ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Effect of Compounds Released During Pretreatment of Wheat Straw on Microbial Growth and Enzymatic Hydrolysis Rates

Gianni Panagiotou, Lisbeth Olsson

Center for Microbial Biotechnology, BioCentrum-DTU, Building 223, Technical University of Denmark, DK-2800 Lyngby, Denmark; telephone: +45-45252677; fax: +45-4588 4148; e-mail: lo@biocentrum.dtu.dk

Received 14 December 2005; accepted 19 June 2006

Published online 24 July 2006 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21100

ABSTRACT: In the process of producing ethanol from lignocellulosic materials such as wheat straw, compounds that can act inhibitory to enzymatic hydrolysis and to cellular growth may be generated during the pretreatment. Ethanol production was evaluated on pretreated wheat straw hydrolysate using four different recombinant *Saccharomyces cerevisiae* strains, CPB.CR4, CPB.CB4, F12, and FLX. The fermentation performance of the four *S. cerevisiae* strains was tested in hydrolysate of wheat straw that has been pretreated at high dry matter content (220 g/L dry matter). The results clearly showed that F12 was the most robust strain, whereas the other three strains were strongly inhibited when the fraction of hydrolysate in the fermentation medium was higher than 60% (v/v). Furthermore, the impact of different lignin derivatives commonly found in the hydrolysate of pretreated wheat straw, was tested on two different enzyme mixtures, a mixture of Celluclast 1.5 L FG and Novozym 188 (3:1) and one crude enzyme preparation produced from *Penicillium brasilianum* IBT 20888. From all the potential inhibiting compounds that were tested, formic acid had the most severe influence on the hydrolysis rate resulting in a complete inactivation of the two enzyme mixtures.

Biotechnol. Bioeng. 2007; 96: 250–258.

KEYWORDS: enzymatic saccharification; inhibition; pretreatment; ethanol; *Saccharomyces cerevisiae*

Introduction

The biodegradation and bioconversion of lignocelluloses into useful products and the biological alleviation of pollution from lignocellulose wastes are an enormous challenge for biotechnology. The utilization of renewable lignocellulosic resources such as wheat straw for ethanol production provides a promising mean to decrease the greenhouse effect and alleviate the shortage of fossil fuel energy (Zaldivar et al., 2001). Conversion of lignocellulosic material to ethanol requires hydrolysis of carbohydrate polymers to their constituent sugars. Enzymatic hydrolysis is a common approach to hydrolysis and offers the benefits of mild reaction conditions and selective hydrolysis (Philippidis and Hatzis, 1997). Untreated lignocellulose is only slowly degraded by enzymes since the crystalline structure of the cellulose notably affects the enzymatic hydrolysis rate, and the lignin impedes access of the enzymes to the cellulose chains.

The wet oxidation process was presented in the early 1980s as an alternative to steam explosion which had become the most widely used pretreatment method (Ahring et al., 1999). Compared with other pretreatment processes, wet oxidation has been proven to be efficient for treating lignocellulosic materials (Bjerre et al., 1996) because the crystalline structure of cellulose is opened during the process and the lignin is decomposed to CO_2, H_2O, and carboxylic acids (Ahring et al., 1999; Klinke et al., 2002; McGinnis et al., 1983). Wet oxidation results in a solid fraction, consisting mainly of lignin and cellulose, and a hydrolysate, which contains hemicellulose-derived sugars, small amounts of other carbohydrates, sugar degradation products, lignin degradation products (aromatic and polyaromatic compounds with a variety of substituents), and other compounds. It is also probable that the solubilization of the hemicellulose fraction of wheat straw in the pretreatment step produces potential inhibitors like furans and organic acids in low concentrations. Such inhibitors can affect enzymes in the hydrolysis step, reduce glucose and xylose conversion during fermentation, and decrease the rate of the fermentation (Palmqvist et al., 1996; Senior et al., 1991; Sewalt et al., 1996; Yourchisin and van Walsum, 2004).

Correspondence to: L. Olsson

Fermentable sugars from the hemicellulose stream of pretreated wheat straw will essentially be glucose and xylose. In order to minimize feedstock costs it is very important to use a microorganism that is able to achieve maximum substrate utilization and a high ethanol yield. In the present study, pretreated wheat straw (in a process combining thermal hydrolysis, wet oxidation, and steam explosion) supplemented with glucose and xylose, was used as a starting material for ethanol production by different recombinant *S. cerevisiae* strains. The examined recombinant *S. cerevisiae* strains of laboratory (CPB.CB4 and CPB.CR4) or industrial (F12) strain background have genes necessary for xylose metabolism introduced (Bro et al., 2005; Roca et al., 2003a; Sonderegger et al., 2004). In the above strains different approaches have been taken in order to modify the redox balance in xylose fermenting *S. cerevisiae*. The fourth investigated strain FLX contains a NADPH-dependent nonphosphorylating glyceraldehyde 3-phosphate dehydrogenase with an ADH1 promotor and it does not harbor the *XYL*-genes. The efficiency of glucose and xylose utilization in the four recombinant *S. cerevisiae* strains was assessed in both defined lab medium and wheat straw hydrolysate with a particular emphasis on the role of the toxic compounds that are present in the hydrolysate on the growth of the yeast and the productivity of ethanol.

Furthermore, in a process where *S. cerevisiae* will be the fermentation microorganism, the external addition of enzyme mixtures for the hydrolysis of the biopolymers to soluble oligosaccharides is necessary. In this study, we also investigated the nature of enzyme inhibition, using pure chemical compounds that previous studies have identified as potential inhibitors present in wet oxidized wheat straw (Klinke et al., 2001).

