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Ethanol Production from Cellulose by Coupled Saccharification/Fermentation using *Saccharomyces cerevisiae* and Cellulase Complex from *Sclerotium rolfsii* UV-8 Mutant*

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ABSTRACT

Using cellulase/hemicellulase complex of *Sclerotium rolfsii* UV-8 mutant and *Saccharomyces cerevisiae* for fermentation, the coupled saccharification/fermentation (CSF) of 15% AT-rice straw was carried out at 40°C, pH 4.5 for the first 24 h and further incubation was performed at 30°C for 72 h. Increasing the amount of cellulase activity from 3–12 IU FPA/g of substrate resulted in increased yields of ethanol from 1.5–3.6% in 96 h. It has been observed that the coupled system was advantageous over the two stage (separate hydrolysis/fermentation) system as it produced higher amounts of ethanol from cellulose (3.6% as compared to 2.3% ethanol from rice straw).

Index Entries: Biomass; cellulase complex; coupled saccharification/fermentation; *Saccharomyces cerevisiae; Sclerotium rolfsii.*

INTRODUCTION

The conversion of cellulose to ethanol requires the use of two biosystems—one to degrade cellulose to glucose, and the other to convert glucose to ethanol. β-Glucosidase, the enzyme that catalyzes the hydrolysis of cellobiose to glucose (the final step in cellulose hydrolysis) shows end product inhibition. One means of reducing inhibition is to use

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cellulase preparation with higher proportions of β-glucosidase. Further improvement can be obtained by using coupled saccharification/fermentation (CSF) process for the conversion of cellulose to ethanol (*1,2*), and thus help in alleviating the problem of end-product inhibition and maintain the reaction at the faster rate for a longer time. In a coupled process for the conversion of pretreated wheat straw to ethanol Spindler et al. (*2*) used commercial cellulase preparation Genencor 150L supplemented with β-glucosidase (Novozym-188) for saccharification, and *Saccharomyces cerevisiae* or *Saccharomyces uvarum* for ethanol fermentation.

From a comparison made with various cellulolytic fungi like *Trichoderma reesei, Sporotrichum pulverulentum, Aspergillus niger* the parent strain of *Sclerotium rolfsii* produces a complete cellulase/hemicellulase complex with exceptionally high glucosidase activity (*3*). While a mutant strain, UV-8 produces 2.5 times more filter paper degrading activity (FPA) and 1.2–1.5 times more endo-glucanase and β-glucosidase activity as compared to the parent strain (*3*). Various factors affecting the cellulase complex of UV-8 mutant during saccharification have also been studied (*4*).

In the present study, *S. rolfsii* UV-8 mutant cellulase complex consisting of high β-glucosidase has been used as a model system for CSF of pretreated rice straw. For fermentation at 40°C, *S. cerevisiae* and *Candida tropicalis* have been used. The results have also been compared with the two stage saccharification/fermentation system using *S. cerevisiae, C. tropicalis,* and *Zymomonas mobilis* for ethanol production at 30°C.

MATERIALS AND METHODS

Enzyme Production

The enzyme used in the studies was produced by growing *S. rolfsii* UV-8 mutant on NM-2 medium with the addition of 2% corn steep liquor for 14 d under shaking conditions at 28°C (*5*). The culture filtrate was adjusted to pH 4.5 with 0.5 *M* Na citrate and stored at 4°C.

Organisms

The yeast strains used in fermentation studies were *S. cerevisiae* NCIM 3078 and NCIM 3095, *C. tropicalis* NCIM 3110, and a bacterium was *Z. mobilis* NCIM 2428. The liquid malt extract-yeast extract- glucose- peptone (MYGP, 0.3%, 0.3%, 1.0% and 0.5%, respectively, pH 4.5) medium was used for inoculum preparation of yeast and *Z. mobilis* (30°C, 24 h).

Substrate

Rice straw (obtained locally) was Wiley-milled and sieved (50 mesh). The alkali treated (2*N* NaOH, 30°C, 48 h) AT-rice straw used in the present investigations had average cellulose (72%) and hemicellulose (20%) contents as determined according to Myhre and Smith (*6*).

