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Comparative fermentability of enzymatic and acid hydrolysates of steam-pretreated aspenwood hemicellulose by *Pichia stipitis* CBS 5776

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Summary. Enzymatic hydrolysates of hemicellulose from steam-pretreated aspenwood were more fermentable than the acid hydrolysate after rotoevaporation or ethyl acetate extraction treatments to remove acetic acid and sugar- and lignin-degradation products prior to fermentation by *Pichia stipitis* CBS 5776. Total xylose and xylobiose utilization from 5.0% (w/v) ethyl acetate extracted enzymatic hydrolysate was observed with an ethanol yield of 0.47 g ethanol/g total available substrate and an ethanol production rate of 0.20 $g \cdot l^{-1}$ per hour in 72 h batch fermentation.

Introduction

For the economic production of ethanol from wood hydrolysates it is essential that the hemicellulosic sugars, in addition to the hydrolysed cellulose, be efficiently fermented. Separation of both hemicellulose and lignin from cellulose following steam-pretreatment of aspenwood has been shown to benefit enzymatic cellulose hydrolysis (Schwald et al. 1988). In addition, the presence of lignin in steam-pretreated aspenwood has been shown to reduce subsequent enzymatic cellulose hydrolysis efficiency by adsorbing cellulases (Sutcliffe and Saddler 1986). Consequently, the separation of steam-pretreated wood into its three major fractions, cellulose, hemicellulose and lignin, prior to enzymatic hydrolysis and fermentation is necessary to improve the utilization efficiency of the total pretreated wood.

When aspenwood hemicellulose is obtained following a non-acid-catalysed steam-pretreatment of 80 s at 240° C, it is primarily in the form of water-soluble pentosan (Brownell and Saddler 1987). Subsequent acid or enzymatic hydrolysis of the pentosan, which is primarily acetylxylan, produces a mixture of sugars, mostly D-xylose, some uronic acids and a significant quantity of acetic acid. The hydrolysates also contain sugar-degradation products such as furfural and hydroxymethylfurfural and a variety of aromatic lignin-degradation products that are generated during the steam-pretreatment and acid hydrolysis. These compounds, together with acetic acid, act cumulatively to inhibit the fermentation activity of both bacteria and fungi (Ando et al. 1986; Beck 1986a; Nishikawa et al. 1988; Puls et al. 1985; Tran and Chambers 1986). The concentration of these water-soluble inhibitors in the hemicellulose hydrolysates makes fermentation to ethanol or other solvents more difficult.

The success of fermentation of the xylose in total hydrolysates of lignocellulosic substrates has been variable (Skoog and Hahn-Hägerdal 1988). Reports have appeared dealing with ethanolic fermentation of hemicellulose hydrolysates from various lignocellulose (Liu et al. 1988; Tran and Chambers 1986; Beck 1986b; van Zyl et al. 1988; Watson et al. 1984), but none has dealt specifically with aspenwood hemicellulose.

In our attempts to improve the ethanolic fermentation of steam-pretreated aspenwood hemicellulose by the yeast, *Pichia stipitis* CBS 5776, we tested both combined enzymatic hydrolysis and fermentation (CHF) and separate hydrolysis and fermentation (SHF) approaches. We also studied the effectiveness of rotoevaporation and ethyl acetate extraction (Clark and Mackie 1984) on removing the fermentation inhibitors in an effort to determine the minimum pre-fermentation treatment of our hemicellulose hydrolysates if they are to be fermented separately from the cellulose hy-

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drolysate. Separate hemicellulose fermentation is desirable to reduce the inhibition (diauxie) of xylose fermentation caused by excess glucose (du-Preez et al. 1986).

Materials and methods

Strain and culture conditions. Pichia stipitis CBS 5776 was obtained from the National Research Council of Canada (NRCC) culture collection as NRC 2893. Cultures were maintained on YPX slants containing 1% w/v yeast extract, 2% peptone, 2% xylose and 2% agar. Inocula were grown in YMPX broth containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 5.0% xylose for 48 h at 30° C and 150 rpm. Cells were harvested at 9800 *g* for 10 min and washed once in 0.1% peptone water prior to inoculation. Inoculum cell density was 6.4 g dry wt·l^{-1}.

