Enzymatic saccharification of cellulosic waste by cellulase system of *Cellulomonas uda* immobilized on tri(4-formyl phenoxy) cyanurate

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Cellulose is one of the most abundant non-degradable organic compound on earth. Near about half of the municipal and agricultural solid wastes contain cellulose or their derivatives. Saccharification or enzymatic hydrolysis of cellulosic wastes liberates glucose. In the present work, cellulase from *Cellulomonas uda* was extracted, partially purified by dialysis and immobilized on an organic support namely tri(4-formyl phenoxy) cyanurate. Percentage saccharification of seven different cellulosic waste materials was studied using native and immobilized cellulase systems. Maximum saccharification for both native and immobilized cellulase was observed for sawmill dust or wood dust (4.9 and 2.4% respectively) as compared to other cellulosic waste substrates.

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Cellulose is a linear homopolymer of glucose units with 1,4-β-D glucosidic linkages, although recent researchers considered it as disaccharide13. It is a product of solar energy, photosynthesized by farm crops, trees and other vegetation. Due to its high insolubility and polymeric nature, much of it is discarded later as waste. It is of no direct food value to animals, but cell free cellulases solubilize the cellulose polymer to provide simple sugars4. Therefore, cellulose in nature as well as in municipal and agricultural wastes has a special significance as an alternative source of fuel and feed stock5. In India, it is predicted that by the year 2047 there will be generation of about 300 million tons per annum of municipal solid wastes containing cellulose and its derivatives5.

The generated cellulosic waste is usually collected by municipal organizations and disposed off in open field or by incineration. These traditional practices emit greenhouse gases like carbon dioxide and methane, polychlorinated *p*-dioxins and dibenzofurans thereby creating problems of health and hygiene including high risk of bladder, brain and hepatobiliary cancers in children27-10. In nature, cellulose remains associated with xylan or lignin. Its density and complexity makes it highly resistant to hydrolysis and requires an association of enzyme activities of cellulases (endo and exo-) acting in concerted manner11-13.

In present investigations, the property of cellulase extracted from *Cellulomonas uda* is exploited to saccharify the cellulose from seven different cellulose containing substrates using tri(4-formyl phenoxy) cyanurate as a support for immobilization to convert these substrates into simple sugar. Reversible binding of enzyme at three different sites to the support, prompted the selection of this compound for immobilization.

**Experimental Procedure**

**Harvesting, extraction and partial purification of cellulase**

The bacterial species *Cellulomonas uda* (NCIM, 2353, Pune) as a source of cellulase was procured from the National Chemical Laboratory, Pune, India. The *C. uda* was cultivated in the half strength nutrient broth (HiMedia M, 244S) containing beef extract 1, NaCl 0.5 and peptone 1% in 100 mL distilled water. In 100 mL nutrient broth, 1 g of solve filter paper strips (Kalpi Paper Industry, Haryana) were also added as a secondary carbon source. The *C. uda* was cultivated in the half strength nutrient broth (HiMedia M, 244S) containing beef extract 1, NaCl 0.5 and peptone 1% in 100 mL distilled water. Temperature of the nutrient broth (transferred in 500 mL conical flask) was maintained constant at room temperature (25 °C)
continuously for 7 days. The pH of the culture media was measured everyday and corrected at 7.2 both by pH paper and by pH meter (Elico) with dropwise addition of alkali (0.1 N NaOH). After the proper enrichment, the nutrient broth was filtered through filter paper (Whatman No 1) and filtrate was subjected to centrifugation in cooling centrifuge (Remi) at 15,000 rpm maintained at 4 °C for 20 min so as to remove bacterial cell mass. The supernatant was collected, to this ammonium sulphate was added slowly (70% w/v fraction) with continuous stirring to obtain saturated solution for the precipitation of protein/enzyme. The precipitate was allowed to settle in a refrigerator for overnight. The remaining supernatant again ammonium sulphate (70% w/v) was added to reprecipitate out the enzyme in the form of pellets16. The pellets were dissolved in 5 mL of sodium citrate buffer (0.1 M, pH 5.2) and dialyzed against the same buffer (0.05 M) for 48 h, so as to purify the enzyme partially. The resulting solution was stored at 4 °C. The protein/enzyme concentration was determined by Folin Lowry method17 and was estimated at 0.80 mg/mL.

