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High Solids Simultaneous Saccharification and Fermentation of Pretreated Wheat Straw to Ethanol

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ABSTRACT

Wheat straw was pretreated with dilute (0.5%) sulfuric acid at 140°C for 1 h. Pretreated straw solids were washed with deionized water to neutrality and then stored frozen at −20°C. The approximate composition of the pretreated straw solids was 64% cellulose, 33% lignin, and 2% xylan. The cellulose in the pretreated wheat straw solids was converted to ethanol in batch simultaneous saccharification and fermentation experiments at 37°C using cellulase enzyme from *Trichoderma reesei* (Genencor 150 L) with or without supplementation with β-glucosidase from *Aspergillus niger* (Novozyme 188) to produce glucose sugar and the yeast *Saccharomyces cerevisiae* to ferment the glucose into ethanol. The initial cellulose concentrations were adjusted to 7.5, 10, 12.5, 15, 17.5, and 20% (w/w). Since wheat straw particles do not form slurries at these concentrations and cannot be mixed with conventional impeller mixers used in laboratory fermenters, a simple rotary fermenter was designed and fabricated for these experiments. The results of the simultaneous saccharification and fermentation (SSF) experiments indicate that the cellulose in pretreated wheat straw can be efficiently fermented into ethanol for up to a 15% cellulose concentration (24.4% straw concentration).

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Above this concentration, the cells lose their viability apparently because of ethanol inhibition. The maximum ethanol concentration achieved of 57 g/L with 20.2% straw concentration (12.5% cellulose) yielded the highest sugar productivity of 1.27 g/Kg·h ethanol productivity of 0.62 g/Kg·h along with a combined ethanol and sugar yield of 70%.

Index Entries: Ethanol; fermentation; wheat straw.

INTRODUCTION

Ethanol production from lignocellulosic biomass is becoming important for both environmental and economic reasons. Ethanol-powered automobiles can reduce emissions of carbon monoxide and smog formation, which are major contributors to urban air pollution. The carbon dioxide released by combustion of petroleum and other fossil fuels may result in global climate change, but this major greenhouse gas does not accumulate in the atmosphere when ethanol from biomass is used because the carbon dioxide produced is needed to replenish the biomass supply. We now import almost 50% of the petroleum we use in the US. Because lignocellulosic biomass is plentiful, ethanol production from biomass can provide a domestic fuel resource that will reduce our dependence on these vulnerable supplies of petroleum-based fuels and cut our balance-of-trade deficit. Lignocellulosic biomass is also low enough in cost to potentially produce ethanol that is competitive in price with conventional fuel without tax subsidies. However, the technology for conversion of these materials into ethanol must be improved to realize this potential.

Production of ethanol from cellulosic biomass requires different conversion systems than those currently used for ethanol production from corn because the carbohydrates are much more difficult to solubilize than for starch in grains (*1*). The SSF process, which was first disclosed in 1977 (*2*), gives high rates and yields for ethanol production from biomass (*3,4*). In SSF, the enzymatic breakdown of cellulose to glucose is coupled with yeast fermentation in one fermenter. This eliminates the need for an extra fermenter, reduces end-product inhibition of cellulase enzymes, and decreases the probability of contamination (*5*). For batch fermentations, the final ethanol concentration is limited by the solids concentrations that can be mixed in the fermentation. For conventional fermenters, the solids content is limited to about 10%, resulting in ethanol concentrations of about 4% (*6,7*). However, if higher solids levels could be processed, it might be possible to achieve higher ethanol concentrations.

Bread, cheese, other fermented foods, and some alcoholic beverages are fermented at high solids concentrations (*8*). In recent years, high solids fermentations have been used extensively by several researchers for ethanol production from sugar-containing substrates such as sorghum

(*9–11*). High solids fermentations have also been studied for anaerobic digestion of solid biomass into biogas or methane (*12*). The advantages of the high solids reactor over a conventional low solids (3–8%) continuous stirred tank reactor (CSTR) can include lower capital costs because of a reduced reactor volume; lower operating costs that result from reduction in heating, cooling, and mixing power; lower downstream processing costs because of a higher product concentration; and reduced residual disposal costs because of less water used. Thus, we designed an inexpensive high solids reactor system to investigate high solids SSF of pretreated wheat straw.

