Evaluation of different pretreatments to enhance degradation of pine needles by *Aspergillus niger* F7 under solid state fermentation

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This study presents degradation enhancement of pine needles by *Aspergillus niger* F7, isolated from soil. A modified alkali pretreatment [NaOH+H$_2$O$_2$ (1M); ratio, 9:1] is found the best among all methods when needles are soaked in this solution for 2 h followed by thorough washing with tap water. Degradation of pine biomass was measured in terms of enzyme (cellulase & xylanase) production, biodegradation index (BI) and hydrolysis (%). There are high enzyme units, BI and hydrolysis (%) in pretreated material as compared to untreated one.

**Keywords:** Biodegradation, Lignocellulose, Pretreatment, Solid State fermentation

**Introduction**

Pine (*Pinus roxburghii* Sarg syn. *P. longifolia* Roxb.) is a predominant forest species, widely scattered in Alpine range globally. Accumulation of pine needles (PNs) on forest floor leads to infertility of soil and forest fire\(^1\). PNs being rich in cellulose can be used as substrate for biodegradation. Among different physical and chemical pretreatment methods, solid state fermentation (SSF) of lignocellulosic material holds several advantages as compared to submerged fermentation (SmF). In SSF, enzymes produced are many folds more than SmF and thus it has direct impact on biodegradation of biomass\(^2\). This study presents pretreated PNs as a substrate for degradation under SSF by a potential isolate to enhance hydrolysis of PNs.

**Experimental Section**

**Extractives of Pine Needles (PNs)**

PNs were collected from different forest site of northern India. Components [holocellulose (cellulose + hemicellulose), lignin and other extractives] of PNs were estimated by following standard methods of Technical Association of Pulp and Paper industry (TAPPI). For alcohol benzene extraction, oven dried PNs (2 g) were placed in a porous thimble and extractives were derived by TAPPI method\(^3\). For holocellulose extraction, oven dried PNs (5 g), pre-extracted with alcohol benzene, were taken in 250 ml conical flask, distilled water (160 ml) was added and holocellulose was estimated following TAPPI method\(^4\). For lignin extraction, oven dried PNs (2 g), pre-extracted with alcohol benzene, were treated with 15 ml of 72% sulphuric acid for 2 h at 18-20°C with constant stirring following TAPPI method\(^5\).

**Pretreatment of Pine Needles (PNs)**

**Grinding**

Chipping of PNs gave small pieces, which were ground (mesh size, 1.5 mm), soaked in water for 24 h, and then air dried for 24 h, followed by drying at 50°C overnight. Completely dried biomass was stored in air tight containers.

**Alkali Pretreatments**

In NH$_3$ pretreatment\(^6\), PNs (10 g) were soaked in 100 ml of 1% ammonia solution. Under NH$_3$ pretreatment (modified), PNs (10 g) were soaked in 100 ml of 5% ammonia solution for 2 h at room temperature (RT) and autoclaved for 15 min. After thorough washing with tap water (until solution became neutral) and dried at 50°C. Under NaOH+ H$_2$O$_2$ pretreatment (modified), PNs (10 g) were soaked in NaOH+ H$_2$O$_2$ solution (9:1) for 2 h at RT followed by washing with tap water and dried at 50°C.

**Acid Pretreatment**

In hydrochloric acid (HCl) pretreatment\(^6\), PNs (10 g) were soaked in 1% HCl solution (100 ml) for 2 h.
Estimation of Reducing Sugars and Soluble Proteins

Reducing sugars produced during degradation of PNs were estimated\textsuperscript{11}. Soluble proteins formed during biodegradation were quantified by Lowry's method\textsuperscript{12}.

Biodegradation Index (BI)

BI\textsuperscript{11} of PNs is calculated as $BI = \frac{[\text{reducing sugar (\% released} + \text{protein (\% formed)}]}{2}$.

Hydrolysis (%)

Hydrolysis\% is calculated on dry matter basis as\textsuperscript{14}

\[
\text{Hydrolysis (\%)} = \frac{\text{Total reducing sugar (g) \times 0.90 \times 100}}{\text{Weight of substrate (g)}}
\]

Statistical Analysis

Completely randomized design was applied. Different regression models (linear, power, exponential and quadratic) were used to predict hydrolysis (\%) and BI activities on the basis of enzyme activities in two mediums (water and modified BSM).

