ELSEVIER

Bioresource Technology 74 (2000) 17–24

BIORESOURCE TECHNOLOGY

Review paper

Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification

Eva Palmqvist [1], Bärbel Hahn-Hägerdal *

Department of Applied Microbiology, Lund University, PO Box 124, 22100 Lund, Sweden

Received 2 February 1999; received in revised form 17 August 1999; accepted 14 November 1999

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 formed or released during hydrolysis. This review describes the effect of various detoxification methods on the fermentability and chemical composition of the hydrolysates. Inhibition of fermentation can be relieved upon treatment with the ligninolytic enzyme laccase, pre-fermentation by the filamentous fungus *Trichoderma reesei*, removal of non-volatile compounds, extraction with ether or ethyl acetate, and treatment with alkali or sulfite. Various fermentation strategies can also be used to improve yield and productivity in lignocellulosic hydrolysates. Batch, fed-batch, and continuous fermentation are discussed in relation to inhibition of fermentation in lignocellulosic hydrolysates.

Keywords: *Saccharomyces cerevisiae*; Ethanol; Lignocellulose; Detoxification; Inhibition

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, such as *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, rapid and efficient fermentation of the hydrolysates is limited because in addition to monomeric sugars a range of toxic compounds is generated during steam pretreatment and hydrolysis of lignocellulosics (for a review, see Palmqvist and Hahn-Hägerdal, 1999). The inhibiting compounds are divided in three main groups based on origin: weak acids, furan derivatives, and phenolic compounds. In the present article detoxification methods and strategies to enhance the efficiency of the fermentation process are reviewed.

Except where explicitly stated, *S. cerevisiae* has been used for fermentation of lignocellulose-derived sugars and in model fermentations in all work reviewed herein. *S. cerevisiae* has been shown to be the best yeast for the fermentation of hexose sugars present in lignocellulose-derived hydrolysates due to its ethanol-producing capacity and high inhibitor tolerance (Hahn-Hägerdal et al., 1991; Olsson and Hahn-Hägerdal, 1993).

2. Detoxification

Biological, physical, and chemical methods have been employed for detoxification (i.e., the specific removal of inhibitors prior to fermentation) of lignocellulosic hydrolysates (for a recent review, see Olsson and Hahn-Hägerdal, 1996). In the following, detoxification methods reported in the literature and their effects on the composition and fermentability of the hydrolysates used will be discussed in relation to the identity of compounds mainly responsible for inhibition. Different detoxification methods cannot be strictly compared when different lignocellulosic hydrolysates and different microorganisms have been used. Lignocellulosic

* Corresponding author.

[1] Present address: Danisco Biotechnology, PO Box 17, DK-1001 Copenhagen K, Denmark.

0960-8524/00/$ - see front matter
PII: S0960-8524(99)00160-1

hydrolysates vary in their degree of inhibition, and different microorganisms have different inhibitor tolerances. The fact that even different strains of *S. cerevisiae* may vary in inhibitor tolerance has been illustrated in a comparison between the performance of *S. cerevisiae*, bakers' yeast, and *S. cerevisiae*, ATCC 96581, isolated from a plant for fermentation of spent sulphite liquor (SSL), in fermentation of SSL (Lindén et al., 1992; Palmqvist et al., 1998). Both glucose consumption and growth were considerably faster for ATCC 96581. Several reports on adaptation to inhibiting compounds in lignocellulosic hydrolysates are found in the literature (e.g., Amartey and Jeffries, 1996; Buchert et al., 1988; Nishikawa et al., 1988; Tran and Chambers, 1986; Yu et al., 1986).

2.1. Biological detoxification methods

Treatment with the enzymes peroxidase and laccase, obtained from the ligninolytic fungus *Trametes versicolor*, has been shown to increase the maximum ethanol productivity in a hemicellulose hydrolysate of willow two to three times (Jönsson et al., 1998). The laccase treatment led 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* has been reported to degrade 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 (Palmqvist et al., 1997). In contrast to the treatment with laccase, treatment with *T. reesei* resulted in a 30% decrease in absorbance at 280 nm, indicating that the mechanisms of detoxification were different. Acetic acid, furfural and benzoic acid derivatives were removed from the hydrolysate by the treatment with *T. reesei*.