MATERIALS AND METHDOS

Materials

The substrate used was pretreated wheat straw (*see* Methods) that contained 64% cellulose, 33% lignin, and 5% xylan. The fermentation organism was *S. cerevisiae* (D5A), which is a NREL strain genetically derived from Red Star baker's yeast. The growth media contained 1% yeast extract (Difco, MI) and 2% peptone (Difco). The cellulase enzyme was *T. reesei* cellulase 150L (Genencor, CA), and β-glucosidase (Novo 188) was from *A. niger* (Novo Inc., Wilton, CT). The stock solution of antibiotic mixture contained penicillin and streptomycin, each 5 g/L.

Methods

The wheat straw was pretreated in a 55-gal Pfaudler reactor with a pH of 1.4 adjusted with sulfuric acid and cooked at 140–160°C for 30–60 min (*13*). Pretreated straw was washed with deionized water, dried to a lower moisture content (50–65%), and frozen for storage. The substrate concentrations on a dry basis are shown in Table 1. The fermentations were performed in 1-L plastic jars equipped with a one-way valve to vent carbon dioxide. At 37°C, the jars were rotated horizontally at 20 rpm using a modified laboratory ball mill (Norton Model #753R109, Mahwah, NJ), which was powered by a variable-speed DC motor (Dayton Manufacturing Co., Chicago, IL). The inoculum was started from a frozen stock culture inoculated into a 50-mL flask containing 20 mL of YEPD (1% yeast extract, 2% peptone, and 2% glucose) medium and then transferred into a 250-mL flask containing 100 mL of YEPD. After the grown culture was centrifuged, the cells were suspended in 5X YEP (1% yeast extract, 2% peptone) and added to the fermentation mixture.

One hundred grams of fermentation mixture contained 20 g of 5X YEP with cell suspension, 0.4 g of antibiotic mixture, the desired amount of straw by weight, 0.24 g cellulase enzyme solution per gram of cellulose

Table 1
Final Ethanol, Sugar, and Cell Concentrations and Estimated Yields After Six Days for SSFs of Pretreated Wheat Straw With and Without β-Glucosidase Supplementation (1:1 IU/IFPU)

Conc. of substrate, %		With β-glucosidase supplementation				Without β-glucosidase supplementation			
		Final ethanol		Final sugar conc.,	Final cell conc.,	Final ethanol		Final sugar conc.,	Final cell conc.,
Straw	Cellulose	g/L	%	g/L	CFU/mL	g/L	%	g/L	CFU/mL
12.1	7.5	38	82.0	1.0	10^7	36	76.5	1.0	10^8
16.1	10.0	48	75.4	1.0	10^4	47	72.2	1.0	10^7
20.0	12.5	57	68.5	16.0	0	52	62.4	3.0	0
24.2	15.0	57	54.5	26.0	0	55	49.9	12.0	0
28.2	17.5	40	33.2	82.0	0	35	27.6	31.0	0
32.3	20.0	34	23.9	83.0	0	32	21.5	52.0	0

[equivalent to 20 international filter paper units (IFPU)/g cellulose], and 0.008 g of β-glucosidase per IFPU of cellulase if necessary (1 IU of β-glucosidase/1 IFPU of cellulase) (*14*). The initial wt of fermentation mixture was measured before starting each experiment. Since the vol of mixture changed during the period of the experiment, all the measurements were done on a wt basis.

Samples were taken on a daily basis and centrifuged; and the supernatant was analyzed for ethanol, sugar, and yeast viability. The ethanol concentration (% w/v) in the supernatant was measured by gas chromatography (Hewlett Packard 5880 A, Porapak Q80/100 column) using 1% isopropanol as an internal standard. Glucose was measured with a model 27 glucose analyzer from Yellow Springs Instruments (Yellow Springs, OH). Yeast viability was measured as colony-forming units (CFU), which were determined by a dilution series method and plated on YEPD plates.

Control experiments were performed with Sigma-Cell 50 cellulose (Sigma Chemical Co., St. Louis, MO) as the substrate over the concentration range of 10, 15, and 20% w/w. Enzyme loading requirements were determined by performing a set of saccharifications. Pretreated wheat straw was hydrolyzed with different enzyme loadings (in IFPU of enzyme/g of cellulose) at 45°C, which is a long-term optimal temperature for Genencor cellulase 150L. Digestibility was monitored by the glucose release measured each day. The optimal loading of the enzyme was the concentration above which there was no increase in the amount of released sugar.

The experiments were carried out with and without β-glucosidase supplementation (1:1 ratio). All the experiments were repeated two to four times to check the accuracy of the data. The ethanol yield and percent conversion of the straw to total sugar was calculated as follows:

$$\text{Percent Ethanol Yield} = E \times 100/(C_o \times 0.568)$$

$$\text{Percent Conversion} = [(S_e + S)/(C_o \times 1.1)] \times 100$$

in which 0.568 is the theoretical yield of ethanol from cellulose and the factor 1.1 is the wt gain in converting cellulose to sugar.

