A sequential pretreatment of lignocelluloses in bamboo biomass to fermentable sugars by acid/enzymatic hydrolysis

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Abstract A sequential pretreatment method for hydrolyzing rigid hemicelluloses and cellulose content in the bamboo biomass was investigated in this study. The effects of different parameters, such as nature of biomass, type of acid, acid and biomass concentration, were studied. Under the optimum condition of 5% (v/v) HCl-treated biomass and biomass concentration (8%, w/v), the maximum yield of sugar (619 mg/g of biomass) was obtained. The enzymatic hydrolysis parameter conditions were further optimized by response surface methodology-based central composite method. According to the results, the highest yield of sugar (515 mg/g of biomass) was obtained at hydrolysis temperature 50 °C, biomass concentration 8.9%, w/v, enzyme concentration (199.8 mg/g of biomass) and time 60 h, respectively. The effects of untreated, pretreated and enzymatically hydrolyzed biomass structure and complexity were investigated by field emission scanning electron microscopy and X-ray diffraction techniques.

Keywords Cellulose · Sequential pretreatment · Enzymatic hydrolysis · Optimization · Bamboo

Introduction

In recent years, the huge consumption of petroleum based fuels has led to increased demand for alternative energy sources (Ferreira et al. 2009; Zaldivar et al. 2001). Ethanol biofuel obtained from renewable resources has attracted lots of research in the past few decades to replace petroleum-based fuels. Currently, renewable fuels such as biodiesel, biohydrogen, biobutanol and bioethanol are obtained by utilizing waste agricultural biomass and lignocellulosic biomass (Cara et al. 2008; Sarkar et al. 2012). Lignocellulosic biomass could be used as a low-cost raw material and an alternative for second-generation biofuel production (Hamzah et al. 2011; Piarpuzán et al. 2011; Gnansounou and Dauriat 2010). Ethanol production using cellulosic biomass is an economical process and large quantity can be obtained from low-cost substrate (Ferreira et al. 2009; Cardona and Sánchez 2007; Cheng et al. 2008; Rass-Hansen et al. 2007). Bamboo is a promising renewable feedstock for ethanol production because of its high growth rate and high content of holocellulose (He et al. 2014; Yamashita et al. 2010; Sathitsuksanoh et al. 2010). The lignocellulosic biomass conversion into ethanol consists of the following steps: pretreatment, enzymatic hydrolysis, fermentation and separation processes (Cara et al. 2008). Pretreatment is the most important process for reducing the complexity of biomass by removing hemicelluloses and lignin and increasing the enzymatic digestibility (Alvira et al. 2010; Kim and Kim 2013; Kim et al. 2012). The physicochemical pretreatment method is one of the cost-effective steps in the process of converting complex biomass to fermentable sugars. In this research work, simultaneous pretreatment methods such as acid combined with pressure have been applied (da Costa et al. 2009; Alvira et al. 2010; Kim and Kim 2013; Kim et al.

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2012). This simultaneous pretreatment process maximizes the polysaccharides conversion into sugars and has low process utility costs (Balat 2011; Sarkar et al. 2012; Ge et al. 2011). Enzymatic hydrolysis is generally adopted to convert the polysaccharides into simple sugar components (Ferreira et al. 2009) and is highly influenced by various factors (Rabelo et al. 2011). The statistical methodology is an effective method to assess the optimal process conditions with minimum number of experiments to improve the yield and reduce the process cost. This method has been successfully applied to the optimization of the enzymatic hydrolysis of the pretreated biomass (Palukurty et al. 2008; Lalue et al. 2009).

In the present study, holocellulose content present in the bamboo biomass was hydrolyzed by the simultaneous pretreatment method. Response surface methodology was applied to evaluate the optimal process conditions of the enzymatic hydrolysis step to obtain maximum fermentable sugars.

Further, pretreated and treated biomass was also characterized by FESEM and XRD techniques.

