Optimization of enzymatic saccharification of microwave pretreated sugarcane tops through response surface methodology for biofuel

Devendra Prasad Maurya, Siddharth Vats, Sudheer Rai & Sangeeta Negi*
Motilal Nehru National Institute of Technology, Allahabad 211 004, India

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The optimization of biomass loading enzyme loading, surfactant concentration and incubation time, using response surface methodology (RSM) and Box Behnken design for enzymatic saccharification of sugarcane tops (SCT) for maximum recovery of fermentable sugars using crude cellulases, resulted in 90.24% saccharification efficiency. Maximum saccharification yield of 0.376 g/g glucose as substrate for ethanol production was observed at optimal conditions of 10% biomass loading (pretreated), 100FPU/g of cellulase loading, 0.04% (w/w) surfactant concentration and 72 h of incubation time.

Keywords: Cellulases, Microwave pre-treatment, Saccharification, Sugarcane top

India is the second largest producer of sugarcane in the world with 350 million metric tons (MMT) of annual production1, and forms important part of staple crops. During harvesting while sugarcane are used for sugar generation, 79.4 MMT of sugarcane tops are left unused in the field and become part of waste biomass. Sugarcane tops (SCT) can be a potent feedstock for bioethanol production especially in tropical countries where sugarcane is cultivated in vast areas2,3. The conversion of lignocellulosic biomass into ethanol involves hydrolysis of cellulose into fermentable sugars by cellulase enzymes and the subsequent conversion of sugars into ethanol. Lignocellulosic materials contain cellulose, hemicellulose and lignin in a complex crystalline structure reducing the efficiency of the hydrolysis by limiting accessibility of the enzymes to cellulose4. Hence, pretreatment processes are required for improving the efficiency of enzymatic hydrolysis by disturbing the crystallinity of cellulose and increasing porosity to make them accessible for the enzymes5. Zhu et al.6,7 reported bioethanol production by combining the microwave radiation with alkali reagents for the pretreatment of rice straw and wheat straw. Enzymatic saccharification of microwave pretreated biomass has been accomplished by using crude cellulases8. Saccharification efficiency depends on several process parameters such as solid loading, enzyme loading, surfactant concentration and incubation time9.

The objectives of this study are to identify the best combination of microwave radiation for the alkali pretreatment of SCT, and its enzymatic saccharification for optimum sugar yield.

Materials and Methods

Determination of biomass composition in SCT—
The compositional analysis of native and microwave assisted alkali pretreated SCT was carried out by two steps dilute acid hydrolysis method following National Renewable Energy Laboratory (NREL) protocol10. Total reducing sugars in the hydrolysates were measured using the dinitrosalicylic acid (DNS) method11.

Microwave assisted alkali pretreatment of biomass—
SCT was obtained from the local market of Allahabad, UP, India. The feedstock was oven-dried (40 °C) to remove moisture and was milled to reduce particle size to pass a 1 mm sieve and stored in sealed plastic bags at room temperature. For microwave assisted alkali pretreatment, samples were mixed with NaOH solution to arrive at a final alkali concentration of 2% (w/v) and biomass loading of 10% (w/v) in a general purpose laboratory microwave oven (LG Electronics India Pvt Ltd, Model MS-2342AE). The apparatus provided microwave radiation using inverter technology. All microwave assisted pretreatments in this study were carried out at optimized power level of 320 W for 10 min, as it

*Correspondent author
Telephone: +91-941501581
E-mail: sn5@mnnit.ac.in
allowed sufficient lengths of pretreatment time without drastic volumetric losses of the liquid phase. After microwave pretreatment slurry was allowed to cool, washed under running tap water till neutralization, and biomass was separated by filtration using a 400 mesh filter screen. Filtered material was air dried at room temperature to remove residual moisture and was used either directly for hydrolysis process or kept at 4 °C until used.