To calculate these values, the following terms must be measured:

W_0 = Initial wt of mixture (gm)
W_1 = Wt of dry straw added to the media (gm)
W = Wt of aqueous media (gm)
$\overline{S}$ = Sugar concentration measured in solution (g/L)
$\overline{E}$ = Ethanol produced (g/l), measured by GC
e = Ethanol produced (W%), which is calculated from Ref. 16
C_0 = Initial cellulose (gm)

In addition, the following terms must be calculated by the given formulas:

$W = W_o - W_1$
$s = \bar{S}/10$ = wt percent of sugar formed
$S = s \times W/(100 - 0.9 \times s - 0.804 \times e)$ = sugar produced (g)
$S_e = E/0.51$ = wt of sugar required to form the measured quantify of ethanol (g)
$E = e \times W/(100 - 0.9 \times s - 0.804 \times e)$ = wt of ethanol produced (g)

In these relationships, 0.9 is the multiplier to determine the amount of cellulose required to form a unit wt of sugar, 0.804 is the coefficient for hydration of cellulose and conversion of cellulose to ethanol, and 0.51 is the theoretical yield of ethanol from sugar.

RESULTS AND DISCUSSION

Simultaneous saccharification and fermentation of pretreated wheat straw was performed with *S. cerevisiae* (D5A) at 37°C. The enzymes used were Genencor 150L *T. reesei* cellulase (*15*) unsupplemented and supplemented with *A. niger* β-glucosidase Novo 188 (1:1). The fermentations were performed under nonaseptic conditions with uncontrolled pH starting at 5.5 at the beginning of the fermentation and dropping steadily to 4.3 at the end. The loading of cellulase enzyme was 20 IFPU/g cellulose, corresponding to the saturation level as determined by performing the set of separate hydrolysis experiments described in the Methods section (data not shown). A standard deviation of 1.0 was calculated for the final ethanol yields in these experiments.

Figure 1 shows, as an example, the results of batch SSFs containing 20.2% straw concentration (equivalent to 12.5% cellulose concentration) with and without β-glucosidase supplementation. The ethanol concentrations in the supernatant at the end of these SSFs (after 6 d) were 57 and 52 g/L, respectively. The viability of the cells, as determined by measuring CFUs, dropped from 10^8 CFU/mL at the start to zero for both runs after approx 4 d.

Table 1 presents final ethanol, sugar, and cell concentrations after 6 d of fermentation for SSFs of pretreated straw with and without β-glucosidase supplementation. The initial concentration of pretreated straw ranged from 12.1 to 32.3% (w/w). As seen in this Table, the ethanol concentration in the liquid reached its highest value of 57 g/L for a straw concentration of 20.2–24.2% with β-glucosidase supplementation and then decreased as the concentration of straw increased beyond this range. The estimated ethanol yield dropped from 82 to 21% with increasing initial concentration of straw. As mentioned above, the cells lost their viability after 4 d for an initial straw concentration of 20.2% or above, and the sugar concentration increased after the cells died, although no further

