Ethanol production from sweet sorghum bagasse through process optimization using response surface methodology

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Abstract In this study, comparative evaluation of acid- and alkali pretreatment of sweet sorghum bagasse (SSB) was carried out for sugar production after enzymatic hydrolysis. Results indicated that enzymatic hydrolysis of alkali-pretreated SSB resulted in higher production of glucose, xylose and arabinose, compared to the other alkali concentrations and also acid-pretreated biomass. Response Surface Methodology (RSM) was, therefore, used to optimize parameters, such as alkali concentration, temperature and time of pretreatment prior to enzymatic hydrolysis to maximize the production of sugars. The independent variables used during RSM included alkali concentration (1.5–4%), pretreatment temperature (125–140 °C) and pretreatment time (10–30 min) were investigated. Process optimization resulted in glucose and xylose concentration of 57.24 and 10.14 g/L, respectively. Subsequently, second stage optimization was conducted using RSM for optimizing parameters for enzymatic hydrolysis, which included substrate concentration (10–15%), incubation time (24–60 h), incubation temperature (40–60 °C) and Celluclast concentration (10–20 IU/g-dwt). Substrate concentration 15%, (w/v) temperature of 60 °C, Celluclast concentration of 20 IU/g-dwt and incubation time of 58 h

led to a glucose concentration of 68.58 g/l. Finally, simultaneous saccharification fermentation (SSF) as well as separated hydrolysis and fermentation (SHF) was evaluated using *Pichia kudriavzevii* HOP-1 for production of ethanol. Significant difference in ethanol concentration was not found using either SSF or SHF; however, ethanol productivity was higher in case of SSF, compared to SHF. This study has established a platform for conducting scale-up studies using the optimized process parameters.

Keywords *Pichia kudriavzevii* HOP-1 · Response surface methodology (RSM) · Simultaneous saccharification and fermentation (SSF) · Separate hydrolysis and fermentation · Sweet sorghum bagasse (SSB)

Introduction

Sweet sorghum bagasse (SSB) is a cheap, widely available resource. It is used as an alternative material for the ethanol production. It contains soluble and insoluble carbohydrates (Mamma et al. 1995). Bagasse is an important residue produced from the processing of sweet sorghum and is a good source of biomass which can be used for saccharification followed by fermentation to produce bioethanol. Sweet sorghum bagasse is a lignocellulosic biomass, mainly composed of cellulose, hemicelluloses and lignin. Cellulose is a linear polymer that is composed of glucose subunits linked by β -1, 4 glycosidic bonds. Cellulose is usually present as a crystalline form, while a small amount of unorganized cellulose chains forms amorphous cellulose. Generally, acids can break down the heterocyclic bonds between sugar monomers in polymeric chain, which are formed by hemicelluloses and cellulose (Sánchez 2009).

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Response surface methodology (RSM) is a statistical technique for the modeling and optimization of multiple variables, which determines optimum process conditions by combining experimental designs with interpolation by first- or second-order polynomial equations in a sequential testing procedure. This technique has been successfully applied in the optimization of parameters for production of ethanol from lignocellulosic biomass (Ferreira et al. 2009; de Freitas Gomes et al. 2012). Response surface methodology is a collection of mathematical and statistical techniques that are useful for modeling and analyzing problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Liu et al. 2003). In this work, pretreatment and enzymatic hydrolysis of SSB were studied simultaneously by employing preliminary tests and experimental designs using central composite design (CCD) of RSM.

The main objective of this work was to determine the optimum process parameters for maximizing production of glucose from SSB by alkali pretreatment and enzyme hydrolysis separately and simultaneously. Simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) for ethanol production were carried out using the sugars obtained through process optimization employed during pretreatment and enzymatic hydrolysis subsequently. Initially, acid and alkali pretreatment was evaluated for sugar production from SSB followed by enzymatic hydrolysis. As alkali pretreatment showed better results, alkali concentration, pretreatment temperature and time were initially optimized using CCD. Response Surface Methodology (RSM) was again employed to optimize four independent variables for enzymatic hydrolysis of alkali-treated SSB. Hydrolysate obtained after enzymatic hydrolysis was subjected to both SSF and SHF for ethanol production using *Pichia kudriavzevii* HOP-1 (Sandhu et al. 2011).

Materials and methods

Pretreatment of SSB

Dried and ground SSB was subjected to acid and alkali treatments, prior to enzymatic saccharification. Lignocellulosic biomass was procured from the fields of ICAR–Indian Institute of Millet Research (IIMR), Hyderabad, India. Bagasse was oven dried to lower the moisture content to about 9–10%, and the dried bagasse was cut with chaff cutters, yielding small pieces of about 1.5 inches each. These pieces were subsequently ground to a fine powder using an electrically operated mill (Perten Instruments AB, Sweden), and the powder was passed through an electrically operated sieve shaker. Material collected in

American Standard Test Sieve Series (ASTM) sieve no. 30 (corresponding to particle size of 600 µm) was used for experiments. The ground material was passed repeatedly through the sieve shaker to ensure uniformity of the sample.

Acid pretreatment

Effect of acid concentration (0.5, 1.0, 1.5 and 2%, w/v) was studied for maximum recovery of glucose from SSB by autoclaving the biomass at 121 °C for 15 min. Hydrolysate and the biomass were separated through filtration. Biomass was repeatedly washed with distilled water to increase the pH to 5.0. Enzymatic hydrolysis of the treated biomass was carried out as per the procedure described elsewhere in this paper.

