Chiang Mai J. Sci. 2013; 40(3) : 307-320
http://it.science.cmu.ac.th/ejournal/
Contributed Paper

A Comparative Study on Simultaneous Saccharification and Fermentation of Agricultural Wastes to Bio-ethanol Using Two *Saccharomyces* Strains

Poludasu Rama Mohan [a], Sake Pradeep [b], and Obulam Vijaya Sarathi Reddy*[a]
[a] Department of Biochemistry, Sri Venkateswara University, Tirupati - 517 502, India.
[b] Department of Microbiology, Yogi Vemana University, Kadapa - 516 003, India.
*Author for correspondence; e-mail: ovsreddy@yahoo.com

Received: 23 February 2012
Accepted:20 September 2012

ABSTRACT

Application of simultaneous saccharification and fermentation (SSF) technique for the conversion of agricultural wastes to bio-ethanol would result in a more cost-effective process. Peracetic acid (PAA) pretreated various lignocellulosic substrates like sugarcane bagasse, rice straw and corn cobs were examined with solka floc as reference in SSF experiments for the production of bio-ethanol using *Asperigillus niger* crude cellulase, *Cladosporium sp.* crude CMCase (Carboxy Methyl Cellulase) and yeast cells. Fermentation temperature was selected as 40 and 43°C for *Saccharomyces cerevisiae* NCIM 3215 and *Saccharomyces bayanus* respectively. Further SSF experiments were compared for ethanol production. The *S. bayanus* was found to perform better and faster fermentation in the SSF process and yielded higher bio-ethanol in pretreated bagasse just after 48 h (17.03% w/v) than the *S. cerevisiae* that gave high yields of bio-ethanol only after 72 h (13.44% w/v). Increased bio-ethanol yields were obtained when the cellulase was supplemented with CMCase of *Cladosporium sp*. The cell viability was lower in *S. bayanus* compared with *S. cerevisiae*. The bio-ethanol yields of the fermented substrates were in the following order: Solka floc > treated sugarcane bagasse > treated rice straw > treated corn cobs.

Keywords: simultaneous saccharification and fermentation (SSF), *Cladosporium sp.*, *Asperigillus niger*, lignocellulose, *Saccharomyces cerevisiae, Saccharomyces bayanus*, bio-ethanol

1. INTRODUCTION

Production of renewable fuels, especially bio-ethanol from lignocellulosics biomass, holds potential to meet the energy demand as well as to mitigate greenhouse gas emissions for a sustainable environment. At present technologies to produce bio-ethanol largely depend on sugarcane, starch based grains and tubers [1]. However, production of bio-ethanol requires the development of an economically feasible and sustainable process utilizing renewable lignocellulosic materials that do not compete with food sources [2].

Agricultural residues (e.g., wheat straw, sugarcane bagasse, corn stover), forest products (hardwood and softwood), and dedicated crops (switchgrass, salix) are

renewable sources of energy [3]. The enzymatic hydrolysis of sugarcane bagasse could be greatly enhanced by peracetic acid (PAA) pretreatment [4], which was achieved mainly by delignification accompanying with the increase of the surface area and exposure of cellulose fibers [5].

Zayed and Meyer [6] carried out single batch bioconversion of wheat straw to ethanol by employing the fungus *Trichoderma viride* and the yeast *Pachysolen tannophilus*. Miyomoto [7] used raw material like sugarcane bagasse, rice straw, and forest wastes in pilot scale unit for the production of ethanol. Nakamura *et al*., [8] reported that rice straw is a potential feed stock for production of power and fuel ethanol. Laser *et al*., [9] used sugarcane bagasse for bioconversion to ethanol.

During enzymatic hydrolysis, cellulase activity is severely inhibited by cellobiose and to a lesser extent by glucose [10]. The competitive product inhibition of the cellobiose can be overcome to some extent by the addition of a surplus of β-glucosidase activity [11]. The cellulose fraction of lignocelluloses can be converted to bio-ethanol by either simultaneous saccharification and fermentation (SSF) or separate enzymatic hydrolysis and fermentation (SHF) processes. SSF is more favoured because of its low potential costs [12]. Large number of publications indicated that the optimum temperature for enzymatic hydrolysis is at 40-50°C [13], while the microorganisms with good ethanol productivity and yield do not usually tolerate this high temperature. This problem has usually been tackled by applying thermo-tolerant yeast [14] and *S. bayanus* [15,16].

Earlier we have reported the high gravity finger millet medium for ethanol production using *S. bayanus* [17]. Recently we have reported the optimization of ethanol production from sugarcane bagasse [18]. In the present study agricultural wastes were considered as inexpensive, abundant and renewable sources for bio-ethanol production using *S. cerevisiae* and *S. bayanus* in SSF.

2. MATERIALS AND METHODS

2.1 Fungal Strains and Their Maintenance

The *Cladosporium sp.* NCIM 901 procured from National Collection of Industrial Microorganisms (NCIM), Pune, India was employed for CMCase production. The *Asperigillus niger* soil isolate was employed for cellulase and β-glucosidase production. Both fungal strains were grown and maintained on potato soluble starch agar at 4°C as agar slant cultures. The actively growing fungal cultures were maintained as such by periodic transfer into fresh potato dextrose agar (PDA) medium at every 2-3 months.

2.2 Preparation of Fungal Inoculum

Both the fungal cultures were grown on PDA slants at 32°C for 4 days and washed with 5 ml sterile distilled water to prepare the spore suspension. Spores were filtered through Whatman No.1 filter paper and resuspended in sterile distilled water to get a spore density of 1×10^8 spores/ml with the aid of a Neubauer counting chamber. The above spore suspension was added (2 ml/g) as inoculum to SSF medium.

