Simultaneous Saccharification and Fermentation of Cellulose with the Yeast *Brettanomyces clausenii*

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Summary

The cellobiose-fermenting yeast *Brettanomyces clausenii* was evaluated for the simultaneous saccharification and fermentation (SSF) of cellulose to ethanol. Three cellulases were used in combination with this yeast: Novo SP-122, Rut C-30, and Genencor 150L; and the results were compared to identical experiments with *Saccharomyces cerevisiae*. *B. clausenii* performed better than *S. cerevisiae* for cellulases low in β-glucosidase activity. The best performance for both SSF and straight saccharification was with the 150L cellulase, and a mixed culture of the two yeasts studied gave better performance at 37°C than either yeast alone or straight saccharification at 50°C.
Keywords: *Brettanomyces clausenii*, cellobiose, cellulose hydrolysis, enzymatic hydrolysis, simultaneous saccharification and fermentation.

INTRODUCTION

Although the value of lignocellulosic materials is limited to the low price of their principal solid fuel competitor, coal, biological conversion processes can directly transform the unique, complex structure of lignocellulose into the most valuable of fuels, substitutes for petroleum. In particular, the cellulosic fraction of this substrate can be hydrolyzed to produce sugars that are fermentable into ethanol, a clean burning fuel that can be blended with gasoline to enhance octane ratings or used directly as a neat fuel. Both acids and enzymes catalyze the breakdown of cellulose into glucose, but enzymes offer two major advantages: less expensive equipment can be used at the milder hydrolysis conditions required and the high selectivity of the enzymes makes very high sugar yields possible. However, sulfuric acid in particular is very inexpensive and rapidly hydrolyzes cellulose. Therefore, for enzymatic processes to compete, the cost of enzymes must be low, the rate of hydrolysis must be rapid, and high yields must be achieved.

The simultaneous saccharification and fermentation (SSF) process was first studied by Takagi et al.[1,2] for cellulose conversion to ethanol. In this configuration, both enzyme and yeast were added to the same fermenter as the cellulosic substrate, allowing one-step production of ethanol in the system. The SSF process offers at least three potential benefits compared to the conventional two-step

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approach: one fermenter and associated equipment are eliminated, the presence of ethanol in the fermentation broth reduces the possibility of contamination, and end-product inhibition of cellulase by the product sugars is substantially reduced.

The last feature offers an attractive route to increasing hydrolysis rates and achieving high ethanol yields. This is particularly beneficial in the case of cellobiose, since it strongly inhibits the cellobiohydrolase activity. Although ethanol has also been shown to inhibit cellulase activity,[3–6] the effect of ethanol is somewhat less than that of either glucose or cellobiose, and since stoichiometrically about half as much ethanol is formed as glucose is consumed, the rates are significantly higher for SSF than for a straight saccharification process operating at the same conditions.

A number of studies of SSF have been conducted since the initial research of Takagi et al. *Candida brassicae* is generally accepted as the yeast of choice,[2,5,7–11] although both *Saccharomyces cerevisiae* and *S. carlsbergensis* have been found to offer similar rates.[1–3,7–9,12–14] Several other yeasts as well as the bacteria *Zymomonas mobilis* have been studied with cellulase from *T. reesei* mutants for SSF processes.[13,15–20] However, although the rates of hydrolysis in SSF for each of these microorganisms have been shown to be greater than for straight saccharification at the same conditions, they nevertheless appear slower than for saccharification at the more optimum temperature for cellulase of 45–50°C. In one study, a strain of yeast was found that is more compatible with the higher temperatures preferred for hydrolysis, but ethanol yields and concentrations were limited.[21] Researchers have also examined several combinations of enzymes with *Z. mobilis, S. cerevisiae,* and other ethanol producers,[13,17,18,22–25] but they have only considered substrate levels lower than necessary to prove economic viability. Since a variety of substrates, pretreatment methods, enzymes, and yeasts were employed in all of the SSF studies, it is difficult to generalize from the results. However, the cellulose conversions achieved in these studies are not significantly better than those possible with the faster-acting and cheaper acids that can be employed, and the substrate levels are typically so low that the processing equipment will be too large and costly.

Unless enzymes costs are lowered markedly, enzyme recycle will be necessary to achieve economic viability for liquid fuels production from lignocellulosic substrates. Current enzyme recovery options generally rely on adsorption of the enzymes on the cellulosic substrate in a countercurrent flow scheme that is not able to recover β-glucosidase. Thus, beyond the goal of improving ethanol yields from the cellulosic component and processing higher substrate levels, the SSF fermentation must be capable of functioning with some portion of the enzyme mixture being recycled, resulting in a lower level of β-glucosidase activity. Although this problem may be overcome by adding supplementary β-glucosidase to the mixture from an organism such as *Aspergillis niger,*[3,15] incorporating a yeast capable of directly fermenting cellobiose and perhaps higher cellodextrins to ethanol would decrease the processing costs.

Several studies of cellodextrin-fermenting yeasts have been performed for fermentation of soluble oligosaccharides,[26–37] and the types of enzymatic activ-

ities have been determined for many such yeasts. Lastick et al.[33,34] examined ten strains of yeast from the genera *Brettanomyces, Candida,* and *Torulopsis* using 10% and 15% concentrations of cellobiose as the substrate. Of those evaluated, *B. clausenii* fermented cellobiose rapidly to the highest ethanol yields at 30 and 37°C, while *Candida lusitaniae* fermented well at 41°C for lower ethanol concentrations. Based on these results, the screening study reported in this paper was directed to determine whether *B. clausenii* would provide the ethanol yields and fermentation rates, as well as process the substrate concentrations, deemed desirable when applied to a SSF process rather than the simple cellobiose fermentations previously examined.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Sigma Chemical Company; prepared media were from Difco with the exception of Sigmacell 50 (Sigma), the substrate. *Brettanomyces clausenii* strain Y-1414 was obtained from the Northern Regional Research Laboratories, U.S.D.A., Peoria, IL. The *B. clausenii* used in these studies was derived from this strain through subculturing in SSF, which resulted in improvement in the initial rate of cell growth. *Saccharomyces cerevisiae* (D_5A) was derived by genetic improvements from commercial Red Star baker's yeast. The SP-122 and 150L cellulase enzymes were from Novo Laboratories, Wilton, CT, and from Genencor, Inc., San Francisco, CA, respectively. Rut C-30 enzyme was prepared in our laboratory from ATCC strain No. 56765 of *Trichoderma reesei* (Rockville, MD). The Sigmacell 50 used for substrate has a crystallinity index of around 85 and is relatively pure cellulose powder containing less than 3% contaminating xylose.

