Cost Effective Fermentation, Purification, and Operational Suitability of Xylanase from *Aspergillus Niger* KR-3 Grown on Agricultural By-Products

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The enzyme super-secreter *Aspergillus niger* KR-3 was isolated from putrefied soil and grown for optimized production of xylanase in Solid State Fermentation (SSF). Xylanase production was carried out by growing the strain for 5 days at 40 ^{o}C on lignocellulosic base material such as wheat bran moistened with mineral salt solution. The extracellular xylanase was purified with 42.16% recovery and 5.3-fold purification using conventional chromatography. The partially purified enzyme followed Michaelis-Menten behavior with K_m and V_{max} values 0.3% and 5UmL^{-1}, respectively for oat spelts xylan. Xylanase was found active over a wide range of *p*H with two apparent optima at 6 and 8. The optimum temperature for enzyme active was 50 ^{o}C, and it was thermostable up to 45 ^{o}C for 1 h. The catalytic activity of enzyme was increased by 3.25-fold in the presence of Mn^{2+}. In the presence of $MnCl_2$, enzyme exhibited broader *p*H profile with a shift in the minor *p*H optima from 8 to 9. With $MnCl_2$ in the assay volume, temperature optima remained unchanged but thermostability improved by +10^{o}C. Enzyme was stable in the presence of 60% acetone and propanol and 3% Sodium Dodecyl Sulphate (SDS).

Keywords: Aspergillus niger; Xylanase; Fermentation; Thermostability

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INTRODUCTION

Large amount of by-products generated annually from the agricultural and industrial processing of crop materials, and generally regarded as waste. Most of these wastes are burnt as fuel alternative or used as animal feed or in soil reclamation. However, such wastes usually have a rich composition, and therefore, they should not be considered "wastes", but could be used as raw materials for other industrial processes. The presence of carbon, nutrients, and moisture in the biomass provides conditions suitable for the growth of microorganisms, and this opens up great possibilities for their usage in solid-state fermentation (SSF) processes, for example agro-industrial wastes can be used as solid support, carbon and/or nutrient source for the production of a variety of value-added compounds (Pal and Khanum 2010; Bhushan *et al.* 2012, 2014).

Large amounts of the agro-industrial wastes are mainly composed by cellulose, hemicelluloses and lignin, being called "lignocellulosic materials". In such materials, the three main components are closely associated with each other, constituting the integral part of the vegetal biomass, and forming a complex structure that act as a protective barrier to cell destruction by bacteria and fungi. The biodegradation of the xylan backbone requires two classes of enzymes. Endoxylanases (EC 3.2.1.8) are able to cleave the xylan backbone into smaller oligosaccharides, which can then be degraded further to xylose by β-xylosidase (EC 3.2.1.37). Endoxylanases differ in their specificity towards the xylan polymer. Some enzymes cut randomly between unsubstituted xylose residues, whereas the activity of other endoxylanases strongly depends on the substituents on the xylose residues neighboring the attacked residues (Bajpai 1997; Subramaniyan and Prema 2002).

Production of xylanases from microorganisms has been reported to be both growth associated (Sa-Pereira *et al.* 2002) and non-growth associated (Archana and Satyanarayana 1997). The production potential of xylanase has been found to be influenced by microbe type and its strain (Collins *et al.* 2005), nutrient type and concentration, and growth conditions (Azin *et al.* 2007). For example *Eubacteria* and *Archaebacteria* produce xylanase having higher temperature optima and better thermostability than those of fungi, but the yield of the enzyme produced by these bacteria is comparatively lower than that produced by fungi (Haltrich *et al.* 1993).

The authors' group is actively engaged in the exploiting natural resources for the industrially important microbial proteins (Pal and Khanum 2010, 2011a,b; Bhushan *et al.* 2012, 2014). The present manuscript, in concerted effort, illustrate an example of utilizing agricultural wastes for bumper production of enzyme from a soil isolate *Aspergillus niger* KR-3 using the solid state mode of fermentation.

EXPERIMENTAL

Materials

Microorganism and supporting material

Isolation was carried out from soil samples collected from different fruit and vegetable markets of Mysore, Karnataka, India. Based on the ability of xylanolysis and potential of xylanase production, a large number of strains were screened. Finally one fungal isolate was selected as the potential producer of xylanase. The isolate was identified at the Department of Virology, Indian Agricultural Research Institute (IARI), New Delhi, India as *Aspergillus niger* using the morphological features and systematic description (Christensen and Raper 1978). The inoculum was prepared in potato dextrose medium (PDM) or malt extract agar medium (MA) containing xylan as the carbon source, by harvesting spores from 120 h old sporulating cultures grown at 40°C. Replacements to synthetic xylan were made by agricultural or industrial by-products such as wheat bran, sugarcane bagasse, aloe-vera skin, pineapple peel, mosambi peel, and banana peel, *etc*. All these materials were washed with water for removal of sugars, dried in hot air oven, and ground into a fine powder.

Methods

Fermentation parameters

Cultivation of the *A. flavus* in solid state fermentation mode was performed while keeping a constant ratio of solid: moisture (10:1) using pure xylan or agricultural wastes such as wheat bran as substrate for xylanase production supplemented with organic nitrogen and mineral salt solution composition of which is given in Table 1. The medium and trace elements were autoclaved separately. *Erlenmeyer* flasks were inoculated with inoculum size of $1x10^5$ spores/mL of moistening agent and incubated at 40°C for 5 days in a BOD incubator (Remi, India). After 5 days, culture broths were centrifuged at 3000×g for 10 min and supernatant was assayed for extracellular xylanase activity.

