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BIOENERGY/BIOFUELS/BIOCHEMICALS

Ethanol production from alkali-treated rice straw via simultaneous saccharification and fermentation using newly isolated thermotolerant *Pichia kudriavzevii* HOP-1

Harinder Singh Oberoi · Neha Babbar · Simranjeet Kaur Sandhu · Sandeep Singh Dhaliwal · Ujjal Kaur · B. S. Chadha · Vinod Kumar Bhargav

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Abstract In this study, simultaneous saccharification and fermentation (SSF) was employed to produce ethanol from 1% sodium hydroxide-treated rice straw in a thermostatically controlled glass reactor using 20 FPU gds^{-1} cellulase, 50 IU gds^{-1} β-glucosidase, 15 IU gds^{-1} pectinase and a newly isolated thermotolerant *Pichia kudriavzevii* HOP-1 strain. Scanning electron micrograph images showed that the size of the *P. kudriavzevii* cells ranged from 2.48 to 6.93 μm in diameter while the shape of the cells varied from oval, ellipsoidal to elongate. *Pichia kudriavzevii* cells showed extensive pseudohyphae formation after 5 days of growth and could assimilate sugars like glucose, sucrose, galactose, fructose, and mannose but the cells could not assimilate xylose, arabinose, cellobiose, raffinose, or trehalose. In addition, the yeast cells could tolerate up to 40% glucose and 5% NaCl concentrations but their growth was inhibited at 1% acetic acid and 0.01% cyclohexamide concentrations. *Pichia kudriavzevii* produced about 35 and 200% more ethanol than the conventional *Saccharomyces cerevisiae* cells at 40 and 45°C, respectively. About 94% glucan in alkali-treated rice straw was converted to glucose through enzymatic hydrolysis within 36 h. Ethanol concentration of 24.25 g l^{-1} corresponding to 82% theoretical yield on glucan basis and ethanol productivity of 1.10 g l^{-1} h^{-1} achieved using *P. kudriavzevii* during SSF hold promise for scale-up studies. An insignificant amount of glycerol and no xylitol was produced during SSF. To the best of our knowledge, this is the first study reporting ethanol production from any lignocellulosic biomass using *P. kudriavzevii*.

Keywords Ethanol productivity · *Pichia kudriavzevii* · Rice straw · Simultaneous saccharification and fermentation · Sodium hydroxide

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H. S. Oberoi (✉) · N. Babbar · S. K. Sandhu · S. S. Dhaliwal · U. Kaur · V. K. Bhargav
Central Institute of Post Harvest Engineering and Technology, Ludhiana 141 004, India
e-mail: hari_manu@yahoo.com

B. S. Chadha
Guru Nanak Dev University, Amritsar, India

Introduction

The high price of oil, limited availability of liquid fuels, and growing energy demands in transportation, industrial and other sectors and concerns over effect of green house gas emissions on environment has led to a search for alternative fuels. Because of limited area under crop cultivation and growing population, cellulosic ethanol could be a key alternative fuel to meet energy requirements of countries like India. Cellulosic ethanol and ethanol produced from other biomass resources have the potential to cut greenhouse gas emissions by 86% [33]. As per the latest FAO statistics, India alone accounts for about 22% of the total rice produced in the world. The availability of rice straw in huge quantities and unavailability of proper infrastructure to handle such a large quantity of biomass leads to burning of rice straw in countries like India, resulting in biomass loss and environmental pollution problems. The best alternative for handling such a huge quantum of biomass is the production of commercially important value-added products like enzymes, ethanol, and organic acids [24].

The bioconversion of lignocellulosic biomass to ethanol is a multi-step process consisting of pretreatment, enzymatic

hydrolysis and fermentation. Among these, pretreatment is particularly crucial, as significant presence of lignin and crystalline nature of cellulose impede the enzymatic hydrolysis of lignocellulosic biomass. The lignin component in lignocellulosic biomass acts as a physical barrier and must be removed or structurally altered by pretreatments to make the carbohydrates available for transformation processes [14]. Pretreatment is required to alter the biomass macroscopic and microscopic size and structure as well as its submicroscopic chemical composition and structure so that hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields. Pretreatment gives high reaction rates and significantly improves cellulose hydrolysis [32, 39]. Alkali pretreatment utilizes lower temperature, pressure and results in lesser sugar degradation, in comparison to acid pretreatment [19]. During alkali pretreatment, the first reactions taking place are salvation and saphonication. This causes a swollen state of the biomass leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkage between lignin and carbohydrates, and disruption of lignin structure making biomass more accessible to action by enzymes [37]. Simultaneous saccharification and fermentation (SSF) has lot of advantages, such as the use of single vessel for saccharification and fermentation; simplicity and ease of operation and completion of process in less time thereby improving the process economics and productivity. In addition, the presence of ethanol causes the medium to be less vulnerable to invasion by undesirable microorganisms [3, 25]. In spite of the economical advantages, SSF suffers from a major drawback, which is the different optimum temperature requirement for the hydrolyzing enzymes and fermenting microbial strain. Previous studies on ethanol production with SSF or separate hydrolysis and fermentation (SHF) from rice straw have reported low ethanol yield or low ethanol productivity [8, 26, 34, 39].

The advantages associated with the production of ethanol at temperatures higher than those used in conventional systems include reduced operating costs with respect to maintaining growth temperature in large-scale systems, reduced risk of contamination, increased productivity and ease with which the product may be recovered [23]. Previous studies have reported the use of *Pichia kudriavzevii* (*Issatchenkia orientalis*) along with *Saccharomyces cerevisiae* in fermentation of grape must and sugarcane juice syrup [11, 15]. We had recently reported ethanol production from sugarcane juice and Kinnow peel using *P. kudriavzevii* [10, 29], but we are yet to come across any literature reporting the use of *P. kudriavzevii* for ethanol production from any lignocellulosic biomass. The present study was thus conducted to analyze the effect of different concentrations of sodium hydroxide (NaOH) on quantitative changes in the rice straw structural components; compositional changes during hydrolysis of pretreated rice straw; characterization of the *P. kudriavzevii* HOP1 strain; comparative evaluation of *P. kudriavzevii* and the conventional *S. cerevisiae* in ethanol production from glucose and also to evaluate the potential of *P. kudriavzevii* HOP1 in ethanol production from alkali-treated rice straw in a SSF process.

