Evaluation of Thermotolerant Yeasts in Controlled Simultaneous Saccharifications and Fermentations of Cellulose to Ethanol

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Simultaneous saccharification and fermentation (SSF) experiments were performed at selected temperatures (37, 41, and 43°C) to obtain comprehensive material balance and performance data for several promising strains of thermotolerant yeast. Parameters measured were ethanol concentration, yeast cell density, and residual sugar and cellulose concentrations. The three yeasts *Saccharomyces uvarum, Candida brassicae,* and *C. lusitaniae* and two mixed cultures of *Brettanomyces clausenii* with *S. cerevisiae* (mixed culture I) and *C. lusitaniae* with *S. uvarum* (mixed culture II) exhibited rapid rates of fermentation, high ethanol yields, strong viability, or high cellobiase activity. Overall, mixed culture II at 41°C performed better than either component yeast by themselves because it combined a cellobiose fermenting capability with the high ethanol tolerance and rapid glucose fermentation of conventional industrial yeasts. Thus, the mixed cultures provide good initial rates by preventing buildup of cellobiose (a strong inhibitor of enzyme activity) while attaining high ultimate yields of ethanol for high cellulase concentrations. However, *C. brassicae* and *S. uvarum* gave similar results to mixed culture II at 37°C.

INTRODUCTION

Simultaneous saccharification and fermentation (SSF) has been investigated as a method of lignocellulosic conversion to ethanol for at least the last 10 years, following the publication of results by Takagi et al.[1] In general, SSF promises to achieve low enzyme loadings, fast hydrolysis reaction rates, and high product yields. If these parameters can be improved sufficiently, ethanol could be produced at prices competitive with that of petroleum fuel.

One approach to improving the rates of the SSF process is to identify fermenting organisms that tolerate temperatures closer to the optimum for cellulase enzyme (about 45–50°C) than typical for most strong glucose fermenters (30–37°C). Thus, this article focused on evaluation of thermotolerant yeasts for SSFs. The microorganisms selected for this study were identified previously through small-scale (100 mL) screening experiments of SSFs.[14] In the original screening tests, the best yeasts identified in the literature were tested over a range of temperatures and enzyme loadings to compare rates of hydrolysis and product yields based on ethanol concentration alone. The purpose of the study reported here was to gather more complete data on all major components in an SSF process for the most promising thermotolerant yeast identified in the previous small-scale study.

The yeasts evaluated have been reported in publications as thermotolerant ethanol producers and as yeasts compatible with the SSF process. *Candida lusitaniae,*[2–5] *Saccharomyces uvarum*[1,6,7] and *Candida brassicae*[1,2,8–12] have been subject of several papers involving the conversion of cellulose to ethanol. In two recent publications,[2,13] the authors have found that mixed cultures of *Brettanomyces clausenii* with *Saccharomyces cerevisiae* and *C. lusitaniae* with *S. uvarum* are favorable for the SSF process.

MATERIALS AND METHODS

Materials

Two of the strains were obtained from the Northern Regional Research Laboratory (NRRL), USDA Peoria, Illinois: *C. lusitaniae* Y-5394 and *B. clausenii* Y-1414. *C. brassicae* 32196 and *S. uvarum* 26602 were obtained from the American Type Culture Collection (ATCC) Rockville, Maryland, while the SERI strain *S. cerevisiae* (D_5A) was derived through genetic improvements from commercial Red Star bakers yeast. Chemicals and the cellulose substrate (Sigmacell-50) were purchased from the Sigma Chemical Company, St. Louis, Missouri and yeast extract and peptone growth media were ordered from Difco, Detroit, Michigan. The cellulase enzyme produced by a mutant of *Trichoderma reesei,* Genencor 150L, was purchased from Genencor Inc., San Francisco, California. Fermentation vessels are 6-L Braun Biostat V fermenters from B. Braun Instruments, Burlingame, California.