Materials and Methods

Pretreated Wheat Straw

Pretreated wheat straw (in a process combining thermal hydrolysis, wet oxidation, and steam explosion) with an initial dry weight of 22% was kindly provided by Prof. B.K. Ahring from EMAB, BioCentrum-DTU.

Yeast Cultivation

Strains and Medium

The strains used in this study were *S. cerevisiae* CPB.CR4, CPB.CB4, FLX, and F12 (Table I). The strains were stored at −80°C in glycerol.

For the preculture a defined medium containing trace metal elements and vitamins was used as described previously (Roca and Olsson, 2003b). A colony taken from an YPD agar plate was inoculated into 500 mL baffled Erlenmeyer flask containing 100 mL medium. Glucose (10 g/L) was used as carbon source and the pH was adjusted to 6.5. The flasks were incubated at 30°C in an orbital shaker operating at 150 rpm for 20–24 h.

Inhibition of Fermentation

All experiments were performed as batch fermentations under anaerobic conditions with 10 mL hydrolysate medium in 30 mL tubes. The tubes were inoculated from the preculture giving a final biomass concentration of 0.24 g DW/L in the tubes. The hydrolysate was supplemented with additional nutrients by adding 10 μL of the defined medium described above per milliliter of hydrolysate. The pH was adjusted to 5.0 and it was not controlled during the fermentation. A test for inhibitory effects of the hydrolysate medium on the ethanol production by the different recombinant *S. cerevisiae* strains was carried out. Hydrolysate from pretreated wheat straw was diluted with distilled water to obtain different concentrations, in the range from 0% to 100% (v/v). Sugars were added in the diluted hydrolysates, such that the final concentrations were 20 g/L of glucose and 15 g/L of xylose in all cases. The tubes were incubated at 30°C in an orbital shaker operating at 150 rpm for 48 h. All cultivations were carried out as triplicate experiments.

Enzymatic Hydrolysis Experiments

Reagents

Stock solutions containing potential enzyme inhibitors were prepared in 150 mM citrate-phosphate buffer pH 5 and were used to determine the relative inhibitory effect on cellulase and hemicellulase system of two enzyme preparations. All experiments were performed with commercially available, reagent grade compounds. Vanillic acid, syringic acid, acetosyringone, syringaldehyde, furoic acid, succinic acid, formic acid, and guaicol were obtained from Sigma-Aldrich (St. Louis, MO). In Table II the examined concentrations of the inhibitors are summarized.

Two enzymic mixtures were employed; one was a gift from Novozymes A/S (Bagsværd, Denmark), a mixture of Celluclast 1.5 L FG and Novozym 188 (3:1) and one crude enzyme preparation produced from *Penicillium brasilianum*

Table I. *Saccharomyces cerevisiae* strains used in the present study

Strain	Relevant genotype	Origin/reference
CPB.CR4	*S. cerevisiae CEN.PK 113–7D MATα SUC2 MAL2-8c pADH-XYL1 pPGK-XYL2pPGK-XKS1gdh1ΔpPgk-GDH2*	Roca et al. (2003a)
CPB.CB4	*S. cerevisiae CEN.PK 113–7D MATa SUC2 MAL2-8 pADH-XYL1 pPGK-XYL2 pPGK-XKS1 pTPIp-gapN*	Bro et al. (2005)
FLX	*S. cerevisiae CEN.PK 113–7D pADH1-gapN*	Bro et al. (2005)
F12	*S. cerevisiae F HIS3::YIploxZEO overexpressing XR, XDH, and XK*	Sonderegger et al. (2004)

Table II. The inhibitory effect of each compound on the hydrolysis of filter paper and xylan was examined in two different concentrations, referred to as low concentration level (LC) and high concentration level (HC)

Inhibitor	Low concentration (LC) (g/L)	High concentration (HC) (g/L)	Inhibition mixture (g/L)
Vanillic acid	0.5	2	0.25
Syringic acid	0.5	2	0.25
Acetosyringone	0.3	0.9	0.11
Syringaldehyde	0.3	0.9	0.11
Furoic acid	0.1	0.5	0.06
Succinic acid	1	4	0.5
Formic acid	4	15	1.8
Guaicol	0.2	0.7	0.09

The synergistic action of these compounds was also tested and the third column shows the concentration of each compound in the inhibition mixture.

IBT 20888. The cultivation of *P. brasilianum* was carried out on a mixture of Solka-Floc, oat spelts xylan, and birchwood xylan. The resulting fermentation broth was concentrated by ultrafiltration (Jørgensen et al., 2005).

Enzyme Assays

As an estimate of the cellulolytic and hemicellulolytic activity, filter paper activity and birchwood xylan activity, respectively, were determined, as described previously (Christakopoulos et al., 1995; Ghose, 1984), in the two enzyme preparations.

Analyses

Extracellular Metabolites

Xylose, xylitol, glucose, glycerol, acetate, pyruvate, succinate, and ethanol were analyzed by high performance liquid chromatography (HPLC) as described previously (Panagiotou et al., 2005).

Oligosaccharide Analysis

Glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, xylose, xylobiose, xylotriose, xylotetraose, and xylopentaose concentrations were determined by HPLC with pulsed amperometric detection (HPAEC-PAD) using a CarboPac PA1 column (Dionex, Sunnyvale, CA). A gradient system was used for the separation: solvent A: 40 mM NaOH and solvent B: 100 mM NaOH, containing 75 mM NaAc, using the following gradient: 100% A and 0% B (0–15 min), a linear decrease of A to 0% and a linear increase of B to 100% (15–40 min), a linear increase of A to 100% and a linear decrease of B to 0% (40–50 min). The flow rate was 1 mL/min. The products were quantified, based on peak areas using standard sugars.

