Use of microwave pretreated Cedrus deodara wood residue as a substrate for enhanced production of cellulase free xylanase from Geotrichum sp. F₃ isolated from rural compost

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ABSTRACT

In the present study, the possibility of production of cost effective cellulase-free xylanase from a newly isolated Geotrichum sp. F₃ using cheap and easily available forest wood residue of Cedrus deodara has been explored in place of conventional and highly expensive oat spelt xylan. The main objective of this research was to determine the best cultivation conditions in the submerged process for xylanase production followed by solid state fermentation (SSF) from Geotrichum sp. F₃ aiming towards optimization of enzyme production. Geotrichum sp. F₃ revealed maximum xylanase titers in Nakamura medium, pH 4.0, temperature 55°C, inoculum size 10%, substrate concentration 2.25% after 7 days of incubation. Optimization of fermentation conditions enhanced the enzyme production to 77.54 IU/ml, as compared to initial production i.e. 10.12 IU/ml. When enzyme production mode was switched over to SSF from submerged fermentation using microwave irradiated sawdust of C. deodara by Geotrichum sp. F₃ using Nakamura medium, pH 4.0 and with inoculum size of 10 % at 25°C, xylanase production was enhanced dramatically to 112.890 U/g.

Keywords: Geotrichum, SSF, xylanase, microwave pretreatment, Cedrus deodara wood residue

INTRODUCTION

Plant cell wall contains hemicelluloses with xylan as a major constituent, and it is composed of a backbone of 1,4-linked D-xylose units, which may be substituted with acetic acid, arabinose, and 4-O-methyl-D-glucuronic acid. Xylanolytic enzymes, a repertoire of hydrolytic enzymes, facilitate the complete hydrolysis of xylan. This enzyme is produced by a variety of microorganisms, including bacteria [1,2], fungi [3], and actinomycetes [4]. Xylan-degrading enzymes have attracted considerable attention because of their widespread application in industrial processes such as biobleaching of pulp [5,2], textile industry [6], production of xylo-oligosaccharides [7], wastewater treatment [8], texture improvement in bakery products [9], clarification of juices and wine [10], debarking process [11], improving the digestibility of animal feed stocks [12] and bioconversion of lignocellulosic wastes into useful economical products such as ethanol, single-cell protein, sugar syrups, and liquid and gaseous fuels [13].

SSF has recently gained importance for the production of microbial enzymes due to several economic advantages over conventional submerged fermentation (SmF) [14]. Among the various groups of microorganisms used in SSF, filamentous fungi are the most widely exploited because of their ability to grow on complex solid substrates and
production of a wide range of extracellular enzymes [15]. Filamentous fungi have been widely used to produce hydrolytic enzymes of industrial importance, including xylanases, whose levels in fungi are generally much higher than those in yeast and bacteria.

Degradation of lignocellulosic wastes by microbial cultivation can be a cost effective approach by which enzymes can be effectively harnessed [16]. But the hydrolysis of plant components pose a major problem because of the complex bonding within the structural matrix. The presence of lignin in lignocellulosic substrates and the firm bonding of hemicellulose with cellulose make it inaccessible for enzyme production. Thus, pretreatment is a requisite step in overcoming biorecalcitrance of lignocellulose and is considered as the core of the lignocellulose bioprocessing because its outcome gives significant impact on overall conversion scheme such as size reduction, hydrolysis, fermentation, product recovery, residue processing, and co-product potentials by hypercellulolytic and xylanolytic microorganisms [17]. Different pretreatment methods including chemical, physical, thermal, and biological have been recommended for effective saccharification of biomass [18,19]. Bonding of hemicellulose with lignin and cellulose restricts its biodegradation in native biomass. Microwave irradiated pretreatment seems to break this strong bonding and release hemicellulosic fractions for saccharification into simple pentose sugars.

The aim of this study was to evaluate the potential of softwood C. deodara wood residue as a carbon substrate for production of industrially important enzyme xylanase by Geotrichum sp. F3 isolated from compost and step wise optimization of various growth conditions for enhanced production of xylanase under SmF and SSF.

**MATERIAL AND METHODS**

**Microorganism**
Geotrichum sp. F3 was isolated along with other 6 fungal isolates from compost collected from different villages of Himachal Pradesh, India. This strain was identified on the basis of its morphology i.e. white colour, septate hyphae and oval arthrospores. The fungus was grown in plates containing malt extract medium and incubated for one week at 28±2°C, in order to get spores. 10 ml of autoclaved distilled water was added to the plate and slightly scratched with a loop to obtain spore suspension. This spore suspension was used as inoculum.

