

Full Length Research Paper

A study of the isolation, purification and characterization of a thermostable alkalophilic xylanase from a fungal source

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The objectives of the present study were Isolation, identification and characterization of xylanase producing fungi, optimization of medium composition and cultural conditions for xylanase enzyme production, production using cheaper sources and extraction and partial purification of extra cellular xylanase enzyme from a potential strain. Xylan has a complex structure consisting of -1, 4-linked xylose residues in the backbone to which short side chains of α - acetyl, -L-arabinofuranosyl, D - - glucuronic and phenolic acid residues are attached. A variety of microorganisms are reported to produce endo xylanases, that can degrade -1,4-xylan in a random fashion, yielding a series of linear and branched oligosaccharide fragments. Totally 69 strains were isolated from Pitchavaram mangroves. In secondary screening, based on the diameter of the clear zone formation in oat spelt xylan agar plates, *Penicillium oxalicum* was selected and optimized for xylanase enzyme production in solid state fermentation using cheaper sources like wheat bran, rice bran, rice straw, sesame oil cake and wood husk. Maximum enzyme activity was observed in wheat bran. (3.89 U/ml) Optimum pH and temperature for xylanase activity were found to be 8 and 45°C at 3% salt concentration. In purification step, 80% ammonium sulphate saturation was found to be suitable giving maximum xylanase activity. The use of wheat bran as a major carbon source is particularly valuable because oat spelt xylan or birch wood xylan are more expensive, Thus the present study proved that the fungal strain *P. oxalicum* used is highly potential and useful for industrial production.

Key words: Xylanases, solid state fermentation, fungi, optimization, purification.

INTRODUCTION

Xylanases show great potential for industrial applications mainly for the bioconversion of lignocelluloses to sugar, ethanol, and other useful substances, clarification of juices and wines, improving the nutritional quality of silage and green feed and the de-inking processes of waste papers (Viikari et al., 2001). The interest in xylan degrading enzyme and its application in the pulp and paper industries had advanced significantly over the past few years (Bajpai et al., 1994; Christov et al., 1999; Garg et al., 1998). Haltrich et al. (1996) gave an overview of fungal xylanases and showed that the enzyme can be produced by a number of microorganisms including bacteria, yeasts and filamentous fungi including *Trichoderma*,

Bacillus, *Cryptococcus*, *Aspergillus*, *Penicillium*, *Aureobasidium*, *Fusarium*, *Chaetomium*, *Phanerochaete*, *Rhizo-*

mucor, *Humicola*, *Talaromyces* etc. These enzymes have been widely detected in fungi and bacteria (Lee et al., 1985; Marques et al., 1998; Shao and Wiegel, 1992).

Chlorinated organic compounds produced from chemical bleaching technologies are harmful to the environment and need to be substituted by eco friendly procedures. Enzymes are the prime concern in processing and waste water treatment now days. Xylanases cleave and solubilize reprecipitated xylan and lignin located on the surface of the microfibrils. This facilitates pulp bleaching and lowers chlorine consumption thereby reducing the discharge of toxic organochlorine compounds in the environment (Senior et al., 1992; Tolan and Canovas, 1992). Moreover, xylanase treatment helps in increasing the brightness of the pulp, which is very much important in developing chlorine free bleaching processes.

Solid-state fermentation can be performed on a variety of lignocellulosic materials, such as wheat straw, wheat bran and corncob, which proved to be highly efficient tec-

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hunique in the production of xylanase (Alam et al., 1994; Hoq and Deckwer, 1995; Haltrich et al., 1996; Gawande and Kamat, 1999; Kang et al., 2004 and Sonia et al., 2005). Solid-state fermentation (SSF) is an attractive method for xylanase production, especially for fungal cultivations, because it presents many advantages, such as the higher productivity per reactor volume as well as the lower operation and capital cost (Purkarthofer et al., 1993; Pandey et al., 1999). The cost of carbon source plays another major role in the economics of xylanase production. Hence, an approach to reduce the cost of xylanase production is the use of lignocellulosic materials as substrates rather than opting for the expensive pure xylans (Haltrich et al., 1996; Beg et al., 2000; Senthilkumar et al., 2005). For the development of suitable xylanase as a pre-bleaching agent, the stability of enzyme at higher optimum pH and temperature is desirable (Beg et al., 2000).

