Production of high level of cellulase-poor xylanases by wild strains of white-rot fungus *Coprinellus disseminatus* in solid-state fermentation

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The production, optimisation and partial characterisation of xylanases from newly isolated wild strains of *Coprinellus disseminatus* was performed in solid-state fermentation. Strains SH-1 and SH-2 showed high xylanase (727.78 and 227.99 IU/mL) with very low CMCase (0.925 and 0.660 IU/mL) and laccase (0.640 and 0.742 U/mL) activities at incubation time seven days, 37°C and initial pH 6.4, using yeast extract as nitrogen source and cheap substrate (wheat bran), which increased the cost effectiveness of the process. Crude cellulase-poor xylanases obtained from test strains showed maximum activities at 55°C and pH 6.4 and retained 32.64% (SH-1) and 35.03% (SH-2) activity at pH 8 and 43.01% (SH-1) and 25.00% (SH-2) activity at 65°C. As test strains produced high level of cellulose-poor xylanases, which were active over a wide range of temperature and pH, these enzymes might be used as pulp biobleaching agents.

Introduction

Xylanases are glycosidases that catalyse endohydrolysis of 1,4-β-D-xylansidic linkages in xylan, a complex, highly branched plant hetropolysaccharide [1]. Xylanases have great potential in various industrial processes, including, manufacture of bread, food and drinks, improvement of nutritional properties of agricultural silage and grain feed, processing plant fibres in the textile industry, pharmaceutical and chemical applications and cellulose pulp and paper [2]. The most potent xylanase producers are fungi, especially wood-rot fungi, because, they secrete enzymes into the medium and their enzyme levels are much higher than those of yeast and bacteria [3]. Among wood-rot fungi, white rotters are the most important as they are able to degrade all the wood wall components, including the highly recalcitrant polymer, lignin [4]. The white-rot fungus, *Coprinellus disseminatus* belongs to the family Coprinaceae (order Agaricales). It is a common mushroom species that fruits in large troops on stumps, buried wood, tree tip-up mounds and logs [5]. Although, white-rot fungi have effective hemicellulose systems, only a few studies have been made on their hemicellulases.

Because biotechnological applications require large amounts of low cost enzymes, an appropriate approach for xylanase production is to search for powerful xylanase producers using cheap lignocellulosic substrates rather than the more expensive xylan. In fact, lignocellulosics appear to be better substrates than xylan for xylanase production [6].

The potential of SSF for the production of xylanases by *C. disseminatus* has not been evaluated extensively. The selections of suitable strain, substrate and process parameters are crucial factors that affect the SSF process [7]. SSF is highlighted by the utilisation of less energy, low wastewater output, high concentration of metabolites at low cost and is technically easier [8]. The stability of produced enzymes at high temperature or extreme pH has also been reported to be better in SSF [9]. This study examines the detailed optimisation of SSF culture for high production of xylanase by indigenously isolated wild strains of *C. disseminatus* (SH-1 and SH-2) using cheap and readily available lignocellulosic substrates.

Materials and methods

Microbial strains

The fungal strains were isolated from decaying wood collected from the vicinity of the Department of Paper Technology, IIT Roorkee, Saharanpur Campus, India, by an enrichment technique.
(using wheat bran as carbon source). Lignocellulosic substrates including wheat bran were purchased from the local market of Saharanpur (India) and were thoroughly washed in hot distilled water, dried in sunlight and milled into powder in a laboratory grinder and a fraction retained on +100 mesh size stored in sealed polythene bags for further use.

The isolates were identified as different strains (SH-1 and SH-2) of white-rot fungus, C. disseminatus at the Pathology Division, Forest Research Institute, Dehradun, India and were deposited under the accession numbers 1163 and 1164, respectively, at the National Type Culture Collection, FRI, Dehradun, India. The isolates were purified on wheat bran–agar medium (2% wheat bran and 2% agar–agar) and incubated at 37°C. Purified cultures were routinely cultured on potato–dextrose–agar (PDA) slants with incubation at 37°C for 72 hours and subsequent storage at 4°C. The cultures were preserved as a suspension of spores and hyphal fragments in 15% (v/v) sterile glycerol at −20°C.

