Enzymatic hydrolysis of chemically pretreated rice straw by two indigenous fungal strains: a comparative study

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This study presents pretreatment of rice straw using ethylene diamine, (EDA 28%), hydrochloric acid (HCl 5%) and sodium hydroxide (NaOH 2%) and on-site enzyme production from two Aspergillus species. Pretreated rice straw was enzymatically hydrolyzed with crude cellulase from A. niger and A. heteromorphus. Enzymatic hydrolysis conditions (pH and temperature) were optimized by RSM model. Maximum activity was found by A. niger (FPase, 7.4 IU/g; CMCase, 97 IU/g) and A. heteromorphus (FPase, 9.5 IU/g; CMCase 125 IU/g) in 72 h with EDA pretreated rice straw followed by NaOH and HCl pretreatment.

Keywords: Cellulase, Lignocellulose, Pretreatment, Aspergillus heteromorphus, A. niger

Introduction
Rice straw is used as animal feed or household fuel, feedstock for paper industry and organic fertilizer. However, most of cellulose content in rice straw cannot be fully and economically utilized in these ways, which also arouses environmental pollution problems subsequently1. Through an enzymatic hydrolysis sub-process, rice straw can be converted to reducing sugars, which can be fermented to ethanol2. If pretreatment is not efficient enough, resultant residue is not easily hydrolysable by cellulase and if it is more severe, result is the production of toxic compounds, which inhibit microbial metabolism3. Cellulase is relatively costly enzyme and it is significant to reduce production cost, cellulase performance improvement for their commercial use4.

This study presents a comparison of chemical pretreatment methods, on-site production of enzyme and enzymatic hydrolysis of pretreated rice straw by Aspergillus spp.

Materials and Methods
All experiments were performed in triplicates and average values were represented. Composition of rice straw or its hydrolyzed residues was expressed on wet basis.

Material
Rice straw [Pusa 44 (Oryza sp.)] was procured from village kalwan, Jind district, Haryana (India). It was sun dried and grinded to fine pieces (1 mm) in grinder and filtered through mesh (1 mm) for further analysis.

Microorganism and Inoculums Preparation
A. niger (Local isolate) and A. heteromorphus MTCC 8625 were maintained at 4°C on potato dextrose agar (PDA) slants, with regular sub-culturing every 3-4 weeks. To prepare inoculums, spores on PDA slant were suspended in 2 ml medium (10^6 spores/ml), and then pipetted into a Erlenmeyer flask (250 ml) containing inoculum growth medium (50 ml) and incubated in an orbital shaker (115±5 rpm) at 30°C. Medium contains: dextrose, 10; peptone, 0.5; MgSO₄.7H₂O, 0.5; and KH₂PO₄, 1 g/1000 ml. Initial pH of medium was adjusted to 7 (1N NaOH or 1 N HCl) before autoclaving at 121°C for 15 min and used for preparation of inoculum for enzymatic hydrolysis.

Pretreatment of Rice Straw
Overnight dried rice straw (size, 1 mm; wt, 10 g) was pretreated with ethylene diamine, (EDA 28%),
hydrochloric acid (HCl 5%) and sodium hydroxide (NaOH 2%) for 72 h at 55°C separately. Solid to liquid ratio is 1:5. Chemically treated rice straw was neutralized with de-ionised water. Chemical solutions were drained and resultant residues dried at 55°C for subsequent analysis.

Experimental Design for Optimization of pH and Temperature

Central composite design (CCD) was used to evaluate main and interaction effects of pH (A) (3-7) and temperature (B) (20-40°C) on release of reducing sugar (Y) obtained from pretreatment and enzymatic hydrolysis experiments (Table 1). A polynomial quadratic equation was fitted to evaluate effect of each independent variable to response as

\[ Y = b_0 + b_1 A + b_2 B + b_{11} A^2 + b_{22} B^2 + b_{12} AB \]

where Y is predicted response; \( b_0 \) is a constant; \( b_1, b_2 \) are linear coefficients; \( b_{11}, b_{22} \) are quadratic coefficients. Response surfaces of variables inside experimental domain were analyzed using Design Expert software.

Enzymatic Hydrolysis

For enzymatic hydrolysis of rice straw with two *Aspergillus* species, inoculum (2 ml) was inoculated into an Erlenmeyer flask (250 ml) containing hydrolysis medium (50 ml), which comprised: pretreated rice straw, 2 g; and Mandel & Strenberg’s mineral media, 50 ml [\( (NH_4)_2SO_4, 1.4 g/l; KH_2PO_4, 2.0 g/l; CaCl_2, 0.3 g/l; MgSO_4, 7H_2O, 0.3 mg/l; FeSO_4.7H_2O, 5.0 mg/l; MnSO_4. H_2O, 1.6 mg/l; ZnSO_4. 7H_2O, 1.4 mg/l; and CoCl_2, 2.0 mg/l]. Initial pH of medium was adjusted to 5 (optimized by RSM method) before being autoclaved at 121°C for 15 min. Culture in Erlenmeyer flask was incubated at 30°C for 5 days. Flasks were removed after regular interval. Culture were filtered through muslin cloth. Filtrate culture broth was centrifuged at 7200 rpm for 10 min and supernatant was analyzed for reducing sugars, FPA (filter paper activity) and CMC (carboxymethylcellulose) values.