Alkali pretreatment

Sweet sorghum bagasse was subjected to pretreatment using different concentrations of sodium hydroxide solution (1, 2, 3, 4 and 5% w/v) and autoclaving them at 121 °C for 15 min. The solids recovered by filtration were repeatedly washed with distilled water until the pH of 5.0 was achieved and the biomass was oven dried at 50 °C. Biomass obtained after alkali pretreatment using different concentrations of alkali was subjected to enzymatic hydrolysis and the concentration of glucose, xylose, and arabinose was analyzed.

Enzymatic hydrolysis and analytical methods

Pretreated SSB was suspended in 0.1 M citrate buffer (pH 5.0) in the capped polycarbonate flasks. Addition of buffer was done in such a way so as to maintain the substrate concentration at 10% (w/v) after enzyme addition. The flasks were autoclave-sterilized for 15 min, cooled and the slurry supplemented with the commercial enzymes. Cel-luclast 1.5 L, Novozym 188 and Pectinase were employed at pectinase loading of 15 International units (IU)/gram dry weight (g-dw), Cellulase loading of 15 filter paper units (FPU)/g-dw and β -glucosidase of 30 IU/g-dw. All the three enzymes used for the hydrolysis studies were purchased from Sigma-Aldrich, MS, USA. Hydrolysis was performed at 50 °C for 48 h at 120 rpm in an incubator shaker. Samples were drawn at regular interval of 12 h. Samples were centrifuged and the supernatants were analyzed for glucose, xylose, and arabinose with HPLC (Ultimate 3000, Dionex Corporation, Sunnyvale, CA, USA) using a Shodex SP-0810 column (300 × 7.8 mm) fitted with an SP-G guard column (Waters Inc., USA). Samples were diluted,

centrifuged, and filtered through Phenomenex 0.45 micron RC membranes before transferring to HPLC vials. Degassed deionised water was used as a mobile phase at a flow rate of 1.0 mL/min. The column oven and refractive index detector were maintained at 80 and 50 °C, respectively. The peaks were detected by the refractive index detector and quantified on the basis of area and retention time of the standard respective sugars. Ethanol was determined with the YSI 2786 ethanol membrane kits using YSI 2700 Select biochemical analyzer (YSI Inc., Buffalo, NY, USA) (Oberoi et al. 2012).

Optimization of alkali pretreatment condition using RSM

Optimization of alkali pretreatment conditions (alkali concentration, pretreatment temperature, and pretreatment time) was performed by RSM using central composite design. Central composite design (CCD) was employed for three factors, consisting of 20 experimental runs with six axial points ($\alpha \pm 2$) and three replications at the central point. The design space consisted of three independent variables: alkali concentration (%), pretreatment temperature and pretreatment time. Each variable was studied at two different levels ($-1, +1$), two axial points ($\alpha = \pm 2$) and a center point (0) which is the midpoint of each factor range. Response variables were R_1 [Glucose (g/L)] and R_2 [Xylose (g/L)]. Actual values and corresponding values of three independent variables are given in Table 1. The central composite design matrix is shown in Table 2.

The relationship of the independent variables and the response was calculated by a second-order polynomial. The behavior of the system was explained by the following quadratic equation (Eq 1):

$$Y = \beta_0 + \sum_{j=1}^k \beta_j X_j + \sum_{j=1}^k \beta_{jj} X_j^2 + \sum_{i < j} \beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted response, β_0 is the intercept term, β_i is the linear effect, β_{ii} is the square coefficient, and β_{ij} is the interaction coefficient; and k is the number of factors (Liu et al. 2003). The regression equation was optimized

for maximum value to obtain the optimum conditions using freely available Design-Expert Version 7.1 (Stat-Ease Inc., Minneapolis, USA). Analysis of variance (ANOVA) was used to estimate the statistical parameters. The statistical significance of the model equation and the model terms was evaluated via the Fisher's test. The predicted values were calculated from the regression model derived from the coefficients of the model and variations were explained by the determination coefficient (R^2 value).

5 g of SSB was suspended in different alkali concentration (w/v) solution in 250 mL Erlenmeyer flasks. Flasks were placed on orbital rotary shaker at 30 °C and 110 rpm for 30 min and autoclave-sterilized at 15 psi, using different temperature and time combinations (Table 2). Hydrolysate was then separated from the pretreated biomass by squeezing through muslin cloth. Biomass was dried, ground and subjected to enzymatic hydrolysis as mentioned elsewhere in this paper.

Validation experiment

Validation experiment for alkali pretreatment was carried out using 10 g of SSB with optimized alkali concentration, pretreatment temperature and time, mentioned elsewhere in this paper. As mentioned previously, the biomass was separated from the liquid and treated with distilled water to bring down the pH to 5.0. Enzymatic hydrolysis of the pretreated biomass was done using the procedure mentioned elsewhere in this paper. Hydrolysate was analyzed for different sugars using HPLC. Validation experiments were conducted in triplicate.

Optimization of enzymatic hydrolysis parameters

Central composite design was used to optimize four different independent variables, viz., substrate concentration, enzyme concentration, temperature and time during hydrolysis (Table 3). Selection of the variables for enzyme hydrolysis was done based on the previously published literature (Oberoi et al. 2012) and the importance of such parameters and their interactions in achieving the desired goal.