Methods

Small-scale 100 mL SSFs were carried out for screening performance in 250 mL flasks with stoppers constructed to vent CO_2 through a water trap. These flasks were agitated at 150–200 rpm in a shaker incubator at 30 and 37°C. To obtain more data on the most promising enzyme yeast combinations, several larger 3 L SSFs were run in Braun Biostat S fermenters agitated at 100–150 rpm. In these fermenters, the pH is controlled by automatic pumps and the temperature maintained via a thermostat vessel. Both small and larger SSFs were run with 1% yeast extract, 2% peptone, 1% glucose, and 0.5% cellobiose, and 10% or 15% (w/v) Sigmacell 50. In most cases, a mixture of penicillin and streptomycin at 10 mg/L was added along with a lipid mixture of ergosterol (5 mg/L) and oleic acid (30 mg/L). All fermentations were inoculated at 1:10 yeast/total volume ratio.

For both the small and large-scale SSFs, the yeasts became anaerobic on their own, using the oxygen left in the system initially to accelerate biomass buildup.

Enzyme loadings were typically 13 IU/g of cellulose, but in small-scale SSFs, they ranged from 7–39 IU/g, where IU refers to International Units of filter paper activity measured in micromoles of glucose equivalent per minute.[38]

The ethanol concentration in the supernatant was measured by gas chromatography (Hewlett Packard 5880 A, Porapak Q80/100 column), using 4% isopropanol as an internal standard. Glucose was measured with a model No. 27 glucose analyzer from Yellow Springs Instruments. Cellobiose measurements were determined as glucose after complete hydrolysis by almond β-glucosidase (Emulsin, Sigma, Type II).

The residual cellulose in the SSFs was determined gravimetrically as follows. Concentrated hydrochloric acid was added to the sample of the culture to a final concentration of 3 v/v%. The mixture was heated for 1 h at 80°C to prehydrolyze yeast cell walls. Solids were recovered by centrifugation, washed with water, and resuspended in 2.5 w/v% sodium hydroxide solution. The yeast cells and other media components were selectively dissolved by heating the mixture to 65°C for 30 min. The solid cellulose residue was recovered by centrifugation and washed twice with deionized water. The cellulose was finally resuspended in deionized water and recovered on a preweighed Millipore 0.45-μm type HA filter. The filter was dried at 45°C until it reached a constant weight. The cellulose content was determined by dry weight difference. Control experiments indicate that the procedure removes 95–98% of dry yeast cell mass but only approximately 5% of cellulose.

Saccharifications of 10 mL samples were carried out in 20 mL scintillation vials with screw caps run at 30, 37, or 50°C on a rotary shaker. Varying amounts of the different cellulase enzymes were added (7–39 IU/g of substrate) at the start along with tetracycline at 100 μg/mL. The substrate was Sigmacell 50 at 10% or 15% (w/v). Digestibility was measured by glucose and cellobiose measurements from samples taken each day.

A central goal of this study was to compare the ethanol yields for SSF processes that employ different fermentation organisms and cellulases at different conditions with each other and with the results for straight saccharification. However, for the small-scale experiments that were run initially to screen performance, only the ethanol concentrations were measured for SSF. To compare these results with the measurements of sugar concentrations and the cellulose conversion calculated from the sugar analysis for straight saccharification, the ethanol concentrations for SSF were transformed into an equivalent conversion of cellulose. Since *S. cerevisiae* actually ferments about 93 to 95% of glucose to ethanol while *B. clausenii* ferments about 90% of glucose to ethanol, transformation to a common basis would be quite difficult for the mixed culture experiments reported here. Therefore, recognizing the limitations of the screening experiments themselves, it was felt that the equivalent conversion of cellulose could be adequately approximated by assuming that 90% of the glucose from cellulose hydrolysis was converted to ethanol stoichiometrically, while the remaining glucose was used for cell growth, maintenance, etc. Furthermore, it was assumed that all of the glucose was used by the yeast as soon as it was formed.

This transformation tends to favor a yeast such as *S. cerevisiae* that ferments glucose to ethanol with higher yields, although it may be compensated by the capability of *B. clausenii* to minimize accumulation of cellobiose in the broth. In any event, the most promising conditions were duplicated in a larger controlled fermenter for which concentrations of glucose, cellobiose, ethanol, and cell mass were measured to ensure reasonable accuracy.

RESULTS

Three enzymes from various strains of *Trichoderma* were selected for this investigation: spray dried SP-122 cellulase from Novo Laboratories, liquid 150L cellulase concentrate from Genencor, Inc., and enzyme produced in our laboratories from the Rut C-30 strain of *T. reesei*. The key properties of these three cellulases are summarized in Table I. As tested, the 150L has the highest volumentric filter paper activity (106 IU/mL) as well as the highest specific activity (0.83 IU/mg protein). The relative CMC activity is also higher for both the 150L and Rut C-30 cellulases than for the SP-122. The relative PnPGU activity is significantly higher for the 150L cellulase than for SP-122 and the Rut C-30, indicating increased β-glucosidase activity. Thus, one would expect the advantage of a cellodextrin-fermenting yeast to diminish for 150L as well as Rut C-30 relative to SP-122.

The performance of the three enzymes was also determined for straight saccharification of Sigmacell 50 cellulose. Enzyme loadings of 7, 13, 26, and 39 IU/g of substrate were tested at 50°C for 10% and 15% (w/v) substrate concentrations. As shown in Table II for 2, 5, and final 6 plus day periods, the 150L cellulase outperformed the SP-122 enzyme, which in turn surpassed the Rut

TABLE I
Characterization of Cellulases

	SP-122	Genencor 150L	Rut C-30
Dry wt (mg/mL)	50	164	81
Protein (mg/mL)	16	127	63
% Protein	31	78	78
CHO (mg/mL)	20	32	18
% CHO	40	19	22
IU/mL	3.5	106	34
PnPGU/mL	1.9	160	15
CMC/mL	84	2500	1100
IU/mg Protein	0.23	0.83	0.53
PnPGU/mg Protein	0.12	1.3	0.24
CMC/mg Protein	5.4	20	17

Nomenclature: CHO, carbohydrate content as determined by phenol-sulphuric acid assay; IU, filter paper assay for saccharifying cellulase expressed in international units; PnPGU, β-glucosidase assay with p-nitrophenyl-β-glucopyranoside substrate expressed in international units; CMC, carboxymethyl cellulose assay for endo-β-1,4 glucanase expressed in international units.

