Industrial Crops and Products 52 (2014) 169–176

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Bioethanol production from dilute acid pretreated Indian bamboo variety (*Dendrocalamus* sp.) by separate hydrolysis and fermentation

Raveendran Sindhu, Mathiyazhakan Kuttiraja, Parameswaran Binod, Rajeev K. Sukumaran, Ashok Pandey*

Centre for Biofuels, National Institute for Interdisciplinary Science and Technology (CSIR), Trivandrum 695 019, India

ARTICLE INFO

Article history:
Received 25 July 2013
Accepted 8 October 2013

Keywords:
Bamboo
Acid pretreatment
Lignocellulosic biomass
Response surface methodology
Bioethanol

ABSTRACT

Bioethanol from lignocellulosic biomass can be utilized for clean and renewable energy production. Bamboo (BM) was used as a feed stock for the production of bioethanol after dilute acid pretreatment and enzymatic saccharification. In this study different mineral and organic acids were screened to select the best pretreatment agent. Dilute H_2SO_4 was selected and the effectiveness of pretreatment was evaluated by enzymatic saccharification. Parameters like acid concentration, biomass loading and incubation time were optimized by adopting a Taguchi design. Under optimized pretreatment conditions 0.319 g/g of reducing sugar was produced. The effect of various process parameters affecting enzymatic saccharification like solid loading, enzyme loading, incubation time and surfactant concentration on enzymatic hydrolysis was studied using a response surface method according to Box–Behnken design. Under optimized hydrolysis conditions – 11.25% (w/w) of biomass loading, 50 FPU of commercial cellulase, 0.125% (w/w) of Tween-80 as surfactant and 42 h of incubation, 0.651 g/g of reducing sugar was produced. Physicochemical characterizations of native and dilute acid pretreated BM were carried out by scanning electron microscopy (SEM) and there were morphological differences between the native and pretreated sample. Model validation showed a good agreement between experimental results and the predicted responses. Fermentation of the enzymatic hydrolysed liquid from the pretreated biomass using *S. cerevisiae* showed bioethanol yield of 1.76% (v/v) with an efficiency of 41.69%.

1. Introduction

Conversion of lignocellulosic biomass to ethanol provides a sustainable energy production system. The price of feed stocks contributes more than 50% to the production cost, inexpensive feed stocks are being considered to make bioethanol more competitive in the open market (Lichts, 2004). Lignocellulosic biomass serves as a low cost feed stock for the production of bioethanol and other value added products. Development of an efficient pretreatment and cost-effective enzymatic conversion is the key issue in conversion of lignocellulosic biomass to bioethanol.

Bioprocessing of lignocellulosic biomass into bioethanol involves pretreatment, hydrolysis, fermentation and distillation. Pretreatment is the most important step in cellulose to ethanol technology because it can remove hemicelluloses, lignin and increase the porosity of materials which improves enzymatic saccharification (Hendricks and Zeeman, 2009; Salvi et al., 2010).

Bamboo is a fast growing woody grass which can be used as a potential feed stock for bioethanol production. Total growing stock of bamboo in India is 80.4 MMT (Million Metric Tons) and the estimated annual harvest is 18.0 MMT. Usually for every 1 MT of bamboo produced around 0.3 MT residue in the form of top portion, branches, twigs, leaves and roots (Pandey et al., 2009). There are several reports on pretreatment of bamboo using alkali (Wen et al., 2011), organic solvent (Sathitsuksanoh et al., 2010), steam explosion (Asada et al., 2005), acid (Leenakul and Tippayawong, 2010) and biological pretreatment using white-rot fungi (Zhang et al., 2007).

Relatively few works have been reported for *Dendrocalamus*, the Indian bamboo variety, which is used in the present study. The objective of the present study was to optimize various process parameters affecting acid pretreatment of BM, optimization of enzymatic saccharification and bioethanol production after fermentation of the hydrolyzate. Compositional analyses as well as physico-chemical characterizations of native and pretreated samples were evaluated by SEM.

2. Materials and methods

2.1. Feed stocks

Bamboo (BM) used in this study was provided by TIFAC-DST, Government of India. The raw materials were dried and milled

* Corresponding author. Tel.: +91 471 2495949/2515279; fax: +91 471 2491712.
E-mail addresses: pandey@niist.res.in, ashokpandey56@yahoo.co.in (A. Pandey).

0926-6690/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.indcrop.2013.10.021

using a knife mill to a particle size less than 1 mm, homogenized and stored at room temperature until used. The compositions of native and dilute acid pretreated samples were determined according to National Renewable Energy Laboratory (NREL) analytical methods (Ruiz and Ehrman, 1994).

2.2. Primary screening

Primary screening for the pretreatment was carried out to select the best pretreatment agent. Bamboo (BM) was pretreated with different mineral and organic acids to select the best pretreatment agent and various process parameters affecting pretreatment like reagent concentration, biomass loading and incubation time were optimized by adopting a Taguchi design. For primary screening different mineral acids – HCl and H_2SO_4 (2%, w/w) and organic acids – acetic acid and formic acid (30%, w/w) were mixed with milled BM with a solid loading of 10% (w/w). The pretreatment was carried out at 121 °C, 15 lb pressure for 60 min. After pretreatment, the samples were neutralized using 1 N NaOH to a final pH of 5.5–6.0. This was followed by washing with tap water to remove salts and the neutralized samples were air dried at room temperature (30 ± 2 °C) to remove moisture.