Methods

SSFs were run in 6-L Biostat V fermenters at 3-L volumes. Medium was 1% yeast extract and 2% peptone (YP) with 10% (w/v) Sigmacell-50 substrate. An enzyme loading of 13 IU/G substrate was employed in all SSFs where

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IU stands for International Units of filter paper activity in micromoles of glucose/minute.[5] A lipid mixture of ergosterol (5 mg/L) and oleic acid (30 mg/L) was also added to the media since they were found to improve ethanol tolerance.[14] Penicillin and streptomycin at 10 mg/L were used to minimize bacterial contamination problems. Inocula were grown in shaker flasks with YP media and 2% glucose at 37°C. An agitator speed of 100–150 rpm was set for growth of inocula and SSFs, and 1/10 v/v yeast culture to total volume of media was added to the fermentation. Media, substrate, and lipids were autoclaved in the fermentation vessels prior to the addition of antibiotics and inoculum.

The ethanol concentration in the supernatant was determined 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 concentrations were determined by incubating the sample with 2 mg/mL almond (β-glucosidase) for 1 h and measuring additional glucose released with the YSI glucose analyzer. Colony forming units (CFUs) were measured via plating serial dilutions on YPD and YPC cellobiose plates.

The residual cellulose was determined as follows: a prehydrolysis of yeast cell walls with dilute acid (HCL 3% v/v) was carried out at 80°C for 1 h followed by several water washes and centrifugation to recover solids. Then the yeast and other media components were selectively dissolved in 2.5% w/v sodium hydroxide by heating at 65°C for 30 min. After a few more washes, the cellulose was recovered on a preweighed Millipore 0.45 m type HA filter, which was dried at 45°C overnight.[13]

Some of the results are reported in percent equivalent conversion of cellulose based on the measured ethanol concentrations. This value is defined as the percent of feed cellulose required to provide the measured ethanol concentration assuming a 90% yeast efficiency, with the remaining 10% of the sugars formed being used for cell growth and maintenance of the yeast. The percent equivalent conversion of cellulose provides an estimate of the total cellulose utilization to produce a given amount of ethanol and can be directly compared to the observed cellulose utilization based on measured cellulose concentrations. This calculation is particularly useful for comparing the results of SSF based on only ethanol concentration to those for straight saccharifications, which produce only sugars.

RESULTS

Genencor 150 L enzyme was found to perform very well in SSF, as reported previously.[2] However, despite its good performance some characteristics of the Genencor 150 L cellulase enzyme vary from one batch to the next as can be seen in Table I. Batch I was consumed in the small-scale screening SSFs, necessitating that the large-scale SSFs reported here be carried out with batch II. In comparison, batch II exhibited about a 30% decrease in β-glucosidase activity, which seemed to decrease the extent of hydrolysis and the ethanol production by about 20% (g/L). The most recent IUPAC revision (1987) "Measurements of Cellulase Activities" points out that the level of β-glucosidase in an enzyme preparation may affect the results of the cellulase assay, especially for filter paper units.[5] Figure 1 demonstrates the increased rate of hydrolysis of batch II with increased β-glucosidase supplementation. Regardless, batch II of Genencor was still valuable in comparing the yeast characteristics on a consistent basis for the larger scale experiments.

Table I. Characterization of cellulases.

	Genencor 150 L Batch I	Genencor 150 L Batch II
Dry wt (mg/mL)	164.0	185.0
Protein Content (mg/ml)[a]	127.0	135.0
Protein (%)	77.7	73.0
CHO (mg/mL)	32.0	44.0
CHO (%)[b]	19.0	23.8
IU/mL[c]	106.0	84.7
PnPGU/mL[d]	160.0	111.0
CMC/mL[e]	2500.0	4500.0
IU/mg Protein	0.83	0.63
PnPGU/mg Protein	1.25	0.82
CMC/mg Protein	19.7	33.3

[a] Protein content was estimated by Lowry's assay.
[b] Carbohydrate content as determined by phenol-sulfuric acid assay.
[c] Filter paper assay for saccharifying cellulase expressed in International Units.
[d] β-glucosidase assay with p-nitrophenyl-β-glucopyranoside substrate expressed in International Units.
[e] Carboxymethyl cellulose assay for endo-β-1,4 glucanase expressed in International Units.