TABLE II

Performance as Percent Cellulose Conversion for Three Cellulases in Small-Scale Straight Saccharification of Sigmacell 50 Cellulose at 10% and 15% Substrate Concentrations, a Temperature of 50°C, and Various Enzyme Loadings

		10% substrate			15% substrate		
Enzyme	Enzyme loading IU/g	48 h	120 h	Final (144+ h)	48 h	120 h	Final (144+ h)
SP-122	7	32%	45%	45%	31%	36%	36%
Rut C-30	7	18	33	35	14	33	34
150L	7	48	54	71	39	50	64
SP-122	13	33%	56%	57%	33%	47%	48%
Rut C-30	13	21	39	40	17	33	35
150L	13	41	71	72	38	60	66
SP-122	26	33%	63%	65%	33%	48%	52%
Rut C-30	26	21	45	48	22	36	39
150L	26	48	71	72	42	66	67
SP-122	39	41%	63%	70%	36%	51%	52%
Rut C-30	39	22	43	49	22	39	40
150L	39	50	72	76	43	70	72

C-30 hydrolysis results by a significant margin. Within the accuracy of the measurements, increasing the enzyme loading beyond about 13 IU/g cellulose resulted in enzyme saturation of the substrate. The maximum cellulose conversion calculated from the sugar analysis was obtained with the 150L cellulase with about a 76% value at 10% substrate and a 72% value at 15% substrate. Although the cellulose conversions for Rut C-30 are lower than expected, this particular preparation serves to illustrate some of the performance features of SSF.

The first SSF runs were performed with SP-122 cellulase, as summarized in Table III for 12-day experiments. The maximum ethanol concentration achieved in these evaluations with a pure culture of *B. clausenii* was 39 g/L, while the maximum equivalent cellulose conversion was 72%. The amount of ethanol was close to the final value within 4 to 6 days. For comparison, the SSF was also run with *S. cerevisiae* as a pure culture and in mixed culture with *B. clausenii*. As shown in Table III, the results were not as good for *S. cerevisiae* as for *B. clausenii,* but the mixed culture performed better than either yeast alone at the same temperature and substrate concentration. Furthermore, a higher ethanol concentration of 46 g/L was achieved for the mixed culture than for previous tests.

To determine whether the decline in ethanol production rate after only a few days was due to enzyme, glucose fermentations were run with *B. clausenii* in the presence of enzyme. For these investigations, yeast was added to a medium containing 10% glucose and 2.0 IU/mL of cellulase, and a control was also run with no enzyme addition. The cell density, sugar utilization, and ethanol concentrations were measured; all three enzyme preparations were evaluated in this manner. The ethanol concentrations plotted in Figure 1 reveal that the 150L and

TABLE III

Ethanol Concentrations in g/L at Various Times Up to 12 Days for SSF Runs with NOVO SP-122 Cellulase and Sigmacell 50 Cellulose for Selected Enzyme Loadings and Temperaures

Yeast	Temperature (°C)	Sigmacell 50	Enzyme Loading IU/g	Time (h)							Final Equivalent Conversion
				24	48	96	144	192	240	288	
B. clausenii	30	10.7	6.5	7.0	12.5	15.5	19.5	20.0	22.0	24.0	44
B. clausenii	30	10.4	26	6.0	13.0	19.5	28.5	29.0	31.0	33.0	62
B. clausenii	30	14.1	13	10.0	28.0	30.0	32.0	37.5	38.0	39.0	54
B. clausenii	30	16.0	8.6	6.0	14.0	20.5	25.0	28.0	29.0	30.0	37
B. clausenii	37	9.0	13	15.5	21.5	27.0	29.0	31.0	32.0	33.0	72
S. cerevisiae	30	15.3	13	9.0	11.5	17.0	19.5	21.5	23.0	24.0	31
Mixed culture	30	15.8	13	6.0	12.1	25.0	36.0	40.0	44.5	46.0	57

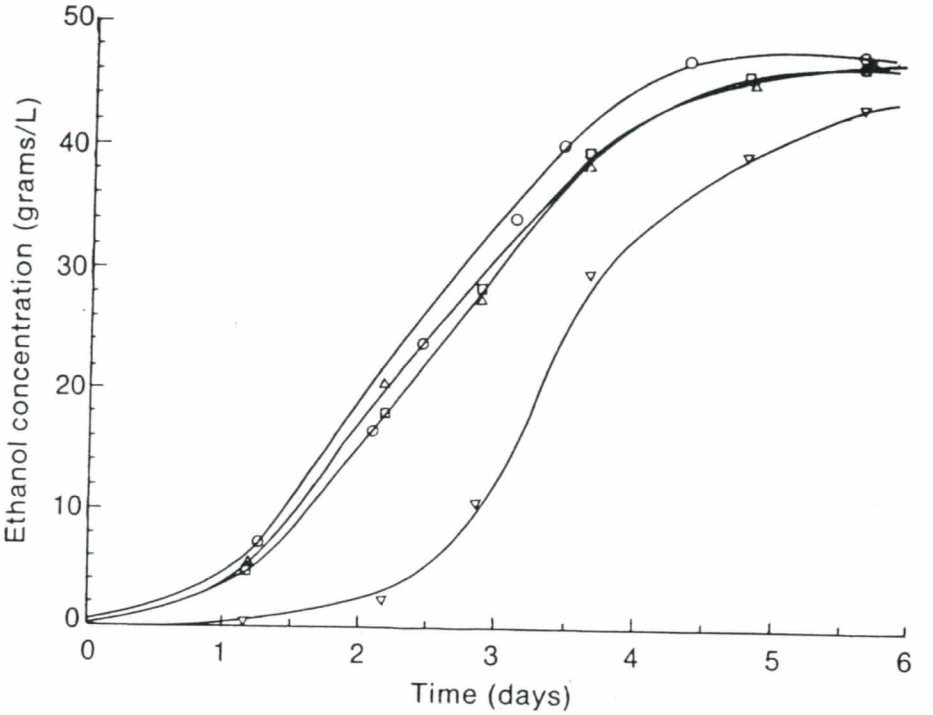

Fig. 1. Ethanol concentrations resulting from fermentation of glucose by the yeast *Brettanomyces clausenii* in the presence of cellulase at 2 IU/mL. Symbols represent: ○–No enzyme control, □–Genencor 150L, △–Rut C-30, and ▽–NOVO SP-122.

fresh Rut C-30 cellulases did not inhibit the glucose fermentation, while the SP-122 enzyme caused a lag of several days in performance. Although these data do not explain the inability to continue the fermentation in SSF after a few days, they do reveal that a component can be present in the enzyme preparation that causes a substantial delay in the fermentation. Sufficient time was not available to pursue the study further to determine the source of inhibition, but the glucose test was run before each set of SSF experiments to be sure inhibitory components were not present in the cellulase before its use. Further research is warranted to evaluate why the fermentation with *B. clausenii* was not maintained in the SSF experiments for SP-122 cellulase.

