Ann Microbiol (2015) 65:423–429
DOI 10.1007/s13213-014-0875-2

ORIGINAL ARTICLE

Simultaneous saccharification and fermentation of pretreated sugarcane bagasse to ethanol using a new thermotolerant yeast

Parul Singh Antil · Rishi Gupta · Ramesh Chander Kuhad

Received: 18 September 2013 / Accepted: 12 March 2014 / Published online: 7 May 2014
© Springer-Verlag Berlin Heidelberg and the University of Milan 2014

Abstract Enzymatic hydrolysis of a cellulosic substrate is the most critical step for the production of bioethanol. In our study, the hydrolysis of steam-exploded sugarcane bagasse (SESB) under optimized conditions (8 % substrate consistency, 22.5 U filter paper cellulase, 0.55 % Tween 80) released a maximum of 461 mg per gram dry substrate sugars. We isolated a thermotolerant yeast strain, *Blastobotrys adeninivorans* RCKP 2012, from sugarcane bagasse collected from the Cooperative Sugar Mill, Sonepat, Haryana that was found to be capable of fermenting the enzymatic hydrolysate of SESB at 50 °C. When grown under simultaneous saccharification and fermentation conditions, this yeast produced 14.05 g L^{-1} ethanol, which corresponds to a theoretical ethanol yield of 46.87 %.

Keywords Bioethanol · Thermotolerant · SSF · Lignocellulose · Steam explosion

Introduction

The rapid increase in industrialization and, consequently, in energy demand have been the driving forces behind the search for alternative energy sources. Among the various potential alternatives, bioethanol derived from lignocellulosics has been considered a good choice due to its renewable nature and carbon-balanced properties (Weng et al. 2008; Gupta et al. 2009). The major constituents of lignocellulosics are cellulose, hemicellulose and lignin (Kuhad and Singh 1993). The first step in the bioconversion of lignocellulosics to ethanol is its conversion into the component sugars. However, the structural recalcitrancy of lignocellulosics hinders its efficient enzymatic conversion to fermentable sugars (Eijsink et al. 2008). Therefore, prior to enzymatic saccharification, it is necessary to pretreat lignocellulosic materials to unlock the structure of lignocellulose and thereby facilitate enzymatic hydrolysis of the target polysaccharides.

Among the various pretreatments studied to date, steam explosion offers several advantages, such as a reduced need of chemicals, or no chemicals at all , reduced generation of fermentation inhibitors and eco-friendliness (Kuhad et al. 2011). During the steam explosion treatment, most of the hemicellulosic fraction is extracted in the condensate, with the residual solid biomass containing mainly cellulose and lignin (Kuhad et al. 2011). The pretreated substrate is then enzymatically hydrolyzed using cellulases (endoglucanase, cellobiohydrolase and β-glucosidase). The factors affecting the enzymatic hydrolysis of cellulose include amount of substrate, cellulase activity, reaction conditions (temperature and pH) and additives (surfactants and metal ions), if any. Optimization of these factors is essential to improve the sugar yield and rate of enzymatic saccharification, which will eventually increase the bioethanol production.

Fermentation of the enzymatic hydrolysate can be carried out using either of the two approaches, i.e. separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF). SSF has been reported to have several advantages over SHF (Soderstrom et al. 2005): (1) improved enzymatic hydrolysis rate and product concentration due to reduced end-product inhibition; (2) reduced production cost due to both the hydrolysis and fermentation reactions being carried out in a single reactor (Chen et al. 2007). However, the difference in the temperature optima of the cellulases (45–50 °C) and that of the fermenting organism (28–35 °C) is a critical factor in SSF (Kadar et al. 2004). Therefore there is an urge to use for thermotolerant yeast for improved performance of SSF system.

P. S. Antil · R. Gupta · R. C. Kuhad (✉)
Lignocellulose Biotechnology Laboratory, Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi 110021, India
e-mail: kuhad85@gmail.com

The aim of our study was to optimize conditions for the enzymatic saccharification of steam-exploded sugarcane bagasse (SESB) and to enhance its saccharification yield. We also attempted to simultaneously saccharify and ferment SESB under optimized saccharification conditions using thermotolerant yeast.