2.2.1. Dilute acid pretreatment of bamboo

Dilute acid pretreatment of BM were carried out by statistical means. A number of factors like reagent concentration, biomass loading, incubation time and particle size influence the pretreatment efficiency. The design and planning of experiments was carried out using Taguchi design experiment (Table 1), to generate experimental data and to understand the interaction between these factors. This method investigates how the different process parameters affect the mean and variance of a process performance. This involves using orthogonal arrays to organize the parameters affecting the process and the level at which it varies. In factorial design all possible combinations were tested while in Taguchi design tests pairs of combinations, hence allows determining which factors affect product quality with a minimum amount of experimentation thus saving time as well as resources. The variables used in this model were acid concentration, biomass loading and pretreatment time. Biomass loading was selected at six levels (5, 10, 15, 20, 25 and 30%, w/w), acid concentration was selected at three levels (1, 3 and 5%, w/w) and pretreatment time at three levels (30, 60 and 90 min). The experiment consists of a total of 21 runs. Eighteen runs are used for modeling and three runs are used for validation. In all the cases, the pretreatment was carried out at 121 °C, 15 lb pressure. The pretreated samples were neutralized with 1 N NaOH, followed by washing with tap water and dried at room temperature (30 ± 2 °C).

2.3. Physicochemical characterization of the feed stock

2.3.1. SEM analysis

The difference in the lignocellulosic structure of BM before and after dilute acid pretreatment was evaluated by scanning electron microscopy (JEOL JSM-5600). The samples were mounted on a double sided conductive tape on precut brass sample stubs and the sputter coated with gold palladium using a JEOL JFC-1200 fine coater. The images of native and dilute acid pretreated BM were acquired with a 10–15 kV accelerating voltage at a magnification of 1500×.

2.4. Enzymatic hydrolysis

Enzymatic saccharification of dilute acid pretreated BM was performed in 150 ml stoppered conical flasks by incubating 1.4 g of dilute acid pretreated biomass in 100 mM citrate buffer (pH 4.8) and 0.1% (w/w) surfactant (Tween-80) which is supplemented with 1% (v/v) of Penicillin-Streptomycin solution (Hi-media, India) to prevent chances of contamination. Hydrolysis was performed using a commercial cellulase (Zytex India Private limited, Mumbai, India). The samples were incubated at 50 °C, 200 rpm for 48 h in a shaking water bath (Julabo, Switzerland). After enzymatic saccharification, the samples were centrifuged to remove the unhydrolyzed residue. The supernatant (hydrolyzate) was used for reducing sugar analysis by 2,5-dinitrosalicylic acid method (Miller, 1959).

2.4.1. Optimization of enzymatic saccharification of acid pretreated BM by RSM

Enzymatic hydrolysis is the key step in the conversion of cellulose into ethanol. Optimization of enzymatic hydrolysis of BM by classical method involve changing one independent variable at a time, while maintaining other parameters at a fixed level which is extremely time consuming and expensive for a number of variables. To improve yield and rate of enzymatic hydrolysis research has been focused on optimization of hydrolytic process. Box–Behnken design and response surface methodologies were employed for optimization of hydrolysis conditions. The effect of biomass loading, enzyme loading, surfactant concentration and incubation time on reducing sugar yield of dilute acid pretreated BM were carried out. Box–Behnken design was used to study the effect of independent variables on the response and factor interactions with different combinations of variables. The effects of four variables were studied at three different levels and a total of 27 runs were used for the study. Biomass loading was selected at three levels (7.5, 11.25 and 15%, w/w), enzyme loading at three levels (20, 50 and 80 FPU/g dry substrate), surfactant (Tween-80) concentration at three levels (0.05, 0.125 and 0.2%, w/w) and incubation time at three levels (24, 42 and 60 h). The software Minitab 15 (Minitab Inc, USA) was used for experimental design, data analysis and quadratic model building. The response surface graphs were obtained using the software to understand the effect of variables individually and in combination, and to determine their optimum levels. The experimental setup of RSM is shown in Table 2.

2.5. Ethanol fermentation

The hydrolyzate obtained after enzymatic saccharification was centrifuged (4 °C, 10,000 g) to remove the un-hydrolyzed residue. Fermentation was carried out in screw capped vials containing 20 ml hydrolysate. This was inoculated with 18 h old seed culture of *S. cerevisiae* and incubated at 30 °C for 72 h. The supernatant was filtered through 0.4 μm filters (Pall, USA) and analyzed by gas chromatography (Chemito, India). The concentrations of ethanol were calculated based on elution time and known concentration of ethanol. The conditions of gas chromatography analysis were oven temperature set at 150 °C, injector temperature at 175 °C and detector temperature at 250 °C. Nitrogen was used as carrier gas and flow rate was maintained at 30 ml min^{-1}. Ethanol concentrations were calculated based on calibration curve drawn with known concentration of standards. Analysis was done in triplicate and the mean value was taken.

3. Results and discussion

3.1. Effect of different process parameters on acid pretreatment of Bamboo

Among the different mineral acids and organic acids used for pretreatment, H_2SO_4 gave higher reducing sugar (0.22 g/g) followed by HCl (0.17 g/g), acetic acid (0.14 g/g) and formic acid (0.13 g/g) (data not shown). Hence dilute H_2SO_4 was selected for

Table 1
Taguchi design for optimization of dilute acid pretreatment of BM.