Table 1. Composition of Mineral Salt Solution - (g/L)

KH_2PO_4	20.0
$(NH_4)SO_4$	13.0
$Co(NH_2)_2$	3.0
$MgSO_4$	3.0
$CaCl_2$	3.0
$FeSO_4$	0.05
$MnSO_4$	0.015
$ZnSO_4$	0.014
$CoCl_2$	0.002
*p*H	6.0

Cultural conditions as process variables

For maximum production of xylanase, various culture conditions *viz*., *p*H, carbon and nitrogen source, were considered as most important and optimized by conventional 'one variable at a time' approach, which involves varying a single independent variable at a time while maintaining the others at a constant level. *A. flavus* was grown in xylanase production medium, containing different carbon and nitrogen sources, at *p*H 6.0 for 120 h. Different nitrogen sources *viz*., peptone, tryptone, beef extract, yeast extract, skimmed milk powder, were supplemented separately to a final concentration of 0.3 % (*w/v*) to study the microbial growth and xylanase activity while wheat bran (WB), sugarcane bagasse (SB), mango peel (MP), pineapple peel (PP), maize involucre (MI), banana peel (BP), aloe vera skin (AS), were used as the basic carbon source. The process was further standardized with respect to cultural conditions *viz*., the inoculum size by inoculating different concentrations ($1\text{-}5x10^6$ spores /mL) of inoculum. Similarly, the temperature and time of incubation were optimized by growing the *A. flavus* at different temperatures (25 to 50°C) for different time periods (72 to 192 h).

Enzyme extraction and recovery

To extract the enzyme from solid state fermentation vessel, in which substrate was mixed with citrate buffer (*p*H 6.0) at the same solid/liquid ratio (1/10), different solvents such as Tween®-20, Triton® X-100, and SDS (Sodium Dodecyl Sulphate) were added in different concentrations ranging from 0.025 to 0.10% to extract the enzyme

from spent biomass in solid state fermentation and stirred at 150 rpm for one hour at 40 °C. Solid mass is separated from the crude extract using filtration and centrifugation to yield a clear extract for *p*H measurement. The supernatant, obtained from fermentations taken as enzyme extract containing extracellular xylanase, was used to assay the enzyme activity.

Isolation and assay

Briefly, 1 mL of 1% xylan solution (in 0.05 M, *p*H 6.0 sodium citrate buffer) was mixed with 0.1 mL enzyme solution and incubated for 15 min at 60 ºC. The reaction was stopped by addition of 1 mL of DNS reagent (Miller 1959). The mixture is heated for 5 min at 100 ºC (boiling water bath) and then cooled in cold water. Absorbance of samples was measured at 540 nm against the substrate blank. A standard curve of xylose ranging from 0 to 1000 µg/mL was prepared and then quantified the released xylose in the samples using a standard curve. One unit of xylanase activity is defined as the amount of enzyme liberating 1 µmole of xylose equivalent under the experimental conditions in 1 min. The protein content was determined according to Lowry's method using bovine serum albumin as the standard.

Purification and characterization of enzyme

The crude xylanase was subjected to 20-60% $(NH_4)_2SO_4$ saturation, centrifuged at 10,000 × g for 10 min, and precipitates were dissolved in 100 mM phosphate buffer (pH 5.5). The enzyme preparation obtained from the above step was further passed through a column (25 × 3 cm.) of activated DEAE-cellulose previously equilibrated with 100 mM citrate-phosphate buffer (pH 5.5). Elution of bound proteins was achieved through applying delayed linear gradient of 0 to 0.5 M KCl in the same buffer at the flow rate of 15 mLh^{-1}. The chosen strategy of delayed gradient application was based on a pre-standardized protocol to elute smaller proteins entrapped in polymer matrix as larger proteins were eluted initially with washing buffer. The active fractions of 3mL each were collected and analyzed for protein (A_{280}) and enzyme activity. The concentrated enzyme preparation obtained after ion exchange chromatography was carefully layered over the top of Sephadex G-100 column (85 × 1.5 cm.) equilibrated with 100 mM citrate-phosphate buffer (pH 5.5), and bound proteins were eluted through same buffer at a flow rate of 12 mLh^{-1}. The active fractions showing enzyme activity were pooled, concentrated using sucrose, and used for further characterization. All steps of enzyme purification were carried out at 0 to 4 °C and stored at this temperature unless in use. The purified xylanase was used for its characterization with respect to reaction parameters. The optimal parameter results were obtained by assaying for ten minutes with the enzyme and substrate mixture in different *p*H for different temperatures. The buffers used were: acetate (*p*H 3.0 to 5.5), MES (*p*H 5.5 to 6.5) and glycine–NaOH (*p*H 7.0 to 8.5). The effect of various additives such as mineral ions, detergents and inhibitory chemicals on enzyme activity was assessed by including them in reaction mixtures and compared with the purified enzyme as control. The kinetic constants, V_{max} and K_m for xylanase were calculated from the double reciprocal plot of Lineweaver and Burk.

Statistical analysis

The analysis was done to determine the effect of variables on production of xylanase using software 'Statistical Package for Agricultural Scientists'.

RESULTS AND DISCUSSION

In recent years, considerable attention has been paid to the up-scaling of microbial fermentation processes, especially for enzyme production. There has been a surge of interest in the production of xylanolytic enzymes for their wide applicability in crop and commodity processing. Microbial cultures, regardless of the nature of the end product and the type of bioprocess, have certain specific requirements for their growth which have to be optimized for their maximum production. The results on the optimization of culture conditions for the maximum production of xylanase by *A. niger* KR-3 are presented here. This study also highlights the characteristic features of the enzyme that favor its commercial utility.

