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Purification and characterization of xylanase from *Aspergillus fumigatus* isolated from soil

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The objectives of the present study were to purify and characterize xylanase enzyme from the fungus obtained from soil. A total of 40 fungi were isolated from 25 soil samples collected after primary screening on Potato Dextrose Agar. In the secondary screening (malt extract agar, 0.5% birch wood xylan), based on the diameter of the clear zone, the fungus was identified as *Aspergillus fumigatus* by microbial type culture collection (MTCC), Chandigarh, India and was selected for xylanase enzyme production in solid state fermentation using wheat bran. Xylanase was subjected to a three-step purification scheme involving ammonium sulphate precipitation, gel filtration chromatography and anion exchange chromatography. Purity was verified by running the extracted protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a single band was observed. When compared with the standard wide range protein molecular markers on SDS-PAGE, it was found to have a molecular weight of 43 KDa. The K_m and V_{max} value of xylanase was 3.12 mg/ml and 2857 µmol/min/mg protein as obtained from a Lineweaver-Burk plot. The optimal temperature and pH was found to be 30 and 10°C, respectively. After 4 h of incubation, enzyme retained 100% activity at 30°C. Xylanase was incubated at various pH levels (2 to 12) for 4 h at 30°C, and the residual activity was measured. More than 65% of the original activity was retained at pH ranging from 4 to 10 after 4 h.

Key words: Xylanase, *Aspergillus fumigatus*, production, enzyme purification, enzyme characterization, Lineweaver–Burk plot.

INTRODUCTION

Xylan, the major hemicellulose component in a plant cell wall, is easily found in solid agricultural and agroindustrial residues, as well as in effluents released during wood processing (Collins et al., 2005). Frequent inappropriate discarding of xylan caused great damage to the ecosystem (Prade, 1995). Xylanase (1, 4-β-D-xylan xylanohydrolase; EC 3.2.1.8) are hemicellulases that hydrolyze xylan, which is a major constituent of the hemicelluloses complex (Khasin et al., 1993). Xylanases have high potential for biotechnological applications. For instance, xylanases can be used to improve the digestibility and nutritional value of ruminant fodder, to facilitate composting process, to improve the quality of bread, to develop environmentally friendly pulp bleaching process and to transform lignocellulosic materials to fermentable products (Singh et al., 2011).

A variety of microorganisms, including bacteria, yeasts and filamentous fungi have been reported to produce xylanases (Stricker et al., 2008). The use of cheaper lignocellulosic residues viz. wheat bran, wheat straw, corn cob and sugarcane bagasse can be used as growth substrates in culture to produce xylanase and also to replace the xylan as an inducer for cost reduction in production. Fungal species known to produce xylanase

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include *Aspergillus, Disporotrichum, Pencillium, Neurospora, Fusarium, Trichoderma*, etc. (Kulkarni et al., 1999). Fungi have been studied widely for alkalistable xylanases over bacteria as they offer many advantages-fungi are biodiverse, produce extracellular enzymes which obviates the need for cell breakage, can efficiently utilize complex agro-industrial wastes as substrates for enzyme production, produce higher amounts of enzymes, are more tolerant and better adapted to changes in the environmental factors for growth and produce several auxiliary enzymes which are important in debranching of substituted xylans (Bakri et al., 2010). However, the main problem faced by the pulp and paper industry while using enzyme treatment is the availability and cost of the enzyme. About 30 to 40% of the production cost of many industrial enzymes is accounted for by the cost of growth substrate (Hinnman, 1994). The use of low cost substrates for the production of industrial enzymes is one of the ways to greatly reduce production costs. This can be achieved using solid agricultural waste materials as substrates (Wizani et al., 1990).

Considering the industrial importance of xylanase, we undertook a study to isolate and identify the strain which secrete extracellular xylanase. However, previously, number research studies were found to have partial, single or two step purification of xylanase. In the present work, we performed three steps purification which can help to get the highly purified form of enzyme; further this can also be used to get the crystallization and structure determination.

MATERIALS AND METHODS

Organism and culture conditions

Twenty-five soil samples were collected from different places of western Uttar Pradesh and screened for the isolation of different species of fungi. The dilution-plate method was employed for this purpose. Potato dextrose agar (PDA) medium containing 0.5% birch wood xylan was used as the isolation medium.