Table 1 Levels of variables used in CCD

Independent variable	Symbol	Coded level				
		$-2 (-\alpha)$	-1	0	1	$2 (\alpha)$
Alkali concentration (%)	A	0.65	1.5	2.75	4.0	4.85
Pretreatment temperature (°C)	B	119.89	125	132.5	140	145.11
Pretreatment time (min)	C	3.18	10	20	30	36.82

Table 2 Central composite design matrix of variables

Run order	Coded values			Actual values		
	A	B	C	A	B	C
1	2	0	0	4.85	132.50	20.0
2	1	−1	1	4.00	125.00	10.0
3	0	−2	0	2.75	119.89	20.0
4	1	1	−1	4.00	140.00	10.0
5	0	−2	0	2.75	119.89	20.0
6	1	1	1	4.00	140.00	30.0
7	−1	−1	−1	1.50	125.00	10.0
8	0	0	0	2.75	132.50	20.0
9	−1	−1	1	1.50	125.00	30.0
10	0	−1	0	2.75	125.00	20.0
11	0	0	0	2.75	132.50	20.0
12	0	2	0	2.75	145.11	20.0
13	0	0	2	2.75	132.50	36.82
14	0	0	0	2.75	132.50	20.00
15	1	−1	1	4.00	125.00	30.00
16	0	0	0	2.75	132.50	20.00
17	−1	1	1	1.50	140.00	30.00
18	−2	0	0	0.65	132.50	20.00
19	−1	1	−1	1.50	140.00	10.00
20	0	0	−2	2.75	132.50	3.18

Table 3 Levels of variables used in CCD

Variable	− α	−1	0	+1	+ α
Substrate concentration (%)	7.50	10.00	12.50	15.00	17.50
Incubation time (h)	6	24	42	60	78
Celluclast IU/mL	5	10	15	20	25
Temperature (°C)	30	40	50	60	70

Central composite design consisting of 27 experimental runs, with eight axial points ($\alpha \pm 2$) and three replications at the central point (0), was employed to optimize the concentration of significant factors (Table 4). The relationship between independent variables and the response (glucose and xylose concentration) was calculated by the second-order polynomial and the behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2$$

where Y is the predicted response; β_0 is the intercept term; β_1 , β_2 , β_3 , and β_4 are the linear effects; β_{12} , β_{13} , β_{14} , β_{23} , β_{24} , and β_{34} are the interaction terms; β_{11} , β_{22} , β_{33} , and β_{44} are the square terms; and X_1 , X_2 , X_3 , and X_4 are the independent variables. Results of the RSM experiment were

analyzed using Design-Expert 7.1 evaluation software. Analysis of variance (ANOVA) was used to estimate the statistical difference among the parameters.

Validation of enzyme hydrolysis of optimized alkali treated SSB process was carried out with 10 g of SSB with optimized substrate concentration, incubation time, temperature and enzyme concentration. The ratio between filter paper cellulase and β -glucosidase was kept as 1:2 in all the runs (Table 4) as well as during the validation studies. Samples were analyzed for glucose, xylose and arabinose concentrations with HPLC as described elsewhere in this paper.

Fermentation

Fermentation was carried out to convert the resulting sugars into ethanol. Pretreated, enzyme-hydrolyzed

Table 4 Central composite design matrix of variables

Run	Coded values				Actual values			
	A	B	C	D	A	B	C	D
1	−1	−1	1	1	10	24	20	60
2	1	−1	−1	−1	15	24	10	40
3	−1	1	1	−1	10	60	20	40
4	0	0	0	0	12.5	42	15	50
5	−1	1	1	1	10	60	20	60
6	−1	−1	−1	1	10	24	10	60
7	0	0	0	0	12.5	42	15	50
8	1	−1	1	1	15	24	20	60
9	0	0	0	0	12.5	42	15	50
10	0	0	0	−2	12.5	42	15	30
11	0	2	0	0	12.5	78	15	50
12	−1	−1	−1	−1	10	24	10	40
13	1	1	1	1	15	60	20	60
14	0	0	−2	0	12.5	42	5	50
15	−1	−1	1	−1	10	24	20	40
16	1	1	−1	−1	15	60	10	40
17	0	0	0	2	12.5	42	15	70
18	−1	1	−1	1	10	60	10	60
19	1	1	1	−1	15	60	20	40
20	1	−1	1	−1	15	24	20	40
21	2	0	0	0	17.5	42	15	50
22	−1	1	−1	−1	10	60	10	40
23	1	−1	−1	1	15	24	10	60
24	1	1	−1	1	15	60	10	60
25	0	0	2	0	12.5	42	25	50
26	0	−2	0	0	12.5	6	15	50
27	−2	0	0	0	7.5	42	15	50

material was transferred to the centrifuge tubes and the hydrolysate was centrifuged at 8000 rpm and 4 °C for 5 min so as to remove the unhydrolyzed material. Supernatant was transferred to capped polycarbonate flasks, supplemented with 0.2% (w/v) each of yeast extract, MgSO₄ and peptone and inoculated with the thermotolerant *Pichia kudriavzevii* HOP-1 (Oberoi et al. 2012). Details about the isolation, identification and characterization of the yeast strain have been described in the previous papers published by one of the co-authors in this paper (Sandhu et al. 2011; Dhaliwal et al. 2011). *Pichia kudriavzevii* HOP1 was used in this experiment because of its multi-stress tolerant characteristics (Oberoi et al. 2012), as these characteristics are desired for industrial ethanol production. The ITS sequence of *P. kudriavzevii* was submitted to GenBank, NCBI with the accession no: HQ 122942 and the isolate is deposited with the ICAR-National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau

Nath Bhanjan, India with accession number NAIMCC-F-02470.