Selection and Identification of Xylanase Producing Microorganism

Aspergillus niger, showing clear zone on agar plates containing xylan, was hypothesized to function as a xylanase producer. The potential of this isolate was evaluated, based on the diameter of clearing zone formation (data not shown) and the maximum production of xylanase activity. The selected fungal isolate, i.e. *A. flavus* in the present investigation, had been identified at Department of Virology, Indian Agricultural Research Institute (IARI), New Delhi and used for further studies for optimizing culture conditions to get maximum production of xylanase.

Optimization of Medium and Culture Conditions

To obtain superfluous xylanase production, *A. flavus* KR-3 was grown on xylanase cultivation medium having xylan as a sole carbon/energy source, with 0.1% xylan under static condition in BOD incubator, and enzyme production was determined. Xylanase production was found maximum with a titer of ~9.87 U/g, when mineral salt solution was used to moisten the solid substrate, whereas the enzyme production was relatively low (7.32 U/g) without mineral salt solution (data not shown). In further studies, mineral salt solution was used. The xylanase production by *A. niger* KR-3 was examined at various *p*H of mineral salt solution ranging from 5.0 to 7.0 with an increment of 0.5 unit. The growth as well as xylanase production was found maximum (11.5 U/g) at *p*H 5.5 while it was minimum at *p*H 7.0 (~ 6.2 U/g), indicating that *A. flavus* grows well and produce maximum xylanase at slightly acidic conditions.

Poorna and Prema (2007) postulated that cultivation of *A. flavus* at an unfavourable *p*H may limit the xylanase production by reducing the bio-availability of the hemicellulosic substrate. The initial *p*H has been observed to influence the transport of enzymes across the cell membrane (Bakri *et al.* 2008). Similar results have been reported in *A. terreus*, where the lowest (*p*H 3.0 or 10.0), moderate (*p*H 4.0), and highest (*p*H 6.0) xylanase activities were observed (Chidi *et al.* 2008). *Aspergillus* sp. RSP-6 was active in xylanase production over a broad range of *p*H from 2.0 to 6.0 with maximum

production at *p*H 3.0 (Laxmi *et al.* 2008). The enzyme production was drastically reduced at neutral *p*H and no enzyme production was noticed in alkaline medium of *p*H 8.0. A *p*H of around 5.0, in general, has been observed to be optimum for xylanase production (Gupta *et al.* 2009, Shah and Madamwar 2005, Sridevi and Charya 2011, Subramaniyan and Prema 2002).

The effect of seven different substrate on xylanase production by *A. niger* was studied (Fig. 1). Agro-residues were tested to make the enzyme production economical, as they are crude and cheaper than pure xylan. Among the substrates used, wheat bran (WB) supported maximum enzyme production, where ~7.5 U/mg enzyme titer was observed. The amount of extracellular protein synthesized was also found to be the highest (6 mg/mL) in wheat bran containing medium components. However, aloe vera skin (AS), musambi peel (MP), pineapple peel (PP), maize involucres (MI), banana peel (BP), and sugarcane bagasse (SB) were poorly utilized by the stain.

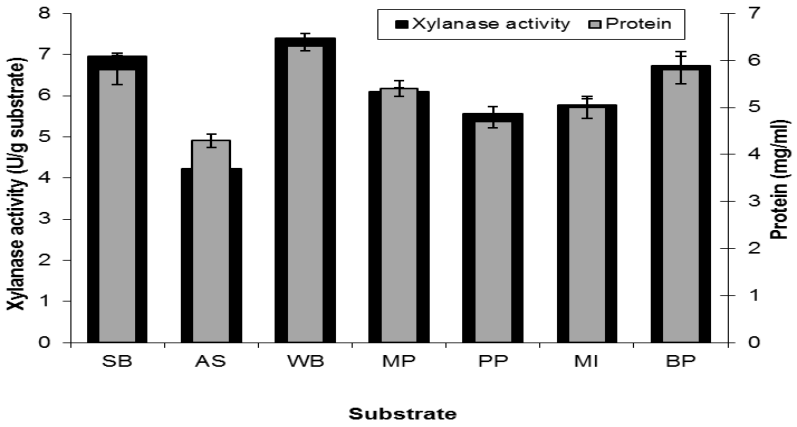

Fig. 1. Effect of carbon source on xylanase production by *A. niger* KR-3

The use of wheat straw and wheat bran as a carbon source for xylanase production has been reported (Bakri *et al.* 2008; Gupta *et al.* 2009; Ghanem *et al.* 2000; Okafor *et al.* 2007). Other agro-residues such as rice straw (Dhillon *et al.* 2000), sugarcane bagasse (Sandrim *et al.* 2005), corncob (Silva *et al.* 2005, Gomes *et al.* 1993), oat spelt xylan (Muthezhilan *et al.* 2007), and Brewer's spent grain (Terrasan *et al.* 2010) have also been reported as suitable substrates for xylanase production. Induction of the synthesis of xylan-degrading enzymes by xylanolytic organisms cultured with xylan as carbon source is well documented (Pal and Khanum 2010; Bhushan *et al*. 2012; Bhushan *et al*. 2014). However, xylanase production in *Aspergillus* sp. RSP-6 appears to be constitutive in nature, and none of the monosaccharide or disaccharide improved the xylanase production compared to palm fiber as carbon source (Laxmi *et al.* 2008). In contrast, xylanase activity in *A. pullulans* Y-2311-1 was induced by xylose (Li *et al.* 1994) as well as by glucose and cellobiose in *T. fusca* and *P. bryantii* (Chen and Wilson 2007; Miyazaki *et al.* 2005). Suppression of xylanase synthesis by readily metabolizable sugars such as glucose and/or xylose has been reported in *Streptomyces* sp. (Beg *et al.* 2000).