Xylanase production in SSF
Nutrient salt solution (NSS) prepared according to Vishniac and Santer [10] and as standardised by Singh and Garg [11], was used. It contained, as g/L, 1.5 KH2PO4, 4.0 NH4Cl, 0.5 MgSO4.7H2O, 0.5 KCl, 1.0 yeast extract (Sigma Chemical Co., St. Louis, MO, USA) and 0.04 trace elements solution. The trace elements solution contained, as μg/L, 200 FeSO4.7H2O, 180 ZnSO4.7H2O and 20 MnSO4.7H2O. Initial pH of NSS was adjusted as desired. 5 g finely powdered wheat bran (100 μm mesh size fraction) and 15 mL NSS were taken in each 250 mL Erlenmeyer flask. After the addition of NSS to wheat bran, the flasks, covered with cotton plugs and aluminum foil, were autoclaved at 121°C and 15 Psi for 15 min. On cooling, flasks were inoculated with 2 discs, each of 5 mm diameter cut from the periphery of three-day-old cultures, for both strains and incubated at 37°C.

Harvesting and storage of enzyme
After desired growth, enzymes were harvested by crushing the contents of the flasks in 15 mL distilled water with a glass rod and then shaking on orbital shaker at 100 rpm for 10 min at room temperature. The filtrate was centrifuged at 5000 rpm at 4°C for 10 min at 4°C after squeezing and filtering the contents through four layers of cheese cloth. The supernatant liquid was treated as crude enzyme and stored at −20°C until use.

Enzyme assays and protein determination
Xylanase activity was determined by incubating 1.6 mL of appropriately diluted enzyme preparation (the filtrate after removing pellets) in a sterile tube, with 0.4 mL of substrate suspension (10 mg of birch wood xylan, Sigma Chemical Co., St. Louis, MO, USA, in 1 mL of 0.1 M potassium phosphate buffer pH 6.0). The assay mixtures were incubated for 15 min at 55°C with constant shaking at 100 rpm. The reducing sugars released were measured by the DNS method [12] at 540 nm [13] and expressed as xylose equivalent. One unit of activity was defined as the amount of enzyme needed to release 1 μmol of xylose equivalents per min at 55°C. CMCase activity was determined by incubating 2 mL of enzyme preparation with 2 mL of 2% (w/v) carboxymethylcellulose (CMC, Sigma Chemical Co., St. Louis, MO, USA) [14] prepared in 0.05 M citrate buffer (pH 4.8) at 50°C for 30 min. CMC is a derivative of cellulose prepared by reaction with alkali and chloroacetic acid. The reducing sugars were measured by DNS method [12] at 575 nm and expressed as glucose equivalent. Enzyme activity was expressed as μmoles of D-glucose equivalents released per min at 50°C and pH 4.8 (IU). Laccase activity was determined by continuous spectrophotometric rate determination [15]. One unit is defined as unit producing A530 nm of 0.001 min⁻¹ at pH 6.5 at 30°C in a 3 mL reaction volume using syringaldazine (Sigma Chemical Co., St. Louis, MO, USA) as substrate. Enzyme activity was expressed as U/mL of the sample. Supernatant protein concentration was determined by the Lowry method [16] using BSA as standard.

Effect of incubation period, initial pH and incubation temperature on xylanase production and supernatant protein concentration
For optimising the incubation period, 250 mL Erlenmeyer flasks were prepared for both the strains with 5 g wheat bran powder and 15 mL NSS in each flask. These were inoculated and then incubated for 2–12 days, at desired initial pH and temperature. Similarly, initial pH for xylanase production was optimised by varying pH from 4.0 to 13 and incubating inoculated flasks at 37°C for seven days. Incubation temperature was optimised by varying from 27, 32, 37, 42 and 52°C and incubating the inoculated flasks at optimum pH and incubation period.

In each case, xylanase activity and supernatant protein concentration were determined as described above.