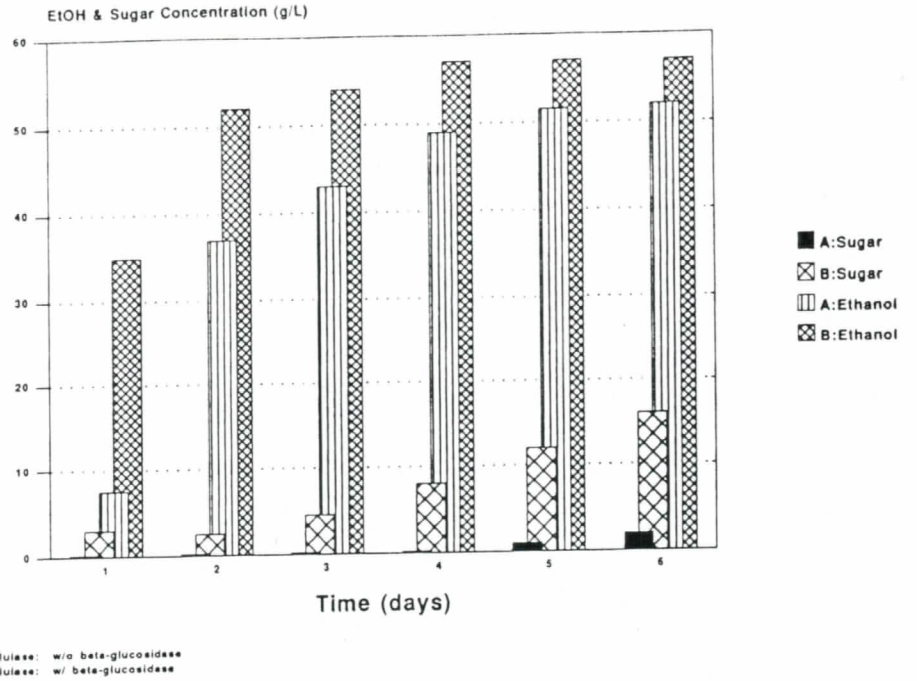

Fig. 1. High solids SSF of 20.2% straw containing 12.5% cellulose and 20 IFPU/g cellulose for a temperature of 37°C with *S. cerevisiae* yeast.

ethanol could be formed. This result suggests that high solids SSFs are not limited by enzymatic saccharification but by yeast viability. For experiments with initial straw concentrations of 12.1 and 16.1%, the cells were still viable after 6 d of SSF experiments, and little sugar accumulated in the broth.

As seen in Fig. 1, SSFs slowed after 3 d, which was the case for all experiments. As a result, the 3-d time period was chosen to analyze data for optimal conditions of SSF. Figure 2 shows the fractional conversion of cellulose to ethanol plus sugar after 3 d. Here, we have defined the fractional conversion as the total sugar released by hydrolysis after 3 d (sum of sugar, in grams, left in solution and stoichiometric quantity of sugar needed for ethanol in grams), divided by the initial cellulose concentrations multiplied by 1.1 to correct for hydration (*see* Appendix for details). These values are plotted against initial concentration of cellulose in the dry straw, for experiments with and without β-glucosidase supplementation (1:1). The cellulose conversion was approx 70% for an initial cellulose concentration 12.5% (20.2% straw) or less, however, above an initial 12.5% cellulose concentration, the conversion decreased sharply.

An important measure of the benefits of high solids fermentation is fermentation productivity. Sugar productivity (g/Kg·h) at 3 d is shown in Fig. 3, in which sugar productivity is defined as the sum of the stoichio-

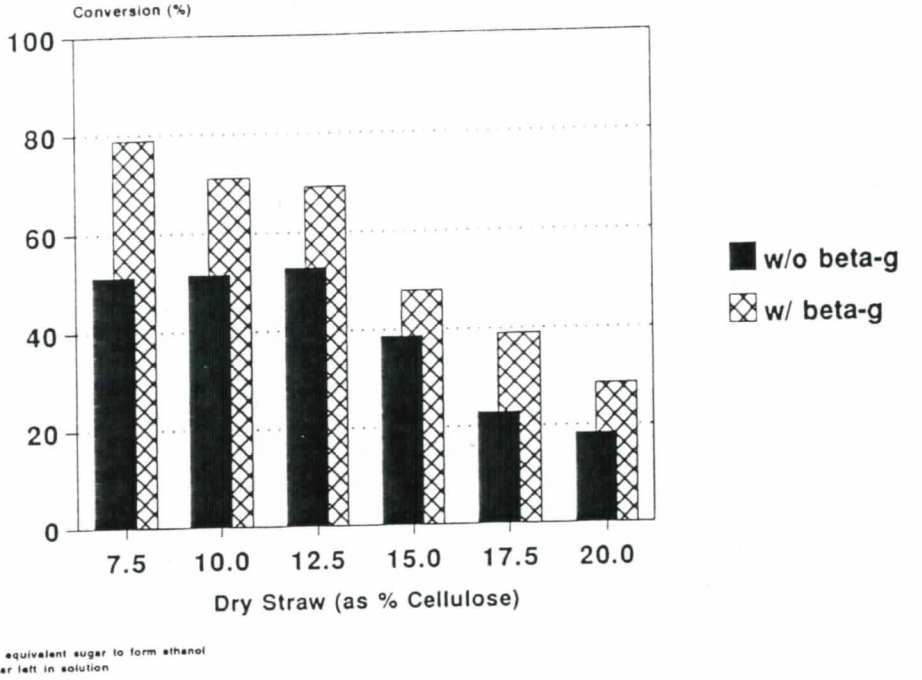

Fig. 2. Percent conversion of cellulose from straw to sugar after 3 d of SSF.