Biomass Estimation

The biomass content was measured by following the increase on the optical density at 600 nm using a Hitachi U-1100 Spectrophotometer. The background absorbance caused by the hydrolysate was substracted (by centrifugation of the sample) from the measurements.

Results and Discussion

Evaluation of the Inhibition of Pretreated Wheat Straw on Growth and Ethanol Production by *Saccharomyces cerevisiae*

To investigate whether the presence of potential inhibitors in pretreated wheat straw hydrolysate has a measurable effect on the rates of fermentation, the following experimental procedure was used. The hydrolysate of the pretreated wheat straw was used as fermentation medium, and it was diluted with distilled water to give final concentrations, based on % hydrolysate, of 100%, 80%, 60%, 40%, 20%, and 0% (v/v) whereas the final concentration of the carbon source in all cases was 20 g/L of glucose and 15 g/L of xylose. The ratio of glucose to xylose in the fermentation medium mimics an industrial process where the pretreated wheat straw (hemicellulose stream and solid fraction) is directly used for fermentation (Bjerre et al., 1996; Garde et al., 2002; Nigam et al., 2001; Thygesen et al., 2003). The growth and the ethanol production by the four different recombinant *S. cerevisiae* strains during fermentation of pretreated wheat straw hydrolysate were investigated (Fig. 1).

The strains CPB.CR4 and FLX were the most inhibited strains as they were unable to utilize glucose when the concentration of the hydrolysate in the fermentation medium was more than 60% (Fig. 1). In contrast, during the fermentation by the strains CPB.CB4 and F12, a significant biomass production was observed, even at the highest concentrations of hydrolysate, and of the two strains F12 was the most tolerant strain (Fig. 1).

The results for all the strains clearly showed that when the hydrolysate concentration in the fermentation medium was in the range of 20%–40% the glucose concentration decreased faster, than in the fermentation without hydrolysate (0%) (data not shown), and the specific growth rate and final biomass concentration were also higher or similar with the control fermentation (Fig. 1). The maximum biomass concentration, for all the strains, was observed after 48 h with 20% hydrolysate as fermentation medium, and it was 3.3, 3.2, 4.1, and 2.7 g/L for CPB.CB4, CPB.CR4, F12, and FLX, respectively. *S. cerevisiae* F12 was the most inhibitor tolerant strain, and it reached the highest biomass concentration. Also in another strain comparison F12 has been shown to be the most tolerant strain (Sonderegger et al., 2004). FLX is not capable of utilizing xylose, and therefore a lower biomass concentration resulted.