Separate hydrolysis and fermentation (SHF)

Separate hydrolysis and fermentation were carried out using the hydrolysate obtained from the validated enzymatic hydrolysis studies. Hydrolysate was collected in the capped polycarbonate flasks and supplemented with the nutrient solution containing 0.2% each of yeast extract, MgSO₄ and peptone. Flasks were autoclave-sterilized, cooled and inoculated with the yeast culture *P. kudriavzevii* HOP-1 at 10% inoculum level with the cell concentration in the inoculum being 1×10^9 cells/mL. Fermentation was carried out at 35 °C and 100 rpm for 72 h. Samples were collected after every 12 h and analyzed by HPLC for ethanol and sugar concentrations.

Simultaneous saccharification and fermentation (SSF)

Alkali-treated SSB was suspended in 0.05 M citrate buffer (pH 5.0) in the capped polycarbonate flasks. Addition of buffer was done in such a way so as to maintain the optimized substrate concentration at 10% (w/v) after enzyme addition. The flasks were autoclave-sterilized for 15 min, cooled and the slurry supplemented with the commercial enzymes, as mentioned previously. Enzymatic hydrolysis was performed at 50 °C and 120 rpm for 12 h in an incubator shaker to achieve partial liquefaction before addition of yeast inoculum. Samples were drawn after 12 h; medium was supplemented with a sterilized nutrient solution containing 0.2% each of yeast extract, MgSO₄ and peptone and inoculated with the yeast culture, using the protocol mentioned under SHF. Fermentation was carried out at 35 °C and 100 rpm for 72 h. Samples were collected after every 12 h and analyzed by HPLC for ethanol and sugar concentrations.

Result and discussion

Pretreatment of sweet sorghum bagasse using chemical methods

Acid pretreatment

A gradual increase in glucose concentration was seen in the enzymatically pretreated biomass with an increase in H₂SO₄ concentration. Glucose concentration of 30.46 g/L was attained using 2% H₂SO₄ (Table 5). As is evident from the results of Table 5, glucose was accompanied with the production of xylose and arabinose at concentrations of 3.17, and 0.97 g/L, respectively (Table 5). In the acid hydrolysate, maximum glucose, xylose, and arabinose were released at a concentration of 16.46, 11.42, and 2.38 g/L, respectively, at 2% acid concentration.

Though the acid hydrolysate comprises a variety of sugars, the mixture has a significant concentration of pentose sugars which are not fermented by most of the

yeasts as is evident from the results of Table 6; therefore, the acid hydrolysate was not further taken up for any experimental work in this study. Acid hydrolysis also has several disadvantages over the other pretreatment processes, such as microbial hydrolysis, alkali pretreatment due to the formation of toxic compounds, such as furfural, hydroxymethylfurfural, acetic acid, formic acid, and levulinic acid. These toxic compounds can inhibit the yeast fermentation in the next step of ethanol production. Removal of these compounds increases the additional costs for the ethanol production (Palmqvist and Hahn-Hägerdal 2000); Table 6.

Alkali pretreatment

Concentration of glucose, xylose, and arabinose varied with different alkali concentrations as is evident from the results of Table 7. A constant increase in the concentration of sugars was seen in the enzymatically hydrolyzed biomass with an increase in alkali concentration until 4% which levelled off or even showed a slight decline with a further increase in alkali concentration. However, biomass loss was substantially high when higher alkali concentrations were used during pretreatment (data not shown). Similar results have been previously reported about loss of biomass and hemicellulose with an increase in alkali concentration (Oberoi et al. 2012). 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% (Bjerre et al. 1996). Pretreatment of substrate with NaOH results in swelling of the particles causing easy removal of the lignin and cellulose depolymerization (Damisa et al. 2008). It has been previously reported that the alkali-treated residues with low concentrations of NaOH showed higher accessibility to enzymatic hydrolysis. Gharpuray et al. (Gharpuray et al. 1983) 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.

Though the higher glucose concentration was seen in the enzymatically hydrolyzed samples obtained using relatively higher alkali concentrations, a significant loss of

Table 5 Effect of acid concentration on sugar profile after enzymatic hydrolysis

S. no.	Experiment (%)	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)
1	0.5	8.94	0.67	0.22
2	1	17.71	1.31	0.65
3	1.5	29.15	3.17	0.97
4	2	30.46	3.09	0.92

Table 6 Acid hydrolysate composition

S. no.	H ₂ SO ₄ conc. (%)	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Fructose (g/L)
1	0.5	6.91	1.85	0.33	1.26
2	1	11.71	5.34	1.65	3.66
3	1.5	14.15	9.97	2.02	6.54
4	2	16.46	11.42	2.38	7.74

Table 7 Effect of alkali concentration on sugar profile after enzymatic hydrolysis

S. no.	Alkali (%)	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)
1	1	16.91	1.57	0.22
2	2	35.72	8.14	1.51
3	3	48.15	12.75	2.09
4	4	55.46	14.91	2.47
5	5	54.08	14.22	2.22

biomass becomes a matter of concern; therefore, it became imperative to conduct the optimization studies for alkali pretreatment using CCD.

Optimization of alkali pretreatment condition by response surface methodology

In the present study, the data for the alkali treatment optimization experiment were analyzed using Design expert 7.1 evaluation software. There were three factors, namely alkali concentration, pretreatment temperature and time and three levels of each parameter were varied as shown in Table 8.

The software analyzed the data and suggested both linear and quadratic models as significant for optimal condition of alkali pretreatment. Experimental results were analyzed by the regression analysis consisting of the effect of variables and their interaction which gave the following regression equation. The results can be predicted using specific combination of three variables by substituting the corresponding values of each variable in Eq. (2).

$$\begin{aligned} \text{Glucose} = & +48.47 + 4.30 \times A + 0.22 \times B - 0.60 \times C \\ & - 4.37 \times A \times B + 5.24 \times A \times C - 3.85 \times B \times C \\ & - 1.62 \times A^2 + 0.76 \times B^2 + 1.97 \times C^2 \end{aligned} \quad (2)$$

where A , B , and C stand for alkali concentration, pretreatment temperature and time, respectively.