The fungal isolates formed were sub cultured to purity and examined for xylanolytic activities. Malt extract agar (MEA) containing 0.5% (w/v) of birch wood xylan was used as the isolation medium. Positive xylanolytic isolates were detected based on the clear zone hydrolysis on xylan. The selected strains were further tested for their abilities to produce extracellular xylanase under solid-state fermentation.

Solid-state fermentation

Wheat bran was used as substrate for xylanase production. Wheat bran was oven dried at 70°C for 48 h and used as a substrate. Fermentation was carried out in Erlenmeyer flasks (250 ml), supplemented with nutrient concentration (g/l, wt/ vol.): KCl 0.5, $MgSO_4.7H_2O$ 0.5 g, $(NH4)_2HPO_4$ 2.5, NaH_2PO_4 0.5, $CaCl_2.2H_2O$ 0.01, $FeSO_4.7H_2O$ 0.01, $ZnSO_4.7H_2O$ 0.002, Birch wood xylan 1.0, pH 5. Media were then autoclaved for 20 min at 121°C (15 lbs). After cooling, the flasks were inoculated. Spores from seven day old slants were harvested by aseptically adding sterile distilled water containing 0.01% Tween-80 so as to get the final concentration of 1×10^6 spores/ml. 1 ml from this solution was taken as inoculum. The sterilized media was inoculated with 1 ml of spore suspension having 1×10^6 spores/ml. After mixing, the flasks were incubated at 28 ± 2°C under static conditions for seven days. After the incubation, the enzyme was harvested by adding minimum amount of sodium citrate buffer (50 mM, pH 5.3) and mixed thoroughly. After 1 h, the fermented slurry was filtered through cheese cloth and the filtrate was centrifuged at 11200 g for 20 min at 4°C. The supernatant was filtered through Whatman No.1 filter paper and the clear filtrate was used as the crude enzyme.

Enzyme assay

The xylanase activity was assayed by estimating the amount of reducing sugar released under assay conditions. Xylanase activity was measured by determining the amount of reducing groups released according to the method described by Bailey et al. (1992).

The enzyme was harvested by adding minimum amount of sodium citrate buffer (50 mM, pH 5.3) and mixed thoroughly. 1% Birch wood xylan solution was prepared by homogenizing 1.0 g of birch wood xylan in 80 ml sodium citrate buffer (50 mM, pH 5.3) at 6°C by continuous stirring and heated to boiling point, cooled with continued stirring, and stirred slowly overnight. 100 ml of solution was prepared with buffer and stored at 4°C.

Dinitrosalicylic acid reagent (DNS reagent) was prepared by dissolving with continuous stirring, 1.0 g 2,4-dinitrosalicyclic acid (DNS), 200 mg crystalline phenol and 50 mg sodium sulphite in 100 ml 1.0% NaOH and stored at 4°C. 0.1% xylose solution (standard) was prepared by dissolving 100 mg xylose in sodium citrate buffer (50 mM, pH 5.3) in a standard flask and was stored at 4°C.

900 µl of xylan solution was taken and incubated at 5°C for 5 min in a water bath. 100 µl appropriately diluted enzyme solution was added and incubated at 50°C for 5 min. 1.5 ml DNS reagent was mixed and removed from the water bath, boiled for 5 min and was cooled in cold water. Absorbance was measured at 540 nm and the reaction was terminated at zero time in the control tubes. Standard graph was prepared with 0 to 500 µg xylose. The amount of xylose released per mL per minute was calculated from standard curve of xylose. One unit of enzyme activity is defined as the enzyme that releases 1 $\mu molmL^{-1}$ min^{-1} xylose under standard assay conditions.

Estimation of total protein

Total soluble protein was measured according to Bradford method (Bradford, 1974). 100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml 95% ethanol. 100 ml 85% (w/v) phosphoric acid was added and diluted it to 1 L when the dye has completely dissolved and filtered through Whatman 1 paper.

Enzyme purification

For purification studies, filtrate of seven days old solid state fermentation (SSF) culture was taken. Three steps purification was done to separate and purify the xylanase viz., ammonium sulphate fractionation, gel filtration chromatography and anion exchange chromatography. Molecular weights of purified proteins were determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis using wide range protein molecular weight markers as standards.

Ammonium sulphate fractionation

The culture filtrate was centrifuged at 11200 g for 20 min at 4°C to remove suspended particles. The clear supernatant was treated

Table 1. Isolates showing maximum growth and clearing zones in mm.