Owing to the increasing biotechnological importance of thermostable xylanases, many thermophilic fungi had been examined for xylanases production (Maheshwari et al., 2000; Singh et al., 2003). Most of the xylanases known to date are optimally active at temperatures below 50 C and are active in acidic or neutral pH (Ryan et al., 2003; Turkiewicz et al., 2000). Enzymes active at high temperatures and alkaline pH values have great potential as they can be introduced at different stages of the bleaching process without requiring changes in pH or temperature (Shoham et al., 1992). Conversely, only a few xylanases are reported to be active and stable at alkaline pH and high temperature (Rani et al., 2000). In view of this, there is a need to search new sources of xylanases and their characterization, especially those with extreme-philic properties. In this present paper, the researcher reports on the isolation, purification and characterization of a thermostable alkalophilic xylanase from a fungal source *Penicillium oxalicum*.

MATERIALS AND METHODS

Collection of samples

The surface water samples and sediment samples were collected using pre-sterilized sample bottles and sterile spatula from Pitchavaram mangrove forest southeast coast of India (Lat. 11°27'N; Long. 79°47'E). Precautionary measures were taken to minimize the contamination. The channels are bordered by heterogeneous mangrove vegetation predominantly by *Rhizophora* spp. followed by *Avicennia* and others in small numbers and the work was carried out in Microbiology laboratory, CAS in Marine Biology, Annamalai University.

Preliminary screening

The xylanase producing fungal strains from water and sediment samples were isolated using dilution-plating technique. One ml of water or one gram of sediment sample was mixed in 9 ml and 99 ml of blank (50% Sea water) respectively. This suspension was serially

diluted to 10⁻⁴. 1ml of the diluted samples from 10⁻³ and 10⁻⁴ dilutions was plated on sterile wheat bran agar (Wheat bran – 1 g, Agar – 15 g, NaNO₃ – 1 g, K₂HPO₄ - 1 g, MgSO₄·7H₂O - 0.5 g, Yeast extract- 1 g, 50% aged sea water – 1000 ml and eliminate the bacterial contamination 8 ml of 1% streptomycin was added to 1 of the medium) surface and incubated at 28 ± 2°C for 3 - 4 days.

Identification of fungi

The fungal isolates were then transferred to fresh plates for purification. Fungi were identified using standard reference manuals (Ellis, 1971, 1976; Raper and Thom, 1949; Raper and Fennell, 1965) and the isolates were preserved on potato dextrose agar slants for further study (Potato infusion – 200 g, Dextrose – 20 g, Agar – 15 g, 50% aged sea water – 1000 ml)

Secondary screening

Those isolated organisms from the preliminary screening were cultured in liquid media containing 5% wheat bran, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O at pH 7 in Erlenmeyer flasks. After incubation on a rotary shaker (37 C, 180 rpm) for 6 days, the culture broth was centrifuged (10,000 rpm for 20 min) and the supernatant was collected for enzyme assay. Its ability to produce xylanase enzyme was further confirmed by the formation of orange coloured digestion halos on oat spelt xylan agar plates when treated with Congo red and washed with 1 M NaCl. The strain *P. oxalicum*, which was isolated from sediment collected from *Avicennia* dominated, zone and showed highest production of xylanase. The strain was maintained on potato dextrose agar and used for further study on xylanase production.

