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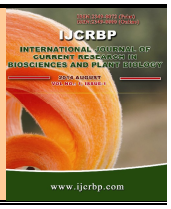

Original Research Article

Production and Optimization of Xylanase Enzyme Activities by Fungal Species in Solid State Fermentation

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A b s t r a c t

The present study was aimed to evaluate the best cultivation conditions and effect of carbon, nitrogen sources for optimizing the production of xylanase by using *Penicillium* sps. The process conditions, such as time, temperature, pH and the levels of the released xylanases were optimized. The time course of xylanase production was investigated and maximum production was observed after 6 days (4.4 U/ml) while minimum was noted at 24 h (2.2 U/ml). 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 (4.2 U/ml).

K e y w o r d s

Bioconversion
Hemicellulose
Penicillium species
Solid state fermentaion
Xylanase

Introduction

Xylan is a major hemicellulose and considered with cellulose and chitin as being among the most abundant polysaccharides in nature. It is composed of a linear backbone of 1,4-β-linked-D-xylopyranosyl units that often has side chains of O-acetyl, arabinosyl and methyl glucuronosyl substituents (Maheshwari et al., 2000). Endo-β-1,4-xylanase (1,4-β- D-xylan xylanohydrolase) is the main enzyme responsible for the cleavage of the linkages within the xylan backbone. Bacterial, fungal and actinomycete xylanases have attracted considerable research interest (Bastawde, 1992; Wong et al., 1988) because of their potential applications in recovery of fermentable sugars from hemicellulose biobleaching of pulp and paper industry and to other industrial applications.

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; 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 micro-organisms including bacteria, yeasts and filamentous fungi *viz.*, *Trichoderma*, *Bacillus*, *Cryptococcus*, *Aspergillus*, *Penicillium*, *Aureobasidium*, *Fusarium*, *Chaetomium*, *Phanerochaete*, *Rhizomucor*, *Humicola*, *Talaromyces*, etc. These enzymes have been widely detected in fungi and bacteria (Marques et al., 1998). Xylanase is one of the microbial enzymes that has aroused great interest recently due to its biotechnological potential in many industrial processes, e.g. in xylitol and ethanol production (Beg et al., 2001), in the cellulose and paper industry (Wong et al., 1988), in the production of oligosaccharides (Pellerin et al., 1991), to obtain cellular proteins, liquid fuels, and other chemical substances, in the food industry (Haltrich et al., 1996), and in poultry, pork, and caprine feeding (Pucci et al., 2003).

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 technique in the production of xylanase (Alam et al., 1994; Haltrich et al., 1996). 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 (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; 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. The objective of this work was to evaluate the best cultivation conditions and effect of carbon and nitrogen sources for optimizing the production of xylanase using *Penicillium* sps. The process conditions, such as time, temperature, pH and the levels of the released xylanases were optimized. Xylanase production using cheaper sources and extraction and partial purification of extra cellular xylanase enzyme were carried out from isolated *Penicillium* sps.

Materials and methods

Collection of samples

The surface soil samples and sediment samples were collected using pre-sterilized sample bottles and sterile spatula from Kancheepuram District, Tamilnadu, India. Precautionary measures were taken to minimize the contamination.

Preliminary screening

The xylanase producing fungal strains from soil and sediment samples were isolated using dilution-plating technique. One gram of soil sample or one gram of sediment sample was mixed in 9 ml and 99 ml of blank (50% distilled) respectively. This suspension was serially diluted to 10^{-4}. 1ml of the samples from 10^{-3} and 10^{-4} dilutions was plated on sterile wheat bran agar.

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.

Secondary screening

Those isolated organisms from the preliminary screening were cultured in Erlenmeyer flasks containing wheat bran liquid medium. 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.

Penicillium oxalicum (as standard)

A culture of *Penicillium oxalicum* was collected from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh and was used as standard.

Chemicals and standard enzyme

All the chemicals used in the present investigation were of analytical grade (AR grade). Standard enzyme – xylanase was procured from Himedia Laboratories Limited, Mumbai.

