Fermentation of Lignocellulosic Hydrolysates: Inhibition and Detoxification

Eva Palmqvist

Department of Applied Microbiology
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To my mother

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Abstract

The ethanol yield and productivity obtained during fermentation of lignocellulosic hydrolysates is decreased due to the presence of inhibiting compounds, such as weak acids, furans and phenolic compounds produced during hydrolysis. Evaluation of the effect of various biological, physical and chemical detoxification treatments by fermentation assays using *Saccharomyces cerevisiae* was used to characterise inhibitors. Inhibition of fermentation was decreased after removal of the non-volatile compounds, pre-fermentation by the filamentous fungus *Trichoderma reesei*, treatment with the lignolytic enzyme laccase, extraction with ether, and treatment with alkali. Yeast growth in lignocellulosic hydrolysates was inhibited below a certain fermentation pH, most likely due to high concentrations of undissociated weak acids. The effects of individual compounds were studied in model fermentations. Furfural is reduced to furfuryl alcohol by yeast dehydrogenases, thereby affecting the intracellular redox balance. As a result, acetaldehyde accumulated during furfural reduction, which most likely contributed to inhibition of growth. Acetic acid (10 g l^{-1}) and furfural (3 g l^{-1}) interacted antagonistically causing decreased specific growth rate, whereas no significant individual or interaction effects were detected by the lignin-derived compound 4-hydroxybenzoic acid (2 g l^{-1}). By maintaining a high cellmass density in the fermentor, the process was less sensitive to inhibitors affecting growth and to fluctuations in fermentation pH, and in addition the depletion rate of biocenvertible inhibitors was increased. A theoretical ethanol yield and high productivity was obtained in continuous fermentation of spruce hydrolysate when the cellmass concentration was maintained at a high level by applying cell recirculation.

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CONTENTS

LIST OF PAPERS

This thesis is based on the following papers[1], referred to by their respective Roman numerals in the text.

I. **Palmqvist, E., Hahn-Hägerdal, B., Galbe, M. and Zacchi, G.** (1996). The effect of water-soluble inhibitors from steam-pretreated willow on enzymatic hydrolysis and ethanol fermentation. *Enz. Microb. Technol.* **19**, 470-476.

II. **Palmqvist, E., Hahn-Hägerdal, B., Szengyel, Z., Zacchi, G., and Rèczey, K.** (1997). Simultaneous detoxification and enzyme production of hemicellulose hydrolysates obtained after steam pretreatment. *Enz. Microb. Technol.* **20**, 286-293.

III. **Jönsson, L. J., Palmqvist, E., Nilvebrant, N. O. N. and Hahn-Hägerdal, B.** Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*. Submitted for publication.

IV. **Palmqvist, E., Grage, H., Meinander, N. and Hahn-Hägerdal, B.** Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. Submitted for publication.

V. **Palmqvist, E. Galbe, M. and Hahn-Hägerdal, B.** Continuous fermentation with cell recirculation of enzymatic hydrolysates of spruce with *Saccharomyces cerevisiae* and on-line monitoring of glucose and ethanol. Submitted for publication.

VI. **Palmqvist, E., Almeida, J. and Hahn-Hägerdal, B.** Influence of furfural on anaerobic glucose metabolism of *S. cerevisiae*. Manuscript.

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1. INTRODUCTION

Lignocellulosic materials, such as wood, provide abundant and renewable energy sources. Lignocellulosics contain sugars polymerised to cellulose and hemicellulose which can be liberated by hydrolysing the material, and subsequently fermented to ethanol by microorganisms, e.g. *Saccharomyces cerevisiae*. Lignocellulose-derived ethanol can be used as an environmentally friendly liquid fuel, the exhaust carbon dioxide being taken up by growing biomass and therefore not making a net contribution to the atmosphere. However, during the hydrolysis of lignocellulosics, a wide range of compounds is generated, in addition to the monomeric sugars. Some of these degradation products are toxic to microorganisms, thereby limiting rapid and efficient fermentation of the hydrolysates. The work presented in this thesis was performed to identify inhibiting compounds and to elucidate the mechanisms involved in the inhibition of fermentation of lignocellulosic hydrolysates, in order to improve the fermentation process. If the inhibitors are identified, the fermentation process can be improved in several ways. Firstly, formation of inhibitors can be minimised through optimisation of the conditions during pretreatment and hydrolysis. Secondly, prediction of fermentability based on analysis of the hydrolysates will be possible, and thirdly, specific detoxification methods can be developed for efficient removal of inhibitors prior to fermentation of strongly inhibiting hydrolysates. A greater understanding of the inhibitory mechanisms of individual compounds and their interaction effects, as well as the influence of environmental parameters such as pH, will allow optimisation of the conditions during fermentation. For optimal design of the fermentation process, rates of bioconversion and the adaptive response of the yeast to the toxic compounds in the hydrolysate also have to be considered.

Throughout the work presented in this thesis, *S. cerevisiae* was used for fermentation of lignocellulose-derived sugars and in model fermentations. Whenever other organisms are considered, this is explicitly stated. *S. cerevisiae* has been shown to be the best yeast for the fermentation of lignocellulose-derived hydrolysates due to its good ethanol-producing capacity and inhibitor tolerance (Hahn-Hägerdal *et al.*, 1991; Olsson and Hahn-Hägerdal, 1993).

2. LIGNOCELLULOSIC MATERIALS AND THEIR DEGRADATION DURING HYDROLYSIS

2.1 Composition of lignocellulosic materials

Lignocellulosic materials include wood, forestry waste, agricultural residues, and municipal solid waste. In the work presented in this thesis, only wood was used as raw material for the production of lignocellulosic hydrolysates. Lignocellulosics are mainly composed of cellulose, hemicellulose and lignin. The average compositions of softwood (e.g. spruce and pine) and hardwood (e.g. willow, aspen and oak) are summarised in Table 1. Cellulose is a high molecular weight linear polymer of β-1,4 linked D-glucose units which can appear as a highly crystalline material (Fan *et al.*, 1982). Hemicelluloses are branched polysaccharides consisting of the pentoses D-xylose and L-arabinose, and the hexoses D-mannose, D-glucose, D-galactose and uronic acids (Saka, 1991). Softwoods and hardwoods differ in structure and composition of the hemicellulose (Saka, 1991). Softwood hemicellulose has a higher proportion of mannose and glucose units than hardwood hemicellulose, which usually contains a higher proportion of xylose units. Furthermore, hemicellulose is more highly acetylated in hardwoods than in softwoods (Fengel and Wenger, 1989). The branched structure of hemicellulose renders it more easily hydrolysed than cellulose.

Table 1. Average composition of softwoods and hardwoods (Saka, 1991).

	Cellulose	Hemicellulose	Lignin
Softwood	38-52%	16-27%	26-36%
Hardwood	37-57%	20-37%	17-30%

Lignin is an aromatic polymer synthesised from phenylpropanoid precursors (Adler, 1977). Lignins are divided into two classes, namely "guaiacyl lignins" and "guaiacyl-syringyl lignins", differing in the substituents of the phenylpropanoid skeleton. Guaiacyl-lignins have a methoxy-group in the 3-carbon position, whereas syringyl-

lignins have a methoxy-group in both the 3-carbon and 5-carbon positions. Softwood and hardwood lignins belong to the first and second category, respectively. Softwoods generally contain more lignin than hardwoods (Table 1).

2.2 Hydrolysis of lignocellulosic materials

Various methods for the hydrolysis of lignocellulosic materials for ethanol production have recently been described (Olsson and Hahn-Hägerdal, 1995). In this thesis, dilute-acid hydrolysis and enzymatic hydrolysis will be considered.

2.2.1 Dilute-acid and enzymatic hydrolysis

Dilute-acid hydrolysis is fast and easy to perform but is hampered by non-selectivity and by-product formation (Fan *et al.*, 1982). The ethanol yields obtained using dilute-acid hydrolysis and fermentation are only 50-60% of the theoretical values (Wyman, 1994). Normally, dilute-acid hydrolysis is carried out using mineral acids such as H_2SO_4 or HCl, at temperatures between 120°C and 200°C (Grethlein and Converse, 1991; Torget and Hsu, 1994).

Enzymatic hydrolysis of cellulose is performed using cellulolytic enzymes. The enzyme cellulase system is a mixture of *endo*-β-1,4-glucanglucanhydrolases, *exo*-β-1,4-glucancellobiohydrolases (cellulases) and β-glucosidase (Parisi, 1989). The cellulases break down cellulose to cellobiose, which is subsequently cleaved to glucose by β-glucosidase. The cellulases and β-glucosidase are inhibited by cellobiose and glucose, respectively (Philippidis *et al.*, 1993). Product inhibition of the enzymes decreases the efficiency of hydrolysis. The rate and yield during enzymatic hydrolysis of willow were also decreased by increased concentrations of non-volatile compounds formed during steam pretreatment, which may accumulate in a process where water streams are recirculated (Paper I). Enzymatic hydrolysis leads to higher yields of monosaccharides than dilute-acid hydrolysis, because cellulase enzymes catalyse only hydrolysis reactions and not sugar degradation reactions (Parisi, 1989). Enzymes are naturally occurring compounds which are biodegradable and therefore environmental friendly.

Prior to enzymatic hydrolysis, the cellulose structure must be made available to the hydrolysing enzymes by subjecting the material to some form of pretreatment (Fan *et al.*, 1982; Parisi, 1989). Wood can be pretreated using high-temperature steam which solubilises the hemicellulose (Galbe and Zacchi, 1986). In order to improve the recovery of hemicellulose-derived sugars, the wood can be impregnated with SO_2 or H_2SO_4 prior to steam pretreatment (Clark and Mackie, 1987). The pretreated wood is then washed to remove the solubilised hemicellulose from the fibrous material.

An overall ethanol yield from the softwood pine, after enzymatic hydrolysis and fermentation of only 20% of the theoretical value has been reported, compared with about 90% for the hardwoods soft maple, American sycamore and red oak under the same conditions (Vinzant *et al.*, 1994). Similarly, a low yield of monosaccharides was obtained during enzymatic hydrolysis of the softwood spruce, leading to a total ethanol yield of only 50% of the theoretical value (Stenberg *et al.*, 1998). These results indicate that hardwoods and softwoods differ in their susceptibility to enzymatic hydrolysis. End-product inhibition of the cellulolytic enzymes probably was partly responsible for the low hydrolysis yields, and also structural differences between hardwoods and softwoods which might contribute to decreased accessibility to the hydrolysing enzymes. Softwoods have a more compact fibrous structure than hardwoods (Vinzant *et al.*, 1994). In addition, softwood lignins are more highly crosslinked than hardwood lignins (due to the absence of a methoxy group in the 5-carbon position of the phenylpropanoid units), and are therefore probably more predisposed to recondensation reactions after hydrolysis (Sakakibara, 1991).

2.2.2 Simultaneous saccharification and fermentation

Using simultaneous hydrolysis (saccharification) and fermentation (SSF), product inhibition of β-glucosidase by glucose is alleviated by the rapid conversion of the glucose to ethanol by the yeast (Takagi *et al.*, 1977). In order to study the effect of product inhibition on the total ethanol yield from spruce, SSF and separate hydrolysis and fermentation (SHF) were compared using the same raw material and enzyme loading. Steam-pretreated spruce (215°C, 5 minutes, 2.4% SO_2 (w/w)) was supplemented with cellulases (celluclast™ 2L, 0.15 g g^{-1} fibrous material), and β-

glucosidase, Novozym 188™, 0.03 g g^{-1} fibrous material). The SSF was carried out at 37°C and pH 5.0 using compressed baker's yeast for ethanolic fermentation. The glucose concentration was virtually zero during the entire process (Figure 1). The overall ethanol yield was 71% of the theoretical value, which is a considerable increase compared with the yield obtained in SHF (50% of the theoretical value) (Stenberg *et al.*, 1998). During the first 47 hours of fermentation, the ethanol concentration increased to 29 g l^{-1}, whereafter it remained constant, indicating that no more fermentable sugars (glucose and mannose) were produced in the hydrolysis. At this point, lactate production by lactic acid bacteria present in the compressed baker's yeast started, yielding a considerable amount of lactate from the sugars not consumed by *S. cerevisiae*.

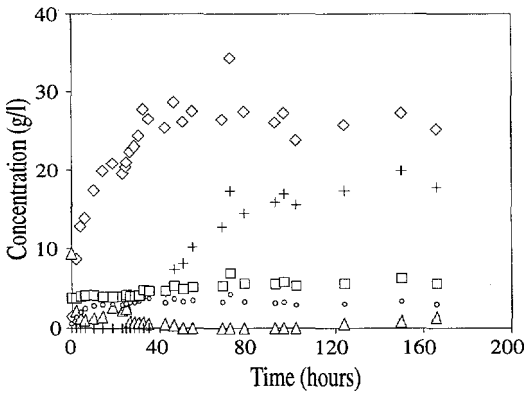

Figure 1. Simultaneous saccharification and fermentation (SSF) of softwood: (△) glucose, (◇) ethanol, (+) lactate, (o) glycerol, (□) acetate.

In addition to obtaining higher ethanol yields in SSF due to alleviation of product inhibition of β-glucosidase, the equipment cost is reduced as only one reactor is required for the conversion of wood to ethanol (Philippidis *et al.*, 1993). However, the enzymes and the yeast do not operate optimally under the same conditions, and thus SSF is run at a pH and temperature that are not optimal, either for fermentation or hydrolysis. Another drawback is that recirculation of the yeast is difficult due the large solid residue (lignin) obtained after hydrolysis, which is difficult to separate from the cells.