Several small-scale evaluations were made with the Rut C-30 enzyme and the yeast *B. clausenii*. Figure 2 presents the ethanol concentrations achieved for enzyme loadings of 7, 13, 26, and 39 IU/g of Sigmacell 50 substrate at 10% and 15% substrate concentrations for 30°C. Although the accuracy of these small-scale screening tests is limited, they do indicate that enzyme loadings above 13 IU/g produce diminishing returns due to substrate saturation. Furthermore, the ethanol concentration does not increase significantly after 10 to 11 days. The highest equivalent conversion after 5 days was about 49% for a 10% substrate level and about 43% for a 15% substrate concentration. These values are about 10% higher than those obtained for straight saccharification at 50°C in the same time period for the Rut C-30 cellulase used in this study. Furthermore, the equivalent conversion after 12 days is as high as 67% at 10% substrate and 58% at 15% substrate, values better than any of those achieved at 30°C with SP-122 enzyme.

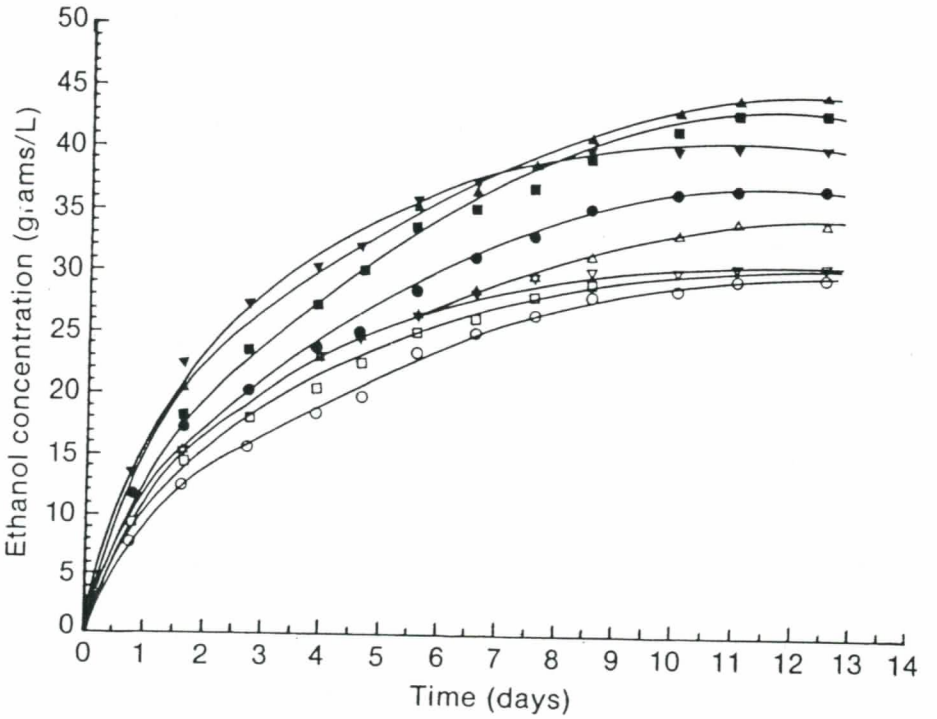

Fig. 2. Small-scale SSF experiments for *B. clausenii* and Rut C-30 cellulase at 30°C. Enzyme loadings are 7 IU/g substrate (○), 13 IU/g (□), 26 IU/g (△), and 39 IU/g (∇). Open symbols represent 10% w/v Sigmacell 50 substrate, while closed symbols are for 15% w/v substrate concentrations.

Small-scale SSF experiments were also run with Rut C-30 cellulase and *B. clausenii* at 37°C with the same enzyme loading and substrate concentration ranges as above. As shown in Figure 3, very little is gained by increasing the enzyme loading beyond about 13 IU/g for either substrate level. In this experiment, the ethanol concentration did not increase much after 7 days, although the highest final ethanol concentrations were the same as for the lower temperature runs. At this point, the equivalent conversions are 67% at 10% substrate and 57% for 15% substrate. The conversions after 5 days are about 59% for 10% substrate and 49% for 15% substrate, values higher than for either the SSF runs at lower temperature or straight saccharification at 50°C in the same time period.

A series of identical experiments was conducted with *S. cerevisiae* at 37°C for the same substrate levels and enzyme loadings as used for *B clausenii*. Figure 4 shows a greater change in rate with enzyme loading than before. Comparison of Figures 3 and 4 reveals that *S. cerevisiae* produced similar results to *B clausenii* for the first 5 to 6 days with higher cellulase loading while lagging behind at lower cellulase levels. However, the ethanol concentration for *S. cerevisiae* continued to increase to higher ultimate ethanol concentrations of about 39 g/L for 10% substrate and 54 g/L at 15% substrate. These values correspond to equivalent conversions of about 78% and 70%, respectively.

Next, the performance of these yeasts was determined by SSF screening runs with the 150L cellulase from Genencor. Figures 5 and 6 present experiments with *B. clausenii* at 30 and 37°C, respectively, for enzyme loadings of 13, 26, and 39 IU/g substrate and the same substrate levels as before. The results at 37°C

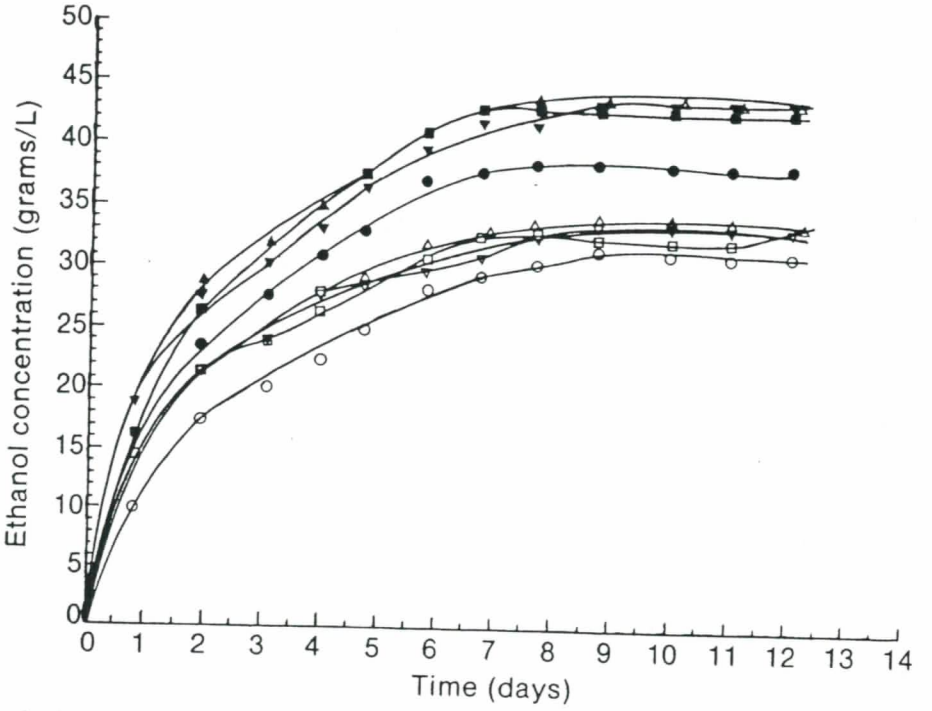

Fig. 3. Small-scale SSF experiments for *B. clausenii* and Rut C-30 cellulase at 37°C. Enzyme loadings are 7 IU/g substrate (○), 13 IU/g (□), 26 IU/g (△), and 39 IU/g (∇). Open symbols represent 10% w/v Sigmacell 50 substrate, while closed symbols are for 15% w/v substrate concentrations.