S/N	Name of isolate	Growth on MEA (mm)	Clear zone (mm)
1	Isolate 1	5.0	1.5
2	Isolate 2	6.5	3.5
3	Isolate 3	4.8	1.0
4	Isolate 4	5.25	2.0
5	Isolate 5	2.8	3.0
6	Isolate 6	4.7	2.4
7	Isolate 7	4.9	1.25
8	Isolate 8	4.8	2.25
9	Isolate 9	4.5	2.5
10	Isolate 10	4.9	2.45

with ammonium sulphate so as to get 30% saturation. Addition of ammonium sulphate was carried out with continuous stirring in an ice water bath, and then it was kept at 4°C for overnight. The precipitated protein was removed by centrifugation at 11200 g for 20 min at 4°C. Ammonium sulphate was again added to get 90% saturation. The precipitated protein was again separated by centrifugation at 11200 g for 30 min at 4°C. The pellet obtained was dissolved in 50 mM sodium acetate buffer, pH 8. The precipitate obtained after treatment with ammonium sulphate was dialyzed extensively against 50 mM sodium acetate buffer, pH 8 with occasional changes of buffer. Cellulose membrane dialysis bags were used for dialysis. The dialyzed sample was concentrated by polyethylene glycol (PEG) 20,000.

Gel filtration chromatography

The concentrated protein was subjected to gel filtration which was carried out by using the column (3.5 × 50 cm). Before loading the column, it was well equilibrated with 50 mM sodium acetate buffer (pH 8). 5 ml of concentrated enzyme was loaded to the top of the column. The enzyme was eluted using 50 mM sodium acetate buffer pH 8 at a flow rate of 20 ml/h and fractions of 5.0 ml were collected. The protein in each fraction was analyzed by taking absorbance at 280 nm.

Anion exchange chromatography

The concentrated protein was loaded to the top of the column. The unbound protein was eluted in 50 mM sodium acetate buffer pH 8, while the bound proteins were eluted with a linear gradient of NaCl (0.1 M) in the same buffer. Flow rate was adjusted to 20 ml/h and fractions of 5.0 ml were collected. Each fraction was analyzed for enzyme activity and protein (at 280 nm). Fractions eluted in the NaCl gradient were dialyzed against 50 mM sodium acetate buffer pH 8. The active fractions were pooled and stored at 4°C for further studies.

Poly acrylamide gel electrophoresis (PAGE)

The purified enzymes were subjected to electrophoretic studies. Sodium dodecyl sulphate (SDS) PAGE was done in 12.5% gels according to Laemmli (1970). Protein bands were stained with Coomassie Brilliant Blue G-250. After the electrophoresis, the gel was immersed in the staining solution (Coomassie Brilliant Blue G-250) for 1 h. To remove excess stain from the gel, the gel was immersed in acetic acid (7.5%) for de-staining. After 2 to 3 changes of de-stain solution, the protein bands become very clear against a white background.

The wide range protein molecular weight marker was used for molecular weight determination of proteins. It contains myosin (205,000 Da), phosphorylase b (97,400 Da), bovine serum albumin (66,000 Da), ovalbumin (43,000 Da) and carbonic anhydrase (29,000 Da).

Characterization of xylanase

The different properties of purified xylanase were studied. The characters studied were the effect of pH on the activity and stability, effect of temperature on the activity and stability, substrate concentration (kinetic parameters such as K_m and V_{max}).

The optimum temperature needed for maximum activity of the purified xylanase was determined by varying the reaction temperature from 30 to 80°C. The thermal stability of xylanase was determined by measuring the residual activity after incubating the enzymes at temperatures ranging from 30 to 80°C for 4 h.