**Enzymatic hydrolysis**—Enzymatic saccharification experiments were performed in duplicate in 150 mL conical flasks containing microwave pretreated SCT, crude cellulases (FPU/g pretreated dry substrate) produced under solid state fermentation by *Trichoderma reesei* RUT C-30 and surfactant (0.1%, w/w Tween 80) in 0.1 M citrate buffer (pH 4.8), which was supplemented with 0.3% (v/v) of sodium azide solution to prevent microbial contamination. Total reaction volume of hydrolysis was made up to 30 mL and component/reaction conditions were altered as appropriate for experiment design. The samples were incubated at 50 °C for 72 h in incubator shaker at (120 rpm). After incubation, samples were centrifuged at 8000 rpm for 10 min to remove the unhydrolysed residues. The hydrolysate was used for reducing sugar analysis by 2, 5-dinitrosalicylic acid method\(^\text{11}\) and yield was expressed in g/g biomass. Filter paper assay was used to estimate total cellulase activity in the crude enzyme preparation according to Ghose\(^\text{12}\) and expressed as filter paper units (FPU).

**Optimization** of saccharification to improve sugar yield—Four independent parameters like biomass loading \((X_1)\), enzyme loading \((X_2)\), surfactant concentration \((X_3)\) and incubation time \((X_4)\) were optimized using RSM\(^\text{13,14}\). A Box–Behnken design was employed to determine the effects of independent variables at three different levels -1, 0 and +1, which corresponded to the low, medium and high values, respectively with 30 experimental runs in three blocks where midpoint is replicated 3 times\(^\text{15}\) (Table 1). The reducing sugar yield was the output factor. System behaviour was analyzed by usually a second order model in response surface methodology\(^\text{16}\).

\[
Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j + \varepsilon
\]

where, \(Y\) is predicted response, \(\beta_0\) is intercept term, \(\beta_i\) is linear coefficient showing linear effect, \(\beta_{ij}\) is quadratic coefficient showing squared effect, \(\beta_{ij}\) is cross product coefficient showing interaction effect, \(X_i\) and \(X_j\) are coded terms for independent input variables under study and \(\varepsilon\) is error factor.

For four variable systems, model equation is given as

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

Regression analysis and coefficient of correlation were analyzed by Design expert software (Version 8.0.7.1, Stat-Ease Inc., Minneapolis, USA). Both the linear and quadratic effects and the possible interactions of the four variables were calculated.

**Results and Discussion**

**Compositional analysis of biomass**—Compositional analysis of sugarcane top was performed in native and pretreated SCT and were cellulose, 31.46, 37.51; hemicelluloses, 21.06, 27.81; and lignin, 23.27%, 7.68% respectively by following NREL protocols. Same study was done by Janu et al.\(^\text{13}\).

**Optimization of enzymatic saccharification of pretreated SCT by Box–Behnken design**—Reducing sugar yields (0.083-0.376 g/g) from different runs represented conversion efficiencies of 21.6 and 90.24% of theoretical maximum (0.382 g/g) respectively. The responses obtained for each experimental run performed according to Box–Behnken design (Table 1) were analyzed by multiple regression analysis and a second order polynomial equation was derived to represent response (reducing sugar yield) as a function of independent variables tested as:

\[
Y = 0.28 + 0.059X_1 + 0.062X_2 - 0.025X_3 + 0.049X_4 - 0.053X_1^2 - 0.068X_2^2 - 0.018X_3^2 - 0.038X_4^2 + 0.021X_1 X_2 + 3.25X_1 X_3 + 0.024X_1 X_4 - 0.038X_2 X_3 + 4.50X_3 X_4 - 0.045X_4
\]

where \(Y\) is predicted response (reducing sugar yield), and \(X_1, X_2, X_3\) and \(X_4\) are coded values of biomass loading, enzyme loading surfactant concentration and incubation time, respectively.

Testing of the model was performed by Fisher’s statical test for analysis of variance (ANOVA) using Design expert software (version 8.0.7.1). ANOVA of quadratic regression model suggested that model was found significant with a computed F value of 11.08 and a \(P\)-value 0.001 (lower than 0.05). \(P\) value of
each of the parameters and their quadratic and interaction terms were also determined by ANOVA test. *P* values less than 0.05 indicates that the model terms were significant and in this case \(X_1, X_2, X_3, X_4, X_3X_4, X_1^2, X_2^2, X_4^2\) (biomass loading, enzyme loading, surfactant concentration, incubation time, interaction effect of surfactant concentration and incubation time, quadratic effect of biomass loading, enzyme loading and incubation time respectively) were found to be significant model terms. There were no statistically significant interactions as could be gauged from *P*-value. Nevertheless, response surface curves were plotted to understand interaction effects of variables and for identifying optimal levels of each parameter for attaining maximal reducing sugar yield. Based on *P*-values and response surface plots, the most important interaction was identified as those between biomass loading and enzyme loading and enzyme loading and incubation time.