Materials and methods

Materials

Rice (*Oryza sativa*) straw of variety Pusa-44 was procured from the Agronomy research fields of Punjab Agricultural University, Ludhiana, Punjab, India. The straw was oven dried, cut into small pieces, milled with a laboratory blender, and screened to a particle size of about 1 mm using different sized sieves [24]. Celluclast 1.5 L (C-2730), Novozyme-188 (C-6105), pectinase (P-2611) and standards for sugars and sugar alcohols, such as those for glucose, xylose, arabinose, glycerol, sorbitol and xylitol used for HPLC determinations were procured from Sigma-Aldrich (St. Louis, MO, USA). The analytical grade chemicals and dehydrated media were procured from Fisher Scientific (Mumbai, India) and Hi-Media Laboratories (Mumbai, India), respectively. The standards for oligosaccharides, such as cellobiose, xylobiose, and xylotriose were procured from Megazyme International, Ireland. The yeast strain used in this study was isolated from sugarcane juice containing 10% (v/v) ethanol as selection pressure. The details about isolation, identification, and propagation are mentioned elsewhere in this paper.

Compositional analysis

The milled rice straw was extracted with ethanol and subjected to the proximate compositional analysis [24]. Ash and moisture contents were determined using muffle furnace and hot-air oven, respectively [2]. Protein content (N $\times$ 6.25) was estimated by the combustion method using autoanalyzer (Leco, FP-2000). Acid insoluble lignin (AIL) was determined according to the laboratory analytical procedure (LAP) of National Renewable Energy Laboratory (NREL) [30]. Acid soluble lignin (ASL) was determined using the previously described procedure [24]. Arabinan, glucan, xylan, and mannan were determined as per the previously described procedure of NREL [30].

Pretreatment of rice straw

The milled rice straw at substrate concentration of 10% (w/v) was treated with 1–5% (w/v) NaOH concentrations in

250-ml Erlenmeyer flasks. The flasks were incubated in an incubator shaker at 150 rpm, 40°C for 1 h and thereafter autoclave-sterilized at 121°C for 30 min. The pretreated biomass (solid residue) was collected by vacuum filtration using Büchner funnel lined with Whatman filter paper. The biomass was washed repeatedly with tap water to a pH of about 5.2. The neutralized alkali-treated straw obtained after treatment with different alkali concentrations was separately dried in a hot-air oven at 70°C to a constant weight. Glucan, xylan, arabinan, lignin, and ash concentrations were determined in the residual biomass obtained after treatment with different alkali concentrations.

Isolation and propagation of yeast cells

Yeasts were isolated from freshly extracted natural sugarcane juice containing 16% (w/v) total sugars. Extraction of genomic DNA was done with the fungal genomic DNA isolation kit (Chromous Biotech Pvt Ltd, Bangalore, India). For the sequence analysis, the ITS1 and ITS4 rDNA region of the yeast was amplified by PCR using primer set pITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and pITS4 (5′-CCTCCGCTTATTGATATGC-3′). Details about the isolation, identification, and propagation of the yeast isolate are discussed in detail in our previous papers [10, 29].

Enzymatic hydrolysis of pretreated rice straw

Alkali concentration that reduced lignin content in rice straw and significantly increased the glucan concentration without a substantial loss of hemicellulosic fraction (xylan, arabinan) as well as pretreated biomass was selected for pretreatment. Enzymatic hydrolysis of pretreated rice straw was performed in 250-ml screw-capped polycarbonate flasks containing 50 mM citrate buffer (pH 5.0) and 10 g pretreated rice straw at a final solid-to-liquid loading of 10% (w/v). Tetracycline and cyclohexamide, each at 50 μg ml^{-1} concentration were added to the flasks prior to hydrolysis. The enzyme concentrations used for hydrolysis were 20 filter paper cellulase units/gram dried substrate (FPU gds^{-1}), 50 international units/gram dried substrate (IU gds^{-1}) β-glucosidase and 15 IU gds^{-1} pectinase. The flasks were incubated in an incubator shaker at 50°C, 120 rpm for 60 h. Flasks were removed from the incubator at 12-h intervals with the first set of flasks analyzed after 6 h of incubation. The contents of the flasks were filtered and clear supernatant was collected after centrifugation. Supernatant was analyzed for sugars while the residue was dried and analyzed for different constituents.

Morphological and biochemical characterization of the *Pichia kudriavzevii* HOP1 cells

Size, shape, number of ascospores, formation of pseudohyphae, hyphal characteristics and presence of ballistoconidia in the yeast isolate were determined as per the procedure of Barnett et al. [4] with the scanning electron microscope (SEM) [S-3400N, Hitachi Corporation, Japan]. The SEM protocol involved primary fixation of samples using 2.5% glutaraldehyde in phosphate buffer (pH 7.2), followed by washing with distilled water, secondary fixation using 1–4% osmium tetraoxide and dehydration with different concentrations of ethanol for different time intervals. The specimens were mounted on the aluminum stub with carbon tape and sputter coated before microscopic evaluation. Colony morphology, such as colony color and colony characteristics was observed from the colonies that appeared on the YPD plates. The biochemical profile of the cells was determined by evaluating the potential of the cells in assimilation of different carbon and nitrogen sources; osmophilic potential of cells; presence of urease in cells and resistance to acetic acid and cycloheximide. Carbohydrate assimilation capability of cells to metabolize different sugars was analyzed using the KB009 HiCarbohydrate kit (Hi-Media Laboratories Pvt Ltd, Mumbai, India). The osmophilic potential of the yeast cells was determined using different concentrations of glucose (20, 40, 60%) and NaCl (5, 10 and 15%). The growth medium for glucose tolerance was composed of 0.2% (w/v) yeast extract, 0.2% (w/v) peptone and varying concentrations of glucose mentioned previously. NaCl was added at concentrations mentioned previously to the growth medium composed of 1% glucose, 0.2% yeast extract and 0.2% peptone (w/v). The plates containing different concentrations of glucose and NaCl were streaked with the yeast cell inoculum and incubated at 30°C for 48 h and observed for growth. The plates showing growth at different concentrations of glucose and NaCl were positive while the plates showing no growth were termed as negative. Different nitrogen sources, such as potassium nitrate, sodium nitrate, and ammonium sulphate at 0.2% (w/v) were added to the yeast carbon base (Hi-Media Laboratories Pvt Ltd, Mumbai, India) to ascertain the potential of the cells in assimilation of such nitrogen sources. Urease enzyme activity for the cells was analyzed using the KB006 HiCandida identification kit (Hi-Media Laboratories Pvt Ltd, Mumbai, India). Resistance to 1% acetic acid, 0.01% and 0.1% (w/v) cycloheximide was assessed by incorporation of acetic acid and cycloheximide at the concentrations mentioned above in the sterilized YM broth flasks which were inoculated with the *P. kudriavzevii* cells. The flasks were incubated at 30°C for 24 h in an incubator shaker. Inoculum from these

flasks was streaked on the sterilized YPD plates, which were incubated at 30°C for 72 h and analyzed regularly for the growth of yeast cells.