DOI 10.1002/bit

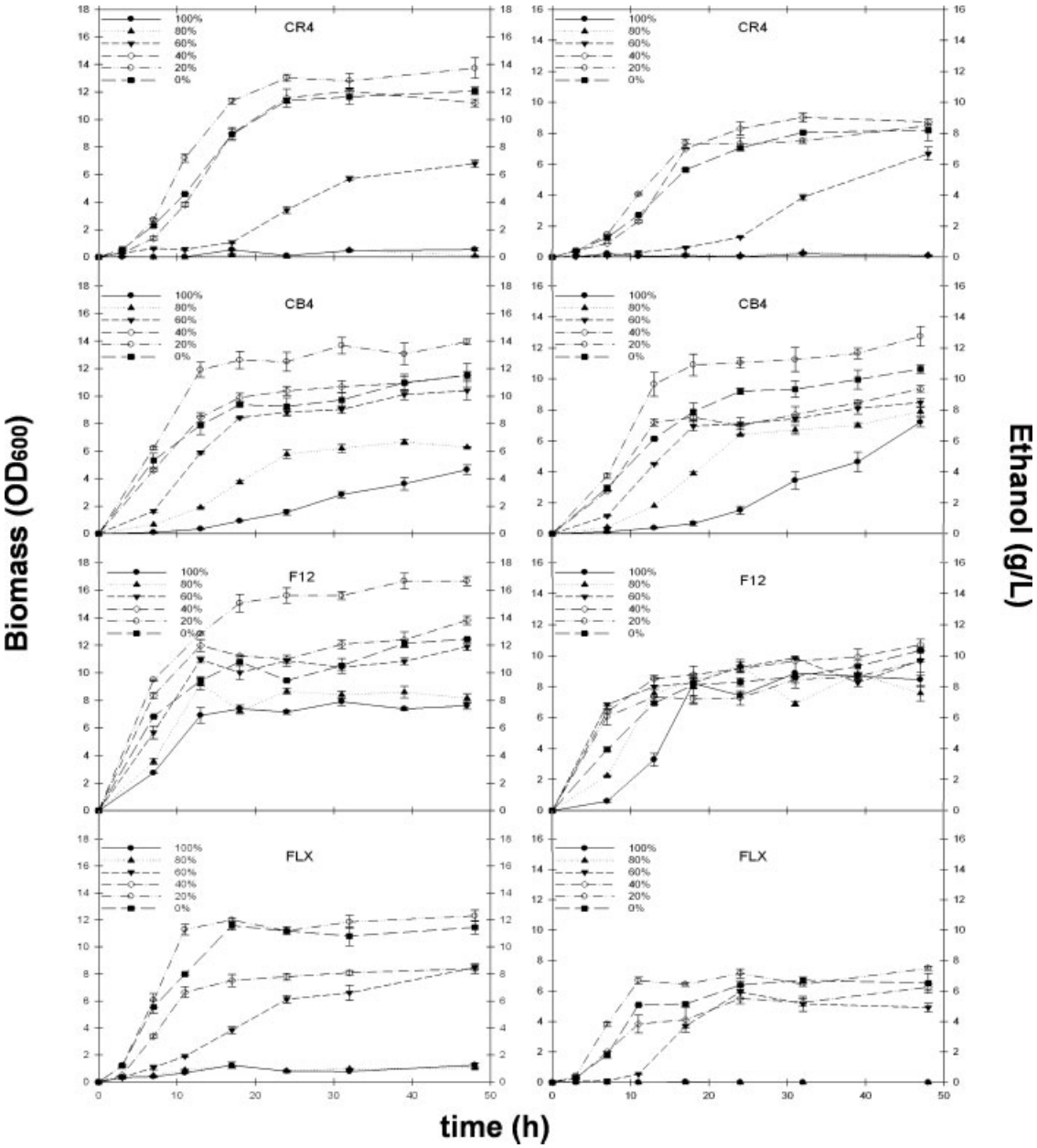

Figure 1. Time course of growth and ethanol production of *Saccharomyces cerevisiae* CPB.CR4, CPB.CB4, F12, and FLX cultivated on increasing concentration of pretreated wheat straw hydrolysate (0%–100%) in the fermentation medium. The initial concentrations of glucose and xylose were 20 and 15 g/L, respectively, in all cases. Values are the mean of three determinations and vertical error bars represent SD (all variations are less than 10%).

Several other researchers have reported problems with microbial inhibition when utilizing aspenwood hydrolysate or birch hemicellulose liquor obtained by different pretreatment techniques, such as steam explosion and acid hydrolysis, even if the concentration of the hydrolysate was very low (Perttunen et al., 1996; Preziosi-Belloy et al., 1997). The fact that in our study the presence of 20% hydrolysate in the fermentation medium had no inhibition effect on the yeast growth is hardly surprising, since during wet oxidation low amounts of toxic compounds such as furfural and 5-hydroxymethyl-furfural are produced (Ahring et al., 1996; Schmidt and Thomsen, 1998).

Ethanolic production by *S. cerevisiae* CPB.CB4 at different concentrations of hydrolysate was also inhibited in a similar way as the growth was (Fig. 1). The maximum ethanol concentration when 100% and 80% of hydrolysate were used, were 7.2 and 7.9 g/L, respectively, and the volumetric productivity was 0.15 and 0.16 g/L/h, respectively, after 48 h of fermentation. The presence of an increasing amount of inhibiting compounds delays significantly the onset of

fermentation in all the strains (Fig. 1). Comparing all the strains that we examined in this study, the highest ethanol concentration, 12.8 g/L, and volumetric productivity, 0.27 g/L/h, were reached by CPB.CB4 during the fermentation of 20% hydrolysate (Fig. 1).

The fact that in our investigation, the ethanol productivity was higher in the hydrolysate, compared to the results when the reference medium (0% of hydrolysate) was used, is not unique, since in similar studies, the ethanol yield has been found to be enhanced when a more concentrated hydrolysate was used as fermentation medium (Klinke et al., 2002). Acetate, present in the hydrolysate, has earlier been shown to reduce the cell mass production, lowering the formation of glycerol and increasing the ethanol yield in *S. cerevisiae* (Palmqvist et al., 1999; Taherzadeh et al., 1997).

In contrast to the growth characteristics of CPB.CB4, the growth of *S. cerevisiae* F12 strongly depended on the hydrolysate concentration, whereas the ethanol productivity did not strongly depend on the hydrolysate concentration. The volumetric ethanol productivity after the 48 h of fermentation was 0.2–0.22 g/L/h in the range of 80%–20% hydrolysate concentrations. Even though that lignocellulosic hydrolysates are far from ideal substrates, *S. cerevisiae* F12 was able to achieve a final ethanol concentration of 8.8 g/L using 100% hydrolysate as fermentation medium.

The other two strains, CPB.CR4 and FLX, which were used in the present study, were according to the fermentation tests more sensitive to the inhibition at concentrations of the hydrolysate between 60% and 100% (Fig. 1). The maximum ethanol volumetric productivity was 0.19 and 0.16 g/L/h for CPB.CR4 and FLX, respectively, during the fermentation of a medium containing 20% hydrolysate whereas the final ethanol concentration was 8.8 and 7.5 g/L, respectively.

CPB.CB4 showed higher maximum ethanol yield compared to CPB.CR4 and F12. Overexpression of GAPN affects both redox and energy metabolism. The introduction of GAPN leads to the production of less NADH in glycolysis, and therefore less NADH needs to be reoxidized via glycerol production. Hence carbon is redirected from glycerol towards ethanol. In addition, less ATP is produced since the GAPN reaction partially by-passes the reaction carried out by the homologous ATP-producing 3-phospho-glycerate kinase. A reduced ATP production leads to reduced biomass as has been experimentally shown and the additional excess carbon can be further directed towards ethanol (Bro et al., 2005).

In our study, a hydrolysate of wheat straw pretreated at high dry matter content was found to be fermentable, however, the degradation products from pretreatment of wheat straw caused a decrease in the volumetric ethanol productivity. Phenol aldehydes, compounds that are usually present in wet oxidized wheat straw (Klinke et al., 2002) are degraded by a special group of enzymes, the oxidoreductases. The oxidoreductases require an electron acceptor cofactor such as NADH or NADPH that must be regenerated (De Wulf et al., 1986) and that can cause alterations in the cellular redox reactions. The cellular conversion of phenol aldehydes to alcohols leads to an increasing demand for cofactor regeneration in the cell. It has been shown that the reductive capacity of $NAD(P)^+$ in *S. cerevisiae* is strain-dependent (Pereira, 1995) and consequently different strains will differ in their capability to convert compounds by redox reactions (Brandberg et al., 2004). In agreement with that, the recombinant strains which were used in our work performed differently under the same cultivation conditions. Whereas the CPB.CB4, CPB.CR4, and FLX strains have a more favorable redox balance, the F12 is not a redox manipulated strain, and this was reflected on the ethanol and biomass production.