The effect of supplementation of different organic nitrogen sources on xylanase production by *Aspergillus niger* KR-3 was studied by incorporating them in mineral salt

solution at a concentration of 1%. Among the 5 nitrogen sources studied, yeast extract favoured maximum xylanase production (17.43 U/mg) followed by tryptone (16.87), peptone (16.32), beef extract (15.67), and skim milk (14.48) (Fig. 2). All if the inorganic nitrogen sources supported the enzyme production, but not as efficiently as yeast extract.

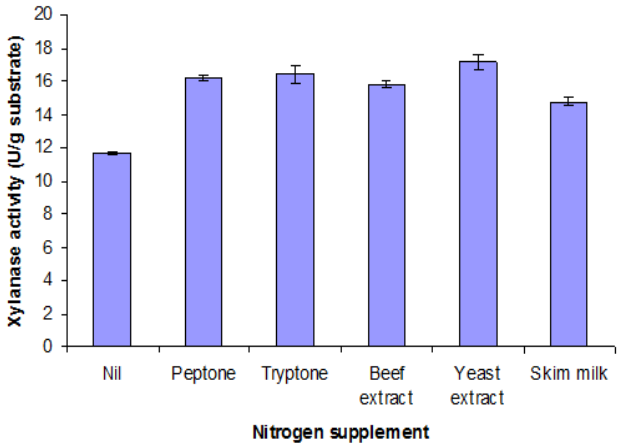

Fig. 2. Effect of nitrogen source on xylanase production by *A. niger* KR-3

The results obtained during the present investigations are in agreement with those reported by Laxmi *et al.* (2008) where yeast extract has been reported to be the best nitrogen source for xylanase production by *Aspergillus* sp. RSP-6, whereas other nitrogen sources such as peptone, beef extract, soybean meal, peanut meal, and corn steep liquor, were found to be poor nitrogen sources. Yeast extract and peptone has been used and recommended by many workers (Sa-Pereira 2002; Bhushan *et al.* 2012, 2014) as nitrogen source. In contrast to our results, peptone as the best source of organic nitrogen for the production of xylanase from *A. niger*, *F. solani* and *T. harzianum* (Bakri *et al.* 2008; Gupta *et al.* 2009). However, when alternative nitrogen sources such as cotton leaf residues and soybean residues were used individually and in combination, lower xylanase activities were observed (Seyis and Aksoz 2005). Inorganic nitrogen such as $NaNO_3$ (0.5%) have been reported to be the best in stimulating xylanase production by *Cochliobolus sativus* (Bakri *et al.* 2008).

Although the physiological changes induced by high temperatures during enzyme production is not very clear, it has been suggested that at high temperatures, microorganisms may synthesize a reduced number of proteins that are probably essential for growth and other physiological processes including enzyme production (Gawande and Kamat 1999). By applying the temperature shift during laboratory cultivation, hydrolytic activity could be almost doubled, whereas the xylanolytic production was three- to five-fold higher in comparison to cultivation at a constant temperature of 30 ºC.

The ambient temperature of 28±3ºC was found to be suitable for maximum production of xylanase in *A. niger* (Kheng and Omar 2005). The best temperature for xylanase production by *A. japonicum* has been reported to be 25ºC (Simoes *et al.* 2005). With cultivation temperature lower and higher than the optimum, a decline in xylanase activity has been reported (Gupta *et al.* 2009; Kheng and Omar 2005). A slightly higher

temperature of 45ºC and 50ºC has been reported to be optimum for xylanase production by *P. oxalicum* and *T. aurantiacus*, respectively (Dhillon *et al.* 2000; Muthezhilan *et al.* 2007).

The medium optimized at this stage for xylanase production by *A. niger* KR-3 was termed as 'xylanase overproduction medium' and finally, optimized conditions were: modified xylanase cultivation medium containing wheat bran and yeast extract; *p*H 5.5 at 45 ºC.

Purification of Xylanase

The purification of xylanase from *A. niger* KR-3 was carried out using conventional techniques such as ammonium sulphate fractionation, dialysis, and gel filtration chromatography through Sephadex G-100. The complete purification procedure was carried out using 0.05 M citrate buffer (*p*H 5.5). One hundred milliliters of crude enzyme were taken for purification of xylanase. The volume was subjected to ammonium sulphate fractionation. The enzyme activity was seen in 25 to 70% pellet. The pellet was dissolved in buffer and dialyzed to remove residual salt. Dialysis was carried out using a 10 kDa cut-off dialysis bag. The total volume of enzyme obtained after dialysis was 15 mL, which was further subjected to gel filtration chromatography (Sephadex G-100).

An elution profile of loaded semi-purified protein and total protein is shown in Fig. 3. All the tubes were assayed for xylanase activity. It is clear from figure that xylanase was eluted in a single peak. The tubes showing xylanase activity were pooled and used in further studies. The final enzyme preparation (30 mL) had a specific activity of 3.15 µmole/min/mg protein. At the end of purification steps, there was 5.3-fold purification and 42.16% recovery of xylanase, and the total protein was reduced to 24.13 mg.

The specific activities of xylanases from one microbial source have been found to vary from 0.28 to 7,600 µmole/min/mg of protein (Shao *et al.* 1995). The results observed are therefore in accordance to literature. The yield is 42.16%, which is more as compared to xylanase from *Bacillus sp.* strain 41M-1, where recovery was only 15.3% (Nakamura *et al.* 1993).