**Selection and microwave pretreatment of lignocellulosic biomass**
The wood residue of C. deodara was selected for xylanase production studies depending upon their wide abundance in local forests of Himachal Pradesh, a hilly state located in Northern Himalayas, India. A large amount of waste of the C. deodara wood is generated in the wood industry due to its wide use in furniture making. The wood waste of C. deodara was collected and chipping of biomass was done to get small pieces. The chipped pieces were dried in oven at 50°C for 72 h. After drying, the chips of each species were ground to fine particles of 1.0-1.5 mm mesh size. 100 g of C. deodara wood residue was taken in a beaker and was microwave (Godrej make) irradiated at 250 V and 50 Hz for 3 min and kept in air tight containers for further use.

**Enzyme production in SmF**
Xylanase production in SmF was carried out using the Riviere’s medium containing (g/l) peptone 5.0; yeast extract 5.0; potassium nitrate (KNO₃) 5.0; potassium dihydrogen phosphate (KH₂PO₄) 1.0; magnesium sulfate (MgSO₄) 0.1, pH 7.0; pretreated C. deodara 0.5. The flasks were autoclaved at 15 psi for 20 min and cooled. They were then inoculated with 10 % inoculum and incubated at 28±2°C for 5 days under shaking at 120 rpm. Contents were centrifuged at 10,000g for 15 min at 4°C, and the clear cell-free supernatant was used for xylanase assay.

**Enzyme assay**
Xylanase activity was measured according to Miller method [20].

**Protein estimation**
The protein was determined by Lowry method [21].

**Effect of media**
The different media i.e. Mandel’s Medium [22], Nakamura Medium [23], Basal Salt Medium [24], Modified Basal Salt Medium [25] and Mandel & Sternberg Medium [26] were studied for the production of xylanase. Each flask
containing 90 ml of production was inoculated with 10 ml of inoculum and incubated at 25±°C with continuous agitation of 120 rpm for 5 days. The xylanase was assayed in the culture filtrate.

**Effect of pH on production**
Xylanase production was studied at pH values ranging from 3.5-6.5. Each flask containing 90 ml Nakamura medium of different pH was inoculated with 10 ml of inoculum and incubated at 25±2°C with continuous agitation of 100 rpm for 5 days. The xylanase was assayed in the culture filtrate after centrifugation.

**Effect of temperature on production**
Xylanase production was studied at temperatures ranging from 15°C- 40°C using optimized pH. Each flask containing 90 ml of production was inoculated with 10 ml of inoculum and incubated at different temperatures with continuous agitation of 100 rpm for 5 days. The enzyme was assayed in the culture filtrate.

**Effect of inoculum size**
To study the effect of inoculum size, 90 ml of production media were inoculated at a level of 5%, 7.5%, 10%, 12.5% and 15% using best conditions from previous experiments. After incubation at 25°C for 5 days, the culture filtrate was centrifuged at 10,000g for 20 min and xylanase activity was determined.

**Effect of substrate concentration**
Cellulase production from *Geotrichum* sp. F3 was examined by varying the different concentrations of *C. deodara* biomass viz. 0.25- 2 % using best parameters optimized in the previous experiments.

**Effect of incubation period**
Flasks containing 50 ml of production medium were inoculated with 10% seed culture and incubated at 25°C with constant shaking at 200 rpm. Following incubation for various time intervals i.e. 1, 3, 5, 7 and 10 days and keeping rest of the conditions optimized in the previous experiments, the culture filtrate was centrifuged and xylanase activity was determined.

**Production of xylanase under SSF**
The 250 ml Erlenmeyer flasks containing 5 g of untreated and pretreated biomass of *C. deodara* and 10 ml of moistening agent (Nakamura medium) was autoclaved at 121°C for 20 min, cooled and then inoculated with 2 ml of spore suspension (10^7-10^8 spores/ml) of *Geotrichum* sp. F3. The inoculated flasks were incubated at 25±2°C for 10 days and control was run without inoculum.