Comparison of *Saccharomyces cerevisiae* and *Pichia kudriavzevii* in ethanol production

Fermentative ability of *P. kudriavzevii* cells was compared with that of *S. cerevisiae* cells in ethanol production from a synthetic medium composed of 15% (w/v) glucose. Dry yeast granules of the industrial *Saccharomyces cerevisiae* strain obtained from Kothari Fermentation and Biochem Ltd, New Delhi, India and loopful of inoculum from *Pichia kudriavzevii* slants were separately inoculated into sterilized YPD broth in 100-ml Erlenmeyer flasks. Flasks were incubated at 30°C for 48 h. Ten percent of inoculum from these flasks was aseptically transferred to 250-ml Erlenmeyer flasks comprising sterilized 100 ml YPD broth separately for the two yeast strains. Incubation conditions remained same as described previously and subsequent transfer of cells into the fresh medium continued until sufficient inoculum concentration was attained. Cell count was determined with the hemocytometer while the culture density was determined at OD_{600} for both the strains. Cell concentration for fermentation experiments was judged on the basis of the cell count for both the strains. Cell concentration for the strain showing higher cell count was appropriately diluted with sterile water to have the same initial cell count for comparative evaluation. Fermentation was done in specially designed 250-ml fermentation flasks fitted with a three-piece air lock system for proper maintenance of fermentation conditions. Flasks containing 150 ml of fermentation medium composed of 15% (w/v) glucose, 0.2% (w/v) yeast extract and 0.2% (w/v) $MgSO_4$ were used for conducting fermentation trials. Initial pH was adjusted to 4.0, 5.0, and 6.0 with the use of 5 N HCl before autoclave-sterilization for 15 min. The flasks were inoculated with 10 ml of inoculum having cell concentration of 1×10^8 cells/ml in the inoculum separately for *S. cerevisiae* and *P. kudriavzevii* cells. Since the idea was to use the yeast cells for SSF process, this comparison was done at 35°C and thus, the flasks were incubated at 35°C for 24 h at 100 rpm. A set of three flasks was removed from the incubator shaker at regular intervals of 6 h until 24 h and analyzed for glucose and ethanol concentrations. A similar experiment was planned by varying the incubation temperature and keeping the other conditions constant for both the strains. In this case, initial pH was adjusted to 5.0 for fermentation and the flasks were incubated at 35, 40, and 45°C. Three flasks were removed from the incubator shaker at regular intervals of 6 h until 24 h and analyzed for glucose and ethanol concentrations.

Simultaneous saccharification and fermentation

The SSF experiments were conducted in a 1.5 l thermostatically controlled glass reactor having provisions for temperature, agitation, and pH control (Bio-Age equipment, Mohali, India). The vessel was also provided with facilities like condensate removal and exhaust valve. Citrate buffer (50 mM, pH 5.0) and alkali-treated rice straw were added to achieve 10% solid-to-liquid ratio. The glass reactor containing alkali-treated rice straw was autoclave-sterilized for 10 min prior to enzymatic hydrolysis. The enzyme concentrations used for hydrolysis remained same as mentioned previously under the section on enzymatic hydrolysis. The agitation speed during enzymatic hydrolysis was maintained at 120 rpm, while pH of the medium was maintained at 5.0 with the use of sterilized 5 N NaOH solution. Enzymatic hydrolysis was carried out for 4 h at 50°C for partial hydrolysis of cellulose and hemicellulose. The temperature of the reactor after 4-h hydrolysis was brought down to 40°C by immersing it in cold water. The medium was then supplemented with the sterilized concentrated nutrient solution composed of 50 g l^{-1}, each of yeast extract, $MgSO_4$, and peptone so as to have a final concentration of 2 g l^{-1}, each of yeast extract, $MgSO_4$, and peptone in the fermentation medium. On the basis of comparative evaluation of the two strains for thermal tolerance and fermentation efficiency at varying pH, *P. kudriavzevii* HOP-1 was selected for the SSF process. The vessel was aseptically inoculated with freshly prepared 80 ml of inoculum of *P. kudriavzevii* HOP-1 cells having a cell concentration of 1×10^9 cells ml^{-1}. Temperature, pH, and agitation speed of 40°C, 5.0, and 100 rpm, respectively were maintained throughout the fermentation process. Temperature of 40°C was selected to accommodate both the enzymes and yeast cells in the same vessel at the same time. Although, optimum temperature requirements for enzymes and the yeast cells are different, a compromise was made in selection of temperature [35]. Samples were drawn at 12-h intervals until 60 h and analyzed for concentrations of sugars, ethanol, glycerol, and xylitol. The experiment was conducted three times in the same vessel and the data presented are average of three replications.

Analytical methods

Reducing sugars liberated were quantified using the dinitrosalicylic acid (DNS) method [20]. Glucose, xylose, arabinose, glycerol, xylitol and oligosaccharides were determined with the high-performance liquid chromatograph (HPLC) [Dionex Corporation, Sunnyvale, CA, USA] using a Shodex SP-0810 column (300 × 7.8 mm) fitted with a SP-G guard column (Waters Inc., Milford, MA, USA). Degassed HPLC grade water was used as a mobile

phase at a flow rate of 1.0 ml min^{-1}. The other parameters, such as temperatures of column oven and refractive index detector were maintained as per the previously described procedure [29]. Ethanol was determined with the YSI 2786 ethanol membrane kits using YSI 2700 Select biochemical analyzer (YSI Inc, Buffalo, NY, USA) [29].

Statistical analysis

All the experiments were conducted in triplicate and mean and standard deviation (SD) values were calculated using MS Excel software. Wherever necessary, the data were analyzed with one-way ANOVA and LSD ($p < 0.05$) for tests of significance with JMP software (SAS Inc, Cary, NC, USA).

Results

Effect of alkali treatment on compositional analysis of rice straw

In cellulosic biomass, glucan is the representative of cellulose content, while xylan and arabinan make up for the hemicellulosic fraction. In addition, galactans, mannans, rhamnans etc. are also present in lignocellulosic biomass, but in very low concentrations. Glucan, xylan, arabinan, galactan, AIL, ASL, ash and protein contents in rice straw were 35.45 ± 1.75%, 23.78 ± 0.97%, 3.20 ± 0.14%, 0.67 ± 0.03%, 12.56 ± 0.95%, 2.5 ± 0.17%, 13.45 ± 0.81% and 3.17 ± 0.19%, respectively. Rhamnans or mannans were not detected in the rice straw used in this study. Pretreatment with 1% NaOH increased glucan by nearly 50%, decreased hemicellulose (xylan and arabinan), lignin, and ash by about 45, 47, and 60%, respectively (Table 1). Increasing the alkali concentration from 1 to 5% increased glucan by about 7% and decreased hemicellulose and lignin by 46 and 16%, respectively (Table 1). Galactan was not observed in the alkali-treated biomass, irrespective of the concentration used for pretreatment.