Materials and methods

Raw materials and chemicals

Steam-exploded sugarcane bagasse was a kind gift from Dr. A.J. Varma, Polymer Chemistry Division, National Chemical Laboratory, Pune, India; the wheat bran was procured locally. 3,5-Dinitrosalicylic acid (DNS) was purchased from Sigma (St. Louis, MO). Ethanol for standard preparation was obtained from Merck India Pvt. Ltd (Mumbai, India). Other chemicals and media components used were purchased locally.

Chemical composition analysis of SESB

The chemical composition of SESB was analyzed for holocellulose, Klason lignin, pentosans, ash and moisture content. The plant material was extracted with alcohol–benzene (1:2, v:v) to remove wax and resin. The extractive-free wood dust was processed for chemical analysis following TAPPI (1992) protocols.

Isolation, screening and identification of the thermotolerant yeast strain

The environmental samples consisted of sugarcane bagasse was collected from the Cooperative Sugar Mill, Sonepat, Haryana, Balma, the starter culture of the traditional beverage of the Bhootiya tribe was procured from Uttrakhand and starter culture of alcoholic beverages was obtained from Kangra Herb Pvt. Ltd, Kangra, Himachal Pradesh. All samples were serially diluted and spread on Sabouraud's agar medium [(g L^{-1}): peptone, 10.0; glucose, 40.0; agar, 15.0; sodium propionate, 3.5; pH 6.0]. The plates were incubated at 30 °C for 3 days. Pure cultures were obtained from the colonies which developed through repeated transfer of the cultures on MGYP agar plates [(g L^{-1}): malt extract, 3; glucose, 20; yeast extract, 3; peptone, 5 agar, 20; pH 6.0].

All yeast isolates were screened for their thermotolerance and fermenting capability by culture in 50-mL culture tubes, each containing 10 mL MGYP broth. The tubes were inoculated with a loop-full of the appropriate yeast isolate and incubated for 72 h at 50 °C and 200 rpm in a rotatory incubator shaker (Innova 4400; New Brunswick Scientific, Nürtingen, Germany). Samples were taken out at 6-h intervals an analyzed for residual sugars, ethanol content and biomass produced.

To identify the potent thermotolerant fermenting yeast isolate (LBLY 2), we amplified a 500-bp region of the 18S rRNA gene in a thermocycler (G-Storm; BMG Labtech, Aylesbury, UK) using the universal primers ITS1 (TCCGTA GGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGAT ATGC). The PCR products were purified and sequenced as described earlier (Khurana et al. 2007), and the nucleotide sequence has been deposited in the GenBank database. The sequence data were analyzed for homology with the similar existing sequences available in the database of the National Center for Biotechnology Information (NCBI) using BLAST.

Cellulase production using *Trichoderma citrinoviridae* RCK2012 under SSF

Trichoderma citrinoviridae RCK2012 was used for cellulase production under solid-state cultivation conditions. The inoculum for enzyme production was prepared by inoculating four fungal discs (diameter 8 mm) removed from the periphery of a 8-day-old potato dextrose agar plate into a 250-mL Erlenmeyer flask containing 100 mL of potato dextrose broth and incubating the flask at 30°C under static culture conditions for 4 days.

The enzyme production process was carried out in 250-mL Erlenmeyer flasks, with each containing 5.0 g of dry wheat bran moistened with a mineral salt solution [(g L^{-1}): soybean meal, 24; $(NH_4)_2SO_4$, 0.3; KH_2PO_4, 0.6; yeast extract, 5.0; pH 5.5] to attain a final substrate-to-moisture ratio of 1:3. The flasks were sterilized by autoclaving at 121°C (15 psi); following cooling to room temperature, they were inoculated with crushed fungal mass (20 % w v^{-1}, on dry weight basis) obtained from the fungal mat in the inoculum flask. The contents of the flasks were mixed well with a sterilized glass rod to distribute the inoculum evenly throughout the substrate and incubated at 30 °C. After the wheat bran had been fermented by the fungus for an appropriate interval, it was aseptically removed from the flasks, suspended in 25 ml of 50 mM citrate phosphate buffer (pH 5.0) and stirred for 10 min. The extrudates were squeezed through muslin cloth for maximizing the enzyme extraction and centrifuged at 10,000 *g* at 4 °C for 10 min. The enzyme solution thus obtained was assayed for cellulase activities.

