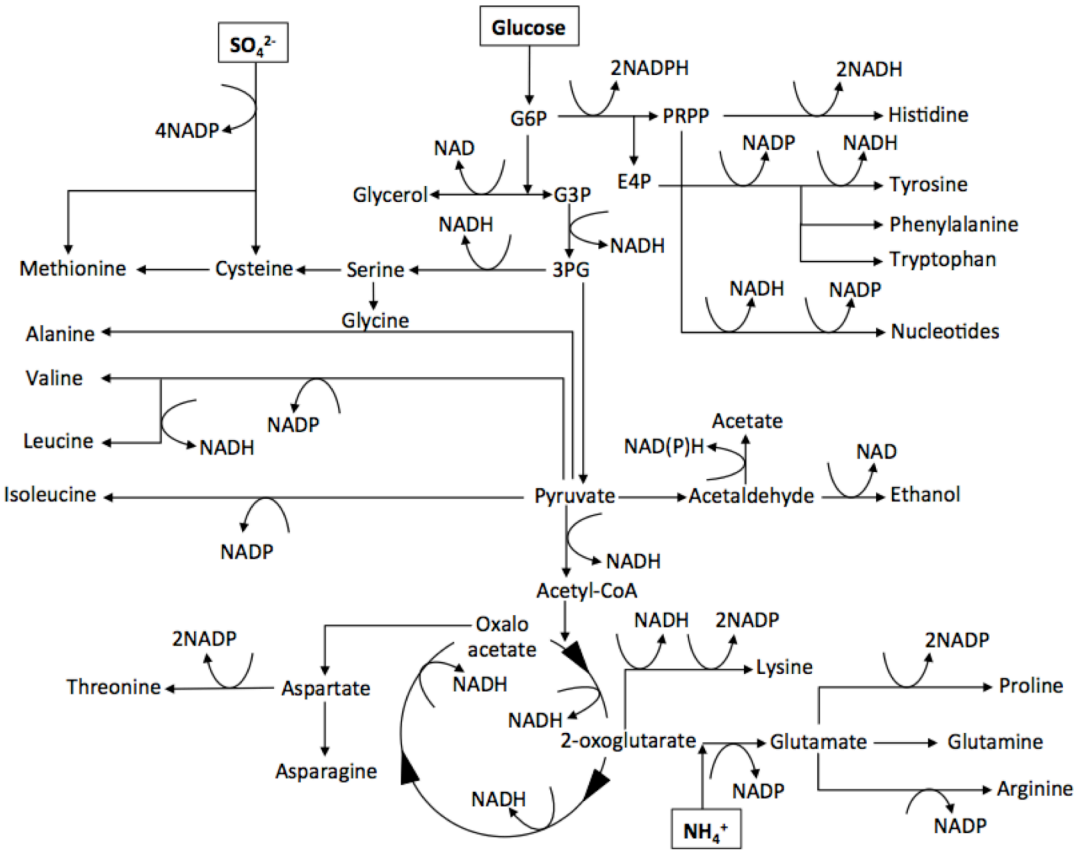

Figure 7. Central NAD(P)H-consuming and -generating reactions in the metabolism of *S. cerevisiae*. For simplicity, several metabolites and reactions were left out. The diagramme is modified from Albers et al. (1996).

While NADH is mainly involved in dissimilatory processes, NADPH is predominantly involved in assimilatory reactions. When ammonia is used as the nitrogen source, the main fraction of the NADPH produced in the cell is utilized in ammonia assimilation by NADPH-

Box 1 continued

dependent glutamate dehydrogenase encoded by *GDH1* (Figure 7). A considerable amount of NADPH is also required in the biosynthesis of lipids and nucleotides (Lagunas and Gancedo, 1973; Nissen et al., 1997). There is turnover of NAD(P)H in both the cytosol and the mitochondria, and as redox cofactors cannot traverse the inner mitochondrial membrane, they must either be reoxidized in the compartment in which they were produced or be transported by redox shuttles such as the glycerol-3-phosphate and ethanol-acetaldehyde shuttles, reviewed by Bakker et al. (2001). Hence, the net NADH produced in amino acid synthesis in mitochondria under anaerobic conditions cannot be reoxidized in the respiration chain but must instead be shuttled out of the mitochondria and reoxidized through glycerol formation in the cytosol. The main route of cytosolic NADPH biosynthesis is the oxidative branch of PPP, whereas mitochondrial NADPH can be produced by the oxidation of isocitrate to 2-oxoglutarate by isocitrate dehydrogenase (Bruinenberg et al., 1983). Cytosolic and mitochondrial NADPH can also be synthesized through the oxidation of acetaldehyde to acetate by $NADP^+$-dependent aldehyde dehydrogenases encoded by *ALD6* and *ALD4*, respectively (Meaden et al., 1997; Tessier et al., 1998; **Paper III**).

Both NAD(H) and NADP(H) are highly interconnected within the metabolic network, meaning that perturbations in the levels of the redox cofactors can lead to a global response (Nielsen, 2003). In fact, NAD^+, NADH, $NADP^+$, and NADPH were included in 78, 65, 86 and 78 reactions, respectively, in a reconstructed metabolic network of *S. cerevisiae* (Forster et al., 2003). In this thesis research, a yeast strain harboring the XR/XDH pathway for xylose utilization was used. Introduction of this heterologous pathway perturbs the redox metabolism of the cell, as the XR/XDH pathway is not redox neutral (as discussed in **Chapter 2**). In the case of the XR/XDH pathway, the NADPH demand of the cell is increased, which leads to higher fluxes in NADPH-generating pathways (Pitkanen et al., 2003). Furthermore, addition of furan aldehydes (the focus of **Papers II-III**) perturbs the redox metabolism as these inhibitors consume NAD(P)H as part of their detoxification. The major consequence of increasing NADH oxidation has been demonstrated to be reduced glycerol production, since the surplus NADH produced in assimilatory reactions can be oxidized by alternative means (Hou et al., 2009; Vemuri et al., 2007). In fact, metabolic engineering strategies aimed at redirecting carbon fluxes towards ethanol at the expense of glycerol and CO_2 production have successfully been demonstrated by increasing NADH oxidation and reducing NADPH production through PPP. Nissen et al. (2000) achieved this through engineering nitrogen assimilation by deleting *GDH1* encoding NADPH-dependent glutamate dehydrogenase and overexpressing the NADH-dependent pathway for nitrogen assimilation catalysed by glutamine synthetase and glutamate synthase encoded by *GLN1* and *GLT1*, respectively, which resulted in a 10% increase in ethanol yield and 28% decrease in glycerol yield.

Increased oxidation of NADPH by external electron acceptors such as furan aldehydes or by introducing NADPH-consuming pathways such as XR/XDH is expected to influence NADPH-requiring reactions such as nitrogen assimilation, nucleotide biosynthesis, and

Box 1 continued

amino acid biosynthesis. Nissen et al. (2001) demonstrated that lowering intracellular NADPH levels resulted in the accumulation of 2-oxoglutarate, which is a precursor of NADPH-dependent glutamate biosynthesis. The accumulation of 2-oxoglutarate suggests a decreased flux towards glutamate, which in turn can influence numerous reactions in amino acid and nucleotide biosynthesis, as glutamate acts as a donor of amino groups in these pathways (Jones and Fink, 1982). Alterations in the redox metabolism have demonstrated to induce changes at the gene expression level. Decreased NADPH demand resulting from the deletion of *GDH1* led to the downregulation of *ZWF1* and *GND1*, which encode the NADPH-generating enzymes in PPP (Bro et al., 2004). In contrast, increased NADPH demand induced by adding the external electron acceptor acetoin, which is enzymatically reduced to 2,3 butanediol with NADPH as the cofactor, resulted in the induced expression of *GND1* and NADPH-consuming pathways such as sulphate assimilation (Celton et al., 2012).

3.2.1 Effect of furfural and HMF on metabolic product distribution in batch and chemostat cultivations

Several metabolic products are produced when glucose and xylose are dissimilated to generate energy in the form of ATP, the major products being biomass, ethanol, CO_2, glycerol, acetate, xylitol, and pyruvate (Figure 3). Determination of the metabolic yields and specific rates of these products ultimately reflects the distribution of metabolic fluxes within the cell. In **Paper III**, furfural and HMF were pulsed in the glucose and xylose consumption phases, respectively (Figure 8).

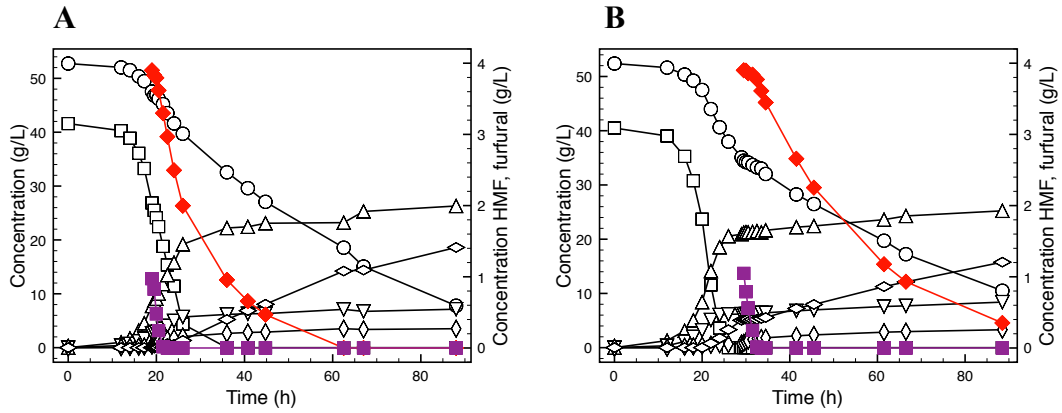

Figure 8. Time courses of sugar, metabolite and inhibitor concentrations after pulsing inhibitors in the glucose (A) and xylose (B) consumption phases. Furfural and HMF were pulsed after 19 and 29.5 hours in the glucose and xylose consumption phases, respectively. Squares: glucose, circles: xylose, triangles: ethanol, inverted triangles: glycerol, elongated diamonds: acetate, compressed diamonds: xylitol, red diamonds: HMF, purple squares: furfural (**Paper III**).

Due to the dynamics of batch cultivations in general and of pulsed batch cultivations in particular, it is crucial to specify the time intervals over which metabolic yields and rates are calculated, as these parameters change with time. In **Paper III**, the response after the inhibitor pulse was divided into three phases, i.e., glucose/xylose phase (period before the pulse), co-conversion phase (period when furfural and HMF were converted simultaneously), and HMF phase (period when furfural had been converted and only HMF remained), in order to describe and understand the response in detail. The metabolic yields from consumed sugars were then calculated for each phase. The directions of changes in the metabolic yields of major fermentation products in the co-conversion phase after adding furfural and HMF are shown in Table 4 together with the results of similar experiments performed elsewhere. Interestingly, I found differences in the yields depending on whether the inhibitors were pulsed in the glucose or xylose consumption phase.

Table 4. Directions of changes of metabolic yields of major metabolic products in the co-conversion phase following addition of furfural (1.2 g L^{-1}) and HMF (3.9 g L^{-1}) determined in this study and in similar experiments performed elsewhere. The arrows indicate the directions of the changes in yields relative to controls without inhibitors.

