Efficient Simultaneous Saccharification and Fermentation of Agricultural Residues by *Saccharomyces Cerevisiae* and *Candida Shehatae*

The D-Xylose Fermenting Yeast

SANJAY S. PALNITKAR AND ANIL H. LACHKE*

Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India

ABSTRACT

Simultaneous Saccharification and Fermentation (SSF) experiments were carried out on agricultural residues using culture filtrate of *Sclerotium rolfsii,* which produces high levels of cellulases and hemicellulases for the saccharification of rice straw and bagasse, and *Candida shehatae*—the D-xylose fermenting yeast, and *Saccharomyces cerevisiae,* both separately and in coculture, for fermenting the released sugars. The coculture system showed efficient utilization of hydrolyzed sugars with 30–38% and 10–13% increase in ethanol production as compared to *C. shehatae* and *S. cerevisiae,* respectively, when cultivated separately. SSF simulation studies were carried out using standard sugar mixtures of glucose, xylose, and cellobiose. Both organisms could not use cellobiose, whereas glucose was used preferentially. *C. shehatae* was capable of utilizing xylose in the presence of glucose.

Index Entries: Simultaneous Saccharification and Fermentation; yeast; *Candida shehatae; Saccharomyces cerevisiae; Sclerotium rolfsii;* xylose fermentation; agricultural residues.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Agricultural residues consist of cellulose, hemicellulose, and lignin in an average proportion of 4:3:3. Efficient utilization of these residues for ethanol production would imply quantitative conversion of the cellulosic as well as the hemicellulosic sugars. The exploitation of cellulosic sugars for ethanol production has been studied in SSF (Simultaneous Saccharification and Fermentation) experiments by several investigators (*1–3*). SSF implies a single-stage process wherein the saccharification of the substrate and the fermentation of the released sugars occurs simultaneously. This has several advantages over SHF (Separate Hydrolysis and Fermentation) as end-product inhibition by glucose and cellobiose is minimized—hence, the concentrations of cellulase/hemicellulase (enzyme loading) required for saccharification is less. Moreover, the total production cost of ethanol by SHF is $2.66/gal, whereas it is $1.78/gal for the SSF method (*4*). However, no data on the fermentation of D-xylose present in the hydrolysates of agricultural residues are available for the SSF system. Only a few yeasts, namely, *Pachysolen tannophilus, Candida shehatae,* and *Pichia stipitis,* capable of fermenting D-xylose to ethanol are known (*5–8*).

In the present study, culture filtrate of *Sclerotium rolfsii,* which produces high activities of cellulases and hemicellulases (*9*), is used for the saccharification of agricultural residues in SSF system. For ethanol production, *Saccharomyces cerevisiae* and *Candida shetatae* have been used separately and also in coculture, the main advantage being efficient bioconversion of the cellulosic as well as the hemicellulosic components present in the agricultural residues. The results are discussed in this chapter.

MATERIALS AND METHODS

Microorganisms and Growth Conditions

Sclerotium rolfsii NCIM 1084 (National Collection of Industrial Microorganisms) was grown on Mandels-Weber medium (*10*) with Solka floc as the carbon source (30°C, 200 rpm). The culture filtrate was collected after 12 d of incubation and filtered through a sterile filter (0.45 μ, Millipore Corp.).

Candida shehatae ATCC 22984 was maintained on MXYP slants. It was cultivated on a liquid medium containing (g/100 mL) D-xylose, 3; KH_2PO_4, 1.87; $(NH_4)_2HPO_4$, 0.6; $MgSO_4 \cdot 7H_2O$, 0.113; and yeast extract, 0.37. The growth was harvested after 48 h (30°C, 200 rpm) by centrifugation. The cells were washed three times with distilled water and finally with phosphate buffer (pH 7.0, 0.05*M*).

Saccharomyces cerevisiae NCIM 3078 was maintained on MGYP slants. It was cultivated on the above medium, except that glucose was used as

the carbon source. The cells were harvested and washed as mentioned earlier.