Optimization of enzymatic saccharification of SESB

Enzymatic saccharification of SESB was carried in a 50 mM citrate phosphate buffer (pH 5.0). Prior to enzyme loading, the slurry was preincubated at 50 °C on a rotatory shaker (Innova 4400; New Brunswick Scientific) at 200 rpm for 2 h. Enzyme and the non-ionic surfactant (Tween 80) were then added to the preincubated slurry and the reaction was allowed to continue at 50°C and 200 rpm.

In order to optimize the enzymatic saccharification of SESB, we performed 17 runs of response surface methodology (RSM)-based Box–Behnkhen design (BBD) experiments (Design Expert, ver. 6.1; Stat-Ease, Minneapolis, MN), with substrate consistency, enzyme dosage and Tween 80 dosage as factors and saccharification yield as response. The range of each variable is shown in Table 1. Samples were withdrawn at 4-h intervals and centrifuged at 10,000 *g* for 10 min in a refrigerated centrifuge (Sigma) at 4 °C; the supernatant was used for further analysis.

SSF of SESB

The primary inoculum was prepared by inoculating a loop full of yeast culture from 24-h-old MGYP agar plates into the inoculation medium [(g L^{-1}): glucose, 30.0; yeast extract, 3.0; peptone, 5.0; $(NH_4)_2HPO_4$, 0.25; pH 6.0±0.2] (Chen et al. 2007; Kuhad et al. 2010). The secondary inoculum was developed by inoculating 2 % of primary inoculum into the inoculation medium and culturing the yeast cells until an optical density (OD_{600}) of 0.6.

The SSF experiments were performed in 250-mL capped conical flasks (SCHOTT DURAN, Mainz, Germany) containing 50 mL reaction volume under the optimum enzymatic saccharification conditions. Nutrients (malt extract, yeast extract and peptone) were added to the medium to a final concentration of 3.0, 3.0 and 5.0 g L^{-1}, respectively. Prior to SSF, a prefermentation saccharification step was carried out for 2 h at 50 °C and 150 rpm, following which 6 % inoculum of the thermotolerant yeast was added. Samples were withdrawn at 6-h intervals, centrifuged at 10,000 *g* for 10 min at 4 ºC and the supernatant was assayed for sugars and ethanol.

Analytical methods

The enzyme assays were carried out using standard International Union of Pure and Applied Chemistry methods (Ghose 1987). Total reducing sugars were estimated by the DNS method (Miller 1959). Ethanol was estimated by gas chromatography Clarus 500; PerkinElmer, Waltham, MA) with an elite-wax (cross bond-polyethylene glycol) column (30.0 m×0.25 mm) at an oven temperature of 90 °C, an injector temperature of 150 °C and flame ionization detection at 200 °C. The ethanol standards were prepared using commercial grade ethanol (Merck, Darmstadt, Germany). Nitrogen at a flow rate of 0.5 mL min^{-1} was used as the carrier gas. The saccharification yield was calculated as:

$$\text{Hydrolysis}(\%) = \left[\frac{(\textit{Amount of reducing sugars released} \times 100)}{\textit{Holocellulose content of pretreated substrate}}\right],$$

While the ethanol yield was calculated as:

$$\text{Ethanol yield}(\%) = \frac{\textit{Ethanol concentration}}{\textit{Holocellulose content}} \times 100$$

All of the experiments were done in triplicate, and the results are presented as the mean ± standard deviation.

Results and discussion

Proximate chemical composition of the SESB

The SESB contained 52.97 % α-cellulose, 7.51 % pentosans, 32.83 % Klason lignin, 4.66 % moisture and 2.02 % ash content (Fig. 1). The carbohydrate content in the SESB is comparable to the holocellulose content of other cellulosic feedstocks used for bioethanol production, such as *Prosopis juliflora* (67 %), *Lantana camara* (61 %) and corn cob (71.6 %) (Gupta et al. 2009, 2011; Kuhad et al. 2010). The considerably high carbohydrate content (holocellulose 60.48 %) of the SESB qualified it as potential feedstock for bioethanol production.