Based on the authors' previous screening experiments in which only ethanol concentrations were measured,[2] the following yeasts were selected for more detailed larger scale tests: *S. uvarum, C. lusitaniae, C. brassicae,* a mixed culture of *B. clausenii* with *S. cerevisiae* (mixed culture I), and another mixed culture of *C. lusitaniae* with *S. uvarum* (mixed culture II). Figure 2 exemplifies the results for mixed culture II at 41°C and shows all the parameters measured for these SSFs. Here low residual sugars are seen, high ethanol production with concurrent degradation of cellulose, and strong cell viability at the start of the SSF followed by some decrease in viable cell density as the fermentation progresses. About half as much ethanol is produced as the cellulose degraded since the weight yield of ethanol is approximately 51% of the glucose consumed. Mixed culture II gave the best fermentation results for all yeast cultures tested. Of the single yeast, Figures 3, 4, and 5 show the details of *C. brassicae, S. uvarum,* and *C. lusitaniae* SSF's at 37°C.

To facilitate comparison of the different yeasts, it is useful to examine some average measures of performance. Figure 6 compares the average amount of residual sugar present for these yeasts in the large-scale SSFs after a 7-day period. *C. lusitaniae* has the highest build up of sugars as its viability drops sharply after four days. The

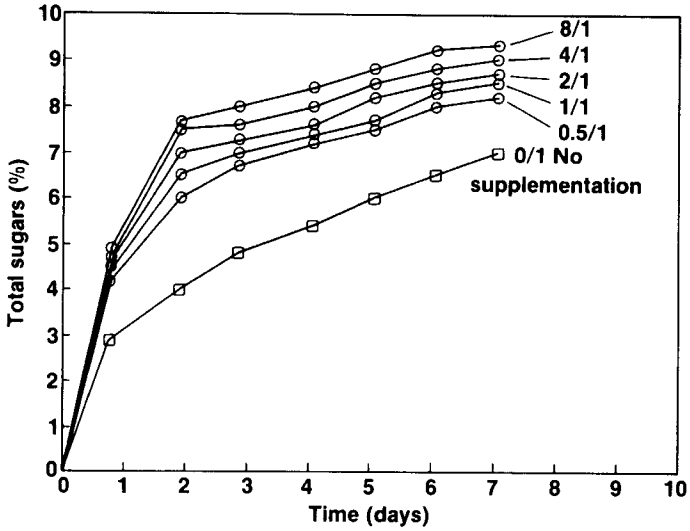

Figure 1. Saccharification of 10% Sigmacell-50 with Genencor cellulase at 13 IU/g substrate, with (○) and without (□) β-glucosidase (NOVO-188) supplementation at given ratios of β-glucosidase activity/IU cellulase enzyme for 50°C.

