

Isolation, screening, characterization and optimization of xylanase production from thermostable alkalophilic *Fusarium* sp. XPF5

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Abstract

Xylanases are the enzymes that hydrolyse xylans, a major constituent of hemicelluloses and have various biotechnological applications. It is produced by various microbial sources including fungi being most promising. In the present study, soil samples rich in plant dead material obtained from various locations Himalayan region around Solan, Himachal Pradesh, India, and screened for xylanase producing fungal isolates. Seven fungal isolates were selected on the basis of clear zone formation around the fungal growth for quantification of xylanase production. Among the seven fungal strains isolated, isolate XPF-5 was selected as hyper producing fungal strains showing highest enzyme activity (0.398 U/ml). The strain was identified microscopically as *Fusarium* sp. XPF-5 on the basis of morphological characteristics. Optimum temperature and pH for XPF-5 xylanase were found as 57°C and 9.0, respectively. The production of xylanase was optimized using different factors such as temperature, pH and time of incubation by 'one variable at a time' approach. The optimum temperature, pH and time of incubation for maximum xylanase production were obtained as 47°C, 8.0 and 96 h, respectively. Based on the results of culture conditions and xylanase activity, the fungal isolate XPF-5 was considered as thermo-alkalophile, and make it a potential candidate for large scale xylanase production and its use in biotechnological processes.

Keywords: Xylan; Xylanase; Optimization; Thermophilic Fungi; *Fusarium* sp.

Introduction

Xylanases are the enzymes that break down the xylans, one of the major constituents of hemicelluloses that constitute almost 20-35% of the dry weight of the woods (Goswami and Pathak 2013). Xylanases are suitable for various applications of biotechnology that involve the bioconversion of plant biomass like in the production of ethanol by delignification (Olson and Hahn 1996) or saccharification of organic waste material from industries and agricultural operations (Sá-Pereira et al. 2002).

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The use of thermostable and alkalotolerant xylanases is of main interest as they are used in various biotechnological important processes mentioned above including the replacement of chemicals such as chlorine and chlorine dioxide used for bleaching of paper pulp (Muthezhilan et al. 2007). Natural producers of the xylanase enzyme, mainly fungi, have been exploited for their use in xylan degrading processes as they secrete enzyme in the nutrient medium and also produce some additional xylanolytic enzymes, that are helpful in breaking down the highly branched structure of xylan (Haltrich et al. 1996; Bakri et al. 2013). The vast usage of xylanases stable at high temperature and pH are the choice of recent biotechnological applications. Therefore, studies using thermophilic and alkalophilic fungi revealed xylanase producing potential of fungal isolates such as *Chaetomium thermophile*, *Humicola insolens*, *Thermomyces lanuginosus* and *Thermoascus aurantiacus* (Ghatora et al. 2006), *Aspergillus Fumigatus* AR1 (Antony et al. 2003), *Penicillium citrinum* (Dutta et al. 2007). Among the various sources reported, *Fusarium* sp. have been suggested to be a promising fungi for the production of xylanases (Jorge et al. 2005; Gupta et al. 2009; Arabi et al. 2011). *Fusarium* fungi are a diverse group of filamentous fungi that are commonly found in the moist soil in major geographic regions of the world rich in plant residues and organic matter. Various species of *Fusarium* have been reported to produce xylanase that include *F. oxysporum* (Christakopoulos et al. 1996), *F. heterosporum* (Heinen et al. 2014) and *F. solani* (Arabi et al. 2011). In present study, the forest soil samples from Himalayan region were used as source to explore presence and potential of fungal isolates for xylanase production and the enzyme was characterized biochemically for its future biotechnological application.

Materials and Methods

Soil samples

Soil samples for the isolation of xylanase producing fungi were collected from various forests around Solan, Himachal Pradesh (Latitude/longitude: 30.9059°N/77.0925°E) where the soil was rich in dead and decaying plant material. The samples were moist with partially degraded organic matter and collected in sterile bags. For further analysis, 10 g of soil sample, 0.5 g xylan (Birchwood Sigma, X-7287) and 1 ml of sterilized water were added and mixed thoroughly. The mixture was incubated

at 37°C with intermittent agitation for one week until maximum fungal spores were produced.