			Reference			
Extracellular compound	Glucose phase (**Paper III**)	Xylose phase (**Paper III**)	(Taherzadeh et al., 2000)[a]	(Palmqvist et al., 1999a)[b]	(Almeida et al., 2009b)[c]	(Wahlbom and Hahn-Hägerdal, 2002)[d]
Ethanol	↑	↑	n.c.	↑	↑	↓
Glycerol	↓	↑	↓	↓	↓	↑
CO_2	n.c.[e]	↑	n.d.	↑	n.d.	n.d.
Acetate	↑	↑	↓	↑	↑	↑
Biomass	↓	-	↓	↓	↓	-
Pyruvate	n.d.[f]	n.d.	↑	↑	n.d.	n.d.
Xylitol	-	↓	-	-	↓	↓

[a]Anaerobic batch cultivation using strain CB8066 with glucose as carbon source; 4 g L^{-1} HMF pulsed in exponential phase.
[b]Anaerobic batch cultivation using baker's yeast with glucose as carbon source; 2.8 g L^{-1} furfural present at start of fermentation.
[c]Anaerobic batch cultivation using strain TMB3320 with glucose and xylose as carbon sources; 2 g L^{-1} HMF present at start of fermentation.
[d]Anaerobic batch cultivation using strain TMB3001with glucose and xylose as carbon sources; 3 g L^{-1} furfural added during xylose consumption phase.
[e]n.c. – no change
[f]n.d. – not determined

As expected, the glycerol yield decreased when furfural and HMF were added in the glucose

consumption phase. The drop in glycerol yield reflects the ability of furan aldehydes to act as electron acceptors, thereby oxidizing surplus NADH generated in biosynthesis (Box 1). Interestingly, the acetate yield increased 7.3-fold in the co-conversion phase, suggesting an increased demand for reducing equivalents, as acetate production is an NAD(P)H-generating pathway (Figure 7). Calculation of the metabolic yields following the pulsing of furan aldehydes in the xylose consumption suggested that carbon was channelled to acetate to generate reducing equivalents. Conversely to the results obtained after pulsing in the glucose consumption phase, the glycerol yield increased after pulsing in the xylose consumption phase. The cause of the increased glycerol yield is not entirely clear, but has been observed in similar studies (Wahlbom and Hahn-Hägerdal, 2002). Xylitol production, which is thought to result from the redox-imbalanced XR/XDH pathway (see section 2.1), ceased completely in the presence of furfural and HMF (**Paper III**). As indicated in Table 4, decreases in xylitol production during furan aldehyde conversion have also been observed in other studies. The decreased xylitol production has been attributed to the increased capacity to reoxidize NADH in the presence of furfural and HMF, as both compounds can act as external electron acceptors (Karhumaa et al., 2007; Wahlbom and Hahn-Hägerdal, 2002). As discussed in section 2.1, NADH is formed in the conversion of xylitol to xylulose and cannot be reoxidized in the XR/XDH pathway since XR prefers NADPH as co-factor. The results of pulsing furfural and HMF in the glucose and xylose consumption phases, respectively, are largely in accordance with previous results from similar studies (Table 4). The few discrepancies possibly result from the use of different strains and different experimental conditions in the cited experiments.

The effects of furfural and HMF were also investigated in chemostat mode in the present work (**Paper II**). As mentioned previously, chemostats make it possible to perform physiological studies at steady state without altering the specific growth rate. As both furfural and HMF are growth-inhibiting compounds, the inhibitors have to be added at concentration levels that allow growth at the selected dilution rate. Thus, the concentrations of furfural and HMF added to the feed medium of the chemostat were three times lower than the concentrations pulsed in the batch cultivations. The lower furan aldehyde concentrations could have explained the generally milder metabolic changes in the chemostats than in the pulsed batch cultures, as only the biomass and xylitol yields changed significantly (Table 5).

Table 5 Directions of changes in metabolic yields of major metabolic products in chemostats following addition of furfural (0.4 g L^{-1}) and HMF (1.3 g L^{-1}) to the feed medium, compared with control cultivations without inhibitors (**Paper II**). Yields determined in similar studies in which furfural and/or HMF were added to the feed medium are also shown.

		Reference	
Extracellular compound	**Paper II**	(Horvath et al., 2001)[a]	(Almeida et al., 2009b)[b]
Ethanol	n.c.[c]	↑	n.c.
Glycerol	n.c.	↓	↓
CO_2	n.c.	n.d.[d]	n.d.
Acetate	n.c.	n.d.	↑
Biomass	↑	↑	n.c.
Pyruvate	n.c.	↑	n.d.
Xylitol	↑	-	n.c.

[a]Chemostat using strain CBS8066 with glucose as carbon source and 3.8 g L^{-1} furfural added to the feed medium.
[b]Chemostat using strain TMB3400 with glucose and xylose as carbon sources and 2 g L^{-1} HMF added to the feed medium.
[c]n.c. – no change
[d]n.d. – not determined

The fact that the biomass yield increased when furfural and HMF were present in the feed medium has been observed before and is probably a result of decreased production of glycerol after adding furan aldehydes (Heer et al., 2009; Horvath et al., 2001). As glycerol production is an ATP-consuming reaction, more ATP would therefore be available for biomass synthesis. However, due to the large standard deviations found in glycerol concentration between the replicate cultivations, no significant difference in glycerol concentration was observed after adding furfural and HMF to the feed (**Paper II**). As opposed to the results obtained when furan aldehydes were pulse added to batch cultivations, the xylitol yield increased when furfural and HMF were present in the feed medium. This result was surprising and may have resulted from limitations downstream from XDH.

3.2.2 Effect of furfural and HMF on intracellular concentrations of redox cofactors and adenonucleotides in batch and chemostat cultivations

Although it is well established that furfural and HMF can be converted to their corresponding alcohols by NAD(P)H-linked oxidoreductases, there are limited data on the actual

concentrations of these cofactors after furan aldehyde stress. As perturbations in the cofactor levels can significantly affect a wide range of metabolic processes (Box 1), the intracellular concentrations of both reduced (NAD(P)H) and oxidized ($NAD(P)^+$) cofactors were determined in batch and chemostat cultivations with and without furfural and HMF present in the medium (**Papers II-III**). In addition, the intracellular concentrations of adenonucleotides and, in particular, ATP give a hint as to the energy status of the cells (Atkinson, 1968). Like the redox cofactors, adenonucleotides are highly interconnected in the metabolic network, which means that perturbations can have widespread effects (Nielsen, 2003). Hence, the influence of furan aldehydes on energy metabolism in terms of the intracellular concentrations of ATP, ADP, and AMP was determined.

Box 2. Anaerobic energy metabolism in *S. cerevisiae*

Under anaerobic conditions, energy derived from glucose is captured in ATP in substrate-level phosphorylation in glycolysis, as described in **Chapter 1**. ATP is used to drive a number of energy-requiring processes, mainly in anabolism. Table 6 shows the ATP requirements of various anabolic processes.

Table 6. ATP requirements for biosynthesis. The values are based on the following cell composition: 47% protein, 37.5% carbohydrates, 7% RNA, 3.5% fatty acids, and 5% ash (Verduyn et al., 1990b).

	ATP requirement (mmol ATP per 100 g biomass
Amino acid synthesis	181
Amino acid polymerization	1776
Carbohydrate synthesis	463
Fatty acid synthesis	0[a]
RNA synthesis and polymerization	135
mRNA turnover	71
NADPH synthesis	44
Ion transport	
Ammonium	629
Potassium, phosphate	240
Total	3539
$Y_{X/ATP}$	28.3 (g biomass per mol ATP)

[a]Assumed to be taken up from the medium.

Amino acid polymerization (protein synthesis) makes up a large portion of the total ATP demand for growth (Table 6). Hence, the protein content of the cells contributes significantly to the ATP yield ($Y_{X/ATP}$), which describes how much biomass is produced from the total amount of ATP produced in catabolism. The ATP yield is defined by $Y_{X/ATP}$ = [biomass]/([ethanol] + [acetate] - [glycerol]), and can therefore be estimated from the concentrations of biomass, ethanol, glycerol, and acetate in the fermentation broth. Measured values of $Y_{X/ATP}$ are typically lower than theoretical estimates.

Box 2 continued

Verduyn et al. (1990b) determined an ATP yield of 14.0 g biomass (mol ATP)$^{-1}$ in anaerobic glucose-limited chemostat cultures, which is 50% lower than the theoretical estimate (Table 6), suggesting that other energy-requiring reactions are involved in biosynthesis, or that the ATP requirements for the biosynthetic reactions have been underestimated.

Like the redox cofactors, the adenonucleotides are highly connected in the metabolic network. In fact, ATP and ADP were involved in 188 and 146 intracellular reactions, respectively, in a reconstructed genome-scale metabolic network, which suggests that perturbations in the levels of these compounds can have cell-wide consequences (Forster et al., 2003). Changes in the [ATP]/[ADP] ratio influence the mass action ratio and thereby the thermodynamics of reactions in which they are involved. Moreover, it has been demonstrated that ATP can act as an allosteric regulator of key glycolytic enzymes such as hexokinase, phosphofructokinase, and pyruvate kinase, and, moreover, that low intracellular concentrations of ATP are correlated with high glycolytic fluxes (Kopetzki and Entian, 1985; Larsson et al., 1997; Larsson et al., 2000a).

The energy charge (E_c) has also been identified as a key factor in energy metabolism. This parameter is defined as $E_c = ([ATP] + 0.5 \times [ADP])/([ATP] + [ADP] + [AMP])$, and gives an indication of the energy status of cells. As the energy charge includes all adenonucleotides in the same parameter, it has been suggested to be a more precise indicator of the energy status than are the individual adenonucleotide concentrations (Atkinson, 1968). The energy charge theory states that at high values of E_c, ATP-regenerating reactions (R in Figure 9) proceed at low rates, whereas ATP-utilizing reactions proceed at high rates (U in Figure 9). If the energy charge is shifted to a low level, this is compensated for by an increased rate of ATP-regenerating reactions. The energy charge of healthy cells has been reported to be in the 0.85-0.90 range (Chapman et al., 1971).

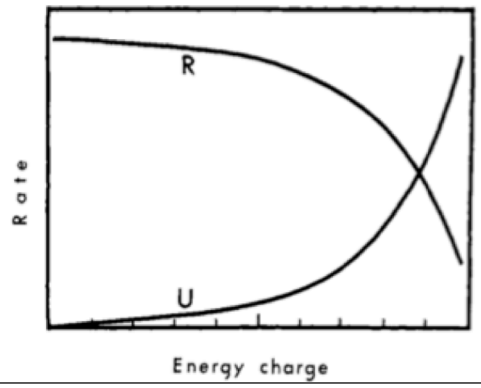

Figure 9. Influence of energy charge on reaction rates. Reproduced from Atkinson (1968) with permission from the American Chemical Society.

In the batch cultivations, samples for redox cofactors and adenonucleotides were taken one

hour after pulsing in the glucose and xylose consumption phases, respectively (Figure 8). The major problem with these measurements is the rapid turnover time of the intracellular metabolites (on the order of seconds) in relation to the long period (1 hour) before sampling, and the fact that only one data point could be obtained for a dynamic process. Dynamic perturbation studies using pulsed additions of compounds such as glucose (Theobald et al., 1997), acetaldehyde (Mashego et al., 2007), and benzoic acid (Kresnowati et al., 2008) have demonstrated that intracellular metabolite levels change at the time scale of seconds. Such short time scales could not be investigated using the manual sampling technique employed in the present study. In fact, the long time that passed before sampling could have explained the apparently unchanging levels of redox cofactors after pulsing furan aldehydes in the glucose consumption phase (Figure 10). On the other hand, after pulsing inhibitors in the xylose consumption phase, the reduced cofactor levels decreased by 62% (NADH) and 85% (NADPH), respectively, compared with those of the control cultivations (Figure 10). As the metabolic fluxes were considerably lower in the xylose consumption phase (**Paper III**), the cells probably had a lower capacity to regenerate the reduced cofactors. Hence, one of the significant findings of **Paper III** was that cells are more susceptible to stress-induced damage from furan aldehydes during xylose consumption due to the lower capacity to generate reducing equivalents for furan aldehyde conversion, as indicated by the dramatically decreased redox cofactor levels. Thus, a possible target for the metabolic engineering of furan aldehyde tolerance in xylose-consuming *S. cerevisiae* would be to improve the xylose conversion rate, thereby increasing the generation rate of NAD(P)H.