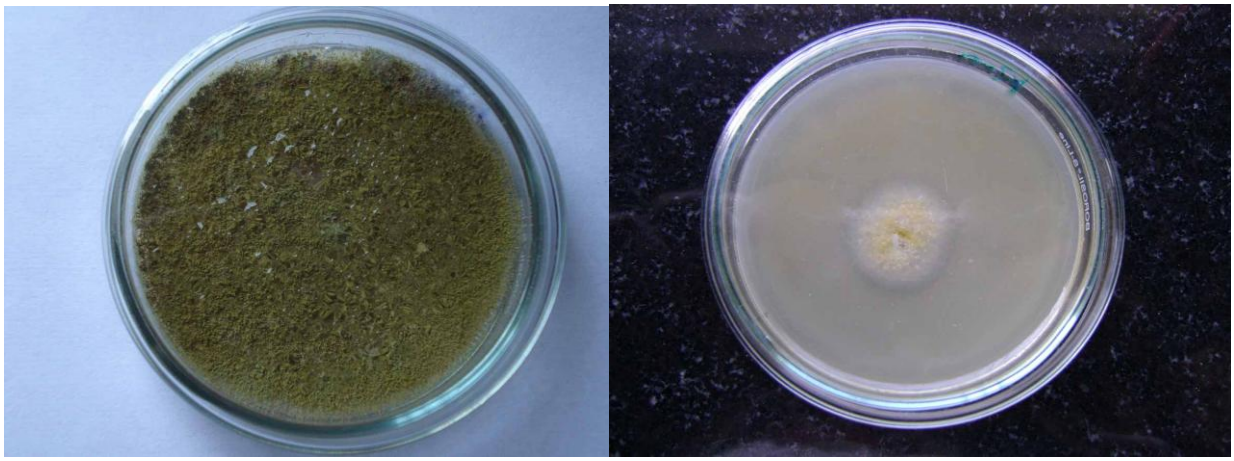
The effect of pH on the activity was determined by incubating xylanase at various pH ranging from 2 to 12. The various buffers used were 50 mM glycine-HCl (2 to 3), 50 mM sodium acetate (4 to 6), 50 mM Tris-HCl (7 to 9) and 50 mM glycine-NaOH (10 to 12). The stability was determined after incubating the enzymes at a pH of 2 to 12 at room temperature for 4 h and the residual activity was determined.

The xylanase was incubated with various concentrations of birch wood xylan (4 to 10 mg/ml) solutions prepared in 50 mM sodium citrate buffer at pH 5.3 and the enzyme activity was estimated. Kinetic parameters K_m and V_{max} were calculated by linear regression from Lineweaver-Burk plots.

RESULTS

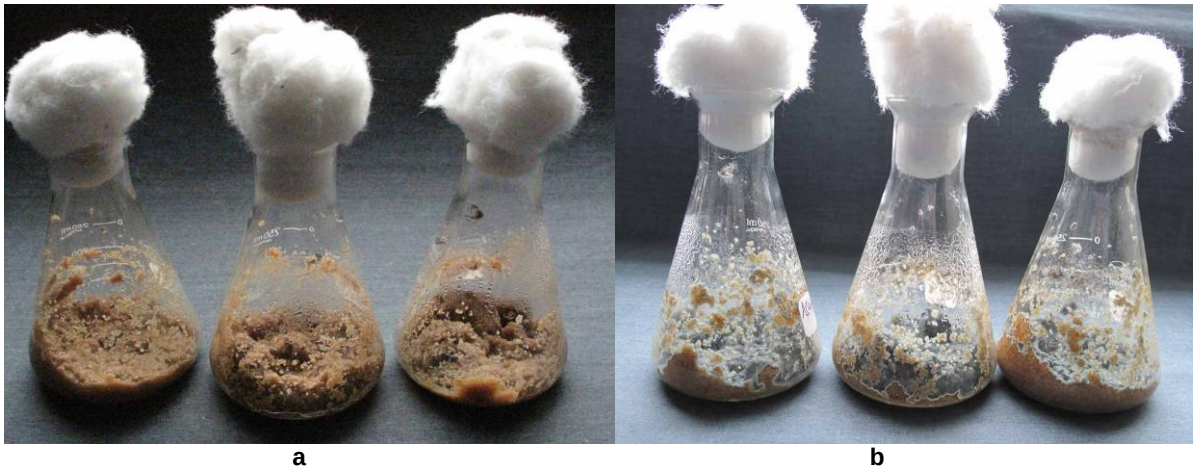
Selection and identification of isolate

A total of 40 fungal strains were isolated from 25 soil samples collected from different areas of western Uttar Pradesh. After primary screening, all the fungal species collected were screened by growing on MEA containing 0.5% birch wood xylan. Among the ten selected fungal species, on the basis of their performance of degrading xylan, the isolate (isolate 2) (Table 1) showing maximum



a b

Figure 1. Isolate 2 of *Aspergillus* sp. showing; **(a)** maximum xylanase activity and **(b)** clear zone.



a b

Figure 2. (a) Each flask with 20 g wheat bran and 50 ml of distilled water; (b) growth of fungi in flask for enzyme production.

activity was identified as *Aspergillus* (Figures 1a and b) at genus level using the guidelines, as detailed in Fundamentals of Diagnostic Mycology (Fisher and Cook, 1998) and Atlas of Clinic Fungi (Hoog et al., 2000). *Penicillium* was the second most common genus. *Trichoderma* represented the third common genus. The isolate was further identified as *Aspergillus fumigates* by IMTECH Chandigarh and selected for further studies.