Isolation, screening and identification of the potent hexose-fermenting thermotolerant yeast isolate

Out of total 41 yeast isolates grown from three types of samples, 17 showed the ability to ferment glucose; of these 17 isolates, isolate LBLY 2 exhibited the ability to ferment glucose at a higher temperature (50 °C). Yeast isolate LBLY 2 was identified based on the results of nucleotide BLAST similarity search against existing 18S rRNA gene sequences in the NCBI database, revealing that the isolate was closely related to the genus *Blastobotrys* and species *adeninivorans* (Fig. 2). The organism was therefore termed *Blastobotrys adeninivorans* RCKP 2012, and the sequence was submitted to the NCBI GenBank as accession no. HE657273.

Table 1 Range of each variable used for response surface methodology-based Box–Behnkhen design experiments

FPU filter paper unit; *g* ds gram dry substrate

Factor	Name	Units	−1 Level	0 Level	+1 Level
A	Enzyme dose	(FPU g ds^{-1})	17.5	20.0	22.5
B	Substrate consistency	(% w v^{-1})	7.5	10.0	12.5
C	Surfactant dose	(% v v^{-1})	0.4	0.5	0.6

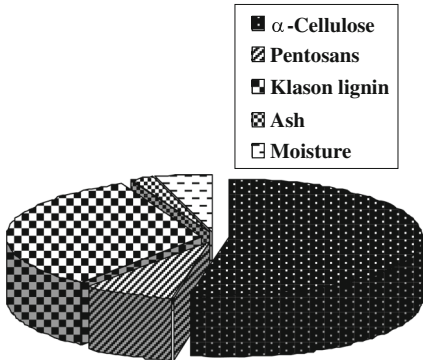

Fig. 1 Composition of steam exploded sugarcane bagasse (SESB)

Cellulase production using *Trichoderma citrinoviridae* RCK2012 during SSF

The soft rot fungus *T. citrinoviridae* RCK2012 when grown under solid state fermentation cultivation condition produced a maximum of 50 U filter paper cellulase (FPase), 75 U carboxymethyl cellulase (CMCase), and 150 U β-glucosidase g ds^{-1} after 6 days of incubation.

Statistical optimization for enzymatic saccharification of SESB using RSM

Response surface methodology was used to study the effect of three independent variables: enzyme dose (A), substrate consistency (B) and surfactant dose (C). From multiple regression analysis, we obtained the quadratic equation that explained the saccharification yield irregardless of the significance of the coefficients:

$$Y = 427.59 + 18.72*A - 18.63*B - 4.39*C - 15.50*A^2 - 17.30*B^2 - 5.45*C^2 - 6.55*A*B + 17.43*A*C - 3.25*B*C$$

where Y is the saccharification yield (mg g^{-1}), and A, B and C represent the coded levels of enzyme dose (U g ds^{-1}), substrate consistency (% w v^{-1}) and surfactant dose (% v v^{-1}), respectively.

The statistical significance of the regression model was checked by the *F* test. The model *F* (269.471) value is a ratio of the mean square due to regression to the mean square due to error and indicates that the influence (significant or not) of each controlled factor on the tested model was significant at a high confidence level. The model was highly significant, as manifested by an *F* value and a probability value ($P_{total} > F$) of <0.0001 (Table 2). The adequate precision of 48.49 signifies that the model has desirable values. The goodness of fit was manifested by the determination coefficient (R^2), and the R^2 value of 0.9971 indicated that the response model could explain 99.71 % of the total variation; in addition, the value of the adjusted R^2 was also sufficiently high (0.9934) to indicate the significance of the model (Table 2). The R^2 values provide a measure of variability in the observed response values that can be explained by the experimental factors and their interactions. The adjusted R^2 corrects the R^2 value for sample size and number of terms in the model. If there is a large number of values in the model and sample size is small, the adjusted R^2 may be significantly smaller than the predicted R^2. The purpose of statistical analysis is to determine the experimental factors which generate signals that are large in comparison to noise. The adequate precision, a measure of signal-to-noise ratio was 48.49 (Table 2). A signal-to-noise ratio greater than 4 is desirable. Based on these results, we concluded that the model was fit and that it could be used to navigate the design space.

