Production of Xyloglucanases from Three Species of Filamentous Fungi - *Fusarium equiseti*, *Aspergillus terreus* and *Cephalosporium sp.*

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**Abstract**

Agro-industrial residues containing ligno-cellulosic material have attracted wide attention in recent years as raw material for the production of many industrial enzymes. Three filamentous fungi viz. *Fusarium equiseti*, *Aspergillus terreus* and *Cephalosporium sp.* capable of producing xyloglucanase, using tamarind seed powder as the sole organic carbon source for growth, were isolated from soil samples collected near Bangalore. The present work aims at production of xyloglucanases from these organisms and alteration of production conditions for enhancement of activity. Czapek Dox medium produced higher activity of 97.2, 67.2 and 65.2 U/hr/ml culture filtrate from *Aspergillus terreus*, *Fusarium equiseti* and *Cephalosporium sp.* respectively. The enzyme activity of *Cephalosporium sp.* and *A. terreus* XG increased by 3.45 and 1.5 folds in the presence of 40 mM Mg²⁺ while in *F. equiseti*, XG activity increased 3.35 fold in the presence of 60 mM Mg²⁺. All three enzymes were secretory while the xyloglucanase from *A. terreus* was identified to be membrane bound.

**Keywords:** Xyloglucanase, tamarind xyloglucan, *F. equiseti*, *Cephalosporium sp.*, *A. terreus*.

**Introduction**

Xyloglucans represent a major group of polysaccharides in the primary cell wall of dicots and several monocots. They have a cellulose-like backbone of β-1, 4-linked β-D-glucopyranose residues. Glucose residues are linked to xylose at O-6 position. The xyloside groups, in turn, can be substituted by residues of D-galactopyranose attached by β-1,2-bonds and L-fucopyranose attached by α-1,2-bonds. The glucose backbone structure is typically substituted by 50 or 75% xylose with varying extents of xylose substitution. This backbone can be cleaved by few endoglucanases which act upon a range of other substrates, including cellulose and various β-glucans. Recently few xyloglucan specific endoglucanases have been reported. Xyloglucanases (XG) have numerous applications viz. conversion of the plant waste, modification of xyloglucans to endow rheological properties in food and feed industries, treatment of the fabrics to change its brightness and color, to remove the fuzz from the surface of textile materials in textile industry and in pulp and paper industry. To facilitate widespread use of this enzyme, there is a need to bring down its cost of production.

Use of cheaper substrates is a common approach to reduce production costs. South India produces several lakhs of tons of tamarind seeds as an agricultural by-product. The seeds are rich in polysaccharide (65-72%). Structurally, tamarind seed polysaccharide is a galactoxyloglucan which is a linear polymer of β-1, 4 linked glucose having 1, 6 – linked galactose and xylose branches. Hence it can be a good substrate for production of specific, efficient and cheap xyloglucanases. The present work aims at comparing XG produced from three different genera of filamentous fungi and altering production conditions to enhance their activity.

**Material and Methods**

**Chemicals and Substrate:** All chemicals and media components used in this study were of analytical grade. For enzyme assay, tamarind xyloglucan was purchased from Megazyme, Ireland.

Tamarind seed powder (TSP), which was used as the carbon source for isolation and production of XG, was prepared as per the procedure described by Rao and Srivastava. It was pretreated by suspending 1.0 g powder in 100 ml of distilled water, boiled for 10 min and centrifuged at 10,000×g for 10 min. The supernatant (TSPS) was used as carbon source for the growth of the fungi. Total carbohydrate content of TSPS was determined by phenol-sulphuric acid method. It varied between 50-60%. One gram of TSP was equivalent to 98 ml of TSPS.