**Enzyme extraction by repeated extraction method [27]**
To each 5 g of untreated and pretreated biomass, 25 ml of phosphate buffer (0.1M, pH 6.9) with 0.1% Tween-80 was added in 250 ml Erlenmeyer flask. The contents were kept in the shaker for 1 h at 120 rpm and contents were then filtered through nylon cloth. The process was repeated twice with 15 and 10 ml of phosphate buffer. After filtration, total contents (50 ml) were centrifuged at 5,400 rpm for 10 min at 4°C. The supernatant of crude xylanase was collected and stored at 4°C for further studies.

**Statistical analysis**
All data are given as the mean ± SD of triplicates (n = 3).

**Results and discussion**
The fungal strain *Geotrichum* sp. F3 was isolated from compost and selected among 5 strains of fungi, due to highest production of xylanase for degradation of biomass. Xylanase activity was also found to be maximum in *Geotrichum* sp. F3 (10.122 IU/ml) and the least enzyme activity was shown by *Trichoderma harzianum* F6 i.e. 0.866 IU/ml (Table 1). Kar *et al.* [28] reported production of 4.75 IU/ml xylanase by *Trichoderma reesei* SAF3.

**Effect of media on xylanase production**
The effect of different media for xylanase production was examined and it was found that Nakamura medium emerged as best medium for xylanase production by *Geotrichum* sp. F3 exhibiting significantly high enzyme activity of 12.353 IU/ml whereas least enzyme production i.e. 1.79 was observed in Mandel’s medium (Fig. 1). Nakamura medium has stimulated growth promotory role as far as production of extracellular xylanase is concerned. Since
nitrogen is a major constituent of protoplasm and building block of the enzymes, the peptone and yeast extract which are the components of the Nakamura medium have helped directly in higher amount of enzyme synthesis. In the present study, though effect of different nutrients has not been tested separately but the significant role of nitrogen sources on enzyme production has been already cited in literature. Yeast extract and peptone have been reported as most balanced and conditioned nitrogen sources in terms of accessibility and composition for getting high yield of xylanase [29].

**Effect of pH on production**

The effect of varying pH of the production medium from 3.5 to 6.5 was studied on xylanase production. *Geotrichum* sp. F3 has expressed significantly higher xylanase production of 18.384 IU/ml at pH 4.0 and only 9.725 IU/ml were produced at pH 6.5 (Fig. 2). da Silva and Carmona [30] reported maximum xylanase activity in the range of 3.5 to 4.5 by *T. inhamatum*.

The medium’s pH is one of the regulatory parameters during fermentation. pH is an important parameter that influences enzyme production [31]. The extracellular pH has a strong influence on the pathways of metabolism and product formation by microorganism. Changes in the external pH alter the ionization of nutrient molecules and reduce their availability to the organism thus lowering their overall metabolic activity [32].

As the pH changes, there is a variation in enzyme units which is logical because the optimum pH for growth rate may be different from that for growth yield and entirely different from the optimum for product formation. Changes of pH of 0.5-1.0 units around the optimum for growth usually have little effect on growth rate or efficiency but may affect product formation [33].

**Effect of temperature on production**

Generally, microbes are known to produce high enzyme titer at their optimum growth temperature. The optimum temperature for xylanase production by *Geotrichum* sp. F3 was found to be 25°C (23.979 IU/ml) whereas minimum enzyme was produced at 40°C (2.864 IU/ml) (Fig. 3). Philipidis [34] reported that some *Aspergillus* strains produce higher enzyme yield at 30°C. Simoes et al. [35] reported that the highest xylanase production occurred at a temperature of 25°C (93.5 IU/ml) by *T. viride*. Rahman et al. [36] stated that the best temperature range for the production of xylanase by fungi is usually from 20°C to 30°C.

Incubation temperature is a critical factor in enzymatic productivity [37]. Maximum enzyme production is produced at optimum temperature and the decrease in enzyme production at lower or higher temperatures is due to the facts that at these temperatures, growth of the organisms was inhibited, causing a decrease in the synthesis of the enzymes [35]. Production of more activity at optimum temperature may be due to the faster metabolic activity and increase in protein content and extracellular enzyme production in culture supernatant. At very low temperatures, membranes solidify and high temperatures damage microorganisms by denaturing enzymes, transport carriers and other proteins thus lowering enzyme activity [32].