Xylanase production in solid state fermentation

Erlenmeyer flasks (250 ml) containing 10 g of wheat bran were added with the Mandels and Sternburg's basal medium (Mandels and Sternburg, 1976) just to wet the wheat bran. Medium contains (g/l) of peptone-1; (NH₄)₂SO₄ 1.4; KH₂PO₄ 2.0; urea 0.3; CaCl₂ 0.3; MgSO₄·7H₂O 0.3 and (mg/l) of FeSO₄·7H₂O 5.0; MnSO₄·H₂O 1.6; ZnSO₄·7H₂O 1.4; CoCl₂ 2.0, and Tween 80 0.1% (v/v) (pH 5.0). The flasks were inoculated with 2 ml of spore suspension prepared from a week old PDA slants of the culture grown at 30°C. Sterile distilled water containing 0.1% (v/v) Tween 80 was used to prepare fungal spore suspension. Inoculated flasks were incubated at 30°C under static conditions for 10 days. The enzyme from each flask was extracted using 50 ml of 0.05M citrate buffer (pH 5.3) and filtered through a wet muslin cloth by squeezing. The extract was centrifuged at 5000 rpm for 20 min. The clear supernatant was partially purified by ammonium sulphate fractionation (40 – 80%) and dialysed using the same buffer for 24 h with three intermittent changes. After lyophilize the protein sample, xylanase activity was measured in oat spelt xylan agar plates.

Xylanase assay

Xylanase activity was assayed using 1% oat spelt xylan, (Himedia, Mumbai, India) as the substrate. Xylan was dissolved in 50 mM glycine– NaOH buffer (pH 9.0). The reaction mixture, containing 10 L of an appropriate dilution of the enzyme and 250 L of the substrate, was incubated for 10 min. in an incubator maintained at 100°C. The amount of reducing sugars liberated was determined by using 3, 5-dinitrosalicylic acid method (DNS method) (Miller, 1959). One unit of xylanase activity was expressed as 1 mol of reducing sugars (xylose equivalent) released in 1 min under the above condi-

Table 1. List of fungi isolated from different areas.

Organism	Rhizosphora area		Avicenna area	
	W	S	W	S
Zygomycotina				
<i>Absidia cylindrospora</i>	-	+	-	+
<i>Mucor hiemalis</i>	+	-	+	+
<i>M. racemosus</i>	+	+	+	+
<i>Rhizopus nigricans</i>	-	+	-	+
<i>R. oxyzae</i>	+	+	+	+
<i>R. stolonifer</i>	+	+	-	+
<i>Syncephalestrum racemosum</i>	-	+	+	+
Hypomycetes (mitosporic fungi)				
<i>Acremonium strictum</i>	+	-	+	+
<i>Alternaria alternata</i>	-	+	+	+
<i>A. citri</i>	+	+	-	-
<i>A. tenuis</i>	+	-	+	+
<i>Aspergillus clavatus</i>	-	+	+	+
<i>A. flavus</i>	+	+	-	+
<i>A. fumigatus</i>	-	-	+	+
<i>A. niger</i>	+	+	+	+
<i>Cephalosporium herbarum</i>	-	+	-	+
<i>Cladosporium herbarum</i>	+	+	-	+
<i>Curvalaria lunata</i>	-	+	+	+
<i>Fusarium solani</i>	+	+	-	+
<i>Penicillium chrysogenum</i>	-	+	+	+
<i>P. oxalicum</i>	+	-	+	+
<i>P. purpurogenum</i>	-	+	+	+
<i>Trichoderma viride</i>	+	+	+	+
<i>Verticillium sp</i>	-	+	-	+

+ denotes xylanase positive. - denotes xylanase negative.

tions. Protein content was estimated by method of Lowry et al. (1951) with BSA (bovine serum albumin, Himedia, Mumbai, India) as the standard.

Optimization

The optimization of composition of medium and cultural conditions was carried out based on stepwise modification of the governing parameters for xylanase production. The effect of various substrates for SSF, consisting of rice bran, wheat bran, sesame oil cake, wood husk and rice straw was examined by adding 10 g of each substrate in a 250 ml Erlenmeyer flask with 10 ml of sterile distilled water, which was added to moisture the substrates. Cultivation was carried out at ambient temperature (28 ± 3 C) for 5 days.