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. Composition of Mandels and Sternburg's basal medium includes peptone, 1.00 g; ammonium sulphate, 1.40 g; dihydrogen potassium, 2.00g; phosphate urea, 0.30 g; calcium chloride, 0.30 g; magnesium sulphate, 0.3 g; ferrous sulphate, 0.50 g; manganous sulphate, 0.16 g; zinc sulphate, 0.14 g; cobalt chloride, 0.20 g; Tween 80, 0.1% (v/v); pH (5.0); and these ingredients were prepared in 1000 ml distilled water and sterilized at 121°C for 15 mins in autoclave.

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 lyophilizing the protein sample, xylanase activity was measured in oat spelt xylan agar plates.

Xylanase assay

Xylanase activity was assayed using 1% oat spelt xylan, as the substrate. Xylan was dissolved in 50 mM glycine – NaOH buffer (pH 9.0). The reaction mixture, containing 10 µL of an appropriate dilution of the enzyme and 250 µL 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).

A reddish brown colour developed after placing the reaction tubes in a boiling water bath for 5 min. After cooling the reaction tubes to room temperature, the O.D. was measured at 575 nm with xylose as the standard. One unit of xylanase activity was expressed as 1 µmol of reducing sugars (xylose equivalent) released in 1 min under the above conditions. Protein content was estimated by method of Lowry et al. (1951) with BSA (bovine serum albumin) as the standard.

Xylanase enzyme assay method

Xylanase activity was estimated by analysis of reducing sugars released during hydrolysis of 0.5% (w/v) starch in 0.05 M phosphate buffer, pH 6.0 at 50°C for 30 min by the dinitrosalicylic acid method (Miller, 1959). The carbohydrates often termed as sugars are the stuff of life. For most organisms they are an important source of energy required for various metabolic activities of living organisms. The energy is being derived as a result of their oxidation. The food stuffs normally contains certain amount of carbohydrates using DNS, the amount of carbohydrates present in the sample can be determined. Several reagents have been employed which assay sugars by using their reducing properties. One such correspond is 3.5 dinitro salicyclic acid (DNS) which in alkaline solution is reduced to 3 amino 5 nitrosalicyclic acid.

Benedict's test

Benedict test was done to identify the presence of sugar present in any solution. It is a routine test done for the diagnosis of urine sugar and rarely for certain body fluids and some chemical solutions. One percent pectin broth was prepared and sterilized using routine sterilization techniques. The test organisms were inoculated and incubated at 37°C for 24 h. Then in a separate test tube benedict test was carried out. Five ml Benedict's reagent was taken in a test tube and 9 drops of test broth was added and then it was heated till boiling near the Bunsen flame. After cooling near the tap water flow, the tube was observed for colour change and the results were compared with standard table for glucose estimation.

Optimization of xylanase

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, sugar cane waste, 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 moisten 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%.

Effect of various carbon sources and nitrogen sources on xylanase production

The effect of supplementation of additional carbon and nitrogen sources to wheat bran was examined using carbon sources. To detect the effect of various carbon-sources on xylanase production 250 ml Erlenmeyer flasks were prepared containing 100 ml of mineral salts medium supplemented with 0.2% (w/v) 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. Xylanase assays were performed daily.

Results

Primary screening of fungal isolates

Fungal species isolated from various sources and sites of different soil samples, were purified and their cultural and morphological characteristics were examined. Morphological study of these isolates permitted to group and more stress was given identify the xylanase producing fungus. Based on the characters fungal sps identified as *Aspergillus sps*, *Penicillium sps*, *Fusarium sps*, and *Mucor sps*. These isolate have been screened in the present studies to select the potent fungi, capable of producing xylanase in solid state fermentation.

Fungal cultures were further screened by Oat spent xylan plate method and the potency index was calculated. These isolates were categorized as high, moderate and low xylanase producers, respectively (Table 1).

Table 1. Xylanase assay in solid media by Oat Spent Xylan plate method.

Name of the organism	Zone of hydrolysis (in mm)	Xylanase producing effect
Aspergillus sps	22	High
Penicillium sps	26	Very High
Fusarium sps	16	Moderate
Mucor sps	12	Low
Control without organisms	No zone	No effect

The results from Benedicts test for reducing sugar estimation and qualitative estimation of reducing groups using the isolated fungal sps such as *Aspergillus sps*, *Penicillium sps*, *Fusarium sps*, and *Mucor sps* were tabulated in Table 2.