Results and Discussion

Estimation of Different components in Pine Needles (PNs)

Analysis of untreated biomass of PNs gave: holocellulose, 57.00; lignin, 23.00; and extractives (alcohol, benzene, fibers, resins etc.), 20.00\% (Table 1). For efficient biodegradation of holocellulose of PNs, different pretreatments were given to wash lignin and extractives out of PNs. Holocellulose was found highest in alkali treated biomass of PNs (5% NH\textsubscript{3}, 88.92; NaOH+H\textsubscript{2}O\textsubscript{2}, 87.10; and 1% NH\textsubscript{3}, 85.87\%). Maximum lignin (19\%) was retained by HCl pretreated material while lowest (4.63\%) was in 1% NH\textsubscript{3} pretreated needles. Extractives were: 1% NH\textsubscript{3}, 9.50; 5% NH\textsubscript{3}, 5.55; and NaOH+H\textsubscript{2}O\textsubscript{2}, 6.00\%. Similar studies showing an increase in cellulose contents and decrease in lignin after pretreating wood biomass has earlier been reported\textsuperscript{15}. Thus different pretreatments temper lignin shield and take it out of lignocellulosic materials, thereby exposing most of the cellulose in active form for better enzymatic digestion.

All pretreated PNs though have shown higher saccharification with enzymes secreted from \textit{A. niger} during degradation but their BI and hydrolysis\% values vary from treatment to treatment (Table 2). SSF of pretreated and untreated materials was carried out under substrate: moisture ratio (1: 2), which has been optimized for other lignocellulosic forest wastes\textsuperscript{7}. Moistening agents (tap water and modified BSM) were used during SSF with an ultimate aim to enhance biodegradation of PNs.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Holocellulose</th>
<th>Lignin</th>
<th>Extractives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>57.00</td>
<td>23.00</td>
<td>20.00</td>
</tr>
<tr>
<td>1%NH\textsubscript{3}</td>
<td>85.87</td>
<td>4.63</td>
<td>9.50</td>
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<tr>
<td>5%NH\textsubscript{3}</td>
<td>88.92</td>
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<tr>
<td>1%HCl</td>
<td>70.15</td>
<td>19.00</td>
<td>10.85</td>
</tr>
<tr>
<td>1%H\textsubscript{2}SO\textsubscript{4}</td>
<td>77.50</td>
<td>12.50</td>
<td>10.00</td>
</tr>
<tr>
<td>1%NaOH+H\textsubscript{2}O\textsubscript{2}</td>
<td>87.10</td>
<td>6.90</td>
<td>6.00</td>
</tr>
</tbody>
</table>

Table 1—Estimation of holocellulose and lignin in untreated and pretreated biomass of pine needles using TAPPI standard method

In sulphuric acid (H\textsubscript{2}SO\textsubscript{4}) pretreatment\textsuperscript{6}, PNs (10 g) were soaked in 1% H\textsubscript{2}SO\textsubscript{4} solution (100 ml) for 2 h.

Biodegradation of Pine Needles (PNs)

\textit{Aspergillus niger} \textit{F}_{1}, capable of producing high amount of hydrolytic enzymes (cellulase and xylanase), was procured from Microbiology laboratory of Basic Sciences, UHF Nauni, Solan (India). Biodegradation of PNs was studied under SSF by using water and modified basal salt medium (BSM) (1: 2). Modified BSM\textsuperscript{7} contained Na\textsubscript{2}HPO\textsubscript{4} (6.0 g), KH\textsubscript{2}PO\textsubscript{4} (3.0 g), NaCl (0.5 g), NH\textsubscript{4}Cl (1.0 g) and separately sterilized solutions of 1 M MgSO\textsubscript{4} (2 ml) and 1 M CaCl\textsubscript{2} (0.1 ml) were added after medium was autoclaved. It was supplemented with urea (2\%), yeast extract (1\%), peptone (0.1\%), NaNO\textsubscript{3} (0.1\%), 1M CoCl\textsubscript{2} (0.2/l) with pH 6.80 to final volume of 1000 ml. To each 20 g of untreated and pretreated biomass of \textit{P. roxburghii}, water (35 ml) and of inoculum (5 ml) containing 1x10\textsuperscript{7} spores/ml of \textit{A. niger} were added in 500 ml of Erlenmeyer flask. Flasks were incubated for 30 days at 28 ± 2°C.