The result of the RSM experiment on the effect of the three independent variables [enzyme dose (A), substrate consistency (B) and surfactant dose (C)] together with the mean predicted series of experiments that were designed and conducted are shown in Table 3. The three-dimensional response surface plots were employed to determine the interaction of the parameters and their effect on saccharification yield. The plots were generated by plotting the response using the *z*-axis against two independent variables while keeping the other independent variables at their 0 level. The coordinates of the central point within the highest contour levels in each of the figures correspond to the optimum concentrations of the respective components.

Figure 3 describes the effects of substrate consistency and surfactant dose on lignocellulose saccharification when the enzyme dose is fixed at its '0' level (20 U FPase g ds^{-1}). The figure reveals that the saccharification yield increased with

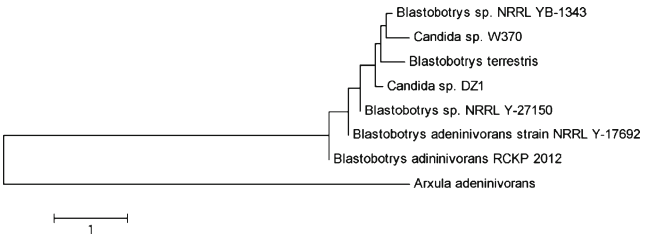

Fig. 2 Dendrogram of *Blastobotrys adeninivorans* RCKP 2012 showing the similarity with other *Blastobotrys* cultures

Table 2 Analysis of variance of the response surface methodology-based model for saccharification yield

Factors	Value
R^2	0.997122
Adjusted R^2	0.993422
Predicted R^2	0.953952
Adequate precision	48.4926
PRESS	451.781
Coefficient of variance.	0.490345
Prob > F	<0.0001
F value	269.471

PRESS predicted residual sums of squares

Table 3 Experimental design and results of the response surface methodology-based model for optimization of saccharification of steam-exploded sugarcane bagasse

Std	Run	Independent variables affecting enzymatic saccharification of SESB			Y: Saccharification yield (mg g^{-1})	
		A: Enzyme dosage (U g ds^{-1})	B: Substrate consistency (%)	C: Surfactant dosage (% v v^{-1})	Actual	Predicted
1	2	17.5	7.5	0.5	389.33	388.16
2	6	22.5	7.5	0.5	436.60	438.69
3	10	17.5	12.5	0.5	366.08	363.99
4	14	22.5	12.5	0.5	387.17	388.34
5	17	17.5	10	0.4	407.30	409.74
6	11	22.5	10	0.4	413.14	412.32
7	13	17.5	10	0.6	365.29	366.11
8	4	22.5	10	0.6	440.85	438.41
9	9	20	7.5	0.4	425.89	424.62
10	15	20	12.5	0.4	394.21	393.86
11	12	20	7.5	0.6	421.98	422.34
12	1	20	12.5	0.6	377.31	378.58
13	16	20	10	0.5	427.59	427.59
14	5	20	10	0.5	427.59	427.59
15	7	20	10	0.5	427.59	427.59
16	8	20	10	0.5	427.59	427.59
17	3	20	10	0.5	427.59	427.59

SESB, Steam-exploded sugarcane bagasse

increased substrate consistency up to 8 % and decreased thereafter. However, the enhancement of surfactant concentration did not significantly increase the saccharification yield (Fig. 3). The interaction between surfactant and enzyme dosage on the saccharification of SESB is shown in Fig. 4. This graph shows a direct proportional relationship between saccharification yield and the amount of enzyme dosage, with saccharification yield increasing regularly with increases in the enzyme dose. Interestingly, surfactant concentration had a significant effect on saccharification yield at higher enzyme levels but not at lower enzyme doses (Fig. 4). The increase in the saccharification yield with the addition of surfactant might be due to the reduced surface tension or reduced thermal deactivation of the enzyme. Similar observations of