The intracellular levels of reduced and oxidized cofactors were also quantified in chemostat cultivations with and without furan aldehydes added to the feed medium (**Paper II**). Interestingly, NADH and NADPH decreased by 40 and 75%, respectively, when furfural and HMF were present in the feed medium, indicating that the cells were drained of reducing power (Figure 10). As the cells were at steady state at the time of sampling, the redox cofactor measurements from the chemostat cultivations were probably more robust with regards to the state of the cells in the presence of inhibitors than were the measurements of samples from the pulsed batch cultivations, which provided only a snapshot of the intracellular levels of redox cofactors in a dynamic process.

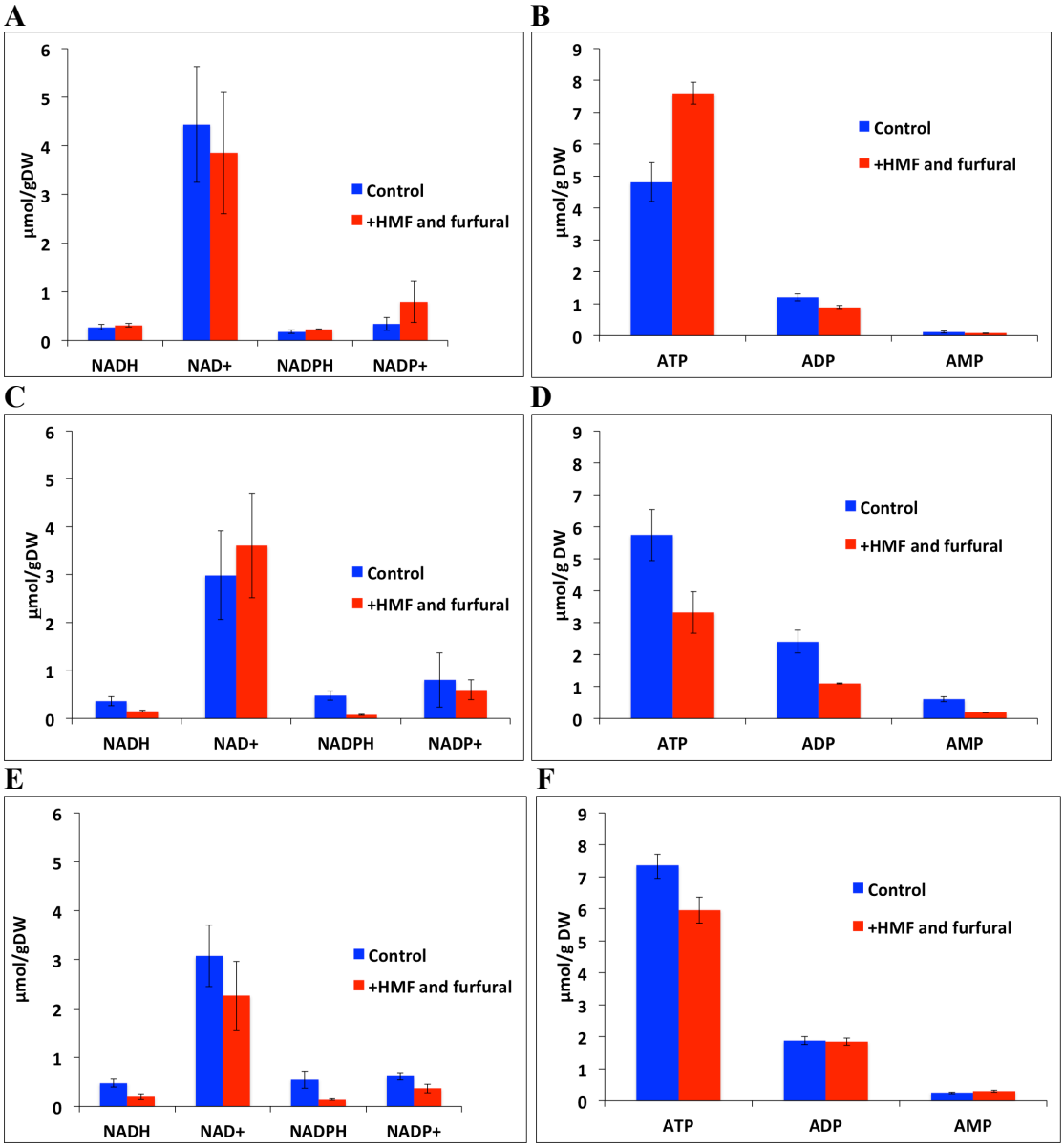

Figure 10. Intracellular concentrations of the redox cofactors $NAD(P)^+$ and NAD(P)H and the adenonucleotides ATP, ADP, and AMP in batch (A-D) and chemostat (E-F) cultivations. The samples from the batch cultivations were taken one hour after pulsing inhibitors in the glucose consumption (A and B) and xylose consumption (C and D) phases. The bars represent data obtained from three independent cultivations and the error bars indicate the standard deviations (**Papers II-III**).

Regarding the effect of furan aldehydes on energy status, it was observed that adding inhibitors in the glucose consumption phase resulted in a 58% increase in the intracellular ATP concentration, which consequently caused an increase in E_c from 0.88 to 0.94 versus the control (Figure 10). This result is probably connected to the decreased specific growth rate and the downregulation of energy-intensive processes such as protein synthesis (see transcriptome results below). In contrast, adding inhibitors in the xylose consumption phase

and to the feed medium of chemostats resulted in decreases of 42 and 19%, respectively, in the intracellular ATP concentrations. A corresponding decrease in E_c from 0.87 to 0.85 was observed in the chemostats, whereas E_c increased from 0.79 to 0.84 after pulsing furan aldehydes in the xylose consumption phase. In both latter experiments, the cells had lost one degree of freedom, as anaerobic growth on xylose is unattainable with the strain used, whereas in the chemostat, alterations in specific growth rate are constrained by the design of the cultivation mode. The lower intracellular concentrations of ATP in the cultivations in which no change in specific growth rate was feasible therefore suggest that energy-requiring repair mechanisms are involved in the stress response to furan aldehydes. These processes remain to be identified, although the transcriptome analysis (as discussed below) did give some hints.

What conclusion can be drawn from the redox cofactor data and how can they be used to elucidate the inhibitory actions of furan aldehydes? The presence of furan aldehydes doubtless alters the intracellular concentrations of redox cofactors, but the ultimate consequences of these perturbations have been difficult to identify. The increased oxidation of NADH should manifest itself in lower glycerol production, which is therefore a positive consequence of furfural and HMF as more carbon can be channelled to ethanol. On the other hand, the increased oxidation of NADPH caused by furan aldehydes competes with the need for NADPH in biosynthetic reactions such as nitrogen assimilation, amino acid synthesis, and lipid biosynthesis. In **Paper II**, glutamate biosynthesis was suggested to be an indirect target of furan aldehyde stress due to the lowering of the intracellular NADPH concentration in stressed cells. NADPH is generally used as a reductant in amino acid synthesis and especially in ammonium assimilation through the production of glutamate from 2-oxoglutarate by Gdh1p (Bro et al., 2004) (Figure 7). Glutamate, which is involved in the biosynthesis of several amino acids, is a highly connected metabolite in the metabolic network. By assuming Michaelis-Menten kinetics, in which the flux through an enzymatic reaction is given by $v = v_{max} \times [S]/([S] + K_m)$, it was found that glutamate biosynthesis proceeds at 65% of v_{max} in the presence of inhibitors versus 86% of v_{max} without inhibitors (**Paper II**). A decreased glutamate flux caused by NADPH depletion is further supported by observations from another study (Bro et al., 2004) in which drainage of NADPH resulted in accumulation of 2-oxoglutarate in the extracellular medium. Although the use of Michaelis-Menten kinetics in this context entails several assumptions regarding the enzyme kinetics (e.g. constant Gdh1p, 2-oxoglutarate and ammonium levels), it serves as an approximate method for elucidating the

effects of perturbations in NADPH levels. In fact, my findings in **Paper II** and, in particular, the influence of furan aldehydes on intracellular NADPH levels may partly explain the observations of Horvath et al. (2001), who demonstrated that when the furfural concentration was increased to a level that completely stopped glycerol production in chemostats, washout occurred. Hence, when no more NADH is available for the conversion of furan aldehydes (as indicated by the decline in glycerol production), competition with NADPH in biosynthetic reactions becomes limiting.

3.2.3 Effect of furfural and HMF on gene expression in batch and chemostat cultivations

The cell has several regulatory control mechanisms that can be activated in response to environmental changes. The control of metabolism is generally considered to have a hierarchical structure that originates at the transcriptional level. Metabolic control at other levels includes translation, post-translational modification, and enzyme activity through allostery. As regulation at these levels does not function in isolation but instead overlaps to an often unknown extent, elucidating the regulatory control mechanisms of a certain process can be complicated (Vemuri and Aristidou, 2005).

Rapid technological advances in recent years have transformed DNA arrays into a routine tool for transcriptome analysis. One week of lab work is required to get a glimpse of the global gene expression changes that follow a change in environmental conditions. From studying the gene expression pattern after exposure to various environmental perturbations, such as heat shock, oxidative stress, osmotic shock, nutrient starvation, and exposure to chemicals such as diamide (sulphhydryl oxidant), DTT (reducing agent), and menadione (ROS inducer), Gasch et al. (2000) identified ~900 genes that shared the same expression pattern under the different conditions in what was referred to as the environmental stress response (ESR). Of this gene set, ~300 genes encoding proteins with functions in carbohydrate metabolism, protein folding and degradation, oxidative stress defence, autophagy and, DNA damage repair were found to be upregulated. On the other hand, ~600 genes functioning in processes such as ribosome synthesis and processing, RNA polymerase I- and III-dependent transcription, and protein translation were found to be repressed. The transcription factors Msn2p and Msn4p have been implicated in the regulation of ESR by binding to stress response elements (STRE-motifs) in the genome, as reviewed by Estruch (2000).

Despite the Msn2p/Msn4p-dependent regulation of ESR, specific responses at the transcriptional level are mediated by other transcription factors, such as Yap1p (oxidative stress) and Hsf1p (heat shock), reviewed by Morano et al., (2012). Through the action of these transcription factors, the cell is capable of producing a tailored response to the insult in question (Gasch et al., 2000). A recent study demonstrated that Yap1p and Hsf1p, together with the pleiotropic drug resistance regulators Pdr1p and Pdr3p (see below), were important regulators of the stress response after pulsing HMF in batch-grown *S. cerevisiae*, suggesting that HMF triggers responses resembling oxidative stress and heat shock (Ma and Liu, 2010b) (Figure 11). The ultimate triggers of these responses can be changes in the intracellular redox potential and the denaturation/damage of proteins, both of which are thought to be consequences of furan aldehyde stress (Allen et al., 2010; Kim and Hahn, 2013).