**Organism and Culture Conditions:** Fungi present in environmental samples collected from Bangalore were isolated by serial dilution method. Samples of wood, leaf litter, compost and cow dung were suspended in sterile distilled water, serially diluted and a suitable dilution was used for inoculation. Czapek Dox (CD) agar (pH 4.5±0.2), where dextrose was replaced by 0.2% TSP as the sole carbon source and amended with yeast extract (0.02 %) was used for isolation. All plates were incubated at 28±2°C for 72 hrs. The isolated colonies were individually subcultured onto the same medium and screened for positive XG producers as per the procedure described by Kasana et al by comparing the enzyme index (EI). Fast growing filamentous fungi that produced halo on agar plate were selected. The fungal isolates were maintained on
Sabouraud’s Dextrose agar slants.

**Fermentation Studies:** Enzyme production was carried out by submerged fermentation using 50 ml medium in 250 ml Erlenmeyer flasks. A spore suspension of $10^5$ spores/ml was used as inoculum and the flasks were incubated at 28 ± 2°C, 150 rpm. The culture filtrate was used as the source of enzyme for estimating activity. All experiments were carried out in triplicates and results presented as mean ± standard deviation.

**Effect of Culture Media on XG Production:** Two different media were used to test for optimal production: CD broth$^1$ and Mandels and Weber (M and W) broth$^3$, pH 4.5 ± 0.2, amended with 1% TSP as the sole carbon source were used and fermentation carried out as described earlier. Results were compared by ANOVA using MSTATC software.

**Optimization of Fermentation Time:** The medium that showed better activity was chosen for further studies. Aliquots of culture filtrate were removed at intervals of 24h for a period of seven days to identify optimal fermentation time for enzyme production.

**Enzyme Localization:** Enzyme localization studies were performed to identify whether the enzyme was membrane bound or intracellular. The entire mycelia obtained after filtration of the fermentation broth, was homogenized with chilled acetate buffer (0.05M, pH 4.5) and centrifuged at 8000 rpm for 10 min. The pellet was resuspended in the buffer. The activity was determined with the supernatant and the resuspended pellet fractions.

**Enzyme Assay:** Tamarind xyloglucan (TXg) (p-Xyloglucan, Amyloid, Megazyme, Ireland) was used as the substrate for all assays. XG activity was assayed by quantifying the reducing sugars using dinitrosalicylic acid in a reaction mixture consisting of 0.4 ml culture filtrate, 0,5 ml of 2% TXg (in distilled water) and 0.1 ml acetate buffer (0.2M, pH 4.5)$^{11}$. One unit of enzyme activity was expressed as micromoles of reducing sugars liberated by the culture filtrate per hour under assay conditions. Glucose was used as the standard.

**Effect of Metal Ions on Enzyme Activity:** Metal ions are reported to positively influence enzyme activity. To detect the effect of Mg ions on XG activity, different concentrations of Mg$^{2+}$ (20, 40 & 60 mM) was incorporated in the buffer and assays were carried out.

**Results and Discussion**

**Screening and Identification of XG Producers:** Isolation was based on the ability of fungi to grow on minimal medium containing TSP as the substrate. A total of 32 different samples were collected and 69 fungal isolates were obtained (Table 1). Screening was carried out by visualizing the zone of clearance that forms on the solid agar when plates were flooded with Gram’s Iodine. It is based on the interaction of the dye with intact β-D-glycans. Hydrolysis of xyloglucan by the organism showed colorless halo around the region of mycelial growth indicating XG activity (Fig. 1). Of the 69 isolates, only 7 showed a zone of clearance indicating their ability to produce XG. XG activity of the different isolates were compared by calculating EI, which is ratio of the radius of the zone of clearance formed on the solid medium to that of the radius of the colony. Efficient xyloglucanolytic activities as well as rapid growing capabilities were used as criteria for selection of isolates. Accordingly, three best isolates having EI 1.9, 1.69 and 1.6 were chosen for this study. They were identified as *Cephalosporium* sp., *Fusarium equiseti* and *Aspergillus terreus* respectively.