Run	Biomass loading (%)	Reagent concentration (H_2SO_4)	Pretreatment time (min)	Reducing sugar (g/g)
1	5	1	30	0.087
2	5	3	60	0.180
3	5	5	90	0.198
4	10	1	30	0.199
5	10	3	60	0.229
6	10	5	90	0.312
7	15	1	60	0.218
8	15	3	90	0.308
9	15	5	30	0.319
10	20	1	90	0.236
11	20	3	30	0.247
12	20	5	60	0.272
13	25	1	60	0.174
14	25	3	90	0.276
15	25	5	30	0.170
16	30	1	90	0.165
17	30	3	30	0.166
18	30	5	60	0.154

further studies. Taguchi design experiments with three factors like biomass loading, reagent concentration and pretreatment time was carried out to optimize the acid pretreatment of BM. Maximum reducing sugar of 0.319 g/g was produced with 5% (w/w) H_2SO_4 concentration, 10% (w/w) biomass loading and 90 min of pretreatment time in a laboratory autoclave. Hence this conditions for selected for further studies including optimization of enzymatic saccharification and characterization.

The interactions of variables on reducing sugar yield were studied by plotting contour plots to determine the optimum level of each variable for maximum response. Contour plots showing interactions of a pair of factors are given in Fig. 1a–c.

Fig. 1a shows the interaction between acid concentration and incubation time on reducing sugar yield. At low level of acid concentration (1–3%, w/w) and low level of incubation time (30–60 min), the reducing sugar yield was low (0.10 g/g). Reducing sugar yield increases with increase in incubation time and reagent concentration. Maximum reducing sugar (0.25 g/g) was produced at high level of acid concentration (5%, w/w) and high level of incubation time (90 min). An identical observation was reported by Leenakul and Tippayawong (2010) for acid pretreatment of bamboo where soft pretreatments lead to sugar rich pre-hydrolyzate. High enzymatic hydrolysis yield was observed with high H_2SO_4 concentration and pretreatment time while pretreatment temperature does not have a significant role. Kabel et al. (2007) reported that acid pretreated wheat straws, the improvements in enzymatic hydrolysis yields were affected by pretreatment temperature, time and acid concentration.

The interaction between biomass loading and incubation time on reducing sugar yield was presented in Fig. 1b. Reducing sugar yield increases with increase in biomass loading and incubation time. Maximum reducing sugar (0.3 g/g) was produced with middle level of biomass loading (15%, w/w) and high levels of incubation time (90 min). In dilute acid pretreatments reported in literature, solid loading usually varies from 5 to 10% (w/w) (Kabel et al., 2007; Kim et al., 2008). A contrary observation was reported by

Table 2
Reducing sugar yields for individual runs of the RSM design in hydrolysis of dilute acid pretreated Bamboo.

Run	Biomass loading (%, w/w)	Enzyme loading (FPU/g)	Surfactant (%, w/w)	Incubation time (hours)	Reducing sugar (g/g)
1	15	50	0.125	24	0.344
2	15	50	0.125	60	0.412
3	7.5	50	0.2	42	0.205
4	7.5	50	0.125	24	0.169
5	15	20	0.125	42	0.539
6	11.25	50	0.125	42	0.295
7	11.25	50	0.2	60	0.293
8	11.25	50	0.2	24	0.319
9	7.5	20	0.125	42	0.214
10	7.5	50	0.05	42	0.197
11	11.25	80	0.05	42	0.424
12	15	80	0.125	42	0.248
13	11.25	20	0.05	42	0.265
14	11.25	50	0.125	42	0.27
15	7.5	50	0.125	60	0.216
16	11.25	50	0.125	42	0.651
17	11.25	80	0.125	24	0.564
18	15	50	0.2	42	0.409
19	15	50	0.05	42	0.532
20	11.25	80	0.2	42	0.408
21	11.25	50	0.05	24	0.179
22	11.25	80	0.125	60	0.251
23	11.25	20	0.125	60	0.309
24	11.25	50	0.05	60	0.249
25	11.25	20	0.125	24	0.385
26	11.25	20	0.2	42	0.31
27	7.5	80	0.125	42	0.187