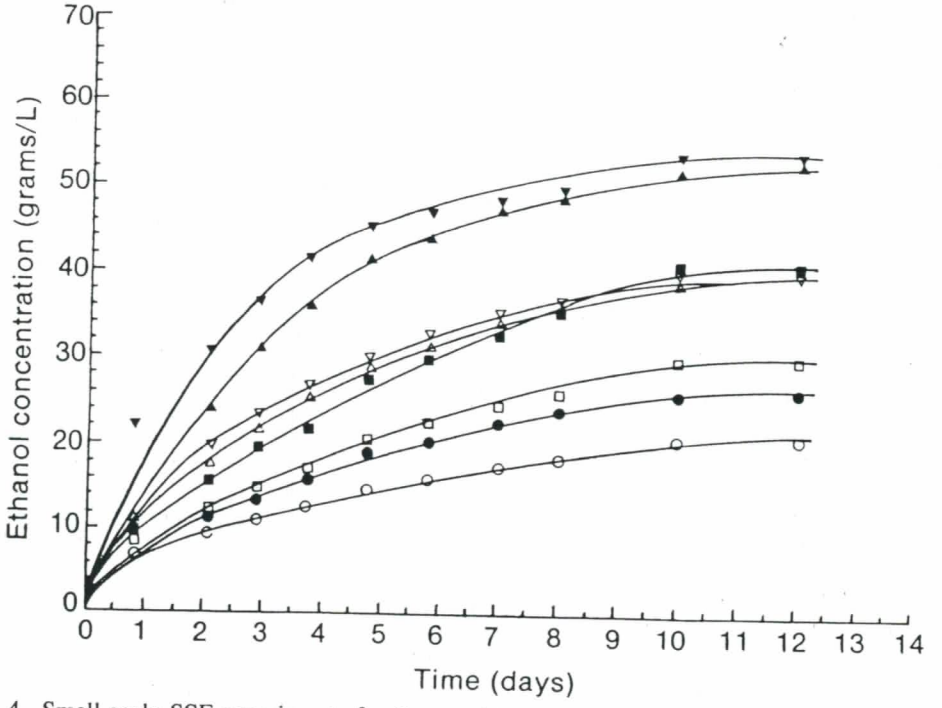

Fig. 4. Small-scale SSF experiments for *S. cerevisiae* and Rut C-30 cellulase at 37°C. Enzyme loadings are 7 IU/g substrate (○), 13 IU/g (□), 26 IU/g (△), and 39 IU/g (∇). Open symbols represent 10% w/v Sigmacell 50 substrate, while closed symbols are for 15% w/v substrate concentraions.

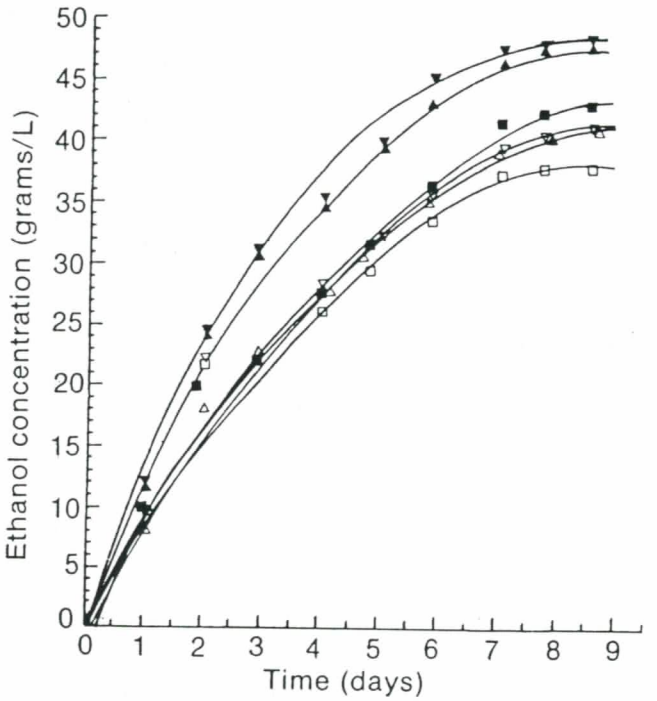

Fig. 5. Small-scale SSF experiments for *B. clausenii* and Genencor 150L cellulase at 30°C. Enzyme loadings are 13 IU/g (□), 26 IU/g (△), and 39 IU/g (▽). Open symbols represent 10% w/v Sigmacell 50 substrate, while closed symbols are for 15% w/v substrate concentrations.