Two Stage Saccharification and Fermentation

Two stage saccharification and fermentation was carried out under the respective temperature optima (50°C for saccharification and 30°C for fermentation). Hydrolysis of the AT-rice straw (15%) was carried out by incubating 12 g substrate with 4 mL 1*M* citrate buffer, pH 4.5, cellulase units (12 IU FPA/g substrate), and water to make final weight 80 g, at 50°C for 48 h under shaking conditions. Supernatants after 48 h (52 mL, containing 104 mg reducing sugars/mL) plus 12 mL nutrient solution (yeast extract, 0.85%; $MgSO_4 \cdot 7H_2O$, 0.01%; $(NH_4)_2SO_4$, 0.132%; $CaCl_2 \cdot 2H_2O$, 0.006%) made to 100 mL with distilled water were used for fermentation. The inoculum size for fermentation was 4×10^8 cells. Fermentation was carried out at 30°C for 96 h in the case of yeast strains, and for 48 h for *Z. mobilis*.

Coupled Saccharification/Fermentation (CSF)

The CSF experiments with 15% AT-rice straw as a substrate were carried out in 250 mL stoppered flasks. The reaction mixture of saccharification [12 g substrate + 4 mL 1*M* citrate buffer, pH 4.5 + 12 IU FPA/g substrate and water to make final weight 80 g] along with 12 mL nutrient solution was inoculated with yeast cells and the flasks were incubated initially at 40°C under shaking conditions for 24 h and further at 30°C for different time periods without shaking.

Analytical Procedures

Cellulase/hemicellulase and β-glucosidase activities in the culture filtrate were determined as described earlier (*5*). To determine ethanol produced from cellulosics, the fermented broth (approximately 50 mL) was diluted to 150 mL with water and then distilled. 100 mL distillate was first collected and then analyzed by the enzymatic method (*7*).

Table 1
Formation of Sugars During Enzymatic Hydrolysis of AT-Rice Straw by *S. rolfsii* UV-8 Cellulase Complex

	Time, h			
	1	4	24	48
Reducing sugars,[a] mg/mL	35.5	44.0	64.0	68.5
Glucose,[b] mg/mL	18.0	30.0	50.0	53.5
Cellobiose,[c] mg/mL	2.5	1.8	1.0	0.2

Saccharification was carried out with 10% substrate, pH 4.5, 50°C.
[a]Reducing sugars were estimated using DNS method (*8*).
[b]Glucose was estimated by glucose oxidase-peroxidase method (*9*).
[c]Purified *S. rolfsii* cellobiase was used for cellobiose estimation. The values also include other soluble cellooligosaccharides.

RESULTS AND DISCUSSION

Cellulase Complex for Saccharification

The cellulase/hemicellulase and β-glucosidase activities determined at 50°C, pH 4.5, of the preparations used were (IU/mL): FPA, 2.16; endoglucanase (CMCase), 195; xylanase, 185; β-glucosidase, 13.9 (when estimated using *p*-nitrophenyl-β-D-glucose as a substrate), and 20.0 (when estimated using cellobiose as a substrate).

Products of Saccharification

The time course estimation of total reducing sugars, glucose and cellobiose produced during the hydrolysis of 10% AT-rice straw by the cellulase/hemicellulase complex of UV-8 mutant has been studied (Table 1). Although the cellobiose accumulation was observed initially, it fell to almost negligible levels in the later stages of hydrolysis. This can be attributed to the high levels of β-glucosidase activity in the culture filtrate. The difference in the values between the total reducing sugars and the sum of the glucose and cellobiose could be caused by the undetermined higher oligomers and pentoses.

Organism

Because of the complexity of the system, optimal conditions for yeast fermentation are not necessarily the same as those optimal for the saccharification. For instance, the optimum temperature range for fermentation is 28–35°C, and the range for saccharification by *S. rolfsii* UV-8 mutant cellulolytic complex is 45–50°C. With this in mind, some of the yeasts and a *Z. mobilis* strain were screened for their ability to grow at 40°C.

Table 2
Study of Alcohol Production in a Two-Stage and Coupled Saccharification/Fermentation System by Yeast and *Z. mobilis*

Organism	% Ethanol, w/v in a two stage process[a]	% Ethanol, w/v in CSF[b] 48 (h)	72 (h)	96 (h)
S. cerevisiae NCIM 3078	2.3	2.5	2.7	3.6
S. cerevisiae NCIM 3095[c]	2.6	—	—	—
C. tropicalis NCIM 3110	2.0	2.4	2.6	3.3
Z. mobilis NCIM 2428[c]	2.6	—	—	—

15% AT-rice straw for saccharification was used.
[a]Saccharification was carried out at 50°C for 48 h. Fermentation was at 30°C for 96 h in the case of yeast strains and for 48 h in the case of *Z. mobilis*.
[b]Initially flasks were incubated at 40°C for 24 h and further continued at 30°C for different time periods as indicated.
[c]The strains could not grow at 40°C.