Substrate preparation. Aspenwood chips were pretreated with saturated steam at 240° C for 80 s and water-extracted to obtain a dilute hemicellulose fraction as described previously (Brownell and Saddler 1987). Water-soluble material was concentrated to 20% w/v solids by rotoevaporation at 55° C and adjusted to pH 5.0 with NH_4OH prior to hydrolysis.

Enzymatic hydrolysis. A hemicellulase preparation from *Trichoderma harzianum* E58 culture filtrate was used for enzymatic hydrolysis (Tan et al. 1987). Concentrated, steam-pretreated aspenwood water-soluble material (14.6% w/v solids) was hydrolysed at 45° C, pH 4.8, using 5000 xylanase units·g^{-1} pentosan. Hydrolysis was stopped after 48 h by boiling the hydrolysate for 15 min.

Acid hydrolysis. Water-soluble material (20% w/v) was mixed with 3% w/v concentrated sulphuric acid and autoclaved for 1 h at 121° C. The resulting hydrolysate was filtered through Whatman No. 1 paper.

Inhibitor removal treatments. Hydrolysates were rotoevaporated under vacuum to near dryness at 55° C to remove acetic acid. Lignin-degradation products were removed by four ethyl acetate extractions (1:1) of the hydrolysates, followed by rotoevaporation to remove the residual solvent.

Combined hydrolysis and fermentation (CHF). Water-soluble material was filter sterilized and diluted to either 2.5% or 5% w/v total solids with sterile fermentation medium containing 0.3% yeast extract and 0.2% diammonium phosphate. The CHF was initiated by simultaneous addition of xylanase and inoculum at the concentrations described above and carried out at 30° C in 250-ml foam-stoppered flasks at 150 rpm. The total reaction volume was 100 ml.

Separate hydrolysis and fermentation (SHF). The same fermentation medium and conditions were used as described for CHF except that the water-soluble material was previously acid-hydrolysed or enzymatically hydrolysed as described above. All fermentations were duplicated and the average results reported. Control fermentations of pure xylose and glucose were also run.

Analytical methods. Acetic acid, ethanol, cellobiose, glucose, xylobiose and xylose were analysed by HPLC (Schwald and Saddler 1988). Sugar- and lignin-degradation products were analysed by HPLC (Burtscher et al. 1987). The dry weight was determined after samples were dried at 105° C for 24 h.

Results and discussion

Combined enzymatic hydrolysis and fermentation (CHF)

This normally offers the advantage of improving enzymatic hydrolysis efficiency by eliminating product inhibiton of xylanase by concomitant xylose fermentation. However, deacetylation of the acetylxylan also occurs during enzymatic hydrolysis, generating acetic acid (Biely et al. 1986). The CHF results obtained during 72 h at 30° C are shown in Table 1. The observed increase in xylo-

Table 1. Combined enzymatic hydrolysis and fermentation of steam-pretreated aspenwood hemicellulose[a]

Substrate (total solids) (% w/v)	Time (h)	Cellobiose (g/l)	Glucose (g/l)	Xylobiose (g/l)	Xylose (g/l)	Ethanol (g/l)	Acetic acid (g/l)
2.5	0	1.0	0.2	1.3	1.8	0.0	2.0
	8	0.6	0.3	5.8	4.0	0.2	2.4
	24	0.4	0.4	5.0	5.4	0.3	2.6
	40	0.3	0.4	4.6	6.3	0.3	2.6
	72	0.2	0.0	3.5	6.3	0.6	2.6
5.0	0	1.6	0.3	1.9	3.7	0.0	4.0
	8	1.2	0.7	11.4	9.4	0.0	4.6
	24	0.9	0.9	9.3	12.7	0.0	5.3
	40	0.5	1.0	7.7	13.9	0.0	5.4
	72	0.3	1.1	6.1	16.0	0.0	5.3
Control (no yeast) 5.0	72	1.8	1.1	6.0	16.0	0.0	5.4

[a] See Materials and methods for details

biose after 8 h, followed by its hydrolysis to xylose indicated both xylanase and xylosidase activities were present during CHF of both hemicellulose concentrations. The hydrolysis of cellobiose to glucose, apparent in the 5% w/v hemicellulose substrate, but not in the control, indicated that β-glucosidase activity was associated with *P. stipitis* and not the *T. harzianum* E58 hemicellulase preparation.