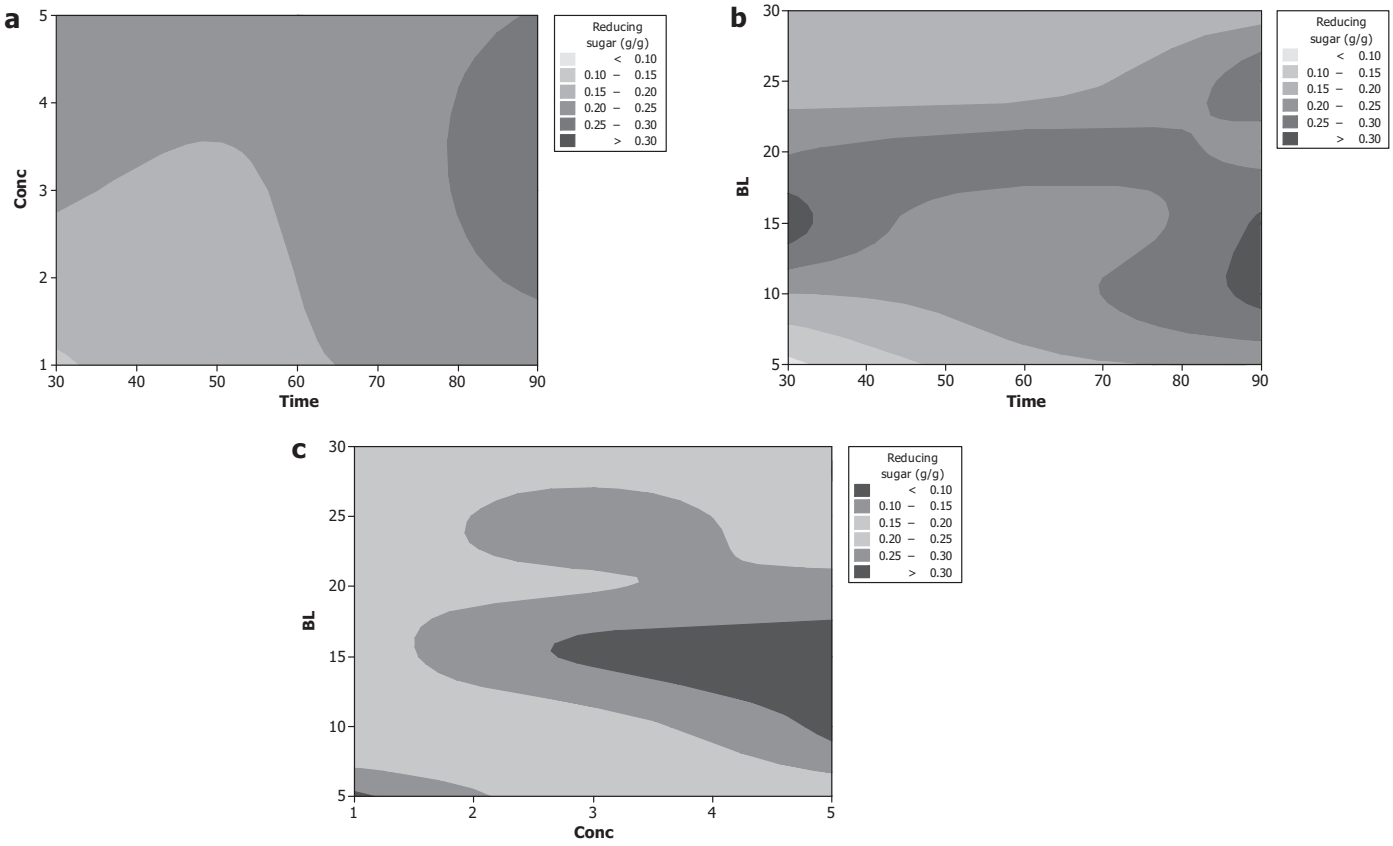

Fig. 1. Effect of various process parameters on reducing sugar yield from acid pretreated Bamboo after enzymatic hydrolysis. (a) Effect of acid concentration and incubation time on reducing sugar yield. (b) Effect of biomass loading and incubation time on reducing sugar yield. (c) Effect of biomass loading and acid concentration on reducing sugar yield.

Sindhu et al. (2011) for dilute acid pretreatment of sugarcane tops where maximum reducing sugar yield was observed with 25% (w/w) biomass loading. Decrease in pretreatment efficiency at high biomass loading was associated with less accessibility of biomass by the pretreatment agent. In the present study 15% (w/w) solid loading was found to be the best and the reducing sugar yield decrease beyond this value. Pretreatment with high biomass loading is challenging due to higher demand for mixing power and equipment. Often high solid loadings were achieved with batch feeding or continuous feeding of biomass or by used of gaseous agent like ammonia vapors or high pressure steam. Increase in biomass loading makes the process economic.

The interaction between reagent concentration and biomass loading was presented in Fig. 1c. At low levels of biomass loading (5–10%, w/w) and low levels of acid concentration (1–3%, w/w), the reducing sugar yield was low (0.2 g/g). Studies with acid concentration at 3–5% (w/w) gave almost the same reducing sugar yield (0.3 g/g). Maximum reducing sugar (0.3 g/g) was produced with middle level (15%, w/w) of biomass loading and medium to high level of reagent concentration (3–5%, w/w). A contrary observation was reported by Leenakul and Tippayawong (2010) were maximum reducing sugar (85 mg/g) were obtained when pretreatment were carried out at 120 °C, 1.2% H_2SO_4 concentration for 60 min.

The adequacy of the model was evaluated using analysis of variance (ANOVA) and the result was shown in Table 3. *p*-Value was used as a tool to check the significance of each of the coefficients. Smaller the *p*-value, more significant is the correlation with the corresponding coefficient. The regression coefficient for reducing sugar yield was found to be best with biomass loading (*p*-value 0.002). The *p*-values verify the significance of each of the coefficients which in turn identifies the pattern of mutual

Table 3
Analysis of variance for reducing sugar (g/g), using adjusted SS for tests.

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
BL	5	0.0398278	0.0398278	0.0079656	10.35	0.002
Conc	2	0.0126114	0.0126114	0.0063057	8.19	0.012
Time	2	0.0093108	0.0093108	0.0046554	6.05	0.025
Error	8	0.0061571	0.0061571	0.0007696		
Total	17	0.0679071				

$S = 0.0277424$ R-Sq = 0.90 R-Sq (adj) = 80.73%

interactions between the selected variables. In this model incubation temperature, acid concentration and pretreatment time were significant factors. The coefficient of determination (R^2) was calculated as 0.90, indicating that the statistical model can explain 90% variability in response.

In order to validate the model, randomly selected three experiments from the various solutions predicted by Taguchi software were performed and the reducing sugar yield was determined. Correlation analysis was performed on the actual responses and predicted values for each solution. The details are shown in Table 4. The experimental and the predicted values lie in the range. The correlation coefficient was 0.917 which indicates that the model is accurate.

3.2. Characterization of native and pretreated biomass

Compositional analysis of native and dilute acid pretreated BM with 15% (w/w) biomass loading, acid concentration 5% (w/w) and incubation time of 30 min (optimum pretreatment conditions) were analyzed by a two stage acid hydrolysis protocol developed by NREL. The cellulose, hemicelluloses and lignin content of native

Table 4
Model predicted value for reducing sugar yield of acid pretreated Bamboo.