**Effect of inoculum size**

Xylanase production was highest (24.35 IU/ml) when 10% inoculum was added to the production medium (Fig. 4). The enzyme titres declined with increase in inoculum size beyond 10%. Enzyme activity is maximum at optimal level because at this point because equilibrium is maintained between inoculum size and availability of substrates while the decline in enzyme yield at larger inoculum size might be due to formation of thick suspensions and improper mixing of substrates in shake flasks. With subsequent increase in inoculum size, competition for carbon source increases which results in rapid depletion of macro and micro nutrients and thus inhibit their growth and enzyme production [38]. Omojasola et al. [38] revealed that optimum glucose from the waste substrates using *T. longibrachiatum* was produced at 10% inoculum size. Several researchers have reported the use of 1.0–5.0% (v/v) inoculum for hyperproduction of xylanase [5, 39].

**Effect of substrate concentration**

Among the various substrate concentration used to enhance xylanase production, 2.25% had a stimulatory effect and exhibited maximum enzyme activity i.e. 72.450 IU/ml. Minimum xylanase production (9.991 IU/ml) was observed at 0.25% substrate concentration (Fig. 5). Substrate concentration is an influencing factor that affects the yield and initial rate of hydrolysis of cellulose [40]. Very low substrate concentration fails to trigger enzyme
production to desirable level because most of the inoculum remains without substrate and hence resulting in minimum secretion of enzymes. Optimum substrate concentration normally results in an increase in the yield and reaction rate of the hydrolysis [41]. However, high substrate concentration can cause substrate inhibition, which substantially lowers enzyme production [42, 43].

**Effect of incubation period**

Xylanase activity was highest (77.54 IU/ml) after 7th day of incubation and declined on further increase in time of incubation (Fig. 6). Minimum enzyme titres were produced on 1st day of incubation (69.99 IU/ml). Jahangeer et al. [44] studied the kinetics of enzyme production from Aspergillus strains and suggested that maximum activity was noted after 7 days of incubation while its activity decreased on further incubation. The optimum production time obtained by Sater and Said [45] with Trichoderma harzianum was on day 8, and Seyis and Aksoz [37], with Trichoderma harzianum, obtained maximum activity on day 7.

Under optimized submerged fermentation conditions, Geotrichum sp. F3 produced 77.54 IU/ml of xylanase, which was higher than the activity under unoptimized Riviere’s medium (10.12 IU/ml). The enzyme titre in our study was much higher than that reported earlier by Chidi et al. [46] in Aspergillus terreus UL 4209 strain (35 IU/ml) under submerged fermentation.

There was a considerable increase in xylanase production escalating up to 666.20 % was observed after optimization of the different growth parameters (Fig. 7). The significant increase in cellulase and xylanase titres is a major achievement as it was one of the main objectives of the present study. The relevant increase in extracellular xylanase from hyperxylanolytic fungus was because of providing best congenial conditions i.e. nutrients, pH, temperature, time, concentration of substrate, etc. for them. The different parameters optimized for enzyme production under submerged mode of fermentation were used for the next experiment i.e. SSF mode to further enhance their units.

**Production of xylanase under SSF**

SSF has gained an importance due to its several advantages over the submerged fermentation (SmF). Biodegradation of biomass was carried out under SSF mode because it has many advantages over submerged fermentation. It reduces the cost of downstream processing, utility of simple and cheap media for the fermentation, lower risk of contamination due to the ability of contaminants to grow in absence of free flowing water, high concentration of the product and simple fermentation equipment as well as low effluent generation and low requirements for aeration and agitation during enzyme production [47]. Here efforts have been made to enhance the production of xylanase using untreated and pretreated C. deodara wood as a substrate under SSF by Geotrichum sp. F3.

In the present study, cheap and easily available lignocellulosic biomass i.e. C. deodara has been used as an efficient carbon source. The use of forest residue for the production of xylanase can replace purified xylan and reduces the cost of the enzyme considerably. The process of biodegradation of lignocellulosic biomass to sugars is a complex process, so to enhance degradation of C. deodara wood, it was grinded followed by microwave pretreatment and SSF of pretreated and untreated biomass with Geotrichum sp. F3 was compared. Table 2 showed the xylanase production during biodegradation of untreated and microwave irradiated sawdust of C. deodara by Geotrichum sp. F3 using Nakamura medium as a moistening agent in the ratio of 1:2 as substrate: moisture, pH 4.0 and with inoculum size of 10 % at 25°C. Maximum xylanase enzyme (112.89 U/g) was observed with specific activity of 6.378. However, in untreated biomass enzyme production of 80.71 U/g (specific activity 5.275) was obtained. Jeya et al. [48] exhibited that xylanase production in SSF by alkali tolerant Aspergillus versicolor MKU 3. The maximum production 960 U/g was obtained in SSF with an optimized media.