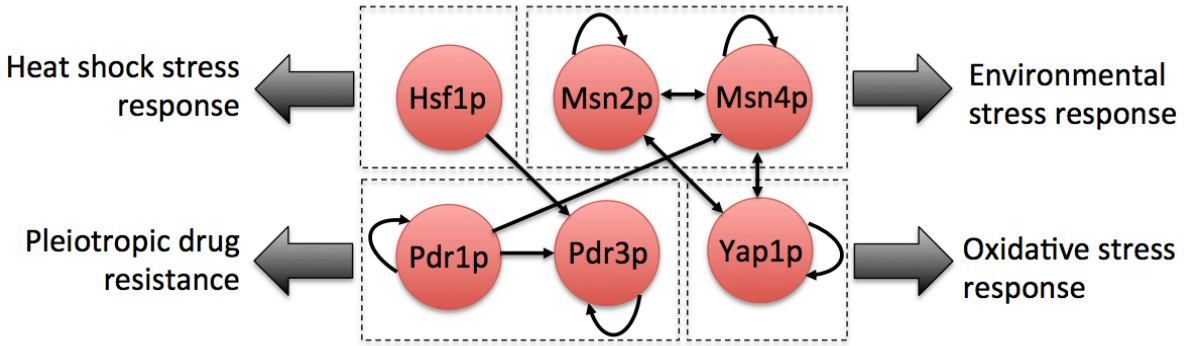

Figure 11. Transcription regulatory network of transcription factors involved in the response to HMF. The arrows indicate direction of transcriptional regulation. Hsf1p is involved in heat shock response, Pdr1p/Pdr3p regulate multidrug transporters, Msn2p/Msn4p are involved in the activation of environmental stress response and Yap1p is essential for protection against oxidative stress.

In **Papers II-III**, chemostats and batch cultivations, respectively, were used to study the responses of *S. cerevisiae* to furfural and HMF when both glucose and xylose were present as carbon and energy sources; key results from these studies will be summarized below. It is important to note that the gene expression changes connected to furan aldehyde stress in these two cultivation modes are expected to differ. In chemostats, where the cells were growing with furan aldehydes present in the feed medium, long-term adaptive effects on gene expression were expected, reflecting the needs for intracellular homeostasis when growing under constant stress. On the other hand, after an abrupt change in environment as imposed by the pulsed addition of furfural and HMF to exponential growing cells in batch cultures, transient effects involving the immediate activation of ESR were expected.

Addition of furan aldehydes to the feed medium of chemostat cultivations resulted in a relatively weak transcriptome response, as only 17 genes elicited a more than twofold upregulation compared with the control. In total, 113 genes were upregulated and 120 were downregulated compared with the cultivation without added inhibitors when considering genes with a false discover rate (FDR) <0.01 and a |log2fold-change| >0.4. Evidence suggesting that cells were stressed was found via a functional enrichment analysis using the MIPS database, which revealed that genes involved in *Cell rescue, Defence, and Virulence* were significantly over-represented (**Paper II**). Nevertheless, genes involved in processes induced by the ESR were few. The absence of ESR induction in the chemostat experiments could possibly be explained by the transient nature of ESR, which is characterized by a strong initial response that levels off over time (Gasch et al., 2000). Hence, the result of the transcriptome analysis of the chemostat experiments possibly reflected genes that were still needed to sustain growth after ESR had diminished. Particularly interesting members of the *Cell rescue, Defence and Virulence* category induced in the presence of furan aldehydes were the ATP-binding cassette (ABC) transporters *PDR5* and *YOR1*. These transporters are known to be involved in xenobiotic resistance and have been implicated before in the context of lignocellulose-derived inhibitors and may be involved in the efflux of furfural and HMF as a means of coping with stress (Alriksson et al., 2010; Ma and Liu, 2010b; **Chapter 4**). The induction of these genes may also partially explain the lower intracellular concentration of ATP in cells growing in the presence of furfural and HMF, thus linking the energy metabolism with the transcriptome response (**Paper II**).

In contrast to the weak response at the transcriptional level in chemostats, 520 genes were found to be upregulated more than two-fold one hour after adding furan aldehydes to cells in the glucose consumption phase growing in batch cultures. Transcription factor analysis using the reporter features algorithm (Patil and Nielsen, 2005), indicated that ESR was initiated, as Msn2p/Msn4p were among the highest scoring transcription factors (**Paper III**). The presence of Pdr1p/Pdr3p, Hsf1p, and Yap1p among the top-scoring transcription factors further corroborated that a strong stress response had been initiated, as these proteins are involved regulating multidrug resistance, heat shock response, and oxidative stress responses, respectively (Figure 11) (Gasch and Werner-Washburne, 2002; Kuge et al., 1997; Moye-Rowley, 2003; Wiederrecht et al., 1988). Downregulated genes included functional categories such as protein synthesis and nucleotide metabolism, which are known to be repressed as a result of ESR (Gasch et al., 2000).

Xylose is not sensed as a fermentable carbon source to the same extent as is glucose by recombinant *S. cerevisiae*. For this reason, genes normally repressed in the presence of glucose have been demonstrated to be induced when xylose acts as the sole carbon and energy source (Salusjärvi et al., 2006). Moreover, it was found that 50% of all genes in the genome were differentially expressed when comparing glucose- and xylose-grown cells in the absence of inhibitors (Ask et al., Unpublished data). There was also a large difference in transcriptional-level response when furan aldehydes were added in the xylose consumption phase rather than the glucose consumption phase (Figure 12). Functional enrichment analysis revealed that processes involved in protein synthesis, such as amino acid metabolism and rRNA processing, were enriched among the upregulated genes following inhibitor pulsing in the xylose consumption phase, whereas these processes were downregulated after inhibitor pulsing in the glucose consumption phase. Furthermore, the Msn2p/Msn4p-mediated ESR was not induced, as indicated by the absence of these transcription factors among the top-scoring reporter transcription factors. The absence of ESR induction may be connected to the fact that ESR is triggered in the diauxic shift-resembling phase after glucose exhaustion. In fact, by comparing the gene expression profiles in the xylose and glucose consumption phases in the absence of inhibitors, Msn2p/Msn4p were found to be the top-scoring reporter transcription factors regulating the induced genes in the xylose consumption phase (Ask et al., Unpublished data), suggesting that ESR was already activated when furfural and HMF were added during xylose consumption.

A key finding of the inhibitor pulse experiment carried out in the xylose consumption phase was the concerted upregulation of several genes encoding proteins involved in sulphur assimilation, nitrogen assimilation, and amino acid biosynthesis. All these processes are known to require reducing equivalents in the form of NADPH, suggesting a link to the low intracellular NADPH concentration after pulsing of furan aldehydes during xylose consumption and gene expression. Since one way for the cell to increase the flux through a metabolic pathway is to synthesize more enzymes, it was hypothesized in **Paper III** that the cell compensates for the low intracellular NADPH levels by inducing genes involved in sulphur assimilation, nitrogen assimilation, and amino acid biosynthesis to maintain a flux in these processes. The influence of NADPH depletion on genes involved in sulphur assimilation has been demonstrated before by using the electron acceptor acetoin (Celton et al., 2012). Limitations in sulphur assimilation have also been implicated in the response of *E. coli* to furfural (Miller et al., 2009), but not in the case of *S. cerevisiae*.

The fact that few changes related to NADPH-consuming processes were observed after pulsing inhibitors in the glucose consumption phase may be due to the cells' ability to reduce the specific growth rate and hence save reducing power otherwise used in biosynthetic processes, instead using it for furan aldehyde detoxification. In the chemostats, changes in specific growth rate were not feasible, so the explanation for the non-induction of NADPH-requiring processes must differ. One possibility is that the stress in terms of NADPH depletion was not severe enough during the chemostat cultivation. In fact, one hour after pulsing in the xylose consumption phase, [NADPH]:[$NADP^+$] was estimated at 0.12, whereas in the chemostat cultivation in the presence of inhibitors, [NADPH]:[$NADP^+$] was estimated at 0.38 (**Papers II-III**), suggesting greater limitation of NADPH in the pulsed batch cultivation.

As is evident from Figure 12, relatively few genes displayed the same expression pattern in the perturbation experiments involving the addition of furan aldehydes in chemostats and in the batch cultivations performed as part of this thesis. Only seven genes were induced in all three experiments, whereas no genes were found to be downregulated. The genes induced in all three experiments were *YGR035C*, *HBN1, PDR5, PDR16, YOR1, ICT1,* and *VHR1,* and their functions are presented in Table 6. In a study by Ma and Liu (2010b), an Δ*ict1* deletion mutant displayed an extended lag phase in the presence of 1.9 g L^{-1} HMF compared with the wild type strain, hinting that at least one of the genes induced in all three experiments is involved in furan aldehyde tolerance. To further evaluate the importance of these genes for increased tolerance to furfural and HMF, each of them should be overexpressed and tested in the presence of inhibitors, preferably in a hydrolysate. Interestingly, 28 genes induced in both the pulsed batch cultivations were involved in unknown processes, suggesting that more work is needed to characterize these genes.

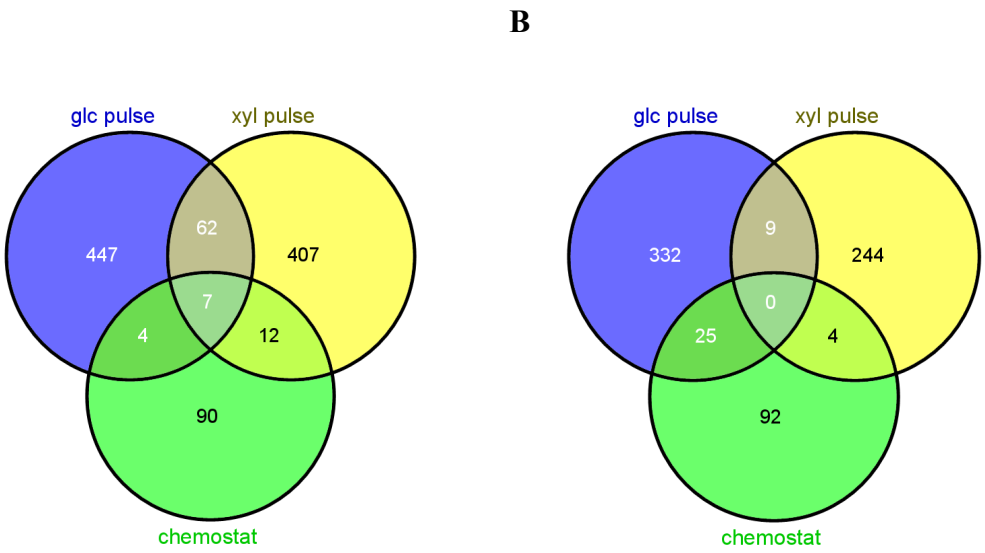

Figure 12. Venn diagrams showing genes upregulated (A) and downregulated (B) in the three perturbation experiments with furan aldehydes performed as part of this thesis research (**Papers II-III**).

Table 6. Functions of genes induced in response to furfural and HMF in chemostats and pulsed batch cultivations.

Gene name	Systematic name	Function
YGR035C	*YGR035C*	Putative protein of unknown function, potential Cdc28p substrate.
HBN1	*YCL026C-B*	Protein of unknown function, similar to bacterial nitroreductases.
PDR5	*YOR153W*	Full-size ABC transporter involved in multidrug resistance.
PDR16	*YNL231C*	Protein involved in lipid biosynthesis and multidrug resistance.
YOR1	*YGR281W*	ABC transporter involved in multidrug resistance.
ICT1	*YLR099C*	Lysophosphatidic acid acyltransferase responsible for enhanced phospholipid synthesis during organic solvent stress.
VHR1	*YIL056W*	Transcriptional activator required for the vitamin H-responsive element mediated induction of VHT1.

