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Fermentation of Xylose and Cellobiose by *Pichia stipitis* and *Brettanomyces clausenii*

Scientific Note

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INTRODUCTION

The direct bioconversion of substrates of cellulosic origin for the manufacture of liquid fuels such as ethanol is a subject of growing interest. One approach to the conversion of cellulosic material to ethanol comprises pretreatment of the lignocellulosics followed by enzymatic saccharification and fermentation of the sugars so produced to ethanol (*1*). Enzymatic saccharification is promising since the process is nonpolluting and requires mild conditions for hydrolysis and inexpensive equipment. Also, total saccharification can be achieved, because of the high selectivity of the enzymes (*2*). However, conversion times are much longer than for acid hydrolysis, and the cost of enzymes is high, thereby requiring recycling of the enzymes for process economy (*3*). These cellulases are also deficient in β-glucosidase, resulting in an accumulation of cellobiose during saccharification in addition to glucose. The accumulation of glucose and cellobiose inhibits the activity of the cellulase complex and decreases the rate of cellulose hydrolysis (*3*). Supplementation of β-glucosidase from other sources to the cellulase complex has been a prerequisite

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to alleviate the inhibition by preventing excessive cellobiose accumulation (*4*).

In addition to cellobiose, hydrolysis of biomass yields varying amounts of D-glucose, D-xylose, D-mannose, D-galactose, and L-arabinose, depending upon the hydrolysis procedure and the nature of the feedstock (*2*). The extent to which these sugars can be fermented to ethanol by yeasts is of paramount importance for commercial exploitation of lignocellulosic biomass to fuel energy.

A few cellobiose-fermenting yeasts have been identified, namely in the *Torulopsis, Brettanomyces,* and *Candida* species (*5,6*). Other investigators have shown that yeasts that can ferment cellobiose to ethanol have a poor capacity for xylose fermentation (*7*). Recently, strains of *Candida tenuis, Candida shehatae,* and *Pichia stipitis* (*9*) have been identified that ferment cellobiose, xylose, and other wood sugars (*8,9*). Also, improved strains of *P. stipitis* and *C. shehatae* have been reported (*10*) that are capable of fermenting pentose-containing lignocellulosic liquors rapidly and efficiently. Such yeasts provide a unique opportunity for integrating the hydrolysis of sugars and their fermentation into a single operation, "Simultaneous Saccharification and Fermentation (SSF)" (*11*).

Although baker's yeast could successfully be applied in eliminating inhibition by glucose in the SSF process, cellobiose accumulation still occurs and inhibits hydrolysis. Since cellulose hydrolysis is the rate limiting step in the SSF process, means of removing cellobiose is desired other than supplementation of β-glucosidase for process economy.

Incorporating yeasts capable of fermenting cellobiose and xylose and other wood sugars directly to ethanol seems an attractive alternative. Such yeasts would allow SSF of the hemicellulose and cellulose fractions, increase the rate of cellulose hydrolysis, and possibly reduce the amount of enzymes. *P. stipitis* CBS 5776 and *P. stipitis* "R" (*10*) offers these merits, however, they are slow cellobiose fermenters. *B. clausenii* (*12*) is a potent glucose and cellobiose fermenter but is deficient in fermenting pentose sugars. The objective of the present study is to evaluate the performance of pentose and cellobiose fermenting yeasts in cocultures for the direct fermentation of wood sugars at rapid and efficient rates, and the possible application of these mixed cultures in the SSF process for lignocellulosics.

We have developed a successful process for producing ethanol utilizing SO_2 prehydrolyzed biomass feedstock coupled with enzymatic saccharification and fermentation using *P. stipitis*. However, the conversion times for the process, including hydrolysis and alcoholic fermentations, were about 96 h (*1*), which is longer than desirable.