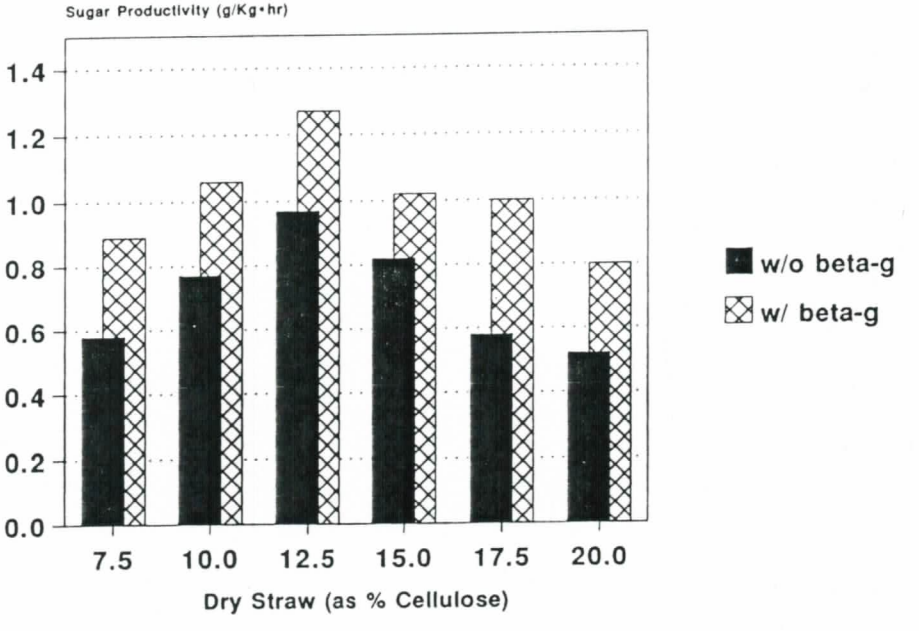

Fig. 3. Total sugar productivity (g/Kg·h) for SSF of straw, after 3 d of SSF run.

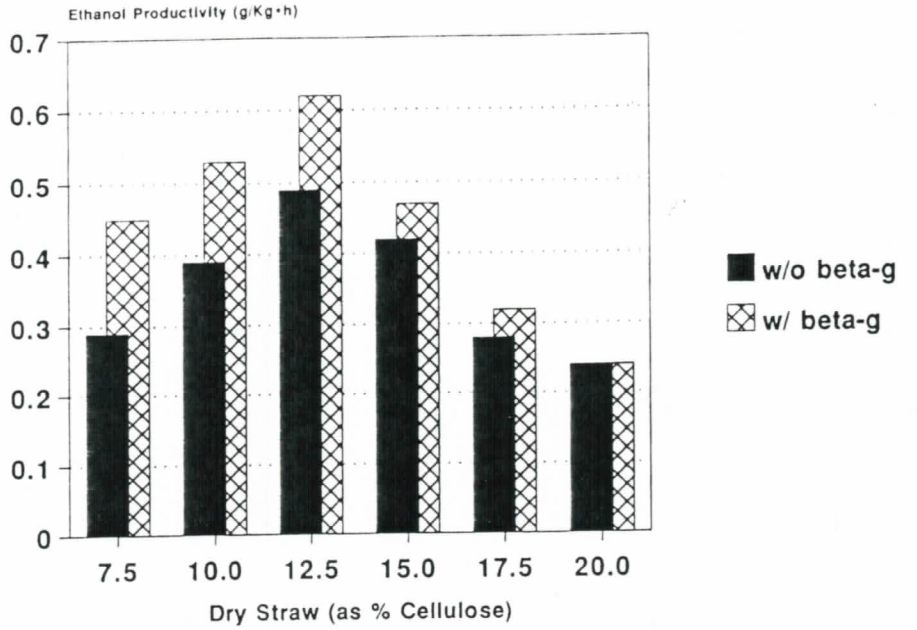

Fig. 4. Ethanol productivity (g/Kg·h) for SSF of straw, after 3 d of SSF run.

metric equivalent of the sugar converted to ethanol plus the sugar left in solution in grams after a period of time (in this case, 3 d) divided by corresponding time. As above, no corrections were made for sugar consumption by the yeast cells. The sugar productivity reached its maximum at around a 12.5% cellulose concentration (20.2% straw), and the sugar productivity with 1:1 IU β-glucosidase supplementation per IFPU cellulase was higher than without. At straw concentrations above 12.5% cellulose, the sugar productivity decreased sharply even though the sugar concentration stayed high. The dropoff in productivity could be attributed to buildup of sugars in the broth that inhibit the enzyme action or loss of cell viability.

The productivity of ethanol only for each experiment is shown in Fig. 4 at 3 d. This figure shows that the ethanol productivity peaked at the same 12.5% initial cellulose concentration found for maximum sugar productivity and ethanol yield. The ethanol productivity was higher for experiments that employed additional β-glucosidase, which confirms that there is an advantage to β-glucosidase supplementation.