2.2. Physical detoxification methods

The most volatile fraction (10% (v/v)) of a willow hemicellulose hydrolysate obtained by roto-evaporation has been shown to only slightly decrease the ethanol productivity compared to a reference fermentation containing glucose and nutrients (Palmqvist et al., 1996b). The non-volatile fraction was found to be considerably more inhibitory, in the referred study. When the non-volatile fraction, obtained by roto-evaporation down to 10% (v/v) of the original volume, 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% and 36%, respectively, of the values in the reference fermentation. At two times dilution only little ethanol was produced (Palmqvist et al., 1996b). The concentrate contained 12 g l^{-1} acetic acid and non-volatile phenolic compounds. In fermentation of an acid hydrolysate of aspen with *P. stipitis* the ethanol yield has been reported to increase 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 a decrease in the concentration of acetic acid, furfural and vanillin 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 strongly inhibiting spruce hydrolysate with diethyl ether at pH 2, the ethanol yield has been reported to be comparable to the value in a reference fermentation containing glucose and nutrients (0.40 g g^{-1}) (Nilvebrant et al., in preparation). The ether extract contained acetic, formic, and levulinic acid, furfural, hydroxymethyl furfural (HMF) and phenolic compounds. Resuspension of the extracted components in fermentation medium decreased the ethanol yield and productivity to 33% and 16%, respectively, of the values obtained in a reference fermentation. In agreement with this result, ethyl acetate extraction has been reported to increase the ethanol yield in fermentation by *P. stipitis* from 0 to 93% of that obtained in a reference fermentation (Wilson et al., 1989) due to removal of acetic acid (56%) and complete depletion of furfural, vanillin, and 4-hydroxybenzoic acid. Ethyl acetate extraction has also been shown to increase 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 suggested to be 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 has again been found inhibitory in fermentation assays, showing that the major inhibitors were relatively soluble both in the aqueous and in the organic phase (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 alkali stable.

2.3. Chemical detoxification 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, has been described as early as 1945 by Leonard and Hajny. $Ca(OH)_2$ 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, ethanol yield and productivity in a solution of the components which had been extracted with ether from a dilute-acid hydrolysate of spruce has been reported to be considerably higher after adjustment to pH 5.5 with $Ca(OH)_2$ than with NaOH (Palmqvist, 1998). After an overliming treatment (pH 10), causing the formation of a large precipitate, the ethanol productivity was further increased. The detoxifying effect of overliming is due both to the precipitation of toxic components and to the instability of some inhibitors at high pH. This has been demonstrated by the fact that preadjustment to pH 10 with NaOH of a strongly inhibiting dilute-acid hydrolysate of spruce prior to fermentation resulted in twice as high ethanol yield (and comparable to the yield in a reference fermentation containing glucose and nutrients) as after only adjustment to fermentation pH (5.5) (Palmqvist, 1998). Preadjustment to pH 10 with NaOH and $Ca(OH)_2$ has been reported to decrease the concentration of Hibbert's ketones in a dilute acid hydrolysate of spruce from 203 to 158 (22% decrease) and to 143 mg l^{-1} (30% decrease), respectively, and the concentration of both furfural and HMF by 20% (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 in the referred study.

In the 1940s, treatment with a reducing agent such as sulphite, or a large yeast inoculum were suggested as means to overcome an 'unfavourable reduction potential' in lignocellulosic hydrolysates (Leonard and Hajny, 1945). In more recent studies treatment of a dilute-acid hydrolysates of spruce with sodium sulphite (Larsson et al., 1999), or using a large cell inoculum (for a review, see Palmqvist and Hahn-Hägerdal, 1999) have been shown to decrease the concentrations of furfural and HMF. A combination of sulphite and overliming has been shown to be the most efficient method to detoxify willow hemicellulose hydrolysate prior to fermentation by recombinant *Escherichia coli* (Olsson et al., 1995). Only 24% of the xylose was fermented in 40 h in the untreated hydrolysate, whereas complete depletion of monosaccharides was obtained in the same time after overliming. When the hydrolysate was supplemented with sodium sulphite (0.1%) and heated (90°C, 30 min), 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 has been 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.