Materials and methods

Substrate preparation

The bamboo wood biomass was used as raw substrate in this work. The wood biomass was washed, dried for 8 h at 60 °C and milled to an average particle size of 80–120 mm using a blade mill. Commercial enzyme cellulase (Nature Biotech) was used in the experiments. All chemicals were purchased from Sisco Research Laboratories Pvt. Ltd, Mumbai, India.

Acid pretreatment

In the first step, the dried biomass was treated with 5% (v/v) HCl using deionized water, followed by samples and autoclaved at 121 °C for 30 min. Then, the contents were filtered and washed with distilled water to remove unwanted impurities present in the surface of the biomass. After washing, the wet biomass was dried at ambient temperature for 6–8 h. In this ambient temperature of the drying process, the biomass structure most probably does not change, but only removes the moisture present on the surface of the biomass (Duan et al. 2013). Further, the dried biomass was subjected to enzymatic hydrolysis.

Enzymatic hydrolysis of the pretreated biomass

The enzymatic hydrolysis process was performed with 2% g (w/v) of pretreated biomass in phosphate buffer

(100 mM, pH 5.0), and (100 mg/g of biomass) cellulase enzyme from *Trichoderma reesei* was added. The flasks were incubated at 35 °C and 120 rpm in an incubator shaker (Orbitek, Scigenics Biotech). The enzymatic hydrolysis was stopped by boiling the hydrolyzate for 10 min. After cooling with cold water, the hydrolyzate was centrifuged at 12,000 rpm for 10 min. The supernatant was analyzed for reducing sugars release during hydrolysis.

Optimization of biomass pretreatment on reducing sugar production

For biomass pretreatment experiments, aqueous stock solutions of the acids (sulfuric, phosphoric, nitric, and hydrochloric acid) were prepared prior to the experiment at room temperature by adding the acids to distilled water until around pH 2 was reached. Then the system was heated in an autoclave at 121 °C, 15 psi for 30 min (Sievers et al. 2009). The washed biomass was dried at 60 °C for 8 h and stored under anhydrous condition. The best acid treatment method was used for further optimization. The effect of varied concentration (0–10%) of acid with different concentrations (2–10%) of the bamboo biomass was studied.

Optimization of enzymatic hydrolysis by response surface methodology

Central composite design (CCD) is one of the most commonly used response surface methodologies for optimizing the process conditions. In our study, the central composite design was used to optimize the enzymatic hydrolysis of the pretreated biomass. The levels of the four independent variables such as cellulase (100–200 mg/g biomass), temperature (30–50 °C), pretreated biomass (3–9%, w/v) and incubation time (36–60 h) were selected and designated as A, B, C and D, respectively, in Table 1. The five levels of the independent variables were coded (+2, +1, 0, −1 and −2) and uncoded (actual) values. All experimental design which include combinations of different levels of the four variables is shown in Table 1. A second-order polynomial model shown in Eq (1) measured response and the independent variables:

$$Y_i = \beta_0 + \sum_{j=1}^k \beta_j A_j + \sum_{j=1}^k \beta_{jj} A_j^2 + \sum_{i=1}^k \sum_{j=1}^k \beta_{ij} A_i A_j, \quad (1)$$

where Y_i is the predicted response, β_0 , β_j , β_{jj} , β_{ij} are regression coefficients for the intercept, linear, quadratic and interaction effects, respectively, and A_i and A_j are the coded independent variables. The obtained data were subjected to multiple regression analysis to estimate the coefficients, and the statistical significance of the