The estimated final conversion of cellulose from straw into sugar (sum of sugar left in solution and sugar converted to ethanol) after 6 d of SSFs with and without β-glucosidase supplementation is shown in Fig. 5. The total sugar concentration is the sum of the sugar left in solution plus the equivalent amount of sugar corresponding to the measured ethanol concentration. This figure confirms that the enzymes were active during the

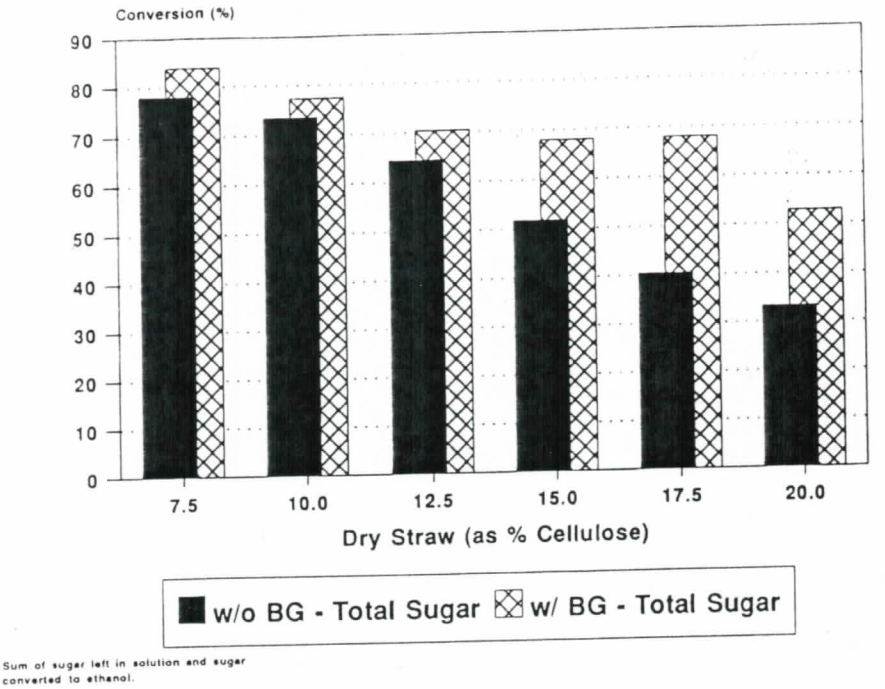

Fig. 5. Comparison of cellulose conversion to sugar in SSF after 6 d with and without beta-glucosidase supplementation (1:1 IU/IFPU).

Table 2
Final Ethanol, Sugar, and Cell Concentrations (After Six Days) and Estimated Yield for Control SSFs of Pure Cellulose (Sigma-Cell 50) with β-Glucosidase Supplementation (1:1 IU/IFPU)

Cellulose conc., %	Ethanol		Final sugar conc., g/L	CFU
	Conc., g/L	Est. yield, %		
10	48	81.5	0.5	10^7
15	55	61.7	12	0
20	54	47.5	18	0

6-d period, and the hydrolysis yield was more than 50% at higher solids concentration with β-glucosidase supplementation.

As a control, SSFs were performed with pure cellulose (Sigma-Cell 50) as a substrate as summarized in Table 2. The initial concentrations of cellulose used were 10, 15, and 20% (w/w), and the same pattern was observed with cellulose as with pretreated wheat straw. Although the cell viability (CFU) remained high at cellulose concentrations of 10%, at 15% or above, the cells lost their viability after 4 d, and the soluble sugar concentration

Table 3
Experiments to Study the Effects of Yeast
and Inoculum Addition on Ethanol Yield of High Solids SSFs

Experiment	Final ethanol yield, %
1. SSF of straw (15% cellulose) + daily addition of yeast extract	52.8
2. SSF of straw (15% cellulose) + daily addition of yeast extract and inoculum	53.9
3. SSF of straw (7.5% cellulose), with more straw added (7.5% cellulose) after 24 h	52.2
4. Hydrolysis of straw (15% cellulose), with additional inoculum after 3 d	51.1
5. SSF of straw (15% cellulose) with additional inoculum after 3 d	51.0

increased. The highest ethanol concentration obtained was approx 55 g/L with 15% cellulose, equivalent to 61.7% yield as calculated here.