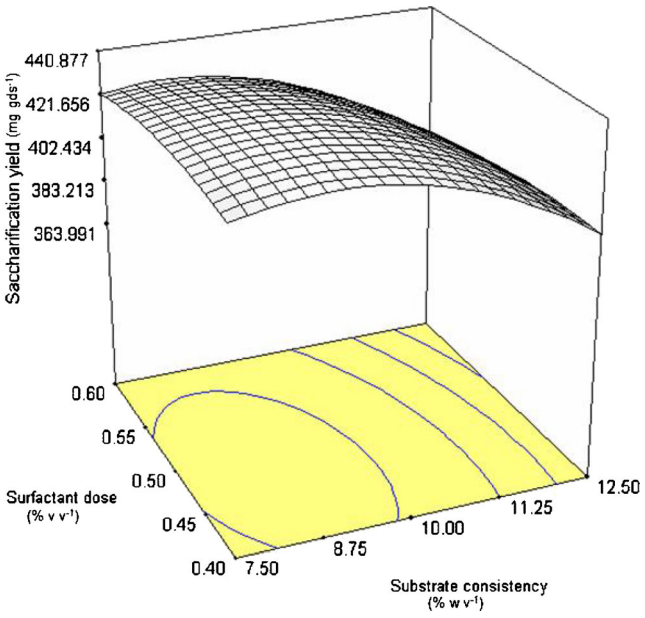

Fig. 3 Response curve of the response surface methodology (RSM)-based experiment showing the effect of substrate consistency (% w v^{-1}) and surfactant dose (% v v^{-1}) on saccharification yield (mg g ds^{-1}) of SESB

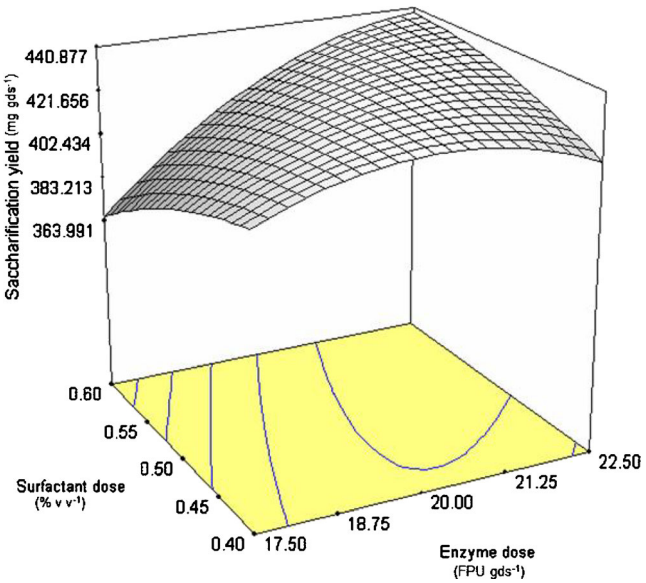

Fig. 4 Response curve of the RSM-based experiment showing the effect of enzyme dose (FPU g ds^{-1}) and surfactant dose (% v v^{-1}) on saccharification yield (mg g ds^{-1}) of SESB. *FPU* Filter paper dose

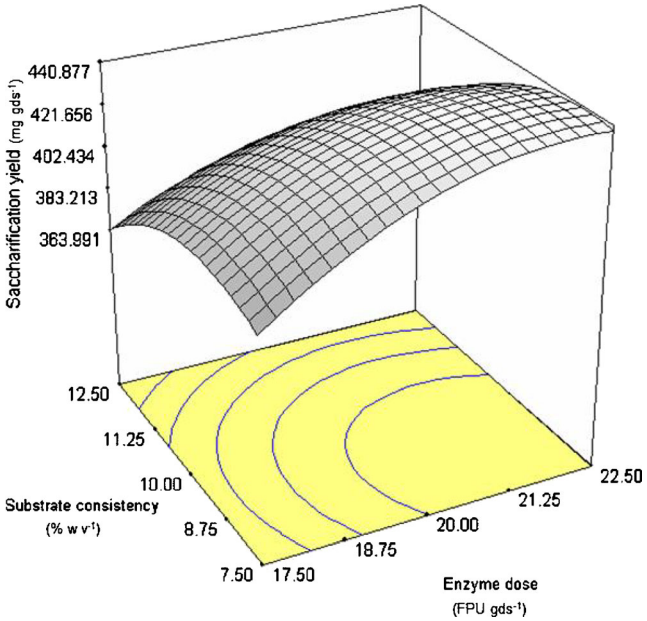

Fig. 5 Response curve of the RSM-based experiment showing the effect of substrate consistency (% w v^{-1}) and enzyme dose (FPU g ds^{-1}) on saccharification yield (mg g ds^{-1}) of SESB

enhancement in saccharification efficiency with the addition of a non-ionic surfactant has also been observed by other researchers (Eriksson et al. 2002; Gupta et al. 2009).