Table 1 Central composite design for the enzymatic hydrolysis of bamboo biomass

S. no	Temperature (°C), A	Pretreated biomass (w/v%), B	Cellulase (mg/g of biomass), C	Incubation time (h), D	Reducing sugars (mg/g of biomass), Y	
					Observed	Predicted
1.	30 (−1)	3 (−1)	100 (−1)	36 (−1)	105.5	48.8
2.	50 (+1)	3 (−1)	100 (−1)	36 (−1)	166.5	106.9
3.	30 (−1)	9 (+1)	100 (−1)	36 (−1)	318.5	296.1
4.	50 (+1)	9 (+1)	100 (−1)	36 (−1)	278.0	246.5
5.	30 (−1)	3 (−1)	200 (+1)	36 (−1)	212.5	214.7
6.	50 (+1)	3 (−1)	200 (+1)	36 (−1)	276.5	239.4
7.	30 (−1)	9 (+1)	200 (+1)	36 (−1)	479.0	474.8
8.	50 (+1)	9 (+1)	200 (+1)	36 (−1)	356.0	391.9
9.	30 (−1)	3 (−1)	100 (−1)	60 (+1)	157.0	76.8
10.	50 (+1)	3 (−1)	100 (−1)	60 (+1)	220.5	212.5
11.	30 (−1)	9 (+1)	100 (−1)	60 (+1)	260.5	285.4
12.	50 (+1)	9 (+1)	100 (−1)	60 (+1)	360.0	313.5
13.	30 (−1)	3 (−1)	200 (+1)	60 (+1)	280.0	299.3
14.	50 (+1)	3 (−1)	200 (+1)	60 (+1)	423.5	401.6
15.	30 (−1)	9 (+1)	200 (+1)	60 (+1)	505.5	520.8
16.	50 (+1)	9 (+1)	200 (+1)	60 (+1)	471.0	515.5
17.	20 (−2)	6 (0)	150 (0)	48 (0)	60.0	82.5
18.	60 (+2)	6 (0)	150 (0)	48 (0)	101.5	135.2
19.	40 (0)	0 (−2)	150 (0)	48 (0)	0.0	92.6
20.	40 (0)	12 (+2)	150 (0)	48 (0)	490.0	453.6
21.	40 (0)	6 (0)	50 (−2)	48 (0)	110.5	222.9
22.	40 (0)	6 (0)	250 (+2)	48 (0)	645.5	590.1
23.	40 (0)	6 (0)	150 (0)	24 (−2)	239.0	297.3
24.	40 (0)	6 (0)	150 (0)	72 (+2)	451.0	448.9
25.	40 (0)	6 (0)	150 (0)	48 (0)	349.0	355.5
26.	40 (0)	6 (0)	150 (0)	48 (0)	371.5	355.5
27.	40 (0)	6 (0)	150 (0)	48 (0)	356.0	355.5
28.	40 (0)	6 (0)	150 (0)	48 (0)	350.5	355.5
29.	40 (0)	6 (0)	150 (0)	48 (0)	346.0	355.5
30.	40 (0)	6 (0)	150 (0)	48 (0)	360.0	355.5

regression model was analyzed by *F* test of ANOVA statistics results.

Analytical methods

The amount of total reducing sugars present was determined by the DNS (3,5-dinitrosalicylic acid) method (Miller et al. 1959). The hydrolysate was filtered, and the filtrates (0.5 ml) were mixed with 1.5 ml of distilled water and then 1 ml of dinitrosalicylic acid (DNS) reagent was added and boiled at 60 °C for 10 min. 1 ml of sodium potassium tartrate was added to the boiled sample and the reducing sugar concentration at 575 nm was estimated using a spectrophotometer. The biomass yield was calculated using the following equation (Eq. 2):

$$\text{Biomass - based sugar yield (g glucose/g dry biomass)} = (C \times V)/m, \quad (2)$$

where *C* is the concentration (g/L) of glucose in the hydrolysis slurry, *V* (L) the total volume of the slurry, and *m* (g) the dry weight of the biomass loaded into the hydrolysis process (Zhang et al. 2014)

Characterization of untreated, pretreated and enzyme-treated biomass

Scanning electron microscopy (SEM) was used to compare the morphological change of biomass before and after treatment of the chemical and enzyme. Samples were imaged by a scanning electron microscope (FEI, USA)

Fig. 1 SEM images showing the surface morphology of untreated, pretreated and enzymatically hydrolyzed bamboo biomass

after coating with gold at an acceleration voltage of 6 keV. The crystallinity of the untreated, pretreated and enzyme-treated biomass was investigated by powder X-ray diffractometry (XRD), using a PAN analytical X-ray diffractometer (X'Pert power, Germany). The diffraction patterns were measured from 4θ to 80θ using Cu Ka radiation at 40 kV and 30 mA. The crystallinity index of cellulose (CrI) was calculated using the formula (Eq. 3):

$$C_I = (I_{002} - I_{am}) / I_{002} \times 100, \quad (3)$$

where I_{002} is the intensity of the peak at 2θ of about 22° and I_{am} the intensity of the baseline at 2θ of about 18° .