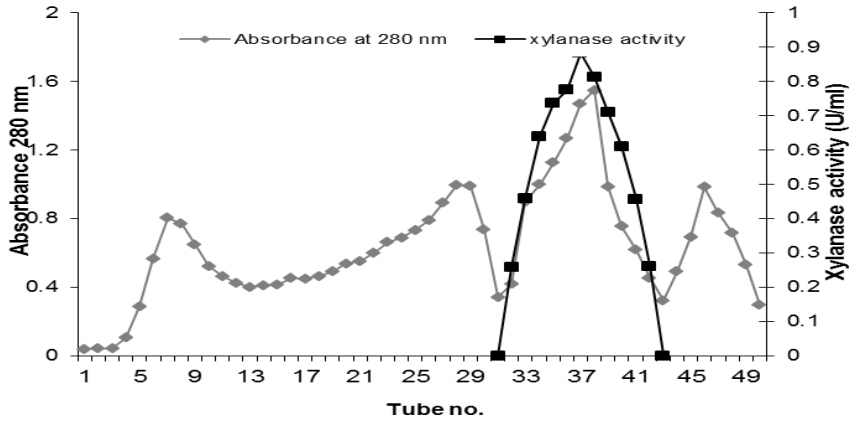

Fig. 3. Elution profile of xylanase from *A. niger* KR-3 on molecular sieve column Sephadex G-100

Characterization of Xylanase

Substrate saturation and velocity

The purified xylanase from *A. niger* showed a hyperbolic response with increasing concentrations of oat spelt xylan (extrapure), in an otherwise standard reaction mixture. The activity of the enzyme increased with increased concentration of the xylan until it reached a maximum at 0.3%, indicating that enzyme follows Michaelis-Menten kinetics (Fig. 4, Inset). With further increase in substrate concentration, the activity remained constant indicating that the active sites are fully occupied with the substrate. From the Lineweaver–Burk plot (Fig. 4), K_m and V_{max} of xylanase were found to be 0.3% and 5U/mL for oat spelt xylan.

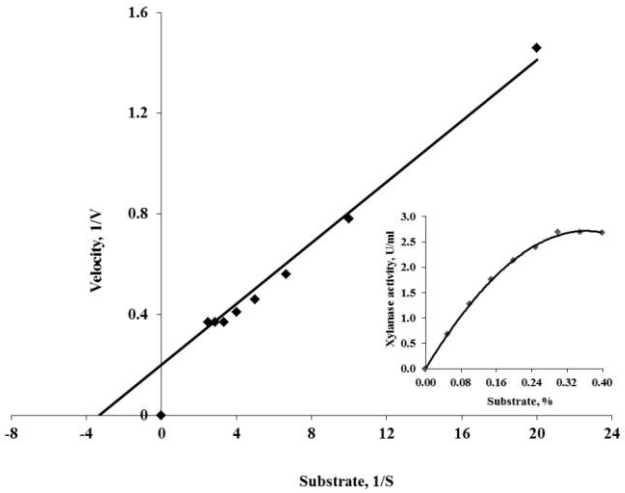

Fig. 4. Lineweaver-Burk plot and substrate saturation curve (inset) of purified xylanase from *A. niger* KR-3 for oat-spelt xylan

Xylanase from *Neocllimastix frontalis* was found to follow Michaelis-Menten kinetics with oat spelt xylan as substrate having a K_m value of 1.22 mg/mL (Gomez *et al.* 1993). Xylanase extracted from *Aspergillus sp.* has K_m and V_{max} values of 8.9 mg/mL and 11,000U/mg protein, respectively (Krisana *et al.* 2005). A K_m value of 0.9 mg/mL was observed for xylanase from *Arthrobacter sp.* towards wheat bran (Khandeparkar and Bhosle 2006).

*Effect of p*H *on enzyme activity*

The activity of xylanase was determined by buffering the reaction mixture in the *p*H range 4.5 to 9.5. Xylanase showed activity over a range of *p*H value with two apparent *p*H optima having maximum activity at *p*H 6, which is considered as the primary *p*H optimum. Another optimum *p*H was observed at *p*H 8, which is considered to be the secondary *p*H optimum (Fig. 5). Ionization of side chains of amino acids in an enzyme molecule has been documented to play significant role in three dimensional structural integrity of enzyme as a whole and its active site. At different *p*H of assay mixture, ionization of side chain is different resulting in different conformation and different activity. At *p*H 6, enzyme shows the maximum activity because of the higher stability of enzyme and its active site. With further increase in *p*H, there was

conformational change leading to loss in activity. But at *p*H 8, due to the ionizing activity, it is highly likely that there is formation of transient secondary active site capable of catalytic activity and as a result showed 75% activity of primary *p*H optima.

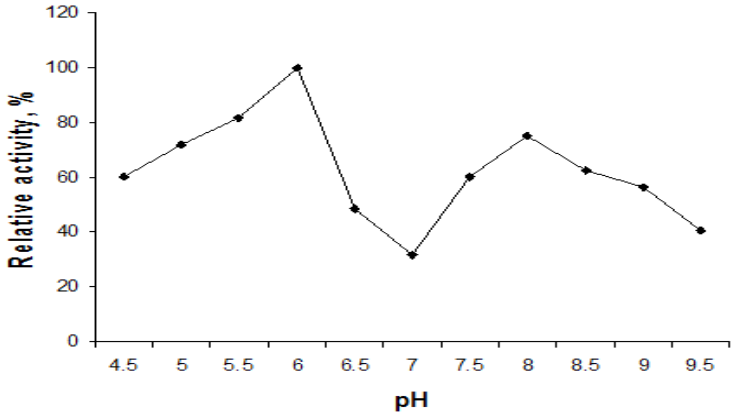

Fig. 5. Effect of pH (at 50 °C) on xylanase activity from *A. niger* KR-3

Similar results have been observed in xylanase from *Rhizopus stolonifera*, which showed optimum activity at *p*H 6 and *p*H 9 (Goulart *et al.* 2005). According to Collmer and Keen (1986), enzymes of fungal origin display highest activity in slightly acidic *p*H range. Xylanase produced from *Aspergillus sp.* showed maximum activity in the range of *p*H 4.0 to 7.0 (Reis *et al.* 2003; Biswas *et al.* 1990), which is in accordance to our result of primary *p*H optima. The minor *p*H optima is interesting from an industrial view-point, as the alkaline xylanases have good scope in bleaching process (Subramaniyan and Prema 2002; Christov *et al.* 1999). Its ability to catalyze activity in both acidic and alkaline conditions can be exploited in both food and pulp bleaching industries.