This research is based on the hypothesis that the hydrolytic action of cellulase is very rapid, that the inhibitory effect of cellobiose is also very rapid, and that the removal of this inhibitor as soon as it is formed by a cellobiose-fermenting yeast would facilitate the speedy conversion of cel-

lulose to ethanol. This hypothesis is illustrated in Fig. 1. The figure is intended to illustrate the formation of cellobiose as the endproduct of cellulose hydrolysis by cellulase, and the reversible or limited nature of that reaction. The hydrolysis of cellobiose by β-glucosidase is limited by the endproduct glucose. The result is that hydrolysis of cellulose by a mixture of these enzymes is slow and incomplete. The figure shows that in the simultaneous saccharification-fermentation (SSF) mode, where both cellulase and *B. clausenii* are present, cellulose is converted directly to ethanol, there being no possibility of endproduct repression since the cellobiose is fermented as formed. Figure 1 also illustrates SSF of biomass, in the form of SO_2-prehydrolyzed wood or agricultural residues. In this case, in addition to the cellobiose-fermenting yeast *B. clausenii*, the pentose-fermenting yeast *P. stipitis* is needed, and the mixed culture together with the enzyme convert the SO_2-prehydrolyzed feedstock directly to ethanol. This is an outline of the hypothesis on which this research report is based.

The optimum temperature for hydrolysis by cellulase is about 50°C, which is too high a temperature for our best cellobiose-fermenting yeast, *B. clausenii*, which ferments best at about 30°C, and a compromise temperature had to be chosen. We found that at 38°C the rates of hydrolysis and of fermentation were compatible. Also, since we used SO_2-pretreated aspen wood as the substrate, there was much xylose present, and this is not fermented by *B. clausenii*. *P. Stipitis* R is an excellent xylose fermenter, but not very efficient in fermenting cellobiose. A mixed cul-

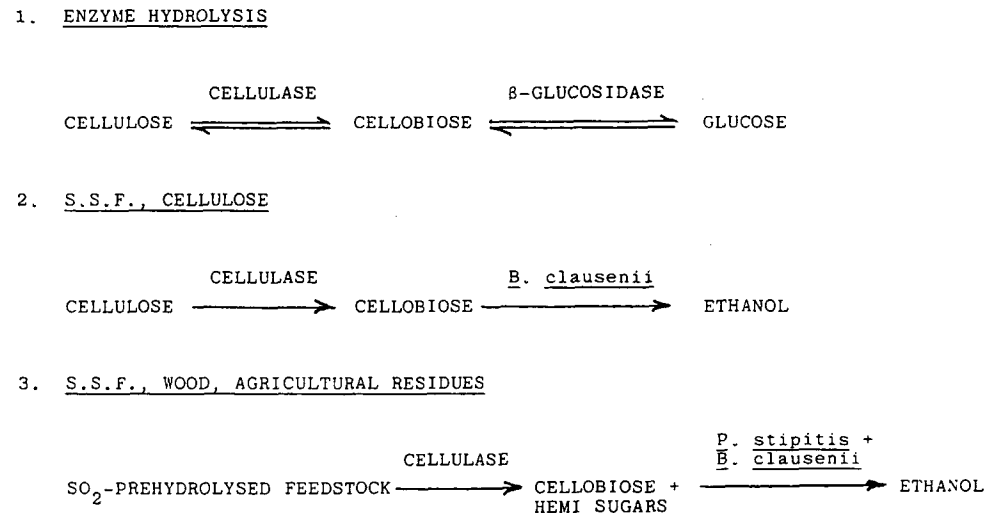

Fig. 1. Enzyme hydrolysis and simultaneous saccharification-fermentation.

ture of this yeast with *B. clausenii* would achieve the double purpose of removing by fermentation cellobiose and of converting xylose rapidly and efficiently to ethanol. The procedure adopted to test this hypothesis was to add to SO_2-pretreated aspen wood—which had not been washed and still had the hemicellulose sugars in place—cellulase, and at the same time a mixed culture of these two yeasts. The results were excellent: in a 48-h simultaneous saccharification and fermentation, 384 L ethanol/t dry wt of aspen were obtained, with 90% of this (345 L/t) being obtained in 24 h. These are the highest yields so far reported of ethanol from aspen wood.