Effect of lignocellulosic substrates and moistening agent level on xylanase production and supernatant protein concentration
The effect of lignocellulosic substrates (sugarcane bagasse, wheat bran, rice bran, and wheat straw), singly and in different combinations, on xylanase production was studied. The dried substrates were ground and 5 g of each substrate (fraction retained on 100 μm mesh size) was placed in different flasks, to which 15 mL of NSS was added and inoculated flasks incubated under optimum temperature, pH and incubation period (Fig. 1).

The effect of moistening agent level on xylanase production was studied by varying wheat bran-to-NSS ratio from 1:2 to 1:5. The prepared flasks were incubated under optimum temperature, pH and incubation period.

In each case, xylanase activity and supernatant protein concentration were determined.

Effect of complex organic nitrogen sources on xylanase production and supernatant protein concentration
Flasks containing 1.0 g/L of different complex nitrogen sources (peptone, beef extract, soybean meal, malt extract and yeast extract) in the NSS were inoculated for each test fungus and incubated at optimum incubation temperature, period and pH. Xylanase activity and supernatant protein concentration were determined.

Effect of glucose and lactose on xylanase production and supernatant protein concentration
Different flasks supplemented with varying concentrations (1–5 g/L) of glucose and lactose were inoculated and incubated at optimum temperature, pH, incubation period and moistening agent
level. Xylanase activity and supernatant protein concentration were determined.

**Effect of pH and temperature on xylanase activity**

To determine pH stability of crude xylanases, the enzymes were incubated in buffers of different pH from 6.0 to 9.0 at 55°C for 15 min. Similarly, crude xylanases were incubated at 55, 65 and 75°C at pH 6.4 for 15 min to assess their temperature stability. Residual xylanase activity was measured in each case.

**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and xylanase activity detection by zymogram analysis**

To determine the protein profile of crude enzyme preparations so obtained, SDS-PAGE was performed using 12.5% acrylamide gel [17]. Gels were stained with Coomassie brilliant blue R-250, destained (30% methanol and 10% acetic acid) [18] and stored in 5% acetic acid. Xylanase zymogram gel was prepared by adding 0.1% oat spelt xylan to the polyacrylamide gel. After running, the
gel was washed four times for 30 min in 100 mM phosphate buffer (pH 6.4), the first two washes contained 25% isopropyl alcohol. Gel was incubated for 20 min at 37 °C before soaking in Congo red solution for 5 min at RT, destained with 1 M NaCl and fixed with 0.5% acetic acid solution. The background turned dark blue and zymograms were observed for clear zones [19].

Statistical analysis
The analysis was replicated three times and results were reported as mean ± standard deviation.

Results and discussion
Effect of incubation period, initial pH and incubation temperature on xylanase production and supernatant protein concentration
Xylanase production (722.98 and 228.62 IU/mL for SH-1 and SH-2, respectively) and supernatant protein concentration (4.829 and 4.621 mg/mL for SH-1 and SH-2, respectively) increased up to seven days of incubation for both strains. A decrease of 42.21 and 7.72% in xylanase activity for strains SH-1 and SH-2 respectively, was observed for eight-day incubation (Fig. 1A). Xylanases are optimally expressed at the end of exponential phase. Thus, their harvesting time must be correlated to their production. Metabolic enzymes like, proteases and transglycosidases might also affect xylanase yield [1].

Xylanase production (723.41 IU/mL for SH-1 and 229.45 IU/mL for SH-2) and supernatant protein concentration (5.000 mg/mL for SH-1 and 4.725 mg/mL for SH-2) were highest at pH 6.4 (Fig. 1B) for both strains. Any enzyme-mediated reaction is influenced by culture pH, which might result in changes in productivity. If pH change is too abrupt, response of microbes might lag behind or overshoot. The pH also determines solubility of some media components [20]. Most of the white-rot fungi have been reported to grow best at slightly acidic pH [21].

Fig. 1C shows the effect of incubation temperature on xylanase production and supernatant protein concentration. Xylanase production (724.45 IU/mL for SH-1 and 230.21 IU/mL for SH-2) and supernatant protein concentration (5.119 mg/mL for SH-1 and 4.800 mg/mL for SH-2) were highest at 37 °C for both strains. At 42 °C, xylanase activity decreased by 79.86 and 73.23% for SH-1 and SH-2, respectively. No growth was observed at 52 °C, thus indicating mesophilic nature of the strains, as already reported for most of the white-rot fungi [21].