Isolation and screening of xylanolytic fungi

One gram of enriched soil sample was serially diluted (10^{-4} to 10^{-6}) with 0.85% sterile saline and fungi was isolated on potato dextrose agar medium (pH 5.5). After serial dilution 0.1 ml of diluted samples were spread on the petriplates containing nutrient medium and incubated at 37°C for one week. The observed fungal colonies were repeatedly streaked and purified on fresh plates containing xylan. The colonies were then screened using the 0.5% congo red dye and then flooding with 1.5 mM sterilized sodium chloride (Teather and Wood, 1982). The colonies with clear ring around were selected as xylanolytic fungi. Selected fungal isolates were identified using microscope and the pure cultures were maintained on the nutrient agar slants and stored at 4°C.

Production and estimation of xylanase activity

For xylanase production, the isolated fungi were grown in 50 ml of potato broth (containing 20% potato; pH 5.5) in 250 ml Erlenmeyer flask containing 0.5% xylan as source of carbon. The xylanase activity was assayed after 5 d of incubation at 37°C with 160 rpm. Xylanase activity was assayed by measuring the release of reducing sugar (xylose) from birchwood wood xylan following the dinitrosalicylic acid (DNS) method at 540 nm (Miller 1959). The absorbance was recorded at 540 nm. Concentration of reducing sugar was calculated from a standard curve of D-xylose. The enzyme activity was expressed in terms of international units per ml. One international units (U) of enzyme activity is defined as μmol of D-xylose released $\text{min}^{-1} \text{ml}^{-1}$ (U ml^{-1}) of culture supernatant under assay conditions.

Biochemical characterization of xylanase

In order to determine the effect of various pH and temperature on xylanase activity, 100 μl of enzyme was used in reaction mixture and the activity was determined at a pH range of 5-10, using 100 mM citrate-phosphate (pH 5.0-6.0), sodium-phosphate (pH 6.0-8.0), Tris-HCl (pH 8.0-9.0), glycine-NaOH (pH 9.0-10.0) buffers. The reaction mixture was incubated at 45°C for 15 min and activity was determined according to given method.

Effect of temperature on enzyme activity was determined by incubation at different temperatures i.e. 37°C, 47°C, 57°C, 67°C, and 77°C and activity was determined as mentioned earlier.. The effect of varying incubation time on enzyme activity was also determined from 10-45 min duration. In addition to above, effect of different substrate concentration (xylan) ranging from 0.25-1% on xylanase activity was also determined according to DNS method.

Optimization of culture conditions for extracellular production of xylanases

In order to determine the optimal conditions for xylanase production, the selected fungal isolate was grown in the Erlenmeyer flasks (250 ml) containing 50 ml of potato broth (pH 5.5) by inoculating 4 disks of 9 mm diameter taken from the 7 d old stock culture. The 'one variable at a time' approach was used for optimization with xylanase activity as an indicator for each step. First, the effect of pH on the growth of fungal isolate for the maximum production of xylanase was determined in the pH range of 5.0-10.0 at 37°C temperature and xylanase activity was determined in supernatant as described earlier. Thereafter, effect of varying incubation temperature (37-77°C) of production media on fungal growth for maximum xylanase activity was determined after

5 d of incubation at 160 rpm. After 5 d, culture contents were centrifuged at 10000 rpm for 15 min at 0-4°C. The supernatant was collected and assayed for xylanase activity. In addition to pH and temperature, time for highest xylanase production at optimum pH and temperature was also determined by recording xylanase activity after each 24 h upto 168 h (1-7 d). The enzyme activity was measured using DNS method described previously at 540 nm using spectrophotometer (Labtronics, LT 2900).

Results and Discussion

The xylanase enzyme can be produced by a number of microorganisms, however, filamentous fungi are considered as more potent producers of xylanases. Seven fungal isolates XPF-1, XPF-2, XPF-3, XPF-4, XPF-5, XPF-6 and XPF-7 were selected for the xylanase production based on their ability to hydrolyze xylan in the medium. The maximum enzyme production was observed in XPF-5 (0.398 U/ml) and selected as hyper xylanase producing fungi for further analysis (Figure 1). The colonies of the fungus was floccose with mycelia and pinnotal type of linear growth. The hyphae were 2.1-4.2 cm and branched. Micoconidia were oval to fusiform, aseptate and measured 4.2-6.3 x 2.4 cm. Chlamydo spores were smooth to rough walled and both intercalary and terminal on short lateral branches in mycelium. Peach yellow to bluish pigmentation was found to be produced by the fungus in medium. The isolate was identified as *Fusarium* sp. XPF-5.