At both concentrations of hemicellulose tested, despite rotoevaporation treatment prior to CHF, the initial acetic acid was at least 2 $g \cdot l^{-1}$, which is sufficient to inhibit xylose fermentation by *P. stipitis* (van Zyl et al. 1988; Parekh et al. 1987). Cellobiose and glucose fermentation occurred at a concentration of 2.5% w/v total solids hemicellulose. For xylose fermentation, however, the CHF approach was unsuccessful at both concentrations of hemicellulose tested. These results identified the need to separately hydrolyse the hemicellulose, using either acid or enzymes, so that the acetic acid generated could be removed prior to fermentation.

Separate hydrolysis and fermentation (SHF)

The SHF results, which compared the fermentability of acid and enzyme hydrolysates before and after inhibitor removal treatments, are summarized in Table 2. Both hydrolysates were fermentable at a concentration of 2.5% w/v total solids. Above 2.5% w/v total solids, the concentration of acetic acid and other inhibitors was apparently sufficient to prevent fermentation.

Both inhibitor removal treatments were capable of improving the fermentability of the 5% w/v hydrolysates. The low initial concentrations of glucose were totally fermented within the first 24 h. The inhibitors that remained following the treatments still significantly decreased the xylose fermentation rates compared to the controls. Analysis of the hydrolysates before and after treatment indicated that furfural and several lignin-degradation products, some of which were identified and quantified, were present in both acid and enzyme hydrolysates. The acid hydrolysate contained significantly higher furfural, indicating that some degradation of pentose sugars had occurred in addition to that resulting from the steam-pretreatment.

The presence of vanillin probably caused some inhibition as Tran and Chambers (1986) reported that 0.09 $g \cdot l^{-1}$ vanillin caused over 50% reduction in ethanol yield from 40 $g \cdot l^{-1}$ xylose

Table 2. Characterization and fermentation of treated hemicellulose hydrolysates from steam-pretreated aspenwood before and after inhibitor removal treatments

Hydrolysate		Inhibitor removal treatment[a]	Initial total xylose[b] (g/l)	Initial total glucose (g/l)	Acetic acid (g/l)	Furfural (g/l)	*p*-Hydroxy-benzoic acid (g/l)	Vanillin (g/l)	Fermentation results[c]			
Type	% (w/v)								Pentose used (%)	$Y_{p/s}$[d]	Q_p[e]	Max. ethanol (g/l)
Acid	2.5	None	12.3	0.9	3.1	0.14	0.53	0.10	100	0.41	0.09	5.4
Acid	5.0	None	23.7	1.7	6.1	0.28	1.07	0.21	0	0	0	0
Acid	5.0	Evaporation	21.8	1.8	2.8	0	1.04	0.15	17	0.13	0.07	3.1
Acid	5.0	Extraction	19.3	1.6	2.7	0	0	0	93	0.41	0.13	8.5
Enzyme	2.5	None	10.5	1.4	2.8	0.02	0.41	0.12	82	0.29	0.03	3.4
Enzyme	5.0	None	20.2	2.6	5.3	0.05	0.82	0.23	0	0	0	0
Enzyme	5.0	Evaporation	21.0	2.8	2.7	0.01	0.81	0.21	77	0.34	0.08	8.0
Enzyme	5.0	Extraction	20.5	2.7	2.7	0	0	0	100	0.47	0.20	11.0
Pure sugar Controls:		None	11.8	2.8	0	0	0	0	100	0.44	0.27	6.4
		None	25.2	5.6	0	0	0	0	100	0.44	0.57	13.6