2.3 Solid State Fermentation for Enzyme Production

It was carried individually in 250 ml Erlenmeyer conical flasks that contained 1g micro crystalline cellulose (solka floc) and 1g of carboxy methyl cellulose as carbon source for *A. niger* and *Cladosporium sp.* NCIM 901 respectively, and 5 ml of basal salt medium

of following composition (g/l) NH_4NO_3 5.0; Peptone, 5; NaCl, 5; Urea, 2; $CaCl_2$,1; Tween 80, 0.5 ml and trace elements: $FeSO_4 \cdot 7H_2O$, 0.005; $MnSO_4 \cdot 7H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001 and $CoCl_2$,0.002 g. The initial moisture content of the medium was adjusted to 70-75%. Prior to sterilization, the initial pH of the medium was adjusted to 5.5. After incubation, 10 ml sodium citrate buffer (50 mM, pH 6.0) was added to flasks, and these flasks were kept at 50°C for 1 h under mild stirring. The fermented slurry was filtered through muslin cloth and centrifuged at 8000 × g for 15 min. The filtrate was concentrated using Amicon cell fitted with a PM-10 membrane (10 kDa cut off) and used for β-glucosidase purification and carboxymethyl cellulase purification [19].

2.4 Crude Enzyme Preparation

Culture filtrate of the crude preparation was precipitated by the addition of ammonium sulphate with 90% saturation. It was kept at 4°C for 24 h and the precipitate was collected by using centrifugation (4000 × g) at 4°C for 30 min. The precipitate was re-dissolved in 10 ml of 0.02 M sodium phosphate buffer and dialysed for 24 h against 0.02 M sodium phosphate buffer, pH 7.0 containing 1 mM EDTA and 1 mM 2-mercaptoethanol [20]. The dialyzed suspension was used for SSF experiments as enzyme source.

2.5 Substrates and Pretreatment

Rice straw was collected from local paddy fields around Tirupati (India). Corn cobs were collected in agricultural market, Tirupati. Sugarcane bagasse was collected at Sri Venkateswara sugar Industries, Renigunta, Tirupati. The peracetic acid (PAA) (5% wt/vol) pretreatment [18] of the substrates were carried out at 90°C for 60 min. Solid-liquid ratio was maintained at 10 g/100 ml. Solka floc SW-40 (microcrystalline cellulose) was employed as the substrate of reference. The solid and liquid hydrolyzate was separated and used for further experiments. The liquid hydrolysate was removed and used for sugar content analysis by Yang and Wyman [21] method.

2.6 Yeast Strains and Preparation of Inoculum

The *Saccharomyces cerevisiae* (NCIM 3215) obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India and *Saccharomyces bayanus* was kindly provided by Dr.Roberto Ambrosoli, University of Turin, Italy. Both strains were used in this study and the inoculum was prepared by transferring the organisms maintained on MGYP medium (malt extract, 3; glucose, 10; yeast extract, 5; peptone, 5 g/l) into 250 ml flask with 100 ml basal medium having 36 g/l glucose. The yeasts were cultured at 33°C on an orbital shaker for 12 h. The inoculum concentration was about 1.5×10^8 yeast cells/ml and the amount of inoculum added was 10% (v/v) of the SSF medium.

2.7 Simultaneous Saccharification and Fermentation (SSF)

The reaction mixture contained PAA pretreated substrate 10% (w/v), crude cellulase (16 FPU) and carboxymethyl cellulase (15 IU), 10% (v/v) yeast inoculum and 87 ml of SSF basal medium [peptone (0.5%, w/v), yeast extract (0.3%, w/v), malt extract (0.3%, w/v) and Tween 80 (0.1%, w/v) supplemented with mineral salts (in g/l) $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2.0, urea 0.3, $CaCl_2$ $2H_2O$ 0.4, $MgSO_4$ $7H_2O$ 0.3; and (in mg/l) $FeSO_4$ $7H_2O$ 5.0, $MnSO_4$ H_2O 1.6, $ZnSO_4$ $7H_2O$ 1.4, $CoCl_2$ $6H_2O$ 3.7. The pH of the medium was adjusted to 4.8 using 0.05 M acetate buffer] and final the volume

made up to 100 ml [22]. The final pH was adjusted to 5.1 with 0.05 M citrate buffer. Reactions were carried out in 250 ml Erlenmeyer flasks with 100 ml working volume on an orbital shaker at 150 rpm. Samples were aseptically taken at regular intervals for the analysis of sugars, reducing sugars and ethanol content.

2.8 Assay Methods

The components (cellulose, hemicellulose and lignin) of pretreated and untreated substrates were analyzed according to the procedures of Goering and Van Soest [23]. Total drymatter, moisture, ash content, crude fibre, crude fat and crude protein were determined by the procedures of AOAC [24]. The amounts of total soluble sugar (TS) of the hydrolysates were tested according to phenol-sulfuric acid [25] method. The reducing sugar concentration was estimated using 3, 5-dinitrosalicylic acid (DNS) method [26]. The xylose and arabinose content was estimated by the method of Khabarov *et al.*, [27]. The reagents (solution 1, 2 and H_2SO_4) were prepared and mixed in test tubes. It was heated on a boiling water bath, the reaction mixture was transferred into a 100 ml volumetric flask, and water was added to the mark. The absorption spectra were recorded on a Spectrophotometer. The calibration plots were constructed and the concentration of pentoses were expressed as mg/g.

Protein content was estimated by method of Lowry *et al*., [28]. Filter paper hydrolase (FPase) and carboxymethyl cellulase (CMCase) activities were assayed as described by Mandels *et al*., [29] at pH 5.0 and 50°C (60 min for FPase and 30 min for CMCase). The β-glucosidase (EC 3.2.1.21) activity was assayed according to Desrochers *et al*., [30] at pH 5.0 and 50°C for 30 min. Glucose produced was assayed by the method of Miller [26] using glucose as the standard. Enzyme activity (U) was expressed either as μmol glucose (for β-glucosidase) or glucose equivalent (for FPase and CMCase) liberated per min.