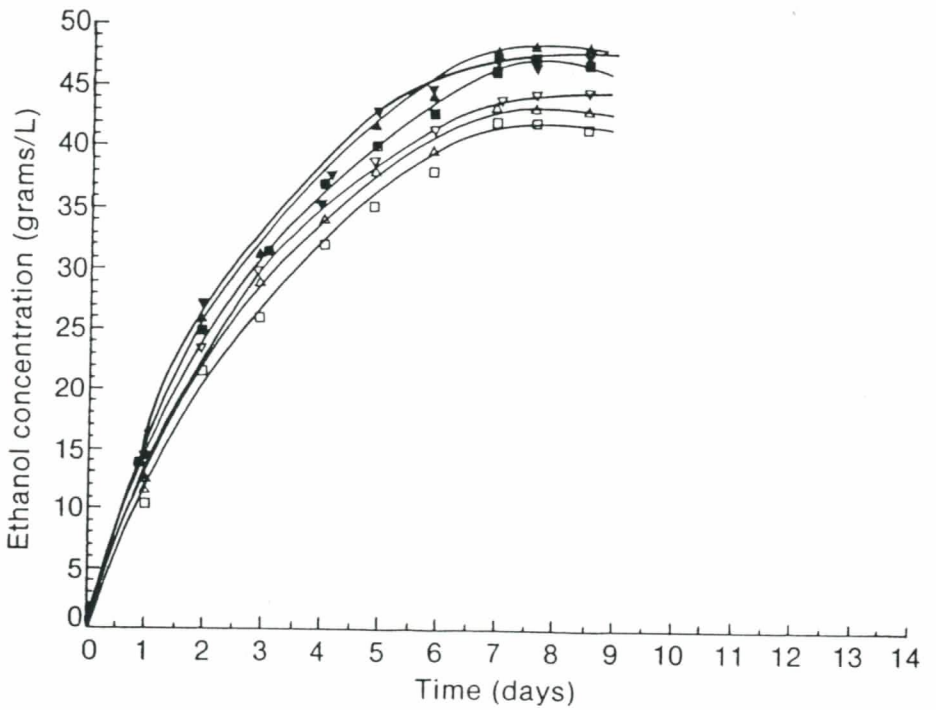

Fig. 6. Small-scale SSF experiments for *B. clausenii* and Genencor 150L cellulase at 37°C. Enzyme loadings are 13 IU/g (□), 26 IU/g (△), and 39 IU/g (▽). Open symbols represent 10% w/v Sigmacell 50 substrate, while closed symbols are for 15% w/v substrate concentrations.

were better than those at 30°C, with little change in ethanol concentration after only 6 to 7 days. The final equivalent cellulose conversions were about 86% at the 10% substrate level and 64% at 15% substrate. For the 5-day period, the conversions were 74% at 10% substrate and 55% for 15% levels, the former being about the same as that for straight saccharification, while the latter is below the 70% value obtained in that study in five days.

A run was also made with *S. cerevisiae* and the 150L cellulase. As shown in Figure 7, the increase in ethanol concentrations slowed considerably after about 6 days. Increasing the enzyme loading above 13 IU/g had some benefit up to about 26 to 39 IU/g. The final yields after 12 days were about 94% for 10% substrate and 83% for 15% substrate levels; the former is somewhat higher than for *B. clausenii,* while the latter is significantly greater. At the 5-day period, the maximum yields are about 76% and 69% for 10% and 15% substrate, respectively, values very close to those for straight saccharification.

Since the SSFs with *B. clausenii* performed well at earlier times while *S. cerevisiae* did better for longer periods, a final set of small-scale screening experiments was conducted for mixed cultures of *B. clausenii* with *S. cerevisiae*. The results of these experiments are represented in Figures 8 and 9 for Rut C-30 and 150L enzymes, respectively. In both sets of experiments, enzyme loadings of 7, 13, 26, and 39 were evaluated for 10% and 15% substrate concentrations at 37°C. For Rut C-30, the maximum equivalent cellulose conversion was about 73% at 10% substrate and about 63% at 15% substrate, values better than those for *B. clausenii* alone but somewhat less than those for *S. cerevisiae* alone. For

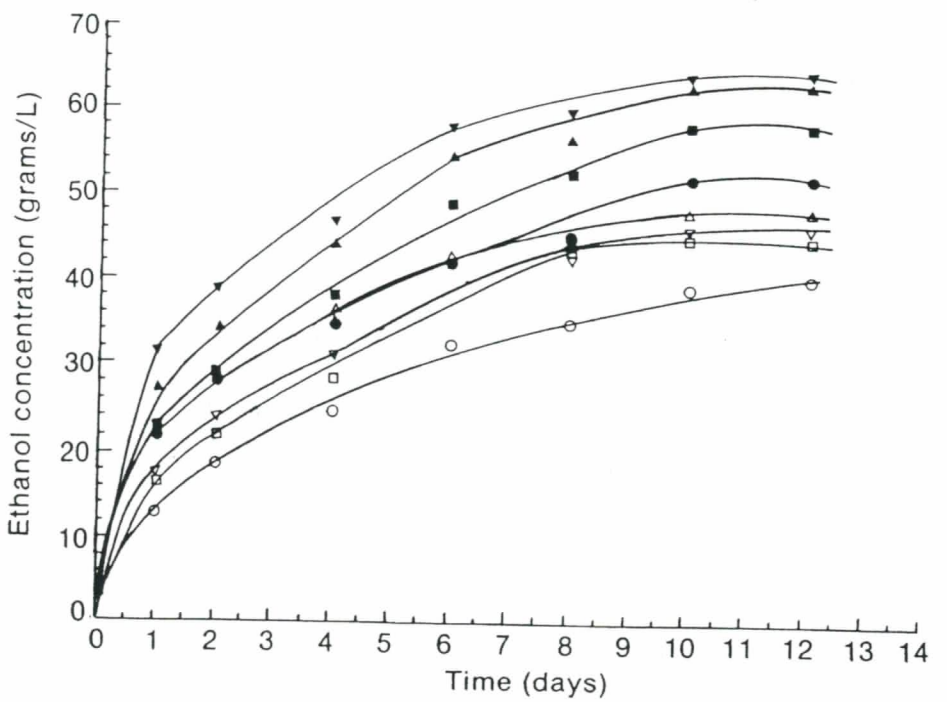

Fig. 7. Small-scale SSF experiments for *S. cerevisiae* and Genencor 150L cellulase at 37°C. Enzyme loadings are 13 IU/g (□), 26 IU/g (△), and 39 IU/g (▽). Open symbols represent 10% w/v Sigmacell 50 substrate, while closed symbols are for 15% w/v substrate concentrations.