Effect of enzymatic hydrolysis on residual biomass composition

Glucan, xylan, and arabinan concentrations decreased significantly leading to an increase in lignin, and ash contents in alkali-treated rice straw during 6-h hydrolysis (Table 2). Although, a decline in concentrations of glucan, xylan, and arabinan was seen until 48 h, hydrolysis rate was relatively higher during the first 12 h. This is corroborated by the increased production of glucose, xylose and arabinose during the first 12 h with the concentration of sugars leveling off at 36 h (Fig. 1). The rate of increase in production of glucose, xylose and arabinose dropped after 12 h of hydrolysis (Fig. 1). The fall in pH during enzymatic hydrolysis could be because of release of the uronic acids from xylan fraction of rice straw (Table 2). Xylan in rice straw is a kind of arabinoglucuronoxylan that is composed of L-arabinofuranose, 4-0-methyl-D-glucopyranuronic acid, D-glucopyranuronic acid, and D-xylopyranose [36]. A continuous decline in pH in the hydrolyzate during hydrolysis might have retarded the efficiency of the hydrolytic enzymes. No cellobiose or cellotriose were detected in the hydrolyzate; however, insignificant amount of xylobiose was formed during hydrolysis. Xylobiose concentration increased from 0.22 to 2.24 g/l during the 6–48 h hydrolysis period. Xylobiose might have been produced because of high endoxylanase activity and low β-xylosidase activity in the enzyme mixture used in this study. The β-xylosidase catalyzes the conversion of xylobiose to xylose and thus the absence or presence of β-xylosidase in low concentrations results in accumulation of xylobiose. The enzymes used in the present study demonstrated activities for xylanase, β-xylosidase, and α-L-arabinofuranosidase in addition to cellulase [28].

Identification and characterization of the isolated yeast strain

Sequencing and analysis of the ITS rDNA region showed that the isolated yeast strain had the highest identity with

Table 1 Composition of rice straw after pretreatment with different concentrations of sodium hydroxide

NaOH concentrations (%)	Glucan (%)	Xylan (%)	Arabinan (%)	Lignin (%)	Ash (%)	Solubilization (%)
0	$35.45^{e} \pm 1.75$	$23.78^{a} \pm 0.97$	$3.20^{a} \pm 0.14$	$12.56^{a} \pm 0.95$	$13.45^{a} \pm 0.81$	–
1.0	$52.34^{d} \pm 2.07$	$13.56^{b} \pm 0.26$	$2.10^{b} \pm 0.11$	$6.60^{b} \pm 0.20$	$5.31^{b} \pm 0.41$	$44^{a} \pm 0.50$
2.0	$57.78^{c} \pm 2.08$	$11.84^{c} \pm 0.14$	$1.92^{c} \pm 0.10$	$5.70^{c} \pm 0.28$	$4.81^{c} \pm 0.31$	$48^{b} \pm 0.40$
3.0	$60.0^{b} \pm 1.90$	$9.70^{d} \pm 0.20$	$1.75^{d} \pm 0.05$	$5.50^{c,d} \pm 0.15$	$3.43^{d} \pm 0.53$	$50^{b} \pm 0.67$
4.0	$60.0^{b} \pm 2.10$	$8.14^{e} \pm 0.4$	$1.60^{e} \pm 0.06$	$5.40^{c,d} \pm 0.30$	$2.58^{e} \pm 0.26$	$51^{c} \pm 0.85$
5.0	$60.54^{a} \pm 1.75$	$8.12^{e} \pm 0.1$	$1.60^{e} \pm 0.05$	$5.10^{d} \pm 0.40$	$1.19^{f} \pm 0.29$	$53^{d} \pm 0.60$
LSD (0.05)	0.17	0.27	0.16	0.45	0.38	1.13

Values depicted are mean ± SD for $n - 3$ and means with superscripts having the same letter are not significantly different

Table 2 Compositional changes in alkali-treated rice straw during enzymatic hydrolysis

Time (h)	Glucan (%)	Xylan (%)	Arabinan (%)	Lignin (%)	Ash (%)	pH
0	$52.34^{a} \pm 2.07$	$13.56^{a} \pm 0.26$	$2.10^{a} \pm 0.11$	$6.6^{f} \pm 0..20$	$5.31^{f} \pm 0.41$	5.2
6	$26.0^{b} \pm 0.15$	$8.0^{b} \pm 0.80$	$1.42^{b} \pm 0.07$	$12.29^{e} \pm 0.29$	$8.42^{e} \pm 0.2$	4.6
12	$19.29^{c} \pm 0.20$	$6.5^{c} \pm 0.50$	$1.21^{c} \pm 0.06$	$17.56^{d} \pm 0.25$	$9.11^{d} \pm 0.2$	3.9
24	$14.42^{d} \pm 0.12$	$5.2^{d} \pm 0.20$	$0.98^{d} \pm 0.05$	$19.13^{c} \pm 0.13$	$12.21^{c} \pm 0.1$	3.8
36	$9.17^{e} \pm 0.17$	$4.1^{e} \pm 0.10$	$0.73^{e} \pm 0.05$	$21.5^{b} \pm 0.10$	$14.41^{b} \pm 0.1$	3.6
48	$5.13^{f} \pm 0.13$	$4.0^{e} \pm 0.10$	$0.64^{e,f} \pm 0.04$	$24.77^{a} \pm 0.04$	$16.45^{a} \pm 0.2$	3.6
60	$5.12^{f} \pm 0.12$	$4.0^{e} \pm 0.10$	$0.60^{f} \pm 0.05$	$24.79^{a} \pm 0.10$	$16.53^{a} \pm 0.2$	3.6
LSD (0.05)	0.34	0.65	0.11	0.31	0.23	

Values depicted are mean ± SD for $n - 3$ and means with superscripts having the same letter are not significantly different

Issatchenkia orientalis [10]. The species ascribed to the genus *Issatchenkia* has been clustered within *Pichia*, and thus, *Issatchenkia orientalis* has been replaced by *Pichia kudriavzevii* as the valid name for that taxonomic entity [17]. The ITS sequence of *P. kudriavzevii* was submitted to GenBank with the accession no: HQ 122942 and the isolate is deposited with the National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau Nath Bhanjan, India (NAIMCC- F-02470). The strain henceforth has been referred to as *Pichia kudriavzevii* HOP-1. The yeast colonies were whitish to creamish in color with raised surface. When observed under microscope, the size of the cells varied from 2.48 to 6.93 μm in diameter. The cell shape varied from oval, ellipsoidal to elongate. Scanning electron microscopic images showed distinct and large pseudohyphae (Fig. 2) with terminal cells shorter than or as long as the adjacent cells. The pseudohyphae formation in *P. kudriavzevii* HOP-1was observed after 5 days of growth and the cells showed extensive pseudohyphae formation. Each asci in *P. kudriavzevii* HOP-1 comprised of two ascospores that were oval, spherical, or globose in shape (not shown). There are generally 1–4 ascospores in the ascus of *Saccharomyces cerevisiae* cells.