*Fusarium equiseti* when first isolated had a floccose white mycelium tinged with peach. Reverse side of the plate showed a prominent deep peach tinge. When observed under light microscope, only macroconidia developing sparsely from simple lateral phialides were observed. The conidiophores had well developed pedicellate foot cell and an attenuated apical cell which curves inwards, a typical characteristic of the species *equiseti*.

*Aspergillus terreus* grew very rapidly compared to the others, producing cinnamon-brown colonies that were marked by shallow radial furrows. The appearance of the colony was velvety with heavy sporulation throughout and consisted of a dense felt of conidiophores. Under light microscope, conidiophores stripes were smooth-walled and hyaline. Conidial heads were densely columnar, conidigenous cells biseriate and conidia were smooth and globose.

*Cephalosporium* sp. grew slowly to produce smooth small colonies of white mycelia with dense sporulation. Under the microscope, solitary phialides arising from thin septate hyphae were clearly visualized. The tapering phialides, arising laterally from the hypha, ended in ellipsoidal conidia arranged in groups. Unicellular intercalary chlamydospores were also observed.

Most of the fungi isolated in this study were filamentous fungi (data not shown). Of these, three representative genera were chosen. All three showed large zones of clearance when grown on xyloglucan rich TSPS, as is evident from the EI, implying their ability to produce good amounts of the enzyme.

**Fermentation Studies:** Most fungal enzymes being inducible, the conditions under which they are cultured play an important role in their production. Studies were carried out to see the effects of nutritional sources, incubation time and metal ions on XG activity. Submerged fermentation has been carried out as it has the advantage of ease of process control and easier sterilization. It is the
sought after method for commercial production of enzymes.

Measurement of enzyme activity by quantification of reducing sugars showed that A. terreus produced the most active enzyme. There are no reports on the use of either F. equiseti or Cephalosporium sp. for production of xylol glucanases, though there are reports on endoglucanases and glycosyl hydrolases production from both.

**Optimization of Medium for Enzyme Production:**
Medium composition had significant effect on XG production. Analysis of variance showed that there is significant difference (p<0.05) in XG activity among the two broth tested and was found to be better in CD broth than in M and W broth for A. terreus and Cephalosporium sp. In the case of F. equiseti, the activity was same when grown in either broth (Table 2). Since there was enhancement of enzyme production in the case of two isolates and no negative impact in the case of the other, CD broth was used for all further experiments.

The organisms produced more active enzyme in CD broth. Lesser activity in M and W broth could probably be attributed to the presence of too many trace elements (Mn, Zn, Co, Fe, Mo) which may have negatively influenced the production of enzyme. Chellapandi and Jani have reported a similar observation where least endoglucanase activity was seen in M and W medium when compared with four different media. Ghosh and Kundu have reported lesser activity from A. terreus when grown on the same substrate. Sipos et al and Yaoi and Mitsuishi have reported significantly lesser activity from Trichoderma reesei and Geotrichum sp. respectively. Though there is one report available on the production of XG in Aspergillus oryzae whose activity is comparable to the XG activity from Cephalosporium sp., the activity of all three XGs from this study, are higher than the above mentioned reports.

**Optimization of Fermentation Time:** XG activity was found to be maximum on the 4th day for A. terreus and Cephalosporium sp. after which the activity gradually declined. F. equiseti showed increased activity on 4th day and maximum activity on 7th day (Fig. 2). Optimum fermentation time for XG production by fungi is usually higher, ranging between 6-8 days. Faster production of the enzyme, as reported here, can reduce production costs.

**Enzyme Localization:** In A. terreus, greater XG activity was found in the reconstituted pellet fraction. Hence the major enzyme could be membrane bound whereas in F. equiseti and Cephalosporium sp., the enzyme may be intracellular as greater activity was found in the supernatant (Table 3).

Most fungal enzymes are reported to be predominantly secretory. But different forms of the enzyme could be localized in different regions of the cell. XG from all three organisms are secretory and the enzyme from A. terreus is membrane bound which accounts for higher activity of A. terreus XG in the CF.