2.9 Glucosamine Determination

For fungal chitin hydrolysis into N-acetyl glucosamine, 20 mg dried biomass was incubated with 2 ml of H_2SO_4 (72%) in a test tube. After continuous shaking (130 rpm) for 60 min at 25°C, it was diluted with 3 ml of distilled water and autoclaved for 2 h. The hydrolyzate was neutralized to pH 7.0 with 10 M and then with 0.5 M NaOH and diluted to 100 ml. Finally, glucosamine was assayed by the colorimetric method described by Tsuji *et al*., [31] and modified by Ride and Drysdale [32]. Hydrolyzate (1 ml diluted) was mixed with 1 ml of $NaNO_2$ (5%) and 1ml of $KHSO_4$ (5%) in a centrifuge tube. After shaking occasionally for 15 min, it was centrifuged at 3500 rpm for 5 min; 2 ml of supernatant was mixed with 0.67 ml of $NH_4SO_3NH_2$ (12.5%) and shaken for 3 min. To the mixture was added 0.67 ml of 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH; 0.5%, prepared daily), and then the mixture was boiled for 3 min and immediately cooled to room temperature; 0.67 ml of $FeCl_3$ (0.5%, prepared within 3 days) was added. After standing for 30 min, the absorbance at 650 nm was measured spectrophotometrically. The chitin content (mg of glucosamine/mg of dry weight of fungal biomass) was calculated in accordance with a standard curve established by measuring the absorbance of known amounts of glucosamine hydrochloride.

2.10 Yeast Cell Viability

The yeast cells viability was determined by the methylene blue staining technique [33].

A 200 µl sterile solution of methylene blue (3.3 mM in 68 mM sodium citrate) was mixed with 200 µl of yeast suspension and diluted to reach an OD of 0.4-0.7 at 620 nm. This mixture was shaken and after 5 min incubation, placed in a Neubauer counting chamber. The number of stained (inactive) and unstained (active) cells were counted in five different fields with a total of 200-300 cells and the percentage of viable (active) cells was noted.

2.11 Ethanol Quantification Using Gas Chromatography

The Agilent systems model 6890 was used and the conditions were as follows: Graphitized packed column 5% carbowax 20M phase, matrix 80/120 carbopack-B, length 6 ft (1.83m) × 2 mm I.D. × 1/4" O.D. Nitrogen was used as carrier gas with flow rate of 20 ml/min and eluted compounds were detected by flame ionization detector (FID). Hydrogen was used as fuel gas, with flow rate 40 ml/min, along with air at a flow rate of 400 ml/min and secondary butyl acetate was used as internal standard [34].

3. RESULTS AND DISCUSSION

3.1 Proximate Analysis of Lignocellulosic Substrates

Chemical composition confirms that lignocellulosic biomass is mainly formed by cellulose, hemicellulose and lignin. All compositions were calculated based on the dry weight of samples. Table 1 represents the proximate and chemical composition of three agricultural residues: rice straw, corn cobs and sugarcane bagasse to highlight the particulate differences in feedstock before and after PAA pretreatment. The chemical pretreatment resulted in the solubilization of different components in three substrates such as lignin, hemicellulose and other soluble extractives. PAA pretreatment of three substrates was carried out at 90°C for 60 min and the chemical composition is presented in Table 1. However, since the sugar yields from raw biomass were very low, the biomass was subjected to a pretreatment step. The increases in cellulose and hemicellulose contents were predominantly attributed to the decreases in lignin and ash. These results indicate that

Table 1. Proximate analyses of untreated and PAA pretreated agricultural wastes (on dry weight basis %).

Composition	RS	PRS	CCB	PCCB	SCB	PSCB
Cellulose	33.4±0.6	58.8±0.8	32.3±0.2	56.8±0.8	42.7±0.5	62.2±0.9
Hemicellulose	18.2±0.12	20.4±0.10	39.8±0.4	42.2±0.4	34.4±0.4	36.4±0.3
Lignin	9.2±0.8	5.4±0.2	13.9±0.2	10.2±0.10	18.2±0.3	7.8±0.6
Dry matter	87.2±0.4	Nd	89.17±0.2	Nd	89.0±0.8	Nd
Moisture	12.8±0.2	Nd	8.5±0.1	Nd	11.0±0.12	Nd
Protein	6.8±0.10	Nd	3.2±0.4	Nd	1.36±0.4	Nd
Crude fibre	26±0.14	18.4±0.3	34.6±0.6	28.2±0.4	51.3±0.2	46.4±0.12
Crude fat	6.0±0.8	Nd	1.47±0.1	Nd	0.89±0.8	Nd
Ash	14.2±0.6	10.3±0.3	1.8±0.2	1.2±0.4	2.73±0.2	1.86±0.6

RS = Rice straw; PRS = Pretreated rice straw; CCB = Corn cob; PCCB = Pretreated corn cob; SCB = Sugarcane bagasse; PSCB = Pretreated sugarcane bagasse. Data shown in above table are the mean ± standard deviations of triplicate experiments; Nd: not determined.

PAA pretreatment could partially disrupt the lignin structure and expose more accessible surface area of cellulose to cellulase. Furthermore, lignin removal could also reduce unproductive binding of cellulase to lignin [35]. Consequently, it improved enzymatic biocatalysis and increased the yields of desired products and recycled more cellulase. Thus, the costs associated with enzymatic saccharification of biomass could remarkably be reduced.