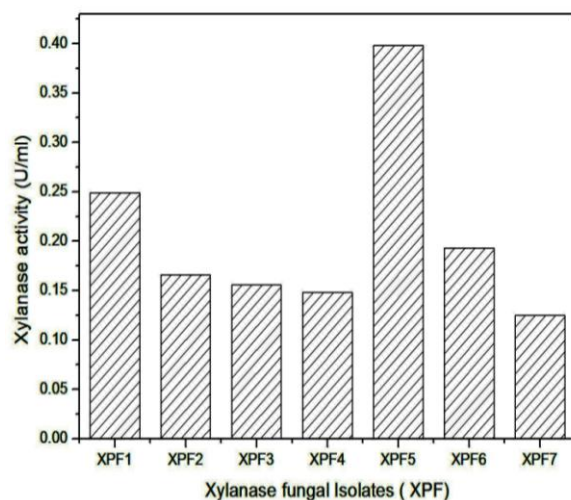


Figure 1: Xylanase activity of various fungal isolates

The biochemical properties (pH, temperature and incubation time optima) of the xylanase produced by *Fusarium* sp. XPF-5 were also determined and results are expressed in Figure 2. Although the enzyme was active (>50% activity) at different temperatures in the range of 37-57°C, but maximum enzyme activity was observed at 57°C. Enzyme activity above 57°C showed an abrupt decline indicating denaturation of the enzyme at a higher temperature. It was higher than the other hyperxylanolytic mutant strain (NTG-19) of *Fusarium oxysporum* with maximum xylanase activity at 50°C (Kuhad et al. 1998). The fungal xylanase was maximum active at pH 9 (0.452 U/ml). The optimum pH for the xylanase activity from other *Fusarium* sp. was reported in the pH range of 4.5 (Ruiz et al. 1997) to 8.0 (Bakri et al. 2013). The observed activity of

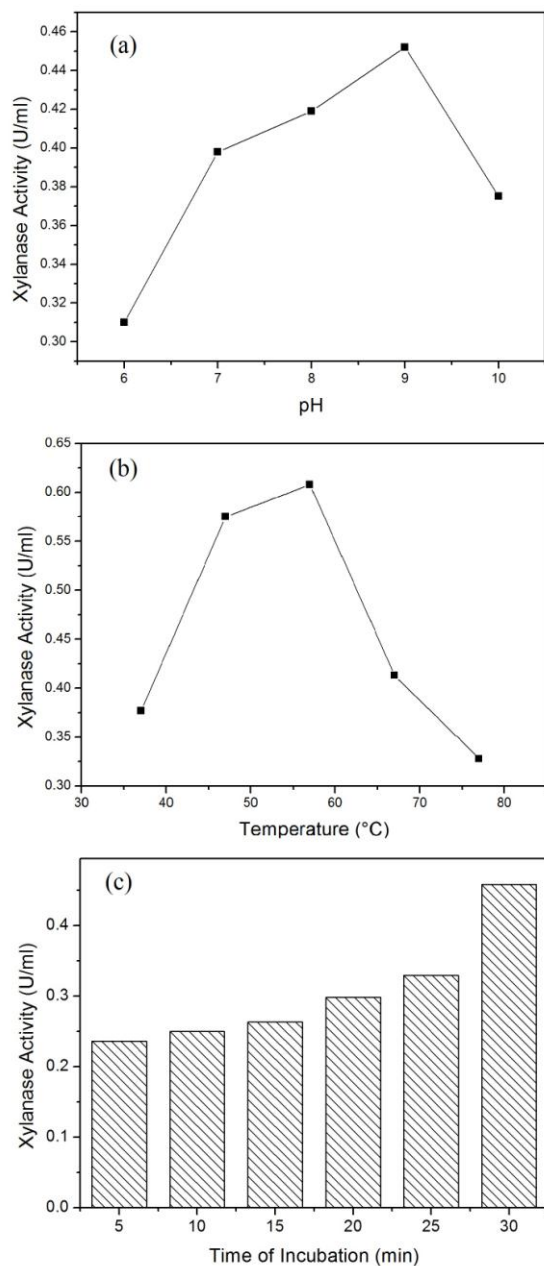


Figure 2: Effect of varying pH (a), temperature (b), and incubation time (c) on xylanase activity

xylanase was highest at 30 min time of incubation is a good prospect considering the possible industrial application of this enzyme. Thereafter, the enzyme activity decreased with increasing time.