2.2.3 Degradation of the lignocellulosic material

Enzymatic and acid hydrolysis procedures which involve treatment of the lignocellulose at high temperature under acidic conditions lead to the formation and liberation of a wide range of compounds, as schematically presented in Figure 2a.

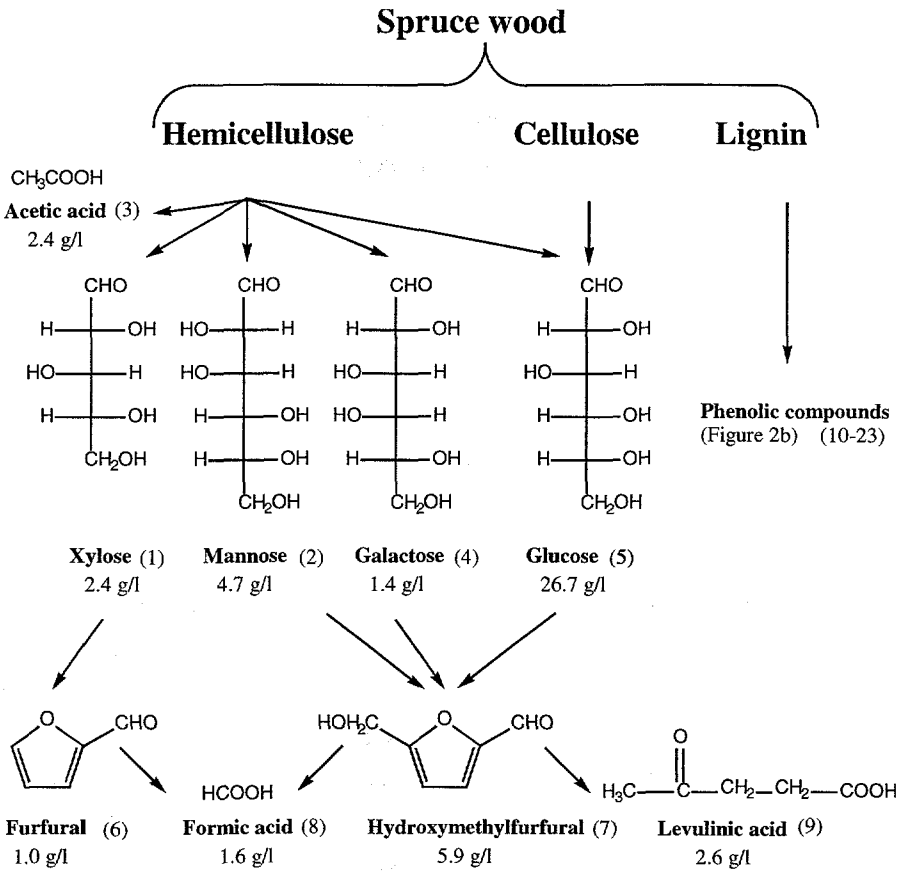

Figure 2a. Reactions occurring during hydrolysis of lignocellulosic materials and concentration in a dilute acid hydrolysate of spruce (g l^{-1}) (222°C, 7 minutes, 2% sulphuric acid (w/w dry matter)). The furan derivatives and phenolic compounds will react further to form some polymeric material (Nilvebrant *et al.*, In preparation).

When hemicellulose is hydrolysed, xylose, mannose, acetic acid, galactose, and glucose (Figure 2a, nos 1, 2, 3, 4 and 5, respectively) are liberated. A hemicellulose hydrolysate obtained after steam pretreatment of willow contained 2.0 g l^{-1} glucose, 5.2 g l^{-1} xylose, 2.6 g l^{-1} acetic acid, 0.6 g l^{-1} mannose and 0.5 g l^{-1} galactose (Paper II).

Cellulose is hydrolysed to glucose. At high temperatures and pressures xylose is further degraded to furfural (Figure 2a, no. 6) (Dunlop, 1948). Similarly, 5-hydroxymethyl furfural (HMF) (Figure 2a, no. 7) is formed from hexose degradation (Ulbricht *et al.*, 1984). Formic acid (Figure 2a, no. 8) is formed when furfural and HMF are broken down (Dunlop, 1948; Ulbricht *et al.*, 1984). Levulinic acid (Figure 2a, no. 9) is formed by HMF degradation (Ulbricht *et al.*, 1984).

In Figure 2b, phenolic monomers identified in the spruce hydrolysate are presented. Phenolic compounds are generated from partial breakdown of lignin (Bardet and Robert, 1985; Lapierre *et al.*, 1983; Sears *et al.*, 1971), and have also been reported to be formed during carbohydrate degradation (Popoff and Theander, 1976; Suortti, 1983).

Hibbert's ketones (Figure 2b, nos 10-14) have previously been detected in the hydrolysate of pine (Clark and Mackie, 1984). Vanillic acid (Figure 2b, no. 17) and vanillin (Figure 2b, no. 18), formed by the degradation of the guaiacylpropane units of lignin, have been reported in hydrolysates from willow (Paper III), spruce (Nilvebrant *et al.*, In preparation), poplar (Ando *et al.*, 1986), red oak (Tran and Chambers, 1985), and pine (Clark and Mackie, 1984). In hardwood hydrolysates, syringaldehyde and syringic acid, formed in the degradation of syringyl propane units, have been reported (Paper III; Tran and Chambers, 1985). Hydroquinone (1,4-di-hydroxybenzene) (Figure 2a, no. 19) has previously not been reported in a lignocellulosic hydrolysate, whereas catechol (1,2-di-hydroxybenzene) (Figure 2b, no. 20) has been identified in hydrolysates of willow (Paper III) and birch (Buchert *et al.*, 1990). 4-Hydroxybenzoic acid (Figure 2b, no. 21) constitutes a large fraction of the lignin-derived compounds in hydrolysates from the hardwoods poplar (Ando *et al.*, 1986), aspen (Bardet and Robert, 1985), and willow (Paper III). A substantial amount of the alcoholic hydroxyl groups of native aspen lignin has been found to be esterified with 4-hydroxybenzoic acid, and it is probable that this compound is liberated during hydrolysis (Bardet and Robert, 1985). No extractives were identified in a dilute acid hydrolysate of spruce, whereas trace amounts were found in the solid residue (Nilvebrant *et al.*, In preparation). In contrast, the extractives caproic acid, caprylic acid, pelargonic acid, and palmitic acid have been reported in dilute acid hydrolysate of red oak (Tran and Chambers, 1985).

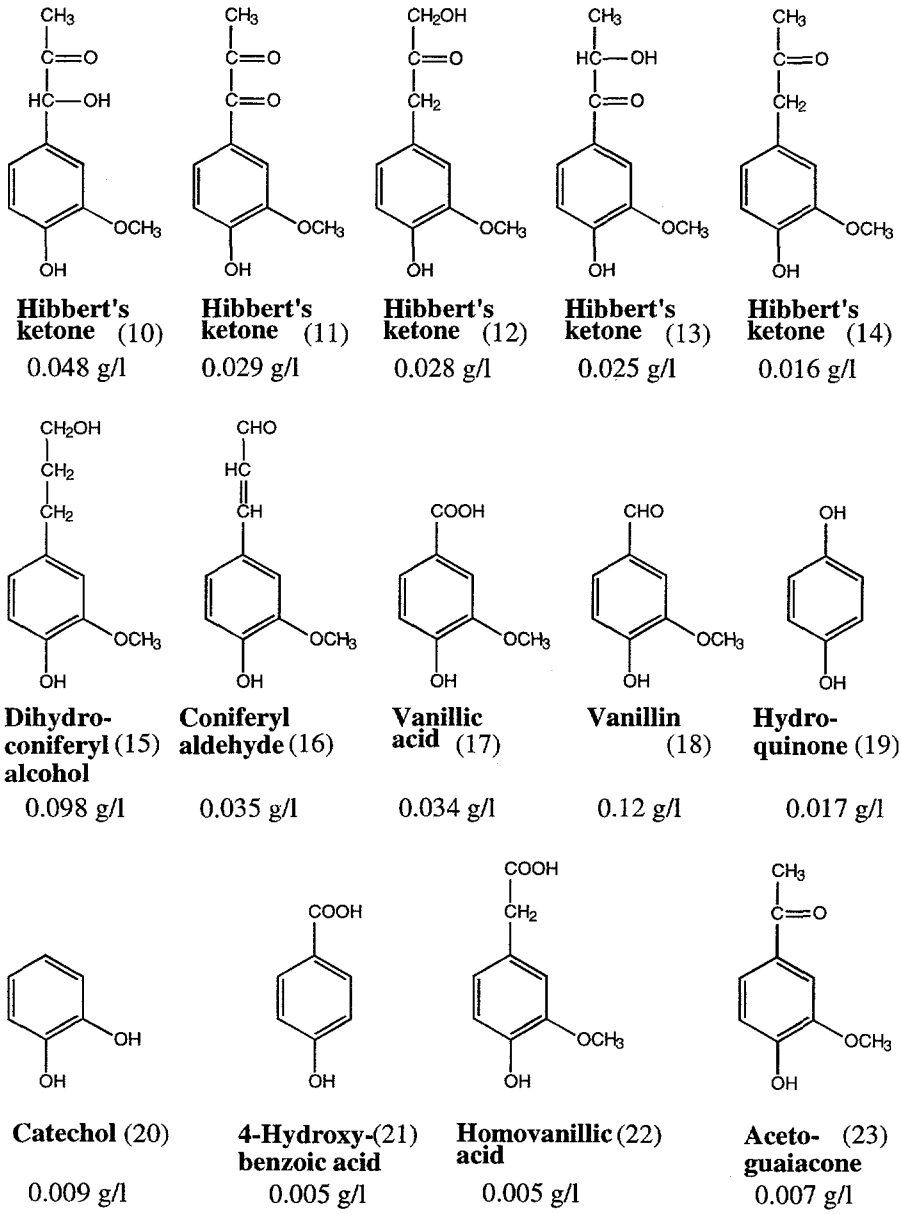

Figure 2b. Phenolic monomers (g l^{-1}) identified in the hydrolysate presented in Figure 2a. Adapted from Nilvebrant *et al.* (In preparation).

The temperature during dilute-acid hydrolysis of spruce strongly influences the concentration of the degradation products of cellulose, hemicellulose, and lignin in the hydrolysates. The compositions of three dilute acid hydrolysates treated at 210°C, 222°C and 232°C (7 minutes, 2% H_2SO_4 (w/w dry matter)) are presented in Table 2.

The concentration of glucose increased from 16.2 g l^{-1} to 27.8 g l^{-1} when increasing temperature from 210°C to 232°C due to more extensive cellulose hydrolysis, whereas the concentrations of mannose, xylose, and galactose from the more easily hydrolysed hemicellulose decreased with increasing temperature due to their degradation. Similar results were obtained in another study where the temperature was varied within the same range (Taherzadeh *et al.*, 1998a). The concentration of furfural remained relatively constant at the three temperatures, despite the decrease in xylose concentration which indicated increased furfural formation (Table 2). This was probably due to further degradation and polymerisation of the produced furfural. The concentration of HMF increased with temperature from 3.7 g l^{-1} to 8.7 g l^{-1} due to more extensive breakdown of glucose.

Table 2. Composition of three dilute acid hydrolysates of spruce, produced at constant residence time (7 minutes), sulphuric acid concentration (2% (w/w dry material)) and increasing temperature (see Table). Adapted from Nilvebrant *et al.* (In preparation).

	Temperature (°C)		
	210	222	232
Hydrolysis product	*Concentration (g l^{-1})*		
Glucose	16.2	26.3	27.8
Mannose	14.6	11.3	7.1
Xylose	6.3	3.4	3.3
Galactose	3.2	2.4	2.1
Furfural	2.4	2.8	2.6
HMF	3.7	5.8	8.7
Acetic acid	4.6	4.6	4.1
Levulinic acid	0.6	2.0	5.1
Formic acid	0.3	1.6	2.5
Total phenolic compounds	0.5	0.5	1.7

The concentration of acetic acid was not significantly affected by the increase in temperature (Table 2). In contrast, the concentrations of formic and levulinic acid (Figure 2a nos 8 and 9), increased from 0.3 to 2.5 g l^{-1} and 0.6 to 5.1 g l^{-1}, respectively, due to more extensive breakdown of HMF and furfural when the temperature was increased from 210°C to 232°C (Table 2). The sum of the concentrations of acetic, levulinic and formic acid was equal to the total concentration of carboxylic acids, determined by titration, in all three hydrolysates (Nilvebrant *et al.*, In preparation). Thus, no unidentified carboxylic acids were present in significant concentrations. The concentration of phenolic compounds increased from 0.5 to 1.7 g l^{-1} when the hydrolysis temperature was increased from 210°C to 232°C (Table 2). The dominating phenolic compounds identified in an ether extract of the hydrolysate treated at 222°C are presented in Figure 2b. The difference in composition between softwood and hardwood hemicellulose (Saka, 1991) was reflected in the composition of the hydrolysates (Taherzadeh *et al.*, 1998a). Hardwood hydrolysates (alder, aspen and birch) contained more xylose and acetic acid and less mannose than softwood hydrolysates (pine and spruce). As a consequence of the higher xylose content, the concentration of the xylose degradation product furfural was higher in the hardwood hydrolysates.

2.2.4 Fermentability

Optimisation of the hydrolysis process requires consideration of not only the yield of fermentable sugars, but also the fermentability of the produced hydrolysate. Some of the compounds formed when lignocellulosic materials are hydrolysed (Figures 2a and b) inhibit microorganisms (Brown and Booth, 1991; Clark and Mackie, 1984), leading to decreased ethanol yield and productivity.