Enzyme production

Enzyme production for secondary screening was done in solid state fermentation medium (Figures 2a and b). Wheat bran proved a suitable substrate along with 0.5% birch wood xylan for the production of xylanase during SSF, as endorsed by various other workers (Gawande and Kamat, 1999; Malarvizhi et al., 2003).

Enzyme purification

The purification procedures of xylanase are given in Table 2. The culture filtrate had a total xylanase activity of 37.44 KU, of which 51.15% of activity was retained in ammonium sulphate fraction. After the three step purification, the xylanase was purified 93.41 times with a yield of 4.49%, respectively. The specific activity of xylanase obtained was 1.17 KU/mg. Purity was verified by running the extracted protein on native PAGE and a single band was observed (Figure 3). When compared with the standard wide range protein molecular markers on SDS-PAGE, it was found that xylanase had a molecular mass of 43 KDa.

Characterization studies of purified xylanase

The optimum temperature of the enzyme was found to be

Table 2. Summary of the three steps purification of xylanse from *A. fumigatus.*

Purification step	Total volume (ml)	Total activity (KU)	Total protein (mg)	Specific activity (KU/mg)	Purification (fold)	Yield (%)
Crude culture filtrate	365	37.44	2996.65	0.0125	1	100
Ammonium sulfate precipitation (30 to 90%)	25	19.150	107.5	0.178	14.26	51.15
Gel filtration (Sephadex G-200)	75	10.35075	45	0.230	18.42	27.65
Anion exchange chromatography (DEAE -Sephadex A-50)	60	1.680	1.44	1.17	93.41	4.49

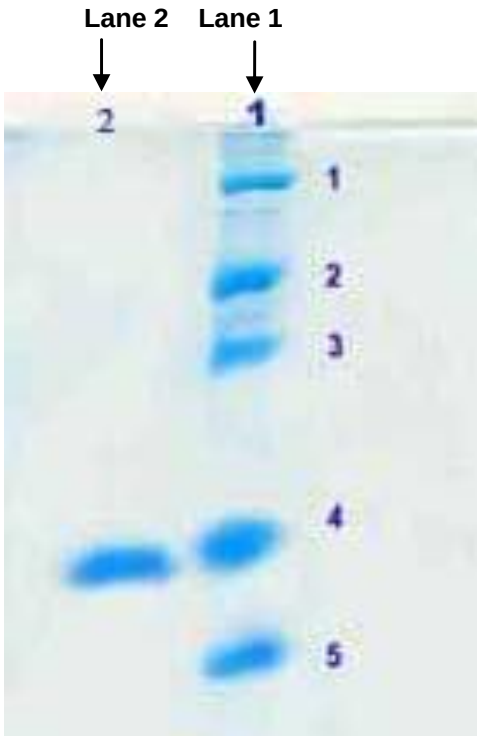


Figure 3. SDS page of xylanase. Lane 1, molecular weight markers; Lane 2, purified enzyme. Molecular marker includes the following proteins, 1, Myosin (205,000 Da); 2, phosphorylase b (97,400 Da); 3, bovine serum albumin (66,000 Da); 4, ovalbumin (43,000 Da); 5, carbonic anhydrase (29,000 Da).

30°C (Table 3). On increasing the temperature, the enzyme activity gradually decreased. At 40°C, enzyme retained 64.82% activity and at 60°C, 38.30% activity was retained. After 4 h of incubation, enzyme retained 100% activity at 30°C. 93.11% activity was retained after 4 h of incubation at 40°C (Figure 4). After 4 h of incubation at 50°C, enzyme retained 50.45% of the original activity. Only negligible activity was retained at higher temperatures (above 70°C).

Xylanase activity was studied at different pH ranging from 2 to 12 and the purified xylanase produced by *A. fumigatus* showed optimum enzyme activity at pH 10 (Table 4). Enzyme xylanase was incubated at various pH levels (2 to 12) for 4 h at 30°C, and the residual activity was measured (Figure 5). More than 65% of the original activity was retained at pH ranging from 4 to 10 after four hours of incubation.