From the results it is evident that in all substrates after pretreatment there was increase in cellulose and hemicellulose contents and decrease in lignin content. The cellulose content of rice straw, corn cob and bagasse increased after pretreatment from 33.4±0.6, 32.3±0.2 and 42.7±0.5% to 58.8±0.8, 56.8±0.8 and 62.2±0.9%, respectively. The hemicellulose content was slightly increased in three substrates after pretreatment. Thus, increased conversion of cellulose would increase the available sugar content in the hydrolyzate. Generally the bagasse contained relatively higher lignin content (18.2±0.3%) than that of corn cob (13.9±0.2%) and rice straw (9.2±0.8%). Bagasse also contains a relatively high amount of lignin (20%) comparable to that in wheat straw [36]. In general lignin strengthens the bagasse structure and influences its suspension texture in water. Moreover, lignin hydrophobic character considerably reduces the bagasse's moisturizing ability [37].

After pretreatment, the level of the crude fiber was significantly reduced in all the studied samples. Compositional changes observed in three substrates before and after pretreatment clearly showed that cellulose, hemicellulose content increased and lignin as well as ash content decreased in all substrates. The increases in cellulose were predominantly attributed to the decreases in lignin and ash. The ash content was decreased in pretreated samples compared to raw untreated samples.

3.2 Production of Enzymes by Solid State Fermentation

The degradation of crystalline cellulose was carried out by synergistic action of FPase, CMCase and β-glucosidase. It has been reported that optimum time for the highest cellulase activity depended upon the substrate and fungus used [38]. In the present study, pure commercial microcrystalline cellulose and carboxy methyl cellulose were used to obtain crude cellulase and carboxymethyl cellulase from *A. niger* and *Cladosporium sp.* respectively, by solid state fermentation. Initially both fungal strains were produced low activities of the enzyme but with the course of time, there was gradual increase in enzyme synthesis and attained maximum activity on the 6th day of incubation in fermentation media (initial pH of 5.5). Further incubation resulted in the gradual decrease of enzyme activity. This decreasing trend of enzyme activity may be due to the reduction in cellulose content and nutrients supplied in the substrate. The decrease in cellulase activity may also be due to accumulation of cellobiose [38]. Cellulolytic enzyme(s) produced from the isolate *A. niger* and *Cladosporium sp.* under solid state fermentation was precipitated by ammonium sulphate. The precipitate obtained from *A. niger* isolate was having high FPase and β-glucosidase activity (15.3 FPU/g and 16.8 IU/g with relative specific activity of 78.46 FPU/g and 86.15 IU/g, respectively) which was higher than the enzymatic activity of *Cladosporium sp.* (Table 2). The CMCase activity of *Cladosporium sp*. was higher than the *A. niger* isolate, with activity of 18.20 IU/g with specific activity of 81.25

Table 2. Enzymes production by two fungal strains in solid state fermentation on pure cellulose.

Fungal strains	Glucosamine (mg/g)[a]	Protein (mg/g)[b]	FPase (FPU/g)	CMCase (IU/g)	β-glucosidase (IU/g)	$^*Y_{FPA}$ (FPU/g)	$^*Y_{CMC}$ (IU/g)	$^*Y_{\beta\text{-Glu}}$ (IU/g)
A. niger	0.195±1.10	52.5±0.26	15.3	12.46	16.8	78.46	63.89	86.15
Cladosporium sp	0.224±1.23	60.6±1.12	14.2	18.20	12.6	63.39	81.25	56.25

A. niger grown on microcrystalline cellulose and *Cladosporium* sp grown on CM cellulose support. Data represents the means ± SEM, n=3; Y_{FP}, Y_{CMC} and $Y_{\beta\text{-Glu}}$ represent the specific enzyme activities of filter paperase, carboxy methyl cellulase and β-glucosidase respectively; $^*Y_{FPA}$ = FPU/g biomass, Y_{CMC} = CMCase/g biomass, $Y_{\beta\text{-Glu}}$ = β-glucosidase/g biomass; [a]Expressed in mg glucosamine per gram of biomass dry weight; [b]Expressed in mg fungal protein per gram of biomass dry weight.

IU/g. The values of CMCase activity were higher than filter paper activity all time intervals studied during the fermentation period. Studies by Jabbari *et al*., [39] stated that, the onset of precipitation of CMCases occurred at 35% and were completed at 75% saturation of ammonium sulphate at 0°C. The purification after ammonium sulphate precipitation was 1.72-fold and 86% CMCases were recovered. After dialysis, specific activity of CMCases was 7.29 U mg^{-1}.

Relationship between fungal protein and fungal biomass indirectly by glucosamine content was estimated on the 6th day. The biomass and fungal protein content of mycelium grown in solid state fermentation were shown in Table 2. The biomass of *A. niger* was 0.195±1.10 (mg/g) with mycelial protein of about 52.5±0.26 (mg/g). The *Cladosporium sp*. biomass was about 0.224 (mg/g) with mycelial protein of about 60.6±1.12 (mg/g). The maximum amount of biomass and fungal protein reached on the 6th day and there after no further increment of these were noticed. Within different carbon sources, the glucosamine and the fungal protein content could be considered as a good indicator of the biomass growth. Furthermore, the glucosamine amount could also be considered as a good indicator of fungal protein. Fungal protein content and the ratio of glucosamine and fungal protein were not influenced by carbon sources, in submerged and in solid state fermentation [40]. The crude cellulase and β-glucosidase preparations were subjected to fermen-tation for ethanol production by utilizing pretreated lignocellulosic materials.

3.3 Saccahrification of Lignocellulosic Substrates

The yields of different reducing sugars after the PAA pretreatment of lignocellulosic substrates were presented in Table 3. Yields of sugars were higher in the pretreated substrates than the untreated ones. However, PAA pretreatment can be performed at relatively low temperature (90°C) under atmospheric pressure. This mild condition avoids the degradation of monosaccharide to the formation of furfural and hydroxymethyl furfural that are strongly toxic to fermentating organisms [41]. This pretreatment removed the hemicellulose and lignin to increase the accessibility of the cellulose. Less carbohydrate was lost under proper optimum conditions and PAA pretreatment could obtain a high recovery yield of carbohydrate as solid phase [42]. Decreasing the lignin content in pretreated substrates allows nearly complete saccharification of

the polysaccharides. A comparison of the residual lignin content with glucose release for all samples during this study showed that as lignin content dropped, glucose release increased.