The probability value (p value) is a tool for evaluating the significance and contribution of each parameter

and the statistical polynomial model equation. The small p -value is an indication of the high significance of the corresponding coefficient (Tébeka et al. 2009). However, the regression coefficients and p value obtained through ANOVA indicated better fit by the quadratic model.

The overall quadratic model showed an R^2 value of 0.9893 and adjusted R^2 value of 0.9797. Lack of fit was non-significant indicating the fitness of the model. Statistical analysis indicated that first-order effects of alkali concentration and pretreatment temperature were statistically significant with a good confidence level. The second-order interaction between alkali concentration and pretreatment temperature, alkali concentration and pretreatment time, pretreatment temperature and time and square of alkali concentration, pretreatment temperature and pretreatment time was significant at a 95% confidence level. The significance of the data is judged by its p value being closer to 0. The p value should be less than 0.1, 0.05 and 0.01 for a 90, 95, and 99% confidence level, respectively, for the factors to be termed as significant (Joglekar and May 1987).

The final response function to predict xylose concentration after eliminating the non-significant terms at the 10% significance level was shown in Eq. (3).

$$\begin{aligned} \text{Xylose} = & +10.30 - 1.91 \times A + 0.039 \times B - 0.084 \times C \\ & - 0.92 \times A \times B + 0.68 \times A \times C - 0.27 \times B \times C \\ & - 0.41 \times A^2 + 0.16 \times B^2 + 0.19 \times C^2 \end{aligned} \quad (3)$$

where A , B , and C stand for alkali concentration, pretreatment temperature and pretreatment time, respectively. From the literature for the good fit of a model R^2 should be at least 80% (Tébeka et al. 2009). The overall quadratic model was significant with an R^2 value of 0.9642 and adjusted R^2 value of 0.9320. Lack of fit was non-significant indicating the fitness of the model. Statistical analysis indicated that the first-order effects of alkali concentration were statistically significant with a good confidence level. The second-order interaction between alkali conc. and pretreatment temperature, and alkali concentration and pretreatment time and square of alkali conc. was significant at a 95% confidence level.

The response surfaces shown in Fig. 1a were based on the final model in which one variable was kept constant at its optimum value and the other two were varied within their experimental range. It is clear from the model graph responses that the interaction of alkali concentration with pretreatment temperature, alkali concentration with pretreatment time, pretreatment time with temperature had a significant effect on glucose production.

Increasing the concentration of both the variables resulted in an increase in glucose concentration. Numerical

Table 8 Experimental design and results of the Central Composite design

Run	Alkali concentration (%)	Pretreatment temperature (°C)	Pretreatment time (min)	Glucose (g/L)	Xylose (g/L)
1	4.85	132.50	20.00	50.25	5.91
2	4.00	125.00	10.00	49.8	8.04
3	2.75	132.5	20.0	48.56	10.5
4	4.00	140.0	10.0	49.94	7.77
5	2.75	119.89	20.00	49.75	10.98
6	4.00	140.0	30.0	50.82	7.65
7	1.50	125.00	10.00	43.39	12.05
8	2.75	132.50	20.00	48.12	10.58
9	1.50	125.00	30.00	38.7	10.28
10	2.75	132.5	20.00	48.34	10.37
11	2.75	132.50	20.00	47.95	9.79
12	2.75	145.11	20.00	50.12	10.21
13	2.75	132.5	36.82	52.85	10.79
14	2.75	132.50	20.00	50.13	10.62
15	4.00	125.00	30.00	57.24	10.14
16	2.75	132.50	20.0	47.98	10.02
17	1.50	140.00	30.00	40.92	12.61
18	0.65	132.50	20.00	36.11	12.1
19	1.50	140.00	10.00	59.83	14.31
20	2.75	132.50	3.18	54.20	10.59

optimization of the parameters was done to maximize glucose concentration in least possible time. The independent variables during numerical optimization were fixed in the range selected. The range for glucose concentration was kept between 36.11 and 67.24 g/L (lowest and highest responses observed during RSM trial). The numerical optimization package suggested 20 different combinations with desirability of 1.0. Thus, after evaluating the model graphs and the combinations suggested by the numerical optimization package, validation experiment was done at alkali concentration 4%, pretreatment temperature of 125 °C and pretreatment time of 30 min, respectively.

The response surfaces shown in Fig. 1 were based on the final model in which one variable was kept constant at its optimum value and the other two were varied within their experimental range. It is clear from the model graph responses that the interaction of alkali concentration with temperature, alkali concentration with pretreatment time, and pretreatment time with temperature had a significant effect on glucose concentration. However, the optimum glucose and xylose concentrations of 67.24 and 10.14 g/L were achieved with the use of alkali concentration,

temperature, and pretreatment time of 4.0%, 140 °C, and 30 min, respectively (Fig. 1a).

Optimization of enzymatic hydrolysis using response surface methodology

Response surface methodology employing CCD was used to optimize the parameters affecting hydrolysis so as to achieve the maximum glucose concentration. We were interested in increasing glucose concentration as the yeast strain used in this study is not capable of fermenting pentose sugars, such as xylose or arabinose. Maximum glucose released during the hydrolysis was 68.41 g/mL (run 19, Table 9). Multiple regression analysis was applied to the data based on the Eqs. 4 and 5.