The effect of various concentrations of birch wood xylan (4 to 10 mg/ml) solution on the activities of xylanase was tested. Kinetic parameters K_m and V_{max} were calculated by measuring the reaction velocity at different concentrations of the substrate. The reciprocal

Table 3. Effect of different temperature on xylanase activity from *A. fumigatus* (enzyme reaction was carried out at different temperatures under assay conditions at pH 8).

Temperature (°C)	Enzyme activity (U/ml)	Relative activity of enzyme (%)
30	27.83±0.04	100
40	18.04±0.03	64.82
50	14.54±0.03	52.24
60	10.66±0.02	38.30
70	7.54±0.03	27.09
80	0.97±0.01	3.48

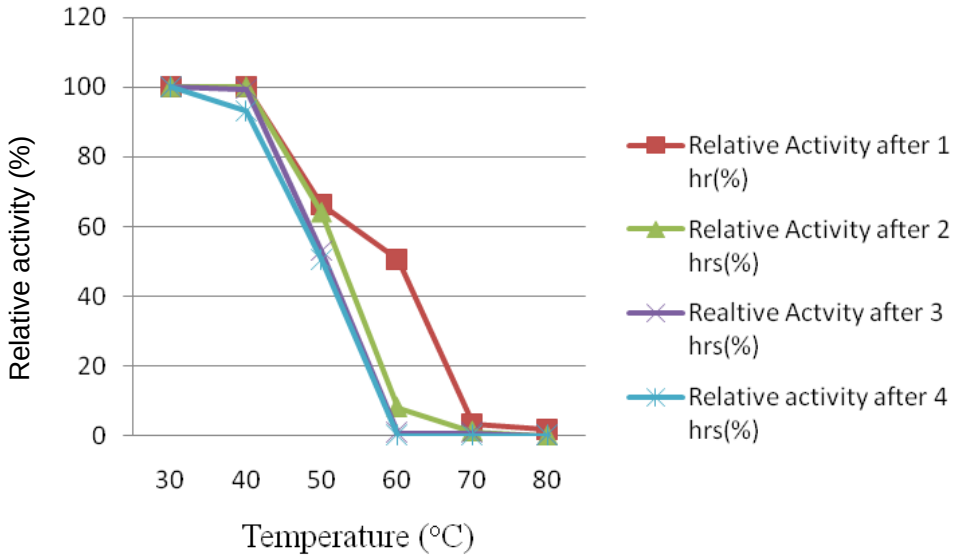


Figure 4. Effect of different temperature on the stability of xylanase from *A. fumigatus*.

Table 4. Effect of different pH on xylanase activity from *A. fumigatus*.

pH	Enzyme activity (U/ml)	Relative activity of enzyme (%)
2	10.93±0.01	39.06
3	26.04±0.04	93.06
4	27.67±0.02	98.89
5	28.67±0.01	102.46
6	27.98±0.02	100
7	27.87±0.01	99.60
8	28.46±0.02	101.71
9	28.27±0.02	101.03
10	29.85±0.02	106.68
11	27.99±0.01	100.03
12	26.03±0.02	93.03

of the reaction velocity (1/V) was plotted against the reciprocal of substrate concentration (1/[S]) to determine the K_m and V_{max} values by the Line-Weaver-Burk plot. The K_m value of xylanase for birch wood xylan was 3.12 mg/ml (Figure 6). The V_{max} of xylanase for birch wood xylan was 2857 µmol/min/mg protein.