The cross-linking between cellulose, hemicelluloses and lignin via ester and ether linkages cause insusceptibility of biomass for enzymatic action [49, 50]. It has been explored that pretreatment of lignocellulosic biomass opens up its capillary size and increases the accessibility of cellulose and hemicelluloses to the action of hydrolytic enzymes substantially producing more titres of enzymes. Pretreatments alter or remove structural and compositional impediments to hydrolysis and subsequent degradation processes in order to enhance digestibility, improve the rate of enzyme hydrolysis and increase yields of intended products [51, 52].

A promising pretreatment method involves the application of microwave radiation to biomass in an aqueous environment. The rationale for microwave pretreatment stems from two aspects. First from a physical aspect,
microwave radiation supplies internal heat to the biomass resulting from the vibrations of polar bonds in the biomass and surrounding aqueous medium. The radiation generates a continuously changing magnetic field causing the polar bonds to vibrate as they align with the magnetic field. This disruption and shock to the polar bonds accelerates chemical, biological and physical processes [53]. Secondly, the thermal treatment of lignocellulosic materials in an aqueous medium is known to release acetic acid, hence providing an acidic environment for auto-hydrolysis [54]. Moreover, microwave pretreatment is an eco-friendly method as no toxic discharge is generated. The earliest known study involving microwave pretreatment examined the effect of microwave radiation on rice straw and bagasse immersed in water [55].

Table 1. Screening of fungal isolates for xylanase production

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Isolate no.</th>
<th>Protein (mg/ml)</th>
<th>Enzyme Activity (IU/ml)</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Rhizopus</em> sp. F1</td>
<td>1.040±0.11</td>
<td>1.865±0.21</td>
<td>1.793</td>
</tr>
<tr>
<td>2.</td>
<td><em>Geotrichum</em> sp. F1</td>
<td>1.440±0.15</td>
<td>10.122±1.02</td>
<td>7.029</td>
</tr>
<tr>
<td>3.</td>
<td><em>Fusarium oxysporum</em> F2</td>
<td>1.120±0.12</td>
<td>0.935±0.23</td>
<td>0.833</td>
</tr>
<tr>
<td>4.</td>
<td><em>Aspergillus</em> sp. F3</td>
<td>1.320±0.17</td>
<td>8.457±0.98</td>
<td>6.407</td>
</tr>
<tr>
<td>5.</td>
<td><em>Trichoderma harzianum</em> F6</td>
<td>1.590±0.14</td>
<td>0.866±0.16</td>
<td>0.545</td>
</tr>
</tbody>
</table>

Table 2. Production of xylanase from *Geotrichum* sp. F3 using untreated and microwave irradiated *C. deodara* wood under SSF

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Protein (mg/g)</th>
<th>Xylanase Activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated biomass</td>
<td>15.30±2.56</td>
<td>80.71±5.87*</td>
<td>5.275</td>
</tr>
<tr>
<td>Pretreated biomass</td>
<td>17.70±2.65</td>
<td>112.89±6.34*</td>
<td>6.378</td>
</tr>
</tbody>
</table>

* Enzyme activity (U/g): µmoles of reducing sugars released / min / g of biomass
** Enzyme activity (IU/ml): µmoles of reducing sugars released / min / ml
ψ protein: mg/ml

Fig. 1 Effect of media on xylanase production

Available online at www.scholarsresearchlibrary.com
Fig. 2 Effect of pH on xylanase production

Fig. 3 Effect of temperature on xylanase production
Fig. 4 Effect of inoculum size on xylanase production

Fig. 5 Effect of substrate concentration on xylanase production
CONCLUSION

The results presented in this study showed that optimization of process parameters under SmF/SSF resulting in enhanced production of xylanase. Microwave pretreated *C. deodara* residue an abundantly available, locally and
widely utilized in wood industry had been successfully used as a carbon substrate by \textit{Geotrichum sp} F3 isolated from compost for xylanase production which had not been reported so far.

**Acknowledgements**

The financial support received from Department of Biotechnology, Govt. Of India, New Delhi, India to carry out this work is highly acknowledged.

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