The results presented in Table 3 show that the initial raw untreated substrates contain low levels of total sugars and reducing sugars. After pretreatment, the reducing sugar concentration was higher in pretreated bagasse (42.8±0.2 mg/g) followed by rice straw (42.6±0.4 mg/g) and corn cob (40.0±0.2 mg/g). The arabinose content was higher in rice straw samples compared with bagasse and corncob. The higher cellulose content of bagasse resulted in increasing the reducing sugar content in pretreated bagasse than the other pretreated samples. Reducing sugar concentration was higher in rice straw (42.6±0.4 mg/g) than in corn cob (40.0±0.2 mg/g) which is due to low lignin content of rice straw than corn cob. The results showed that after pretreatment the availability of reducing sugars from pretreated substrates were increased from their respective carbohydrate content. In comparison the bagasse contained higher cellulose content than the other substrates. The levels of reducing sugars in the sample would determine to some extent the percentage of ethanol that would be produced in the fermenting medium. Yield of sugars from their corresponding substrates were presented in Table 3. Cellulose conversion of PAA pretreated samples after 60 min were shown in Figure 1. The hydrolysis of PAA pretreated bagasse samples resulted in the highest glucose from glucan conversion at 35.97%, arabinose from arabinan conversion low at 22.22% compared with pretreated corn cob sample, higher than the pretreated rice straw sample.

The highest xylan and arabinan conversion rate at 36.50 and 31.11% was observed in pretreated corncob than the other substrates. The xylan and arabinan conversion rate at 27.90 and 21.42% respectively was noted in the pretreated rice straw which is more than the pretreated corn cob.

3.4 Simultaneous Saccharification and Fermentation (SSF)

The saccharification of pretreated substrates by crude cellulase preparations along with fermentation process was allowed to occur simultaneously for 96 h duration by employing *S. cerevisiae* and *S. bayanus*, which could be cultured very near to the optimum temperature (50°C) for cellulase. Though 50°C was found to be optimum, the range of 40-50°C did not affect the yield significantly, and hence SSF

Table 3. The yields (mg/g) of different sugars after PAA pretreatment.

Substrate	Total sugars	Reducing sugars	Glucose	Xylose	Arabinose
RS	12.46±0.2	10.82±0.8	7.4±0.2	2.8±0.1	1.6±0.3
PRS	48.2±0.2	42.6±0.4	30.5±0.1	8.6±0.2	4.2±0.1
SCB	12.6±0.6	10.6±0.12	8.2±0.2	5.4±0.2	3.2±0.1
PSCB	52.4±0.6	42.8±0.2	32.8±0.4	10.6±0.6	3.6±0.4
CCB	14.2±0.4	12.4±0.6	8.6±0.3	2.4±0.3	2.5±0.2
PCCB	46.4±0.5	40.0±0.2	26.9±0.2	12.6±0.3	4.5±0.2

The values are based on dry-weight basis (%); Data represents the mean ± SEM, n = 3.

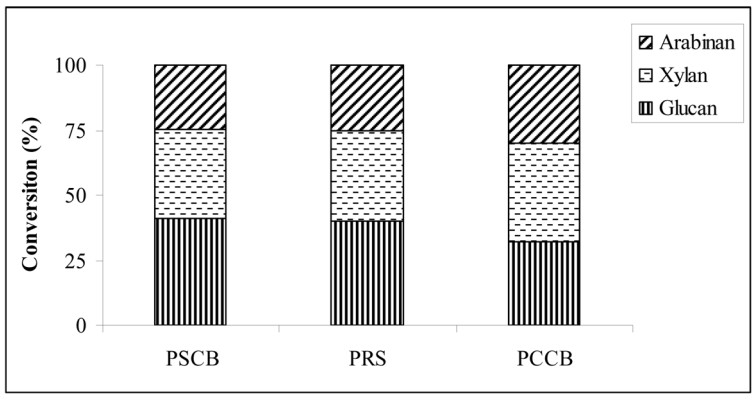

Figure 1. Variation in glucan, xylan and arabinan conversion (%) with PAA pretreatment: pretreated sugarcane bagasse (PSCB), pretreated rice straw (PRS) and pretreated corn cob (PCCB).

temperature was chosen at 40 and 43°C for *S. cerevisiae* and *S. bayanus*, respectively. Crude cellulase (16 FPU) preparation of *A. niger* (higher cellulase and β-glucosidase) and crude CMCase (15 IU) of *Cladosporium sp.* per gram of substrate was selected. The Table 4 summarize the results of ethanol yield obtained with all the substrates (sugarcane bagasse, rice straw, corn cobs and solka floc) using both *S. cerevisiae* and *S. bayanus*. After 48 h the concentration and yield of ethanol was increased in all cultures fermented by *S. bayanus*. It should be noted that *S. bayanus* proved to be superior to *S. cerevisiae*, as the yields obtained with *S. bayanus* were distinctly higher. *S. bayanus* was found to perform better and faster in the SSF process and resulted in higher yields of bio-ethanol in pretreated bagasse just after 48 h (17.03% w/v) than to *S. cerevisiae* as it gave

Table 4. Ethanol yield (g/l) in simultaneous saccharification and fermentation.