A method of evaluating the effect of inhibitors on yeast fermentation was developed (Paper IV). A modified version of this method has proven to be a rapid and reliable tool for assessing inhibition in large numbers of differently treated hydrolysates (Larsson *et al.*, Submitted; Stenberg *et al.*; Submitted; Taherzadeh *et al.*, 1998a). Glucose was added to the hydrolysates to obtain the same initial concentration of fermentable sugars in all fermentations, and the fermentability was evaluated in terms

of ethanol yield (produced ethanol during the fermentation/ initial concentration of fermentable sugars) and productivity (ethanol production rate). Commercial compressed baker's yeast was used for fermentation. High cell-mass inocula were used order to obtain rapid fermentation with little or no net growth, in order to reduce the effect of differences in viability of the yeast on ethanol yield and productivity. The ethanol yield obtained after 24 hours' fermentation decreased from 0.41 g g^{-1} to 0.19 g g^{-1} (pH adjustment with $Ca(OH)_2$) in the hydrolysates treated at increasing temperature from 210°C to 232°C (Table 2), showing that the generation of fermentation inhibitors increased with increasing hydrolysis temperature (Nilvebrant *et al.,* In Preparation). A less accurate but faster way of assaying the toxicity is to use a simple sensor, for instance using yeast cells immobilised in agarose gel for detection of fermentation inhibitors (Palmqvist *et al.*, 1994). By immobilising enzymes instead of whole cells, the selectivity of a biosensor is greatly increased (Palmqvist *et al.*, 1995), and it could be used to determine the presence and concentration of specific inhibiting compounds.

Not only the hydrolysis temperature, but also the reaction time and acid concentration influence the generation of fermentation inhibitors. The severity of different pretreatment conditions can be compared by calculating a severity parameter, where the reaction temperature, T (°C), and residence time, t (minutes), variables are combined into a single reaction ordinate. The severity factor, $\log R_0$, is defined by $R_0 = t\, e^{(T-100)/14.75}$ (Overend and Chornet, 1987). The influence of hydrolysis pH (reflecting the amount of acid used), is taken into consideration by the combined severity, CS, defined as $\log R_0$-pH (Chum *et al.*, 1990). The influence of CS during dilute-acid hydrolysis of spruce on the concentration of fermentable sugars and sugar degradation products, and on the fermentability, was investigated by varying the parameters T (150-240°C), t (1-30 minutes) and the sulphuric acid concentration (0.5-4.4% H_2SO_4 (w/w dry matter)) (Larsson *et al.*, Submitted). The maximum concentration of mannose from the relatively easily hydrolysed hemicellulose occurred at a lower CS than the maximum glucose concentration. When the CS was increased beyond the value which generated maximal mannose and glucose concentrations, hemicellulose sugars and glucose were broken down. The decrease in the concentration of fermentable sugars coincided with the formation of furfural and HMF which, in turn, were degraded to levulinic and formic acid when the CS was

further increased. The fermentability drastically decreased around the value of CS where furfural and HMF started to accumulate. It was concluded that the CS should be kept at about 3 in order to obtain both high yields of fermentable sugars and high fermentability. The best hydrolysis conditions were 0.5% H_2SO_4, 225°C, 5 minutes and 0.5% H_2SO_4, 210°C, 10 minutes. Comparison between hydrolysis using SO_2 or H_2SO_4 at the same CS showed that the sugar yields obtained were equal, whereas the fermentability was better when SO_2 was used, indicating that less inhibitors were formed in this case (Tengborg *et al.*,1998).

3. INHIBITION IN LIGNOCELLULOSIC HYDROLYSATES

The compounds formed or released during hydrolysis of lignocellulosic materials belong to three major groups: weak acids, furans and phenolic compounds. The effects of compounds from these three groups on microorganisms in general, and on *S. cerevisiae* in particular, are summarised in this chapter. In addition, inhibitory effects of ethanol, important during continuous fermentation and the late stages of batch fermentation are discussed. The influence of fermentation pH is considered in relation to weak acids. In the last section interaction effects between compounds or groups of compounds in lignocellulosic hydrolysates are discussed.

3.1 Inhibition of *Saccharomyces cerevisiae* metabolism

3.1.1 Growth and viability of Saccharomyces cerevisiae

The viability of the yeast cell population is crucial for the fermentation process. A microorganism is viable if it is capable of replication in conditions that are optimal for the species and strain considered (Postgate, 1969). The value of the specific growth rate (μ) may vary depending on the method used to measure the cell-mass concentration. Only when growth is completely balanced (coupled growth), i.e. all cellular constituents are manufactured at the same specific rate (Prescott *et al.*, 1996), will μ be independent of the measurement method (Pamment, 1989). In batch fermentation, the cellular composition adjusts continuously to the changing medium composition, and therefore both the initial state of the inoculum and the method used to determine the biomass concentration will influence the value of μ. Non-viable cells include dead cells as well as replicatively inactivated cells, still capable of metabolic activity (Pamment, 1989). A cell is glycolytically active but non-replicating when it has entered the stationary phase (Willison and Johnston, 1985), e.g. in response to nutrient starvation (de Winde *et al.*, 1997) or in the presence of high ethanol concentrations (Brown *et al.*, 1981; Jones and Greenfield, 1985; Melzoch *et al.*, 1991). Three kinds of cells may thus exist in a population: actively replicating, metabolically active but non-replicating, and dead.

The biomass concentration can be determined in several ways. The plate count method records viable cells only, optical density measurements and vital staining techniques determine the concentration of viable as well as inactivated cells, and dry-weight measurements quantify replicating, inactivated and dead cells. The apparent specific growth rate based on the three latter methods thus reflects the processes of growth, inactivation of replication and death, and will be less than the true specific growth rate.

In experimental work, the variation in viability between different yeast populations must be considered. In Figure 3, the specific growth rates (calculated from optical density measurements) in nine batch fermentations containing various inhibitor combinations, for two different inocula preparations and run on different days, are presented. There is a systematic variation of 0.069 ± 0.015 h^{-1} between the growth rates for the two inocula preparations, in spite of the fact that the inocula were prepared using exactly the same procedures and the cells harvested in the exponential phase (Paper IV). The fermentations were included in a modified central composite design containing in total 45 experiments, used to evaluate statistically significant individual and interaction effects of acetic acid, furfural, and 4-hydroxybenzoic acid on growth and volumetric ethanol productivity. In order to exclude the systematic variation between different inocula preparations from the experimental error, the fermentations were divided into 5 sets of nine fermentations each (blocks), corresponding to different inocula run on different days. The experiment was then designed in such a way that much of the anticipated variation between blocks did not contribute to the experimental error (Montgomery, 1997).

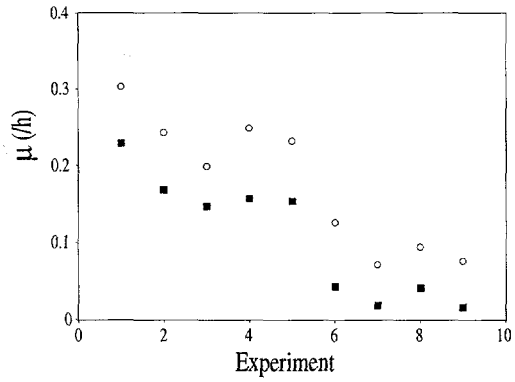

Figure 3. Specific growth rates in nine fermentations with *S. cerevisiae* using two different inocula preparations (○ and ■). Different combinations of acetic acid, furfural, and 4-hydroxybenzoic acid were added to the nine fermentations. Adapted from Paper IV.

3.1.2 Energetics of growth

During anaerobic conditions the ATP production is calculated from substrates that are degraded through known catabolic pathways (Verduyn *et al.*, 1990a). ATP is used for biomass production, transport, and cell maintenance (Pirt, 1965; Stouthammer, 1979). Uncoupled (unbalanced) growth, leads to a larger fraction of the substrate used for ATP generation, decreased growth rate and biomass yield. Uncoupled growth is induced by synthetic uncouplers such as dinitrophenol (Stouthammer, 1979), by nitrogen limitation (Larsson *et al.*, 1995), or by weak acids (Verduyn *et al.*, 1990a; Stouthammer, 1979). Inhibition by compounds affecting energy metabolism can be measured in terms of differences in growth yield compared with a control. The growth yield decreases when additional energy is required for maintenance purposes in the presence of an inhibitor (Warth, 1988).

3.1.3 Stress response mechanisms

Microorganisms respond to adverse conditions (stress) in various ways (Mager and Ferreira, 1993). The purpose of stress response mechanisms is to protect the cells and to repair any molecular damage. Cells in the stationary phase have been shown to be more resistant to heat and other forms of stress than exponentially growing cells (Werner-Washburne *et al.*, 1993). Two different transcriptional control systems activated by heat stress have been demonstrated in *S. cerevisiae* (Piper, 1997), the

Heat Shock Element (HSE) and the general STress Responsive Element (STRE). HSE controls genes encoding chaperons and proteins functionally cooperating with chaperons. This system provides a basal level of factors required for proper protein folding. HSE is activated by heat shock leading to the expression of heat-shock proteins (hsps), and this is considered to reflect the important function of hsps as chaperons (Siderius and Mager, 1997). STRE-activated genes appear to be important for survival under severe stress (for a recent review, see (Ruis and Shuller, 1995)). STRE is activated by a wide range of stress conditions, in addition to heat, e.g. nutrient starvation, high osmolarity, oxidative stress, low pH, weak acids and ethanol, and induces a different set of stress proteins than HSE. It has been suggested that intracellular pH (pH_i) plays an essential role in triggering the stress response (Coote *et al.*, 1991). Compounds in lignocellulosic hydrolysates acting as stress-inducing factors and suggested effects on the yeast are summarised in Table 3. These factors will be further discussed in Section 3.2.

Table 3. Summary of stress factors and reported effects on yeast cells/ suggested cell responses (•) (increase (↑), decrease (↓)), see Section 3.2 for details.

	Stress factor					
Effect on cells/ response	Weak acids	Low pH	Furfural	Phenolic compounds	EtOH	Osmotic stress
Low pH_i	•	•			•	
Enzyme inhibition	•	•	•			
Membrane effects	•			•	•	
↑ATPase activity	•	•			•	
↑ ADH activity			•		•	
Synthesis of stress proteins	•	•			•	•
Glycerol production			↓			↑
Decreased cell volume	•				•	•

3.2 Mechanisms of inhibition

3.2.1 Weak acids

Acids are classified as either strong or weak, depending on their dissociation constant, K_a, the negative logarithm of which is denoted pK_a (Atkins, 1990). pK_a is the pH value at which the concentrations of the undissociated and dissociated form of the acid are equal, and the buffering capacity of the acid therefore is highest. The concentration of undissociated acid is a function of pH and pK_a, and increases with decreasing pH (Henderson-Hasselbach equation (Atkins, 1990)). Weak acids have rather high pK_a values, e.g. the values of pK_a at zero ionic strength for acetic, formic, octanoic and levulinic acid are 4.75 (25°C), 3.75 (20°C), 4.89 (25°C) (Weast, 1975-1976) and 4.66 (25°C) (Soni *et al.*, 1982), respectively.

Weak acids inhibit cell growth and are used as food preservatives to prevent microbial growth (Brown and Booth, 1991). Undissociated weak acids are liposoluble and can therefore diffuse across the plasma membrane. The growth-inhibiting effect on microorganisms has been proposed to be due to the inflow of undissociated acid into the cytosol (Axe and Bailey, 1995; Stouthammer, 1979; Verduyn *et al.*, 1990a; Verduyn *et al.*, 1992; Warth, 1988). In the cytosol, dissociation of the acid occurs due to the neutral intracellular pH, thus decreasing the cytosolic pH (Pampulha and Loureiro-Dias, 1989).

Low fermentation pH inhibits cell proliferation and viability also in the absence of weak acids, due to the increased proton gradient across the plasma membrane, resulting in an increase in the passive proton uptake rate (Verduyn *et al.*, 1990a). Maintaining a neutral intracellular pH is crucial for cell viability. The cell replicative activity has been found to decrease linearly with decreasing intracellular pH (Imai and Ohono, 1995). The optimal external pH range for growth of *S. cerevisiae* is 5.0-5.5 (Verduyn *et al.*, 1990a). Growth has been detected at a pH as low as 2.5 in the absence of acetic acid in model fermentations, while the minimum pH for growth increased to 4.5 in the presence of acetic acid (10 g l^{-1}) (Taherzadeh *et al.*, 1998b).

In Figure 4, the titration curves of three dilute acid hydrolysates of spruce are shown. A horizontal line is drawn at pH 5.5, the normal fermentation pH. The hydrolysates had a high buffering capacity until approximately pH 5.5 due to partial dissociation of acetic, formic, and levulinic acid (the compositions of the hydrolysates are given in Table 2). It can be concluded from the figure that the concentration of undissociated acids is very sensitive to small decreases in pH from 5.5. Fermentation pH thus appears to be a crucial variable during fermentation of lignocellulosic hydrolysates. This was confirmed by the fact that growth in two different dilute acid hydrolysates of spruce was initiated by increasing the pH from 4.6 to 5.0 and from 5.4 to 5.7 after long lag phases (Paper V).