A similar linear increase in saccharification yield was observed with increased enzyme dose (Fig. 5). Moreover, optimum saccharification was observed at 8 % substrate consistency, and deviations from this level resulted in decreased saccharification yield (Fig. 5). The decrease in saccharification yield with increased substrate consistency might be due to an increase in viscosity or rheological problems, such as improper mixing of substrate or improper temperature control (Hodge et al. 2009; Gupta et al. 2012).

The optimal conditions (enzyme dose of 22.5 FPU g ds^{-1}, 8 % substrate consistency, surfactant dose of 0.55 %) for the maximum predicted saccharification yield (448 mg g ds^{-1}) were validated experimentally. The validation results showed that the maximum saccharification of 461 mg g ds^{-1} was in close agreement with the predicted values.

SSF of SESB

During the time course of SSF of SESB, we noted a regular increase in ethanol production up to 90 h, which remained almost constant thereafter (Fig. 6). However, a residual sugar content of approximately 17 g L^{-1} was also observed after 90 h of incubation. The high amount of glucose available during the early stage of fermentation was due to the pre-saccharification of SESB (Fig. 6). The maximum ethanol production (14.05 g L^{-1}) that was obtained after 96 h of fermentation corresponds to a theoretical yield of 46.87 % based on total carbohydrates present in the SESB, which is an ethanol conversion of 14 g ethanol/100 g ds. This yield agrees with those reported earlier by Ballesteros et al. (2006) and Linde et al. (2008). Linde et al. (2008) observed an ethanol conversion of 18 g/100 g steam exploded wheat straw, while Ballesteros et al. (2006) achieved an ethanol conversion of 10 g/100 g acid-pretreated wheat straw. Interestingly, during the late phase of fermentation, a continuous decline in the rate of fermentation was also observed, which subsequently resulted in the accumulation of glucose in the fermentation broth (Fig. 6). This trend was also reported by Tomás-Pejó et al.(2009), who observed a decline in fermentation rate after 48 h and subsequent enhancement in the accumulated residual sugars.

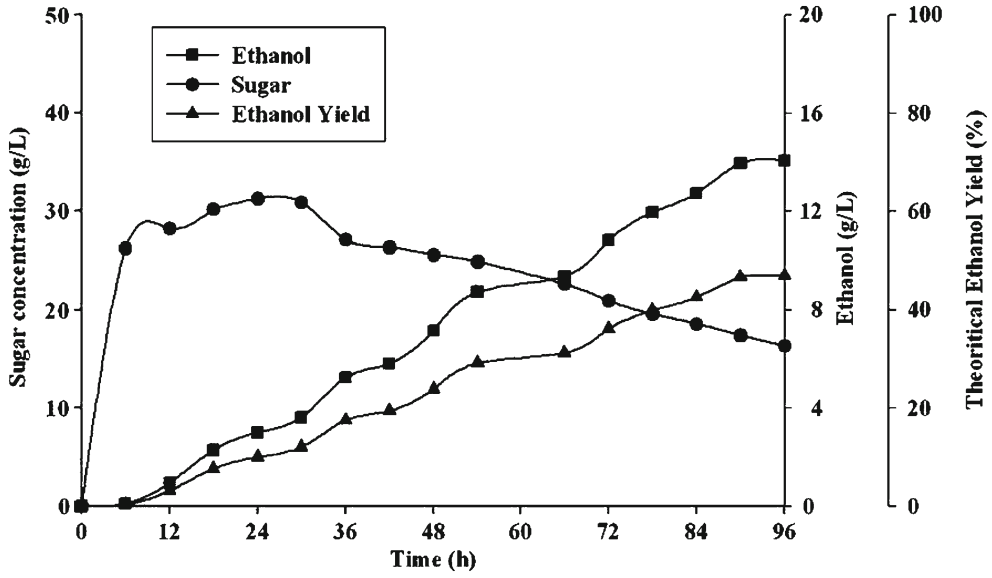

Fig. 6 Time courses of ethanol concentration, glucose concentration and ethanol yield during the simultaneous saccharification and fermentation process

Conclusion

Optimization of enzymatic saccharification using a statistical approach allows maximum utilization of the substrate, which will ultimately improve the economics of the process. We have shown that the thermotolerant yeast *Blastobotrys adeninivorans* RCKP2012 has a good potential for fermenting sugars in the SSF process in a single reactor. This may be a better approach to enhance the process efficiency. However, further detailed studies on the bioprocessing of ethanol fermentation are needed.