The cellulose crystallite size was determined using the Scherrer equation (Eq. 4):

$$\text{Crystallite size} = \frac{0.89 \times \lambda}{\beta \cos \theta}, \quad (4)$$

where λ is the wavelength of the incident X-ray (1.5418 Å), θ the Bragg angle corresponding to the (0 0 2) plane, and β the half-height width of the peak angle of the (0 0 2) reflection.

Results and discussion

Morphology analysis of untreated and treated biomass

The surface morphology of untreated and pretreated and enzymatic hydrolysis biomass is shown in Fig. 1a–c. Based on the observation of SEM analysis, the untreated biomass showed (Fig. 1a) compact, rigid and long fiber structures. After acid pretreatment, the biomass changed into a flaky structure with disordered surface (Fig. 1b). The rigid structure of the biomass, probably with high cellulose and low hemicellulose content, was efficiently disordered by acid

treatment. Similar observations were obtained by Duan et al. (2013) after pretreatment of poplar solid with peroxide combined with acetic acid. After enzymatic hydrolysis, the flaky structure of the biomass changed into a clear strip fiber structure (Fig. 1c), which was confirmed effectively by the removal of the cellulose content in the pretreated biomass.

Effect of the pretreatment method on reducing sugar production

Comparison of various state biomass for reducing sugar production

Chemical pretreatment can efficiently reduce the hemicellulose and lignin content in the biomass and enhance the sugar yield production. It is a sequential method of approach, in which chemical pretreatment is followed by enzymatic hydrolysis to produce sugar from the biomass. Similar reports in literature show that empty palm fruit bunch treated with acid/alkali followed by enzymatic saccharification increased the fermentable sugar production (Kim et al. 2012). The effect of various state biomass for reducing sugars was studied with the addition of a fixed amount of biomass as shown in Fig. 2. In this experiment, it was found that in the biomass after pretreatment with acid, the sugar yield was remarkably enhanced with the increase of hydrolysis time. However, enzymatic hydrolysis of untreated biomass released a low yield of sugar. These results indicated that pretreatment improved the enzyme accessibility to cellulose from biomass and rapid release of reducing sugars. Prolonging the reaction time could enhance the reducing sugar yield from the pretreated biomass (Duan et al. 2013). The effect of various acid-pretreated biomass used to produce reducing sugars is shown in Fig. 3a. The results showed that hydrochloric

Fig. 2 Time course profile for untreated and acid-treated biomass for obtaining reducing sugar

acid pretreatment has a stronger acid strength that gives a better digestibility of hemicelluloses and cellulose in bamboo biomass than other acid pretreatments. The effect of various concentrations of hydrochloric acid on biomass pretreatment for reducing sugar production in the enzymatic hydrolysis process is shown in Fig. 3b. The reducing sugar yield was substantially increased for up to 5% acid-treated biomass, and approximately 433 (mg/g of biomass) of reducing sugars was produced in the enzymatic hydrolysis process. However, a higher concentration of acid-treated biomass significantly decreased reducing sugar yield in the enzymatic hydrolysis process. Similarly, a previous study observed that monomer sugar yields decreased at high acid concentration, due to degradation of the product monomers by the acid (Palmarola-Adrados et al. 2005). The effect of various concentrations of pre-treated biomass used for enzymatic hydrolysis is shown in Fig. 3c. Figure 3c shows that increasing the biomass concentrations from 3 to 6% at constant enzyme concentrations led to an initial increase in glucose yield. A further increase in biomass concentrations does not lead to any appreciable increase in the yield. At low enzyme and high biomass concentrations, the ratio of enzyme to biomass concentration is low enough to limit the yield of glucose. High contents of cellulose in biomass and high glucan digestibility are desirable bioenergy traits. A similar work on transgenic poplar lines treated with acid, followed by enzymatic digestion steps have reported significant enhancement in glucose production. (Duan et al. 2013).