Effect of temperature on enzyme activity

Xylanase activity was monitored at different temperatures of assay mixture. Enzyme showed maximum activity at 50 ^{o}C, and further increase in temperature caused a sharp decrease in enzyme activity (Fig. 6).

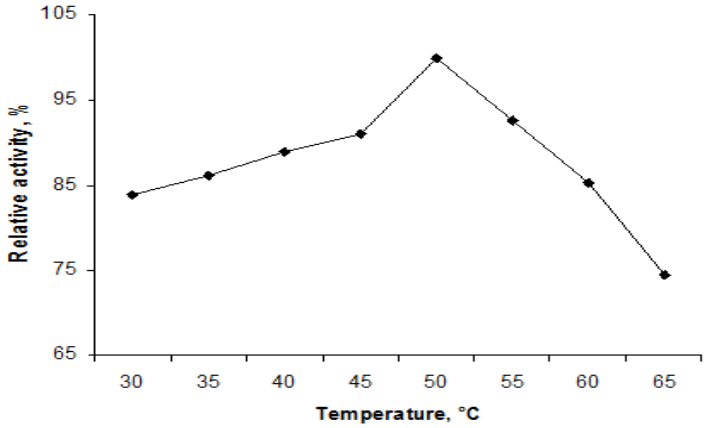

Fig. 6. Effect of temperature (*p*H 6.0) on xylanase activity from *A. niger* KR-3

The present finding on optimum temperature is supported by various authors. Similar results were reported (Sandrim *et al.* 2005; Georis *et al.* 2000). The optimum temperature for xylanase activity in the range of 60 to 80°C have been reported (Anand *et al.* 1990; Breccia *et al.* 1998). Xylanase produced by most of the *Aspergillus sp.* showed highest activity in the range of temperature 50 to 60°C (Haltrich *et al.* 1993; Bajpai 1997). The optimum temperature for both bacterial and fungal sources has been found to vary between 40 and 60 °C (Kulkarni *et al.* 2003).

Thermostability of xylanase

No loss of activity of xylanase was found when incubated for 60 min at 45 °C. The enzyme was stable for 30 min at 50 °C, and after 60 min incubation it was able to retain ~91% of its original activity. The enzyme could retain only 52 and 42% of its activity after 60 min incubation at 55 and 60 °C, respectively (Fig. 7).

Usually xylanase obtained from fungus is less stable than that of bacterial xylanase (Coral *et al.* 2002). Xylanase from *Marasmius sp.* was also stable up to 50 °C (Ratanochomsri *et al.* 1999). In contrast to our result, the xylanase from *Aspergillus nidulans* was found to be stable up to 55 °C (Reis *et al.* 2003). Crude enzymes have been found to be more stable than the purified enzyme (Krisana *et al.* 2005), showing the sensitiveness of purified xylanase to temperature.

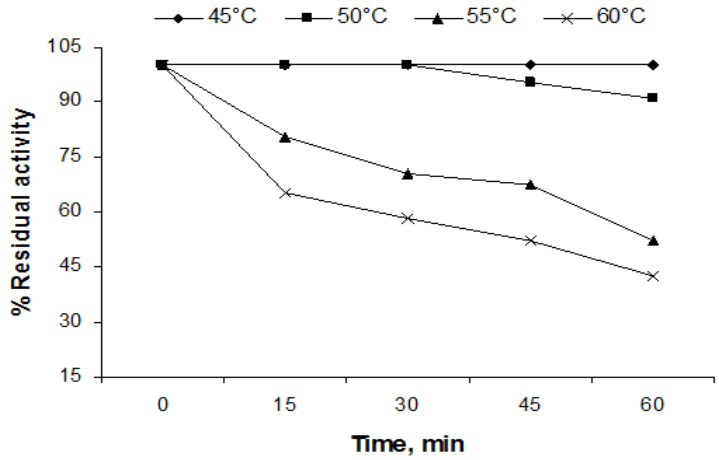

Fig. 7. Temperature stability of xylanase from *A. niger* KR-3

Effect of metal ion and EDTA

Xylanase could have a requirement for a divalent metal cation, which we tried to satisfy using Mn^{2+}. The enzyme showed increased activity with increasing concentrations of $MnCl_2$. Other divalent metal ions such as Ca^{2+} and Mg^{2+} had inhibitory action on the activity. Among monovalent metal ions, Na^+ had neutral effect, while Li^+ was found to be an activator and K^+ had slight negative effect on the activity. EDTA, being the chelator of metal ions, had an inhibitory effect on enzyme activity (Table 2).

Table 2. Effect of Metal Ions and EDTA on Xylanase Activity

Metal ions	Concentration (mM)			
	1	5	10	20
Li^{+}	109.5±0.61	118.8±0.42	119.4±0.41	122.5±0.37
Na^{+}	98.2±0.81	98.8±0.83	103.5±0.91	104.7±0.84
K^{+}	92.3±0.29	92.3±0.75	91.7±0.82	88.7±0.94
Mg^{+2}	72.5±0.57	70.7±0.72	62.5±0.79	55.8±0.81
Ca^{+2}	59.1±0.79	55.7±0.99	51.4±0.83	49.5±0.71
Mn^{+2}	216.4±0.94	244.8±0.19	270.5±0.76	325.5±0.89
EDTA	62.1±0.59	58.2±0.81	55.3±0.69	49.4±0.60

The stimulation of fungal xylanase activity by Mn^{2+} has been previously reported (Cesar and Mrsa 1996; Fialho and Carmona 2004; Faulet *et al.* 2006). In contrast to the results obtained, Na^{+} was found to be a potent effector of xylanase obtained from *A. niger* BCC14405 (Krisana *et al.* 2005). Xylanases obtained from *Bacillus sp.* did not have any requirement of metal ion and EDTA did not affect its activity (Nakamura *et al.* 1993; Bataillon *et al.* 2000).