Acknowledgments The authors acknowledge the support from the University of Delhi for providing the facilities and finances to carry out the work. The authors also acknowledge the technical support of Mr. Subhojit Chakraborty. Rishi Gupta also acknowledges the Council of Scientific and Industrial Research for providing a Senior Research Fellowship.

References

Ballesteros I, Negro MJ, Liva JM, Cabanas A, Manzanarest P, Ballesteros M (2006) Ethanol production from steam-explosion pretreated wheat straw. Appl Biochem Biotechnol 129–132:496–508

Chen M, Xia L, Xue P (2007) Enzymatic hydrolysis of corncob and ethanol production from cellulosic hydrolysate. Int Biodeterior Biodegrad 59:85–89

Eijsink VGH, Vaaje-Kolstad G, Varum KM, Horn SJ (2008) Towards new enzymes for biofuels: lessons from chitinase research. Trends Biotechnol 26:228–235

Eriksson T, Börjesson J, Tjerneld F (2002) Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzym Microb Technol 31:353–364

Ghose TK (1987) Measurement of cellulase activities. Pure Appl Chem 59:257–268

Gupta R, Sharma KK, Kuhad RC (2009) Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*-NCIM 3498. Bioresour Technol 100:1214–1220

Gupta R, Khasa YP, Kuhad RC (2011) Evaluation of pretreatment methods in improving the enzymatic saccharification of cellulosic materials. Carbohydr Polym 84:1103–1109

Gupta R, Kumar S, Gomes J, Kuhad RC (2012) Kinetic study of batch and fed-batch enzymatic saccharification of pretreated substrate and subsequent fermentation to ethanol. Biotechnol Biofuel 5:16

Hodge DB, Karim MN, Schell DJ, McMillan JD (2009) Model-based fed-batch for high-solids enzymatic cellulose hydrolysis. Appl Biochem Biotechnol 152:88–107

Kadar Z, Szengyel Z, Reczey K (2004) Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. Ind Crop Prod 20:103–110

Khurana S, Kapoor M, Gupta S, Kuhad RC (2007) Statistical optimization of alkaline xylanase production from *Streptomyces violaceoruber* under submerged fermentation using response surface methodology. Indian J Microbiol 47:144–152

Kuhad RC, Singh A (1993) Lignocellulose biotechnology: current and future prospects. Crit Rev Biotechnol 13:151–172

Kuhad RC, Gupta R, Khasa YP, Singh A (2010) Bioethanol production from *Lantana camara* (red sage): pretreatment, saccharification and fermentation. Bioresour Technol 101:8348–8354

Kuhad RC, Gupta R, Khasa YP (2011) Bioethanol production from lignocellulosics: an overview. In: Lal B, Sarma PM (eds) Wealth from waste. TERI Press, New Delhi, pp 53–106

Linde M, Jakobsson EL, Galbe M, Zacchi G (2008) Steam pretreatment of dilute H_2SO_4-impregnated wheat straw and SSF with low yeast and enzyme loadings for bioethanol production. Biomass Bioenergy 32:326–332

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428

Soderstrom J, Galbe M, Zacchi G (2005) Separate versus simultaneous saccharification and fermentation of two-step steam pretreated softwood for ethanol production. J Wood Chem Technol 25:187–202

Technical Association of Pulp and Paper Industry (TAPPI) (1992) TAPPI Coating Conference. TAPPI Press, Atlanta

Tomás-Pejó E, Oliva JM, González A, Ballesteros I, Ballesteros M (2009) Bioethanol production from wheat straw by the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875 in a simultaneous saccharification and fermentation fed-batch process. Fuel 88:2142–2147

Weng JK, Li X, Bonawitz ND, Chapple C (2008) Emerging strategies of lignin engineering and degradation for cellulosics biofuel production. Curr Opin Biotechnol 19:166–172