Changing the pH 3 to 10 in the production medium the effect of pH was observed. The effect of cultivation temperature on the enzyme production was examined at different temperatures starting from 25 to 60 C with 5 C intervals. The effects of incubation period were evaluated by 24 h interval by checking the enzyme activity. The optimization of salt concentration was carried out by adding of different salt concentration, varying from 1 to 6%.

The effect of supplementation of additional carbon and nitrogen sources to wheat bran was examined using carbon sources like glucose, sucrose, galactose, maltose, lactose, oat spelt xylan, sorbitol, fructose and cellulose. Nitrogen sources like were peptone, urea,

yeast extract, sodium nitrate, ammonium sulphate, ammonium nitrate, meat extract and beef extract, were also tried.

RESULT AND DISCUSSION

A total of 13 fungal strains belonging to 13 species coming under 9 genera were isolated from the water samples in *Rhizophora* dominated area, Likewise 19 fungal strains belonging of 19 species under 14 genera were isolated in sediment samples of that area. A total 15 fungal strains belonging 15 species under 11 genera were isolated from the water sample of *Avicennia* dominated area and 22 fungal strains belonging of 14 species under 14 genera were isolated from sediment samples of that area. Collectively only and 7 species 4 genera were found to be members of Zygomycotina where as 17 species in 11 genera were of Deutero-mycotina group represented by Mito-sporic fungi (Table 1) *Aspergillus* occurred in large numbers followed by *Rhizopus*, *Alternaria*, *Mucor* and *Penicillium*. However the total number of fungi isolated in the sediment sample was relatively more than water sample in-