The results of different SSFs showed that the viability of the cells decreased as the concentration of straw or cellulose increased, and the maximum concentration of ethanol that could be accumulated in the supernatants was 55–57 g/L, even though glucose was still present in solution. To investigate the cause of these phenomena, experiments were designed to study variables such as nutritional deficiency and inocula age, which may have affected the ethanol production process. Table 3 outlines the set of experiments and the ethanol yields for each. It can be seen from Table 3 that the ethanol yield was not increased by supplementing daily with yeast extract or yeast inoculum. Breaking the fermentation into two fed-batch steps using 7.5% cellulose feed, which individually achieved more than 80% yield, did not improve the yield either. The final yield and productivity for the fed-batch system was 52.2% and 0.3 g/Kg·h, respectively. These results show that there is no advantage to fed-batch SSF over batch SSF for high solids concentrations.

Because the ethanol production ceased when its concentration reached approx 55–57 g/L, the results of these experiments suggested that ethanol concentration was affecting the process, and the effect of ethanol on glucose fermentation was studied as a control. Five experiments were designed. Experiment 1 was a 40 g/L glucose control fermentation that yielded approx 20 g/L ethanol at complete conversion; in experiments 2–5, 40 g/L glucose was fermented in the presence of 20, 40, 49, and 63 g/L ethanol, respectively. The final ethanol concentrations in solution (the amount added initially plus that produced) and the glucose concentration left in solution are shown in Table 4. It can be seen from this Table that ethanol did not affect the fermentation of glucose by this strain for an ini-

Table 4
Effect of Ethanol on Glucose Fermentations by *S. cerevisiae* (D5A) at 37°C

Glucose conc., g/L	Ethanol added*, g/L	Final ethanol conc., g/L	Final glucose concentration, g/L	Remarks
40.0	0.0	20.0	0.1	Control
40.0	20.0	40.0	0.1	No inhibition
40.0	40.0	58.0	1.0	No inhibition
40.0	49.0	62.0	12.0	Some inhibition
40.0	63.0	63.0	40.0	Total inhibition

*Actual values of ethanol measured at the beginning of the experiment.

tial concentration of less than 40 g/L but that higher concentrations had deleterious effects. At final ethanol concentrations above 63 g/L, the fermentation stopped, and unfermented sugar was left in solution.

The results of these experiments showed that an ethanol concentration of about 60 g/L kills yeast cells even with abundant glucose in solution. For SSFs, less sugar is available to support yeast viability, and the ethanol production in high solids SSFs ceased as the concentration of ethanol reached around 55 g/L, even with some free sugar in solution. At higher initial concentrations of straw (more than 24.2%), the low water content in the growth media may be responsible for the loss of viability, which results in even lower ethanol yield.

To assess the influence of ethanol on SSF yields, a set of experiments was designed using the supernatant from a 6-d-old SSF of 24.4% straw, which was shown previously to contain high concentrations of both sugar and ethanol at the end of the cycle. The flow chart of these experiments is shown in Fig. 6, and the results are shown in Fig. 7. One portion of supernatant from SSF was directly subjected to fermentation without any treatment. As can be seen by comparing experiments 1 and 2 in Fig. 7, there was no increase in ethanol concentration, which confirms that ethanol concentration above 55 g/L inhibits ethanol production. Another portion of supernatant was distilled under vacuum to lower the ethanol concentration to 8.7 g/L and then divided into two parts. The first part was fermented, and ethanol concentration increased from 8.7 to 25 g/L with very little sugar (1.3 g/L) left in solution. Since phenolic compounds that are inhibitory to yeast may be released from lignin during pretreatment of wheat straw, the other portion was treated with charcoal to remove possible phenolic compounds in the SSF solution from the pretreated wheat straw. This solution was then fermented as well. The amount of ethanol produced was 24 g/L, and no sugar was left in solution, indicating complete fermentation. A comparison between experiments 3, 4, and 5 in Fig. 7 shows that the fermentation continued after the ethanol was removed from solution, and the extractives from pretreated straw did not have any effect on fermentation. This set of experiments confirmed the inhibitory effect of the final product, ethanol, on the yeast.