2.4. Concluding remarks regarding detoxification

Weak acids, furan derivatives, and phenolic compounds inhibit fermentation of lignocellulosic hydrolysates as discussed above. In addition, negative interaction effects between compounds leading to increased inhibition exist (Palmqvist et al., 1999b). Inhibitors also increase the environmental stress for the fermentative organism due to decreased water activity and increasing ethanol concentrations. 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, furan derivatives, phenolic compounds, and thereby the 'general stress level'.

The fact that inhibition has been shown to decrease considerably by specifically removing the phenolic compounds with the enzyme laccase (Jönsson et al., 1998), implies that the phenolic compounds are major inhibitors in lignocellulosic hydrolysates. Supporting this is the observation that a dilute-acid hydrolysate of spruce has been shown to be considerably more inhibitory than a model fermentation containing the corresponding concentrations of weak acids, furfural and HMF, but no phenolic compounds (Larsson et al., 1998). Instead of decreasing the overall inhibitor concentration, or 'stress level' by non-specific detoxification treatment, it thus seems to be possible to obtain an equivalent alleviation of inhibition by specifically removing the low molecular weight phenolic compounds by, for instance, laccase treatment. Different detoxification methods have recently been compared, using the same dilute acid hydrolysate of spruce and fermentation with *S. cerevisiae* (Larsson et al., 1999). Among the investigated methods, only anion exchange at pH 10 and overliming resulted in higher ethanol productivity in the fermentation than laccase treatment. Both laccase treatment and anion exchange led to more than 80% decrease of total phenolics, and the anion treatment also removed virtually all levulinic, acetic, and formic acids, and 70% furfural and 5-HMF. Anion exchange however also led to a considerable loss of fermentable sugars, and

is therefore not a feasible method. The effect of the overliming treatment, also investigated in the referred study, is less easily understood. The weak acids were not affected, and furfural, 5-HMF, and total phenolics only decreased by 20% each. These inhibitor concentrations were almost exactly the same as analysed after preadjustment with NaOH to pH 10, and yet the overliming treatment resulted in almost three times as high ethanol productivity as the NaOH treatment. Thus there seem to be yet unknown inhibitors in lignocellulosic hydrolysates, the identification of which presents a challenge for future work.

2.5. Fermentation strategies

Fermentation is performed in batch, in fed-batch, or in continuous mode. The ethanol productivity is determined by cell-specific productivity and cell mass concentration, 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 in relation to the fermentation configuration.

2.6. 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. Cell growth in lignocellulosic hydrolysates has been found to be strongly dependent on pH (Palmqvist et al., 1998). In batch fementation of enzymatic hydrolysate of spruce (initial cell-mass concentration 0.8 g l^{-1}) no growth occurred during 17 h at pH 4.6. pH adjustment from 4.6 to 5.0 was reported to initiate cell replication, and increased the volumetric ethanol productivity to 0.17 g $(l\ h)^{-1}$, in the referred study. The strong pH dependency of growth in lignocellulosic hydrolysates is due to the large concentration of undissociated weak acids at low pH.

The productivity is increased and the sensitivity of the process to low pH decreased when the initial cell-mass concentration was 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} have been reported during fermentation of enzymatic hydrolysate of willow at pH 5.5 (Palmqvist et al., 1996a). An ethanol productivity of 3.0 g $(l\ h)^{-1}$ and a final yield of 0.44 g g^{-1} have been 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 $(l\ h)^{-1}$ has been reached in fermentation of bagasse hydrolysate (Ghose and Tyagi, 1979). However, in rapid batch fermentation, high rates of yeast cell inactivation have been observed (Nagodawithana and Steinkraus, 1976). In addition, the apparent specific growth rate, and thus the viability, has been reported to decrease with increasing inoculum size, and at a certain cell density no net increase in cell-mass concentration was detected (Ghose and Tyagi, 1979; 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 increases. Cell recirculation is beneficial for the process since it may lead to adaptation as discussed previously.

Monitoring of the fermentation is important in order to detect process disturbances. Off-line sampling and HPLC analysis of substrate and product concentrations is labour intensive and time consuming. By applying online sampling using a microdialysis probe and automatic HPLC analysis, the frequency of sampling can be greatly increased (Palmqvist et al., 1998). 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., 1981; Stanley and Pamment, 1992) and low aeration (Cysewski and Wilke, 1978; Ghose and Tyagi, 1979; 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 has been reported to decrease when a high initial cell density has been used (Chung and Lee, 1984; Nishikawa et al., 1988; Tran and Chambers, 1986), partly due to 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 by the presence of weak acids (Palmqvist et al., 1999b; Taherzadeh et al., 1997), or due to decreased glycerol production in the presence of furfural (Palmqvist et al., 1999a).