Enzyme	Units/g substrate	Time (h)	PSCB		PRS		PCCB		CEL	
			A	B	A	B	A	B	A	B
Crude cellulase	16 FPU	12	3.5	4.48	2.72	3.63	1.78	2.49	4.68	5.04
		24	5.73	7.17	4.77	5.90	2.85	4.09	6.30	7.20
		48	9.86	16.13	7.27	10.9	5.16	8.02	10.9	17.6
		72	12.54	16.13	9.77	10.9	7.13	8.02	13.5	17.6
		96	12.54	Nd	9.77	Nd	7.13	Nd	13.5	Nd
		12	4.48	5.37	3.63	4.31	2.49	2.85	5.76	6.48
Cellulase	16 FPU	24	6.81	8.06	5.27	6.81	3.56	4.45	7.56	8.64
+	+	48	10.39	17.03	8.63	12.5	6.41	9.26	11.3	18.3
Carboxy	15 IU	72	13.44	17.03	10.45	12.5	7.84	9.26	14.7	18.3
methyl		96	13.44	Nd	10.45	Nd	7.84	Nd	14.7	Nd
cellulase										

Data represents mean of two experiments; A represents *S. cerevisiae* (NCIM 3215) and B represents *S. bayanus* (PSCB= Pretreated sugarcane bagasse; PRS= Pretreated rice straw; PCCB= Pretreated corn cob; CEL= Solka floc).

high yields of bio-ethanol only after 72 h (13.44% w/v). The time period 72 h was required for *S. cerevisiae* to attain maximum ethanol concentration with corresponding ethanol yield. This result is attributed to the thermo-tolerance of *S. bayanus*. But at 50°C, the ethanol yield was found to be reduced with both the yeasts and significant amount of sugar was found unmetabolized. This was due to the thermo-lability of both the yeasts at the higher temperature. However, *S. bayanus* generally tolerated a higher ethanol and temperature than *S. cerevisiae*. Addition of crude preparation of *Cladosporium sp.* was found to favor faster reactions with both the yeasts. This might be due to low activities of CMCase in the crude preparation of *A. niger* and supplementing it from external source gave better yields since the cellulose degradation was initiated by the action of CMCase. Pretreatment of substrates has been found to have significant influence on ethanol production. The levels of reducing sugar in the sample would determine to some extent the high percentage of ethanol produced in the fermenting medium. The higher ethanol yield in the fermented pretreated bagasse was due to high amount of reducing sugars than the others in the fermenting medium.

Thermo-tolerant yeasts capable of growth and ethanol production at temperatures above 40°C have been investigated in the past for suitability in SSF [43]. The ethanol yields of substrates under SSF by yeasts were in the order of: solka floc > sugarcane bagasse > rice straw > corncobs.

3.5 Yeast Cell Viability and Cell Count

The Figures 2 and 3 represent the yeast cell (*S. cerevisiae and S. bayanus*) viability and total number of cells during fermentation of pretreated substrates along with solka floc (reference culture medium). During initial stages of fermentation, there was no increase in yeast cell numbers, as they were in lag phase. After that there was a gradual increase in cell numbers indicating that the yeast cells were utilizing the sugars present in the medium. The cultures grown in pretreated bagasse had higher cell numbers than the culture grown on other pretreated substrates. This is due to the availability of higher reducing sugars in the pretreated bagasse culture medium than the others. Generally, yeast performance may be affected both by very low glucose concentration resulting in metabolic stress conditions and high ethanol presence in fermenting media. The lower viability shown by *S. bayanus* at same sugar concentrations seem to be due to a fast accumulation of internal ethanol over short fermentation periods. At 48 h, 80% viability of *S. bayanus* cells on bagasse medium was compared with 86% viability of *S. cerevisiae* cells. At short fermentation periods (48 h), *S. bayanus* has a great fermentation activity. Despite the very low viability of *S. bayanus*, it fermented glucose at temperatures ≥ 42°C; increments in biomass were obtained due to the contribution of intact but non viable cells at the end of the fermentation. High values of final viability were obtained due to the use of a high cell density. It has been reported that the maintenance of viability during fermentation is favoured by high cell densities [44]. The limiting effect of increasing temperatures on the fermentation capacity of *S. bayanus* is due to losses in cell viability. Fast fermentation is one of the causes for the drops in cell viability, due to a high internal ethanol concentration that reaches inside the cells at increasing sugar concentrations. On the other hand, *S. cerevisiae* was able to maintain higher

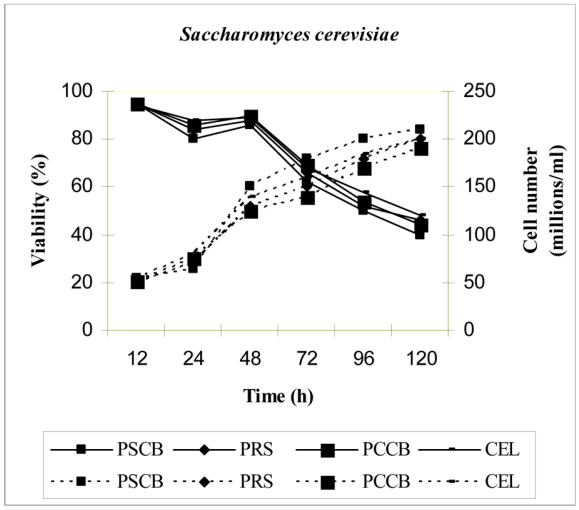

Figure 2. Viability and cell number of *S. cerevisiae* during SSF process.