Effect of different lignocellulosic substrates and moistening agent levels on xylanase production and supernatant protein concentration
Xylanase production (724.21 IU/mL for SH-1 and 229.81 IU/mL for SH-2) and supernatant protein concentration (5.098 mg/mL for SH-1 and 4.754 mg/mL for SH-2) were highest with wheat bran as substrate. The order of substrate suitability for SH-1 was: wheat bran > rice bran + wheat bran > wheat bran + bagasse > wheat bran + wheat straw > rice bran + wheat straw > rice bran + wheat straw > bagasse > wheat straw + bagasse. For SH-2, substrate suitability was: wheat bran > wheat bran + wheat straw > wheat straw + rice bran + rice bran + rice bran + wheat straw > bagasse > wheat straw + bagasse > wheat straw + bagasse. The presence of activator or inhibitor, surface area, catabolite diffusion, pretreatment, content and sugar composition influences the effect of substrate on enzyme production [22]. Xylanases are generally induced by xylan, xylobiose, xylose and lignocellulosics that contain xylan [23]. Low molecular mass xylan induces transferases for their translocation. Thus, level of inducers and/or required enzymes in the culture filtrate affects xylanase synthesis [1]. Wheat bran contains sufficient nutrients and remains loose in moist conditions, thus, providing a large surface area for xylanase production [24]. Its cell wall contains about 40% xylans and 28% proteins, which might serve as carbon and nitrogen source for the microbe [25]. The fungi might have different lag phase, regarding to some of these substrates, and if that substrate was sampled later, higher enzyme concentration might have been achieved. However, it was in our interest to compare different substrates with wheat bran, which had produced enzymes in seven days of fermentation.

The maximum xylanase production (719.98 IU/mL for SH-1 and 225.00 IU/mL for SH-2) and supernatant protein concentration (4.890 mg/mL for SH-1 and 4.587 mg/mL for SH-2) were obtained at substrate to NSS ratio of 1:3 (Table 1). A higher than optimum moistening agent level might decrease porosity and lower oxygen transfer, while a lower than optimum level might reduce solubility and swelling of substrate [26]. Nutrient availability might also influence xylanase activity at varying moistening agent level.

Effect of complex organic nitrogen sources on xylanase production and supernatant protein concentration
The maximum xylanase production (723.78 IU/mL for SH-1 and 230.00 IU/mL for SH-2) and supernatant protein concentration (4.981 mg/mL for SH-1 and 4.888 mg/mL for SH-2) were obtained for yeast extract. The order of suitability of complex nitrogen sources was: yeast extract > beef extract > malt extract > soybean meal > peptone, for SH-1 and yeast extract > malt extract > soybean meal > peptone > beef extract, for SH-2. The differences in enzyme activity for different nitrogen sources could be because of their varying contents of amino acids, peptides, vitamins, trace elements and/or mineral salts [27]. The highest xylanase production observed with yeast extract might also be because of better absorption of its amino acids directly through mycelia of the strains [3].

Effect of glucose and lactose on xylanase production and supernatant protein concentration
Xylanase production and supernatant protein concentration decreased in presence of both glucose and lactose for both the strains. The repression was found to be concentration dependent as, the higher the concentration of glucose or lactose, the greater the repression in xylanase yield. Smith and Wood [28] have also reported similar findings. Xylanase repression in the presence of easily metabolisable carbon sources suggested that enzyme synthesis is controlled by transition state regulators and catabolite repression [1].

Enzyme production under optimised SSF conditions
Under optimum SSF conditions, xylanase activity was 727.78 and 227.99 IU/mL, cellulase activity 0.925 and 0.660 IU/mL, laccase
activity 0.640 and 0.742 U/mL and supernatant protein concentration 5.480 and 4.900 mg/mL, respectively for strains SH-1 and SH-2. Although an overestimation of soluble proteins in crude extracts because of interferences with other compounds was possible, its profile agreed with increase in enzyme activities.