Analytical Methods

Cellulose and lignin content was estimated by reported method. Organic carbon was determined by dry combustion method. Total nitrogen was estimated by standard Kjeldahl method. FPA for total cellulose activity in cultural filtrate and endoglucanase activity (carboxymethylcellulose, CMCase) were determined using method of IUPAC. Reducing sugar was determined by DNS method.

Results and Discussion

Characterization and Effect of Pretreatment on Rice straw

Rice straw contained: cellulose 38; lignin, 7; and total organic carbon, 42%. Similar results were found out by Zhang & Zhang and Zhanga & Cai. Pretreatment affects structure of biomass by solubilizing hemicellulose, reducing crystallinity and increase available surface area and pore volume of substrate. Pretreated rice straw was softer than untreated one, coinciding with reported results. Acid pretreatment involves use of sulfuric, nitric, or hydrochloric acids to remove hemicellulose components and expose cellulose for enzymatic digestion. Use of acid to remove hemicellulose has been tried on a wide range of feedstocks (hardwoods, grasses

### Table 1—Experimental design in term of coded factors and results of Box Behnken model

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>A</th>
<th>B</th>
<th>Reducing sugar, µg/g</th>
<th>A. heteromorphus</th>
<th>A. niger</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1.000</td>
<td>-1.000</td>
<td></td>
<td>475.73</td>
<td>386.56</td>
</tr>
<tr>
<td>2</td>
<td>1.000</td>
<td>-1.000</td>
<td></td>
<td>442.37</td>
<td>395.83</td>
</tr>
<tr>
<td>3</td>
<td>-1.000</td>
<td>1.000</td>
<td></td>
<td>445.87</td>
<td>385.91</td>
</tr>
<tr>
<td>4</td>
<td>1.000</td>
<td>1.000</td>
<td></td>
<td>293.51</td>
<td>230.28</td>
</tr>
<tr>
<td>5</td>
<td>-1.414</td>
<td>0.000</td>
<td></td>
<td>496.19</td>
<td>431.45</td>
</tr>
<tr>
<td>6</td>
<td>1.414</td>
<td>0.000</td>
<td></td>
<td>365.55</td>
<td>326.75</td>
</tr>
<tr>
<td>7</td>
<td>0.000</td>
<td>-1.414</td>
<td></td>
<td>463.69</td>
<td>381.64</td>
</tr>
<tr>
<td>8</td>
<td>0.000</td>
<td>1.414</td>
<td></td>
<td>344.05</td>
<td>273.61</td>
</tr>
<tr>
<td>9</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td>980.58</td>
<td>865.25</td>
</tr>
<tr>
<td>10</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td>981.09</td>
<td>869.15</td>
</tr>
<tr>
<td>11</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td>984.78</td>
<td>868.56</td>
</tr>
<tr>
<td>12</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td>982.01</td>
<td>864.98</td>
</tr>
<tr>
<td>13</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td>981.17</td>
<td>866.09</td>
</tr>
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</table>
EDA is an organic solvent and breaks internal lignin and hemicellulose bonds, also solubilizes both fractions, while cellulose remains as solid\textsuperscript{16,17}. Alkali pretreatments remove acetyl and uronic acid substitutions on hemicellulose that lower accessibility of enzyme to hemicellulose and cellulose surfaces\textsuperscript{18}. EDA pretreatment showed highest increase in cellulose content and maximum decrease of lignin and total organic carbon compared to HCl, and NaOH. After EDA pretreatment, cellulose content increased (25.20%) and there was decrease in lignin (70.0%) and total organic carbon (14.2%) in rice straw (Fig. 1). Detroy et al\textsuperscript{19} reported reduction in lignin (60%) and increase in cellulose (30%) by EDA pretreatment and similar trend was observed by Thompson et al\textsuperscript{17}. HCl pretreatment showed cellulose increase (17.13%) and delignification (31.43%). NaOH pretreatment showed cellulose increase (13.70%) and delignification (35.72%).