Effect of reducing agents

Xylanase was assayed for activity in the presence of various reducing agents. L-ascorbic acid was found to be a potent stimulator of the enzyme with nearly 5-fold increase in the activity. Vanillin had a neutral effect on enzyme activity, while glutathione, cysteine, and ellagic acid were also found to have positive effects on enzyme activity, to a varying extent. Under influence of tannic acid, there was stimulation at lower concentration, and at higher concentrations precipitates were observed (Table 3).

Reducing agents are used in the assay mixture to reduce the disulphide bonds (Faulet *et al.* 2006). The stimulation of activity suggests that sulfhydryl groups play an essential role in the native conformation of xylanase.

Table 3. Effect of Reducing Agents on Xylanase Activity

Reducing agents	Concentration (mM)			
	1	5	10	20
Ascorbic acid	342.1±0.71	367.8±0.42	512.5±0.61	513.8±0.39
Vanillin	99.8±0.94	98.9±0.69	101.3±0.47	100.8±0.84
Glutathione	155.4±0.45	237.7±0.75	283.6±0.67	181.9±0.69
Cysteine	136.7±0.57	152.4±0.72	131.3±0.74	114.5±0.84
Tannic acid	171.5±0.79	113.3±0.99	-	-
Ellagic acid	182.1±0.94	193.4±0.19	206.8±0.76	208.1±0.89

Effect of organic solvents

Organic solvents are used in enzyme purification for selective protein precipitation, as they decrease the dielectric constant of the media and increase the electrostatic forces between the protein molecules, thereby causing them to aggregate. So before employing this step, resistance of enzyme to organic solvents must be ensured. Carrying out enzymatic reactions in organic media also is advantageous so as to avoid the problems of solubility in water. Keeping this in view, residual xylanase activity was estimated in the presence of organic solvents in the assay mixture. Enzyme was stable to acetone and propanol treatment when used up to 60% concentration, while methanol and ethanol had negative effect on enzyme activity (Fig. 8a). Based on the present findings, it can be easily concluded that acetone and propanol can be used as first step in xylanase purification to reduce the working volume and also to achieve certain levels of purification.

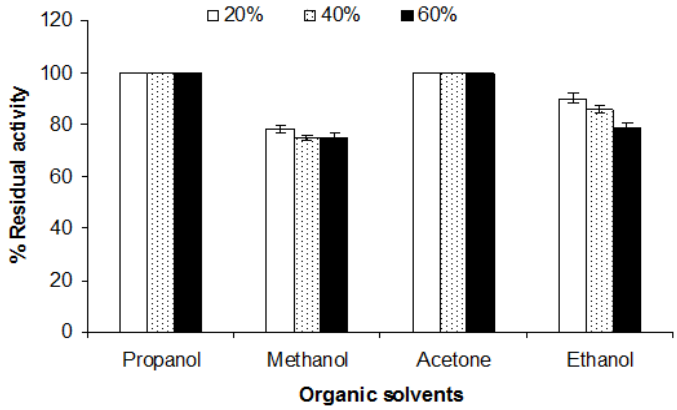

Fig. 8 a. Effect of solvents on enzyme activity from *A. niger* KR-3

In case of xylanase from *Termitomyces sp.*, acetone was found to be the activator of the enzyme (Faulet *et al.* 2006).

The activity of xylanase was assayed in the presence of varying concentrations of glycerol in the reaction mixture. Glycerol was found to have a positive effect, and ~10% increase in enzyme activity was observed at 20 mM concentration.

It has been suggested that polyhydroxy alcohols, such as glycerol, stabilize proteins by decreasing water activity by forming strong hydrogen bonds with the water. The protein molecules preferentially bind water, and the structure so formed is less able to unfold against the structured glycerol solvent than it would be with water alone. It may also work through the interaction with active site of the enzyme for stabilization. In case of xylanase from *Arthrobacter sp.* polyhydroxy additives like sorbitol, mannitol *etc.* had a positive effect on enzyme activity (Khandeparkar and Bhosle 2006).

Effect of surfactants

The activity of xylanase was assayed in the presence of Triton® X-100, Tween®-20, and SDS of various concentrations in the assay condition (1 to 4%). Tween®-20 had

a negative effect on enzyme activity, while Triton® X-100 was nearly neutral in its action. The enzyme exhibited full stability in the presence of SDS (Fig. 8b).

In contrast to the results obtained, SDS was found to inhibit xylanase activity from *Melanocarpus albomyces* 1168, and Triton® X-100 was found to inhibit xylanase activity from *Termitomyces sp.* (Faulet *et al.* 2006). Surfactants are known to increase the enzyme secretion by decreasing the phospholipids content of cell membrane of fungal mycelium coupled with increased permeability. Therefore, SDS being neutral to xylanase can be added in the production medium to improve xylanase production.