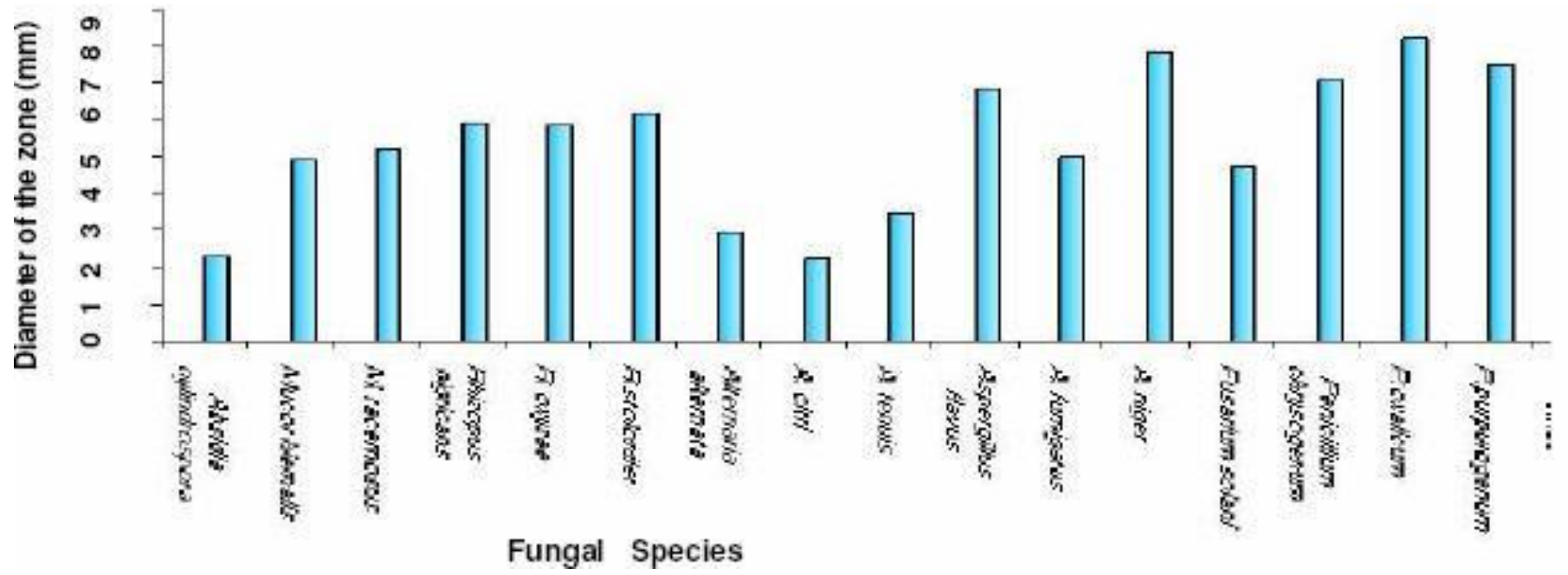


Figure 1. Clear zone formation by xylanase activity.

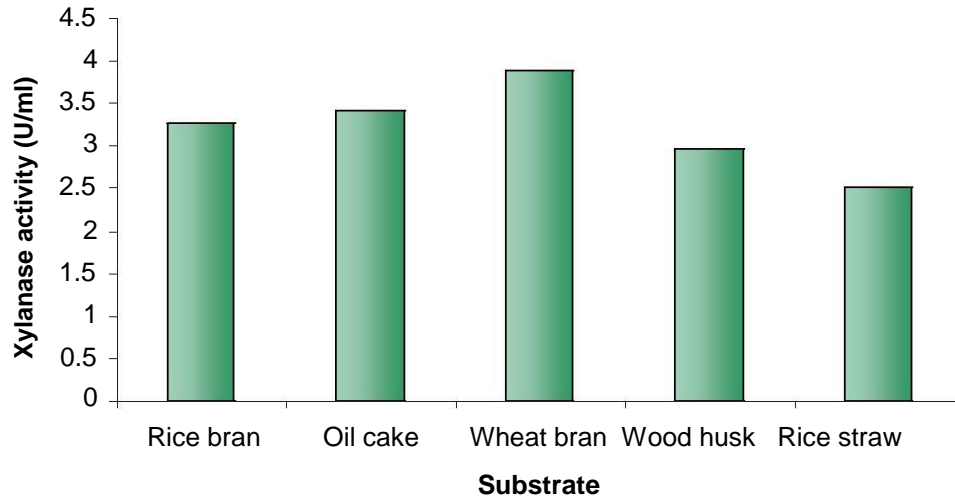


Figure 2. Xylanase activity in different substrates.

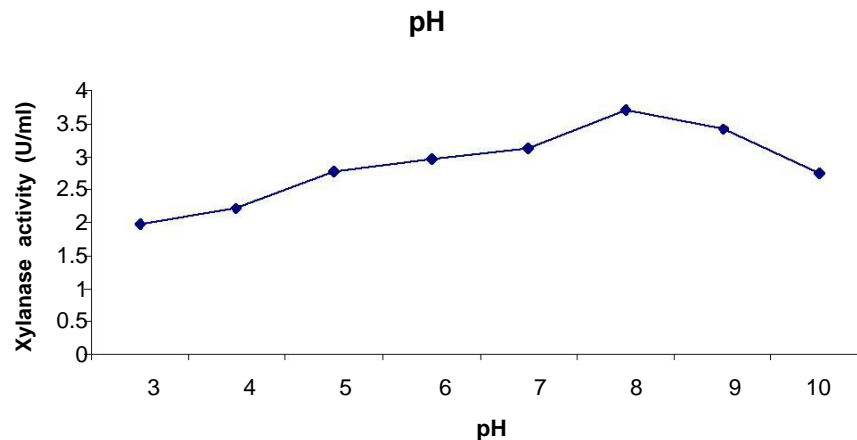


Figure 3. Effect of pH on the production of xylanase by *Penicillium oxalicum*.

indicating that the sediment act as a reservoir of nutrient materials needed for the fungal growth. All these 69 strains were inoculated in to oat spelt xylan agar plates and 17 strains were found to produce the xylanase enzyme is secondary screening (Figure 1). Based on the diameter of the clear zone formation and enzyme activity *P. oxalicum* was selected and optimized for xylanase enzyme production and characterization. Generally filamentous fungi have been widely used to produce xylanases for industrial applications, of which xylanase levels are generally much higher than those produced by yeast or bacteria (Haltrich et al., 1996).