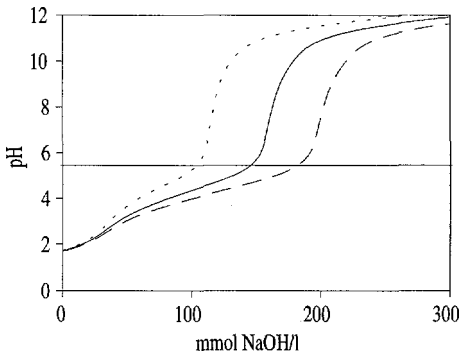

Figure 4. Titration curves for acid hydrolysates of spruce treated at (---) 210°C, (—) 222°C, and (— — —) 232°C. Adapted from Nilvebrant *et al.* (In preparation). The horizontal line is drawn at fermentation pH 5.5

The influence of acetic, formic, and levulinic acid on the ethanol yield was studied in model fermentations (Larsson *et al.*, Submitted). Low acid concentrations (<100 mmol l^{-1}) stimulated the ethanol yield at pH 5.5, whereas the yield decreased at higher concentrations (Figure 5a). In agreement with this result, high concentrations of aliphatic carboxylic acids (>200 mmol l^{-1}) were found to be correlated with inhibition of fermentation by principal component analysis of chemical characterisation and fermentability data from a large number of dilute acid hydrolysates of spruce, hydrolysed under a broad range of conditions (Nilvebrant *et al.*, 1997). The influence of the undissociated form of the three acids on the ethanol yield is illustrated in Figure 5b. The ethanol yield decreased with increasing concentration of undissociated acid for each acid, as has been reported previously (Pampulha and Loureiro-Dias, 1989), but a clear difference was observed in toxicity between the acids, which may be due to

differences in membrane permeability or in toxicity of the anionic form of the acids once they have entered the cell.

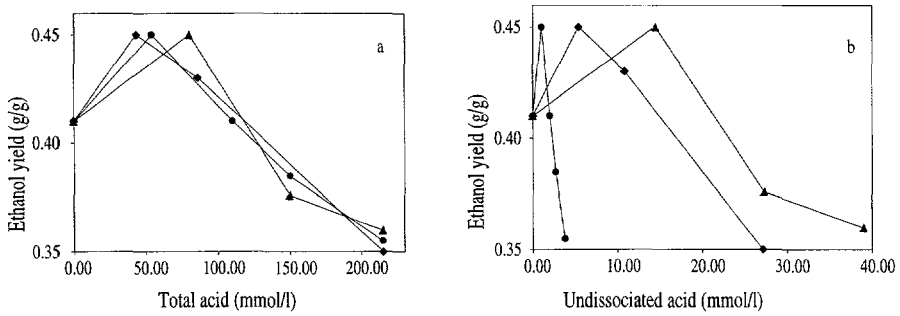

Figure 5. Influence of weak acids on the ethanol yield obtained in fermentation with *S. cerevisiae* at fermentation pH 5.5: acetic acid (▲); formic acid (•); levulinic acid (◆): (a) total acid concentration, (b) concentration of undissociated acid. Adapted from Larsson *et al.* (Submitted).

The reason for the slightly higher toxicity of undissociated levulinic acid (Figure 2a, no. 9) compared with acetic acid might be its greater hydrophobicity. Levulinic acid may also form even more lipophilic intramolecular esters, so-called Angelica lactones. The uptake rate of medium-chain-length linear monocarboxylic acids in *Zygosaccharomyces bailii,* has been found to increase with increasing chain length and thus greater hydrophobicity (Warth, 1989). In agreement with this, inhibition by propionate was more pronounced than by acetate in *S. cerevisiae* (Verduyn *et al.*, 1990b). However, undissociated formic acid appears to be considerably more toxic than undissociated levulinic acid, in spite of its lower hydrophobicity. This is in accordance with what has been reported previously (Maiorella *et al.*, 1983). The high toxicity of formic acid might be due to the small size of the molecule or specific effects of the anion.

The effect of medium-chain-length fatty acids is probably not only due to proton inflow into the cytoplasm, but also to a direct effect on membrane integrity (Viegas *et al.*, 1989; Heipieper *et al.*, 1994). Insertion of the hydrophobic chains into the membrane may alter the membrane structure and hydrophobicity, leading to increased permeability. The liposoluble fermentation by-products decanoic and octanoic acid were found to be inhibitory already at 10-20 mg l^{-1}, probably because the concentration of acid in the plasma membrane was high enough to affect the activity

of membrane-bound enzymes (Viegas and Sá-Correia, 1991). However, the inhibition caused by decanoic acid was less significant than would have been expected on the basis of its hydrophobicity. This was suggested to be due to that the size of the molecule had surpassed the critical dimension for easy penetration of the membrane. Consistent with this observation, alcohols with chain lengths greater than 10 carbon atoms have been found to be relatively inefficient microbial inhibitors (Ingram and Buttke, 1984).

Two theories have been proposed to explain the inhibitory effect of weak acids: uncoupling (see Section 3.1.2), and intracellular anion accumulation (Russel, 1992). According to the uncoupling theory, the drop in intracellular pH resulting from inflow of weak acids is neutralised by the action of the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis (Stouthammer, 1979; Verduyn *et al.*, 1992). Growth in the presence of sorbic acid (0.45 mM, pH 4.5) was associated with a 10-fold decrease in cellular ATP levels (Holoyaki *et al.*, 1996). Additional ATP must be generated in order to maintain the intracellular pH, and under anaerobic conditions this is achieved by increased ethanol production at the expense of biomass formation (Taherzadeh *et al.*, 1998a; Viegas and Sá-Correia, 1991). At high acid concentrations, the proton pumping capacity of the cell is exhausted, resulting in depletion of the ATP content, dissipation of the proton motive force, and acidification of the cytoplasm (Imai and Ohono, 1995). Synthetic uncouplers, such as dinitrophenol, can move cross the plasma membrane in both dissociated and undissociated form, causing a high rate of proton importation (Russel, 1992). In contrast, the anionic forms of acetic, propionic, and formic acid, are lipophobic, and do not traverse the plasma membrane during growth on glucose (Casal *et al.*, 1996). Since each molecule of undissociated acid therefore leads to the importation of only one proton, dissipation of the proton motive force by weak acids has been questioned (Russel, 1992). It was instead suggested that the toxicity of weak acids was due to intracellular anion accumulation.

According to the anion accumulation theory, the anionic form of the acid is captured in the cell and undissociated acid will diffuse into the cell until equilibrium is reached (Rottenberg, 1979). Since the equilibrium concentration of undissociated acid is a function of pH, the extent of intracellular anion accumulation will be a function of the

pH gradient over the plasma membrane (Russel, 1992). At low extracellular pH, intracellular anion accumulation reaches high levels in *S. cerevisiae* as the yeast maintains a neutral intracellular pH. In media with mixtures of glucose and acetic acid at low pH the accumulation ratio increased by a factor of 10 to 1000 when the pH was decreased from 6.0 to 3.5 (Casal *et al.*, 1996). The activity of glycolytic enzymes in the presence of acetic acid has been investigated, showing that enolase was the most sensitive enzyme, and that the inhibition was due to both internal acidification and direct interference with the acid (Pampuhla and Loureiro-Dias, 1990).

The results presented in Figure 5b indicate that the mechanism of inhibition by weak acids is a combination of uncoupling and specific effects of the anion or anion accumulation. In accordance with this, it was recently concluded that the influence of lactic acid on *Lactobacillus rhamnosus* was not only due to the undissociated form of the acid but also to ionic inhibition (Goncales *et al.*, 1997).

S. cerevisiae has been reported to respond in different ways to weak acids and decreased intracellular pH, as shown in Table 3. Growth in the presence of octanoic acid (Viegas and Sá-Correia, 1991), sorbic acid (Holoyaki *et al.*, 1996), and low intracellular pH (Eraso and Gancedo, 1987), have been shown to activate the plasma membrane ATPase, thereby increasing the proton pumping capacity of the cell. The production of succinic and acetic acid has been reported to decrease during cell growth in the presence of octanoic acid, thereby decreasing the total acid stress experienced by the yeast (Viegas and Sá-Correia, 1995). The cell volume has also been reported to decrease with increasing concentration of octanoic acid in the medium, thereby increasing the buffering capacity of the cytoplasm due to a higher concentration of cellular compounds (Viegas and Sá-Correia, 1995). In addition, low internal pH (Coote *et al.*, 1991) and sorbic acid (Cheng and Piper, 1994) have been reported to enhance thermotolerance in *S. cerevisiae* by inducing certain heat-shock genes.

There is a large difference in resistance to weak-acid preservatives between yeast species (Warth, 1988). Yeasts resistant to benzoic acid exhibited lower uptake rates of the acid. Adaptation by growth in the presence of benzoic acid (0.25 mM) decreased the accumulation of benzoate by the yeast and increased its tolerance to the

preservative. This was suggested to be due to changes in membrane permeability (Warth, 1988). Gradual adaptation of *S. cerevisiae* to benzoic acid (10 mM) and stimulation of respiration has been observed in aerobic chemostat culture, whereas a pulse addition of the same amount inhibited respiration (Verduyn *et al.*, 1992). Adaptation of *S. cerevisiae* to sorbic acid (up to 90 mmol l^{-1}) has also been reported (Holoyaki *et al.*, 1996). The lag phase in growth, reflecting the time of adaptation, increased with increasing acid concentration and decreasing pH.

3.2.2 Furfural and HMF

Furfural is metabolised by *S. cerevisiae* under aerobic (Taherzadeh *et al.*, Submitted), oxygen-limited (Fireoved and Mutharasan, 1986; Navarro, 1994) and anaerobic conditions (Paper VI). During fermentation furfural reduction to furfuryl alcohol with high yields occurs (Paper VI; Diaz de Villegas *et al.*, 1992; Taherzadeh *et al.*, Submitted; Villa, 1992). Product inhibition of aerobic growth of *Pichia stipitis* by furfuryl alcohol has been reported (Weigert *et al.*, 1988), whereas only slight inhibition of anaerobic growth of *S. cerevisiae* was detected (Paper VI). Furfural oxidation to furoic acid occurred to some extent, primarily under aerobic conditions (Paper VI; Taherzadeh *et al.*, Submitted). A metabolite which may be a reaction product of furfural and pyruvate was recently discovered in a fermentation with furfural (Taherzadeh *et al.*, Submitted). The furfural reduction rate increased with increasing inoculum size (Boyer *et al.*, 1992; Chung and Lee, 1984; Navarro, 1994), and with increasing specific growth rate in chemostat (Fireoved and Mutharasan, 1986) and batch cultures (Taherzadeh *et al.*, Submitted). The reduction rate also increased with increasing furfural concentration up to 42.0 mmol l^{-1}, and then decreased again, probably due to cell death at high furfural concentrations (Figure 6).

Furfural has been reported to reduce the specific growth rate (Azhar *et al.*, 1981; Boyer *et al.*, 1992; Navarro, 1994; Navarro, 1994), the cell-mass yield on ATP (Paper VI), the volumetric (Azhar *et al.*, 1981; Navarro, 1994), and specific ethanol productivities (Paper VI, Taherzadeh *et al.*, Submitted). When the furfural concentration was increased to 53 mmol l^{-1} in batch fermentation, the specific ethanol productivity decreased to 13% of the value in a reference fermentation and the specific growth rate was reduced to zero (Figure 6).

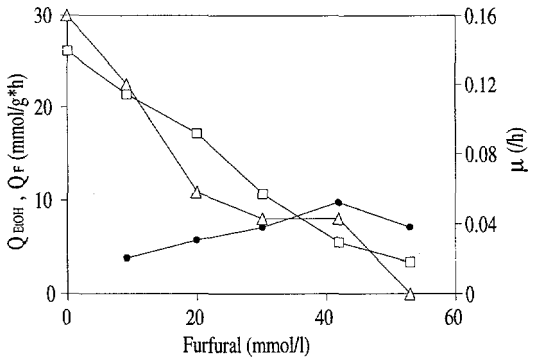

Figure 6. Influence of furfural on the specific rates of growth (μ) (5 hours) (△), ethanol production (Q_{EtOH}) (5 hours) (□), and furfural reduction (Q_F) (3 hours) (•) in batch fermentation. Adapted from Paper VI.

NADH-dependent yeast alcohol dehydrogenase (ADH) is believed to be responsible for furfural reduction (Diaz de Villegas *et al.*, 1992; Taherzadeh *et al.*, Submitted; Weigert *et al.*, 1988). Under anaerobic conditions, glycerol is normally produced to regenerate excess NADH formed in biosynthesis (Oura, 1977). No glycerol was produced during furfural reduction (Figure 7) suggesting that furfural reduction regenerated NAD^+ (Paper IV and VI). The fact that less carbon was consumed by glycerol production in the presence of furfural resulted in an increased ethanol yield in the presence of 29 mmol l^{-1} furfural compared with fermentation in the absence of furfural (Paper VI). Elevated concentrations of acetaldehyde were excreted in the beginning of the fermentation (Figure 7), probably due to a decreased NADH concentration in the cell during furfural reduction. Furfural inhibition of glycolytic enzymes *in vitro* has also been reported (Banerjee *et al.*, 1981b), and direct inhibition of ADH might have contributed to acetaldehyde excretion. Intracellular acetaldehyde accumulation probably was the reason for the lag-phase in growth in the presence of furfural, for a review on the toxicity of acetaldehyde, see Jones, (1989).

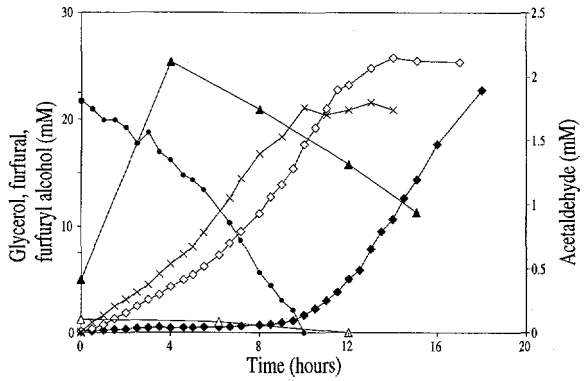

Figure 7. Influence of furfural on the production of glycerol and acetaldehyde: glycerol in reference fermentation containing no furfural (◇), glycerol in fermentation with furfural (◆), acetaldehyde in reference fermentation (△), acetaldehyde in fermentation with furfural (▲), furfural (•), furfuryl alcohol (X). Adapted from Paper VI.