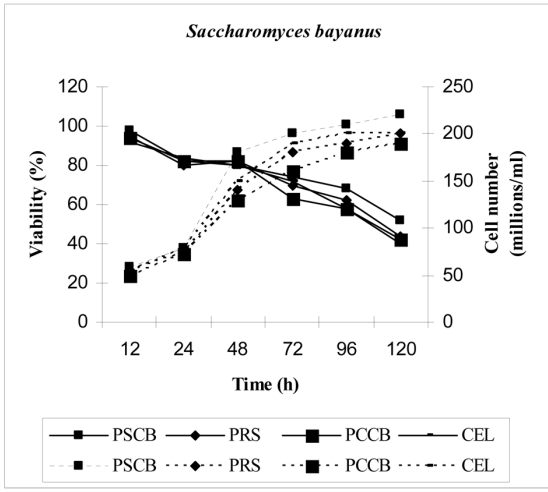

Figure 3. Viability and cell number of *S. bayanus* during SSF process.

viability values than *S. bayanus* at similar concentration of sugar fermented (Figures 2 and 3). This seems to be caused by the slower fermentation rate of *S. cerevisiae* and utilized more nutrients for its growth compared to *S. bayanus* that resulted low ethanol yields (Table 4). Based on the experimental results, it is suggested that the thermo-tolerant ethanol-producing yeast strain tested may be useful in simultaneous saccharification and conversion of cellulosic substrates to ethanol.

4. CONCLUSIONS

Low cost lignocellulosic agricultural residues such as bagasse, rice straw and corn cobs are potential renewable biomass for the production of bio-ethanol. A promising lignin-selective reagent like peracetic acid is used to treat the biomass which could be efficiently fermented by yeast to make bio-ethanol. In order to reduce the fermentation cost and make it more feasible, the crude cellulase preparations of *A. niger* and *Cladosporium sp.* were used in the solid state fermentation. Further improvements

should focus on increasing the viability rate of *S. bayanus* strain to obtain higher ethanol yield by adding the special nutrients like finger millet [17] medium for rapid fermentation.

ACKNOWLEDGEMENTS

We are thankful to UGC, New Delhi for providing funds. We also wish to thank Dr. S.C. Basappa, former Deputy Director and Scientist, Central Food Technological Research Institute (CFTRI), Mysore, for his encouragement and critical comments on the manuscript.

REFERENCES

[1] Joshi B., Bhatt M.R., Sharma D., Joshi J., Rajani M. and Lakshmaiah S., Lignocellulosic ethanol production: Current practices and recent developments, *Biotechnol. Mol. Biol. Rev.*, 2011; **6(8):** 172-182.

[2] Hayes D.J., An examination of biorefining processes, catalysts and challenges, *Catal. Today*, 2009; **145:** 138-151.

[3] Yat S.C., Berger A., Shonnard D.R., Kinetic characterization of dilute surface acid hydrolysis of timber varieties and switchgrass, *Bioresource Technol.*, 2008; **99:** 3855-3863.

[4] Mosier N., Wyman C., Dale B., Elander R., Lee Y.Y. and Holtzapple M., *et al*. Features of promising technologies for pretreatment of lignocellulosic biomass, *Bioresource Technol.*, 2005; **96:** 673-686.

[5] Teixeira L.C., Linden J.C. and Schroeder H.A., Simultaneous saccharification and cofermentation of peracetic acid-pretreated biomass, *Appl. Biochem. Biotechnol.*, 2000; **84-86:** 111-27.

[6] Zayed G. and Meyer O., The single batch conversion of wheat straw to ethanol employing the fungus *Trichoderma viride* and the yeast *Pachysolen tannophilus*, *Appl. Microbiol. Biotechnol.*, 1996; **45:** 551-555.

[7] Miyomoto K., Production of fuel alcohol from cellulosic biomass, *FAO Agricultural Services Bulletin*, 1997; **128:** 19-52.

[8] Nakamura Y., Sawada T. and Inoue E., Enhanced ethanol production from enzymatically treated steam exploded rice straw using extractive fermentation, *J. Chem. Technol. Biotechnol.*, 2001; **76:** 879-884.

[9] Laser M., Schulman D., Allen S.G., Lichwa J., Antal M.J. and Lynd L.R., A comparison of liquid hot water and steam pretreatments of sugarcane bagasse for bioconversion to ethanol, *Bioresource Technol.*, 2002; **81:** 33-44.

[10] Haki G.D. and Rakshit S.K., Developments in industrially important thermostable enzymes: A review, *Bioresource Technol.*, 2003; **89:** 17-34.

[11] Shen Y., Zhang Y., Ma T., Bao X., Du F. and Qu Y., Simultaneous saccharification and fermentation of acid-pretreated corncobs with a recombinant *Saccharomyces cerevisiae* expressing beta-glucosidase, *Bioresource Technol.*, 2007; **99:** 5099-5103.

[12] Wyman C.E., Ethanol from lignocellulosic biomass: Technology, economics and opportunities, *Bioresource Technol.*, 1994: **50:** 3-15.

[13] Qurat-ul-ain., Baig S. and Saleem M., Production and characterization of cellulases of *Asperigillus niger* by using rice husk and saw dust as substrates, *Pak. J. Bot.*, 2012; **44:** 377-382.

[14] Spindler D.D., Wyman C.E., Mohagheghi A. and Gorhmann K., Thermotolerant yeast for simultaneous saccharification and fermentation of

cellulose to ethanol, *Appl. Biochem. Biotechnol*., 1988; **17:** 279-293.

[15] Miller K.P., *Biofuel Ethanol Production by Saccharomyces bayanus*, *The Champagne Yeast*, Ph.D. thesis, Clemson, South Carolina. 2010.

[16] Barros M.R., Cabral J.M. and Novais J.M., Production of ethanol by immobilized *Saccharomyces bayanus* in an extractive fermentation system, *Biotechnol. Bioeng*., 1987; **29(9):** 1097-1104.

[17] Pradeep P., Krishna Goud G.K. and Reddy O.V.S., Optimization of very high gravity (VHG) finger millet (*ragi*) medium for ethanolic fermentation by yeast, *Chiang Mai J. Sci*. 2010; **37:** 116-123.