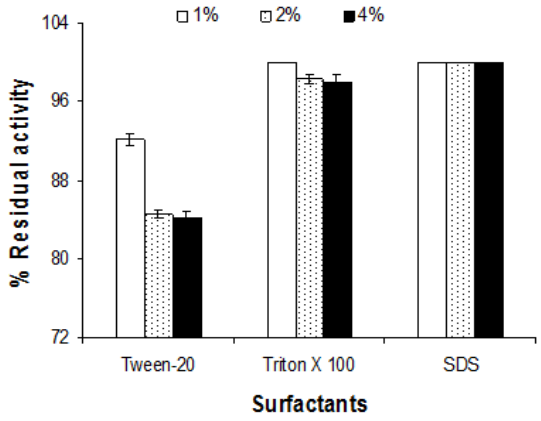

Fig. 8 b. Effect of detergents on enzyme activity from *A. niger* KR-3

Effect of Mn^{+2} on pH profile, temperature profile and thermostability

$MnCl_2$, which was found to act as an enzyme activator in an earlier experiment, was used in the assay mixture to study its effect on *p*H and temperature profile as well as on the thermostability of the enzyme. In the presence of $MnCl_2$ in the assay mixture, the primary or major *p*H optima remained the same (*p*H 6), whereas the secondary or minor *p*H optima shifted forward by one unit, from 8 to 9 and enzyme showed activity in a broader *p*H range (Fig. 9).

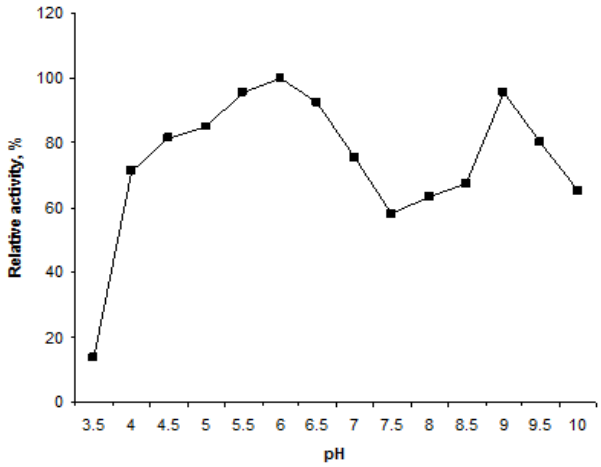

Fig. 9. Effect of $MnCl_2$ on *p*H optima of purified xylanase by *A. niger* KR-3

This shift and broadening of the *p*H profile may be due to the change in ionization behavior of side chain of amino acids near or at the active site in the presence of Mn^{+2} in the assay condition. The optimum temperature for enzyme activity remained the same with which was 45 ^{o}C without metal ion, and thermostability for short duration was enhanced by +10^{o}C (Fig. 10a & b).

In case of *Aspergillus ochraceus*, K^{+} was found to increase the thermostability of xylanase (Biswas *et al.* 1990).

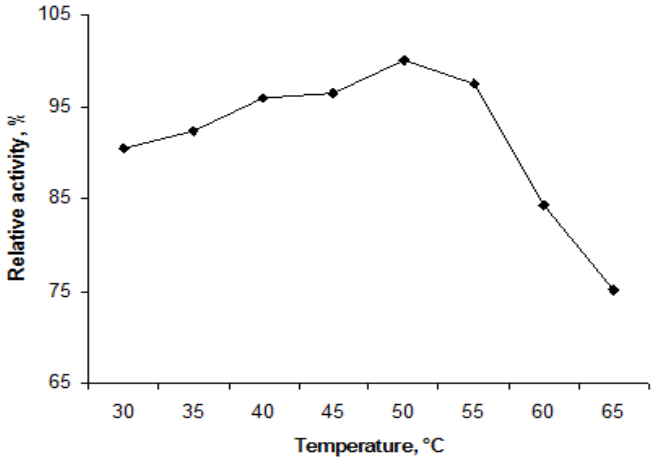

Fig. 10a. Effect of $MnCl_2$ on temperature optima of purified xylanase by *A. niger* KR-3

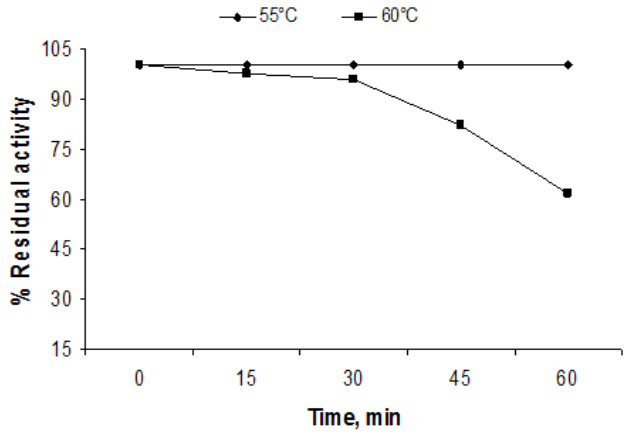

Fig. 10b. Effect of $MnCl_2$ on thermostability of purified xylanase by *A. niger* KR-3

CONCLUSIONS

Isolation and two-step screening method employed in the study led to the isolation of one efficient strain of *Aspergillus* for production of xylanase. Results obtained on optimization of process variables under SSF revealed that by-products including wheat bran could be effectively used for xylanase production. About five fold enhancement in xylanase production by *A. niger* KR-3 was achieved when trace metal solution moistened wheat bran was inoculated and incubated at *p*H 6.0 and 45°C for 144 h. The enzyme was

purified to near homogeneity and characterized with respect to its industrial robustness, such as stability at high temperatures, resistance to organic solvents and detergents. The enzyme also exhibited peculiar pH optima at 6, 8, and 9. The enzyme activity can also be modulated through exogenous modulators. The low cost of its production, thermophillic nature of microorganism, and stable nature of the enzyme may further broaden the scopes for its use in fruit juice industries.

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