A mechanistic model describing the specific rates of growth, glucose consumption, ethanol and glycerol formation, and furfural reduction in batch fermentation was developed. The model was based on the following assumptions: *i*) furfural reduction to furfuryl alcohol by NADH dependent dehydrogenases had a higher priority than reduction of dihydroxyacetone phosphate to glycerol, and *ii*) furfural caused inactivation of cell replication. The model accurately described the experimental data in the absence and in the presence of furfural (Figure 8), strongly suggesting that the model assumptions were correct.

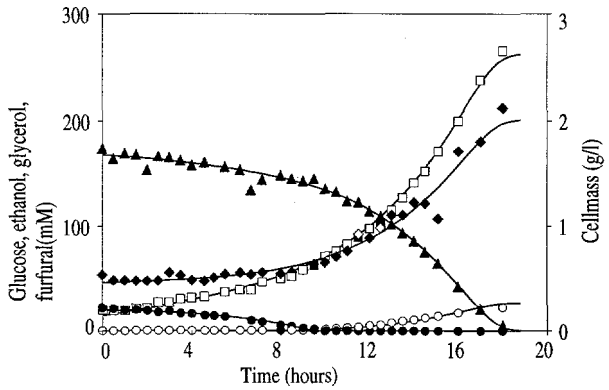

Figure 8. Model predictions (lines) and experimental data (symbols) for carbon fluxes in batch fermentation with *S. cerevisiae* in the presence of furfural: glucose (▲), ethanol (□), glycerol (○), furfural (•), cell dry weight (◆). Adapted from Paper VI.

Adaptation of *S. cerevisiae* to furfural by pre-adaptation has been reported in batch culture (Banerjee *et al.*, 1981a), fed-batch culture (Nutek Report, 1997; Villa, 1992),

and in continuous culture (Chung and Lee, 1984; Fireoved and Mutharasan, 1986), leading to increased growth and volumetric ethanol productivity. The adaptation might be due to the synthesis of new enzymes or co-enzymes for furfural reduction (Boyer *et al.*, 1992). Supporting this hypothesis, the ADH activity in anaerobic fermentation increased by 78% after 48 hours' fermentation with an initial furfural concentration of 2 g l^{-1} (Banerjee *et al.*, 1981b).

HMF was also metabolised by *S. cerevisiae* (Nutek Report, 1997; Larsson *et al.*, Submitted; Sanchez and Bautista, 1988). This compound was degraded at a lower rate than furfural, which might be due to lower membrane permeability, and caused a longer lag phase in the volumetric ethanol productivity (Figure 9) (Larsson *et al.*, Submitted). It is probable that HMF is reduced in a manner similar to that of furfural, either directly or after cleavage of the ring (Sanchez and Bautista, 1988), thereby suggesting similar inhibitory mechanisms of HMF and furfural toxicity.

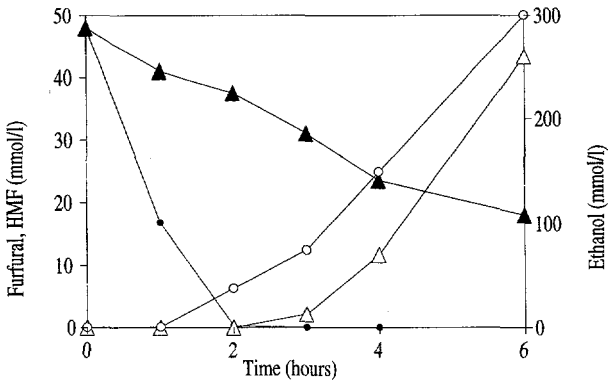

Figure 9. Conversion of furfural and HMF by *S. cerevisiae* and influence on ethanol production: furfural (•), HMF (▲), ethanol in fermentation with furfural (o); ethanol in fermentation with HMF (△). Adapted from Larsson *et al.* (Submitted).

3.2.3 Phenolic compounds

Phenolic compounds partition into biological membranes and cause loss of integrity, thereby affecting their ability to serve as selective barriers and enzyme matrices (Heipieper *et al.*, 1994). Phenolic compounds have been suggested to exert a considerable inhibitory effect in the fermentation of lignocellulosic hydrolysates, the low molecular weight phenolic compounds being most toxic (Buchert *et al.*, 1989;

Clark and Mackie, 1984). However, the mechanism of the inhibiting effect has not been elucidated, largely due to a lack of accurate qualitative and quantitative analyses. Model studies of the inhibitory action of phenolic compounds have been performed using far higher concentrations than are actually present in the hydrolysates (Clark and Mackie, 1984; Delgenes *et al.*, 1996b; Mikulásová *et al.*, 1990). When the results of those studies are interpreted, it should be borne in mind that the water solubility of phenolic compounds is limited. The solubility depends on the composition of the liquid and can be different in hydrolysates and in pure solution. When a high concentration of a certain compound has been used, it is therefore possible that the concentration actually experienced by the organism has been lower.

Inhibition of fermentation decreased when phenolic monomers and phenolic acids were specifically removed from a willow hemicellulose hydrolysate by treatment with the lignin-oxidising enzyme laccase (Paper III). 4-Hydroxybenzoic acid, vanillin, and catechol were identified as major constituents in the untreated hydrolysate. 4-Hydroxybenzoic acid was chosen to study the influence of phenolic compounds on fermentation (Paper IV). This choice was based on the abundance of 4-hydroxybenzoic acid in hardwood hydrolysates (Paper III, Ando *et al.*, 1986; Bardet and Robert, 1985) and its reported inhibitory effect on fermentation with *S. cerevisiae* (1 g l^{-1} caused a 30% decrease in ethanol yield compared to a reference fermentation) (Ando *et al.*, 1986). However, no significant effects on either growth or volumetric ethanol productivity were detected during fermentation with 2 g l^{-1} 4-hydroxybenzoic acid (Paper IV). Vanillin constitutes a large fraction of the phenolic monomers in hydrolysates of spruce (Nilvebrant *et al.*, In preparation), pine (Clark and Mackie, 1984) and willow (Paper III). Vanillin has been found to be less toxic than 4-hydroxybenzoic acid (1 g l^{-1} caused a 25% decrease in the ethanol yield), and vanillic acid had no effect at concentrations up to 1 g l^{-1} (Ando *et al.*, 1986). The concentration of catechol in a willow hemicellulose hydrolysate was 0.44 g l^{-1} (Paper III), and this compound was slightly inhibitory at 0.2 g l^{-1} in model fermentations (Larsson *et al.*, In preparation). In contrast, the structurally similar hydroquinone, identified in a spruce hydrolysate (Figure 2b, no 19), had a slightly stimulating effect on the cell yield at concentrations up to 2 g l^{-1}, whereas the specific ethanol yield was decreased (Larsson *et al.*, In preparation). It is possible that oxidation of hydroquinone and catechol to quinones in the hydrolysates generates more toxic compounds, as indicated by

drastically increased inhibition after oxygen treatment of a mixture of these compounds (Larsson *et al.*, In preparation). Benzoquinone, which is the oxidation product of hydroquinone, was more inhibitory than catechol (Larsson *et al.*, In preparation). *S. cerevisiae* assimilated vanillin, hydroxybenzaldehyde, and syringaldehyde during fermentation (Delgenes *et al.*, 1996b), and growth has been reported on catechol, recorcinol, salicylic acid, and p-hydroxybenzoic acid (Mills *et al.*, 1971).

3.2.4 Ethanol

The action of alcohols on both eucaryotic and procaryotic organisms shares the same general mechanisms, which are due to the physico-chemical properties of the alcohols affecting cytosolic and envelop components, for a review, see Ingram and Buttke, (1984). In a study of *Escherichia coli* it was suggested that the interaction between ethanol and biological membranes decreased membrane fluidity, which is essential for the function of many membrane-associated enzymes (Ingram, 1976).

Adaptation to increasing ethanol concentrations (up to 4.7% v/v) was observed in continuous culture (Arneborg *et al.*, 1995). In batch fermentations it has been suggested that the cells continuously adapt to the increasing ethanol concentration (Jones and Greenfield, 1985; Loueiro and van Uden, 1986). Upon the addition of ethanol (10% v/v) to growing cultures, growth was inhibited and did not resume until the cells had begun to change their plasma membrane fatty acid composition (Lloyd *et al.*, 1993). Adapted cells, exhibiting greater ethanol tolerance, contained an increased proportion of unsaturated fatty acids, and a decreased sterol content, where ergosterol was the predominant sterol (Alexandre *et al.*, 1994; Arneborg *et al.*, 1995; Walker-Caprioglio *et al.*, 1990). Sugar and amino acid uptake in *S. cerevisiae* were inhibited by ethanol, possibly due to inhibition of membrane proteins involved in the transport of these solutes (Thomas and Rose, 1979). The detected inhibition was less for cells grown in the presence of linoleic rather than oleic acid, probably due to the greater mobility of the doubly unsaturated fatty-acyl residues which counteracted the decrease in membrane fluidity caused by ethanol. Adaptive responses in membrane composition of *E. coli* also occurred when the cells were exposed to the by-products of ethanolic fermentation, acetaldehyde and acetic acid (Ingram, 1976). An increased

ADH level might also be involved in the adaptive response to high ethanol concentrations (Gokhale *et al.*, 1986; Lafon-Lafoucade and Riberau-Gayon, 1984).

The effect of ethanol on cell growth has been found to be a combination of true growth inhibition (decrease in μ) and decreased viability (replicative activity) (Brown *et al.*, 1981); in accordance with this, the biomass yield on consumed glucose has been shown to decrease with increasing ethanol concentration in batch fermentation (Thatipamala *et al.*, 1992). The yeast cells in any given population have been found to be variable in their susceptibility to ethanol (Herman *et al.*, 1980). Some cells stopped growing completely, some slowed down, and others were not affected, depending on which growth phase they were in when the ethanol was added. With the ethanol production rates normally found in batch fermentation, partially reversible inactivation of replication has been suggested to occur since the culture is in a process of continuous adaptation to an increasing ethanol concentration (Jones and Greenfield, 1984). High rates of inactivation have been observed in rapid batch fermentation, and several reasons for this have been suggested, including nutrient deficiency (Dasari *et al.*, 1990), intracellular acetaldehyde accumulation (Jones, 1989) and inability of the microorganism to adjust to the rapidly increasing ethanol concentration (Dasari *et al.*, 1990; Nagodawithana and Steinkraus, 1976; Tilak *et al.*, 1976). The highest ethanol concentration permitting cell growth is approximately 100 g l^{-1}, whereas inhibition of ethanol production occurs at a higher concentration (Bazua and Wilke, 1977; Dauglis and Swaine, 1987; Ghose and Tyagi, 1979a; Thatipamala *et al.*, 1992).

The plasma membrane ATPase content (Piper *et al.*, 1994) and activity (Alexandre *et al.*, 1994) decreased when a pulse of ethanol was added to the incubation medium. In contrast, cultivation of *S. cerevisiae* in the presence of ethanol resulted in activation of the plasma membrane ATPase (Alexandre *et al.*, 1994; Monteiro *et al.*, 1994; Rosa and Sá-Correia, 1996). The activity increased at ethanol concentrations up to 7-8% (v/v) with maximum activity obtained at about 5-6% (v/v) (Rosa and Sá-Correia, 1996; Monteiro *et al.*, 1994). During growth in the presence of ethanol in concentrations above 7-8% the activity of the ATPase decreased again (Rosa and Sá-Correia, 1996). Sub-lethal concentrations of ethanol have been reported to induce heat-shock proteins (Piper *et al.*, 1994).

An increased proton influx into the cytoplasm has been reported in the presence of ethanol (Juroszek and Charpentier, 1987; Leão and van Uden, 1984; Rosa and Sá-Correia, 1996). At ethanol concentrations above 6% (v/v) the intracellular pH was found to decrease (Rosa and Sá-Correia, 1996). As during growth in the presence of weak acids, the cell volume decreased with increasing ethanol concentration in the medium; this effect probably serves to increase the buffering capacity in the cytoplasm in response to the increased inflow of protons.

3.2.5 Interaction effects

Acetic acid (10 g l^{-1}) and furfural (3 g l^{-1}) interacted antagonistically on growth (i.e. the observed decrease in μ in the presence of both compounds was greater than the sum of the decreases caused by the individual compounds (Myers and Montgomery, 1991)) when 0.2 g l^{-1} initial cell mass was used (Figure 10a). The effect on the volumetric ethanol productivity, on the other hand, was purely additive (Figure 10b). Using 10 g l^{-1} initial cell mass, only slightly decreased ethanol yield was obtained compared to the reference fermentation in the presence of 5 g l^{-1} acetic acid, 10 g l^{-1} formic acid, 23 g l^{-1} levulinic acid, 1.2 g l^{-1} furfural, and 1.3 g l^{-1} HMF. This result was consistent with the fact that these compounds inhibit cell growth more than ethanol productivity, and virtually no net growth occurred when the initial cell mass was 10 g l^{-1}, even in the absence of inhibitors. However, the fact that almost no ethanol production was observed in a spruce hydrolysate containing the same concentrations of weak acids and furans as the model fermentation, even at high initial cell-mass concentration, showed that other compounds, possibly the lignin degradation products, contributed to the inhibition in the spruce hydrolysate (Larsson *et al.*, Submitted). Thus, the phenolic compounds are probably responsible for considerable inhibition, despite the fact that no significant effects of 4-hydroxybenzoic acid were detected up to 2 g l^{-1}, either individually or in combination with acetic acid and furfural in model fermentations (Paper IV).