Enzymatic hydrolysis optimization by central composite design

Preliminary experiments were carried out to evaluate the pretreatment of biomass with acid to enhance the

Fig. 3 Effect of (a) acids, (b) HCl concentration and (c) bamboo biomass concentration on the hydrolysis process

enzymatic hydrolysis process. RSM-based CCD is generally used to investigate a combined effect of several variables on enzymatic hydrolysis and to find optimum conditions for a multivariable system. The combined cellulase (100–200 mg/g biomass), temperature (30–50 °C), pretreated biomass (3–9%, w/v) and

incubation time (36–60 h) was determined by analyzing the reducing sugar production after conducting 31 CCD experiments as presented in Table 1. The responses of reducing sugar production were subjected to regression analysis and the corresponding coefficients and the estimated p and F values are represented in Table 2. By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was obtained as in Eq. (5):

$$\begin{aligned} \text{Reducing sugars (mg/gofbiomass)} (Y) &= 355.50 + 13.19A + 90.27B + 91.98C \\ &+ 37.90D - 26.91AB - 8.34 AC - 19.41 AD \\ &+ 3.22BC - 9.66BD + 14.16CD - 61.65A^2 - 20.59B^2 \\ &+ 12.66C^2 + 4.41D^2. \end{aligned} \quad (5)$$

The analysis of variance (ANOVA) of the quadratic regression model indicated that the model fitted well with

Table 2 Estimated coefficients, and F and p values for reducing sugar production using coded units

Term	Coefficients	Standard error	F value	p value
Intercept	355.50	24.54	11.63	<0.0001
A—temperature	13.19	12.27	1.16	0.2995
B—pretreated biomass	90.27	12.27	54.13	<0.0001
C—cellulase	91.98	12.27	56.19	<0.0001
D—incubation time	37.90	12.27	9.54	<0.0001
AB	−26.91	15.03	3.21	0.0936
AC	−8.34	15.03	0.31	0.5869
AD	19.41	15.03	1.67	0.2161
BC	3.22	15.03	0.046	0.8333
BD	−9.66	15.03	0.41	0.5302
CD	14.16	15.03	0.89	0.3611
A^2	−61.65	11.48	28.85	<0.0001
B^2	−20.59	11.48	3.22	0.0930
C^2	12.66	11.48	1.22	0.2873
D^2	4.41	11.48	0.15	0.7061
$R^2 = 0.915$				

Table 3 ANOVA for enzymatic hydrolysis of bamboo biomass

Factors	DF	SS	MS	F value	p value
Y: reducing sugars (0.915)					
Model	14	5.882E+005	42,014.11	11.63	<0.0001
Residual error	15	54,199.90	3613.33		
Lack of fit	10	53,765.90	5376.59	61.94	0.0001
Pure error	5	434.00	86.80		
Total	19	6.424E+005			

SS sum of squares, DF degree of freedom, MS mean squares

the experiment, as the model possesses a high F value (11.63) (Table 3). The fitness of the model was also expressed by the coefficient of determination; R -squared value of 0.915 was in good agreement with the adjusted R -squared of 0.836. In this case, the R^2 value closer to unity denotes the goodness of fit, showing that good relation existed between the experimental and predicted model. The p value for the model <0.05 indicated that the model was highly significant and insignificant p value for lack of fit indicated that experimental data obtained showed better mutual interactions between the variables (Banik et al. 2007). The response reveals that B , C , D and A^2 are significant model terms with p values of less than 0.05 for reducing sugar production.