When different substrates were used in the solid state fermentation medium, the highest enzyme activity was obtained in wheat bran (Figure 2) (3.89 Unit/ml) and minimum at rice straw (2.52 Unit/ml). Wheat bran is an inexpensive by product, which contains a lot of xylan. Therefore, it is one of the most popular components of complex media for xylanase production (Deschamps and Huet,

1985; Ghosh et al., 1993; Fujimota et al., 1995; Hoq et al., 1994; Sapereira et al., 2002). However, the wheat bran particles suspended in the cultivation medium have to be decomposed to form soluble compounds to be used by the fungus and also protects the fungal mycelium from the shear forces.

The pH ranging from 3 - 10 was studied for the detection of optimum pH with high xylanase production was found to be pH 8 (3.72 Unit/ml) and minimum was observed at pH 3 (1.98 Unit/ml) (Figure 3). The xylanase has pH optima around 8 but the activity at pH value of 7 – 9 makes it suitable for bio-bleaching applications. Among the xylanases from *Bacillus* species, highest pH optima had been reported for *Bacillus* Tar-1, C-125 and *Bacillus* sp. NCL-86-6-10 (Beg et al., 2001; Techapun et al., 2003). However, several alkaline-tolerant fungal xylanases have also been characterized recently (Bim and Franco, 2000; Tseng et al., 2002; Duarte et al., 1999; Dhillon et al., 2000).

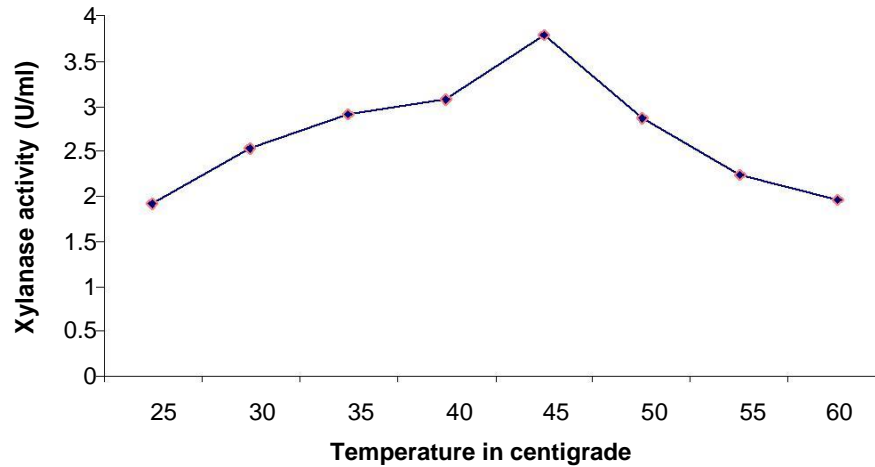


Figure 4. Effect of temperature on the production of xylanase by *Penicillium oxalicum*.

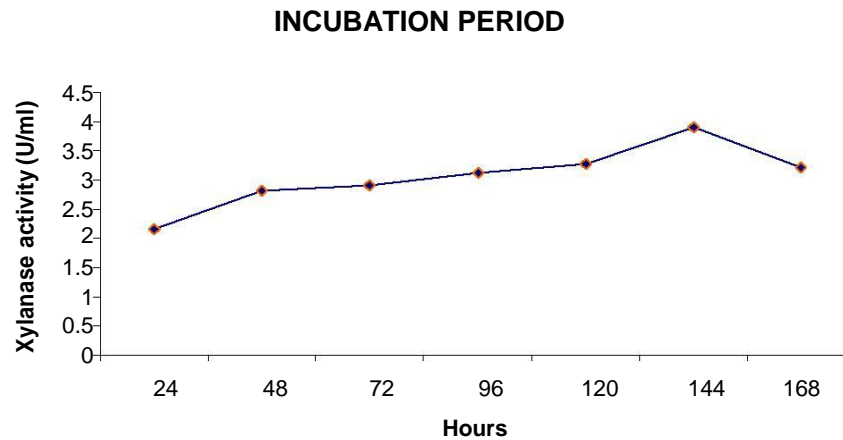


Figure 5. Effect of incubation period on the production of xylanase by *Penicillium oxalicum*.