In our study we investigated in detail the response of four different recombinant *S. cerevisiae* strains to compounds produced or released during the pretreatment of wheat straw; however, the exact biochemistry involved in the inhibition or toxicity is not yet fully understood and investigated.

Degree of Inhibition on Cellulolytic and Hemicellulolytic Activity

To investigate the influence of potential inhibitory substances on the enzymatic hydrolysis, experiments using single chemical components were performed. A set of hydrolysis experiments was carried out in the presence of two concentration levels of vanillic acid, syringic acid, acetosyringone, syringaldehyde, furoic acid, succinic acid, formic acid, and guaicol, respectively (Table II). The potential synergism was also investigated adding the entire pool of chemicals under the same conditions as for the individual inhibitory experiments. The range of the examined concentrations was based on previous studies on the characterization of degradation products produced by wet oxidation of 60 g/L wheat straw (Klinke et al., 2003).

A second series of experiments was also carried out in order to study the direct influence of the hydrolysate obtained after the pretreatment of the wheat straw, on the enzymatic hydrolysis rates.

The mechanism of inhibition of the commercial enzyme mixture of Celluclast + Novozyme (referred from now on in the text as enzyme preparation CN) in a ratio 3:1 (Tengborg et al., 2001) and the crude enzyme mixture of *P. brasilianum* (referred from now on in the text as enzyme preparation Pb) was studied in two different ways. First, the inhibition effects were investigated by filter paper and xylan assays (as described in Materials and Methods), which were performed in the presence of different inhibitors, individually and in a combination or in the presence of hydrolysate. Second, the inactivation effect was investigated by measuring the remaining cellulolytic and hemicellulolytic activity of the enzyme mixtures CN and Pb after preincubation at 30°C for 24 h with the same concentration of the inhibitory compounds or hydrolysate. In all the experiments a reference without the addition of any chemical compound was used.

DOI 10.1002/bit

Effects on Cellulolytic Activity

Figure 2A and B show the relative amount of glucose which was produced when the different inhibitory compounds were added during the filter paper assay. With the exception of formic acid, most of the examined inhibitors did not reduce the filter paper activity of the preparations CN and Pb, even at the high inhibitor concentrations. However, the presence of formic acid during the hydrolysis of filter paper had a strongly negative effect on the released glucose. The addition of 4 g/L formic acid caused a 20% reduction in the glucose concentration whereas an addition of 15 g/L almost totally inactivated the enzymes of the cellulose complex (Fig. 2A and B). Klinke et al. (2002) quantified the degradation products in the hemicellulose liquid fraction of wet oxidised wheat straw (60 g/L) and the concentration of formic acid was found to be 4.7 g/L. However, due to that higher dry matter content has been used pretreating the wheat straw (220 g dry matter/L) in the hydrolysate investigated here, higher concentrations of formic acid could be expected in the hydrolysate. The observed formic acid inhibition of enzymatic cellulose digestion is in agreement with previous studies where the hydrolysis of pretreated poplar wood by commercial available enzymes was strongly inhibited by the presence of 11.5 g/L formic acid (Cantarella et al., 2004). The combined effect of the addition of all the potential inhibitors was investigated and the experimental results presented in Figure 2A and B seem to exclude the presence of factors such as synergism and cumulative inhibitory effects. However, when the enzymatic assay performed in the presence of the pretreated wheat straw hydrolysate, a 34% and 36% decrease on the hydrolysis rates for the CN and Pb mixtures, respectively, was observed (data not shown).

Filter paper activity of the enzyme mixtures CN and Pb was determined after preincubation of the enzyme preparations with phenols, acids, and furans for 24 h to quantify the direct inactivation of these compounds on the cellulase enzyme system. None of the individual phenol or acids appeared to significantly effect the saccharification of filter paper either when the commercial enzyme mixture (CN) or the enzyme mixture Pb was used. The substance with the greatest inactivation effect was again found to be formic acid. However, preincubation with all the potential inhibitors was detrimental to cellulose hydrolysis, resulting in minimal filter paper activity. Similar inactivation effect of mixed lignin derivatives on commercially available cellulase has been reported earlier (Sewalt et al., 1996). In order to further investigate which of the enzymes of the cellulase system was most affected, the samples were analyzed by HPAEC-PAD. During the analysis no cellobiose was detected (data not shown) and these data indicate that the enzymatic system contains sufficient β-glucosidase to cleave almost immediately all of the cellobiose produced. No accumulation of cellotriose, cellotetraose, and cellopentaose was observed indicating that the enzyme load was enough to efficiently convert lignocellulosic raw material to glucose and cellobiose.