Agricultural Residues and Pretreatment

Rice straw and bagasse (obtained locally) were Wiley-milled and sieved (50 mesh). They were treated with 2*N* NaOH for 48 h at 30°C. The residues were washed free of alkali and dried.

Saccharification and Fermentation

The pretreated agricultural residues (5 g each) were taken in 250-mL Erlenmeyer flasks with 120 mL of culture filtrate of *S. rolfsii*, containing (g/100 mL) yeast extract, 0.85; $MgSO_4 \cdot 7H_2O$, 0.01; $(NH_4)_2HPO_4$, 0.132; and $CaCl_2 \cdot 2H_2O$, 0.006. *S. cerevisiae* and *C. shehatae* cells were added separately and together (1:1 proportion) to a final concentration of 10×10^{10} cells/mL.

The flasks were shaken at 40°C (100 rpm) for 24 h and then at 30°C (200 rpm) for 96 h. Samples were collected periodically to determine the sugars and alcohol produced. Fermentation of standard sugar mixtures by *S. cerevisiae* and *C. shehatae* were studied using D-glucose, D-xylose, and D-cellobiose (2% each) and 0.67% Yeast Nitrogen Base (YNB)(Difco). The YNB was filter sterilized. The final pH of the medium was 5.0. Fermentation was carried out in 250-mL Erlenmeyer flasks at 30°C, 200 rpm.

Analytical Procedures

Alcohol was determined by the enzymatic method (*11*). Reducing sugars were determined by the Somogyi-Nelson method (*12*). Paper chromatography of sugars and sugar alcohols was carried out using butanol:acetic acid:water (3:1:1) as the solvent system. Standard sugars were quantified by HPLC (HP Model 1084B, Waters' Sugar-Pak column with water at 75°C as the eluent). Cellulase and hemicellulase units in the culture filtrate were determined according to IUPAC (*13*). The assay of D-xylose reductase was carried out as described previously (*14*).

RESULTS AND DISCUSSION

SSF with *C. shehatae* and *S. cerevisiae* (Separate Cultivation)

Table 1 shows the enzyme activities used in the SSF experiments. SSF was carried out at 40°C for 24 h, although the optimum temperature of these enzymes is 50°C because of the poor growth of the two microorganisms at 50°C. After 24 h, the flasks were kept at 30°C. The ethanol production and profile of reducing sugars from pretreated rice straw and bagasse are shown in Figs. 1 a and b, respectively.

Table 1
Enzyme Activities from Culture Filtrate
of *S. rolfsii* Used in the SSF Experiments

Enzyme	Activity, IU/mL
CMCase	25.0
Xylanase	32.5
FPA	2.6
β-Glucosidase	2.7
β-Xylosidase	0.15

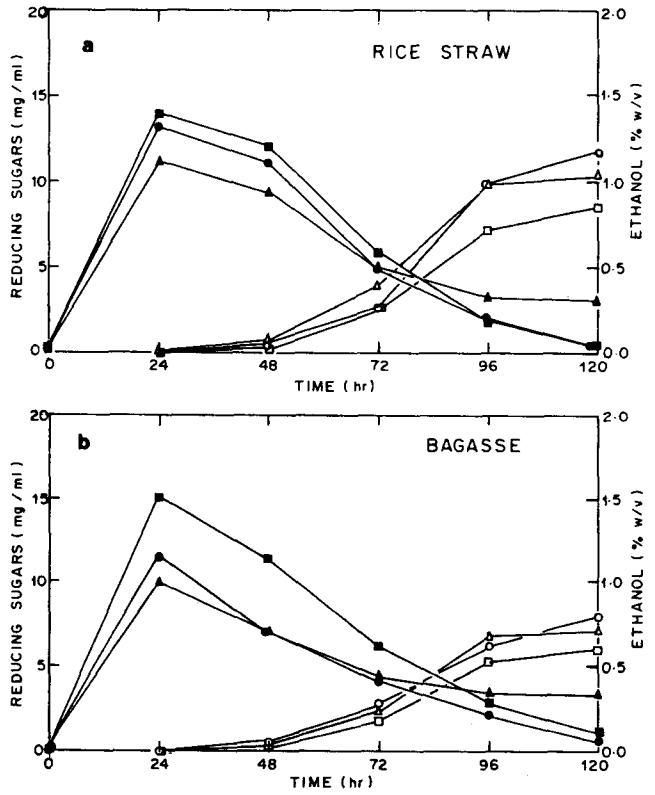

Fig. 1. (a) and (b) show the profile of reducing sugars [(▲) *S. cerevisiae*, (■) *C. shehatae* and (●) co-culture] and the alcohol produced [(△) *S. cerevisiae*, (□) *C. shehatae* and (○) co-culture] from Rice straw and Bagasse respectively.