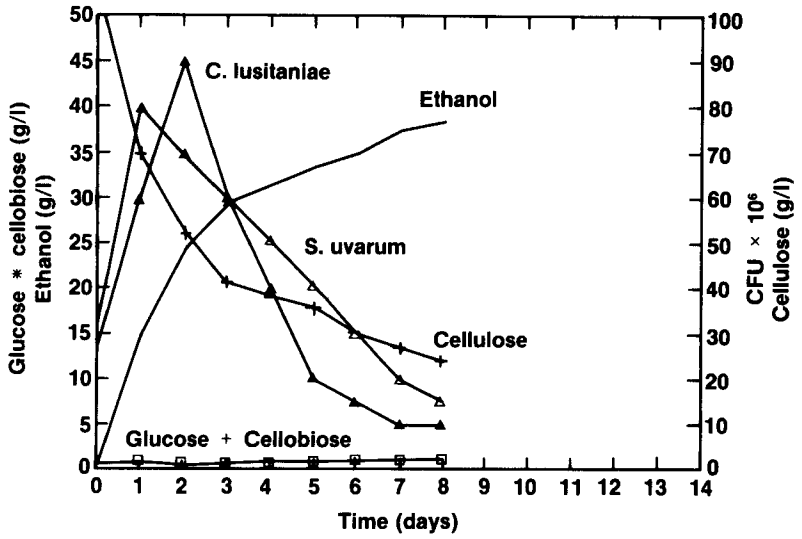

Figure 2. Large-scale SSF with mixed culture II at 41°C with Genencor cellulase loading of 13 IU/g substrate Sigmacell-50 at 10% w/v.

viability of *C. brassicae* also decreases as temperature is increased, hence increasing the residual sugars. Mixed culture I at 37°C maintains the lowest sugar concentration in comparison to the rest but cannot be run at higher temperatures because *B. clausenii* dies above 37°C. Mixed culture II also does well in utilizing the sugars, although there is a slight increase in amount of residual sugars as the temperature is raised in increments from 37°C to 41°C. *S. uvarum* is unique relative to the other yeasts in that it manages to utilize the residual sugars just as well at 41°C as it does at 37°C. However, at 43°C, *S. uvarum* did not grow in SSF. Residual sugars are important as they will inhibit the cellulase enzyme activity.[15]

The average residual sugars over the 7-day-time period showed an inverse relationship to the average colony forming units as seen in Figures 6 and 7, since residual sugars increase as the viability decreases. Factors affecting the yeast viability are ethanol tolerance, temperature tolerance, and the pH of the fermentation. From the results shown in Figure 7, it is apparent that the mixed cultures have the highest total yeast content since they have two yeasts inoculated at the start of the fermentation. For the single cultures of *C. brassicae* and *S. uvarum*, strong growth is seen at 37°C, which decreases with an increase in temperature. *C. lusitaniae's* total cell density is reduced due to its low ethanol tolerance,[2] yet *C. lusitaniae* shows good via-

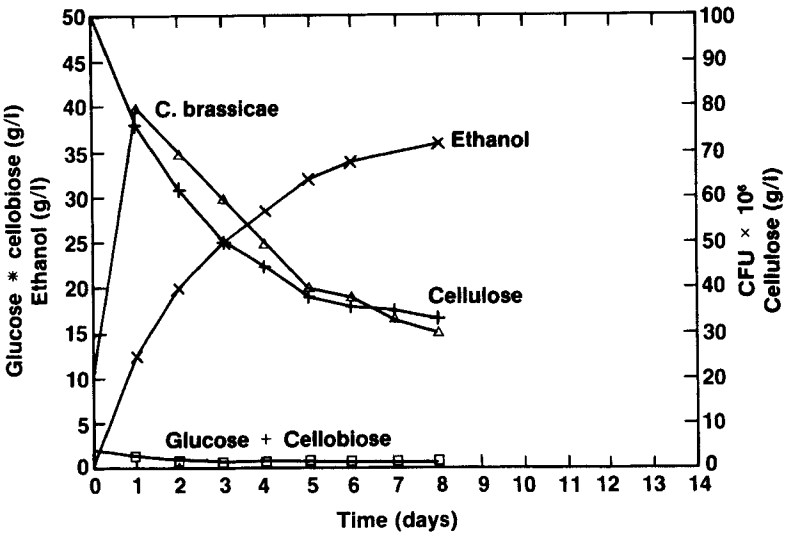

Figure 3. Large-scale SSF with *C. brassicae* at 37°C with Genencor cellulase loading of 13 IU/g substrate Sigmacell-50 at 10% w/v.