Pichia kudriavzevii HOP-1 cells could grow at 5% NaCl concentration but did not show any growth at 10 or 15% NaCl concentrations. Cells showed growth at 20 and 40% glucose concentrations but glucose concentration as high as 60% inhibited the cell growth completely. Thus, the *P. kudriavzevii* HOP-1 strain could be considered as partially osmotolerant strain. Cycloheximide at both 0.01 and 0.1% concentrations inhibited the cell growth indicating the vulnerability of cells to cyclohexamide even at a low antibiotic concentration. Although, the cells could assimilate ammonium sulphate, they did not show growth in medium containing potassium or sodium nitrate. *Pichia kudriavzevii* HOP-1 cells also did not possess urease enzyme and thereby could not hydrolyze urea. Acetic acid concentration of 1% was lethal to the *P. kudriavzevii* cells.

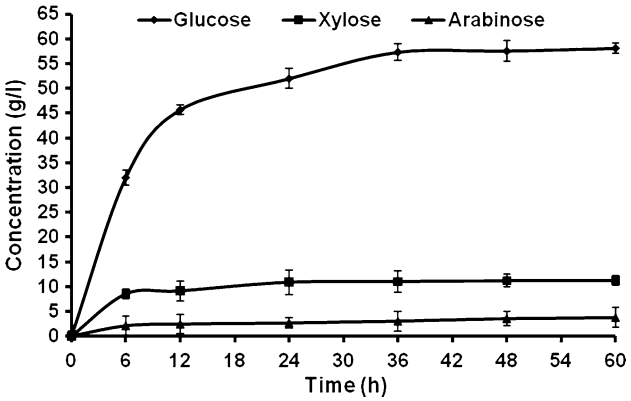

Fig. 1 Production of glucose, xylose, and arabinose during hydrolysis of 1% alkali-treated rice straw

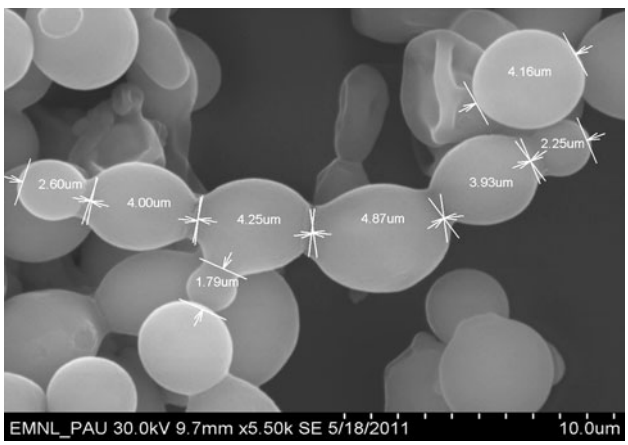

Fig. 2 Scanning electron micrograph of pseudohyphae in *Pichia kudriavzevii* HOP-1 cells

Among the carbohydrates tested, the yeast cells could grow in presence of sugars, such as glucose, sucrose, fructose, and mannose but they weakly assimilated galactose and could not metabolize sugars like maltose, xylose, arabinose, cellobiose, raffinose, or trehalose.

Comparative evaluation of fermentative ability of *S. cerevisiae* and *P. kudriavzevii* cells

It is evident from the results of Figs. 3 and 4 that *P. kudriavzevii* HOP-1 cells showed better adaptability at varying temperature and pH ranges, respectively than the *S. cerevisiae* cells. Although the two yeasts showed a similar pattern of ethanol production, *P. kudriavzevii* cells produced about 10% more ethanol than the *S. cerevisiae* cells at 35°C (Fig. 3). Irrespective of the temperature, rate of increase in ethanol production was highest during 6–12 h for both yeasts. Although ethanol concentration increased from 12 to 18 h and then leveled off, rate of increase in ethanol concentration was lower during this period, compared to the 6–12 h period (Fig. 3). This could be attributed to the cells entering into the exponential phase during the 6–12 h phase, accompanied with availability of glucose in higher quantities. We observed a similar pattern in ethanol production with *P. kudriavzevii* cells in one of our previous studies [10]. The *P. kudriavzevii* cells produced about 35% and 200% more ethanol at 40 and 45°C, respectively in 18 h than the *S. cerevisiae* cells. *Pichia kudriavzevii* cells also showed better fermentative ability at varying pH when compared to the *S. cerevisiae* cells, though substantial difference as seen in case of temperature was not observed at pH of 5.0 and 6.0 (Fig. 4). However, about 20% more ethanol was produced by the *P. kudriavzevii* HOP-1 cells, compared to ethanol produced by *S. cerevisiae* cells at pH of 4.0 (Fig. 4). On the basis of these results and our recently published work [10, 29], we conducted the fermentation of alkali-treated rice straw with the *P. kudriavzevii* HOP-1 strain.

Simultaneous saccharification and fermentation for ethanol production

The 4 h pre-hydrolysis during SSF resulted in 10.45 g l^{-1} glucose and 16.27 g l^{-1} reducing sugars. Xylose and arabinose concentrations during pre-hydrolysis were 3.8, and 1.1 g l^{-1}, respectively. Sukumaran et al. [31] obtained fermentable sugar concentration of 16.3 g l^{-1} during pre-hydrolysis of pretreated rice straw. An increase in glucose and RS concentrations was observed during 12 h with production of only 6.8 g l^{-1} ethanol (Fig. 5). No xylitol and only an insignificant amount of glycerol were produced during SSF in 60 h. Some glucose was left after 36 h during SSF, suggesting that although glucose was being produced through enzymatic hydrolysis of cellulose, it could not be fermented to ethanol (Fig. 5). Ethanol concentration did not show a significant increase after 24 h and leveled off after 36 h (Fig. 5). Reducing sugars left after 36 h SSF were mainly xylose and arabinose, which could not be fermented by the *P. kudriavzevii* strain used in this study (Fig. 5). Ethanol concentration at the end of 24 h was 24.25 g l^{-1}, which corresponds to nearly 82% theoretical yield on glucan basis. Ethanol concentration of 24.25 g l^{-1} in 24 h corresponds to ethanol yield on biomass basis and ethanol productivity of 0.24 g g^{-1} and 1.1 g l^{-1} h^{-1}, respectively, which holds potential for scale-up studies.