The production of reducing sugars was maximum at 24 h with a steady decline until 120 h. The concentration of reducing sugars at 24 h was less for *S. cerevisiae* as compared to *C. shehatae* because of the capability of strain 3078 to grow at 40°C. All the sugars were not utilized by *S. cerevisiae.* Paper chromatograms revealed D-xylose to be the major unutilized sugar in the case of *S. cerevisiae.* Ethanol production started after 36 h and reached a maximum at 120 h. Both the cultures produced more alcohol from rice straw as compared to bagasse.

SSF in Coculture

Figure 1 (a and b) shows the sugar utilization profile and the alcohol production by the coculture. Alcohol production started after 36 h, but was less than that produced by *S. cerevisiae* alone. However, after 90 h, it increased and reached 1.18% (w/v) at 120 h. This is the result of fermentation of D-xylose by *C. shehatae* as revealed on paper chromatograms.

It can be observed from Fig. 1 (a and b) that, after 24 h, the amounts of reducing sugars were similar for rice straw and bagasse. However, the final ethanol concentration was higher in the case of rice straw. This could be the result of several factors; extraneous constituents (such as gums, resins, waxes, and so on) that are present in the hemicellulosic substrate may affect the final yield of ethanol. Moreover, the Crystallinity Index (CrI) of the cellulose and the extent of heterogenicity in the hemicellulose present in a particular substrate are known to limit the enzymic hydrolysis process during the later stages of saccharification.

Fermentation of rice straw and bagasse with the coculture yielded increased ethanol levels (Fig. 2). Table 2 summarizes the percent increase in alcohol production in the coculture experiment.

Fermentation of Sugar Mixtures

The major sugars in the hydrolysates of rice straw and bagasse were D-glucose, D-xylose, and D-cellobiose. Their utilization was simulated for SSF conditions by employing 2% standard sugars in mixture. Figures 3 a and b show the sugar utilization profile by *C. shehatae* and *S. cerevisiae,* respectively, along with the ethanol production. Cellobiose was not utilized by both the yeasts, whereas glucose was used preferentially. The results indicated that *C. shehatae* could use xylose concurrently when glucose concentration dropped below 1%, whereas *S. cerevisiae* did not ferment xylose. Utilization of xylose in minor quantities by *S. cerevisiae* was accounted for by the production of equivalent amounts of xylitol.

A significant finding of the simulation studies was that D-xylulose accumulated during the fermentation by *C. shehatae.* This may be the result of the repression of D-xylulokinase (EC 2.7.1.17) by glucose (*15,16*). Similarly, accumulation of xylitol by *S. cerevisiae* suggests the presence of D-xylose reductase (EC 1.1.1.27). The presence of this enzyme was