MATERIALS AND METHODS

Microorganisms

Brettanomyces clausenii NRRL Y-1414 was obtained from Northern Regional Research Laboratories, USDA, Peoria, IL. It was maintained on YM slant with 2% glucose as the carbon source. *Pichia stipitis* CBS 5776 was obtained from Centraal Bureau Voor Schimmelcultures (CBS), Yeast Division, Delft, The Netherlands. The R strain of *P. stipitis* was obtained by adapting the parent strain (CBS 5776) to the wood hydrolysates by semiaerobic cultivation for 24 h, centrifuging and recycling the yeast many times. The adapted strain is referred to here as *P. stipitis* R (*10*). Both *Pichia stipitis* strains were maintained on YM agar slant with xylose as the carbon source.

Culture Media

Du Preez's medium CA containing casamino acids (Difco, Vitamin free), added vitamins and minerals and adjusted to pH 5.0 was used (*9*). When added, cellobiose was used at 20 gL^{-1}. The wood sugar mixtures used as substrate contained 20 gL^{-1} D-glucose, 20 gL^{-1} D-xylose, 10 gL^{-1} each of D-cellobiose, D-galactose, and D-mannose. The sugars were autoclaved separately from the medium constituents.

Inoculum and Fermentation Conditions

The inoculum was prepared in 250 mL Erlenmeyer flasks with 100 mL of the medium containing 40 gL^{-1} xylose (in case of *P. stipitis* strains) and 40 gL^{-1} glucose (in case of *B. clausenii*), as the carbon source, and grown for 48 h at 30°C on a rotary shaker at 150 rpm. Cells were harvested by centrifugation, washed twice with saline, and inoculated in shake flasks at the dry cell concentrations described in the text. The fermentation conditions were the same when evaluating the performance of the parent and the R strains of *P. stipitis* and the mixed cultures on simulated wood sugar fermentation. All shake flask fermentations having xylose sugar were performed under semiaerobic conditions at 30°C, as described earlier, (*10*) and under anaerobic conditions by *B. clausenii* with cellobiose.

Simultaneous Saccharification and Fermentation (SSF) of SO_2 Prehydrolyzed Aspen Wood

Aspen wood chips were prehydrolyzed using 3% SO_2 (w/w) as the hydrolyzing catalyst at 150°C for 30 min (*13*). The pH of the prehydrolysates containing residual fiber in suspension was raised to 4.8 with $Ca(OH)_2$, the liquid/solid ratio being 10:1. The enzyme system contained cellulase (Meiji Seika), and hemicellulase (Miles) along with vitamin B_{12} and trace elements as promoters (*10*). No addition of β-glucosidase was made. The total enzyme added was 15% (w/w) enzyme on fiber. SSF studies with added cellobiose at 20 gL^{-1} were also made. Saccharification without any microorganism was also performed in parallel to monitor the concentrations of sugars produced on hydrolysis. All SSF experiments were performed in duplicate in a constant temperature water bath at 38° and 125 rpm.

Analysis

Individual wood sugars were determined by HPLC (Waters Sugar Analyzer 1) using an Aminex HPX-87P column (Bio-Rad) with water as the mobile phase at 65°C, flow rate .5 mL/min, using a refractive index detector (Waters 401 R1). Ethanol was determined by GC.

RESULTS AND DISCUSSION

Fermentation of Xylose

Since the economic feasibility of using hydrolyzed biomass in an industrial fermentation process depends not only on the successful and rapid fermentation of all the wood sugars but also on the tolerance of these yeasts to toxic substances, adaptation of *P. stipitis* 5776 to these hydrolysates by repeated recycling was carried out to get strains that would overcome these inhibitions and limitations (*10*). Improvement in ethanol yields utilizing recycled cells in the fermentation of lignocellulosics by *Pachysolen tannophilus, Candida tropicalis,* and *C. shehatae* have also been reported (*7,10*).