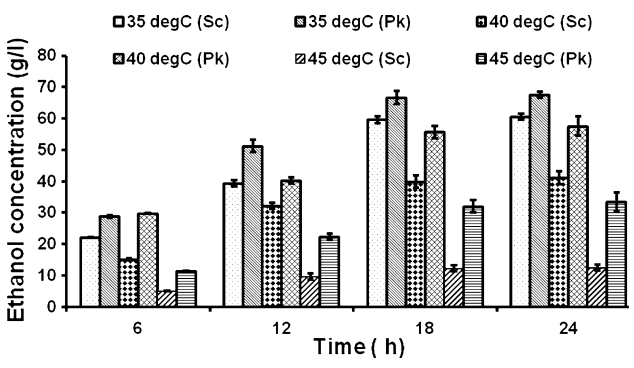

Fig. 3 Comparison of *Saccharomyces cerevisiae* and *Pichia kudriavzevii* HOP-1 for ethanol production using 15% (w/v) glucose at different temperatures

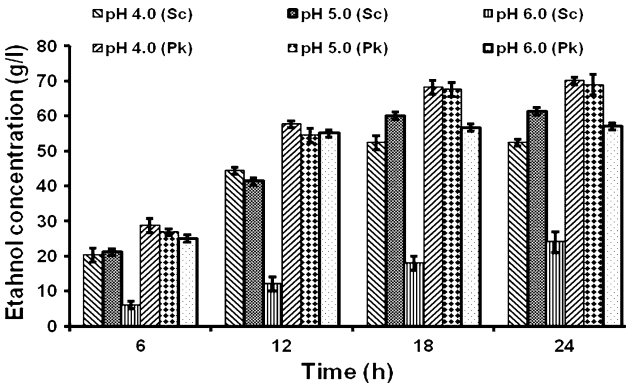

Fig. 4 Comparison of *Saccharomyces cerevisiae* and *Pichia kudriavzevii* HOP-1 for ethanol production using 15% (w/v) glucose at different pH

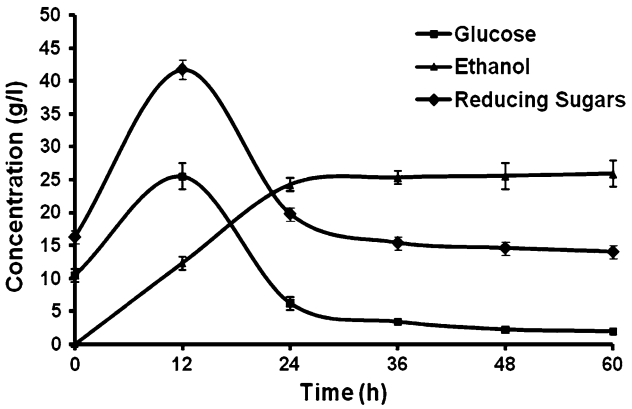

Fig. 5 Ethanol production and sugar consumption during simultaneous saccharification and fermentation

The scanning electron micrographs revealed that the rice straw structure was damaged because of enzymatic hydrolysis (Fig. S1a), while an extensive disruption in the structure was seen in the rice straw structure after SSF (Fig. S1b). The voids in the structure of rice straw sample after SSF are because of the fermentation of the sugars obtained during SSF to ethanol (Fig. S1b). In our previous study we had shown the intact cellulose and hemicellulose layers in untreated ground rice straw particles [24]. More studies at molecular levels are needed to arrive at the metabolic pathway used by *P. kudriavzevii* for carbon and energy metabolism and formation of glycerol during mixed-sugar fermentation. Ethanol concentration and productivity obtained during the present study were comparable or better than the results reported from alkali-treated rice straw by previous authors [8, 26, 34, 38]. Although, it may not be a reliable estimate, it is likely that the size reduction and alkali pretreatment cost for rice straw would cost approximately 0.50–0.55$ to produce one liter of ethanol based on the ethanol concentration and yield obtained in this study.

Discussion

Alkali concentration above 1% neither increased glucan nor decreased lignin content substantially. However, the quantity of treated biomass and hemicellulose decreased significantly when alkali was used at concentrations higher than 1% for pretreatment (Table 1). It is possible that galactan solubilized during the alkali treatment and was removed in the hydrolyzate. The reduction in lignin and ash concentrations and partial solubilization of hemicellulose during alkali pretreatment led to concentration of glucan in the pretreated rice straw. Hemicellulose is the second most important polymer after cellulose in rice straw and is rich in pentose sugars, such as xylose. Previous studies have reported successful conversion of xylose to ethanol using pentose-fermenting yeasts such as *Pichia stipitis*, *Pachysolen tannophilus*, and *Candida tropicalis* [1, 13, 24]. Therefore, fermentation of mixed sugar stream to ethanol is likely to improve ethanol concentration necessary for commercial success of ethanol production process from lignocellulosic materials. The use of low concentrations of NaOH for pretreatment has been reported as an effective pretreatment method for lignocellulosic materials with relatively low lignin content of 10–18% [6]. Pretreatment of substrate with NaOH results in swelling of the particles causing easy removal of the lignin and cellulose depolymerization [9]. Same authors reported that the alkali-treated residues with low concentrations of NaOH showed higher accessibility to enzymatic hydrolysis. Gharpuray et al. [12] concluded in their study that when certain delignification percentage has been achieved, further disruption of the lignin/carbohydrate linkage is not necessary to increase the accessibility to enzymes. Thus, after data analysis and on the basis of previously published literature, we decided to use 1% alkali for pretreatment of rice straw.

The fall in rate of glucose, xylose, and arabinose production after 12 h could largely be attributed to the reduced enzyme accessibility due to concentration of lignin and ash, and also the limited availability of cellulose and hemicellulose after 12 h hydrolysis. Previous studies have reported a decline in hydrolytic efficiency of cellulase because of catabolite repression and increase in lignin concentration [5, 21, 22]. Berlin et al. [5] showed an increased glucose production during the first 10 h using combination of cellulase and pectinase for hydrolysis of acid-treated corn stover.

Gallardo et al. [11] observed the pseudohyphae formation for non-*Saccharomyces* isolates after 3 days of growth. Although the mechanism behind formation of pseudohyphae or their role in cell metabolism is not clear, it is thought that the pseudohyphae are formed during depletion of nutrients, especially the nitrogen source. Lo and Dranginis [18] reported that the diploid yeasts develop pseudohyphae in response to nitrogen starvation, while haploid yeasts produce invasive filaments that penetrate the agar in rich medium. They also identified a gene FLO11 that encodes a cell wall protein, which is critically required for both invasion and pseudohyphae formation in response to nitrogen starvation. Most of the morphological and physiological characteristics and the ability of the yeast cells used in the present study to assimilate specific sugars and nitrogen sources resembled the characteristics shown by the group-I non-*Saccharomyces* isolates belonging to *I. orientalis* reported previously [11].