[a] Inhibitor removal treatments: evaporation — rotoevaporation under vacuum at 55° C; extraction — ethyl acetate extraction followed by rotoevaporation (see text)
[b] Expressed as xylose equivalents; xylose was the only pentose detected in acid hydrolysate; initial xylose:xylobiose ratio was 1.8:1 in enzymatic hydrolysate
[c] Control results after 24 h; acid and enzyme hydrolysate results after 72 h
[d] Ethanol yield (g ethanol/g total fermentable substrates)
[e] Maximum volumetric ethanol production rate (g ethanol/l/h)

by *P. stipitis* CBS 5776. However, even in the presence of 0.21 $g \cdot l^{-1}$ vanillin and other inhibitors, we observed 77% utilization of the xylose in the rotoevaporated enzymatic hydrolysate. Tran and Chambers (1986) also reported that 1.3 $g \cdot l^{-1}$ furfural had little effect on fermentation. For this reason, the low levels of furfural reported here were not considered inhibitory.

The separate inhibitory effect of 0.8–1.0 $g \cdot l^{-1}$ *p*-hydroxybenzoic acid was not determined; however, 1 $g \cdot l^{-1}$ has been shown to inhibit glucose fermentation by *Saccharomyces cerevisiae* (Ando et al. 1986). The synergistic effect of a combination of low levels of these known inhibitors, and other unidentified lignin-derived compounds may cause a greater inhibition than when present individually at less than their inhibitory concentrations (Beck 1986a).

The lower ethanol yield ($Y_{p/s}$) from the rotoevaporated acid hydrolysate compared to the similarly treated enzymatic hydrolysate ($Y_{p/s} = 0.13$ and 0.34 respectively) may be a result of using NH_4OH instead of $Ca(OH)_2$ to adjust the acid hydrolysate to pH 5.0 for fermentation as suggested by others (van Zyl et al. 1988). Other unidentified, non-volatile inhibitors created during the acid hydrolysis may also be responsible for the reduced fermentability.

Ethyl acetate extraction was more effective in removing the inhibitors than rotoevaporation alone, even though residual acetic acid levels were equal. Rotoevaporation removed furfural and most of the acetic acid but, as expected, this treatment did not reduce lignin-derivative levels. Ethyl acetate extraction removed all of the inhibitory compounds except for the residual acetic acid. This level of acetic acid (2.7 $g \cdot l^{-1}$) is sufficient to cause a slower fermentation (i.e., 60–72 h). The control fermentations of similar levels of initial sugars, with no acetic acid present, were completed within 24 h, producing near theoretical ethanol yields and maximum volumetric ethanol production rates (Q_p) almost triple those observed when acetic acid was present.

The fermentation profile for the 5% ethyl acetate extracted acid hydrolysate showed that the fermentation was still incomplete after 72 h (Fig. 1). An 8 h diauxic lag in xylose fermentation was observed as the 1.6 $g \cdot l^{-1}$ initial glucose was fermented (data not shown). The xylose uptake rate was estimated from Fig. 1 to be 0.24 $g \cdot l^{-1}$ per hour.

The 5% ethyl-acetate-extracted enzymatic hydrolysate fermentation profile showed that all fermentable sugars were consumed before 72 h

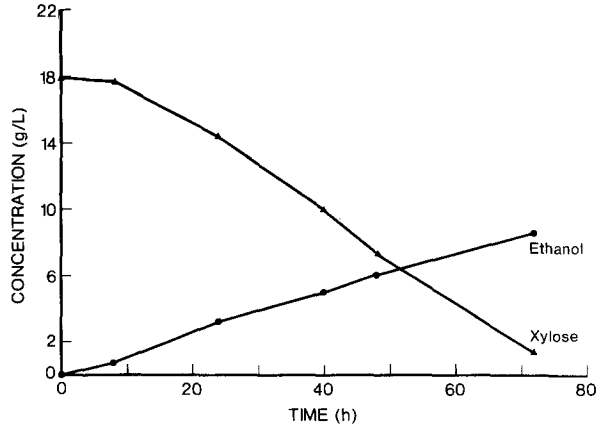

Fig. 1. Fermentation of 5% w/v steam-pretreated aspenwood hemicellulose *acid* hydrolysate by *Pichia stipitis* after ethyl acetate extraction to remove fermentation inhibitors