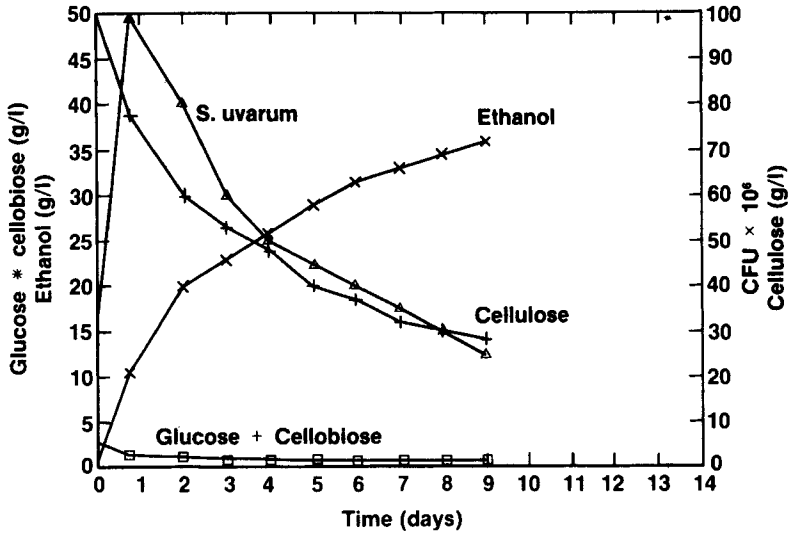

Figure 4. Large-scale SSF with *S. uvarum* at 37°C with Genencor cellulase loading of 13 IU/g substrate Sigmacell-50 at 10% w/v.

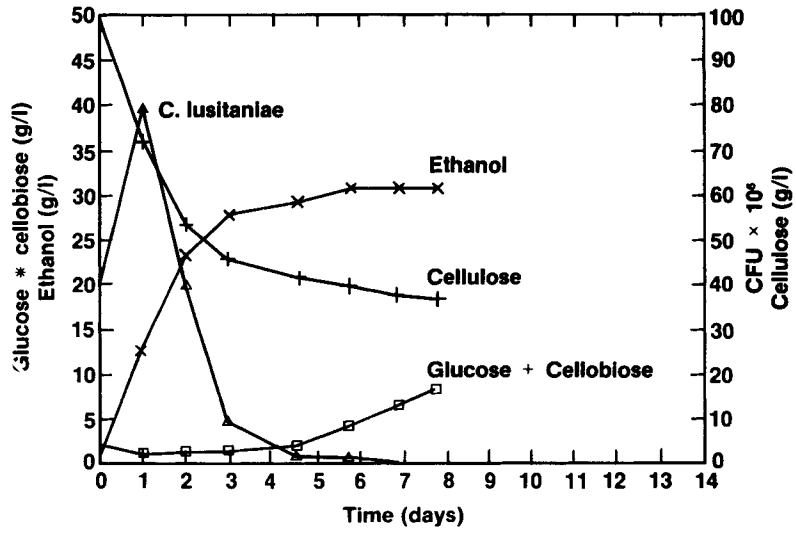

Figure 5. Large-scale SSF with *C. lusitaniae* at 37°C with Genencor cellulase loading of 13 IU/g substrate Sigmacell-50 at 10% w/v.

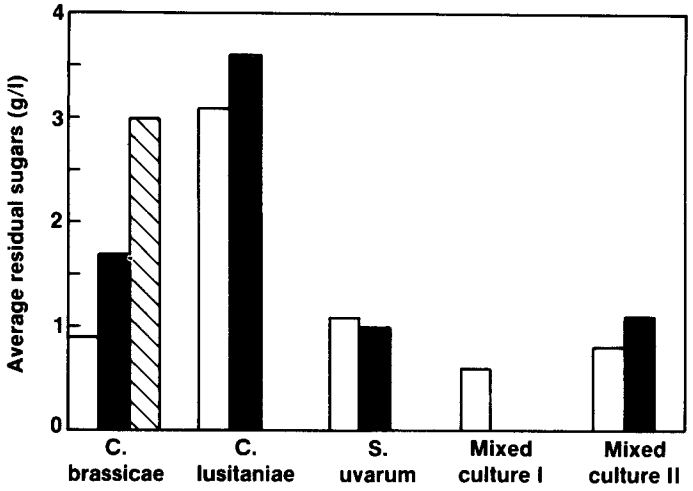

Figure 6. Comparison of average residual sugars for large-scale SSFs at 37° (□), 41° (■) and 43°C (▨) over a 7-day period and a cellulase loading of 13 IU/g Sigmacell-50 cellulose substrate (10%).