3.3 Concluding remarks on the physiological responses of *S. cerevisiae* to furfural and HMF

In my thesis research, two cultivation modes were applied to dissect the influence of furan aldehydes on the redox and energy metabolism and the gene expression of xylose-utilizing *S. cerevisiae*. In my opinion, chemostat cultivations are the best methodology for such studies, since confounding influences on intracellular metabolite and transcript levels originating from changes in specific growth rate can be circumvented, which is crucial as furfural and HMF inhibit growth. Nevertheless, if concentrations of furan aldehydes as high as those present in non-diluted lignocellulosic hydrolysates are the focus of the study or if carbon sources that cannot be utilized for growth are used (e.g. xylose), batch cultivations have to be performed. Caution should then be taken before drawing conclusions involving genes and metabolites known to change with specific growth rate, and the batch cultivation results should be compared with those of studies investigating genes and metabolites displaying such characteristics. Although gene expression analyses of *S. cerevisiae* in response to furfural and HMF have been performed before, **Papers II-III** represent the first studies in which xylose is included as carbon source, which is highly significant for lignocellulosic bioethanol production. In addition, the quantification of the intracellular redox cofactors and adenonucleotides in response to furan aldehydes represents the first of its kind. Moreover, an important connection between the NADPH drainage caused by furfural and HMF and gene expression related to NADPH-consuming processes was found when cells were utilizing xylose as the sole carbon source. By comparing the gene expression analyses of the three cultivations presented in **Papers II-III**, I found incentives for further research into both characterized and uncharacterized genes with great potential to serve as metabolic engineering targets for more robust yeast strains. Strain engineering for improved robustness will be the subject of the next chapter.

CHAPTER 4

IMPROVING STRAIN ROBUSTNESS OF *S. CEREVISIAE* TO LIGNOCELLULOSIC INHIBITORS

In **Chapter 3,** I discussed how one can gain insights into the mechanisms of inhibitory action on cellular metabolism by performing detailed analyses of the physiological responses of cells to stress imposed by furan aldehydes. The current chapter will cover how the knowledge obtained in such studies can be harnessed to design *S. cerevisiae* strains with improved inherent capacity to resist high concentrations of inhibitors. Both non-targeted and targeted engineering approaches to improving furan aldehydes tolerance will be reviewed, including the strategy developed as part of my thesis research (**Paper IV**).

Before proceeding, it is worth mentioning that there are other alternatives for dealing with toxic hydrolysates that will not be further considered in this chapter. Besides improvement of the microorganism itself, two other options have been described in the literature: removing inhibitory substances using various detoxification methods, as reviewed by Jönsson et al. (2013), and developing improved fermentation strategies. Although hydrolysate detoxification has produced promising results, it adds to the process cost and should be avoided in industrial settings. The improvement of fermentation strategies includes improving feeding strategies (Olofsson et al., 2010), cell retention in continuous cultures (Brandberg et al., 2007), and microorganism encapsulation (Talebnia and Taherzadeh, 2006), which all have shown promise at the lab scale, though their industrial-scale performance remains to be elucidated.

4.1 Definition of robustness

As this chapter will be about microbial robustness, a definition of this concept is required for the coming discussion. Stelling et al. (2004) defined robustness as: "The persistence of a system's characteristic behaviour under perturbations or conditions of uncertainty". In the context of the present chapter, this definition implies that the parameters relevant to lignocellulosic bioethanol production should remain constant even in the presence of inhibitors. In the case of furan aldehydes, the inhibitory effects include an extended lag

phase, decreased fermentation rate, lowered specific growth rate, and decreased viability, as reviewed by Almeida et al. (2009a). Thus, a microorganism that displays improvements in any of these parameters compared with those of a control strain is deemed more robust.

4.2 Non-targeted methodologies for strain improvement

The common denominator of non-targeted methods is that they all include a component of randomness. Depending on the specific methodology, the randomness arises from the direct introduction of mutations in the genome, genetic drift of actively dividing cells, or random effects involved in mating. The random effects in mating arise from meiotic recombination through chromosomal crossover during sporulation (see 4.2.1). Two important advantages of non-targeted methods are that no *a priori* knowledge of the specific toxicity mechanisms of the inhibitory compounds is required, and that often-polygenic traits such as tolerance to multiple inhibitory compounds can be addressed simultaneously (Nevoigt, 2008). The accumulation of undesirable mutations is often considered a drawback of these methods, especially in the case of prolonged selection during evolutionary engineering, as a more fit phenotype does not necessarily retain other beneficial genetic traits, as exemplified by the loss of xylose utilization capacity during selection for improved robustness to lignocellulosic inhibitors (described in 4.2.2). Non-targeted methods for strain improvement will be described below, and examples of applications for improving strain robustness in the context of lignocellulosic ethanol production will be presented. This section is not intended to give a complete overview of the data available in the literature, but instead to provide some illustrative examples of the advantages and drawbacks of non targeted-methodologies for strain improvement.

4.2.1 Classical strain improvement

Classical strain improvement relies on repeated rounds of mutagenesis and screening. Mutagenesis is performed to increase the genetic diversity of a cell population and can be achieved by exposure to radiation (i.e. gamma, x-ray, and UV), chemicals (e.g. base analogues, alkylating and intercalating agents), and biological agents (e.g. DNA transposons) (Parekh et al., 2000). A wide range of genetic rearrangements is created by these methods, including small-scale mutations such as insertions or deletions (indels) and the introduction of single-nucleotide polymorphisms (SNPs), or large-scale mutations such as gene duplication/deletion and chromosomal translocation. Increased genetic diversity can also be

achieved by sexual recombination. There are two mating types of *S. cerevisiae* (MAT**a** and MATα), and two haploid strains of opposite mating type can combine sexually to produce an **a**/α diploid. The diploid strain can be induced to undergo sporulation (by nitrogen starvation in the presence of a non-fermentable carbon source), during which genetic recombination occurs through chromosome crossover, creating four spores with unique genotypes (reviewed by Attfield and Bell 2003). After mutagenesis or mating, screening to find the desired phenotypes ensues. The screening step can be laborious, since great many clones usually have to be screened to find an improved phenotype, although the development of high-throughput screening methods and automation have resolved some of these issues (Patnaik, 2008). Classical strain improvement has been used in combination with both targeted and other non-targeted methods such as evolutionary engineering for enhancing traits such as xylose utilization ability (Koppram et al., 2012; Wahlbom et al., 2003).

4.2.2 Evolutionary engineering

Evolutionary engineering, or directed evolution, is performed by applying a selection pressure for the desired phenotype, and allowing the organism to adapt to the prevailing conditions by natural evolution over extended periods ranging from weeks to years. Mutagenesis by any of the methods mentioned in section 4.2.1 is often performed at the start of the evolutionary process to increase the genomic diversity of the population. Moreover, rational metabolic engineering (section 4.3) has also been preceding evolutionary engineering, mainly to broaden the substrate range to include xylose and arabinose (Garcia Sanchez et al., 2010; Kuyper et al., 2004; Wahlbom et al., 2003; Wisselink et al., 2009). Cultivating the cells under a selection pressure for extended periods results in the enrichment of phenotypes with improved ability to endure the imposed conditions.

Most evolutionary engineering experiments have been performed in either repetitive batch cultivations or chemostats (Dragosits and Mattanovich, 2013). In repetitive batch cultivations, the cells are allowed to pass through lag and exponential growth phases into the stationary phase before a small fraction of the culture is transferred to fresh medium, after which the procedure is repeated until the desired phenotype is obtained. Over time, the selection pressure (e.g. concentration of inhibitors) is increased to select for improved phenotypes. Repetitive progress through the growth stages has been reported to result in a selection bias favouring certain characteristics (Sauer, 2001). These characteristics include a shorter lag phase and increased specific growth rate at non-limiting substrate concentrations

(see section 3.1.1), as phenotypes possessing these properties have the potential to divide more often in each round of batch cultivation and consequently become enriched (Wright et al., 2011). Evolutionary engineering by repetitive batch cultivation has successfully been performed to improve the robustness of *S. cerevisiae* to both hydrolysates such as pretreated corn stover (Almario et al., 2013) as well as specific inhibitors such as furfural (Heer and Sauer, 2008) and HMF (Liu et al., 2005). The improvements generally consisted of a shorter lag phase and improved specific ethanol productivity, which were attributed mainly to an increased furan aldehyde conversion capacity (Table 7).

Evolutionary engineering can also be performed in continuous cultivations by cultivating the cells in the presence of inhibitory compounds for extended periods. Despite the increased time required to set up the cultivation compared with repeated batch cultivations in shake flasks, the continuous cultivation mode requires less maintenance during the course of the experiment. An important difference from repeated batch cultivations in which selection occurs in an excess of nutrients is that chemostat cultivations are performed with one essential nutrient at its limiting concentration (see section 3.1.2). Interestingly, the nutrient limitation results in a selection pressure for phenotypes with an increased affinity for the limiting substrate and consequently a higher specific growth rate at suboptimal concentrations of the limiting nutrient (Jansen et al., 2005; Wright et al., 2011). Moreover, as cells are continuously removed from the fermentation broth, cells adhering to the reactor walls are also selected for (Sauer, 2001). Evolutionary engineering in chemostat cultivations has been performed to improve the robustness of *S. cerevisiae* to both pretreated sugar cane bagasse and pretreated spruce hydrolysates (Koppram et al., 2012; Martin et al., 2007) (Table 7).

As described at the beginning of this section, two important advantages of non-targeted methods for strain improvement are that minimal *a priori* knowledge of the molecular inhibition mechanisms is required, and that polygenic properties such as tolerance to acid stress, high temperatures, and whole hydrolysates can be addressed. Nevertheless, hundreds of generations are often required before improved phenotypes evolve, making evolutionary engineering a time-consuming enterprise (Dragosits and Mattanovich, 2013). When an improved phenotype arises, isolating that particular clone can be time-consuming due to the heterogeneity of the population. The stability of evolved strains should also be properly addressed by sub-cultivating the improved clones in non-inhibitory medium and thereafter

reassessing the superiority of the phenotypes. Trade-offs in evolved strains have also been reported. Koppram et al. (2012) observed a several-fold improvement in the specific furan aldehyde conversion rate after >400 generations in repeated batch cultures (Table 7). Concomitant with this improvement, the inhibitor-tolerant phenotype lost its ability to consume xylose, suggesting that a careful selection programme should be designed so that no essential traits are lost. This can supposedly be obtained by sub-cultivating the cells with xylose as the sole carbon source occasionally throughout the course of the experiment, as proposed by Wisselink et al. (2009) for xylose and arabinose utilization.

Table 7 Summary of results of a selection of experiments in which *non-targeted* methods were used to improve strain robustness to lignocellulosic inhibitors.

Method	Conditions	No. of generations	Effects	Reference
Chemostat	Pretreated spruce hydrolysate	97	Four-fold increase in specific conversion rates of furfural and HMF	(Koppram et al., 2012)
Chemostat	Pretreated sugar cane bagasse	51	Doubling of specific ethanol productivity and volumetric furfural conversion rate	(Martin et al., 2007)
Repeated batch	Synthetic hydrolysate	429	83% increase in μ_{max}, 100% decrease in lag phase, xylose utilization lost	(Koppram et al., 2012)
Repeated batch	Pretreated corn stover	463	12-57% increase in μ_{max} in presence of corn stover hydrolysate, furfural, HMF and acetic acid	(Almario et al., 2013)
Repeated batch	0-20 mM furfural	300	Thirteen-fold decrease in lag phase in the presence of furfural and spruce hydrolysate	(Heer and Sauer, 2008)
Repeated batch	Increasing concentrations of furfural and HMF	100[a]	Nine-fold decrease in lag phase in the presence of 30 mM HMF; adapted strains able to grow in 30 mM furfural whereas the wild type was not	(Liu et al., 2005)

[a]This figure refers to the number of serial transfers.