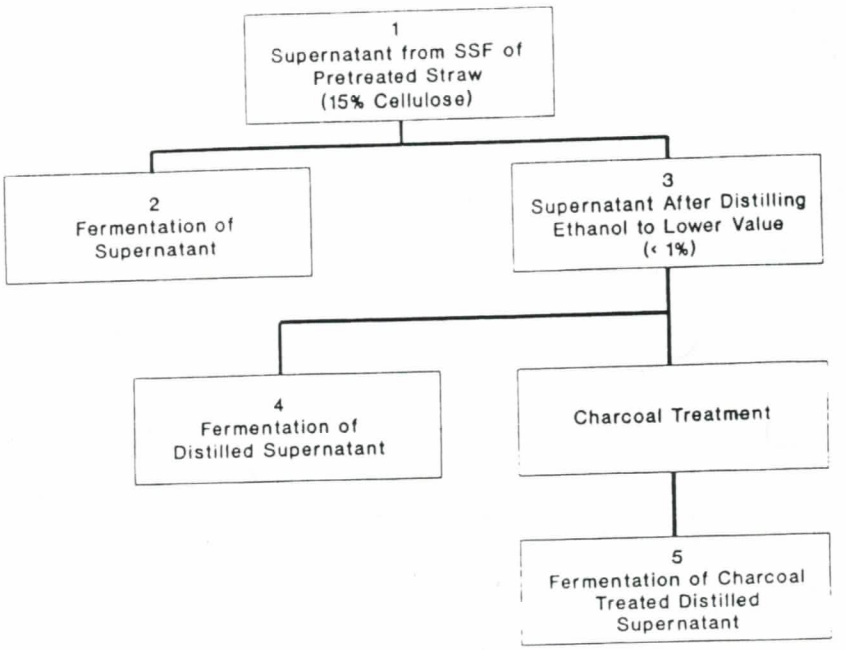

Fig. 6. Flow chart for experiments to check the effect of ethanol or other compounds on the SSF process for conversion of wheat straw to ethanol.

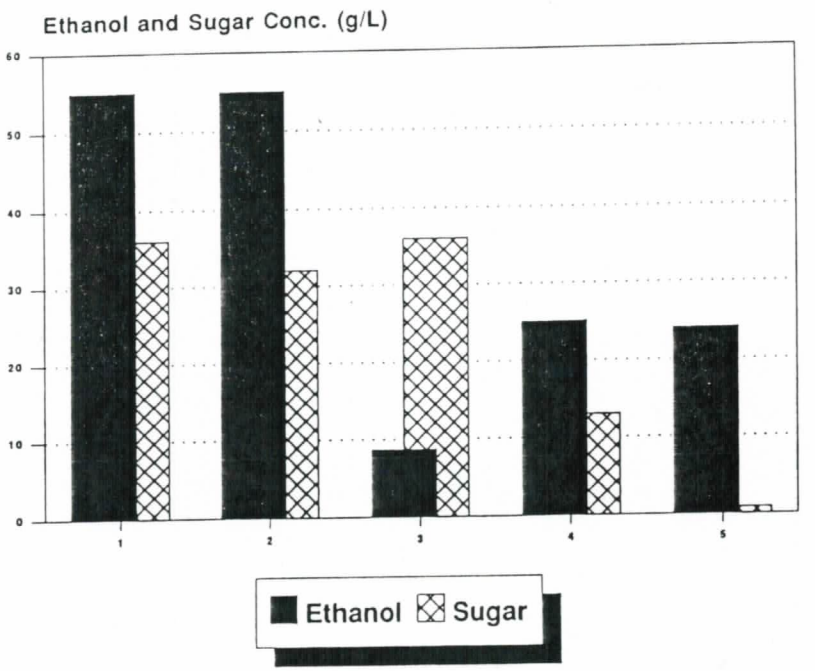

Fig. 7. Ethanol and sugar concentrations for fermentation of supernatant from SSF of straw to ethanol as shown in Fig. 6.

The overall results show that the cellulose in pretreated wheat straw can be efficiently fermented into ethanol at up to a 15% cellulose concentration corresponding to a 24.4% pretreated straw concentration. Above this concentration, the yeast cells lose their viability because of ethanol inhibition. The maximum ethanol concentration obtained was 55–57 g/L in the liquid.

CONCLUSIONS

The maximum ethanol concentration achieved was 57 g/L for a 20.2% straw concentration (12.5% cellulose). At this substrate level, the highest sugar productivity of 1.27 g/Kg·h and ethanol productivity of 0.62 g/Kg·h was also achieved along with a fractional conversion to combined ethanol and sugar of 70%. It should be noted that these small-scale SSFs were not optimized in terms of mixing or pH control. Overall, the high solids SSFs run were limited by yeast cell viability and not by enzymatic saccharification. Saccharification continued after ethanol production stopped at very high concentrations of solids and resulted in an overall fractional conversion of more than 50% for a 32.3% straw concentration (20% cellulose). Finally, high solids SSFs resulted in higher ethanol concentrations and productivities than low solids SSFs. Fed-batch SSFs started with low solids (12.1% straw concentration equivalent to 7.5% cellulose) did not show any advantages over high solids batch SSFs.

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