The ability of *P. stipitis* to ferment xylose before and after adaptation to wood hydrolysate is shown in Table 1 and Fig. 2. The designation *R* is assigned to the adapted strain, (*10*) which has quite different genetic characteristics from the unadapted strain (*14*). The adapted strain utilizes more xylose than the unadapted strain, according to the results in Table 1, resulting in higher ethanol concentration, but the efficiency in terms of ethanol production from the sugar utilized is not greater. When glucose is also present, as shown in Fig. 2, which illustrates fermentation of a 70:30 glucose-xylose mixture, fermentation by the R strain is much faster. In Fig. 2, the sugar solution was about 140 gL^{-1}, which was reduced to 20 gL^{-1} in 24 h by the R strain, a level not attained in 72 h by the

Table 1
Fermentation of D-Xylose and Aspen Wood Hydrolysates by Adapted *Pichia stipitis*

Substrate	Strain	Substrate utilized, %	Ethanol, gL^{-1}	Yield, g ethanol/g sugar utilized
Xylose[a]	*P. stipitis* CBS 5776	73	34	.44
	P. stipitis R	88	40	.44
Aspen wood hydrolysate[b]	*P. stipitis* CBS 5776	78	29	.41
	P. stipitis R	95	41	.47

[a]Initial xylose was 105 gL^{-1}, results reported at 96 h.
[b]Initial total reducing sugars was 92 gL^{-1}, results reported at 48 h.

CBS strain. When aspen wood hydrolysate, obtained by SO_2-prehydrolysis followed by enzyme hydrolysis, was fermented by these yeasts, substrate utilization, efficiency, and ethanol concentration were all much improved by the R strain. In this case, 95% of the sugars were utilized of an

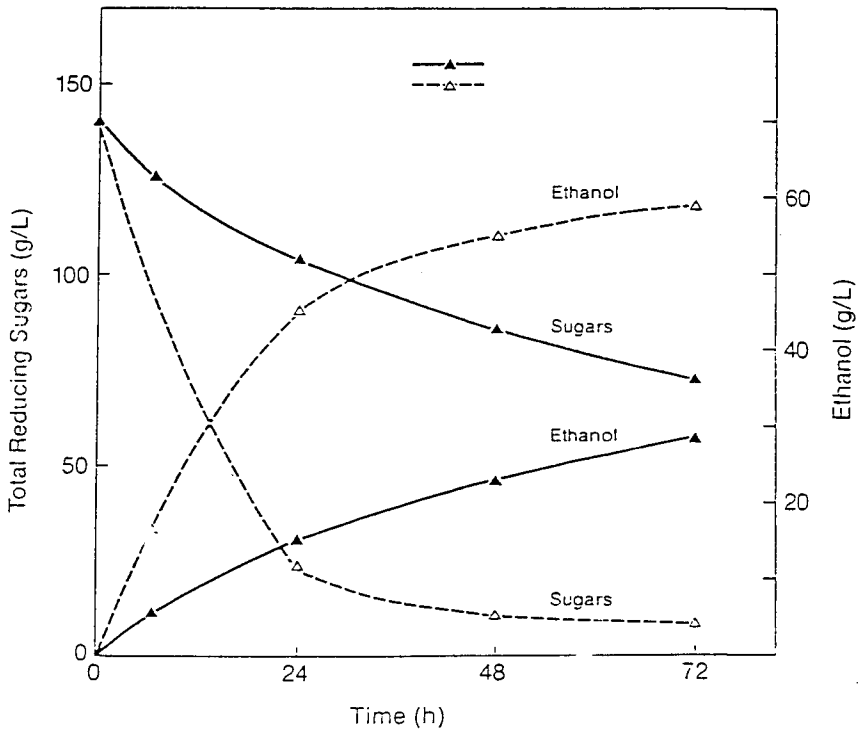

Fig. 2. Comparison of ethanol fermentation of 70:30 glucose:xylose mixtures by *Pichia stipitis* CBS5776 and *P. stipitis* R, cell concentration 8.5 g dry cell L^{-1}

initial concentration of 92 gL^{-}, and ethanol concentration was 5.3% by vol.