Effect of temperature and pH on xylanase activity

The maximum xylanase activity was at 55°C for both the strains. Xylanase retained 43 and 12% of its activity for SH-1 and 25 and 20.22% of its activity for SH-2 at 65 and 75°C, respectively. While proceeding towards optimum temperature for enzyme activity, increased kinetic energy of reacting molecules increase the reaction rate. On further rise in temperature, protein denaturation occurs, leading to loss of enzyme activity.

Xylanase activity was highest at pH 6.4 for both the strains. For SH-1, enzyme retained 49.03, 32.64 and 5.70% of its activity and for SH-2, enzyme retained 95.30, 35.03 and 5.60% of its activity at pH 7.0, 8.0 and 9.0, respectively. At pH 6.0, the enzyme retained 97.47 and 89.48% of its activity for strains SH-1 and SH-2, respectively. The results revealed that these xylanases have slightly acidic pH optima, but show activity over a wide pH range (6–9). Strain SH-2 xylanase seems to have slightly better pH stability than SH-1. Most fungal xylanases are reported to produce the highest activity over pH range of 6–6.5[29]. Enzymes tend to lose their basic structure under harsh conditions like, change in pH. This might lead to loss in enzyme activity. The pH activity profiles of enzymes also depend on pKₐ (ionisation constant) of catalytic residues. Lower the pKₐ value, higher is the pH stability. Amino acid residues contributing positive charges and hydrogen bonds lower the pKₐ values with shorter bonds having a more definite effect [30].

SDS-PAGE analysis and xylanase activity detection by zymogram analysis

SDS-PAGE profile (Fig. 2A–C) of crude enzymes shows that major protein bands for both strains were almost similar in number and

![FIGURE 2](image-url)

**TABLE 1**

<table>
<thead>
<tr>
<th>Test isolates</th>
<th>Solid substrate: NSS</th>
<th>Protein concentration (mg/mL)</th>
<th>Protein concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain SH-1</td>
<td>Xylanase activity (IU/mL)</td>
<td>Strain SH-2</td>
<td></td>
</tr>
<tr>
<td>1:2.0</td>
<td>555.98 ± 7.23</td>
<td>4.107 ± 0.18</td>
<td>161.06 ± 7.15</td>
</tr>
<tr>
<td>1:2.5</td>
<td>628.22 ± 11.19</td>
<td>4.297 ± 0.32</td>
<td>189.45 ± 11.15</td>
</tr>
<tr>
<td>1:3.0</td>
<td>719.98 ± 13.08</td>
<td>4.890 ± 0.21</td>
<td>225.00 ± 10.08</td>
</tr>
<tr>
<td>1:3.5</td>
<td>475.88 ± 9.43</td>
<td>3.396 ± 0.09</td>
<td>138.62 ± 9.10</td>
</tr>
<tr>
<td>1:4.0</td>
<td>449.86 ± 11.21</td>
<td>3.127 ± 0.07</td>
<td>122.20 ± 6.13</td>
</tr>
<tr>
<td>1:4.5</td>
<td>391.54 ± 10.31</td>
<td>2.760 ± 0.24</td>
<td>105.45 ± 12.00</td>
</tr>
<tr>
<td>1:5.0</td>
<td>318.27 ± 5.12</td>
<td>2.321 ± 0.10</td>
<td>89.21 ± 10.21</td>
</tr>
</tbody>
</table>

**Figure 2**

SDS-PAGE and zymogram analysis: (A) crude xylanase from strain SH-1, (B) medium range protein marker (kDa), (C) crude xylanase from strain SH-2, (D) zymogram for strain SH-1 and (E) zymogram for strain SH-2.
mol wt, corresponding to about 20, 29 and 66 kDa, except for, one more major band of size >97 kDa for SH-2. For both strains, a single active band corresponding to xylanase activity was observed (Fig. 2D–E) as clear zones against dark blue background. This corresponded to a mol wt of about 66 kDa for SH-1 and 29 kDa for SH-2.

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