Biomass loading (%, w/w)	Reagent concentration (%)	Incubation time (minutes)	Reducing sugar yield (g/g)	
			Predicted	Experimental
15	5	30	0.325	0.319
10	5	90	0.299	0.312
20	5	60	0.275	0.272

Table 5
Composition of native and H_2SO_4 pretreated BM.

Components	Total (%)	
	Native	Pretreated
Cellulose	42.2	55.8
Hemicellulose	16.1	5.75
Total lignin	27.8	25.03

and pretreated samples were presented in Table 5. Native BM contained 42.2% cellulose, 16.1% hemicelluloses and 27.8% lignin. The mass balance analysis shows that there was a loss of 30% biomass during pretreatment. Considering the mass balance, the cellulose content in the acid pretreated BM was 55.8%; the hemicelluloses and lignin content were 5.75% and 25.03%, respectively.

Physical properties of cellulose microstructure are one of the potential factors that affect enzymatic hydrolysis. SEM images of native and acid pretreated BM was presented in Fig. 2. Native BM exhibited a highly ordered structure, while the acid pretreated BM showed a highly distorted surface, which cause fiber separation and which in turn increases the accessible surface area and porosity of BM. The increase in porosity, improves saccharification efficiency by increasing the accessible surface area. An identical observation was earlier reported by Wei et al. (2009) for pretreatment of rice hulls by acid followed by alkali.

3.3. Optimization of enzymatic saccharification of acid pretreated BM by Box–Behnken design

RSM has been successfully used for the optimization of maize starch (Kunenmani and Singh, 2005); steam exploded soft wood (Tengborg et al., 2001), Sugarcane tops (Sindhu et al., 2011) and Corn stover (Lu et al., 2007). Response surface optimization is more advantageous than the traditional one parameter at a time optimization in that it saves time, space and raw material. There were a total of 27 runs for optimizing the four individual parameters in the current Box–Behnken design. Experimental design and experimental reducing sugar yields are presented in Table 2. The data were analyzed by multiple regression analysis using the Minitab software and the following polynomial equation was derived to represent reducing sugar yield as a function of the variables tested. Polynomial equation for the model was as below:

$$\text{predicted reducing sugar yield}\,(g/g) = 0.648 + 0.108X_1 + 0.005X_2 + 0.008X_3 - 0.019X_4 - 0.183X_1^2 - 0.129X_2^2 + 0.168X_3^2 - 0.180X_4^2 - 0.066X_1X_2 - 0.032X_1X_3 + 0.005X_1X_4 - 0.0152X_2X_3 - 0.059X_2X_4 - 0.024X_3X_4$$

where X_1, X_2, X_3 and X_4 are biomass loading, enzyme loading, surfactant concentration and incubation time respectively. The regression coefficient for reducing sugar yield was found to be best with biomass loading (p-value 0.003). The p-values verify the significance of each of the coefficients which in turn identifies the pattern of mutual interactions between the selected variables. In this model biomass loading was the significant factor. The coefficient of determination (R^2) was calculated as 0.80, indicating that the statistical model can explain 80.06% variability in response. The details were presented in Table 6.

Contour plots were plotted to find out the interaction of variables and to determine the optimum level of each variable for maximum response. Contour plot showing the interaction between a pair of factors on hydrolysis of acid pretreated BM are given in Fig. 3a–e. Fig. 3a indicates reducing sugar yield as a function of enzyme loading and biomass loading. This plot explains that at low to middle level of enzyme loading ((20–50 FPU) and high level of biomass loading (13–15%, w/w), the reducing sugar yield was high (0.5 g/g). At low levels of biomass loading (8–10%, w/w), the reducing sugar yield was low (0.3 g/g). With increase in biomass loading, there was a significant increase in reducing sugar yield. An identical observation was reported by Sindhu et al. (2012) were 15% (w/w) biomass loading was found to be optimum for hydrolysis of acetone pretreated rice straw. A contrary observation was reported by Sindhu et al. (2011) for hydrolysis of acid pretreated sugarcane tops, where maximum reducing sugar yield was observed at middle level of biomass loading (11.25%, w/w). Biomass loading is one of the critical factors which affects yield and enzymatic hydrolysis of cellulose. The decrease in hydrolysis efficiency with increase

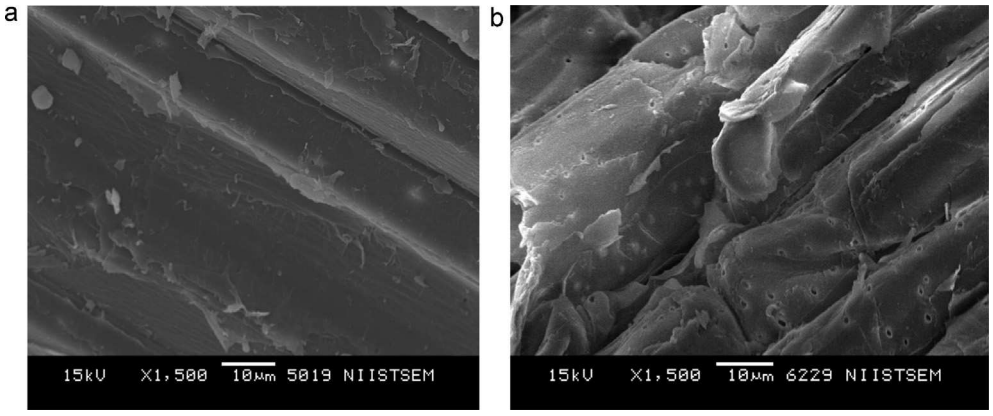

Fig. 2. Scanning electron micrographs of (a) native and (b) acid pretreated Bamboo.