While preincubation of the enzyme preparations CN and Pb with the mixture of the inhibitor compounds had fatal results in their activity, the preincubation with the wheat

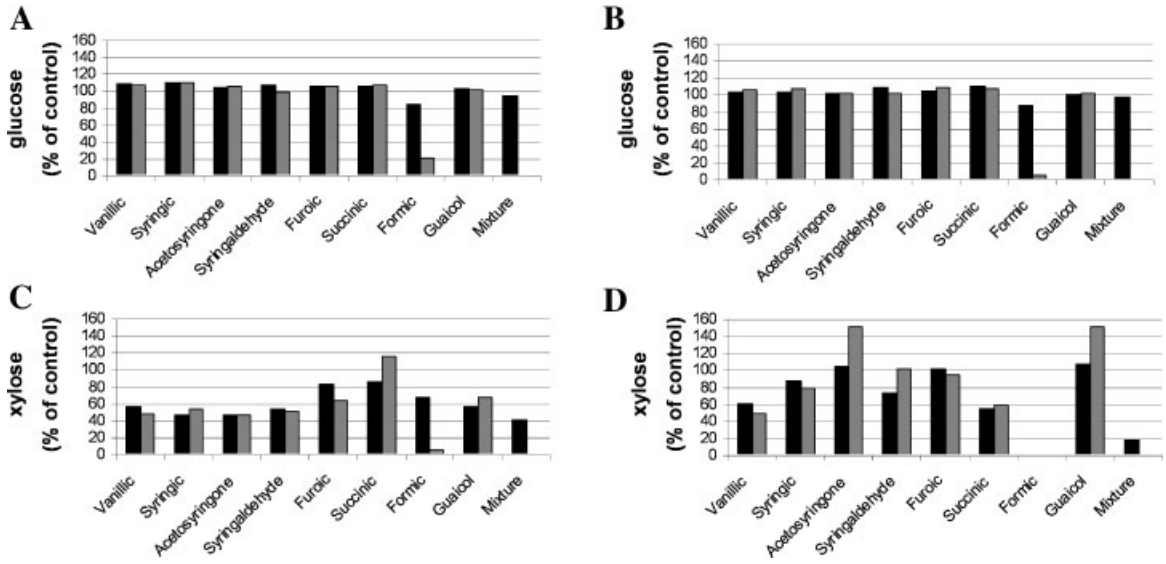

Figure 2. Investigation of the inhibition of filter paper and xylan hydrolysis. **Panels** (**A**) and (**B**) show the hydrolysis of filter paper using the enzyme solutions CN and Pb, respectively, after addition of different compounds during the enzymatic assay. **Panels** (**C**) and (**D**) show the hydrolysis of xylan using the enzyme solutions CN and Pb, respectively, after addition of the same compounds during the enzymatic assay. The results are expressed as percent of the glucose or xylose released in the control (no addition of inhibitors) compared to the release of glucose or xylose in the enzymatic assay including the respective inhibitors. (■) low concentration level of the inhibitors, (■) high concentration level of the inhibitors, (Mixture) the entire pool of the inhibitors.

straw hydrolysate decreased the glucose production 49% and 64% for CN and Pb, respectively (data not shown).

Effects on Hemicellulolytic Activity

In order to evaluate the possible inhibition on xylanases caused by inhibitory compounds that are usually present in the hemicellulose hydrolysate, the same set of experiments as in the last chapter was carried out using the xylanase assay. In our work, the xylanase activity by the CN enzyme preparation was inhibited when assayed in the presence of different potential inhibitor compounds (Fig. 2C). The inhibition level ranged between 40% and 60% for most of the examined inhibitors and the inhibition level was not dependent on the concentration level of the inhibitor, with the exception of formic acid. The inhibition effects in our study were more severe than the xylanase inhibition of Novozym 188 when lignin preparation (soluble lignin compounds eluted from the pulp) from spent sulfite liquor was added during the enzymatic assay (Senior et al., 1991). This divergence could be due to the different inhibiting compounds that are present in the two hydrolysates. No oligosaccharides of higher degree of polymerization (xylotriose, xylotetraose, xylopentaose) were observed in any of these hydrolysis experiments (data not shown).

In contrast, the enzyme mixture Pb was less inhibited in the presence of syringic acid, syringaldehyde, and furoic acid than the CN-enzyme cocktail was (Fig. 2D). In the presence of 0.5 g/L vanillic acid strong inhibition was observed and 4 g/L of formic acid totally inactivated the hemicellulolytic enzyme system in Pb (Fig. 2D). However, the addition of 0.9 g/L acetosyringone or 0.7 g/L guaicol had a positive effect to the released xylose (Fig. 2D). The results from the analysis of the oligosaccharide profile indicated an activation of β-xylosidase in both cases resulting in higher concentration of xylose. In previous studies, Pecarovicova et al. (1989) have shown that several phenolic compounds have a similar activating effect on the xylanases from *Cryptococus albidus* and on the cellulases from *Trichoderma reesei* QM 9414.

When the xylan digestion was studied in the presence of the entire pool of the inhibiting compounds, a 60% and 80% decrease on the hydrolysis rates for the CN and Pb mixtures, respectively, was observed (Fig. 2C and D), whereas an 85% decrease in the xylose production was obtained in the presence of wheat straw hydrolysate for both enzyme preparations (data not shown). This difference between the entire pool of chemicals and the wheat straw hydrolysate on the inhibition level indicates that more compounds with inhibitory action could be present in the hydrolysate.

The different compounds were also added to enzyme incubations, either separately or as a mixture, and the extent of the xylan hydrolysis was then evaluated. It was found that all the tested compounds had an inactivation effect on the hemicellulase system of the enzyme mixture CN. The decrease on the production of xylose ranged from 40% to 100% supporting Sanderson et al. (1965), which showed that phenols might inactivate enzymes by causing their denaturation and precipitation. Oligosaccharides of higher degree of polymerization (xylobiose, xylotriose, xylotetraose, xylopentaose) were also observed in all cases, in lower concentrations than the control, indicating an inactivation of the total hemicellulolytic activity (data not shown). Xylose:xylooligosaccharides ratio was highest in the control indicating a greater inactivation of β-xylosidase activity than xylanase activity.

However, the influence of compounds on the enzyme mixture Pb was significantly different from the effects on enzyme mixture CN. Preincubation with low concentration of vanillic acid, syringaldehyde, and furoic acid resulted in higher concentration of xylose during the hydrolysis of xylan than the control. However, the results from the oligosaccharides analysis showed that higher concentrations of xylotetraose and xylopentaose were produced compared to the control, whereas less xylobiose was detected (data not shown).