4.3 Targeted methodologies for improvement of strain robustness

Growing knowledge of microbial metabolism has paved the way for targeted approaches to strain improvement commonly referred to as metabolic engineering. One of the pioneers of the discipline, James E. Bailey, defined metabolic engineering as "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with recombinant DNA technology" (Bailey, 1991). Several metabolic engineering strategies have been applied to improve cell robustness to lignocellulose-derived inhibitors

(Table 8). As most targeted strategies have aimed to increase the tolerance to furfural and HMF, the main focus in this section will on various methods for improving tolerance to furan aldehydes. As will become evident, these metabolic engineering strategies can roughly be divided into methods that deal directly with the inhibitors by converting them to less toxic compounds or by transporting the toxic compounds out of the cells and methods that aim to protect the cells against damage caused by the inhibitors. The latter strategy involves "protective metabolites" that represent a novel direction in engineering yeast strains with improved robustness to lignocellulosic inhibitors developed as part of this thesis research (**Paper IV**).

4.3.1 Oxidoreductases

As the *in situ* detoxification of furan aldehydes to their less toxic corresponding alcohols is supposedly mediated by NAD(P)H-dependent oxidoreductases, several trials have been attempted to increase the oxidoreductase activity. At the time of writing, 284 genes have been annotated to the Gene Ontology (GO) term "oxidoreductase activity", and many of these genes can be potential mediators of furan aldehyde tolerance. In fact, several genome-wide studies (Heer et al., 2009; Ma and Liu, 2010a; Petersson et al., 2006; **Paper III**) have identified the induction of genes encoding enzymes with oxidoreductase activity in response to furan aldehyde stress.

Petersson et al. (2006) have identified NADPH-dependent *ADH6* as a potential candidate for increased HMF tolerance in a genome-wide study. Upon overexpression of *ADH6*, higher conversion rates of HMF to HMF alcohol both *in vitro* and *in vivo* were obtained compared with the control. Xylose reductase from *S. stipitis*, which is commonly overproduced to confer the ability to utilize xylose on *S. cerevisiae*, has also been demonstrated to increase the ability to convert HMF to the corresponding alcohol form (Almeida et al., 2008a). By comparing a strain obtained by evolutionary engineering in the presence of furfural with its non-evolved parent, Heer et al. (2009) have identified several genes with oxidoreductase activity that were induced in the evolved strain. Overexpression of two of these genes, *ADH7* and *YKL071W*, resulted in earlier exit from the lag phase in the presence of furfural than in the control. The identification of *ADH6, ADH7,* and *YKL071W* as metabolic engineering targets represents an example of inverse metabolic engineering, which is a strategy to identify previously unknown targets for strain improvement (Bailey et al., 2002).

Furan aldehyde tolerance has also been addressed by enzyme engineering. Using a directed enzyme evolution approach, Moon and Liu (2012) obtained two mutated forms of the NADPH-dependent methylglyoxal reductase-encoding gene *GRE2*. Clones encoding the altered versions of *GRE2* displayed improved *in vitro* activity towards both HMF and furfural and a shorter lag phase of growth than did the wild type. Laadan et al. (2008) identified and overexpressed a mutated form of an NADH-dependent *ADH1*, which increased the *in vivo* HMF conversion capacity compared with the non-modified control. It was later demonstrated that overexpression of the mutated *ADH1* also resulted in improved resistance in a spruce hydrolysate (Almeida et al., 2008b).

Although probably not directly involved in converting furan aldehydes, PPP and, in particular, *ZWF1* encoding glucose-6-phosphate dehydrogenase were identified as important for strain robustness by screening a genome-wide deletion mutant library (Gorsich et al., 2006). Glucose-6-phosphate dehydrogenase catalyses the conversion of glucose-6-phosphate to 6-phosphogluconolactone with the concomitant production of NADPH (Figure 3), indicating the importance of a direct supply of NADPH for tolerance to furan aldehydes. Overexpression of *ZWF1* allowed growth in the presence of furfural concentrations toxic to the wild type (Gorsich et al., 2006).

While many of the above-mentioned strategies have led to improved yeast robustness to furan aldehydes, the increased oxidoreductase activity has in some cases resulted in an altered distribution of fermentation products. For example, the increased tolerance of the strains overexpressing *ADH6* came at the expense of higher glycerol and acetate yields compared with the wild type (Almeida et al., 2008b; Petersson et al., 2006). Hence, other strategies not directly connected to reducing furan aldehydes to their corresponding alcohols could be of interest, as will be discussed next.

4.3.2 Transporters

Like the oxidoreductases, multidrug resistance (MDR) transporters of the major facilitator superfamily (MFS) and ATP-binding cassette (ABC) transporters involved in the pleiotropic drug response (PDR) have been found in genome-wide studies to be up-regulated in response to furfural and HMF (Ma and Liu, 2010a; **Papers II-III**). These transporters are known to be involved in resistance to a wide range of xenobiotic compounds by catalysing the efflux of the compounds out of the cell (reviewed by Ernst et al. 2010). Although not directly related

to bioethanol production, but still in a biofuel context, overexpression of the PDR transporters *PDR5* and *SNQ2* resulted in increased tolerance to C10-C11 alkanes, which was manifested in a shorter lag phase of the recombinant strains than the wild type strains (Ling et al., 2013).

As a consequence of the upregulation of MDR transporters in response to furan aldehydes, it has been hypothesized that these transporters also act to transport lignocellulose-derived inhibitors to the extracellular medium. In fact, transformants overexpressing the MFS proteins *ATR1* and *FLR1* could sustain growth at higher concentrations of coniferyl aldehyde and HMF than could the wild type. Moreover, deletion of the transcription factors controlling the expression of PDR genes (Δ*pdr1* and Δ*pdr3*) resulted in a longer lag phase of growth in the presence of HMF, further corroborating the importance of multidrug transporters for tolerance to lignocellulosic inhibitors (Ma and Liu, 2010a). Detailed physiological studies of the distribution of fermentation products of strains overproducing multidrug transporters have not yet been reported in the literature.

4.3.3 Protective metabolites: glutathione

The two targeted strain engineering strategies described so far have involved enhancing enzymatic activities such as oxidoreductase activity and multidrug transporter activity in order to improve inhibitor tolerance. Whereas the rationale for improving oxidoreductase activity involves the conversion of furan aldehydes, the multidrug transporters could have a broader role in protecting against weak acids and high salt concentrations, although the performance of strains with improved multidrug transporter activity remain to be evaluated under industrial conditions. **Paper IV** introduced the concept of protective metabolites, which is a novel strain engineering strategy for improving robustness to lignocellulose-derived inhibitors. Protective metabolites do not necessarily function directly in detoxifying inhibitors, as do oxidoreductases, but could also protect the cells against damage caused by inhibitors (see section 2.2). Glutathione serves as the main redox buffer in the cell due to its low redox potential and high intracellular concentration (for a more thorough description, see Box 3). With the specific properties of the glutathione system in mind, we hypothesized that glutathione has the potential to function as a protective metabolite in the cell (**Paper IV**). As furfural and HMF have been demonstrated to reduce the intracellular redox potential by depleting the intracellular NAD(P)H pool (**Papers II-III**) and the glutathione pool (Kim and Hahn, 2013), increased glutathione concentrations were hypothesized to protect primarily

against furan aldehydes, though, as will be discussed later, alternative mechanisms cannot be excluded.

Box 3. Glutathione metabolism and its importance in redox processes

γ-glutamylcysteinylglycine, or glutathione (GSH), is the most abundant non-protein thiol in yeast. Its high intracellular concentration (~10 mM) and low redox potential (-240 mV) have given GSH the status of an intracellular redox buffer (Schafer and Buettner, 2001). The redox potential measures the tendency of a redox pair (GSSG/2GSH in the case of glutathione) to donate electrons, and can be estimated from the Nernst equation ($E_{hc} = E_0 - \frac{RT}{nF} \ln \frac{[GSH]^2}{[GSSG]}$ where E_0 is the standard redox potential, R is the gas constant, T is the absolute temperature, n is the number of electrons, and F is the Faraday constant), assuming that the concentrations of the individual components of the redox couple are known. The low intracellular redox potential maintained by GSH is thought to protect oxidation-sensitive cysteine residues in proteins (Grant, 2001). GSH is often implicated in the oxidative stress response induced by reactive oxygen species (ROS) such as H_2O_2, $\bullet O_2^-$, and $OH\bullet$, but compounds such as diamide, furfural, and HMF are also known to oxidize glutathione (Kim and Hahn, 2013; Kosower et al., 1969). ROS are formed as byproducts in mitochondria during respiratory metabolism, and GSH acts to scavenge these reactive compounds before they can damage membranes, DNA, or proteins (Grant et al., 1996).

GSH may act either non-enzymatically or as a cofactor of glutathione peroxidases, glutaredoxins, or glutathione-S-transferases. GSH oxidized by ROS is re-reduced by NADPH-dependent glutathione reductase (Glr1p), linking the glutathione system to the generation of NADPH in PPP. Notwithstanding the importance of GSH as an antioxidant during respiratory metabolism, it has also been demonstrated to be essential even in the absence of oxygen, suggesting other roles for this molecule, which has been partly attributed to its role in iron-sulphur cluster assembly (Toledano et al., 2007). Moreover, GSH has prominent roles in xenobiotic detoxification, sulphur and nitrogen metabolism, and protein folding, as reviewed by Penninckx (2002).

GSH is synthesized in two consecutive ATP-dependent steps from its constituent amino acids cysteine, glutamate, and glycine by the enzymes γ-glutamylcysteine synthetase (encoded by *GSH1*) and glutathione synthetase (encoded by *GSH2*) (Inoue et al., 1998; Ohtake and Yabuuchi, 1991) (Figure 13). Both *GSH1* and *GSH2* are transcriptionally regulated by the redox-sensitive transcription factor Yap1p, which is activated in response to oxidative stress through the formation of a disulphide bond masking a nuclear export signal, resulting in accumulation in the nucleus (Delaunay et al., 2000). Gsh1p is feedback-inhibited by GSH (Wheeler et al., 2002; Wheeler et al., 2003).

Box 3 continued

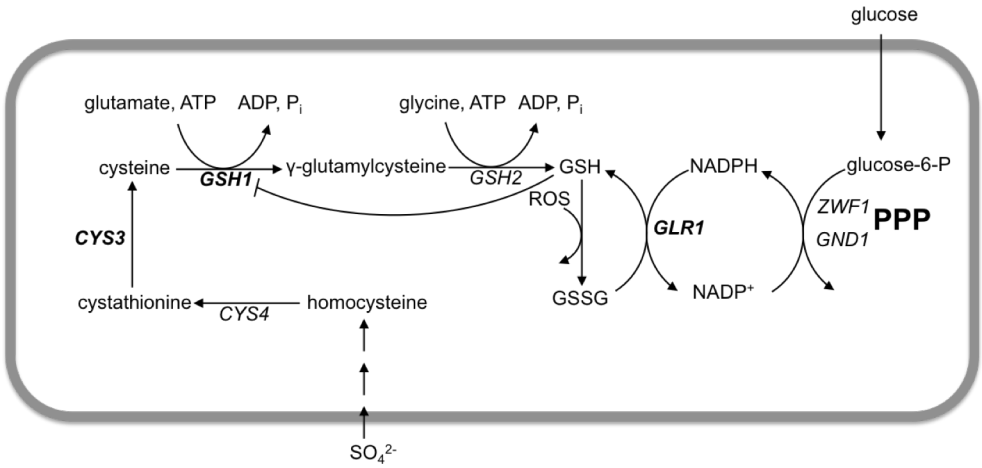

Figure 13. Schematic of glutathione biosynthesis. The genes overexpressed in the research reported in **Paper IV** are marked in bold.