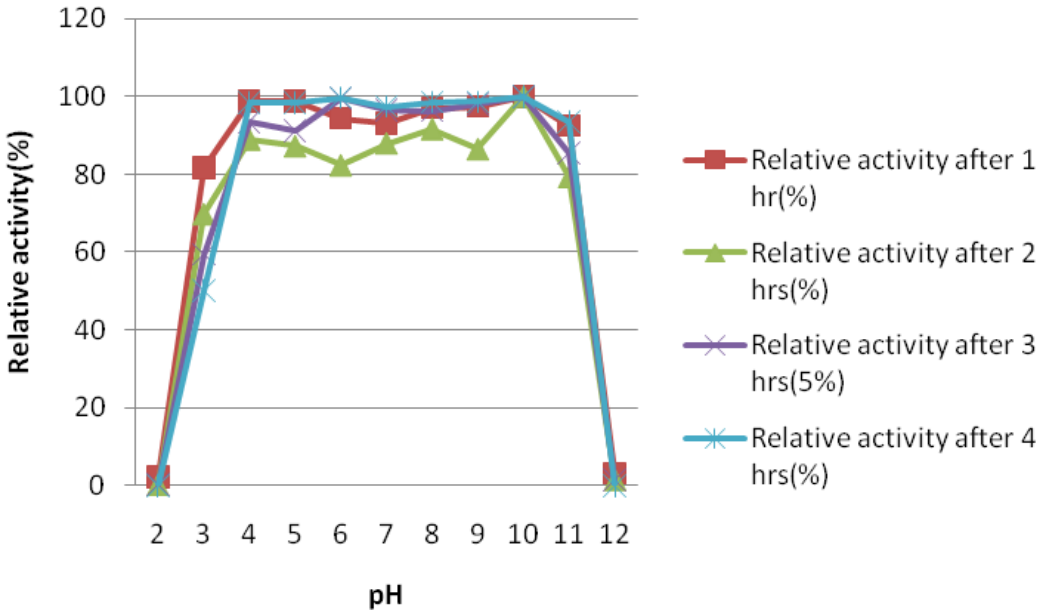


Figure 5. Effect of different pH on stability of xylanase activity from *A. fumigatus*.

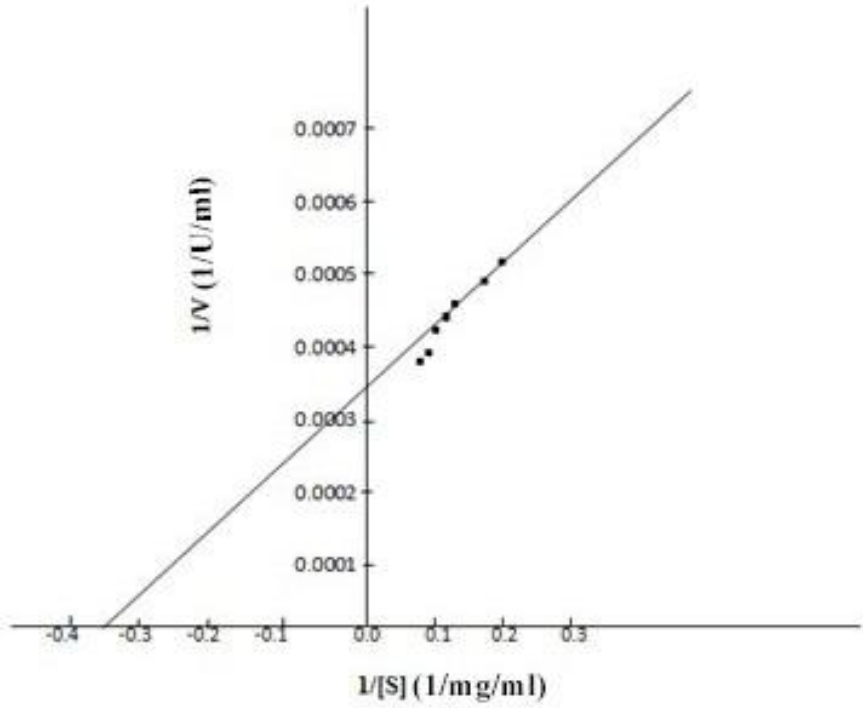


Figure 6. Lineweaver Burk plot determining the V_{max} and K_m values for xylanase from *A. fumigatus* using Birchwood xylan as substrate.

DISCUSSION

Soil is a rich source for collection of xylanolytic fungi (Pang Pei and Ibrahim, 2005). We have isolated 40 fungal species from 25 soil samples collected from different parts of western Uttar Pradesh and were subsequently grown on MEA containing 0.5% birch wood xylan. The suitability of MEA for the screening of

xylanolytic fungi has well been reported earlier by several workers (Bhalla and Joshi, 1993; Kvesitadze et al., 1999). Among the ten selected fungal species, on the basis of their performance of degrading xylan, the isolate showing maximum activity was identified as *Aspergillus. Penicillium* was the second most common genus. *Trichoderma* represented the third common genus. Species of *Aspergilli*, *Penicillium* and *Trichoderma* were the best xylanase producers among ten fungal species isolated from decomposing wood (Medeiros et al., 2003). The xylanase production in SSF was much higher than that of submerged fermentation. Malarvizhi et al. (2003) observed 30-fold enhancement of xylanase production in solid state fermentation than liquid culture when wheat bran was used as the substrate for a culture of *Ganoderma lucidum*.