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 remain low, and the inhibiting effect therefore decreases. 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 (Taherzadeh et al., 1999). The

productivity in fed-batch fermentation is limited by the feed rate which, in turn, is limited by the cell-mass concentration.

2.7. Continuous fermentation

Process design studies of molasses fermentation have shown that the investment cost was considerably reduced when continuous rather than batch fermentation was employed, and that the ethanolic productivity could be 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. The productivity is a function of the dilution rate, and since the growth rate is decreased by the inhibitors, the productivity in continuous fermentation of lignocellulosic hydrolysates is low (Lee et al., 1996; Palmqvist et al., 1998).

By employing cell retention systems, the cell-mass concentration in the fermentor, the maximum dilution rate, and thus the maximum ethanol productivity increase. 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 (Damiano and Wang, 1985; Lee and Chang, 1987; Lee et al., 1996; Melzoch et al., 1991; Palmqvist et al., 1998), settling (Ghose and Tyagi, 1979; Kuriyama et al., 1985), or centrifugation (Cysewski and Wilke, 1978). The investment cost for a continuous process with cell recirculation has been found to be less than for continuous fermentation without cell recirculation (Cysewski and Wilke, 1978). The ethanol productivity has been reported to increase three to four times when cell recirculation was employed in the fermentation of an enzymatic hydrolysate of spruce (Palmqvist et al., 1998), in fermentation of molasses (Cysewski and Wilke, 1978), and in fermentation of bagasse hydrolysate (Ghose and Tyagi, 1979).

A precipitate, probably calcium oxalate, causing problems in the filter unit has been reported to appear after one week of continuous fermentation of spruce hydrolysate with cell recirculation by filtration (Palmqvist, 1998). In industrial-scale operation cell recirculation by centrifugation would preferably be used and this problem thus circumvented. However, after long-term operation calcium oxalate will probably accumulate in the fermentor and ultimately cause problems in the cell recirculation step. Therefore calcium salts should be avoided for pH adjustment.

Theoretical ethanol yields on fermentable sugars have been 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 density (Palmqvist et al., 1998). Cell growth has previously been reported to decrease at high cell density in continuous fermentation with cell recirculation (Melzoch et al., 1991). The specific ethanol productivity has also been reported to decrease with increasing cell-mass concentration (Lee and Chang, 1987; Palmqvist et al., 1998). Ideally, the cell density should be kept at a level providing maximum ethanol productivity and yield and slow cell growth only to compensate for cell death. A low bleed stream avoids accumulation of biomass in the fermentor.

2.8. Recycling 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. The ethanol concentration in the feed to the distillation stage can be increased by recirculating part of the dilute ethanol stream from the fermentor. A higher ethanol concentration in the feed decreases the energy requirement in the distillation stage. The energy demand has been reported to be high when the ethanol concentration in the feed is below 1% (Busche, 1983), and to decrease drastically with increasing ethanol concentration to about 3%. Above 4% ethanol in the feed, the energy requirement is rather constant for increasing ethanol concentrations, according to the referred study. However, computer simulations have shown that recirculation of streams leads to the accumulation of non-volatile inhibitory compounds in the process (Galbe and Zacchi, 1991; Palmqvist et al., 1996b).

A bench-scale unit has been designed for the development and testing of a process for ethanol production from lignocellulosics based on enzymatic hydrolysis (Palmqvist et al., 1996a). In the pretreatment unit, 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. The filamentous fungus *T. reesei* produced cellulolytic enzymes for the hydrolysis of the fibrous material (40°C, pH 4.8). Ethanolic fermentation with *S. cerevisiae* was carried out in batch and continuous mode with and without cell recirculation. Ethanol was purified by distillation.