Three genes involved in glutathione biosynthesis (*GSH1, CYS3,* and *GLR1*) were overexpressed to increase the intracellular concentration of glutathione and to improve the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), respectively. Consequently, five strains overexpressing *GSH1, GSH1/CYS3, GLR1, GSH1/GLR1,* and *GSH1/CYS3/GLR1* were constructed. The highest intracellular concentration of total GSH was achieved by the overexpression of *GSH1*, which resulted in a 42% increase compared with the wild type. Surprisingly, overexpression of *GLR1* did not result in an increased [GSH]:[GSSG] ratio and/or decreased redox potential. The recombinant strains were subsequently tested in SSF of pretreated spruce to investigate whether increased intracellular GSH levels conferred increased tolerance under process-relevant conditions. In my opinion, pretreated lignocellulosic hydrolysates should be the primary substrate for evaluating strains engineered for improved robustness, as such substrates are representative of the conditions that prevail in industrial media for lignocellulosic bioethanol production. There are surprisingly few reports of strains claimed to possess increased tolerance, constructed by rational engineering that have been evaluated in hydrolysates. As can be seen in Table 8, Almeida et al. (2008b) were the only researchers who tested their engineered strains in a hydrolysate. Their approach consisted of the pulsed addition of spruce hydrolysate during the exponential phase, which can hardly be considered an approach adoptable in industry. Strikingly, all strains carrying the *GSH1* overexpression-construct and consequently ~40% higher intracellular GSH levels were able to consume glucose for a longer period than was the wild type, which resulted in an approximately 70% increase in ethanol yield for the

recombinant strains (Figure 14). Hence, **Paper IV** provides the first demonstration of a successful rational metabolic engineering strategy for improved robustness under process-relevant conditions in SSF of pretreated spruce.

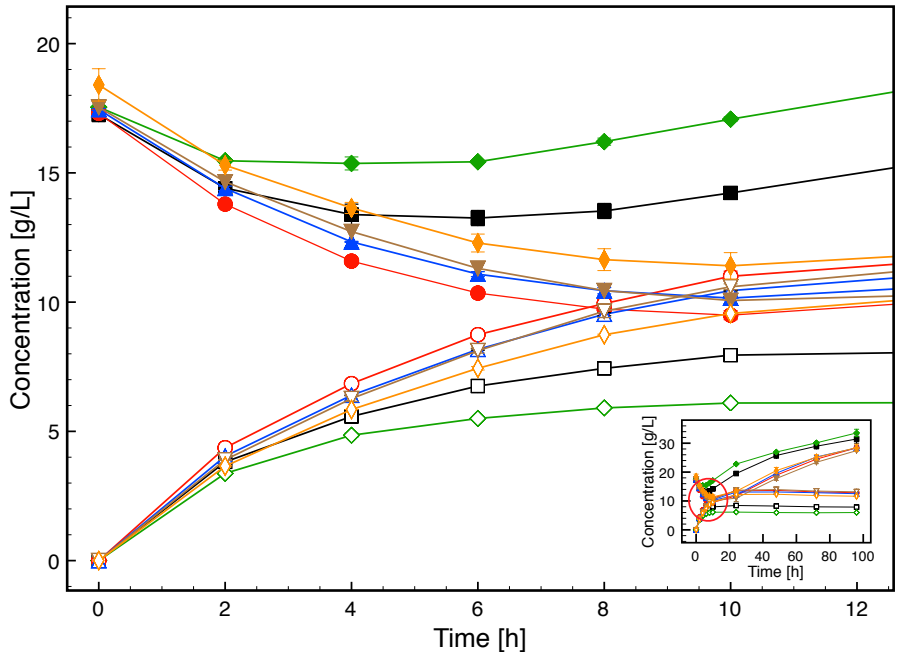

Figure 14. Time course of glucose and ethanol concentrations in SSF of spruce with the strains investigated in the present study. Black squares: CEN.PK 113-7D, red circles: GSH1, blue triangles: GSH1/CYS3, green diamonds: GLR1, brown reversed triangles: GSH1/GLR1, orange stretched diamonds: GSH1/CYS3/GLR1. The filled and open symbols represent glucose and ethanol, respectively. The data points are means from two independent cultivations. The error bars indicate the maximum and minimum values (**Paper IV**).

The beneficial effect of an increased intracellular glutathione concentration in SSF of pretreated spruce supposedly results from the ability of the *GSH1*-overexpressing strains to maintain a low redox potential for longer than the wild type, despite the presence of inhibitory compounds, such as inhibitory aldehydes, that deplete the intracellular GSH and NAD(P)H pools. During normal growth, a low redox potential is maintained in the cytosol, conceivably to counteract oxidative stress events and to maintain oxidation-sensitive cysteine residues in a reduced state (Rietsch and Beckwith, 1998). In fact, abrupt decreases in the redox potential have been implicated in the triggering of apoptosis (Cai and Jones, 1998). Nevertheless, one should not rule out other possible beneficial actions of GSH, such as providing an additional sulphur source. In fact, sulphur limitation has been suggested to result from furfural stress in *E. coli* (Miller et al., 2009), thus contributing to the inhibitory action of furan aldehydes. Such indications were also discovered after pulsing furfural and HMF in *S. cerevisiae* cultures containing xylose as the sole carbon source (**Paper III**), in which

NADPH limitation was hypothesized to cause an upregulation of genes involved in sulphate assimilation. Another possibility is that increased intracellular GSH levels can provide an extra source of glutamate, glycine, and cysteine, the constituent amino acids of glutathione (Mehdi and Penninckx, 1997). Indeed, in a fed-batch cultivation of the wild-type and the strain overexpressing *GSH1*, the wild-type adopted an elongated morphology indicative of nitrogen starvation, whereas the strain overexpressing *GSH1* retained its round morphology (Ask et al., Unpublished data). Thus, although overproduction of glutathione was demonstrated to increase strain robustness in SSF of pretreated spruce, the detailed mechanisms of the beneficial effects remain to be elucidated.

Table 8 Summary of results of a selection of experiments in which *targeted* methods were used to improve strain robustness to lignocellulosic inhibitors.

Targeted gene(s)[a]	Origin	Identification	Activity/process enhanced	Demonstrated effects	Reference
ADH6	*S. cerevisiae*	Microarray analysis	Oxidoreductase.	Three- to four-fold increase in specific HMF uptake rate under anaerobic and aerobic conditions, respectively.	(Petersson et al., 2006)
ADH7, YKL071W	*S. cerevisiae*	Microarray analysis	Oxidoreductase	30% reduction of lag phase in presence of 11 mM furfural.	(Heer et al., 2009)
Mut-*GRE2*	*S. cerevisiae*	Microarray analysis	Oxidoreductase	50% reduction of lag phase in presence of 30 mM HMF.	(Moon and Liu, 2012)
Mut-*ADH1*	*S. cerevisiae*	Protein purification	Oxidoreductase	Reduced lag phase and increased volumetric ethanol productivity in presence of 20 mM HMF. Five-fold increased specific HMF uptake rate in spruce hydrolysate.	(Almeida et al., 2008b; Laadan et al., 2008)
XYL1	*S. stipitis*	Protein purification	Oxidoreductase	20% increase in specific HMF conversion rate.	(Almeida et al., 2008a)
ZWF1	*S. cerevisiae*	Screening of gene disruption library	Oxidoreductase/ increased NADPH supply	Cell growth attainable in presence of 50 mM furfural for strain overexpressing *ZWF1,* whereas no growth was observed for the wild type.	(Gorsich et al., 2006)
GSH1, GLR1, CYS3	*S. cerevisiae*	Reports of antioxidant activity	Glutathione overproduction	Overexpression of *GSH1/GLR1* resulted in 70% improvement in ethanol yield in the SSF of pretreated spruce compared with the wild type.	**Paper IV**
FLR1, ATR1	*S. cerevisiae*	Screening of gene disruption library	Multidrug resistance transporter	OD reached after 48 hours was 62 and 44% higher than in the wild type in the presence of 10 mM HMF and 1 mM coniferyl aldehyde, respectively.	(Alriksson et al., 2010)

[a]All modifications include overexpression of the target genes.

4.4 Is there a "magic bullet" for strain improvement?

This chapter presented several methodologies for improving strain robustness to inhibitory compounds in pretreated lignocellulosic raw materials. Evidently, no "magic bullet" that remedies the inhibitory action of the wide range of inhibitory compounds exists, as most methods have drawbacks. Evolutionary engineering has the advantage of being able to address complex traits such as tolerance to weak acids or to the combination of inhibitors present in hydrolysates. Nevertheless, trade-offs in terms of loss of other beneficial traits (e.g. xylose utilization) due to selection in highly specialized environments have to be considered. Although the knowledge of yeast metabolism is increasing continuously, targeted methods for strain improvement are still hampered by the incomplete knowledge of how microbes respond to various kinds of inhibitors and combinations thereof. To overcome the challenges encountered in targeted and non-targeted engineering strategies, a combination of evolutionary engineering and detailed physiological studies of the responses to specific inhibitors spanning various "omics" – transcriptomics, proteomics, metabolomics, and fluxomics – would be of great value in finding novel metabolic engineering targets. Beneficial traits could be discovered using methods such as inverse metabolic engineering or pooled-segregant whole-genome sequence analysis (Swinnen et al., 2012) and transferred to the strain background of choice. The latter method relies on classical strain breeding protocols in which superior and inferior phenotypes are mated, after which the mated cells are induced to sporulate. Linkage analysis is subsequently performed on a pool of superior haploid segregants by means of comparative genomics to find the loci in the genome where the beneficial mutations are located. Once a tolerant strain has been obtained, it is as stated previously of significant importance that the improved strain is evaluated in pretreated lignocellulosic hydrolysates by applying process configurations relevant to industry, such as SSF or SHF.

CONCLUSIONS

To construct rational metabolic engineering strategies for improving strain robustness in the context of lignocellulosic bioethanol production, it is crucial to identify the inhibitory compounds, whose molecular actions constitute bottlenecks in the production process. When this goal has been achieved, the next step is to elucidate the molecular mechanisms connected to the specific inhibitors addressed. The accomplishments presented here span the entire path from identifying challenges encountered when fermenting industrial substrates to harnessing knowledge of the physiological responses of *S. cerevisiae* to inhibitory compounds for the metabolic engineering of more robust strains. As my PhD project was part of the collaborative EU-funded NEMO project, I conducted case studies of two lignocellulosic raw materials, *A. donax* and spruce, representing feasible feedstocks for lignocellulosic bioethanol production in Europe. Based on the results of my work, conclusions have been formulated to answer four key questions summarizing my achievements.

What challenges in terms of lignocellulose-derived inhibitors were identified in the conversion of *Arundo donax* and spruce to bioethanol using SHF and SSF?