It is now clear from the results of Table 9 that increasing temperature and incubation time resulted in a higher glucose yield. However, increasing hydrolysis time would lead to a fall in the sugar productivity. As our major goal was to maximize yield in minimum time, it would not be advisable to carry on with the experiment beyond 60 h. Equation (4) describes the correlation between the significant variables and the glucose releasing rate for pretreated SSB in terms of decoded values when using the reduced model.

$$\begin{aligned}
 \text{Response} = & -79.06569 - 8.69683 \times \text{sub conc} + 0.60336 \\
 & \times \text{Incubation time} - 2.42442 \times \text{Celluclast} \\
 & + 7.26112 \times \text{Temperature} - 0.015514 \\
 & \times \text{sub conc} \times \text{Incubation time} + 0.066150 \\
 & \times \text{sub conc} \times \text{Celluclast} + 0.058475 \\
 & \times \text{sub conc} \times \text{Temperature} + 8.12500\text{E}-004 \\
 & \times \text{Incubation time} \times \text{Celluclast} - 1.28819\text{E} \\
 & -003 \times \text{Incubation time} \times \text{Temperature} \\
 & + 0.025913 \times \text{Celluclast} \times \text{Temperature} \\
 & + 0.22925 \times \text{sub conc}^2 - 1.29533\text{E} \\
 & -003 \text{Incubation time}^2 + 0.022462 \\
 & \times \text{Celluclast}^2 - 0.084997 \times \text{Temperature}^2.
 \end{aligned}
 \tag{4}$$

Analysis of variance indicates that the models were statistically valid with p values lower than 0.0001 for model terms and its significance (p values lower than 0.05) indicating that the model terms were significant. This means that the linear effects of substrate concentration, incubation time, Celluclast concentration and temperature were considerably higher than other effects ($p < 0.0001$) demonstrating that these are the most significant factors affecting enzymatic hydrolysis of pretreated SSB. The absence of interactions between factors ($p > 0.05$) may lead to the assumption that factors have an additive effect on the response.

The proportion of total variation attributed to each fit can be evaluated by the value of *R*-squared (a value of *R*-square >0.75 indicate the fitness of the model) (Gould 1985). For glucose, the regression equation obtained after ANOVA indicating *R*-squared value of 0.9066 was in good agreement with the adjusted *R*-squared of 0.8652. This ensured a satisfactory adjustment of the theoretical values to the experimental data by this model. The lack of fit was significant however; *R*-squared value is high (0.9066 for SSB) indicating that the models are well adapted to the responses. Therefore, the model is suitable to predict enzymatic hydrolysis of pretreated SSB. Equation (5) describes the correlation between the significant variables and the xylose releasing rate for pretreated SSB in terms of decoded values when using the reduced model.

$$\begin{aligned} \text{Response} = & -15.98380 - 1.38500 \times \text{sub conc} + 0.18650 \\ & \times \text{Incubation time} - 0.13250 \times \text{Celluclast} \\ & + 1.11375 \times \text{Temperature} - 4.02778\text{E}-003 \\ & \times \text{sub conc} \times \text{Incubation time} - 3.50000\text{E}-003 \\ & \times \text{sub conc} \times \text{Celluclast} + 8.25000\text{E}-003 \\ & \times \text{sub conc} \times \text{Temperature} + 6.94444\text{E}-005 \\ & \times \text{Incubation time} \times \text{Celluclast} - 2.04861\text{E}-003 \\ & \times \text{Incubation time} \times \text{Temperature} - 1.12500\text{E}-003 \\ & \times \text{Celluclast} \times \text{Temperature} + 0.043833 \\ & \times \text{sub conc}^2 - 1.96116\text{E}-004 \\ & \times \text{Incubation time}^2 + 8.95833\text{E}-003 \\ & \times \text{Celluclast}^2 - 0.011135 \times \text{Temperature}^2 \end{aligned} \quad (5)$$

The overall quadratic model was significant with R^2 value of 0.9799 and adjusted R^2 value of 0.9565. Lack of fit

Fig. 1 A Response surface plots of glucose production by alkali pretreatment. **a** Interaction of temperature with alkali conc. **b** Interaction of time with alkali conc. **c** Interaction of temperature with time.

B Response surface plots of xylose production by alkali pretreatment. **a** Interaction of temperature with alkali conc. **b** Interaction of time with alkali conc. **c** Interaction of time with temperature

Fig. 1 continued

was non-significant, indicating no reason to doubt the fitness of the model. The relationship between the response and variables is visualized by the response surface or contour plot to analyze the influence of the parameters. The quadratic polynomial equations (Eqs. 4 and 5) can be described by the response surface plots for released glucose and xylose by enzymatic hydrolysis of pretreated SSB (Fig. 2) as a function of two factors at a time, maintaining all other factors fixed at level zero.

Analysis of variance (ANOVA) was performed for the evaluation of the effects of the variables and their possible interactions. The coefficients of a full model were evaluated by regression analysis and tested for their significance. The non-significant coefficients were excluded from the model to conduct a thorough validation study. Response

surface curves depicting the effect of interaction between different variables on glucose and xylose production are shown in Fig. 2a, b, respectively. These plots show the type of interaction between the tested variables and hence allow us to obtain the optimum conditions. The response surfaces shown in Fig. 2a were based on the final model in which two variables were kept constant at their optimum values and the other two were varied within their experimental range. It is clear from the model graph responses that the interaction of incubation time with substrate concentration, Celluclast with substrate concentration, Celluclast with incubation time and incubation time with temperature had a significant effect on glucose production. Increasing the concentration of both the variables resulted in an increase in glucose concentration. Numerical optimization of the

Table 9 Effect of interactions between independent variable on production of glucose and xylose