There have been several reports of compounds present in complex substrates that exert inhibitory effects on saccharifying enzymes. The phenolic monomer *p*-coumaric acid has been found to inhibit the xylanase from *Bacteriodes succinogenus.* In contrast, ferulic and vanillic acid had activating effects on the same xylanase when used at concentrations of 0.15 and 0.10% (w/w), respectively (Martin and Akin, 1988). Unidentified compounds produced during enzymatic hydrolysis of *Eucalyptus regnans* pulps have been found to have an inhibitory effect on the β-glucosidase of *T. reesei* C30, but not on the β-glucosidase, β-xylanase, or β-xylosidase enzymes from *Aspergillus niger* (Dekker, 1988). The aforementioned results of these previous investigations support the conclusions of this work that the inactivation of xylanases, when incubated in the presence of different compounds, varied dramatically depending on the source of the enzyme and the type of inhibitors involved.

Preincubation of the enzyme preparations CN and Pb with the mixture of the inhibitor compounds resulted in their complete deactivation as also did preincubation with the wheat straw hydrolysate (data not shown).

Conclusions

In order to investigate whether the pretreatment of wheat straw at high dry matter content (220 g/L) by a combination of thermal hydrolysis, wet oxidation, and steam pretreatment produces a suitable substrate for different recombinant *S. cerevisiae* strains, a set of experiments was carried out using increasing concentrations of hydrolysate. *S. cerevisiae* F12 was found to be the most robust strain, whereas strong inhibition was observed on the growth and ethanol production of the strains CPB.CR4, CPB.CB4, and FLX when the concentration of the hydrolysate exceeded 60% in the fermentation medium. These results indicate that the toxicity is dependent not only on the concentration of the

DOI 10.1002/bit

inhibitors, but also on the tolerance of the strain employed. The striking differences between different strains in terms of growth and ethanol production in the hydrolysate show that the choice of strain is of utmost importance when designing a process for the fermentation of wheat straw hydrolysate.

Furthermore, the addition of three major groups of potential inhibitors, furans, phenols, and low molecular weight acids, during enzymatic digestion of filter paper and xylan, provides evidence that some of these compounds exert a strong negative effect on the cellulolytic and hemicellulolytic activity. Indeed, just the presence of formic acid was capable to cause a decrease at the level of hydrolysis or totally inactivate the enzyme mixtures, pointing out that the selective removal of one or two compounds may be sufficient to yield a hydrolysate that can be efficient degraded by enzymes.

The work of bioethanol production at CMB, is part of the activities in Danish Center for Biofuels. The financial support from the Energy Research Programme, Danish Energy Authority, is gratefully acknowledged. The authors thank Prof. B.K. Ahring and Dr. F. Haagensen at BioCentrum-DTU for supplying the pretreated wheat straw hydrolysate, Novozymes A/S (Denmark) and H. Christensen, BioCentrum-DTU for providing us with the two enzyme preparations. We thank Dr. C. Roca, Dr. C. Bro BioCentrum-DTU, and Dr J. Forster. Fluxome Sciences for providing us with the recombinant *S. cerevisiae* strains.

References

Ahring BK, Jensen K, Nielsen P, Bjerre AB, Schmidt AS. 1996. Pretreatment of wheat straw and conversion of xylose and xylan to ethanol by thermophilic anaerobic bacteria. Biores Technolol 58:107–113.

Ahring BK, Licht D, Schmidt AS, Sommer P, Thomsen AB. 1999. Production of ethanol from wet oxidized wheat straw by *Thermoanaerobacter mathranii*. Biores Technol 68:3–9.

Bjerre AB, Olesen AB, Fernqvist T, Ploger A, Schmidt AS. 1996. Pretreatment of wheat straw using alkaline wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. Biotechnol Bioeng 49:568–577.

Brandberg T, Franzen CJ, Gustafsson L. 2004. The fermentation performance of nine strains of *Saccharomyces cerevisiae* in batch and fed-batch cultures in dilute-acid wood hydrolysate. J Biosci Bioeng 98:122–125.

Bro C, Regenberg B, Forster J, Nielsen J. 2005. In silico aided metabolic engineering of *Saccharomyces cerevisiae* for improved bioethanol production. Metabolic Eng 8:102–111.

Cantarella M, Cantarella L, Gallifuoco A, Spera A, Alfani F. 2004. Effect of inhibitors released during steam-explosion treatment of poplar wood on subsequent enzymatic hydrolysis and SSF. Biotechnol Progr 20:200–206.

Christakopoulos P, Kekos D, Kolisis F, Macris BJ, Claeyssens M, Bhat M. 1995. Purification and characterization of non randomly acting endo-1,4-β-D-glucanase from the culture filtrates of *Fusarium oxysporum*. Arch Biochem Biophys 316:143–146.

De Wulf O, Thonart P, Gaignage P, Marlier M, Paris A, Paquot M. 1986. Bioconversion of vanillin to vanillyl alcohol by *Saccharomyces cerevisiae*. Biotechnol Bioeng Symp 17:606–616.

Dekker RFH. 1988. Inhibitors of *Trichoderma reesei* beta-glucosidase activity derived from autohydrolysis-exploded eucalyptous-regnans. App Microbiol Biotechnol 29:593–598.

Garde A, Jonsson G, Schmidt A, Ahring BK. 2002. Lactic acid production from wheat straw hemicellulose hydrolysate by *Lactobacillus pentoses* and *Lactobacillus brevis*. Biores Technol 81:217–223.