### Optimization of pH and Temperature

Experimental design for release of reducing sugar indicated (Table 1) that treatments (9-13) showed highest level of total reducing sugar from A. niger and A. heteromorphus at pH 5 and temperature 30°C. Statistical significance of a second order polynomial model equation was evaluated by F-test analysis of variance (ANOVA), which showed that regression is statistically significant in both cases (Table 2) as

Reducing sugar by A. heteromorphus = \( +981.93-46.31*A-44.49*B-276.28*A^2-289.78*B^2-29.75*A*B \) ...(1)

F value implies that model was significant, and coefficient of variation (\( R^2 = 0.99 \)) indicated a high correlation between observed and predicted values. Lack-of-fit statistics, indicated that P-value (0.1291) was not significant. No abnormality was observed from diagnoses of residuals. Thus, model was statistically sound. P-value denoting significance of coefficients was also important in understanding pattern of mutual interactions between variables. Additionally, P-values suggest that independent variables A and quadratic term of B had significant effects on release of reducing sugar by A. niger as

### Table 2—Analysis of variance (ANOVA) for reducing sugar (mg/ml) by A. heteromorphus and A. niger

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares A. heteromorphus</th>
<th>Sum of squares A. niger</th>
<th>DF</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>1.022</td>
<td>8.503</td>
<td>5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>A</td>
<td>17154.66</td>
<td>10769.92</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>B</td>
<td>15130.46</td>
<td>12790.12</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>A(^2)</td>
<td>5.310</td>
<td>4.170</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>B(^2)</td>
<td>5.841</td>
<td>5.093</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AB</td>
<td>3540.25</td>
<td>6724.08</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>40.67</td>
<td>72.41</td>
<td>7</td>
<td>0.1291</td>
</tr>
<tr>
<td>Lack of fit</td>
<td>29.43</td>
<td>57.57</td>
<td>3</td>
<td>0.0731</td>
</tr>
<tr>
<td>Pure error</td>
<td>11.23</td>
<td>14.84</td>
<td>4</td>
<td>Non-significant</td>
</tr>
<tr>
<td>Cor total</td>
<td>1.022</td>
<td>8.504</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

\( R^2 = 0.99 \) (A. heteromorphus), \( R^2 = 0.99 \) (A. niger)
Reducing sugar by *A. niger* =

\[ +866.81 - 30.55 \times A - 39.98 \times B - 244.84 \times A^2 - 270.58 \times B^2 - 41.00 \times A \times B \]  

...(2)

Isoresponse contour and surface plots of responses using Eqs (1) & (2) for release of reducing sugar by *A. heteromorphus* (Fig. 2) and *A. niger* (Fig. 3) depict interactive effects of independent variables on responses. Shapes of response surfaces and contour plots indicate nature and extent of interaction between different factors. Most of fungal growth and their metabolites are suitable for pH 4-6 and at temperature of 30°C.

Enzymatic Hydrolysis of Rice straw by *A. niger* and *A. heteromorphus*

There were four different periods (lag phase, exponential phase, stationary phase and death phase) appearing during production of FPA, CMCase and reducing sugars, same as that during microbial growth. As for the production of FPA, CMCase and reducing sugars came into exponential phase up to 72 h and then entered into stationary phase. When rice straw was used as a substrate, both fungi grew better at pH 5 and at 30°C. When rice straw was pretreated with different reagents, amount of total reducing sugar, FPA and CMCase activity increased. Maximum reducing sugar was released after 72 h of enzymatic hydrolysis from substrate pretreated with EDA (2856.21 µg/g with *A. heteromorphus* and 2665.65 µg/g with *A. niger*) followed by HCl and NaOH (Fig. 4). Reducing sugar released
after 72 h by HCl and NaOH pretreatments, respectively, was: *A. heteromorphus*, 2598.2, 2489.9 µg/g; and *A. niger*, 2355.43, 2243.54 µg/g. There was gradual increase in FPA up to 72 h, thereafter decrease in activity (Fig. 5). Maximum FPA was shown by EDA pretreated rice straw followed by HCl and NaOH pretreatments. Same pattern was shown by CMCase activity (Fig. 6). Maximum FPase activity was by *A. heteromorphus* (9.5 IU/g) and by *A. niger* (7.4 IU/g) in 72 h with EDA pretreatment. Maximum CMCase by *A. heteromorphus* (125 IU/g) and by *A. niger* (97 IU/g) in 72 h with EDA pretreatment followed by NaOH and HCl pretreatment. *A. heteromorphus* showed higher amount of total reducing sugar, FPA and CMCase activity as compared to *A. niger*.

**Conclusions**

Rice straw could provide an economical advantage as a solid substrate as well as carbon source for production of cellulase enzyme by using fungus strain, *A. heteromorphus* and *A. niger*. Factors affecting enzymatic hydrolysis [pH (5) and temp. (30°C)] were optimized by RSM method. In optimum conditions, production of FPA, CMCase, reducing sugar and saccharification in hydrolysis process of different pretreated rice straw showed best results with EDA pretreatment.

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**References**