Table 2. Benedicts test for reducing sugar estimation in fungal species.

Name of the organism	Colour development	Range of increase
Aspergillus sps	Dark green	++
Penicillium sps	Dark green	++
Fusarium sps	Green	+
Mucor sps	Slight green colour	+
Control without organisms	No change	Nil

High xylanase producing fungal species were further screened semi-quantitatively by different xylanase assay methods. The maximal xylanolytic activity was observed in isolates of mixed cultures such as *Aspergillus sps*, *Penicillium sps*, *Fusarium sps* and *Mucor sps*. The maximum activity was seen in *Penicillium sps* (0.966). Minimum enzyme activity was observed in *Aspergillus sps* (0.912) (Table 3 and Fig. 1).

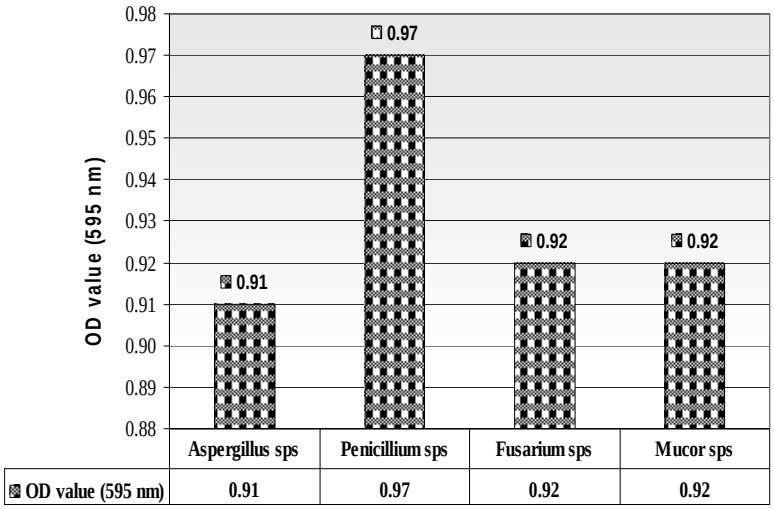
Production of xylanase by isolated organisms:

The production profile of xylanase on xylan containing basal medium without sucrose was studied. It was found that maximal production of xylanase (4.2 U/ml) was obtained in *Penicillium sps* after 48 and 72 h of incubation, respectively. Further incubation resulted in the decline in enzyme activities up to 120 h.

Table 3. Estimation of xylanase activity of fungal species by DNS method.

Name of the organism	OD value
Aspergillus sps	0.912
Penicillium sps	0.966
Fusarium sps	0.922
Mucor sps	0.916
Control without organisms	Nil

Fig. 1: Estimation of xylanase activity in fungal species by DNS method.



Production of xylanase by isolated organisms

It was found that maximal production of xylanase (4.2 U/ml) was obtained in *Penicillium sps* after 48 and 72 h of incubation, respectively. Further incubation resulted in the decline in enzyme activities up to 120 h.

Effect of pH

A pH of 7.0 was found more suitable for xylanase (4.2 U/ml) activity. Either increase or decrease in pH beyond the optimum value showed decline in enzyme activities. From the above experiments it was concluded that the *Penicillium sps* showed maximum xylanolytic activities after two to three days of incubation in a medium containing wheat bran (Fig. 2).

Effect of temperature

The results showed that the flasks containing basal medium supported maximal xylanase (4.2 U/ml) activities at 45ºC produced by *Penicillium sps*. The production of the enzyme however, was drastically affected at incubation temperatures of 50 or 55ºC (Fig. 3).

Effect of assay temperature (45°C) and assay incubation on xylanase activity

At assay temperature of 45ºC for 144 h was most suitable for maximum xylanase activity (4.4 U/ml). With the increase in incubation period, a constant decrease in enzyme activity was observed. At 168 h incubation, only 20% activity was quantified (Fig. 4).