As discussed previously, weak acids and ethanol influence intracellular pH, and the effect of these compounds is therefore more severe at low fermentation pH. In addition, the inhibiting effect of ethanol has been reported to increase in the presence

of octanoic, decanoic (Sá-Correira, 1986) and acetic acid (Pampulha and Loureiro-Dias, 1989; Ramos and Madeira-Lopes, 1990).

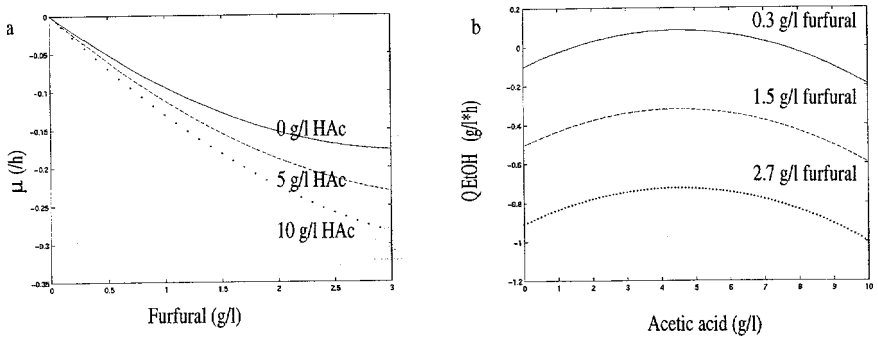

Figure 10. Effect of furfural at three acetic acid concentrations showing a) the antagonistic interaction between acetic acid and furfural on μ, and b) the additive effect of acetic acid and furfural on the volumetric ethanol productivity (Q_{EtOH}). Adapted from Paper IV.

Specific inhibitors have been discussed in the previous sections. In addition to these, the fermenting organism will be exposed to an aqueous solution containing sugars, amino acids, ions and ethanol, resulting in decreased water activity. The salt content was estimated to be 200 g l^{-1} in spent sulphite liquor, and 20 g l^{-1} in an enzymatic hydrolysate (Olsson and Hahn-Hägerdal, 1993). Osmotic stress causes a decrease in cell volume, for a review see Hohmann (1997), and induces stress tolerance in *S. cerevisiae* (Ruis and Shuller, 1995). Glycerol is produced in eucaryotic microorganisms in response to osmotic stress, in order to increase internal osmolarity and to more efficiently retrieve water from the environment (Brown, 1976). It is therefore possible that the reduction of furfural, which leads to decreased glycerol production, interferes with the cell response to osmotic stress in lignocellulosic hydrolysates.

4. DETOXIFICATION

Detoxification of lignocellulosic hydrolysates (i.e. the removal of inhibitors prior to fermentation) improves the fermentability, for a recent review, see Olsson and Hahn-Hägerdal (1995). Representative examples of detoxification methods are presented in Table 4, together with the microorganisms used for fermentation.

Table 4. Methods for detoxification of lignocellulosic hydrolysates. In the cases of alkali treatment to pH 10, the pH was readjusted to pH 5.5 with H_2SO_4 prior to fermentation.

Method	Test organism	Fermentation pH/ maximal pH and base used for adjustment	Reference
Enzymatic	*S. cerevisiae*	5.5 NaOH	Paper III
Trichoderma reesei	*S. cerevisiae*	5.5 NaOH	Paper II
Roto-evaporation	*S. cerevisiae*	5.5 NaOH	Paper I
Ether extraction	*S. cerevisiae*	5.5 NaOH	(Nilvebrant *et al.*, In preparation)
Ethyl acetate extraction	*S. cerevisiae*	5.0 NaOH	(Clark and Mackie, 1984)
Alkali	*S. cerevisiae*	5.5/ 10 NaOH / $Ca(OH)_2$	(Nilvebrant *et al.*, In preparation)
Sulphite	*S. cerevisiae*	4.8 $Ca(OH)_2$	(Leonard and Hajny, 1945)
Alkali and sulphite	*E. coli*	6.0/ 10.5 $Ca(OH)_2$	(Olsson *et al.*, 1995)
Anion resin	*P. stipitis*	6.5 $Ca(OH)_2$	(van Zyl *et al.*, 1991)
Mixed-bed ion resin	*G. oxydans*	5.5-6.5 $Ca(OH)_2$	(Buchert *et al.*, 1990)

Detoxification methods cannot be strictly compared when different lignocellulosic hydrolysates and different microorganisms have been used. Lignocellulosic hydrolysates vary in their degree of inhibition, and different microorganisms have different inhibitor tolerances. The fact that even different strains of *S. cerevisiae* vary in inhibitor tolerance was illustrated in a comparison between the performance of *S.*

cerevisiae, baker's yeast, and *S. cerevisiae,* ATCC 96581, isolated from a plant for fermentation of spent sulphite liquor (SSL) in fermentation of SSL (Figure 11). Both glucose consumption and growth were considerably faster for ATCC 96581, the adapted strain. Biological, physical, and chemical methods have been employed for the detoxification of lignocellulosic hydrolysates. In the following, various detoxification methods reported in the literature will be discussed in reference to their effects on the composition of the hydrolysate in order to identify the compounds mainly responsible for the inhibition.

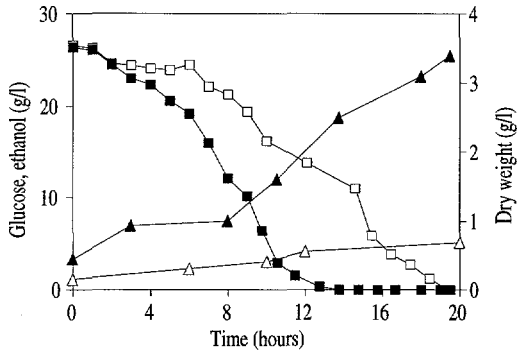

Figure 11. Fermentation of spent sulphite liquor with *S. cerevisiae*, baker's yeast (open symbols) and *S. cerevisiae*, ATCC 96581, isolated from a spent sulphite liquor fermentation plant (closed symbols): glucose (□),cell dry weight (△). Adapted from Paper V.

4.1 Biological methods

Treatment with the enzymes peroxidase and laccase, obtained from the lignolytic fungus *Trametes versicolor*, increased the maximum ethanol productivity in a hemicellulose hydrolysate of willow two to three times (Paper III). Laccase treatment lead to selective and virtually complete removal of phenolic monomers (2.6 g l^{-1} in the crude hydrolysate) and phenolic acids. The absorbance at 280 nm, indicative of the presence of aromatic compounds, did not decrease during the laccase treatment, whereas an increase in absorbance for the large-sized material and a decrease for the small-sized material were observed for all wavelengths tested. Based on these observations, the detoxifying mechanism was suggested to be oxidative polymerisation of low molecular weight phenolic compounds.

The filamentous soft-rot fungus *Trichoderma reesei* degraded inhibitors in a hemicellulose hydrolysate obtained after steam pretreatment of willow, resulting in around three times increased maximum ethanol productivity and four times increased ethanol yield (Paper II). Treatment with *T. reesei* resulted in a 30% decrease in absorbance at 280 nm, in contrast to the treatment with laccase, indicating that the mechanisms of detoxification were different. In accordance with this, to the best of our knowledge, neither laccase nor peroxidase have been characterised from *T. reesei.* Acetic acid, furfural and benzoic acid derivatives were removed from the hydrolysate by the treatment with *T. reesei.*

4.2 Physical methods

The most volatile fraction (10% (v/v)) of a willow hemicellulose hydrolysate obtained by roto-evaporation contained 4 g l^{-1} acetic acid (Paper I). The concentration of furfural was not determined in the volatile fraction, however, it was most likely higher than the concentration in the original hydrolysate. The concentration of furfural in the most volatile 10% of a spruce hydrolysate was 2.5 times higher than in the original hydrolysate (Nilvebrant, personal communication). When the volatile fraction of the willow hemicellulose hydrolysate was fermented the same yield as in a reference fermentation was obtained, but the productivity was decreased to 80%. Roto-evaporation down to 10% (v/v) of the original volume, and subsequent dilution ten times in fermentation medium to restore the concentration of non-volatiles into that of the original hydrolysate caused the ethanol productivity to decrease slightly compared with a reference fermentation containing glucose and nutrients, whereas the yield was not markedly affected (Paper I). However, when the non-volatile fraction was diluted five times to obtain twice the concentration of non-volatile compounds in the original hydrolysate, the ethanol yield and productivity decreased to 46% to 36%, respectively, of the values in the reference fermentation, and at two times dilution only little ethanol was produced. Thus, the non-volatile components in the willow hydrolysate were considerably more toxic than the volatile components. The concentrate contained 12 g l^{-1} acetic acid together with non-volatile phenolic compounds. The ethanol yield obtained in fermentation of an acid hydrolysate of aspen with *P. stipitis* increased from 0 to 13% of that in a reference fermentation containing no inhibitors after roto-evaporation almost to dryness and subsequent resuspension of the residue in

fermentation medium (Wilson *et al.*, 1989). The detoxification was ascribed to decreases in the concentrations of acetic acid, furfural and vanillin which were decreased by 54% (down to 2.8 g l^{-1}), 100%, and 29%, respectively, compared with the concentrations in the hydrolysate.

After continuous overnight extraction of a spruce hydrolysate (Table 2, 222°C) with diethyl ether at pH 2, the ethanol yield was similar to the value in a reference fermentation (0.40 g g^{-1}) (Nilvebrant *et al.*, In preparation). The ethanol yield obtained in the crude hydrolysate was 0.1 g g^{-1} after NaOH adjustment (Larsson, personal communication). The ether extract contained acetic, formic, and levulinic acid, furfural and HMF (nos 3, 8, 9, 6, and 7 in Figure 2a) and phenolic compounds (nos 10-23 in Figure 2b). Resuspension of the extracted components in fermentation medium caused the ethanol yield and productivity to decrease to 33% and 16% of the values obtained in a reference fermentation containing glucose and nutrients (Nilvebrant *et al.*, In preparation). Ethyl acetate extraction increased the ethanol yield in fermentation by *P. stipitis* from 0 to 93% of that obtained in a reference fermentation (Wilson *et al.*, 1989). The reported effects of the treatment were acetic acid removal (56%) and complete depletion of furfural, vanillin, and 4-hydroxybenzoic acid. Ethyl acetate extraction also increased the glucose consumption rate in a hydrolysate of pine by a factor of 12 (Clark and Mackie, 1984). The low molecular weight phenolic compounds were identified as the most inhibiting compounds in the ethyl acetate extract.

After extraction of the inhibitory ether fraction of a dilute acid hydrolysate of spruce with water (three times), the water phase was again found inhibitory in fermentation assays (Figure 12), showing that the major inhibitors were relatively hydrophilic compounds (Nilvebrant *et al.*, In preparation). When 0.5 mol l^{-1} $NaHCO_3$ was used for extraction, in order to further fractionate the inhibitor containing ether extract, neither the ether phase nor the water phase caused inhibition of fermentation. This indicated that the inhibitors were not stable in an alkaline environment.

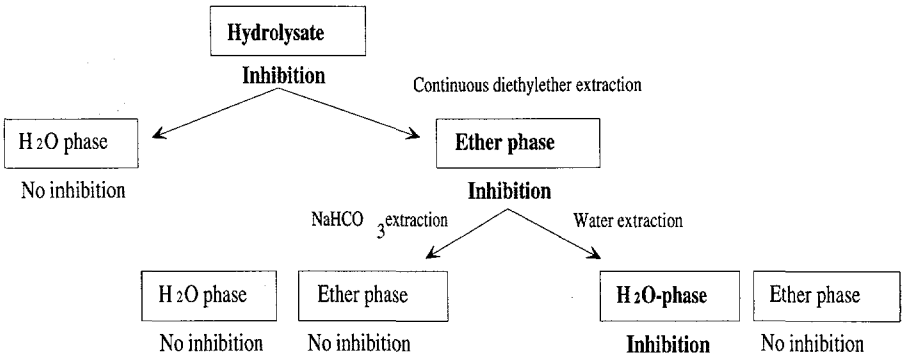

Figure 12. Extraction of a dilute acid hydrolysate of spruce. Adapted from Nilvebrant *et al.* (In preparation).

4.3 Chemical methods

Detoxification of lignocellulosic hydrolysates by alkali treatment, i.e. increasing the pH to 9-10 with $Ca(OH)_2$ (overliming) and readjustment to 5.5 with H_2SO_4, was described already in 1945 (Leonard and Hajny, 1945). $Ca(OH)_2$ adjustment of pH has been reported to result in better fermentability than NaOH adjustment due to the precipitation of "toxic compounds" (van Zyl *et al.*, 1988). In agreement with this, fermentation of a suspension of the components extracted with ether from a dilute-acid hydrolysate of spruce treated under harsh conditions was better after $Ca(OH)_2$ than after NaOH adjustment to fermentation pH 5.5. The productivity increased from zero after NaOH adjustment to pH 5.5 to 0.16 g $(g\ h)^{-1}$ after adjustment to pH 5.5 with $Ca(OH)_2$. After an overliming treatment (pH 10) the productivity was further increased to 0.50 g $(g\ h)^{-1}$.