In **Paper I**, I used two principal process technologies, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF), to identify challenges related to bioethanol production from *A. donax*. The high xylose content of this feedstock is a problem that has to be dealt with using efficient xylose-consuming *S. cerevisiae* strains. I found that the formation of xylooligomers during the pretreatment process must be considered, as the cellulolytic enzymes were inhibited in the presence of these compounds. In **Paper I**, I proposed that the production of high amounts of xylooligomers could be circumvented either by increasing the pretreatment harshness or by including a mild acid hydrolysis step before the enzymatic hydrolysis. Xylose utilization was inhibited by acetic acid, a fact that calls for the development of strains with improved inhibitor tolerance.

Pretreated spruce was identified as more toxic than pretreated *A. donax*, based on the lower specific ethanol production rate obtained in the SHF of spruce than when *A. donax* was used as substrate. I suggest that this discrepancy is related to the high concentrations of furfural and HMF in the spruce material.

Concerning the best process option, I demonstrated that SHF resulted in a 13% higher final ethanol yield than did SSF of *A. donax*, whereas the opposite was observed when spruce was used. The different results regarding the choice of process configuration for these two feedstocks are related to the difference in composition of the pretreated raw materials. Despite the higher ethanol yields achieved in SHF of *A. donax*, the ultimate choice between SHF and SSF should be based on a complete techno-economic evaluation.

The furan aldehydes furfural and HMF were identified as a serious challenge in the conversion of pretreated spruce to ethanol. In general, assuming you have a robust yeast strain capable of growing despite high concentrations of inhibitors, spruce would be the raw material of choice due to its considerably higher amounts of rapidly consumable hexoses, resulting in higher specific ethanol productivities than if a xylan rich material is used, as xylose is consumed at a significantly slower rate than hexoses.

What can be learned from studying the responses of lignocellulose-derived inhibitors in chemostat cultivations?

A wide range of inhibitory compounds is formed or released when pretreating lignocellulosic raw materials. As the furan aldehydes furfural and HMF were identified as key inhibitors in the pretreated spruce material, they were investigated as a case study in this thesis. In **Paper II,** I used chemostat cultivations as a tool to study the long-term influence of furan aldehydes on the redox and energy metabolism and gene expression of a xylose-utilizing *S. cerevisiae* strain. The advantages of chemostat cultivations are that the specific growth rate can be kept constant and a steady state can be maintained in the reactor, making this cultivation mode extremely suitable for physiological investigations. However, the concentration of inhibitors has to be carefully chosen to avoid cell washout. Adding furan aldehydes to the feed medium of a chemostat brought about small changes in the distribution of extracellular metabolites. On the other hand, I found that the levels of NADH and NADPH were reduced by 40 and 75%, respectively, suggesting that the furan aldehydes drain the cells of reducing power. I also found that the intracellular ATP concentration decreased by 19% in the presence of inhibitors compared with inhibitor-free medium, which indicated that energy-requiring maintenance mechanisms were activated. A genome-wide analysis of gene expression suggested that some energy might be utilized in the export of inhibitors, as ATP-binding multidrug transporters were induced in response to the stress. Thus, I identified that the redox

metabolism and multidrug transporters constitute metabolic engineering targets for improved tolerance to furfural and HMF.

What can be learned from studying the responses of lignocellulose-derived inhibitors in batch cultivations?

In **Paper III**, I investigated the influence of furfural and HMF on the redox and energy metabolism of xylose-utilizing *S. cerevisiae* in batch cultivations by pulsing the inhibitors during either the glucose or xylose consumption phase. Pulsed batch cultivations are a valuable tool for studying short-term dynamic effects following metabolic perturbations. In contrast to the responses observed in chemostat cultivations (**Paper II)**, inhibitor pulses in either the glucose or xylose consumption phase resulted in perturbed fluxes of redox-related metabolites such as glycerol and acetate. The concentrations of the intracellular redox cofactors were unchanged one hour after pulsing the inhibitors in the glucose consumption phase, whereas the intracellular levels of NADH and NADPH decreased by 58 and 85%, respectively, one hour after pulsing in the xylose consumption phase, compared with the control. In **Paper III,** I hypothesized that the discrepancy between the results from pulsing in the glucose versus xylose consumption phases was connected to the lower metabolic flux during xylose consumption, resulting in a longer period needed to restore the cofactors to pre-stress levels. I suggest that additional studies investigating responses in redox cofactor levels following the pulsing of furan aldehydes in the glucose consumption phase on considerably shorter time scales should resolve the bases of this discrepancy. Concerning the perturbations in energy state in terms of changes in the intracellular concentration of ATP, I found that pulsing inhibitors in the glucose phase resulted in a 58% increase in intracellular ATP levels; conversely, intracellular ATP levels decreased by 42% after pulsing in the xylose consumption phase. I concluded that different responses in the intracellular ATP levels could be a consequence of the fact that the cells can respond by reducing the specific growth rate in the glucose phase, redirecting ATP to energy-requiring maintenance mechanisms. I found further evidence for this hypothesis in the gene expression data, which indicated that genes involved in energy-consuming processes such as protein synthesis and nucleotide metabolism were downregulated. After pulsing in the xylose consumption phase, induction of genes that encode proteins involved in NADPH-utilizing processes such as glutamate biosynthesis and sulphur assimilation was observed, further corroborating the fact that furan aldehydes drain the cells of reducing power, as was first discovered in **Paper II**. Based on a comparison of genes upregulated after inhibitor treatment in both phases, I identified multidrug transporters

and several oxidoreductases as potential metabolic engineering targets for improved tolerance to furan aldehydes.

Is there a "magic bullet" for improving strain tolerance?

Increasing the tolerance to the process environment is crucial in lignocellulosic bioethanol production. As concluded at the end of **Chapter 4**, no "magic bullet" exists for improving strain robustness, as both non-targeted and targeted strategies have trade-offs. Non-targeted methods such as evolutionary engineering have successfully been applied to improve tolerance to both individual inhibitors and pretreated lignocellulosic raw materials, but the problem of losses of essential traits due to metabolic trade-offs is imminent. Targeted metabolic engineering strategies have been used mainly to improve tolerance to furan aldehydes by overexpressing endogenous oxidoreductases. Several of these modifications reportedly result in perturbed byproduct distribution, such as increased glycerol production. In **Paper IV,** I presented a novel strategy for improving strain robustness in the context of lignocellulosic bioethanol production, a strategy that exploits the overproduction of protective metabolites, and in particular, glutathione, to increase the redox buffering capacity of the cells. By using a substrate known to be challenging in terms of its toxicity (pretreated spruce) and a process configuration (SSF) applicable in an industrial setup, I demonstrated that cells harbouring the *GSH1* overexpression-construct and hence an increased intracellular concentration of reduced glutathione sustained ethanol production for longer than did the wild type, resulting in a 70% increase in ethanol yield. Although the specific mechanisms of the beneficial role of glutathione remain to be elucidated, to the best of my knowledge, this is the first time a successful rational engineering approach to obtaining improved tolerance to lignocellulose-derived inhibitors has been demonstrated in SSF.

FUTURE DIRECTIONS

Lignocellulosic bioethanol production is approaching commercialization after years of intense research into the various aspects of the production process. Even so, room for improvement remains in all steps of the process, as is evident from the research presented here. In **Paper I**, I highlighted the importance of the pretreatment process as a key step on which many other unit operations are dependent. Failure to remove the hemicellulose fraction and lignin could result in a pretreated raw material with very low enzymatic digestibility. On the other hand, overly harsh conditions during the pretreatment result in sugar loss and the formation of fermentation inhibitors. Novel pretreatment processes with minor byproduct formation that make it possible to fractionate the raw material would benefit a future biorefinery, as the raw material could then be used more efficiently, having favourable effects on the process economy.

The use of high solid loading during the fermentation process is another potential way to reduce the final cost of ethanol, as less process water would have to be used, while higher ethanol concentrations could be achieved due to the elevated sugar concentrations. Increasing the solid consistency in lignocellulosic fermentation results in a concomitant increase in the amount of lignocellulose-derived inhibitors, which can be dealt with by developing more robust production hosts – an area where my thesis work can provide a basis for future work. However, it is important to note that high solid loading also results in mass transfer issues that calls for further development of the SHF and SSF process configurations used here by, for example, implementing substrate and enzyme feeding strategies.

In **Papers II-III**, I investigated the influence of furan aldehydes on the redox and energy metabolism. Both these articles have contributed significantly to our understanding of how these inhibitory compounds exert their action, especially in terms on their effect on the drainage of reducing equivalents. However, because both the redox cofactors and the adenonucleotides participate in a multitude of metabolic processes in both anabolism and catabolism, an integrated approach is needed involving modelling the intracellular fluxes in response to perturbations in these highly connected metabolites. Such modelling experiments have been hampered by incomplete knowledge of the concentrations of intracellular metabolites, but advances in the field of metabolomics could address this challenge. In **Paper II**, ammonia assimilation through NADPH-dependent glutamate dehydrogenase was

suggested as particularly sensitive to the drainage of intracellular NADPH levels, which was further corroborated in **Paper III.** Utilization of systems biology tools such as thermodynamics-based metabolic flux analysis and metabolic control analysis permit the more systematic identification of key enzymes affected by perturbations in redox cofactor and adenonucleotides (Soh et al., 2012).

Global gene expression analysis functioned as a key tool in **Papers II-III**. Analysing the entire transcriptome after adding furan aldehydes in both chemostat and batch cultivations, revealed that hundreds of genes were differentially expressed. It is an intriguing thought that some of these genes might encode proteins with key roles in mediating strain robustness. For example, of the 69 genes upregulated in both the glucose and xylose consumption phases after adding furfural and HMF, 28 were coding for proteins with unknown functions (**Paper III**). Hence, considerable work remains to conduct a functional analysis of these proteins, as they could be important to improving strain robustness. Furthermore, by comparing the genes induced upon adding furfural and HMF in both chemostat and batch cultivations, genes belonging to the multidrug transporters of the pleiotropic drug response family were found to be induced in all experiments. These transporters have received recent attention in efforts to improve tolerance to alkanes (Ling et al., 2013), but their applicability in the context of furfural and HMF tolerance remains to be investigated.

In **Paper IV** I demonstrated that overproduction of the intracellular protective metabolite glutathione significantly improved the strain robustness in SSF of pretreated spruce. This study constituted a proof of concept using a laboratory strain background. However, as strains with an industrial background have been demonstrated to be more tolerant of pretreated lignocellulosic raw materials (Panagiotou and Olsson, 2007), the glutathione overproduction strategy should be tested in an industrial strain background. Although a redox potentiating effect was suggested as the mechanism of action for the beneficial effects of increased intracellular glutathione concentrations, further studies are required to investigate this hypothesis more thoroughly. Clues could possibly be found by time-lapse measurements of the intracellular redox potential following inoculation in a hydrolysate. Such studies should preferably be performed using non-invasive redox sensors such as rxGFP, as invasive methods do not capture compartment-specific redox potentials (Ostergaard et al., 2004). It would also be interesting to investigate the effects of increasing the intracellular NADPH

availability by overexpression of *ZWF1* in a strain engineered for improved glutathione recycling capacity, such as the one overexpressing *GLR1* and *GSH1* discussed in **Paper IV**.

Since the ultimate goal of strains engineered for improved robustness is in applications involving industrial substrates, the improved strains should be evaluated under industrial conditions. There are several examples in which researchers have used evolutionary engineering strategies for strain improvement and later demonstrated improvements in pretreated lignocellulosic hydrolysates, whereas such demonstrations are virtually absent when rational metabolic engineering methods have been used. Thus, I hope a trend shift is to come, in which metabolic engineers aim to construct strains that display enhanced performance under process-relevant conditions. In my thesis research, I used SSF to demonstrate the improved robustness of strains overproducing the protective metabolite glutathione (**Paper IV),** which represents a step in the right direction.

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