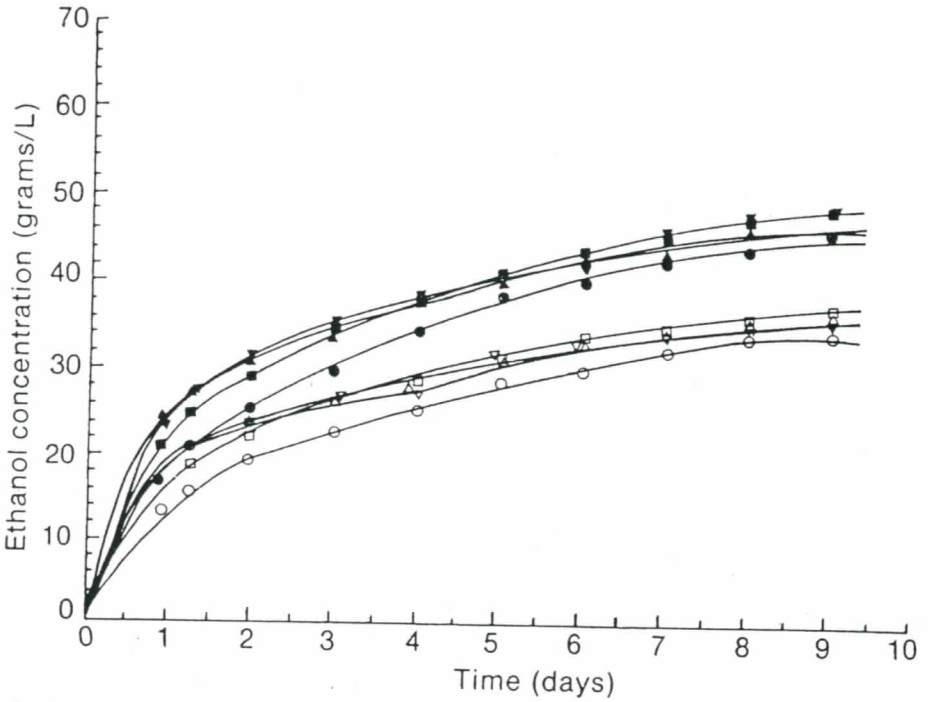

Fig. 8. Small-scale SSF experiments for a mixed culture of *B. clausenii* and *S. cerevisiae* with Rut C-30 cellulase at 37°C. Enzyme loadings are 7 IU/g substrate (○), 13 IU/g (□), 26 IU/g (△), and 39 IU/g (▽). Open symbols represent 10% w/v Sigmacell 50 substrate, while closed symbols are for 15% w/v substrate concentrations.

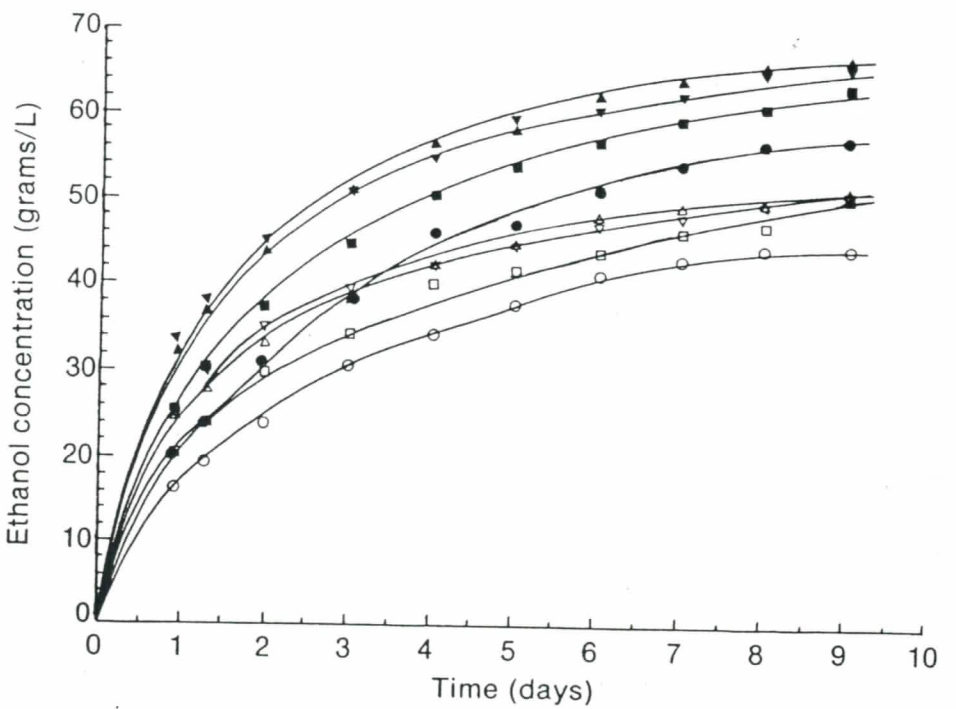

Fig. 9. Small-scale SSF experiments for a mixed culture of *B. clausenii* and *S. cerevisiae* with Genencor 150L cellulase at 37°C. Enzyme loadings are 7 IU/g substrate (○), 13 IU/g (□), 26 IU/g (△), and 39 IU/g (▽). Open symbols represent 10% w/v Sigmacell 50 substrate, while closed symbols are for 15% w/v substrate concentrations.

the Genencor 150L cellulase, the maximum equivalent conversions reached over 95% for 10% substrate and about 86% for 15% substrate over the 9-day period. At the 5-day period of the straight saccharification (Table II), the conversions for the mixed culture SSFs were 88% at 10% substrate and 78% for 15% substrate, compared to 72% and 70%, respectively, for straight saccharification at that time.

Following the small-scale screening experiments, the 3-L Braun controlled fermenters were run at the most promising conditions to obtain more complete and accurate data than possible in the shake flasks. The first such run with Genencor 150L cellulase was for straight saccharification of a 10% w/v concentration of Sigmacell 50 cellulose with an enzyme loading of 13 IU/g substrate and a temperature of 50°C. As shown in Figure 10, the total glucose and cellobiose reached about 78 g/L in 9 days, a cellulose conversion of about 70%. The conversion at the 5-day period used in Table II is about 61%, somewhat lower than for the small screening tests.

The mixed culture of *B. clausenii* and *S. cerevisiae* was also run in the 3 L Braun fermenters. Again Genencor 150L was used at a loading of 13 IU/g of substrate with a 10% w/v charge of Sigmacell 50 and a temperature of 37°C. As shown in Figure 11, the ethanol concentration reached about 46 g/L in 7 days, corresponding to an equivalent cellulose conversion of 90% according to our definition. The cellulose concentration drops to 1% from the initial 10% charge, resulting in a 90% actual conversion. This result is about the same as the value derived in the screening tests for a loading of 13 IU/g and considerably better than for the large-scale or small-scale straight saccharification tests. It is worth