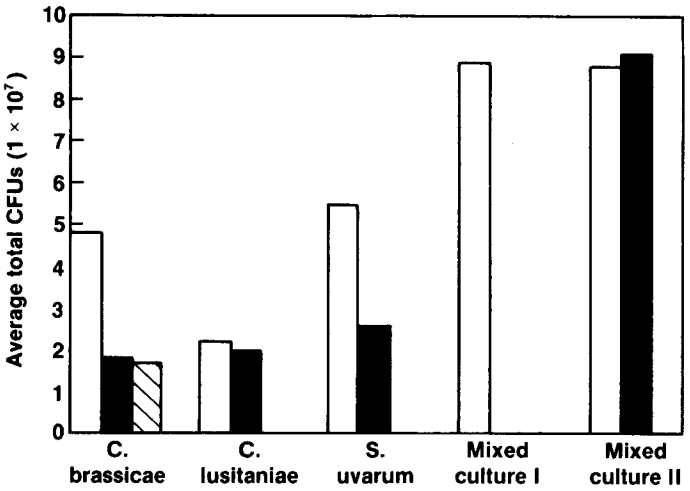

Figure 7. Average total colony forming units for large-scale SSFs at 37° (□), 41° (■) and 43°C (▨) with a cellulase loading of 13 IU/g Sigmacell-50 cellulose substrate (10%) over a 7-day period.

bility in mixed culture II. All the cultures grow best at the 37°C temperature with the exception of mixed culture II, which grows just as well at 41°C because of the enhanced viability of *S. uvarum* in this system.

Cellulose degradation is another parameter measured in the SSF fermentations that reflects ethanol production. Figure 8 illustrates the conversion of cellulose in percent degraded in seven days for all of the SSFs. Any increased rate of hydrolysis beyond straight saccharification by the cellulase enzyme is due to the yeast performance in sugar uptake and ethanol production. This data would suggest that mixed culture II at 41°C performs best with this enzyme preparation.

Figure 9 reveals the 2-day, 4-day, and final ethanol (approximately seven days) percent equivalent conversions of cellulose based on ethanol concentrations for all the cultures in the large-scale SSFs. Mixed culture II at 41°C again performs best in rates and final yield of ethanol with a final percent conversion of 74.5%, followed by 70.5% for *C. brassicae*, and *S. uvarum* at 37°C. Mixed culture I comes next with a percent equivalent conversion of 68.5%. *C. brassicae* and *S. uvarum* perform the same at 41°C, as does mixed culture II at 37°C, with a final percent cellulose conversion of 66.5%. *C. lusitaniae*, with a high initial rate, drops off in yield giving a final percent conversion of 61.0% for both 37°C and 41°C SSFs. All of the yeasts tested are within 5% cellulose conversion efficiency.

The mixed cultures appear to perform well due to their cellobiose fermenting capability combined with a strong glucose fermenter. This is no doubt due to the initial reduction in accumulation of cellobiose, a strong enzyme inhibitor, and the hardy continued performance as SSF time continues. Furthermore, mixed culture II at 41°C demonstrates the advantage of the higher temperature, increasing the enzyme activity over the SSFs at 37°C. However, *C.*