Run	A: subconc (%)	B: incubation time (h)	C: celluclast (IU/mL)	Temperature (°C)	Response1 Glucose (g/L)	Response2 Xylose (g/L)
1	10.00	24.00	20.00	60.00	45.00	5.8
2	15.00	24.00	10.00	40.00	46.5	4.5
3	10.00	60.00	20.00	40.00	50.81	7.00
4	12.50	42.00	15.00	50.00	54.5	6.2
5	10.00	60.00	20.00	60.00	51.4	6.1
6	10.00	24.00	10.00	60.00	40.8	5.3
7	12.50	42.00	15.00	50.00	53.7	6.3
8	15.00	24.00	20.00	60.00	60.00	5.7
9	12.50	42.00	15.00	50.00	51.17	6.1
10	12.50	42.00	15.00	30.00	50.8	1.5
11	12.50	78.00	15.00	50.00	61.6	6.4
12	10.00	24.00	10.00	40.00	50.5	4.9
13	15.00	60.00	20.00	60.00	68.00	5.3
14	12.50	42.00	5.00	50.00	35.00	6.4
15	10.00	24.00	20.00	40.00	50.5	5.4
16	15.00	60.00	10.00	40.00	56.24	5.2
17	12.50	42.00	15.00	70.00	52.00	1.6
18	10.00	60.00	10.00	60.00	51.61	5.4
19	15.00	60.00	20.00	40.00	68.41	6.1
20	15.00	24.00	20.00	40.00	63.5	4.9
21	17.50	42.00	15.00	50.00	66.5	6.6
22	10.00	60.00	10.00	40.00	53.41	7.00
23	15.00	24.00	10.00	60.00	35.4	5.8
24	15.00	60.00	10.00	60.00	61.3	5.5
25	12.50	42.00	25.00	50.00	58.7	7.4
26	12.50	6.00	15.00	50.00	35.59	5.1
27	7.50	42.00	15.00	50.00	51.5	7.6

parameters was done to maximize glucose concentration in least possible time. The numerical optimization package suggested 20 different combinations with desirability of 1.0. Thus, after evaluating the model graphs and the combinations suggested by the numerical optimization package, validation experiment was done at substrate concentration 15%, temperature 60 °C, Celluclast concentration of 20 IU/g-ds and incubation time of 58 h, respectively.

Simultaneous saccharification and fermentation using optimized parameters

A continuous increase in ethanol production, ethanol yield and fermentation efficiency was seen from 12 to 60 h of incubation using the optimized conditions (Table 10). During fermentation, sugar consumption pattern showed a drastic reduction after 36 h. As the glucose that was being

produced was converted to ethanol by yeast cells, glucose concentration was almost negligible at the end of 48 h. Since the yeast was not able to ferment xylose or arabinose, their concentrations increased with time during SSF. The dried residual biomass after 24 h SSF was composed of (% w/v) xylose 11.32, arabinose 1.73 and 1.29 cellobiose. Glycerol concentration though low was observed after 24 h of fermentation. Similar results have been previously reported on simultaneous production of ethanol and glycerol (Dhaliwal et al. 2011). Since ethanol concentration did not increase beyond 48 h, the process can be terminated after 48 h, which means that ethanol productivity at the end of 48 h was 0.56 g/L/h. The remaining constituents like fats, starch, polyphenols, and alkaloids were not analyzed in the residual biomass. Since the residual biomass is rich in protein and minerals and does not contain lignin in high concentrations, it could be ideally used for cattle feed (Table 11).

The highest ethanol concentration of 26.81 g/L was observed at 48 h of incubation. El-Abayad et al. (El-Abayad et al. 1992) have reported maximum fermentation efficiency of 77% after 48 h while fermenting beet molasses by *S. Cerevisiae* Y-7. However, Sharma (Sharma 2000) have reported the maximum ethanol yield and fermentation efficiency of 0.397 g/L and 77.84%, respectively, after 36 h of incubation at the 30 °C using mixed culture of *S. cerevisiae* G and *P. tannophilus* MTCC 1077 to a large extent. This study could establish the optimized fermentation parameters for effective utilization of SSB for ethanol production.

Separate hydrolysis and fermentation

Enzymatic hydrolysis of alkali-treated SSB was carried out using the optimized parameters as described elsewhere in this paper. Glucose, xylose, arabinose and cellobiose did not increase significantly beyond 48 h of hydrolysis. Maximum glucose, xylose, arabinose and cellobiose concentrations of 53.02, 14.70, 2.10 and 0.97 g/L, respectively, were achieved after 48 h of hydrolysis. Although the higher glucose concentration was obtained during the validation process in 58 h, but from the commercial and scale-up perspective, it was decided to terminate the hydrolysis process at the end of 48 h as the total time of hydrolysis and fermentation has a drastic effect on the ethanol volumetric productivity. Ethanol concentration and

Fig. 2 A Response surface plots of the central composite design for the optimization of the enzymatic hydrolysis of pretreated SSB. Effect of **a** incubation time and substrate concentration; **b** cellulase concentration and substrate concentration; **c** cellulase concentration and incubation time; **d** temperature and incubation time. B Response surface plots of the central composite design for the optimization of

the enzymatic hydrolysis of pretreated SSB. Effect of **a** incubation time and substrate concentration; **b** cellulase concentration and substrate concentration; **c** temperature and substrate concentration; **d** cellulase concentration and Incubation time; **e** temperature and incubation time; **f** temperature and cellulase concentration

Fig. 2 continued

productivity are the two most critical factors for any industrial fermentation process.