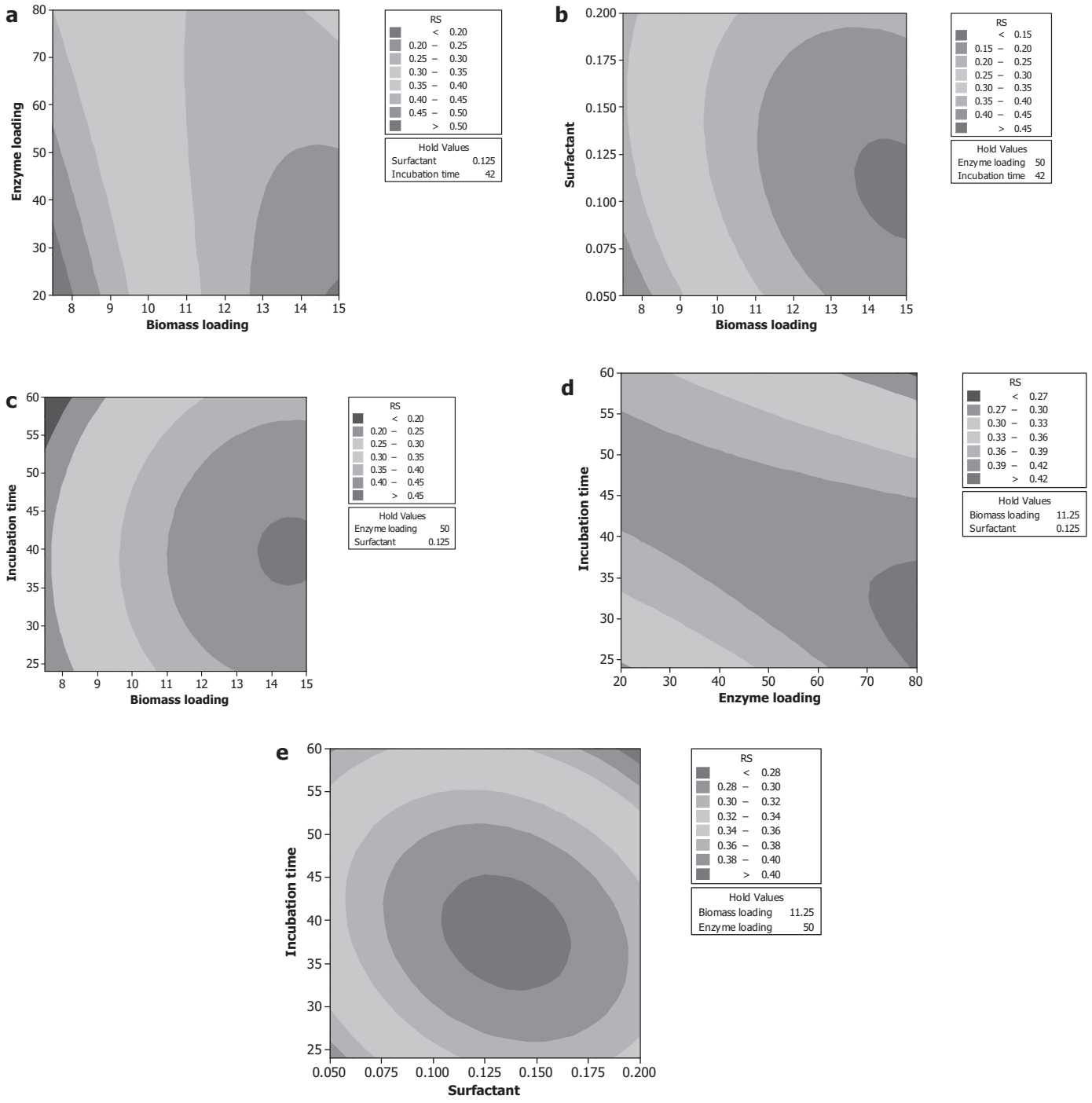

Fig. 3. Effect of various parameters on hydrolysis of acid pretreated bamboo on reducing sugar yield after enzymatic saccharification. (a) Effect of biomass loading and enzyme loading on reducing sugar yield. (b) Effect of surfactant concentration and biomass loading on reducing sugar yield. (c) Effect of incubation time and biomass loading on reducing sugar yield. (d) Effect of incubation time and enzyme loading on reducing sugar yield. (e) Effect of incubation time and surfactant concentration on reducing sugar yield.

in biomass loading is due to unavailability of free water and also due to product inhibition of the cellulolytic enzyme system (Binod et al., 2011).

Fig. 3b shows reducing sugar yield as a function of surfactant concentration and biomass loading. It was observed that at low levels of surfactant concentration (0.05–0.075%, w/w) and low levels of biomass loading (8–12%, w/w), the reducing sugar yield was low. At middle level of surfactant concentration (0.125%, w/w) and high level of biomass loading (15%, w/w), the reducing sugar yield was high (0.45 g/g). Substrate concentration is one of the most important factors affecting enzymatic saccharification. A contrary observation was reported by Qi et al. (2009) where a high conversion rate of wheat straw was obtained at lower substrate concentration with high level of surfactant. Surfactants have a positive effect on enzymatic hydrolysis. Surfactants improve hydrolysis efficiency by surface modification, preventing non-productive adsorption of enzymes or by acting as enzyme stabilizers by preventing

Table 6
Regression co-efficient for the model for reducing sugar yield after enzymatic saccharification of acid pretreated Bamboo.