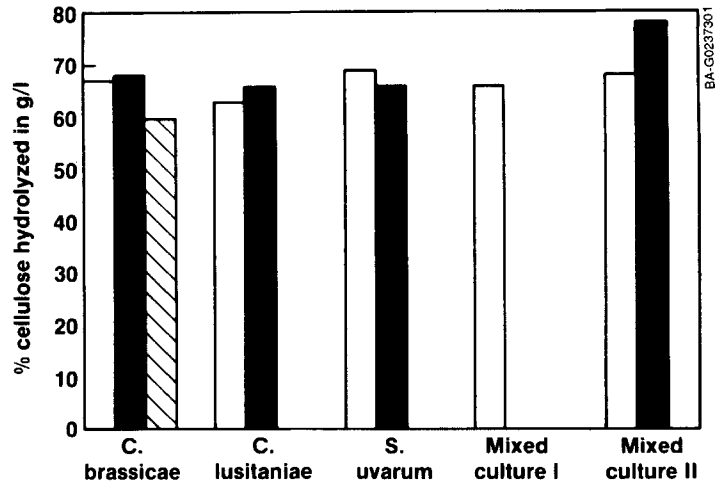

Figure 8. Cellulose hydrolyzed in grams per liter for large-scale SSFs at 37° (□), 41° (■) and 43°C (▨) with a cellulase loading of 13 IU/g Sigmacell-50 cellulose substrate (10%) over a 7-day period.

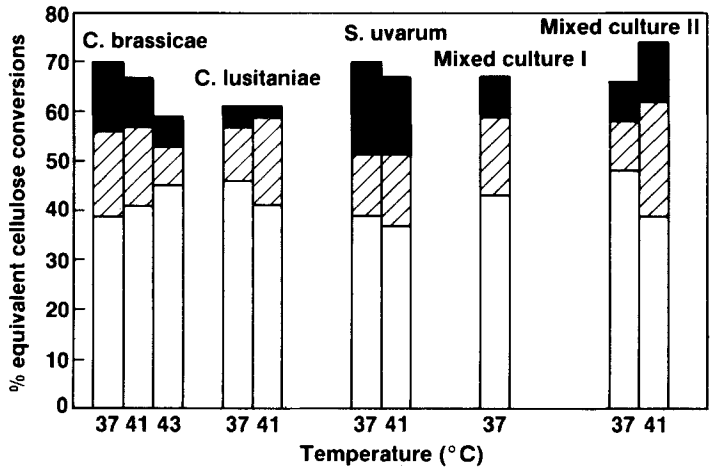

Figure 9. Percent equivalent cellulose conversions as a function of temperature for large-scale SSFs with cellulase enzyme loading of 13 IU/g of Sigmacell-50 substrate (10%) at two days (□), four days (▨) and final (■).

brassicae and *S. uvarum* outperform all of the other fermenting organisms tested for the 37°C SSFs.

DISCUSSION

Thermotolerant yeast were screened with the goal of improving the rate-limiting hydrolysis step in SSF since cellulase enzyme from *T. reesei* saccharifies optimally at approx. 45°C. These thermotolerant yeast evaluations revealed very little difference between 37°C and higher temperature SSFs for both rate and product yield. In fact, for all the yeast tested except mixed culture II, a decrease in final percent conversions is seen when the higher (41°C) temperature was used. It seems that the buildup of sugars due to reduced yeast viability will reduce enzyme performance more than can be compensated by increased temperatures.

In the previous screening experiments of thermotolerant yeast, mixed culture I at 37°C outperformed mixed culture II at 41°C.[2] In this study, the opposite relationship is seen. The only real difference between the two studies besides the scale of the SSF is the change in enzyme, in particular the reduction in β-glucosidase for the large scale. One difference between the two cellobiose fermenters is that *C. lusitaniae* has a faster initial rate of conversion compared to *B. clausenii*. This gives it an edge, especially when an insufficient amount of β-glucosidase is present. Without the fast fermentation capability, cellobiose builds-up causing end-product inhibition of the enzyme.

A minor difference was observed in saccharification rates between 37°C and 41°C, thus deemphasizing the higher temperature advantages, at least for the combinations of yeast and cellulose tested here. Since residual sugars accumulate with loss of yeast viability due to increases in temperature and a low ethanol tolerance, the inhibition of cellulase by these sugars apparently counters any rate improvements with increasing temperature. Finally, all of the yeast evaluated performed about the same, resulting in close final ethanol yields and cellulose degradation.

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