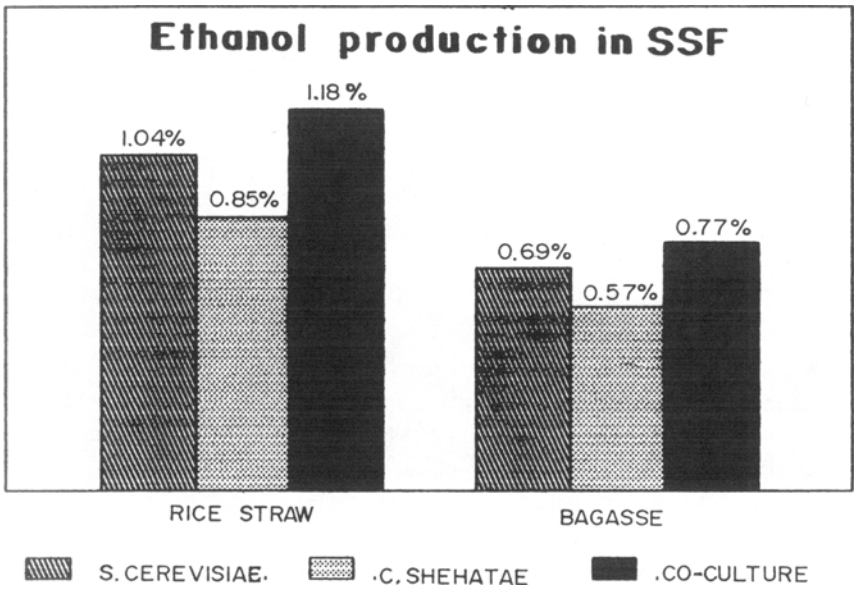

Fig. 2. shows the final alcohol concentration produced from Rice straw and Bagasse by *S. cerevisiae, C. shehatae* and their co-culture.

Table 2
Percent Increase in Alcohol Concentration Produced by Coculture of *S. cerevisiae* and *C. shehatae* as Compared to That Produced by the Two Organisms Separately

	% increase in alcohol concentration with coculture as compared to	
Residue	*C. shehatae*	*S. cerevisiae*
Rice straw	38	13
Bagasse	35	11

demonstrated earlier by Batt et al. (*17*). This finding was confirmed by detecting the intracellular activity of D-xylose reductase in *S. cerevisiae* cells, but the activities were about 10-fold lower than those of *C. shehatae.* D-xylose reductase activity in both the cultures was found to be linked with NADH as well as NADPH as cofactors.

Thus, it can be concluded that a coculture of *C. shehatae* and *S. cerevisiae* can efficiently utilize the cellulosic and hemicellulosic sugars from rice straw and bagasse with enhanced ethanol production. *S. rolfsii* produces

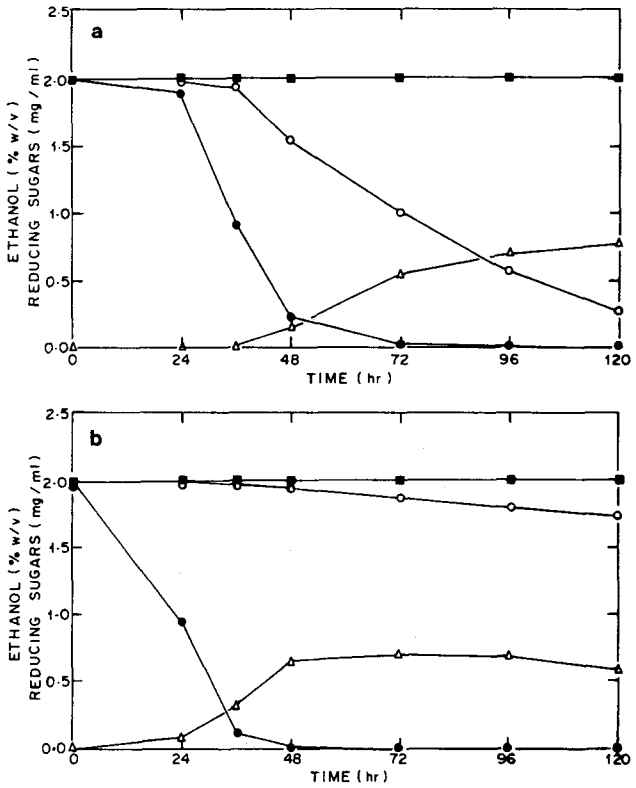

Fig. 3. (a) and (b) show the utilization of glucose (●), xylose (○) and cellobiose (■), and the alcohol produced (△) by *C. shehatae* and *S. cerevisiae* respectively.

high levels of cellulase/hemicellulase, which are fairly stable (9), and this enzyme preparation can also be used in a continuous fermentation system, but would require standardization. Further experimentation on standardization of the SSF conditions for improvement in ethanol yields is in progress.

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