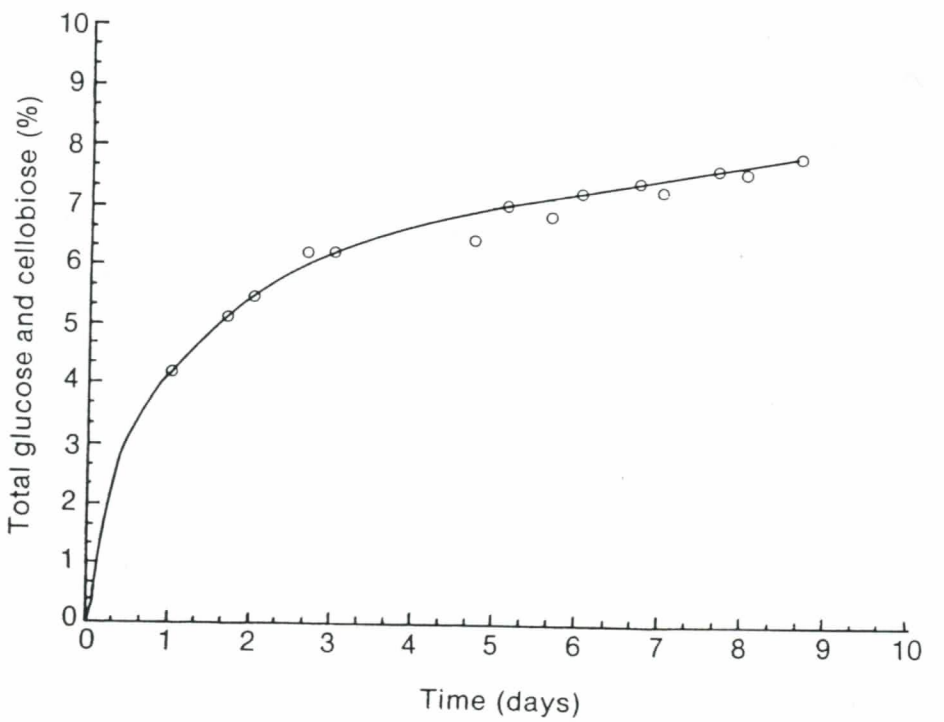

Fig. 10. Saccharification of Sigmacell 50 cellulose at 50°C for 10% w/v substrate concentration with 13 IU/g loading of Genencor 150L cellulase.

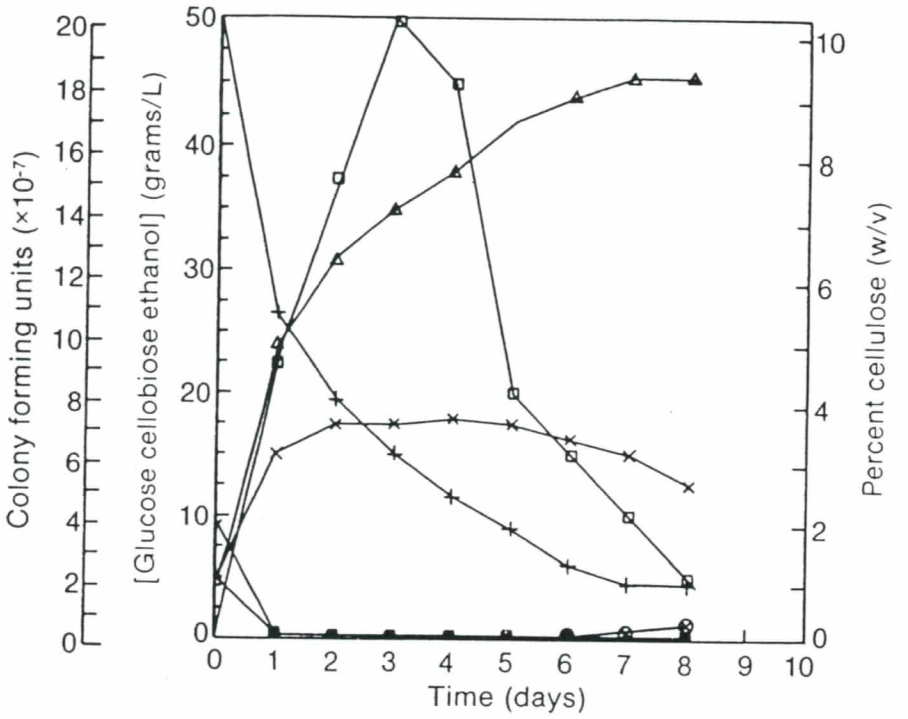

Fig. 11. Larger scale 3 L SSF experiment for a mixed culture of *B. clausenii* and *S. cerevisiae* with Genencor 150L cellulase at 37°C, 10% (w/v) Sigmacell 50 cellulose concentration, and 13 IU/g of substrate. Ethanol represented (△), cellulose (+), *B. clausenii* (□), *S. cerevisiae* (×), glucose (⊗), and cellobiose (■).

noting that the viable cell density of *B. clausenii* rises more rapidly than that of *S. cerevisiae* but falls much more quickly to a lower final value. Furthermore, the maximum number of growing cells for both yeasts occurs shortly after the glucose and cellobiose concentrations attain low levels. Thus, although the ability of *B. clausenii* to ferment cellobiose is beneficial initially, the better viability of *S. cerevisiae* sustains the fermentation at longer times.

DISCUSSION

The Genencor 150L enzyme evaluated in this study performed the best of the three cellulases tested for both straight saccharification and for SSF with either yeast employed. With this cellulase, yields of about 70% of theoretical could be achieved in 5 days for straight saccharification for both 10% and 15% substrate levels. Thus, end-product inhibition of the cellulase is considerably less than for other cellulases such as the Rut C-30 prepared for this test, and improved enzymes such as 150L present stiff competition for the SSF process that relies on simultaneous yeast fermentation to improve kinetic rates and cellulose conversion.

For SP-122 cellulase, *B. clausenii* produced higher concentrations of ethanol than *S. cerevisiae,* while the latter yeast achieved higher ethanol levels than the former for both Rut C-30 and 150L cellulases. Apparently, the ability to ferment cellobiose is far more beneficial with a cellulase that is low in β-glucosidase,

such as SP-122, than with cellulase that has higher levels of that activity, such as Rut C-30 or 150L. However, with Rut C-30, SSF at 37°C improved the conversion rates of cellulose relative to straight saccharification at 50°C. On the other hand, the kinetic performance for SSF was about the same as that of straight saccharification for both SP-122 and 150L cellulases in combination with a pure culture of either yeast tested. This result clearly illustrates the attribute of SSF to reduce end-product inhibition for a cellulase such as the Rut C-30 prepared for this study, which demonstrated severe rate reductions with increasing conversion for straight saccharification.

B. clausenii achieved better or virtually identical results to *S. cerevisiae* over the first few days of SSFs, but after 6 days, the latter yeast performed best under most conditions. It is suspected that *B. clausenii* loses viability, perhaps due to ethanol toxicity or starvation for the low sugar levels available in SSF. In these runs, a temperature of 37°C gave the highest rates with the cellobiose-fermenting yeast, and cellulase loadings above 13–26 IU/g substrate did not give proportionally better results for either yeast.

Since *S. cerevisiae* maintained fermentation rates longer than *B. clausenii*, while the ability to ferment cellobiose appears beneficial initially, mixed cultures of the two yeasts were studied in an effort to combine the attributes of these yeasts. For Rut C-30 and 150L cellulases, the ethanol yields were generally better for SSF with mixed cultures than for any other SSF and as good or better than straight saccharification with these enzymes. The performance was particularly good with the 150L cellulase. In fact, the mixed culture with 150L achieved the highest combination of yields and ethanol concentrations known for high cellulose loadings.

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