In the present study the culture parameters were optimized as these were suggested to enhance the production of enzyme. The parameters studied include the pH, temperature and time of incubation (Figure 3). The culture showed maximum xylanase production at pH 8.0 (0.420 U/ml), indicating that the isolate was alkalophilic in nature. Various studies show the production of xylanases in pH range of 5-9 in species such as *Cephalosporium* sp and *Aspergillus* sp. (Bansod et al. 1993; Chandra and Chandra 1995;

Anthony et al. 2003). However for *Fusarium* sp. the xylanase production at pH 8 in submerged culture conditions has not been reported in the previous studies. The optimum production of xylanase was observed at 47°C (0.462 U/ml). At 37°C temperature, the xylanase production was 33% lower than in 45°C, respectively. The production decreased to almost negligible amount at 77°C (0.087 U/ml). In previous studies with thermostable fungi the optimum temperature for xylanase production was varied between 45 and 60°C (Coral et al. 2002;

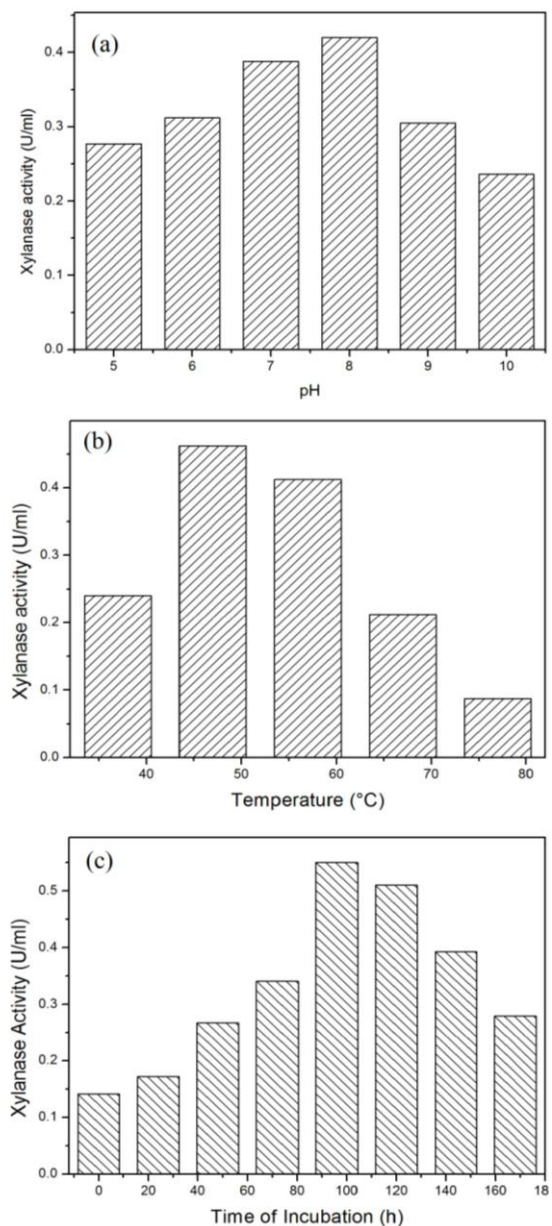


Figure 3: Optimization of xylanase production at different pH (a), Temperature (b), and Incubation time (c)

Isil and Nilufer 2005). As the fungus could best grow upto 47°C therefore it is classified as thermotolerant fungi. To study the effect of time of incubation on the growth and release of extracellular xylanase the growth profile of isolate was observed by monitoring enzyme activity after every 24 h

interval and revealed maximum xylanase production at 96 h. The xylanase activity was enhanced to 0.550 U/ml after optimizing the culture conditions.

Conclusions

The present study reveals that the thermostable alkaline enzyme produced by *Fusarium* sp. XPF-5 is thermo-alkalophile in nature and can be a promising source for thermophilic xylanase. The biochemical properties of characterized enzyme are suitable for its industrial application and a large scale production using fermenter based on optimization results may be carried out in future studies.

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