Low conversion yields and ethanol concentrations found with unadapted fresh cultures were easily overcome by "strain improvement" using the cell recycling technique. How these high ethanol producing yeast strains are generated upon repeated recycling is not known. It may be that recycling results in selection of an adapted strain with derepression of the enzymes required for efficient pentose fermentation (*14*).

Fermentation of Cellobiose

The ability of various strains of *Candida shehatae*, *P. stipitis*, and *B. clausenii* to produce ethanol from 25 gL^{-1} cellobiose is presented in Table 2. Four of the strains shown, *C. shehatae* ATCC 22984, *P. stipitis* CBS 5776, *P. stipitis* Y633, and *B. clausenii* Y1414 were able to utilize all of the cellobiose present. Of these, the *B. clausenii* was the most rapid and most efficient, 100% cellobiose fermentation occurring in 20 h, giving a yield of .47 g ethanol/g sugar consumed (84% of theory). In the case of *P. stipitis*, some rate limiting step of cellobiose utilization resulted in slower ethanol production. Disaccharides are either split outside or may be taken up intact and hydrolyzed inside the cell. Either extracellular hydrolysis or uptake or both could thus be the rate limiting step in cellobiose fermentation by *P. stipitis* 5776.

Fermentation of a Simulated Wood Sugar Mixture

The five wood sugars, namely D-glucose, D-mannose, D-galactose, D-xylose, and D-cellobiose were used in a mixture simulating wood

Table 2
Ethanol Production from 25 g/L Cellobiose by Various Yeasts

Yeast strains	Fermentation time, h	Substrate utilized, %	Final ethanol concentration, g/L	Ethanol yield, g ethanol/ g substrate utilized
C. shehatae	65	22	0	0
C. shehatae 57	64	15	0	0
C. shehatae ATCC 22984	48	100	5.8	.23
P. stipitis CBS 5776	48	100	10.3	.41
P. stipitis CBS 5773	64	92	2.0	.09
P. stipitis Y633	48	100	7.4	.30
B. clausenii Y1414	200	100	11.7	.47

hydrolysate in order to determine the utilization pattern that could be expected during SSF of lignocellulosics. The results are shown in Table 3. Both yeasts initially utilized glucose preferentially, and the presence of glucose appeared to retard the fermentation of mannose and galactose more severely in case of *B. clausenii* than with *P. stipitis* 5776 (data not shown). Also, with *P. stipitis*, utilization of the other sugars commenced after glucose was depleted and thereafter simultaneous fermentation of galactose, mannose, xylose, and cellobiose occurred. The ethanol yield coefficient was similar for both yeasts (.43–.44 g/g sugar consumed), but the failure of *B. clausenii* to ferment xylose is reflected by the 29 gL^{-1} ethanol produced by *P. stipitis* 5776 compared to 21 gL^{-1} by *B. clausenii* under the same conditions.

Using mixed cultures of *B. clausenii* and *P. stipitis* 5776, each at 2.5 gL^{-1} initial cell concentration, almost complete fermentation of simulated wood sugars was achieved, with a yield of .48 or 92% of theoretical. Ethanol yield was higher in the mixed culture (.48) compared to .42 with *P. stipitis* or .31 with *B. clausenii*. These results reflect the advantage of cocultures of *B. clausenii* and *P. stipitis* in SSF for successful and rapid fermentation of lignocellulosics.

SSF Process

In order to overcome the inhibiting influence of cellobiose on cellulase, wood sugar hydrolysates were fermented simultaneously with saccharification. Neither the optimum temperature for cellulase activity, 50°C, nor for yeast growth, 30°C, were used; instead, these SSF runs were made at 38°C, as high as thought feasible for the fermentation. Cellulase, hemicellulase, and either *P. stipitis* CBS 5776 or mixed cultures were added. The results are shown in Tables 4 and 5, and Fig. 3.