Extraction of Enzymes

Hydrolytic enzymes and other fermented products produced during biodegradation of PNs were extracted by Repeated Extraction Method\textsuperscript{8}. To 5 g of biomass, 50 ml of phosphate buffer (0.1M, pH 6.9) was added in 250 ml Erlenmeyer flask and contents were kept at 120 rpm for 1 h and then filtered through muslin cloth. The process was repeated twice with additional 25 x 2 ml of phosphate buffer making final volume of extracted products to 100 ml. After filtration, contents were centrifuged at 5000 rpm for 5 min at 4°C. Supernatant was collected to estimate enzymes, biodegradation index (BI) and hydrolysis (\%). Enzyme assays were performed to quantify CMCase\textsuperscript{9}, FPAAse\textsuperscript{8} and ß-glucosidase of cellulase\textsuperscript{10} and xylanase\textsuperscript{11} activities.

Statistical Analysis

Completely randomized design was applied. Different regression models (linear, power, exponential and quadratic) were used to predict hydrolysis (\%) and BI activities on the basis of enzyme activities in two mediums (water and modified BSM).

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When water was used as moistening agent, degradation was very low in case of untreated biomass as follows: enzyme production, 28.16 U/g; BI, 7.16; and hydrolysis, 0.89%. Among pretreatments, NaOH+H₂O₂ pretreated PNs had led to maximum values as follows: cellulase and xylanase production from *A. niger*, 96.14 U/g; BI, 27.76; and hydrolysis, 7%. Biodegradation carried out with HCl pretreated biomass of PNs gave minimum values as follows: enzyme production, 44.91 U/g; BI, 8; and hydrolysis, 1.22%. When modified BSM was used as moistening agent, NaOH+H₂O₂ pretreated PNs have led to maximum cellulase and xylanase production from *A. niger* (259.6 U/g), thus resulting in highest BI (53.30) and hydrolysis 12.87%. Acid pretreatments showed comparatively lower production of enzymes, BI and hydrolysis% as compared to alkali pretreatments. On the other hand, untreated biomass in modified BSM also has shown least production of enzyme as compared to all pretreated biomass of PNs, consequently resulting in marginally low degradation as follows: enzyme production, 95.40 U/g; BI, 13.45; and hydrolysis, 1.85%.

Since PNs are exceptionally inert biomass for biodegradation, therefore alkali pretreatment of PNs [NH₃ (1%, 5%) and NaOH + H₂O₂] has been chosen with an idea of removing maximum lignin and other hindering substances like resins etc. Alkali pretreatment is reported to decrease crystallinity of cellulose, remove lignin shield around cellulose and increase pore size of biomass, thus increasing digestibility of lignocelluloses. Compared with acid or oxidative reagents, alkali pretreatment appears to be the most effective methods in breaking ester bonds between lignin, hemicellulose and cellulose and avoiding fragmentation of hemicellulose polymers. Alkaline pretreatment in combination with H₂O₂ (NaOH + H₂O₂) additionally promotes to loosen the linkage of hydrogen bonds, resulting in easy enzymatic hydrolysis of biomass. Alkaline pretreated biomass is hydrolyzed 40% faster than native cellulose. Under acid treatment of lignocellulosic materials, sulphuric acid is the most applied acid and found most effective in dissolving lignin, and thus increasing cellulose’s susceptibility to enzymatic attack. Pretreatment consists of collection, transportation, manipulation, storage, grinding or chipping to reduce particle size and opening fibrous material in order to transform it into a suspension that can be pumped and enable further penetration of chemical hydrolysis agents.