It was not possible to carry out CSF with *Z. mobilis* because the available strain could not grow at 40°C. *S. cerevisiae* NCIM 3095 also failed to grow at 40°C, however, both of these strains produced higher amounts of ethanol from rice straw in a two stage process at 30°C as compared to the other strains examined. *C. tropicalis* NCIM 3110 showed good growth at 40°C, but it was a less potent ethanol producer in CSF compared to *S. cerevisiae* NCIM 3078. Table 2 summarizes the results of the two stage process and CSF studied under optimal conditions. Unless otherwise mentioned, all of the experiments in CSF were carried out using the NCIM 3078 strain.

Effect of Factors Governing CSF

Size of Yeast Inoculum

The seed culture of the NCIM 3078 strain was grown in 1% MYGP medium for 24 h at 30°C, harvested, and centrifuged at $2000 \times g$ for 15 min. The pellet was resuspended after two washings with sterile 0.9% KH_2PO_4 in approximately 0.1 of the original volume. In CSF, using AT-rice straw as a substrate, increasing the yeast inoculum size from 1×10^8 to 4×10^8 cells/12 g substrate raised the ethanol yields from 3.1–3.6% (w/v) in 96 h. Therefore, 4×10^8 cells were used in further studies.

Cellulase Units

Increasing the amount of cellulase activity from 3–12 IU FPA/g substrate resulted in increased yields of ethanol from 1.5–3.6% in 96 h. Similar observations were noted by Wright et al. (*10*) when they used not less than 15% initial cellulose concentration. According to that study, the extent of reaction was controlled by the ethanol tolerance of the yeast and not by the enzyme concentration.

Effect of Ethanol Concentration on Cellulase Complex

It has been shown that ethanol inhibits the cellulase activity (*11*), and this inhibition may be the effect of ethanol on the adsorption of enzymes on the substrate. Different concentrations of ethanol (up to 10%) were used to study the inhibition of cellulase, xylananse, and β-glucosidase activities, and saccharification measured as reducing sugars produced. Ethanol concentrations studied inhibited the production of reducing sugars in saccharification of rice straw, but it did not affect FPA and CMCase activity in concentrations up to 10%. However, xylanase activity was stronglyl inhibited. AT-Rice straw contained almost 20% hemicellulose that contributed in the estimation of reducing sugars because of the high xylanase activity in the culture filtrate. Therefore, in the presence of ethanol the decrease in the reducing sugars formation may be caused by the inhibition of xylanase activity (Fig. 1). β-Glucosidase, although showing inhibition above 5% ethanol concentration, enhancement in the activity was observed with lesser concentrations. However, as seen from Table 1, cellobiose concentration was decreased during the hydrolysis of rice straw. Therefore, the further increase in β-glucosidase activity in the presence of ethanol (<5%) may not be beneficial to the system (Fig. 1).

Adsorption of Cellulase on Substrate

Since the cellulase system hydrolyzing crystalline cellulose is a balanced complex of three different types of enzymes, the availability of each enzyme component should be the same in relative terms (*2,12*). Therefore, particular attention was given to the amount of enzymes adsorbed onto the solid material during saccharification.

The adsorption of cellulase, β-glucosidase, and hemicellulase on substrate was studied by incubating a known amount of the culture filtrate with varying amounts of straw (3–15%) for 20 min at 40°C (Fig. 2). All the activities were rapidly adsorbed, however, the increase in substrate concentration above 5% did not show any further decrease in CMCase activity in the supernatant. FPA, xylanase, and β-glucosidase showed more adsorption in the presence of higher substrate concentrations (>6%). The ratio of β-glucosidase to FPA, which has an influence on effective saccharification (*2*), remained constant (6.4:1) under studied substrate concentrations.