Sugar production profiles from SO_2-prehydrolyzed aspen wood are shown in Fig. 3. Over 80% of the total sugars in aspen wood were hydrolyzed in the first 24 h, with a very slow increase thereafter. This slow rate may be a result of the low temperature of saccharification, together with the lack of β-glucosidase. At the end of 48 h, about 680 kg total sugars/t of aspen wood were obtained, or about 90% of theoretical yield.

The results of ethanol production using mixed cultures of *P. stipitis* and *B. clausenii* in the SSF mode are presented in Table 4. All fermentations were 90% complete in the first 24 h and slower thereafter, possibly because of inhibition of cellulase by ethanol. Mixed cultures of *P. stipitis* R and *B. clausenii* gave better fermentation rates and a yield of 384 L ethanol/t aspen wood in 48 h (85% of theory). This is believed to be the highest yield yet reported from aspen wood. Mixed cultures of *P. stipitis* 5776 and *B. clausenii* were slightly inferior, giving 365 L/t. *P. stipitis* 5776 in pure culture was less efficient, resulting in only 73% theoretical ethanol yield. This incomplete fermentation is attributed to the lack of toler-

Table 3
Ethanol Yields from Simulated Wood Hydrolysate by *P. stipitis* and *B. clausenii* and the Mixed Cultures of Both Organisms

Yeast cultures	Cell concentration, gL^{-1}	Fermentation time, h	% substrate utilized[a]	Cellobiose utilized, %	Ethanol, gL^{-1}	Ethanol yield, g/g sugar consumed	Final yield, g ethanol/g total sugar present
B. clausenii	2.5	48	71	100	21.4	.43	.31
P. stipitis CBS 5776	2.5	48	95	90	29.0	.44	.42
P. stipitis and *B. clausenii*	2.5 + 2.5	48	98	100	33.0	.48	.48

[a]70 gL^{-1} wood sugars (D-xylose 20 gL^{-1}, D-glucose 20 gL^{-1}, D-mannose 10 gL^{-1}, D-cellobiose 10 gL^{-1}, D-galactose 10 gL^{-1}).

Table 4
Ethanol Concentrations at Various Times, SSF with Mixed Cultures of *P. stipitis* and *B. clausenii* on SO_2 Prehydrolyzed Aspen Wood, Containing 70 gL^{-1} Total Reducing Sugars

Yeast culture	Cultural conditions	Ethanol, gL^{-1}						% Substrate utilized	Ethanol yield, g/g sugar consumed	Final ethanol conversion, L/t aspen	Ethanol yield on wood % of theory
		T = 2	8	10	24	28	48				
P. stipitis CBS 5776	18 gL^{-1} cells[a]	1.6	15	18	23	24	26	85	.42	328	73.2
P. stipitis CBS 5776 + *B. clausenii*	16 gL^{-1} cells[a]	1.8	17	20	23	28	29	92	.44	365	81.5
P. stipitis R + *B. clausenii*	16 gL^{-1} cells[a]	2.3	19	21	28	29	30	93	.46	384	85.0
P. stipitis CBS 5776 + *B. clausenii*	18 gL^{-1} cells[b]	2.0	20	23	29	31	36	90	.44	461[c]	79.0

[a]5 g SO_2 prehydrolyzed aspen wood, 10% dry solids, du Preez salts, 15% enzyme loading (hemicellulase + cellulase).
[b]The above conditions plus 20 gL^{-1} cellobiose or 20% on wood.
[c]Of which 96 L are attributable to cellobiose.

ance of this yeast to inhibitors in the wood hydrolysate. Addition of cellobiose at 20 gL^{-1} or 20% on wood in the SSF process also resulted in high overall ethanol yields and confirmed that if cellobiose accumulated during the course of saccharification in absence of β-glucosidase it would be rapidly converted to ethanol by these cellobiose-fermenting yeasts. No cellobiose was detected in the fermented hydrolysate at the end of 48 h.