Experimental simulations of various recycling configurations have been 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 has been shown to be reduced by 50% by recirculation of the stillage stream or part of the liquid stream from the fermentor without either enzymatic hydrolysis or fermentation being inhibited. However, when the amount of freshwater was further 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, containing 23 g l^{-1} EtOH, was shown to decrease the energy demand in the distillation stage by 42%, without influencing hydrolysis or fermentation.

2.9. 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 recirculation of streams, or when a fermenting organism with low inhibitor tolerance is used. The detoxification method should selectively remove inhibitors, and be cheap and easy to integrate into the process.

Enzymatic detoxification using the lignolytic enzymes laccase or peroxidase (Jönsson et al., 1998) could be performed directly in the fermentation vessel prior to fermentation, and would thus not require an additional process step. Immobilization would facilitate enzyme recovery and reduce cost. Simultaneous detoxification and enzyme production has been reported to occur when the inhibitor-containing hemicellulose hydrolysate from the pretreatment stage was used as substrate for *T. reesei* (Palmqvist et al., 1997). The enzyme-containing, inhibitor-free liquid can then be used to hydrolyse the cellulose fraction. This detoxification method would improve the process economy since all wood-derived sugars are utilised.

Overliming in combination with sulphite 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% when overliming was used (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 sulphite, and incubating the hydrolysate at 90°C for 30 min (Olsson et al., 1995). This method resulted in a four times higher fermentation rate than detoxification by overliming only, whereas only little ethanol was produced in the untreated hydrolysate. Overliming could however be a cheap and simple detoxification method. The amount of OH^- needed to adjust a spruce hydrolysate from pH 1.7 after dilute-acid hydrolysis to fermentation pH 5.5 has been reported to be 146.3 mmol l^{-1}, and the additional amount required to increase the pH to 10 to be only 32.5 mmol l^{-1}, due to the low buffering capacity of the hydrolysate above pH 5.5 (Palmqvist, 1998). The cost for the detoxification treatment using a base would thus only increase the cost of neutralisation to fermentation pH by approximately 20%. 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 above.

Through the introduction of an evaporator after the distillation unit the stillage stream could be evaporated and the inhibitor-free volatile fraction recirculated (Palmqvist et al., 1996a). 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.

In the present review mainly hexose fermentation using *S. cerevisiae* was considered as a model system. In an economically feasible process complete fermentation of not only hexoses, but also of pentose sugars must be obtained. The detoxification method chosen in the final process depends on the requirements of the microorganism used for fermentation in this process.

Acknowledgements

This work was supported by grants from the Swedish National Board for Industrial and Technical Development (NUTEK), Swedish Natural Science Research Council (NFR), the Knut and Alice Wallenberg Foundation, and the Carl Trygger Foundation.

References

Amartey, S., Jeffries, T., 1996. An improvement in *Pichia stipitis* fermentation of acid-hydrolysed hemicellulose achieved by overliming (calcium hydroxide treatment) and strain adaptation. Wo J. Microbiol. Biotechnol. 12, 281–283.

Banerjee, N., Bhatnagar, R., Viswanathan, L., 1981. Inhibition of glycolysis by furfural in *Saccharomyces cerevisiae*. Eur. J. Appl. Microbiol. Biotechnol. 11, 226–228.

Buchert, J., Puls, J., Poutanen, K., 1988. Comparison of *Pseudomonas fragi* and *Gluconobacter oxydans* for production of xylonic acid from hemicellulose hydrolysates. Appl. Microbiol. Biotechnol. 28, 367–372.

Busche, R.M., 1983. Recovering chemical products from dilute fermentation broths. Biotechnol. Bioeng. Symp. 13, 597–615.

Chung, I.S., Lee, Y.Y., 1984. Ethanol fermentation of crude acid hydrolyzate of cellulose using high-level yeast inocula. Biotechnol. Bioeng. 27, 308–315.

Clark, T., Mackie, K.L., 1984. Fermentation inhibitors in wood hydrolysates derived from the softwood *Pinus radiata*. J. Chem. Biotechnol. B 34, 101–110.

Cysewski, G.R., Wilke, C.R., 1978. Process design and economic studies of alternetive fermentation methods for the production of ethanol. Biotechnol. Bioeng. 20, 1421–1444.

Damiano, D., Wang, S.S., 1985. Improvements in ethanol concentration and fermentor ethanol productivity in yeast fermentations using whole soy flour in batch and continuous recycle systems. Biotechnol. Lett. 7 (2), 135–140.