Ghose T. 1984. Measurement of cellulose activities. Commission on Biotechnology, International Union of pure and applied chemistry, Delhi, India, p 1–13.

Jørgensen H, Mørkeberg A, Krogh KBR, Olsson L. 2005. Production of cellulases and hemicellulases by three *Penicillium* species: Effect of substrate and evaluation of cellulose adsorption by capillary electrophoresis. Enzym Microb Technol 36:42–48.

Klinke HB, Thomsen AB, Ahring BK. 2001. Potential inhibitors from wet oxidation of wheat straw and their effect on growth and ethanol production by *Thermoanaerobacter mathranii*. Appl Microb Biotechnol 57:631–638.

Klinke HB, Ahring BK, Schmidt AS, Thomsen AB. 2002. Characterization of degradation products from alkaline wet oxidation of wheat straw. Biores Technol 82:15–26.

Klinke HB, Ahring BK, Olsson L, Thomsen AB. 2003. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae*: Wet oxidation and fermentation by yeast. Biotechnol Bioeng 81:738–747.

Martin SA, Akin DE. 1988. Effect of phenolic monomers on the growth and beta-glucosidase activity of *Bacteroides ruminicola* and on the carboxylmethylcellulase, beta-glucosidase and xylanase activities of *Bacteroides succinogenes*. Appl Environ Microbiol 54: 3019.

McGinnis GD, Wilson WW, Mullen CE. 1983. Biomass pretreatment with water and high pressure oxygen. The wet-oxidation process. Ind Eng Chem Prod Res Dev 22:352–357.

Nigam JN. 2001. Ethanol production from wheat straw hemicellulose hydrolysate by *Pichia stipitis*. J Biotechnol 87:17–27.

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

Palmqvist E, Grage H, Meinander N, Hahn-Hagerdal B. 1999. Main and interaction effects of acetic acid, furfural and 4-hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnol Bioeng 63:46–55.

Panagiotou G, Christakopoulos P, Olsson L. 2005. The influence of different cultivation conditions on the metabolome of *Fusarium oxysporum*. J Biotechnol 118:304–315.

Pecarovicova A, Luzakova V, Bystricky S, Pecarovic J, Kosik M. 1989. An activation of effect of some phenolics on enzymatic hydrolysis of polysaccharides. Cellulose Chem Technol 23:225.

Pereira RS. 1995. Baker's yeast. Some biochemical aspects and their influence in biotransformations. Appl Biochem Biotechnol 55:123–132.

Perttunen J, Sohlo J, Maentausta O. 1996. The fermentation of birch hemicellulose liquor to lactic acid. In Proceedings of the 6th Conference on Biotechnology in the Pulp and Paper Industry. Chemical Abstracts, CAN 683172, p 295–298.

Philippidis GP, Hatzis C. 1997. Biochemical engineering analysis of critical process factors in the biomass-to-ethanol technology. Biotechnol Prog 13:222–231.

Preziosi-Belloy L, Nolleau V, Navarro JM. 1997. Fermentation of hemicellulosic sugars and sugar mixtures to xylitol by *Candida parapsilosis*. Enzym Microb Technol 21:124–129.

Roca C, Olsson L. 2003b. Increasing ethanol productivity during xylose fermentation by cell recycling of recombinant *Saccharomyces cerevisiae*. App Microbiol Biotechnol 60:560–563.

Roca C, Nielsen J, Olsson L. 2003a. Metabolic engineering of the ammonium assimilation in xylose fermenting *Saccharomyces cerevisiae* results in improved ethanol production. Appl Environm Microbiol 69:4732–4736.

Sanderson GW. 1965. The action of polyphenolic compounds on enzymes. Biochem J 94:24–25.

Schmidt AS, Thomsen AB. 1998. Optimization of wet oxidation pretreatment of wheat straw. Biores Technol 64:139–151.

Senior DJ, Mayers PR, Saddler JN. 1991. The interaction of xylanases with commercial pulps. Biotechnol Bioeng 37:274–279.

Sewalt VJH, De Olivera W, Glasser WG, Fontenot JP. 1996. Lignin impact on fiber degradation. 2. A model study using cellulose hydrogels. J Sci Food Agric 71:204–208.

Sonderegger M, Jeppsson M, Larsson C, Gorwa-Grauslund MF, Boles E, Olsson L, Spencer-Martins I, Hahn-Hagerdal B, Sauer U. 2004. Fermentation performance of engineered and evolved xylose-fermenting *Saccharomyces cerevisiae* strains. Biotechnol Bioeng 87:90–98.

Taherzadeh MJ, Niklasson C, Liden G. 1997. Acetic acid-friend or foe in anaerobic batch conversion of glucose to ethanol by *Saccharomyces cerevisiae*. Chem Eng Sci 52:2653–2659.

Tengborg C, Galbe M, Zacchi G. 2001. Reduced inhibition of enzymatic hydrolysis of steam pretreated softwood. Enzym Microb Technol 28:835–844.

Thygesen A, Thomsen AB, Schmidt AS, Jorgensen H, Ahring BK, Olsson L. 2003. Production of cellulose and hemicellulose-degrading enzymes by filamentous fungi cultivated on wet-oxidised wheat straw. Enzym Microb Technol 32:606–615.

Yourchisin DM, van Walsum PG. 2004. Comparison of microbial inhibition and enzymatic hydrolysis rates of liquid and solid fractions produced from pretreatment of biomass with carbonic acid and liquid hot water. Appl Biochem Biotechnol 113-116:1073–1086.

Zaldivar J, Nielsen J, Olsson L. 2001. Fuel ethanol production from lignocellulose: A challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol 56:17–34.

DOI 10.1002/bit