Temperature varying from 25 to 60 C were examined for the detection of optimum temperature required for the production of enzyme, and the results showed optimum to be 45 C (3.79 Unit/ml) and minimum was observed at 25 C (1.91 Unit/ml) (Figure 4). At 50 C, a significant decline in xylanase activity was evident. The optimum temperature for xylanase production by *P. oxalicum* was similar to some thermophilic fungi, such as *Thermomyces lanuginosus* (Purkarthofer et al., 1993), *Thermoascus aurantiacus* (Kalogeris et al., 1998), and *Sporotrichum thermophile* (Topakas et al., 2003) which were grown in SSF. The results clearly indicated the thermophilic nature of the fungus.

The time course of xylanase production was investigated and maximum production was observed after 6 days (3.89 Unit/ml) while minimum was noted at 24 h (2.17 Unit/ml) (Figure 5). Further incubation after this did not show any increment in the level of enzyme production. When different carbon sources were used in the production medium, the highest enzyme activity was obtained in

oat spelt xylan (Figure 6) (3.79 Unit/ml). Xylanase production of *Penicillium canescens* in SSF was obtained by using the mixture of wheat straw and xylan as substrate (Bakri et al., 2003). Several substances have been indicated in the literature as suitable carbon sources for xylanase-producing micro-organisms, oat wheat (Carmona et al., 1998), birchwood xylan (Rani and Nand, 2001; Tseng et al., 2002; Salles et al., 2000; Duarte et al., 1999), oat spelt xylan (Chan et al., 2002; Chivero et al., 2001; Georis et al., 2000; Sa-pereira et al., 2000; Sa-pereira et al., 2002; Ohta et al., 2001; Saha, 2002; Teotia et al., 2001), bagasse xylan (Breccia et al., 1998), wheat bran arabinoxylan (Bataillon et al., 2000), wheat bran (Liu et al., 1998; Fujimota et al., 1995; Carmona et al., 1998; Zhao et al., 2002; Taneja et al., 2002; Gawande and Kamat, 1998; Gawande and Kamat, 1999), sugar cane bagasse (Martinez-Trujillo et al., 2003) and rice bran (Dhillon et al., 2000) are few recommended sources.

Maintaining the physical factors and the carbon source at optimized condition, nitrogen sources of varying nature

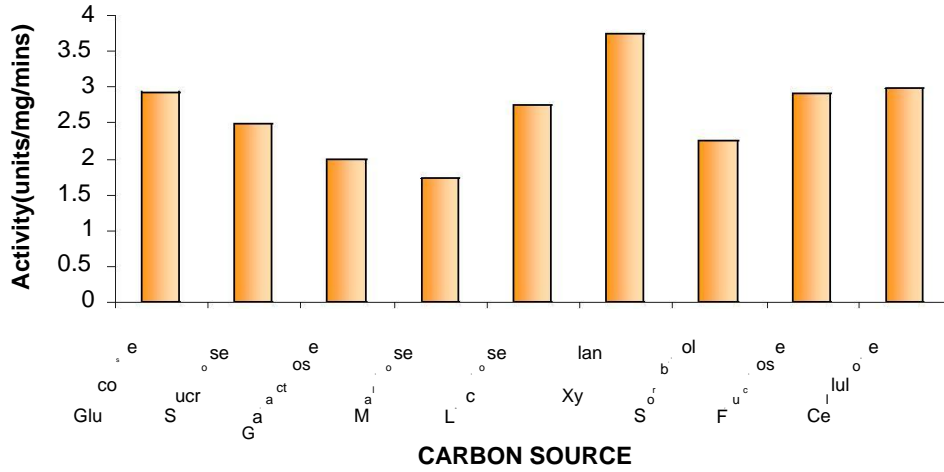


Figure 6. Effect of carbon sources on production of xylanase by the isolate *Penicillium oxalicum*.