Interactions among the factors for reducing sugars production

The 3D response surface plots represent graphically the optimal conditions of each variable for the maximized response (reducing sugar production). The response surface plots shown in Fig. 4a–f represent the interaction effects of cellulase, temperature, pretreated biomass and incubation time on reducing sugar production. As can be observed from Fig. 4a, the maximum sugar production was observed with a relatively high pretreated biomass and the optimum temperature was around the center point. Figure 4b shows that the medium temperature and high cellulase concentration in the hydrolysis medium led to the production of maximum reducing sugars. In other reports, optimal conditions for reducing sugars were obtained at a middle level cellulase concentration (Harinder et al. 2012). Figure 4c indicates that the maximum reducing sugar production was obtained at medium temperature and long incubation time, while other factors were at fixed level. Maximum reducing sugars were obtained at a temperature from 30 to 50 °C with a long hydrolysis time of 72 h. It is possible to increase the yield continuously with biomass as reported in previous studies (Ferreira et al. 2009). When the biomass and cellulase concentrations increase from a low to high level, the productivity also increases, whereas the other factors remain at a fixed level as shown in Fig. 4d. Figure 4e demonstrates that increase in both biomass and incubation time improves the sugar production. The maximum reducing sugar production was observed with a higher level of cellulase concentration and incubation time (Fig. 4f). This result had good agreement with the results reported by Ferreira et al. (2009). The response optimizer tool of Design Expert software was used to identify the optimal conditions: hydrolysis temperature (50 °C), biomass concentration (8.9% w/v), enzyme concentration (199.8 mg/g biomass) and time (60 h) with the maximum reducing sugar recovery of 515 mg/g biomass. The

Fig. 4 3D response surface plots representing the interaction effect of independent variables (cellulase concentration; temperature; pretreated biomass; and incubation time) on the dependent variable (reducing sugar concentration)

Fig. 5 XRD patterns of the untreated, pretreated and enzymatically hydrolyzed biomass

obtained reducing sugars in this study highly correlated with the results of sugar yield from Indian bamboo biomass (Sindhu et al. 2014).

Crystallinity of untreated and treated biomass

The XRD patterns of the untreated, pretreated and enzymatically hydrolyzed biomass are shown in Fig. 5. In all the samples, the characteristic peaks for cellulose in bamboo were observed at 16° and 22° (Jin et al. 2014). The cellulose crystallinity index and crystallite size were

Table 4 Calculated crystallinity index and crystallite size of untreated and treated bamboo biomass from XRD data

Parameter	Untreated	Pretreated	Enzyme treated
Crystallinity index (%)	53.09	56.62	61.06
Crystallite size (nm)	22.8	19.3	10.4

calculated for untreated, pretreated and enzymatically hydrolyzed samples and represented in Table 4. The crystallinity indices of untreated, pretreated and enzymatically hydrolyzed samples were 53.09, 56.62 and 61.06%, respectively. This increasing trend of crystallinity index of cellulose confirmed that the bamboo biomass was modified during the pretreatment process. The crystal size of the cellulose given in Table 4 explained that the crystallite size was reduced from untreated to enzyme-hydrolyzed bamboo biomass. Decrease in the cellulose crystal size revealed that the amorphous content of the cellulose was hydrolyzed during the pretreatment process (see Table 4).

Conclusion

A simultaneous pretreatment method for cellulose hydrolysis was successfully studied. HCl acid pretreatment of bamboo biomass was found to be a better method than other acid treatments with the maximum sugar yield of

619 mg/g of the biomass. RSM optimization of enzymatic hydrolysis conditions showed that acid-treated biomass concentration, cellulase concentration and time had significant effect in this study. The maximum sugar yield of 515 mg/g of biomass was obtained at a hydrolysis temperature of 50 °C, biomass concentration 8.9%, w/v, enzyme concentration 199.8 mg/g biomass and time 60 h, respectively. Further characterization of untreated, pretreated and enzymatically hydrolyzed biomass by XRD showed that enzymatic hydrolysis of acid-treated biomass highly influenced the crystallinity index and crystallite size. The results of this study revealed that simultaneous acid followed by enzymatic hydrolysis of bamboo biomass gives better reducing sugars recovery and can be utilized effectively for biofuel production.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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