D'Amore, T., Stewart, G.G., 1987. Ethanol tolerance of yeast. Enz. Microb. Technol. 9, 322–330.

Galbe, M., Zacchi, G., 1991. Simulation of ethanol production processes based on enzymatic hydrolysis of lignocellulosic materials using ASPEN PLUS. Appl. Biochem. Biotechnol. 34/34, 93–104.

Ghose, T.K., Tyagi, R.D., 1979. Rapid ethanol fermentation of cellulose hydrolysate I Batch versus continuous systems. Biotechnol. Bioeng. 21, 1387–1400.

Hahn-Hägerdal, B., Lindén, T., Senac, T., Skoog, K., 1991. Ethanolic fermentation of pentoses in lignocellulosic hydrolysates. Appl. Biochem. Biotechnol. 28/29, 131–144.

Inoles, D.S., Taylor, D.P., Stanley, N.C., Michaels, A.S., Robertson, C.R., 1983. Ethanol production by *S. cerevisiae* immobilized in hollow-fiber membrane bioreactors.. Appl. Environ. Microbiol. 46 (1), 264–278.

Jönsson, L.J., Palmqvist, E., Nilvebrant, N.O., Hahn-Hägerdal, B., 1998. Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*. Appl. Microbiol. Biotechnol. 49, 691–697.

Kriz, D., Berggren, K., Palmqvist, E., 1998. SIRE-technology part III: glucose monitoring during a fermentation of a lignocellulosic hydrolysate by *Saccharomyces cerevisiae* employing a differential amperometric biosensor. Instrument. Sci. Technol. 26, 69–79.

Kuriyama, H., Seiko, Y., Murakami, T., Kobayashi, H., Sonoda, Y., 1985. Continuous ethanol fermentation with cell recycling using flocculating yeast. J. Ferment. Technol. 63 (2), 159–165.

Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., Nilvebrant, N.O., 1998. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enz. Microb. Technol. 24, 151–159.

Larsson, S., Reimann, A., Jönsson, L., 1999. Comparison of different methods for the detoxification of lignocellulosic hydrolysates of spruce. Appl. Microbiol. Biotechnol. 77-79, 91–103.

Lee, C.W., Chang, H.N., 1987. Kinetics of ethanol fermentations in membrane cell recycle fermentors. Biotechnol. Bioeng. 24, 1105–1112.

Lee, W.G., Lee, J.S., Park, B.G., Chang, H.N., 1996. Continuous ethanol production from wood hydrolysate by chemostat and total cell retention culture. Korean J. Chem. Eng. 13 (5), 453–456.

Leonard, R.H., Hajny, G.J., 1945. Fermentation of wood sugars to ethyl alcohol. Ind. Eng. Chem. 37, 390–395.

Lindén, T., Peetre, J., Hahn-Hägerdal, B., 1992. Isolation and characterisation of acetic acid tolerant galactose-fermenting strains of *Saccharomyces cerevisiae* from a spent sulfite liquor plant. Appl. Environ. Microbiol. 58, 1661–1669.

Melzoch, K., Rychtera, M., Markvichov, N.S., Pospichalova, V., Basarova, G., Manakov, M.N., 1991. Application of a membrane recycle bioreactor for continuous ethanol production. Appl. Microbiol. Biotechnol. 34, 469–472.

Nagashima, M., Azuma, M., Noguchi, S., Inuzuka, K., Samejima, H., 1984. Continuous ethanol fermentation using immobilised yeast cells. Biotechnol. Bioeng. 26, 992–997.

Nagodawithana, T.W., Steinkraus, K.H., 1976. Influence of the rate of ethanol production and accumulation on the viability of *Saccharomyces cerevisiae* in "rapid fermentation". Appl. Environ. Microbiol. 31 (2), 158–162.

Navarro, A.R., 1994. Effects of furfural on ethanol fermentation by *Saccharomyces cerevisiae*: mathematical models. Curr. Microbiol. 29, 87–90.

Nilvebrant, N.O., Reimann, A., Kleen, M., Larsson, S., Palmqvist, E. Chemical characterisation of fermentation inhibiting dilute acid hydrolysates from spruce. in preparation.