Ethanol produced after 18 h fermentation with *S. cerevisiae* at 35°C was comparable to the ethanol produced by *P. kudriavzevii* HOP-1 cells at 40°C, which indicates the thermotolerant ability of the *P. kudriavzevii* strain and its adaptability at relatively higher temperatures. Thermotolerant nature of the cells is probably because of the composition of the cellular components and range of different molecular mechanisms and thus, molecular level studies are needed to arrive at the rationale behind adaptability of *P. kudriavzevii* cells to varying temperatures. Yeast cells exhibit a rapid molecular response when exposed to elevated temperatures. Kitagawa et al. [16] reported that *Issatchenkia orientalis* MF-121 strain has the potential for ethanol production from lignocellulosic biomass because of its acid tolerant, ethanol tolerant and thermotolerant characteristics. It can be inferred from the above discussion that *P. kudriavzevii* HOP-1 strain was better adapted to varying temperature and pH conditions, compared to the *S. cerevisiae* strain used in this study.

The data in Fig. 5 shows that the hydrolysis rate was higher than the fermentation rate during the first 12 h. It is noteworthy to mention here that unlike SHF, where the fermentation medium has sugars freely available for fermentation; sugars were initially bound to the straw during the SSF process. Enzymatic hydrolysis during SSF led to liquefaction of the medium, which subsequently favored fermentation of sugars to ethanol. Low glycerol production during SSF could be because of the continuous availability of glucose through enzymatic hydrolysis of pretreated rice straw [24]. Since the yeast strain used in this study did not assimilate xylose as mentioned previously, xylitol production from xylose could be ruled out. Although, ethanol concentration during SSF increased until 36 h, the fermentation rate drastically dropped after 24 h. We observed a similar trend during the SSF of dilute acid pretreated rice straw using hydrolyzate-adapted *Candida tropicalis* [24]. Previous studies have shown that the cellulolytic enzymes are highly active at temperatures in the vicinity of 50°C, whereas yeast cells perform well in the vicinity of 30°C [7, 27]. Therefore, a compromise was made in selecting a temperature during SSF to accommodate both the enzymes and yeast cells in the same vessel, which might have resulted in a lower hydrolytic activity for enzymes. Since *P. kudriavzevii* HOP-1 was isolated at relatively higher temperatures from cane juice containing ethanol and propagated at 40°C, it is not likely to get affected by temperatures in the vicinity of 40°C or presence of ethanol as is the case with conventional *Saccharomyces cerevisiae* strains [35]. It is therefore important to use a thermotolerant and ethanol tolerant yeast strain in the SSF process so that hydrolysis and fermentation could be successfully accomplished at the same temperature. It is possible that because of the increase in lignin and ash concentrations during hydrolysis as described previously, sugars produced in latter half during SSF were bound by the insoluble lignin and ash fractions and hence could not be converted to ethanol by the yeast strain. Since significant concentrations of xylose and arabinose were also produced during SSF, it is important to include pentose sugar fermenting yeast in the medium or use a recombinant, which could effectively ferment hexose and pentose sugars simultaneously. One could expect about 15% more ethanol using the SSF process described in this paper, if pentose sugars are also fermented even with 70% fermentation efficiency.

The commercial success of ethanol production for its use as bio-fuel depends on high ethanol concentration and high ethanol productivity. High ethanol volumetric productivity also means that many batches can be produced in a short time, which would offset the explicit and implicit costs involved in the ethanol production process. We have recently attempted SSF of alkali-treated cotton stalks with *P. kudriavzevii* at the shake flask level (unpublished data). We are now attempting to use *P. kudriavzevii* HOP-1 in conjunction with pentose-fermenting yeasts for further improving ethanol concentration. We also intend to conduct studies to evaluate the role of aeration in ethanol production using this strain of *P. kudriavzevii* along with the pentose-fermenting yeast in mixed-sugar fermentation with pure sugars as well sugars obtained in the hydrolyzates of acid- and enzymatically hydrolyzed pretreated biomass.

Conclusions

This probably is the first study in which *P. kudriavzevii* was used for ethanol production from any lignocellulosic material. This study demonstrated that the treatment of native straw with 1% NaOH effectively decreased lignin by about 47% and increased glucan by about 50%. Increasing alkali concentration beyond 1% for pretreatment led to higher loss of biomass and relatively higher solubilization of hemicellulose. Simultaneous saccharification and fermentation was conducted using 1% alkali-treated rice straw and a thermotolerant strain of *P. kudriavzevii*. The SSF process could be completed in 24 h and thus could be a likely choice of the users as ethanol volumetric productivity of 1.1 g l^{-1} h^{-1} suggests that more batches could be completed in a relatively short time. The use of a single vessel during SSF also has an impact on the overall economic feasibility of the process. The use of a thermotolerant and ethanol tolerant yeast strain offers a tremendous potential in ethanol production from lignocellulosic biomass, especially during the SSF process. As the use of ethanol as a bio-fuel from abundantly available cellulosic biomass is gaining importance, the results obtained from the present study hold promise for pilot scale studies. However, more studies are needed to further exploit the potential of *P. kudriavzevii* in improving ethanol production from lignocellulosic biomass.

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References

1. Agbogbo FA, Coward-Kelly G, Tory-Smith M, Wenger KS (2006) Fermentation of glucose/xylose mixtures using *Pichia stipitis*. Process Biochem 41:2333–2336
2. AOAC, Official Methods of Analysis of AOAC international (2000) Association of official analytical chemists. Gaithersburg, Maryland, USA, 17th edn, p. 14
3. Ballesteros M, Oliva JM, Negro MJ, Manzanares P, Ballesteros I (2004) Ethanol from lignocellulosic materials by simultaneous saccharification and fermentation process with *Kluveromyces marxianus* CECT 10875. Process Biochem 39:1843–1848