The two major cellulose hydrolysate sugars, glucose and cellobiose, were each fermented by *B. clausenii* or *P. stipitis*. However, the higher ethanol production rate on cellobiose obtained with *B. clausenii*, which has β-glucosidase activity, is advantageous, and the ability to ferment cellobiose rapidly improves hydrolysis and ethanol yields.

P. stipitis 5776 poses the seemingly rare ability to ferment both xylose and cellobiose effectively and compares favorably with other pentose fermenting yeasts used in lignocellulosic fermentation. Some particular problems are sometimes encountered when attempting to ferment all major wood sugars. For example, with *C. tropicalis*, depletion of glucose and mannose results in consumption of ethanol before the onset of galactose and xylose utilization (*15*). Diauxie has also been shown to cause a considerable lag period during the fermentation of sugar mixtures by *P. tannophilus* and appears to be a common phenomenon during the fermentation of xylose or cellobiose in presence of glucose (*16*). However, *P. stipitis* 5776 does not exhibit such an effect, and fermentation of xylose and cellobiose commences immediately after glucose depletion.

Experiments with mixed cultures of *P. stipitis* and *B. clausenii* in simulated wood sugar fermentations (Table 4) produced 12% more ethanol than *P. stipitis* 5776 alone, suggesting that the use of such mixed cultures would be an advantage in a rapid SSF process.

Table 5 shows the results of the application of mixed culture fermentation of SO_2-prehydrolyzed woods where the pretreatment was carried out on the pilot scale. Aspen wood was prehydrolyzed with SO_2 in a Wenger reactor at the rate of 100 kgh^{-1}, treatment with 2.5% SO_2 being at 200°C for 45 s. Mixed coniferous woods (*1*) were prehydrolyzed in a Stake reactor with 2.6% SO_2 at 208°C for 2 min. Neither of these runs can be considered optimized, being short runs with little variation in conditions. Parallel tests were carried out in the laboratory for sugar production and for ethanol production. The enzyme used in all cases contained no β-glucosidase, but was a mixture of cellulase and hemicellulase. Sugar production in 48 h was 693 and 717 kg/t for the two feedstocks. Upon SSF with a mixed culture of *B. clausenii* and *P. stipitis*, ethanol production from the SO_2-prehydrolyzed wood was 369 and 360 h/t in 48 h, and was about 80% complete in 24 h.

We have earlier reported a process for producing ethanol utilizing SO_2 prehydrolyzed biomass feedstock coupled with enzymatic hydrolysis and the use of pentose fermenting yeast (*1*). However, the total time