Since the results from the ether extraction (Section 4.2) indicated that adjustment to high pH caused detoxification, the effect of NaOH adjustment was evaluated in fermentation assays. A dilute-acid hydrolysate of spruce was pre-adjusted to pH 10 with NaOH and readjusted to pH 5.5 with H_2SO_4. The yield was twice as high after adjustment to pH 10 as after adjustment to pH 5.5, and comparable to the yield in a reference fermentation containing glucose and nutrients. Thus, the improvements of fermentation caused by overliming is due both to precipitation of toxic components, and to the pH adjustment to around 10. Treatment to pH 10 with NaOH and $Ca(OH)_2$ decreased the concentration of Hibbert's ketones (Figure 2b, nos. 10-14) in the

hydrolysate from 203 mg l^{-1} to 158 mg l^{-1} (22% decrease) and to 143 mg l^{-1} (30% decrease), respectively, and the concentrations of furfural and HMF were reduced by 20% using both bases (Nilvebrant *et al.*, In preparation). In contrast to what has been reported previously (Amartey and Jeffries, 1996; van Zyl *et al.*, 1988), the concentration of acetic acid was not affected by either treatment.

Already in the 1940's, an "unfavourable reduction potential" in lignocellulosic hydrolysates was overcome by using a large yeast inoculum or a reducing agent such as sulphite (Leonard and Hajny, 1945). A combination of overliming and sulphite was the most efficient method of detoxifying willow hemicellulose hydrolysate prior to fermentation by *E. coli* (Olsson *et al.*, 1995). Only 24% of the xylose was fermented in 40 hours in the untreated hydrolysate, whereas complete depletion of the monosaccharides was obtained in the same time after an overliming treatment. When the hydrolysate was supplemented with sodium sulphite (0.1%) and heated (90°C, 30 minutes), in addition to the overliming treatment, the fermentation time was reduced by a factor three. Similarly, adjustment to pH 10 with KOH, readjustment to pH 6.5 with HCl and addition of 1% sodium sulphite at room temperature was found to be the most efficient method of improving fermentation of a hemicellulose hydrolysate of sugar cane bagasse with *P. stipitis* (van Zyl *et al.*, 1988). The effect of the combined treatment was probably due to decreased concentrations of Hibbert's ketones and aldehydes, and the removal of volatile compounds when a heat treatment was employed. Recent data show that treatment of a dilute-acid hydrolysate of spruce with sodium sulfite caused a significant decrease in the concentration of furfural and HMF (Nilvebrant, personal communication).

Several other chemical detoxification methods and their mechanisms have been discussed in the literature. Treatment of bagasse hydrolysate with anion resins removed 84% of the acetic acid and improved the yield and productivity in fermentation with *P. stipitis* by 37% and 270%, respectively, to values similar to those in a reference fermentation containing only xylose and nutrients (van Zyl *et al.*, 1991). Treatment of a red oak hydrolysate with mixed-bed resins decreased the concentration of acetic acid by 20%, furfural by 20% and the concentration of soluble phenolic compounds (Tran and Chambers, 1986). The untreated hydrolysate was not fermentable by *P. stipitis*, whereas 83% of the yield obtained in a reference

fermentation containing only xylose and nutrients was obtained after the treatment. Mixed-bed resin treatment of a hemicellulose hydrolysate of birch decreased the content of UV-absorbing monomers and dimers (total concentration in crude hydrolysate 1.4 g l^{-1}) by 94% thereby improving the xylose fermentation by *Gluconobacter oxydans* (Buchert *et al.*, 1990). The major UV-absorbing monomers were HMF, syringaldehyde and vanillin.

4.4 Comparison between different detoxification methods

As discussed in Chapter 2, three major groups of compounds are formed during the hydrolysis of lignocellulosic materials: weak acids, furans, and phenolic compounds. From Section 3.2 it can be concluded that compounds from all three groups contribute to the inhibition of fermentation, and that antagonistic effects between compounds exist. The inhibitors increase the general stress level in ethanolic fermentation caused by decreased water activity and ethanol. Microorganisms can survive stress up to a certain limit, but cell death results if the capacity of the cell to respond to the stress is exceeded. In the previous sections several detoxification methods were discussed where the effect was due to a decreased overall concentration of weak acids, furans and phenolic compounds "general stress level" (Figure 13).

The fact that inhibition was considerably decreased by specifically removing the phenolic compounds with the enzyme laccase, implies that the phenolic compounds are major inhibitors in lignocellulosic hydrolysates. Further supporting this is the observation that a dilute-acid hydrolysate of spruce was much more inhibitory than a model fermentation containing the corresponding concentrations of weak acids, furfural and HMF, but no phenolic compounds (Larsson *et al.*, Submitted). Instead of decreasing the overall inhibitor concentration, or "stress level" by non-specific detoxification treatment, it thus appears possible to obtain a similar alleviation of inhibition by specifically removing the low molecular weight phenolic compounds by, e.g., laccase treatment.

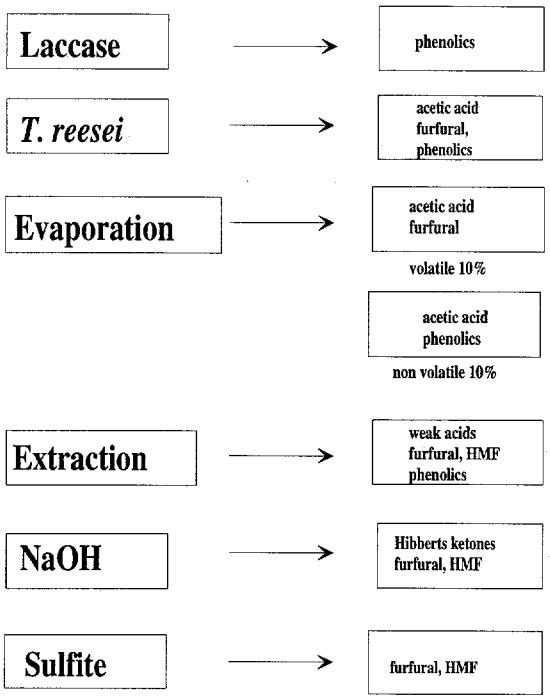

Figure 13. Summary of detoxification methods investigated in this work and compounds removed by the various forms of treatment. For details see the text.

5. PROCESS DEVELOPMENT

5.1 Fermentation strategies

Fermentation is performed in batch or in continuous mode. The ethanol productivity is determined by product inhibition and cell-mass concentration (Kolot, 1980), and in lignocellulosic hydrolysates also by lignocellulose-derived inhibitors. When configuring the fermentation process, several parameters must be considered. The ethanol yield and productivity should be high, and the equipment cost should be low. The need for detoxification must be evaluated for each fermentation process. In this chapter different fermentation strategies for lignocellulose hydrolysates are compared (Table 5), and the integration of a detoxification step into a process is considered. The fermentation of pentoses in lignocellulosic hydrolysates is also discussed.

Table 5. Fermentation strategies. See text for details.

Mode	Advantages	Disadvantages
Batch	Low risk of contamination	Labour intensive Low productivity High investment cost
Batch, high cell density	Increased productivity Decreased inhibition	Labour intensive High investment cost
Batch, recirculation of streams	Reduced water usage Reduced energy demand	Inhibitor accumulation
Fed-batch	Decreased inhibition	As for batch fermentation
Continuous	Reduced equipment cost Decreased inhibition Adaptation of yeast	Contamination Low productivity in hydrolysates
Continuous, cell recirculation	High productivity Decreased inhibition	

5.1.1 Batch and fed-batch fermentation

The volumetric ethanol productivity is low in lignocellulosic hydrolysates when low cell-mass inocula are used due to poor cell growth, as discussed previously. In the batch fementation of enzymatic hydrolysate of spruce (initial cell-mass concentration 0.8 g l^{-1}) no growth occurred during 17 hours at pH 4.6. An increase in pH from 4.6 to 5.0 initiated cell replication (Figure 14). The strong pH dependency of growth in lignocellulosic hydrolysates is due to the large pH dependency of the concentration of undissociated weak acids below pH 5.5 as discussed in Section 3.2.1. After pH adjustment to 5.0, the volumetric ethanol productivity was 0.17 g $(l\ h)^{-1}$.

The productivity is increased and the sensitivity to low pH decreased when the initial cell-mass concentration is increased. Using an initial cell-mass density of 6.0 g l^{-1} (d.w.), a productivity of 3.4 g $(l\ h)^{-1}$ and a final yield of 0.41 g g^{-1} were obtained during fermentation of enzymatic hydrolysate of willow at pH 5.5 (Palmqvist *et al.*, 1996). A productivity of 3.0 g $(l\ h)^{-1}$ and a final yield of 0.44 g g^{-1} were obtained when an enzymatic hydrolysate of spruce was fermented without pH adjustment (pH 4.8) using an initial cell-mass concentration of 10 g l^{-1} (d.w.) (Stenberg *et al.*, 1998). By using an even higher initial cell-mass density (23.6 g l^{-1}), an initial ethanol productivity of 16 g $(l\ h)^{-1}$, was obtained in fermentation of bagasse hydrolysate (Ghose and Tyagi, 1979b). However, in rapid batch fermentation, high rates of inactivation of the yeast cells have been observed (Nagodawithana and Steinkraus, 1976). In addition, the apparent specific growth rate, and thus the viability, decreased with increasing inoculum size, and at a certain cell density no net increase in cell-mass concentration was detected (Ghose and Tyagi, 1979b, Navarro, 1994).

The seed culture for batch fermentation is either grown in a separate fermentor for each fermentation, or more economically, the cells are recirculated after completed fermentation. If the cells are recirculated, it is important that cell viability remains high. If the fermentor is aerated during the initial phase of fermentation, the viability of the recirculated cells is increased. Cell recirculation is beneficial for the process since it leads to adaptation to inhibiting compounds in the hydrolysate (Amartey and Jeffries, 1996; Buchert *et al.*, 1988; Nishikawa *et al.*, 1988; Tran and Chambers, 1986; Yu *et al.*, 1986).

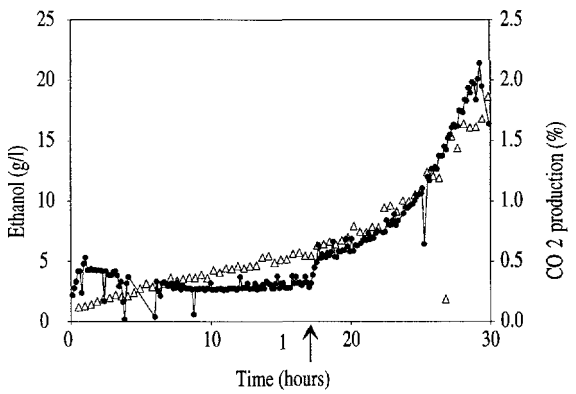

Figure 14. On-line measurement of CO_2 and ethanol using microdialysis sampling and automatic HPLC analysis in the fermentation of spruce hydrolysate. The arrow indicates the time of pH adjustment from 4.6 to 5.0 (17 hours): (△) ethanol, (•) CO_2. Adapted from Paper V.

Monitoring of the fermentation is important to detect process disturbances. Off-line sampling and HPLC analysis of substrate and product concentrations is labour intensive and time consuming. By applying on-line sampling using a microdialysis probe and automatic HPLC analysis, the frequency of sampling can be greatly increased (Figure 14). It is possible to increase the analysis frequency even further by using a biosensor for detection, or by mounting a sensor directly in the fermentor (Kriz *et al.*, 1998). Through the rapid detection of process disturbances it is possible to minimise losses in yield and productivity due to decreased cell viability or cell death. By supplementation of the hydrolysate with lipids, proteins, vitamins, peptone or casein extract (D'Amore and Stewart, 1987; Banerjee *et al.*, 1981b; Stanley and Pamment, 1992) and low aeration (Cysewski and Wilke, 1978; Ghose and Tyagi, 1979b; Lee *et al.*, 1996) growth and ethanolic fermentation are stimulated. As discussed above, a well controlled pH is crucial for cell growth.

The need for detoxification decreased when a high initial cell density was used (Chung and Lee, 1984; Nishikawa *et al.*, 1988; Tran and Chambers, 1986), probably partly due to an increased depletion rate of bioconversible inhibitors, and partly to that growth is more affected by inhibitors like weak acids and furfural than volumetric ethanol productivity. The "inhibitors" may even lead to increased ethanol yield and productivity due to uncoupling in the presence of weak acids (Paper IV; Taherzadeh *et*

ethanol productivity. The "inhibitors" may even lead to increased ethanol yield and productivity due to uncoupling in the presence of weak acids (Paper IV; Taherzadeh *et al.*, 1998b), or due to decreased glycerol production in the presence of furfural (Papers IV and VI). The need for detoxification can also be decreased by choosing an adapted yeast strain (Figure 11)(Paper V; Lindén *et al.*, 1992) or by strain adaptation by recirculation in lignocellulosic hydrolysate, as discussed above.

By adding the substrate at a low rate in fed-batch fermentation the concentrations of bioconvertible inhibitors such as furfural and HMF in the fermentor remains low, and the inhibiting effect is therefore decreased. Complete fermentation of an acid hydrolysate of spruce which was strongly inhibiting in batch fermentation has been achieved in fed-batch fermentation without any detoxification treatment (Nutek Report, August 1997). The productivity in fed-batch fermentation is limited by the feed rate which, in turn, is limited by the cell-mass concentration.

5.1.2 Continuous fermentation

Process design studies showed that the investment cost was considerably reduced when continuous rather than batch fermentation of molasses was employed, and that the ethanolic productivity increased by more than 200% (Cysewski and Wilke, 1978). In continuous fermentation, as in fed-batch fermentation, the substrate is added at a low rate, thereby ensuring a low concentration of bioconvertible inhibitors in the fermentor. In contrast to the situation in fed-batch fermentation, however, cell growth is necessary at a rate equal to the dilution rate in order to avoid wash-out of the cells. As the growth rate is decreased by the inhibitors, the productivity in continuous fermentation of lignocellulosic hydrolysates is low (Paper V; Lee *et al.*, 1996). The productivity can not be increased by maintaining a high cell-mass concentration in the fermentor, as the steady-state concentration of cells is determined by the concentration of fermentable sugars in the feed.