4. Barnett JA, Payne RW, Yarrow I (2000) Laboratory methods for identifying yeasts (Chapter-4) In Yeasts: Characteristics and identification, 3rd edn. Cambridge University Press, UK
5. Berlin A, Maximenko V, Gilkes N, Saddler J (2007) Optimization of enzyme complexes for lignocelluloses hydrolysis. Biotechnol Bioeng 97:287–296
6. Bjerre AB, Olesen AB, Fernavist T (1996) Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. Biotechnol Bioenerg 49:568–577
7. Castellanos F, Sinitsyn P, Vlasenkko EY (1995) Comparative evaluation of hydrolytic efficiency toward microcrystalline cellulose of *Penicillium* and *Trichoderma* cellulases. Bioresour Technol 52:119–124
8. Chadha BS, Kanwar SS, Garcha HS (1995) Simultaneous saccharification and fermentation of rice straw to ethanol. Actamicrobiol Immunol Hung 42:71–75
9. Damisa D, Ameh J, Umoh VJ (2008) Effect of chemical pretreatment of some lignocellulosic wastes on the recovery of cellulase from *Aspergillus niger* AH3 mutant. Afr J Biotechnol 7:2444–2450
10. Dhaliwal SS, Oberoi HS, Sandhu SK, Nanda D, Kumar D, Uppal SK (2011) Enhanced ethanol production from sugarcane juice by galactose adaptation of a newly isolated thermotolerant strain of *Pichia kudriavzeii*. Bioresour Technol 102:5968–5975
11. Gallardo JCM, Souza CS, Cicarelli RMB, Oliveiara KF, Morais MR, Laluce C (2011) Enrichment of a continuous culture of *Saccharomyces cerevisiae* with the yeast *Issatchenkia orientalis* in the production of ethanol at increasing temperatures. J Ind Microbiol Biotechnol 38:404–414
12. Gharpuray MM, Lee YH, Fan LT (1983) Structural modification of lignocellulosics by pretreatment to enhance enzymatic hydrolysis. Biotechnol Bioeng 25:157–172
13. Jeffries TW, Jin YS (2000) Ethanol and thermotolerance in the bioconversion of xylose by yeasts. Adv Appl Microbiol 47:221–268
14. Kadam KL, Forrest LH, Jacobson WA (2000) Rice straw as a lignocellulosic resource: collection, processing, transportation and environmental aspects. Biomass Bioenergy 18:369–389
15. Kim DH, Hong YA, Park HD (2008) Co-fermentation of grape must by *Issatchenkia orientalis* and *Saccharomyces cerevisiae* reduced the malic acid content in wine. Biotechnol Lett 30:1633–1638
16. Kitagawa T, Tokuhiro K, Sugiyama H, Kohda K, Isono N, Hisamatsu M, Takahashi H, Imaeda T (2010) Construction of a beta-glucosidase expression system using the multistress-tolerant yeast *Issatchenkia orientalis*. Appl Microbiol Biotechnol 87:1841–1853
17. Kurtzman CP, Robnett CJ, Basehoar-Powers E (2008) Phylogenetic relationships among species of *Pichia*, *Issatchenkia* and *Williopsis* determined from multigene sequence analysis, and the proposal of Barnettozyma gen.nov., Lindnera gen.nov. and Wickerhamomyces gen.nov. FEMS Yeast Res 8:939–954
18. Lo WS, Dranginis AM (1998) The cell surface flocculin Flo11 is required for pseudohyphae formation and invasion by *Saccharomyces cerevisiae*. Mol Biol Cell 9:161–171
19. Merino ST, Cherry J (2007) Progress and challenges in enzyme development for biomass utilization. Adv Biochem Eng Biotechnol 108:95–120
20. Miller G (1959) Dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 31:426–428
21. Murray WD (1987) Effects of cellobiose and glucose on cellulose hydrolysis by growing and resting cells of *Bacteroides cellulosolvens*. Biotechnol Bioeng 29:1151–1154
22. Mussatto SI, Fernandes M, Milagres AMF, Roberto IC (2008) Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain. Enz Microbial Technol 43:124–129
23. Nolan AM, Barron N, Brady D, McAree T, Smith D, McHale L, McHale AP (1994) Ethanol production at 45°C by an alginate-immobilized strain of *Kluyveromyces marxianus* following growth on glucose containing media. Biotechnol Lett 16:849–852
24. Oberoi HS, Vadlani PV, Brijwani K, Bhargav VK, Patil RT (2010) Enhanced ethanol production via fermentation of rice straw with hydrolysate-adapted *Candida tropicalis* ATCC 13803. Process Biochem 45:1299–1306
25. Olofsson K, Bertilsson M, Liden G (2008) A short review on SSF, an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol Biofuels 1:1–14
26. Punnapayak H, Emert GH (1986) Use of *Pachysolen tannophilus* in simultaneous saccharification and fermentation (SSF) of lignocellulosics. Biotechnol Lett 8:63–66
27. Sa-Correia I, Van Uden N (1982) Temperature profiles of ethanol tolerance: effects of ethanol on the minimum and the maximum for growth of the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces fralilis*. Biotechnol Bioeng 25:1665–1667
28. Saha BC, Iten LB, Cotta MA, Wu YV (2005) Dilute-acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochem 40:3693–3700
29. Sandhu SK, Oberoi HS, Dhaliwal SS, Babbar N, Kaur U, Nanda D, Kumar D (2011) Ethanol production from Kinnow mandarin (*Citrus reticulata*) peels via simultaneous saccharification and fermentation using crude enzyme produced by *Aspergillus oryzae* and the thermotolerant *Pichia kudriavzevii* strain. Ann Microbiol, doi:10.1007/S13213-011-0302-x
30. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D (2008) Determination of structural carbohydrates and lignin in biomass. Laboratory analytical procedure, Technical report NREL-TP/510–42618. National Renewable Energy Laboratory, Colorado
31. Sukumaran RK, Singhania R, Mathew GM, Pandey A (2009) Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bioethanol production. Renew Energy 34:421–424
32. Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1–11
33. Wang M (2007) Life cycle energy and greenhouse gas emission impacts of different corn ethanol plant types. Environ Res Lett 2:1–13
34. Wati L, Kumari S, Kundu BS (2007) Paddy straw as substrate for ethanol production. Indian J Microbiol 47:26–29
35. Wilkins MR, Widmer WW, Grohman K (2007) Simultaneous saccharification and fermentation of citrus peel waste by *Saccharomyces cerevisiae* to produce ethanol. Process Biochem 42:1614–1661
36. Yoshida S, Kusakabe I, Matsuo N, Shimzu K, Yasui T, Murakami K (1990) Structure of rice straw arabinoglucuronoxylan and specificity of *Streptomyces* xylanase towards the xylan. Agric Biol Chem 54:449–457
37. Zhao XB, Wang L, Liu DH (2008) Peracetic acid treatment of sugarcane bagasse for enzymatic hydrolysis; a continued work. J Chem Technol Biotechnol 83:950–960
38. Zhu S, Wu Y, Yu Z, Zhang X, Wang C, Yu F, Jin S, Zhao Y, Tu S, Xue Y (2005) Simultaneous saccharification and fermentation of microwave/alkali pre-treated rice straw to ethanol. Biosyst Eng 92:229–235
39. Zhu SD, Wu YX, Yu ZN, Chen QM, Wu GY, Yu F (2006) Microwave-assisted alkali pretreatment of wheat straw and its enzymatic hydrolysis. Biosyst Eng 94:437–442