Effect of carbon sources on production of xylanase by the isolate *Penicillium sps*

The results of different carbon sources used in the production medium showed the highest enzyme activity in oat spent xylan (4.2 U/ml). Xylanase production of *Penicillium sps* in SSF was obtained by using the mixture of wheat straw and xylan as substrate (Fig. 5).

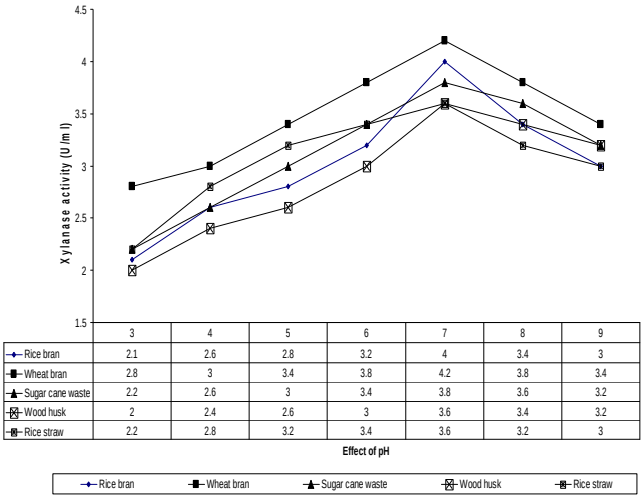
Effect of nitrogen sources on production of xylanase by the isolate *Penicillium sps*

Maintaining the physical factors and the carbon source at optimized condition, nitrogen sources of varying nature were studied, in which yeast extract gave the maximum enzyme activity (4.0 /ml) (Fig. 6).

Discussion

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).

Fig. 2: Effect of pH on xylanase activity in different substrates by *Penicillium* sps.



	3	4	5	6	7	8	9
Rice bran	2.1	2.6	2.8	3.2	4	3.4	3
Wheat bran	2.8	3	3.4	3.8	4.2	3.8	3.4
Sugar cane waste	2.2	2.6	3	3.4	3.8	3.6	3.2
Wood husk	2	2.4	2.6	3	3.6	3.4	3.2
Rice straw	2.2	2.8	3.2	3.4	3.6	3.2	3

Fig. 3: Effect of temperature on xylanase activity in different substrates by *Penicillium* sps

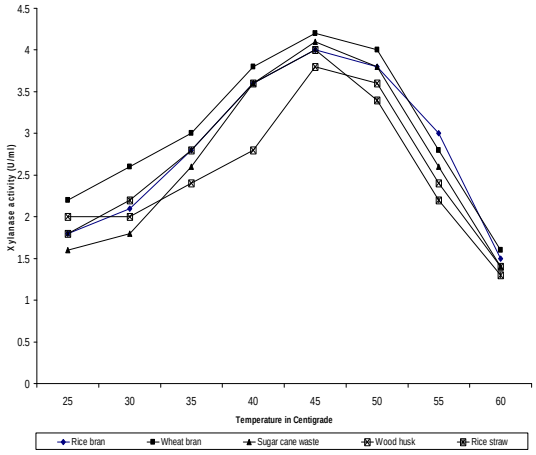


Fig. 4: Effect of assay temperature (45°C) and assay of incubation period on xylanase activity in different substrates by *Penicillium sps*