Saccharification of different cellulosic waste as substrate by cellulase

The different cellulosic substrates selected for enzyme assay were wood dust (sawmill dust), dried and fresh grass, newspaper, cellulose powder, carboxy methyl cellulose and cotton. Cellulose powder and high viscosity carboxy methyl cellulose (s.d. Fine Chemicals Ltd., Mumbai) were used without further purification. The enzymatic assay for native cellulase was performed using 50 mg of each of the above mentioned substrates. 1 mL partially purified enzyme was added to the substrate (presoaked in 5 mL of 0.1 M sodium citrate buffer at room temperature) taken in series of 25 mL capacity beakers and the beakers were kept in shaking water bath (at 100 rpm) for 90 min maintained at temperature 50 °C. After the incubation period, the reaction mixture was filtered to remove unreacted substances. The resulting filtrate of each substrate was used for the estimation of sugar by standard method18 (Table 1).

Immobilization of cellulase on tri(4-formyl phenoxy) cyanurate

2 g of tri(4-formyl phenoxy) cyanurate was mixed with 13.5 mL enzyme solution (0.80 mg/mL) and stirred continuously for 30 min. The amino group of enzyme was made to bind reversibly to the aromatic aldehyde groups of aromatic tri(4-formyl phenoxy) cyanurate to form Schiff base21.

The mixture was filtered through (Whatman No 1) filter paper. The protein concentration of unbound enzyme was estimated and found to be 0.14 mg/mL22. The amount of immobilized enzyme bound to 2 g of support was estimated as 0.66 mg. For the assay of immobilized cellulase approximately 1 g of immobilized enzyme was incubated with 50 mg of different cellulosic substrates with 2 mL of 0.1 M sodium citrate buffer (pH 5.2) for 90 min at 50 °C in a shaking (100 rpm) water bath. After stipulated time, the mixture was filtered and filtrate was used to estimate the amount of sugar produced by the immobilized cellulase system (Table 2).

Results and Discussion

The experimental results and observations for saccharification of seven different cellulose containing substrates using native and immobilized enzyme are depicted in Tables 1 and 2 respectively. The saccharification of wood dust (sawmill dust) was observed more 4.9 and 2.45% as compared to other substrates for both the native and immobilized enzymes, respectively. The high percentage of saccharification in saw mill dust may be attributed to the presoaking of substrate with sodium citrate buffer that make the substrate to swell, thereby, expanding.
the capillary structure and increase the surface area of cellulose fibers and make the substrate more accessible to enzyme. Another reason for high saccharification seems to be related to amorphous nature of sawmill dust (depending on type of wood) rendering susceptibility to hydrolysis\(^2\). Saccharification of fresh and dried grass revealed that fresh grass (wet) is more susceptible to hydrolysis than dried grass (2.17 and 1.67\% respectively). In dried condition, the cellulose fibers become densely packed and render crystalline nature to the substrates which may lower the hydrolysis rate.

Carboxy methyl cellulose (CM-cellulose) revealed decrease in percentage saccharification (0.92\%). This is due to the fact that this substrate was highly viscous in which primary and secondary hydroxyl groups are not replaced completely with more reactive groups. Thus, instead of rendering amorphous nature to the substrate the crystallinity may be more, thereby, making CM cellulose resistant to hydrolysis.

Newspapers contain the high content of short chain length oligomers and hemicelluloses. These short oligomers and less crystalline structure may disrupt the enzyme substrate interactions thereby lowering the rate of hydrolysis. Another reason may be attributed to the fact, that, newspapers may contain non-glucose sugars like arabinose, galactose, xylose which are resistant to hydralysis\(^2\).