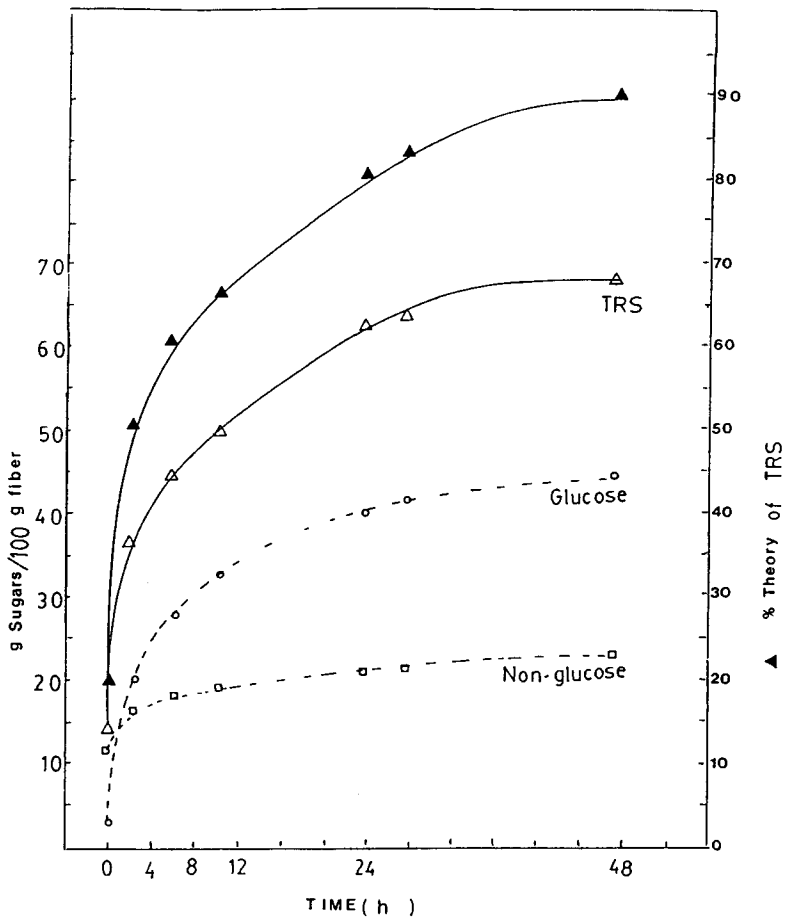

Fig. 3. Time course of saccharification of SO_2-prehydrolyzed aspen wood by cellulase and hemicellulase at 38°C, pH 4.8.

for the process was 96 h. The present work has shown that yeasts capable of robust cellobiose and pentose fermentation perform faster and better in coupled wood saccharification and fermentation systems. Applying the SSF process to SO_2 prehydrolyzed aspen wood with cellulase and hemicellulase but without β-glucosidase, and employing mixed cultures of *P. stipitis* R and *B. clausenii* not only produced ethanol at a faster rate but also resulted in 5–14% more ethanol compared to *P. stipitis* 5776, and *B. clausenii*, and to *P. stipitis* 576 alone. Added cellobiose in the SSF process (Table 4) also fermented without difficulty, indicating that cellobiose inhibition can be overcome by use of a potent cellobiose fermenter. Also, no problems were encountered during the operation at 38°C, thereby indicating the possibility of the application of these yeasts in SSF of lignocellulosics.

Table 5
Ethanol Yields from SO_2 Prehydrolyzed Aspen and Coniferous Woods: Simultaneous Saccharification-Fermentation with Mixed Cultures[a]

	Total sugars, parallel saccharification, kg/t		Ethanol, L/t	
	24 h	48 h	24 h	48 h
Aspen	658	693	287	369
Coniferous woods	580	717	297	360

[a]Enzyme saccharification: Meicelase + hemicellulase, 12%/fiber, 12% solids; mixed culture: *Brettanomyces clausenii* + *Pichia stipitis* R, 16 gL^{-}; pH 4.8, temperature 38°C; aspen: 2.5% SO_2, 200°C, 45 s, Wenger reactor; coniferous woods: 2.6% SO_2, 208°C, 2 min, Stake reactor.

CONCLUSIONS

This paper draws attention to the fact that successful SSF processing of lignocellulosics with high pentosans can be achieved rapidly and efficiently without β-glucosidase supplementation when pentose fermenting yeast is coupled with a cellobiose fermenter.

B. clausenii was identified as a potent cellobiose fermenter. All the major sugars that would be released on saccharification of biomass were fermented by *P. stipitis* 5776. Strain improvement of *P. stipitis* through recycling represented a useful technique and resulted in rapid fermentation of lignocellulosic hydrolysates. A mixed culture of *P. stipitis* R and *B. clausenii* in a single vessel in a SSF mode converted the SO_2 prehydrolyzed aspen wood to monomeric wood sugars and the resulting wood sugars to ethanol at a rapid and efficient rate, with yields that are higher than any reported to date. This also shows for the first time the application of coupled pentose and cellobiose fermentation in SSF process, eliminating the need for β-glucosidase supplementation.

Further investigation aimed at eliminating the use of hemicellulase and reducing the amount of cellulase are in progress.

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