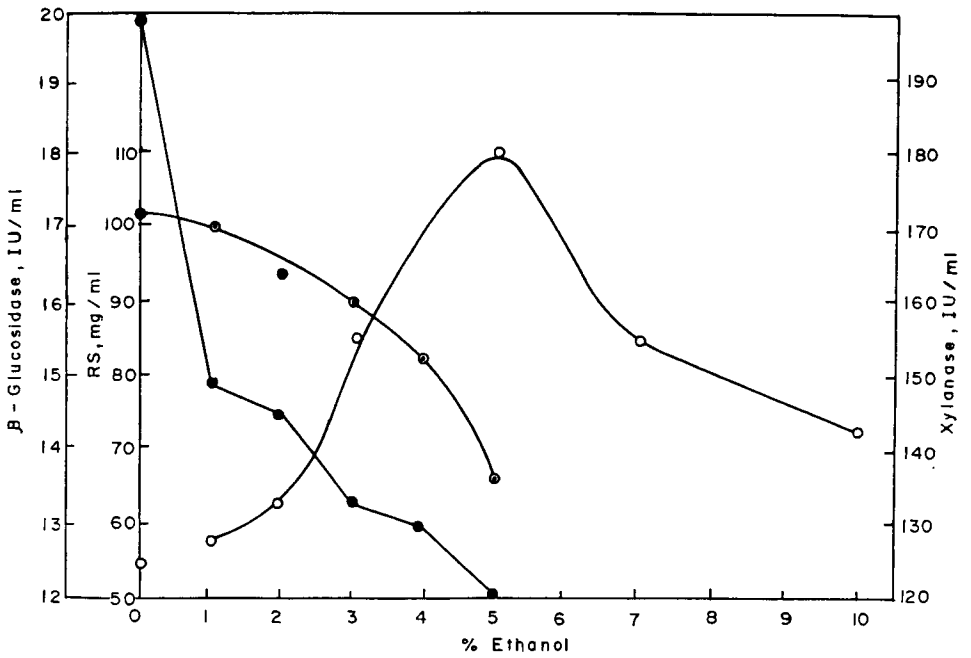

Fig. 1. Effect of addition of ethanol on enzyme activities and on saccharification of 15% AT-rice straw. The enzyme was incubated at room temperature for 10 min with different concentrations of ethanol and the assays were carried out. Key: ⊙, Reducing sugars produced in 48 h; ●, Xylanase; ○, β-Glucosidase.

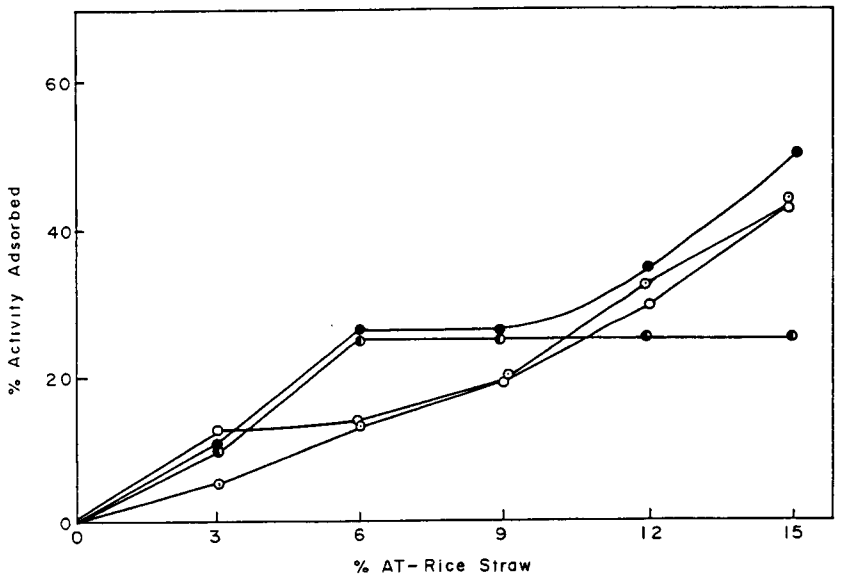

Fig. 2. Adsorption of cellulase/hemicellulase activities of *S. rolfsii* UV-8 mutant on the AT-rice straw incubated at 40°C for 20 min. Key: ○, FPA; ◐, CMCase; ⊖, Xylanase; ⊙, β-Glucosidase.

The hydrolysate obtained after 48 h saccharification of AT-rice straw contained 7.6% fermentable sugars as glucose. In the coupled system, glucose or cellobiose, except in the initial stages of the process, could not be detected, indicating that it helps to alleviate the problem of end product inhibition of cellulolytic enzyme complex. In CSF the amount of ethanol produced from 15% rice straw was 36 g/L in 96 h (that is, 55% conversion of theoretical). In addition, as the AT-rice straw contains almost 20% hemicellulose, the expected xylose in the hydrolysate could be used in the ethanol fermentation. In this regard, co-culture of *S. cerevisiae* and *Candida shehatae*, a xylose fermenting yeast, can be used. Alternatively, the supplementation with glucose(xylose) isomerase of cellulase complex and subsequent fermentation with *S. cerevisiae* that can utilize both glucose and xylulose can be used (*13*). The strain of *Z. mobilis* used in the present studies could not grow at 40°C, therefore it was not possible to carry out CSF. However, using *Z. mobilis* strains that can grow at 40–45°C for fermentation, it is possible to obtain higher ethanol production in CSF using *S. rolfsii* UV-8 mutant cellulase complex that has high amounts of β-glucosidase activity.

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