Estimated regression coefficients for RS				
Term	Co-ef	SE Co-ef	*T*	*P*
Constant	0.648333	0.05827	11.127	0.000
Biomass loading	0.108000	0.02913	3.707	0.003
Enzyme loading	0.005000	0.02913	0.172	0.867
Surfactant	0.008167	0.02913	0.280	0.784
Incubation time	−0.019167	0.02913	−0.658	0.523
Biomass loading*biomass loading	−0.183000	0.04370	−4.188	0.001
Enzyme loading*enzyme loading	−0.129000	0.04370	−2.952	0.012
Surfactant*surfactant	−0.168250	0.04370	−3.850	0.002
Incubation time*incubation time	−0.180750	0.04370	−4.136	0.001
Biomass loading*enzyme loading	−0.066000	0.05046	−1.308	0.215
Biomass loading*surfactant	−0.032750	0.05046	−0.649	0.529
Biomass loading*incubation time	0.005250	0.05046	0.104	0.919
Enzyme loading*surfactant	−0.015250	0.05046	−0.302	0.768
Enzyme loading*incubation time	−0.059250	0.05046	−1.174	0.263
Surfactant*incubation time	−0.024000	0.05046	−0.476	0.643

S = 0.100924 PRESS = 0.703964
R-Sq = 0.80 R-Sq (pred) = 0.00% R-Sq (adj) = 56.81%

Table 7
Experimental validation for reducing sugar yield after enzymatic saccharification of dilute acid pretreated Bamboo.

Biomass loading (%, w/w)	Enzyme concentration (FPU)	Surfactant concentration (%)	Incubation time (hours)	Reducing sugar yield (g/g)	
				Predicted	Experimental
11.25	50	0.125	42	0.658	0.651
11.25	80	0.125	24	0.589	0.564
15	50	0.2	42	0.412	0.409

enzyme denaturation during enzymatic hydrolysis (Binod et al., 2011).

The effect of incubation time and biomass loading on the hydrolysis of BM with the other two factors kept at constant are shown in Fig. 3c. It was observed that at low levels of incubation time (25–35 h) and low levels of biomass loading (8–12%, w/w), the reducing sugar yield was low (0.35 g/g). At middle level of incubation time (42 h) and high level of biomass loading (15%, w/w), the reducing sugar yield was high (0.45 g/g). Most of the reported literatures show that 10–15% biomass loading is good for hydrolysis (Sindhu et al., 2010; Satyanagalakshmi et al., 2011).

Fig. 3d indicates the interaction between incubation time and enzyme loading. It was observed that low to middle levels of incubation time (24–35 h) and high levels of enzyme loading (70–80 FPU), the reducing sugar yield were high (0.42 g/g). Increase in hydrolysis time can cause inhibition from substrate and product, which considerably lowers the hydrolysis rate. An identical observation was reported by Thongkheaw and Pitiyont (2011) for enzymatic hydrolysis of acid pretreated sugarcane shoot. The highest yield of reducing sugar was observed at 48 h of incubation.

Fig. 3e shows reducing sugar yield as a function of incubation time and surfactant concentration. Incubation time is an important factor affecting enzymatic hydrolysis. At low levels of surfactant concentration (0.05–0.1%, w/w) and low levels of incubation time (25–30 h), the reducing sugar yield is low (0.3 g/g). Reducing sugar yield increased with increase in surfactant concentration and incubation time up to 0.15% (w/w) of surfactant concentration and 45 h of incubation time, beyond that level there was a decrease in reducing sugar yield. This plot explained that, middle level of surfactant concentration (0.125%, w/w) and middle level of incubation time (45 h), the reducing sugar yield was high (0.40 g/g). Surfactants act as an enzyme stabilizer contributing to better performance of the enzyme. The decrease in reducing sugar yield with high level of surfactant concentration, micelles were formed which in turn prevent cellulase from contacting cellulose substrate, thereby reducing sugar yield (Jonstromer and Strey, 1992; Schomacker and Strey, 1994).

Validations of the model were carried out under conditions predicted by the software. The experimental and the predicted values showed good correlation and the correlation coefficient was found to be 0.996, hence the empirical models developed were reasonably accurate. The details are shown in Table 7.

3.4. Ethanol fermentation

Ethanol fermentation was carried out in screw capped vials containing 20 ml hydrolysate with sugar concentration of 65 mg/ml. Maximum ethanol concentration of 1.758% (v/v) was obtained after 72 h of fermentation of the hydrolyzate obtained after enzymatic saccharification of acid pretreated BM by *S. cerevisiae*. The overall efficiency of the process was 41.69% which was calculated based on the theoretical ethanol yield from glucose.

4. Conclusions

Environmental and economic concerns as well as increase in oil prices are the major drivers for the development of strategies for biofuel production. Dilute acid pretreatment of BM based on Taguchi method has been evaluated and the optimum conditions of pretreatment was found to be 15% (w/w) biomass loading, 5% acid concentration and pretreatment time for 30 min. ANOVA of pretreatment of BM revealed that biomass loading is the most significant factor. Dilute acid pretreatment of BM could increase the enzymatic saccharification rate by removing hemicelluloses and lignin. The optimum conditions for hydrolysis were 11.25% (w/w) biomass loading, 50 FPU of commercial cellulase, 0.125% (w/w) surfactant concentration and incubation time for 42 h. Fermentation of the enzymatically saccharified hydrolyzate by *S. cerevisiae* produced 1.758% (v/v) ethanol. The study revealed that BM can be used as a potent feed stock for the production of bioethanol.

Acknowledgements

The authors are grateful to Technology Information, Forecasting and Assessment Council (TIFAC), Department of Science and Technology, Government of India and Council of Scientific and Industrial Research (CSIR), New Delhi for financial support to Centre for Biofuels.