(probably closer to 60 h if we interpolate between sample points) (Fig. 2). Both xylose and xylobiose were fermented concurrently after an 8 h diauxic lag caused by 0.8 $g \cdot l^{-1}$ glucose initially present (data not shown). The xylose uptake rate estimated from Fig. 2 (0.32 $g \cdot l^{-1}$ per hour) was 2.7 times that of xylobiose (0.12 $g \cdot l^{-1}$ per hour) during the first 40 h, but the uptake rates became near equal after 40 h when both xylose and xylobiose concentrations were 3.6 $g \cdot l^{-1}$.

The 1.8 $g \cdot l^{-1}$ cellobiose present in the hemicellulose was probably a contaminant from the cellulose water-extraction step or the hemicellulase enzyme preparation. Cellobiose fermentation occurred after 40 h, at which point both xylose and xylobiose concentrations had been reduced to 3.6 $g \cdot l^{-1}$ (Fig. 2).

This is the first report of xylobiose fermentation by *P. stipitis*. Xylobiose has been shown to be

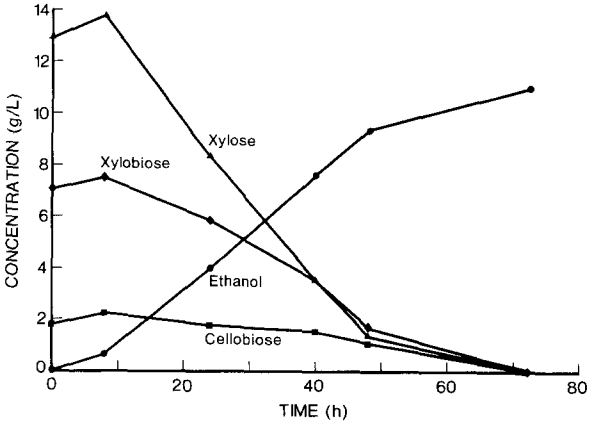

Fig. 2. Fermentation of 5% w/v steam-pretreated aspenwood hemicellulose *enzymatic* hydrolysate by *P. stipitis* after ethyl acetate extraction to remove fermentation inhibitors

an intracellular inducer of endo-1,4-β-xylanase in the yeast *Cryptococcus albidus* (Biely et al. 1980). *Pichia stipitis* CBS 5776 has been shown to produce low levels of endo-1,4-β-xylanase activity when grown on xylan (Lee et al. 1986). The ability to ferment xylobiose indicates that *P. stipitis* produces an intracellular β-xylosidase activity similar to that reported for *C. albidus* (Biely et al. 1980).

In conclusion, the results of this study confirm that *P. stipitis* CBS 5776 is capable of fermenting the hemicellulose hydrolysates of steam-pretreated aspenwood to produce theoretical ethanol yields, provided that the hydrolysates are suitably treated to remove fermentation inhibitors. Ethyl acetate extraction was more effective than rotoevaporation alone for reducing fermentation inhibition. Enzymatic hydrolysis of the hemicellulose produced fewer inhibitors than acid hydrolysis and allowed a faster fermentation to theoretical ethanol yields. Despite incomplete enzymatic hemicellulose hydrolysis, the xylobiose in the hydrolysate was fermented by *P. stipitis* concurrently with xylose, although at a reduced rate.

Our results with aspenwood support the conclusion that the wood pretreatment and inhibitor removal methods used in preparing hemicellulose hydrolysates should be capable of minimizing the level of residual acetic acid and lignin-degradation products. This is especially important if higher concentrations of hemicellulose hydrolysates are to be fermented by *P. stipitis* to produce economically recoverable ethanol concentrations in the final beer.

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