**Effect of Metal Ions:** Fungal enzymes can be sensitive to the presence of bivalent metal ions in the reaction mixture. Performance of XG in the presence of these ions is important for its applicability in various processes. The presence of Mg improved the activity of all the three XG (Fig. 3). Mg ion concentration up to 40mM increased the activity of A. terreus and Cephalosporium sp. XG by 1.5 and 3.45 folds respectively. In the case of F. equiseti XG, enhancement of activity was observed upto 60mM Mg with 3.35 fold increase.

Presence of Mg ions was shown to enhance the catalytic activity of XG. Sinitsyna et al have reported that Cu, Mg, and Fe had no effect on XG from Penicillium sp. which could be due to the very low concentration (1mM) of ions used by them. Not many reports are available on the effects of metal ions on XGs specifically but Mg ions improve the activity of most reported endoglucanases.

**Conclusion**
This study led to the laboratory scale production of xylolglucanases from a strain of Fusarium and Cephalosporium. All the three isolates showed appreciable amounts of enzyme activity which could be further enhanced by making simple amends like change in the medium composition and use of activating salts (MgSO4). The enzyme is extracellular, which is advantageous because it reduces the extraction procedure. Also, the enzyme is produced using a cheap, unprocessed substrate viz. tamarind seed powder and hence reduces cost of production of the enzyme.

**Acknowledgement**
The authors would like to thank Jain University for their patronage and gratefully acknowledge the help of Savitha J., Dept. of Microbiology, Bangalore University for her help in identification of cultures.

**References**


### Table 1

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>No. of Samples Collected</th>
<th>No. of Isolates</th>
<th>No. of Positive Isolates</th>
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<tbody>
<tr>
<td>Wood</td>
<td>13</td>
<td>33</td>
<td>2</td>
</tr>
<tr>
<td>Leaf litter</td>
<td>10</td>
<td>24</td>
<td>5</td>
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<tr>
<td>Compost</td>
<td>3</td>
<td>10</td>
<td>0</td>
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<tr>
<td>Cow dung</td>
<td>6</td>
<td>2</td>
<td>0</td>
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### Table 2

<table>
<thead>
<tr>
<th>Isolate</th>
<th>CD Medium</th>
<th>M and W Medium</th>
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<tbody>
<tr>
<td><em>F. equiseti</em></td>
<td>3360a</td>
<td>3360b</td>
</tr>
<tr>
<td><em>A. terreus</em></td>
<td>4860a</td>
<td>570b</td>
</tr>
<tr>
<td><em>Cephalosporium sp.</em></td>
<td>3270a</td>
<td>1080b</td>
</tr>
</tbody>
</table>

*Average of four replicates; The alphabets represent the order of the means within a column which are significantly different at *P* = 5% by Duncan’s Multiple Range Test (DMRT). Means in a column with different superscripts are significantly different.*

### Table 3

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Enzyme Activity in Supernatant</th>
<th>Enzyme Activity in Pellet</th>
<th>Enzyme Activity in CF</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. equiseti</em></td>
<td>49.2±1.0</td>
<td>35.4±0.6</td>
<td>3360±10</td>
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<tr>
<td><em>A. terreus</em></td>
<td>60±0.6</td>
<td>222±9.1</td>
<td>4860±12</td>
</tr>
<tr>
<td><em>Cephalosporium sp.</em></td>
<td>82.8±1.0</td>
<td>21.6±0.6</td>
<td>3270±10</td>
</tr>
</tbody>
</table>

Fig. 1: Zone of Clearance produced by (a) *F. equiseti* (b) *A. terreus* (c) *Cephalosporium sp.* when flooded with Gram’s Iodine
Fig. 2: Effect of Fermentation Time on Xyloglucanase Production in CD Medium


Fig. 3: Effect of Mg$^{2+}$ ions on Xyloglucanase Activity


(Received 30th June 2011, revised 20th October 2011, accepted 10th January 2012)

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