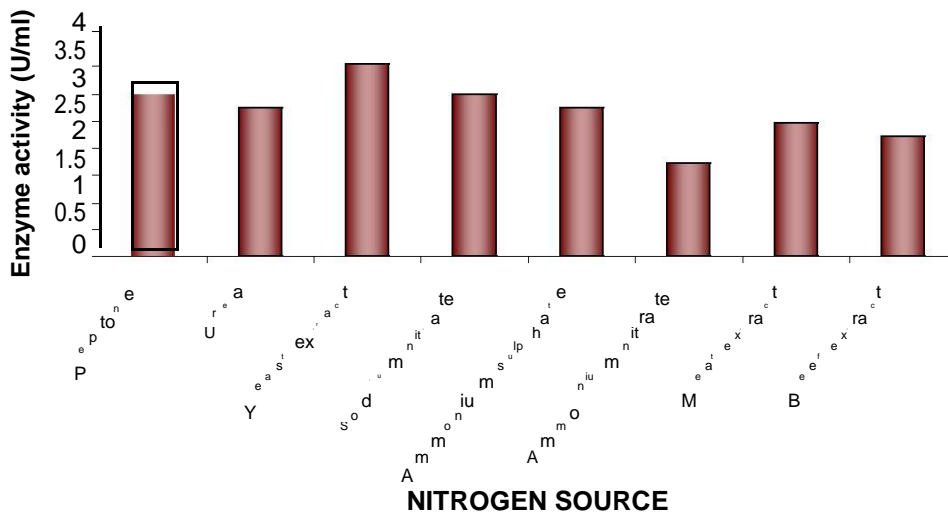


Figure 7. Effect of nitrogen sources on production of xylanase by the isolate *Penicillium oxalicum*.

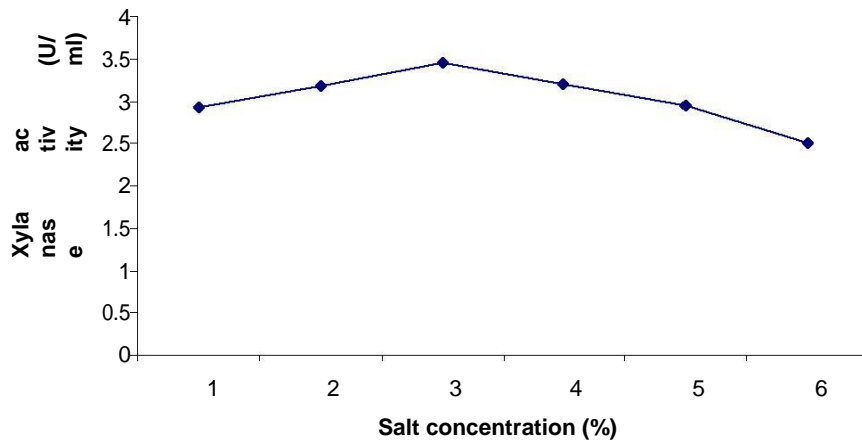


Figure 8. Effect of salt concentration on the production of xylanase by *Penicillium oxalicum*.

were studied, in which yeast extract gave the maximum enzyme activity (3.56 Unit/ml) (Figure 7). These results are in agreement with those reported in the literature (Purkarthofer et al., 1993; Lemos et al., 2001; Bakri et al., 2003). Varying percentage of salt concentration was used to determine the optimum, and enzyme production was confirmed to be highest at 3% of salt concentration (3.46 Unit/ml) (Figure 8). To the best of our knowledge, there is no literature available on the xylanase production by *P. oxalicum*. Hence the present study is an important piece of work regarding the xylanase production from an estuarine fungus.

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