Fermentation of the hydrolysate obtained during hydrolysis proceeded vigorously during the first 12 h with nearly 95% of sugars getting consumed with a corresponding increase in cell biomass and ethanol

concentration (Table 12). This could be attributed to the early entry of cells into the log phase because of the use of high initial inoculum. It is possible that the cells might have reached the stationary phase around 18–24 h, after which the fermentation rate declined. The maximum ethanol yield was obtained 26.02 g/L at 24 h.

Table 10 Ethanol production and sugar consumption pattern during different time intervals

S. no.	Sugar profile in liquid hydrolysate after fermentation	12 h	24 h	36 h	48 h	60 h
1	Glucose (g/L)	22.1 ± 0.23	15.1 ± 0.45	4.12 ± 0.29	1.05 ± 0.39	–
2	Xylose (g/L)	4.62 ± 0.78	11.3 ± 0.25	13.65 ± 0.28	14.12 ± 0.89	14.07 ± 0.1
3	Arabinose (g/L)	0.51 ± 0.29	1.73 ± 0.98	2.46 ± 1.2	2.81 ± 0.69	2.93 ± 0.25
4	Cellobiose (g/L)	0.70 ± 1.6	1.29 ± 0.59	0.57 ± 0.36	–	–
5	Ethanol (g/L)	0	14.6 ± 0.89	23.39 ± 0.17	26.87 ± 0.35	26.4 ± 0.02
6	Glycerol (g/L)	0	1.48 ± 0.09	2.98 ± 0.65	3.59 ± 0.25	3.70 ± 0.68

Values represented are for Mean ± SD for *n*-3

Table 11 Sugar profile during enzymatic hydrolysis of alkali-treated SSB

S. no.	Sugar profile in hydrolysate after enzymatic hydrolysis	0 h	12 h	24 h	36 h	48 h
1	Glucose (g/L)	0	21.25 ± 0.65	40.62 ± 0.8	52.10 ± 0.36	53.02 ± 0.2
2	Xylose (g/L)	0	4.55 ± 0.83	11.88 ± 0.2	14.64 ± 0.51	14.70 ± 0.2
3	Arabinose (g/L)	0	0.47 ± 0.15	1.65 ± 0.02	2.18 ± 0.09	2.10 ± 0.02
4	Cellobiose (g/L)	0	0.58 ± 0.12	1.31 ± 0.55	1.24 ± 0.67	0.97 ± 0.15

Values represented are for Mean ± SD for *n*-3

Table 12 Ethanol production and sugar consumption pattern during different time intervals in an SHF process

S. no.	Sugar profile in liquid hydrolysate after fermentation	0 h	6 h	12 h	18 h	24 h
1	Glucose (g/L)	53.02 ± 0.21	37.18 ± 0.1	15.03 ± 0.2	0.71 ± 0.1	0.0
2	Xylose (g/L)	14.70 ± 0.1	14.66 ± 0.23	14.60 ± 0.1	14.52 ± 0.06	14.19 ± 0.2
3	Arabinose (g/L)	2.10 ± 0.26	2.22 ± 0.65	2.25 ± 0.36	2.19 ± 0.51	2.07 ± 1.2
4	Cellobiose (g/L)	0.97 ± 0.23	–	–	–	–
5	Ethanol (g/L)	0	8.14 ± 0.3	19.28 ± 0.1	26.59 ± 0.03	26.62 ± 0.1
6	Glycerol (g/L)	0	0.77 ± 0.8	1.45 ± 0.56	3.18 ± 0.16	3.75 ± 0.45

Values represented are for Mean ± SD for *n*-3

During the SHF process, ethanol concentration of 26.59 g/L was obtained after 18 h of fermentation, indicating that the total time for ethanol production from SSB was 66 h, which corresponds to the ethanol volumetric productivity of 0.4 g/L/h. Though the comparable ethanol production was seen during both SSF and SHF, volumetric ethanol productivity was substantially higher in case of SSF. Therefore, it is evident from the results of the fermentation process that because of the ability of the *P. kudriavzevii* HOP-1 to grow and ferment at relatively higher temperature than the ones usually employed for industrial process, SSF could be a better option for ethanol production from SSB. Ethanol concentration produced through statistical optimization at two stages was higher than the one reported previously either through SSF or SHF (Shen et al. 2012; Li et al. 2013).

Conclusion

This study has clearly shown that SSB can serve as an ideal substrate for ethanol production after process optimization. Alkali pretreatment was more effective than the acid pretreatment in deconstruction of the biomass as evident from the enzymatic hydrolysis results. Enzymatic hydrolysis of optimized alkali pretreatment parameters (4% alkali concentration, 125 °C and 30 min) led to the production of about 57 and 10 g/L glucose and xylose, respectively. Subsequent optimization of enzymatic hydrolysis process through RSM using alkali-treated SSB resulted in an increase in glucose concentrations by about 20%. Validation of enzymatic hydrolysis process was carried out using 15% substrate concentration, 60 °C, Celluclast concentration of 20 IU/g-ds and 58 h. Ethanol production studies

using SSF and SHF revealed that though both the processes led to comparable production of ethanol, SSF could be a better option for achieving higher ethanol productivity. Advantages of using *P. kudriavzevii* HOP-1 in an SSF process because of its thermo-tolerant ability make this process more attractive commercially. This study has shown that statistical optimization at two different stages of ethanol production from SSB improved the overall ethanol concentration and productivity over the process which is not statistically optimized. This study has created a platform for further scale-up studies to explore the possibility of use of SSB for ethanol production at a commercial level.

Compliance with ethical standards

Conflict of interest The authors declare that there is no potential conflict of interest.

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