Biomass loading is one of the major factors affecting efficiency of enzymatic hydrolysis (Fig. 1a). At low levels of biomass loading, efficiency of enzymatic hydrolysis was higher, since more enzymes were available per unit biomass to be hydrolyzed. It may be due to more free water content, efficient enzyme transport and mass transfer of intermediates. However, the yield of sugars will be less at lower biomass loading, since the maximum available cellulose and hemicellulose was less. Thus a higher biomass loading to an optimum limit was found better for attaining higher sugar concentration and consequently higher ethanol production. But with

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the increase in biomass loading there might be chance of decrease in saccharification efficiencies due to product inhibition and enzymatic inactivation on cellulosic substrate\textsuperscript{19}. Therefore, choice of biomass loading and enzyme loading has to be made very sensibly to improve sugar yield. At low levels of biomass loading and low levels of enzyme loading, the reducing sugar yield was low. Middle level of biomass loading (10\%) and middle level of enzyme loading (100 FPU/g) showed maximum sugar yield.

At middle level of enzyme loading, a higher incubation time was desirable for better sugar yield, but at low levels of enzyme loading, sugar yield was low and with increase in enzyme loading, there was a significant increase in sugar yield till certain level and further increase in the enzyme loading resulted in a decreased sugar yield. This might be due to the feedback inhibition of the product (Fig. 1b). The best yield of sugars was obtained at enzyme loading (~100 FPU/g) and an incubation time of 72 h. Optimal conditions of hydrolysis were predicted using numerical optimization function in Design Expert software. Top five solutions provided by the software (Table 2) had very similar levels of parameters and less error suggesting that the model generated in this study can be applied for studying the hydrolysis of microwave assisted alkali pretreated SCT and other agricultural wastes for production of reducing sugars for bio-ethanol. The results of the present study were compared with other studies for BBD (Table 3) and strengthened the fact that the optimum sugar yield can be achieved through RSM efficiently.

**Conclusions**

Microwave alkali (NaOH) pretreatment of sugarcane top at 320 watts was able to remove 67\% of lignin from biomass, thereby increasing cellulose and hemicelluloses content and making it more susceptible to saccharification. Optimization of saccharification conditions resulted in a high sugar yield (0.376 g/g), which represent 90.24\% of theoretical maximum based on cellulose and hemicellulose content of pretreated biomass. It can be

**Table 2**—Optimal conditions for enzymatic hydrolysis of microwave pretreated SCT.

<table>
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<th>S. No.</th>
<th>Biomass loading (%w/w)</th>
<th>Enzyme loading (FPU/g)</th>
<th>Surfactant concentration (% w/w)</th>
<th>Incubation time (h)</th>
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**Table 3**—Comparative study of hydrolytic yield of different biomass by crude enzymes.

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<th>References</th>
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<td><em>A. awamori</em> + <em>T. reesei</em></td>
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<td>[20]</td>
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<td>Corn stover</td>
<td><em>A. niger</em> + <em>T. reesei</em></td>
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</tr>
<tr>
<td>Sugarcane bagasse</td>
<td><em>Penicillium funiculosum</em> + <em>Trichoderma harzianum</em></td>
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<td>[22]</td>
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<tr>
<td>Wheat straw</td>
<td><em>A. flavus</em> + <em>T. reesei</em></td>
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<td><em>T. reesei RUT C-30</em></td>
<td>90</td>
<td>Present study</td>
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</table>
concluded from the present results that sugarcane top can serve as a potent and sustainable feedstock for bioethanol production due its high content of cellulose and ample availability.

References
15 Box GEP & Behnken DW, Some new three level designs for the study of quantitative variables, Technometrics, 2 (1960) 455.