By employing a cell retention system, the cell-mass concentration in the fermentor, the maximum dilution rate, and thus the maximum ethanol productivity are increased. Different cell-retention systems have been used, e.g. immobilization (Inoles *et al.*,

1983; Nagashima *et al.*, 1984; Williams and Munnecke, 1981), cell-recycling by filtration (Paper V; Lee and Chang, 1987; Lee *et al.*, 1996; Melzoch *et al.*, 1991; Damiano and Wang, 1985), settling (Ghose and Tyagi, 1979b; Kuriyama *et al.*, 1985), or centrifugation (Cysewski and Wilke, 1978). The investment cost for a continuous process with cell recirculation was found to be less than for continuous fermentation without cell recirculation (Cysewski and Wilke, 1978). The ethanol productivity was increased by three to four times when cell recirculation was employed in the fermentation of enzymatic hydrolysate of spruce (Paper V), in fermentation of molasses (Cysewski and Wilke, 1978), and in fermentation of bagasse hydrolysate (Ghose and Tyagi, 1979b).

A precipitate appeared after one week of continuous fermentation with cell recirculation of spruce hydrolysate, which caused problems by clogging the filter unit. This might have been due to precipitation of calcium oxalate (the hydrolysate had been adjusted with $Ca(OH)_2$ prior to enzymatic hydrolysis). The concentration of oxalic acid in a dilute-acid hydrolysate of spruce was 0.04 g l^{-1} (Nilvebrant, personal communication) and this amount would have caused precipitation of about 1.2 g l^{-1} calcium oxalate in the fermentor during the entire fermentation period (12 days). In large-scale operation cell recirculation by centrifugation is used (Paper V), thereby avoiding the problem of clogging of the filter unit. However, after long-term operation calcium oxalate will probably accumulate in the fermentor and cause problems in the cell recirculation step. NaOH should therefore preferentially be used for pH adjustment.

The theoretical ethanol yield from fermentable sugars was achieved in continuous fermentation with cell recirculation of a spruce hydrolysate at a cell-mass concentration of about 25 g l^{-1}, indicating that the cells were not growing at this cell-mass concentration (Paper V). In accordance with this, cell growth has been reported to decrease at high cell-mass density in continuous fermentation with cell recirculation (Melzoch *et al.*, 1991). The specific ethanol productivity also decreased with increasing cell-mass concentration (Paper V; Lee and Chang, 1987). The cell-mass density should be kept at a level providing maximum ethanol productivity and yield

and only slow cell growth, to compensate for cell death. A low bleed stream avoids accumulation of biomass in the fermentor.

5.1.3 Closing of process streams

In an environmentally sustainable process the use of fresh water, the amount of waste water and the energy consumption must be minimised. The water consumption is decreased by recirculating process streams for use in the washing and hydrolysis steps (Galbe and Zacchi, 1991). Computer simulations have shown that recirculation of streams might lead to the accumulation of large amounts of non-volatile inhibitory compounds in the process (Paper I; Galbe and Zacchi, 1991). The energy requirement in the distillation stage can be reduced by increasing the ethanol concentration in the feed. The energy demand was high when the ethanol concentration in the feed was below 1% (Busche, 1983), and decreased drastically with increasing ethanol concentration to about 3%. Above 4% ethanol in the feed, the energy requirement was rather constant for increasing ethanol concentrations.

A bench-scale unit was designed for the development and testing of a process for ethanol production from lignocellulosics based on enzymatic hydrolysis (Figure 15) (Palmqvist *et al.*, 1996). In the pretreatment unit (**1**), the lignocellulosic material, impregnated with SO_2 or H_2SO_4, was treated with steam (T_{max} = 235°C, 30 bar). The solubilised hemicellulose was separated from the fibrous material in a filter press (**2**). The filamentous fungus *T. reesei* produced cellulolytic enzymes (**3**) for the hydrolysis of the fibrous material (40°C, pH 4.8) (**4**). Ethanolic fermentation with *S. cerevisiae* was carried out in batch and continuous mode with and without cell recirculation (**5**). The ethanol was purified by distillation (**6**).

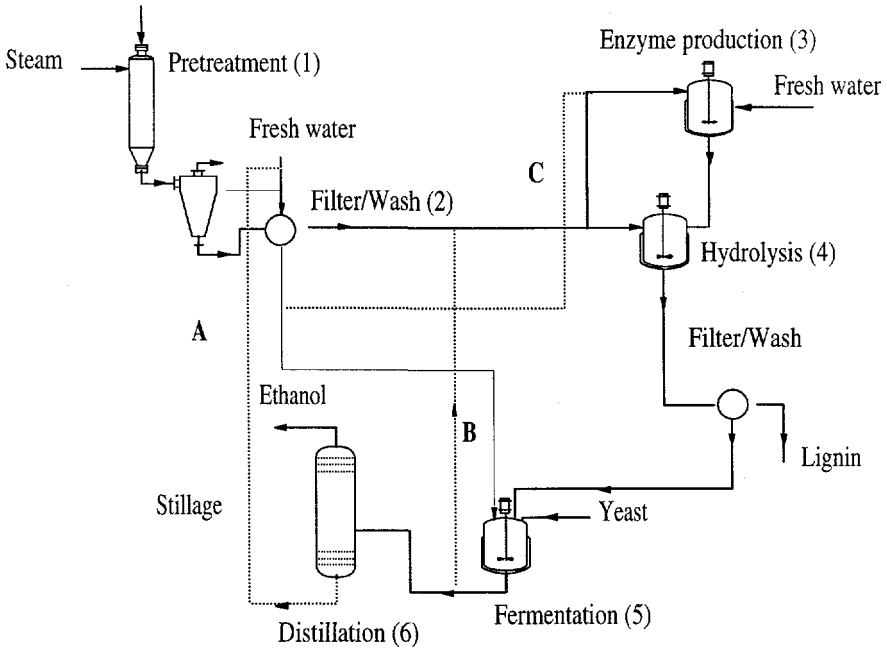

Figure 15. Bench-scale process development unit for ethanol production from lignocellulosic materials. Dotted lines represent various recirculation alternatives (see text). Adapted from Palmqvist *et al.* (1996).

Experimental simulations of various recycling configurations were performed in the bench-scale unit to study the effect of the accumulation of non-volatile compounds and ethanol in the hydrolysis and fermentation steps (Stenberg *et al.*, 1998). During ethanol production from softwood, the fresh water demand, and thus also the amount of waste water produced was reduced by 50% by recirculation of the stillage stream or part of the liquid stream from the fermentor (Figure 15, recirculation alternative **A** or **B**) without any negative effects due to inhibitor accumulation either on enzymatic hydrolysis or fermentation. However, when the amount of freshwater was reduced to 25% of the total liquid added to the hydrolysis stage the productivity and yield in the fermentation decreased. Recirculation of the dilute ethanol stream after fermentation (Figure 15, recirculation alternative **B**), containing 23 g l^{-1} EtOH, decreased the energy demand in the distillation stage by 42%, without influencing hydrolysis or fermentation.

5.2 Integration of a detoxification step

Detoxification may be necessary when strongly inhibiting hydrolysates are fermented, if high concentrations of inhibitors accumulate in the fermentation unit due to the recirculation of streams, or when a fermenting organism other than *S. cerevisiae* is used. The detoxification method used should selectively remove inhibitors, and be cheap and easy to integrate into the process.

When the inhibitor containing hemicellulose hydrolysate from the pretreatment stage (Figure 15, alternative **C**) was used as substrate for *T. reesei*, simultaneous detoxification and enzyme production was obtained (Paper II). In the case of a process using enzymatic hydrolysis (Figure 15), the enzyme-containing, inhibitor-free liquid can be used to hydrolyse the cellulose. This detoxification method would not require the introduction of an additional process step, and complete utilisation of all wood-derived sugars would improve the process economy. Enzymatic detoxification using the lignolytic enzymes laccase or peroxidase (Paper III) could also be performed directly in the fermentation vessel prior to fermentation. Immobilization would facilitate enzyme recovery and reduce the cost of this method.

Overliming in combination with sulfite addition is so far the only detoxification method for which an economic analysis has been made. It has been reported that the cost of ethanol produced from willow hydrolysates using recombinant *E. coli* increased by 22% (von Sivers *et al.*, 1994). The treatment consisted of increasing the pH from 3.1 to 10.5 with 53 mmol l^{-1} $Ca(OH)_2$ (106 mmol l^{-1} OH^-), adding 1 g l^{-1} sodium sulfite, and incubating the hydrolysate at 90°C for 30 minutes (Olsson *et al.*, 1995). This method resulted in four times higher fermentation rate than detoxification by overliming only, whereas only little ethanol was produced in the untreated hydrolysate. Detoxification by overliming would however be a cheap and simple method. To adjust a spruce hydrolysate from pH 1.7 after dilute-acid hydrolysis to fermentation pH 5.5, 146.3 mmol l^{-1} OH^- were needed, and the additional amount required to increase the pH to 10 was only 32.5 mmol l^{-1}, due to the low buffering capacity of the hydrolysate above pH 5.5 (Figure 4, 222°C). The additional cost for detoxification treatment by using a base would thus only be around 22% of the cost of neutralisation to fermentation pH. Adjustment with $Ca(OH)_2$ generates a precipitate of

$CaSO_4$ which must be removed by centrifugation prior to fermentation, and adjustment with this base would therefore require an additional process step. In addition, precipitation of calcium oxalate is likely to cause problems during long-term continuous fermentation, as discussed in Section 5.3.3. Similar improvements in fermentability were observed after adjustment of a spruce hydrolysate to 10 with NaOH or $Ca(OH)_2$ (Larsson, personal communication), and thus NaOH should preferably be used in this case.

Through the introduction of an evaporator after the distillation unit (Figure 15 (**6**)) the stillage stream could be evaporated and the inhibitor-free volatile fraction recirculated (Palmqvist *et al.*, 1996). The non-volatile, inhibitor-containing residue could be used as a solid fuel to provide energy for the process. This detoxification method would require an additional process step and increase the energy requirement of the process.

5.3 Pentose fermentation

In the present work, hexose fermentation with *S. cerevisiae* has been studied to acquire knowledge concerning inhibitors, to identify optimal hydrolysis conditions, and to compare different fermentation modes. Less attention has been paid to the fermentation of pentoses from the hemicellulose. Efficient xylose-fermenting organisms have been found among bacteria, yeasts, and fungi (for a recent review, see (Olsson and Hahn-Hägerdal, 1995)). The fermentation of glucose-xylose mixtures with recombinant *S. cerevisiae* harbouring genes encoding for xylose reductase (XR), xylitol dehydrogenase (XDH), and transaldolase (TAL) been studied in defined medium (Meinander *et al.*, 1995).

To evaluate the inhibitor tolerance of recombinant, xylose-utilising *S. cerevisiae,* the fermentation performance in spent sulphite liquor (SSL) was evaluated. SSL was supplemented with 3 g l^{-1} $(NH_4)_2HPO_4$ and fermented under anaerobic conditions in batch mode without any detoxification treatment (Figure 16). The effects of cell-mass concentration and cell recirculation on the xylose consumption were studied.

Xylose consumption increased with increasing cell-mass concentration (1.2 to 2.3 g l^{-1}). Xylose was mainly converted to xylitol, indicating that gene expression must be optimised for ethanol production. The XR activity, and therefore the xylose consumption rate, decreased with the number of cell recirculation steps. The loss of XR activity has also been observed in continuous culture (Meinander *et al.*, 1995). The instability problems would be alleviated by integration of the genes into the chromosome. A genetically engineered strain of *S. cerevisiae*, converting pentose and hexose sugars from corn fibre to ethanol, has recently been presented (Moniruzzaman *et al.*, 1997). However, the instability of the plasmid harbouring the enzymes necessary for xylose fermentation thus far limits the use of this organism. Recombinant *E. coli* has also been used to ferment pentose and hexoses (Olsson *et al.*, 1995). However, *E. coli* was less resistant to inhibitors in lignocellulosic hydrolysates that *S. cerevisiae.*

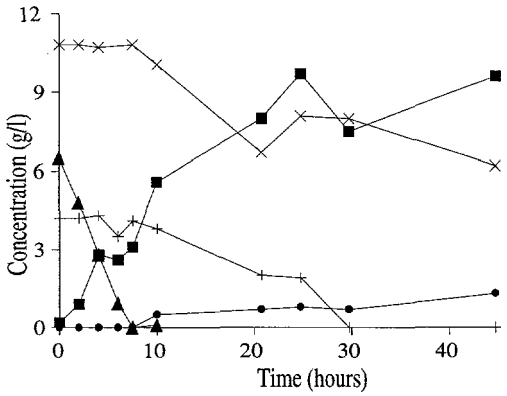

Figure 16. Fermentation of spent sulphite liquor with recombinant *S. cerevisiae*: glucose (▲), mannose (+), xylose (×), ethanol (■) and xylitol (●).

Complete utilisation of sugars (i.e. pentoses and hexoses) can also be achieved by using separate fermentation of pentoses and hexoses using two organisms, or by employing co-fermentation using a hexose fermenting organism (e.g. *S. cerevisiae*) and a pentose-fermenting organism (e.g. *P. stipitis*) (Delgenes *et al.*, 1996a). The fermentation conditions cannot be optimised for both organisms in the latter case. Some aeration is necessary for the pentose fermentation of *P. stipitis* and the maximal ethanol concentration obtained in the fermentation is limited by the low ethanol tolerance of this yeast.

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