Nishikawa, N.K., Sutcliffe, R., Saddler, J.N., 1988. The influence of lignin degradation products on xylose fermentation by *Klebsiella pneumoniae*. Appl. Microbiol. Biotechnol. 27, 549–552.

Olsson, L., Hahn-Hägerdal, B., 1993. Fermentative performance of bacteria and yeasts in lignocellulosic hydrolysates. Process biochem. 28, 249–257.

Olsson, L., Hahn-Hägerdal, B., 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme Microb. Technol. 18, 312–331.

Olsson, L., Hahn-Hägerdal, B., Zacchi, G., 1995. Kinetics of ethanol production by recombinant *Escerichia coli* KO11. Biotechnol. Bioeng. 45, 356–365.

Palmqvist, E., Hahn-Hägerdal, B., Galbe, M., Stenberg, K., Szengyel, Z., Tengborg, C., Zacchi, G., 1996a. Design and development of a bench-scale process development unit for the production of ethanol from lignocellulosics. Bioresour. Technol. 58, 171–179.

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

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

Palmqvist, E., 1998. Fermentation of lignocellulosic hydrolysates: inhibition and detoxification. Ph.D. thesis, Lund University, Sweden.

Palmqvist, E., Galbe, M., Hahn-Hägerdal, B., 1998. Evaluation of cell recycling in continuous fermentation of enzymatic hydrolysates of spruce with *Saccharomyces cerevisiae* and on-line monitoring of glucose and ethanol. Appl. Biochem. Biotechnol. 50, 545–551.

Palmqvist, E., Hahn-Hägerdal, B., 1999. Fermentation of lignocellulosic hydrolysates: inhibitors and mechanisms of inhibition. Bioresour. Technol. submitted.

Palmqvist, E., Almeida, J., Hahn-Hägerdal, B., 1999a. Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. Biotechnol. Bioeng. 62, 447–454.

Palmqvist, E., Q. Meinander, N., Grage, H., Hahn-Hägerdal, B., 1999b. Main and interaction effects of acetic acid, furfural and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnol. Bioeng. 63, 46–55.

Stanley, G.A., Pamment, N.B., 1992. Transport and intracellular accumulation of acetaldehyde in *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 42, 24–29.

Stenberg, K., Tengborg, C., Galbe, M., Zacchi, G., Palmqvist, E., Hahn-Hägerdal, B., 1998. Recycling of process streams in ethanol production from softwoods based on enzymatic hydrolysis. Appl. Biochem. Biotechnol. 70/72, 697–707.

Taherzadeh, M., Niklasson, C., Lidén, G., 1997. Acetic acid-friend or foe in anaerobic conversion of glucose to ethanol. Chem. Eng. Sci. 52, 2653–2659.

Taherzadeh, M.J., Niklasson, C., Lidén, G., 1999. Conversion of dilute-acid hydrolyzates of spruce and birch to ethanol by fed-batch fermentation. Bioresour. Technol. 69, 59–66.

Tran, A.V., Chambers, R., 1986. Ethanol fermentation of red oak acid prehydrolysate by the yeast *Pichia stipitis* CBS 5776. Enz. Microb. Technol. 8, 439–444.

van Zyl, C., Prior, B.A., du Preez, J.C., 1988. Production of ethanol from sugar cane bagasse hemicellulose hydrolysate by *Pichia stipitis*. Appl. Biochem. Biotechnol. 17, 357–369.

von Sivers, M., Olsson, L., Hahn-Hägerdal, B., Zacchi, G., 1994. Cost analysis of ethanol production from willow using recombinant *Escherischia coli*. Biotechnol. Prog. 10, 555–560.

Williams, D., Munnecke, D.M., 1981. The production of ethanol by immobilised yeast cells. Biotechnol. Bioeng. 23, 1813–1825.

Wilson, J.J., Deschatelets, L., Nishikawa, N.K., 1989. Comparative fermentability of enzymatic and acid hydrolysates of steam-pretreated aspenwood hemicellulose by *Pichia stipitis* CBS 5776. Appl. Microbiol. Biotechnol. 31, 592–596.

Yu, S., Wyman, M., Parek, S.K., 1986. Fermentation to ethanol of pentose-containing spent-sulfite liquor. Biotech. Bioeng. 29, 1144–1150.