Table 1—Enzymatic saccharification of different cellulosic waste substrates by native cellulase (pH 5.2, Temp. 50 °C, incubated for 1.5 h at 100 rpm).

<table>
<thead>
<tr>
<th>Sr no</th>
<th>Cellulosic waste substrates</th>
<th>Sugar yield/50 mg dry weight of substrate (mg)</th>
<th>% Saccharification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wood dust (sawmill dust)</td>
<td>2.45</td>
<td>4.91</td>
</tr>
<tr>
<td>2</td>
<td>Fresh grass</td>
<td>0.96</td>
<td>1.93</td>
</tr>
<tr>
<td>3</td>
<td>Dried grass</td>
<td>0.52</td>
<td>1.04</td>
</tr>
<tr>
<td>4</td>
<td>Carboxy methyl cellulose</td>
<td>0.46</td>
<td>0.92</td>
</tr>
<tr>
<td>5</td>
<td>Newspaper</td>
<td>0.42</td>
<td>0.84</td>
</tr>
<tr>
<td>6</td>
<td>Cellulose powder</td>
<td>0.41</td>
<td>0.83</td>
</tr>
<tr>
<td>7</td>
<td>Cotton</td>
<td>0.38</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Table 2—Enzymatic saccharification of different cellulosic waste substrates by cellulase immobilized on tri (4-formyl phenoxy) cyanurate (pH 5.2, Temp. 50 °C; incubated for 1.5 h at 100 rpm)

<table>
<thead>
<tr>
<th>Sr no</th>
<th>Cellulosic waste substrates</th>
<th>Sugar yield/50 mg dry weight of substrate (mg)</th>
<th>% Saccharification</th>
<th>Immediate</th>
<th>After 20 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wood dust (sawmill dust)</td>
<td>1.22</td>
<td>2.45</td>
<td>2.40</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Fresh grass</td>
<td>1.08</td>
<td>2.17</td>
<td>2.14</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Dried grass</td>
<td>0.83</td>
<td>1.67</td>
<td>1.62</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Carboxy methyl cellulose</td>
<td>0.60</td>
<td>1.20</td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Newspaper</td>
<td>0.48</td>
<td>0.97</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Cellulose powder</td>
<td>0.48</td>
<td>0.97</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Cotton</td>
<td>0.48</td>
<td>0.97</td>
<td>0.87</td>
<td></td>
</tr>
</tbody>
</table>

Cotton remain associated with variety of polysaccharides like hemicelluloses, xylan and lignins. The microfibrils of cotton have protective layers of lignin and xylan which may have the property of resistance to enzymatic hydrolysis\(^2\). Repetitive use of immobilized cellulase

The cellulase immobilized on tri(4-formyl phenoxy) cyanurate was stable for 20 days without significant loss of activity. After 20 days, however, this preparation demonstrated a rapid drop in activity. This may be due to the discharge of enzyme from the supporting material during the course of reactions\(^2\).

Conclusions

Experimental results revealed that cellulose hydrolysis is not possible in a single step by single enzyme. For complete hydrolysis/saccharification synergistic action of complex enzyme system like endo-1,4, \(\beta-D\) - glucanase exo-1,4- \(\beta-D\) glucanase and \(\beta-D\)-glucosidases is necessary.

The tri(4-formyl phenoxy) cyanurate have multipoint attachment sites for the reversible binding of enzyme and can be used as a carrier material for immobilization of cellulase. This immobilized system can be extrapolated for continuous production of glucose from cellulose containing substrates and at the same time can be recycled for longer duration as compared to native cellulase.
The immobilized preparation can be applied economically if much better ability of repetitive use are obtained, after knowing the origin of activity loss. The work is in progress to find out the reasons of activity loss.

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References