References

Asada, C., Nakamura, Y., Kobayashi, F., 2005. Waste reduction system for production of useful materials from un-utilized bamboo using steam explosion followed by various conversion methods. Biochem. Eng. J. 23, 131–137.

Binod, P., Janu, K.U., Sindhu, R., Pandey, A., 2011. Hydrolysis of lignocellulosic biomass for bioethanol production. In: Pandey, A., Larroche, C., Ricke, S.C. (Eds.), Biofuels: Alternative Feedstocks and Conversion Processes. Elsevier Inc., USA, pp. 229–250.

Hendricks, A.T.W.N., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. 100, 10–18.

Jonstromer, M., Strey, R., 1992. Nonionic bilayer in dilute solutions – effect of additives. J. Phys. Chem. 96, 5993–6000.

Kabel, M.A., Bos, G., Zeevalking, J., Vorageri, A.G.J., Schols, H.A., 2007. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. Bioresour. Technol. 98, 2034–2042.

Kim, Y., Hendrickson, R., Mosier, N.S., Ladisch, M.R., Bals, B., Balan, V., Dale, B.E., 2008. Enzyme hydrolysis and ethanol fermentation of liquid hot water and AFEX pretreated distillers' grains at high-solids loadings. Bioresour. Technol. 99, 5206–5215.

Kunenmani, A., Singh, S., 2005. Response surface optimization of enzymatic hydrolysis of maize starch for higher glucose production. Biochem. Eng. J. 27, 179–190.

Leenakul, W., Tippayawong, N., 2010. Dilute acid pretreatment of bamboo for fermentable sugar production. J. Sustain. Energ. Environ. 1, 117–120.

Lichts, F.O., 2004. Ethanol Production Costs: a Worldwide Survey. Special Report No. 132.

Lu, X.B., Zhang, Y.M., Yang, J., Liang, Y., 2007. Enzymatic hydrolysis of corn stover after pretreatment with dilute sulfuric acid. Chem. Eng. Technol. 30, 938–944.

Miller, G.M., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.

Pandey, A., Biswas, S., Sukumaran, R.K., Kaushik, N., 2009. Study on the Availability of Indian Biomass Resources for Exploitation: a Report Based on Nationwide Survey. TIFAC, New Delhi.

Qi, B., Chen, X., Shen, F., Su, Y., Wan, Y., 2009. Optimization of enzymatic hydrolysis of wheat straw pretreated by alkaline peroxide using response surface methodology. Ind. Eng. Chem. Res. 48, 7346–7353.

Ruiz, R., Ehrman, T., 1994. Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography Laboratory Analytical Procedure #002, Revision 12/08/1996. National Renewable Energy Laboratory.

Salvi, D.A., Aita, G.M., Robert, D., Bazan, V., 2010. Ethanol production from sorghum by dilute ammonia pretreatment. Braz. J. Ind. Microbiol. Biotechnol. 37, 27–34.

Sathitsuksanoh, N., Zhu, Z., Hoc, T., Bai, M., Zhang, Y.P., 2010. Bamboo saccharification through cellulose solvent-based biomass pretreatment followed by enzymatic hydrolysis at ultra-low cellulase loadings. Bioresour. Technol. 101, 4926–4929.

Satyanagalakshmi, K., Sindhu, R., Binod, P., Janu, K.U., Sukumaran, R.K., Pandey, A., 2011. Bioethanol production from acid pretreated water hyacinth by separate hydrolysis and fermentation. J. Sci. Ind. Res. 70, 156–161.

Schomacker, R., Strey, R., 1994. Effect of ionic surfactants on nonionic bilayers-bending elasticity of weakly charged membranes. J. Phys. Chem. 98, 3908–3912.

Sindhu, R., Binod, P., Satyanagalakshmi, K., Janu, K.U., Sajna, K.V., Kurien, N., Sukumaran, R.K., Pandey, A., 2010. Formic acid as a potential pretreatment agent for the conversion of sugarcane bagasse to bioethanol. Appl. Biochem. Biotechnol. 162, 2313–2323.

Sindhu, R., Kuttiraja, M., Binod, P., Janu, K.U., Sukumaran, R.K., Pandey, A., 2011. Dilute acid pretreatment and enzymatic saccharification of sugarcane tops for bioethanol production. Bioresour. Technol. 102, 10915–10921.

Sindhu, R., Binod, P., Janu, K.U., Sukumaran, R.K., Pandey, A., 2012. Organosolvent pretreatment and enzymatic hydrolysis of rice straw for the production of bioethanol. World J. Microbiol. Biotechnol. 28, 473–483.

Tengborg, C., Galbe, M., Zacchi, G., 2001. Influence of enzyme loading and physical parameters on the enzymatic hydrolysis of steam-pretreated softwood. Biotechnol. Prog. 17, 110–117.

Thongkheaw, S., Pitiyont, B., 2011. Enzymatic hydrolysis of acid-pretreated sugarcane shoot. World Acad. Sci. Eng. Technol. 60, 454–458.

Wei, G., Gao, W., Jin, H., Yoo, S., Lee, J., Chung, C., Lee, J., 2009. Pretreatment and saccharification of rice hulls for the production of fermentable sugars. Biotechnol. Bioprocess Eng. 14, 828–834.

Wen, J., Xiao, L., Sun, Y., Sun, S., Xub, F., Sun, R., Zhang, X., 2011. Comparative study of alkali-soluble hemicelluloses isolated from bamboo (*Bambusa rigida*). Carbohydr. Res. 346, 111–120.

Zhang, X.Y., Xu, C.Y., Wang, H.X., 2007. Pretreatment of bamboo residues with *Coriolus versicolor* for enzymatic hydrolysis. J. Biosci. Bioeng. 104, 149–151.