[18] Mohan P.R., Ramesh B. and O.V.S. Reddy., Production and optimization of ethanol production from pretreated sugarcane bagasse using *Saccharomyces bayanus* in simultaneous saccharification and fermentation, *Microbiol. J*., 2012; **2:** 52-63.

[19] Kristian B.R.M.K., *Biomass degrading enzymes from Penicillium-cloning and characterization*, Ph.D. Thesis. 2008.

[20] Bakare M.K., Adewale I.O., Ajayi A. and Shonukan O.O., Purification and characterization of cellulase from the wild-type and two improved mutants of *Pseudomonas fluorescens*, *Afr. J. Biotechnol*., 2005; **9:** 898-904.

[21] Yang B. and Wyman C.E., Effect of xylan and lignin removal by batch and flow through pretreatment on the enzymatic digestability of corn stover cellulose, *Biotechnol Bioeng*., 2004; **86:** 88-95.

[22] Takagi M., Abe S., Suzuki S., Emert G.H. and Yata N., A method for production of alcohol directly from cellulose using cellulase and yeast. In Ghose T.K. (ed.), Proceedings of bioconversion of cellulosic substances into energy, chemicals and microbial protein, I.I.T, New Delhi, 1997; 551-571.

[23] Goering H.K. and Van Soest P.J., Forage fibre analysis. USDA-ARS agricultural hand book. Government printing office, Washington DC, 1971.

[24] AOAC., Official methods of analysis of the association of official chemists, 15th ed. In: Helrich K., ed., AOAC Inc., Arlington, VA., USA, 1990.

[25] Dubois M., Gills K.A., Hamilton J.A. and Rebers P.A., Colorimetric method for determination of sugars and related substances, *Anal. Chem*., 1956; **28:** 350-356.

[26] Miller G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem*., 1959; **31:** 426-428.

[27] Khabarov Y.G., Kamakina N.D., Gusakov L.V. and Veshnyakov V.A., New spectrophotometric method for determination of furfural and pentoses, *Russ. J. Appl. Chem*., 2006; **79:** 103-106.

[28] Lowry O.H., Rosenrough N.J., Farr A.L. and Randell R.J., Protein measurement with the folin phenol reagent, *J. Biol. Chem*., 1951; **193:** 265-275.

[29] Mandels M., Andreotti R. and Roche C., Measuring of saccharifying cellulose, *Biotechnol. Bioeng. Symp*., 1976; **6:** 21-33.

[30] Desrochers M., Jurasek L. and Paige M.G., Production of cellulase, β-glucosidase, and xylanase by *Schizophyllum commune* grown on a cellulose-peptone medium, *Dev. Ind. Microbiol*., 1981; **22:** 675-684.

[31] Tsuji A., Kinoshita T. and Hoshino M., Analytical chemical studies on amino sugar, II. Determination of hexosamines using 3-methyl-2-benzothiazolone hydrazone hydro chloride, *Chem. Pharm. Bull.*, 1969; **17:** 1505-1510.

[32] Ride J.P. and Drysdale R.B., A rapid method for the chemical estimation of filamentous fungi in plant tissue, *Physiol. Mol. Plant. Pathol.*, 1972; **2:** 7-15.

[33] Postgate J.P., Viable counts and viability, In: Norris, J.R., Ribbons, D.W. (Eds.), *Methods in Microbiology*, New York Academic Press, 1967: 611-628.

[34] Anthony J.C., Malt beverages and malt brewing materials: Gas chromatographic determination of ethanol in beer, *J. Assoc. Anal. Chem.*, 1984; **67:** 192-193.

[35] Lu C.G., Yang B., Gregg D., Saddler J.N. and Mansfield S.D., Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues, *Appl. Biochem. Biotechnol.*, 2002; **98:** 641-654.

[36] Rémond-Zilliox C., *Etude des mécanismes de dégradation enzymatique des parois végétales, Application á l'hydrolyse de la paille de blé par une endoxylanase*, Ph.D. Thesis, Reims, France, 1996.

[37] Boussarsar H., Roge B. and Mathlouthi M., Optimization of sugarcane bagasse conversion by hydrothermal treatment for the recovery of xylose, *Bioresource Technol.*, 2009; **100:** 6537-6542.

[38] Singh A., Singh N. and Bishnoi N.R., Production of cellulases by *Aspergillus heteromorphus* from wheat straw under submerged fermentation, *Int. J. Civil Environ. Eng.*, 2009; **1:** 1-4.

[39] Jabbari A., Rashid M.H., Malana M.A., Amanullah, Saleem M., Niaz M. and Yasin M.Z., Production, partial purification and proteolysis of carboxymethyl cellulases from *Arachniotus citrinus, Int. J. Agric. Biol.*, 2002; **6:** 615-619.

[40] Chen Z., Wang Z. and Zhou A., Fungal protein estimation of *Aspergillus oryzae* growing in solid state cultivation conditions, *Can. J. Pure Appl. Sci.*, 2010; **4:** 1127-1131.

[41] Mussatto S.I. and Roberto I.C., Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: A review, *Bioresource Technol.*, 2004; **93:** 1-10.

[42] Zhao X.B., Peng F., Cheng K.K. and Liu D.H., Enhancement of the enzymatic digestibility of sugarcane bagasse by alkali-peracetic acid pretreatment, *Enz. Microb. Technol.*, 2009; **44:** 156-164.

[43] Szezodrak J. and Targonski Z., Selection of thermotolerant yeast strains for simultaneous saccharification and fermentation of cellulose, *Biotechnol Bioeng.*, 1988; **31:** 300-303.

[44] Ueno R., Urano N. and Kimura S., Effect of temperature and cell density on ethanol fermentation by a thermotolerant aquatic yeast strain isolated from a hot spring environment, *Fisheries Sci.*, 2002; **68:** 571.