In several studies, liquid and solid state cultivation procedures have been compared. Wheat bran proved a suitable substrate along with 0.5% birch wood xylan for the production of xylanase during SSF, as endorsed by other various workers (Gawande and Kamant, 1999; Malarvizhi et al., 2003). The enzyme was purified to homogeneity, in the present study, a three step purification strategy had been adopted, that is, ammonium sulphate predpitation (30 to 90%), gel filtration on Sephadex G-200 followed by anion exchange chromatography on DEAE Sephadex A-50. Different workers purified xylanases with different purification factor and yield. Georgi and Boriana (2012) purified xylanase from *Aspergillus niger* B03 and obtained a purification folds 12.9 and 9.67% yield. A purification factor of 5.0 with 20.7% yield was reported for a xylanase purified from *Aspergillus* cf*. niger* by Asano et al. (2005). A xylanase from *Aspergillus terreus* was purified by ammonium sulphate fractionation, anion exchange chromatography on DEAE-Bio-Gel A followed by gel filtration on Sephadex G -75 (Ghanem et al., 2000).

In the present study, 93.41 fold purification and 4.49% yields were obtained for xylanase from *A. fumigatus*. A single band of protein was obtained for xylanase during the SDS-PAGE on a 12.5% acrylamide gel showing the purity of the enzyme. A single band indicating the high purity of the isolated enzyme with a molecular mass of approximately 43 kDa was observed. This was similar to those isolated from alkalitolerant *A. fumigates* MKU1 (Thiagarajan et al., 2006).

Purified xylanase was characterized, and the xylanase isolated from *A. fumigatus* showed maximum activity at 30°C and retained 27.87% activity after 1 h of incubation at 40°C. After 2, 3 and 4 h of incubation maximum enzyme activity at 30°C was shown. Duarate and Costa-Ferreira (1994) reported that xylanase isolated from the members of the genus *Aspergillus* shows maximum activity at 30°C.

The suitability of xylanase for biobleaching application is generally decided with respect to the enzyme stability at high optimum pH. In the present study, effects of pH on enzyme activities were studied by varying the pH from 2 to 12 and maximum activity was obtained at pH 10. Bijender and Massarat (2011) isolated an alkaliphilic xylanase from *A. fumigates* MA28 and observed the optimum activity at pH 8.

The K_m value of xylanase for birch wood xylan was 3.12 mg/ml. The V_{max} of xylanase for birch wood xylan was 2857 µmol/min/mg protein. Bansod et al. (1993) reported that K_m values of xylanase lies in the range between 0.5 and 19.6 mg/ml. Nair et al. (2008) studied the kinetic parameters of two xylanases from *Aspergillus sydowii* SBS 45 and reported that Km value of xylanase I for birch wood xylan was 3.18 mg/mL, while the K_m value of xylanase II for birch wood xylan was 6.51 mg/mL. V_{max} of xylanase I and II for birch wood xylan was 1,191 and 1,587 µmol/min/mg protein, respectively. The xylanase from *Aspergillus niveus* RS2 had K_m and V_{max} values of 2.5 mg/mL and 26 µmol/ mg/min, respectively (Sudan and Bajaj 2007).

Sandrim et al. (2005) isolated two xylanases (X I and X II) from *Aspergillus caespitosus* having K_m values of 2.5 and 3.9 mg/ml and V_{max} values of 1679 and 113 µmol/min/mg protein. The K_m and V_{max} values were found to be 3.85 mg/ml and 570 µmol/mg/min of xylanase from *Aspergillus foetidus* (Shah and Dutta, 2005).

In conclusion, the results obtained from this work strongly indicate the significant amount of alkaline xylanase production from an isolate of *A. fumigatus* using SSF system and wheat bran as substrate, which is an economical method for the production of xylanase at extremely low operational cost based on the fact that wheat bran is one of the cheap and abundant agrowaste by-product. Hence, it can be used for large-scale production of xylanase using such agroresidual substrates. Unlike other microorganisms that produce multiple xylanases (Saha and Bothast, 1999), only one xylanase was isolated from this strain. The three step purification results in producing more purified form of xylanase hence it may be capable of producing high-quality xylo-oligosaccharides, indicating its application potential not only in pulp biobleaching processes but also in the nutraceutical industry (Nidhi, 2010).

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