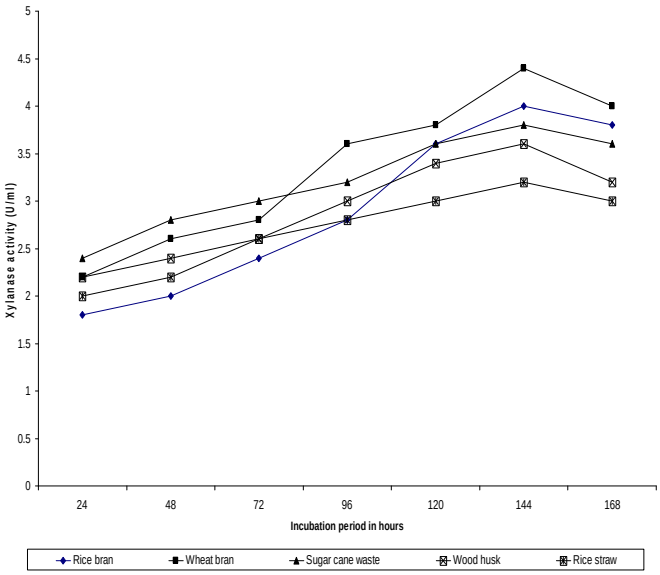
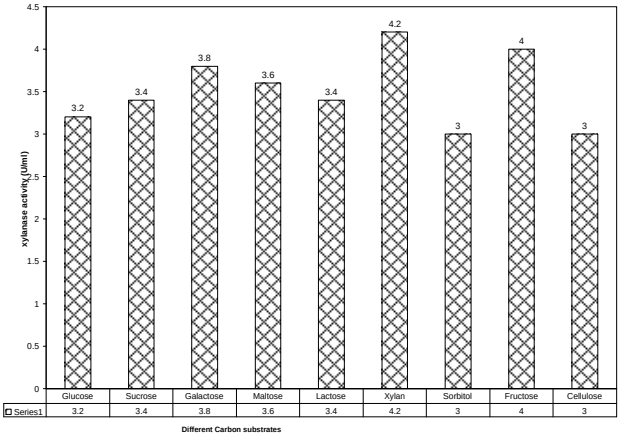


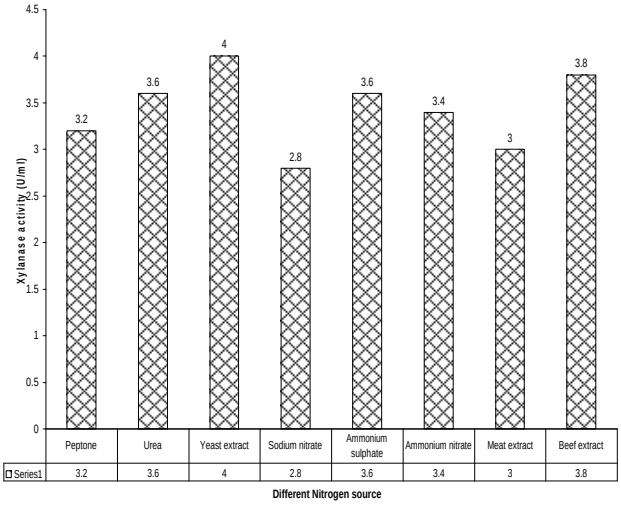
Fig. 5: Effect of carbon sources on production of xylanase by the isolate *Penicillium sps*



All these 4 strains were inoculated in to oat spent xylan agar plates and were found to produce the xylanase enzyme in secondary screening. Based on the diameter of the clear zone formation and enzyme activity *Penicillium* sps 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 (4.2 U/ml) and minimum at rice bran (3.2 U/ml). Wheat bran is an inexpensive by product, which contains lot of xylan. Therefore, it is one of the most popular components of complex media for xylanase production (Ghosh et al., 1993; 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.

Fig. 6: Effect of nitrogen sources on production of xylanase by the isolate *Penicillium sps*



The pH ranging from 3-10 was studied for the detection of optimum pH with high xylanase production was found to be pH 7.0 (4.2 U/ml) and minimum was observed at pH 3 (2.6 U/ml) (Fig. 2). The xylanase has pH optima around 7 but the activity at pH value of 6–9 makes it suitable for bio-bleaching applications. Among the xylanases from fungal species, highest pH optima had been reported for *Penicillium sps* (Beg et al., 2001; Techapun et al., 2003). However, several fungal xylanases have also been characterized recently (Bim and Franco, 2000; Tseng et al., 2002; Duarte et al., 1999; Dhillon et al., 2000).

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 (4.2 U/ml) and minimum was observed at 25°C (2.2 U/ml) (Fig. 4). At 50°C, a significant decline in xylanase activity was evident. The optimum temperature for xylanase production by *Penicillium* sps was similar to some 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 time course of xylanase production was investigated and maximum production was observed after 6 days (4.4 U/ml) while minimum was noted at 24 h (2.2 U/ml). 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 (Fig. 5) (4.2 U/ml). Xylanase production of *Penicillium sps* in SSF was obtained by using the mixture of wheat straw and xylan as substrate (Bakri et al., 2003).

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