Overall an appreciable increase has been observed in pretreated PNs as compared to untreated ones. When water was used as moistening agent, in acidic pretreatment (HCl & H₂SO₄), increase was observed as follows: enzyme activity, 59.48, 81.85%; BI, 11.73, 25.69% and hydrolysis, 37.07, 57.30%. In NaOH + H₂O₂ pretreated PNs, increase was observed as follows: enzyme, 241.40; BI, 287.70; and hydrolysis, 686.51%. When modified BSM was used, in NaOH + H₂O₂ pretreated PNs, increase was observed as follows: enzyme production, 172.11; BI, 296.28; hydrolysis, 595.67% (Fig. 1). A positive correlation has been derived between enzyme activity, BI and hydrolysis% of PNs. Thus when enzyme activity increases, BI and hydrolysis% also increases (Fig. 2). Parameters of various regression models and R² for estimation of BI, hydrolysis% and enzyme activities show a direct correlation between these parameters (Table 3). Different regression models were tried and higher value of R² was found in quadratic model ($Y = a + bx + cx^2$) for prediction of BI activities on the

<table>
<thead>
<tr>
<th>Treatments</th>
<th>H₂O as moistening agent</th>
<th>BSM as moistening agent</th>
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<tr>
<td></td>
<td>Total Enzyme</td>
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<tr>
<td></td>
<td>U/g</td>
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<tr>
<td>NaOH+H₂O₂</td>
<td>259.60</td>
<td>53.30</td>
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</table>

U/g (on dry matter) of hydrolytic enzymes.
Fig. 1—Comparison in pretreated pine needles by *Aspergillus niger* F, using water and modified BSM as medium over untreated pine needles of increase in: a) Total enzyme activity; b) BI; and c) Hydrolysis%
Fig. 2—Correlation between enzyme activity, B.I. and per cent hydrolysis of pine needles after SSF with *Aspergillus niger* F using: a) water as medium; and b) Modified BSM as medium.

Table 3—Parameters of various models to predict enzyme activity, BI and hydrolysis%.

| Characters | Prediction model for BI activities and per cent hydrolysis (Y) on the basis of enzyme activities | R² | a | b | a | b | a | b | a | b | a | b | a | b | a | b | a | b | a | b | a | b | a | b |
| Water     | Y=+bX                                                                         | R² | -6.48 | 4.37 | 0.95 | 5.11 | 1.21 | 0.92 | 2.264 | 1.183 | 0.915 | 1.204 | 1.444 | 0.968 | 0.196 | 0.920 |
| BSM       | Y=+bX                                                                         | R² | -2.66 | 1.121 | 0.90 | 1.82 | 0.66 | 0.85 | 0.01 | 0.22 | 0.86 | -0.289 | 0.339 | 0.93 | 1.82 | 0.664 | 0.85 |

X=Enzyme activity, Y=BI/hydrolysis, R²=COefficient of determination

Thus quadratic model can be used for the prediction of enzyme activities, BI and hydrolysis%. Thus it has been established that with increase in extracellular cellulase and xylanase production from hydrolytic microorganisms, biodegradation of PNs is enhanced.

Though biodegradation of lignocellulosic wastes like agricultural biomass (corn cob, corn straw, bagasse) and
other forest residues has already been reported\(^2\), but successful biodegradation of PNs are rarely reported. This study strongly proves that pretreated PNs under SSF with \textit{A. niger} F\(_7\) can serve as an inexpensive substrate for its saccharification into fermentable sugars, which in turn can be fermented to ethanol to be used as biofuels.

**Conclusions**

PNs, which are highly resistant to biodegradation, can be degraded successfully with \textit{A. niger} after suitable pretreatment. Modified alkali pretreatment [NaOH+H\(_2\)O\(_2\) (1M); ratio, 9:1] in 2 h followed by steam explosion for 15 min has been found the best pretreatment for PNs hydrolysis by a hypercelulolytic isolate, \textit{A. niger} F\(_7\). Modified BSM mediated SSF was found better over tap water. A positive correlation is drawn in three parameters (enzyme activity, BI and hydrolysis%) and has been proved statistically by using regression model.

**References**