Pak. J. Agri. Sci., Vol. 50(3), 433-437; 2013
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
http://www.pakjas.com.pk

PARTIAL PURIFICATION AND CHARACTERIZATION OF XYLANASE PRODUCED FROM *Aspergillus niger* USING WHEAT BRAN

Zulfiqar Ahmad[1,*], Masood Sadiq Butt[2], Muhammad Riaz[3]

[1]University College of Agriculture and Environmental Sciences, The Islamia University of Bahawalpur, Pakistan;
[2]National Institute of Food Science and Technology, University of Agriculture, Faisalabad, Pakistan;
[3]Department of Food Sciences and Technology, University college of Agriculture, BZ University Multan;
[*]Corresponding author's e.mail: zulfiqar2233@gmail.com

In present exploration, purification and characterization of xylanase was carried out to find its optimum conditions for maximum functionality. The xylanase (EC 3.2.1.8) synthesized by *Aspergillus niger* in submerged fermentation was partially purified and characterized for different parameters like temperature, pH & heat stability. The molecular mass determined through SDS-PAGE was found 30 kDa. The specific activity of the enzyme was raised from 41.85 to 613.13 with 48.63% yield just in a two step partial purification comprising ammonium sulphate precipitation and Sephadex gel filteration column chromatography. The partially purified enzyme was found to be optimally active at 60°C and 7.5 pH. Conclusively, for the application of xylanase in food, feed or paper manufacturing processes, it is necessary to consider its optimum pH and temperature.

Keywords: Xylanase, enzyme, purification, characterization, SDS-PAGE, wheat bran, *Aspergillus niger*.

INTRODUCTION

The agricultural waste materials like wheat bran, ground nut cake, rice bran, rice straw, sugarcane bagasse, cotton leaf scraps, fruit and vegetable wastes if not properly handled cause environmental issues. The proper management of these wastes not only reduces their detrimental impact to environment, but also transforms them into value added products of industrial and commercial potential (Bhosale *et al.*, 2011; Javed *et al.,* 2011). *Aspergillus niger* when grown on agricultural waste materials produces sufficient activities of xylanase (Haq *et al.,* 2002).

Xylanase is an extracellular enzyme which hydrolyses β-1, 4 D-xylosidic linkages of highly polymerized and substituted β-1, 4 linked D-xylobiose, xylotriose and glucuronosyl residues. The enzyme holds potential for the degradation of plant cell wall material (Omar *et al.,* 2008). Microbial xylanases (β-1, 4 D-xylan xylanohydrolase, EC 3.2.1.8) are being used in various industries including food, feed, textile and paper processing (Ahmad *et al.,* 2012). In food and feed, they liberate nutrients by hydrolyzing the non-degradable hemicellulose fibers; thus, make the nutrients available (Leisola *et al.*, 2002; Walk *et al.,* 2011). On account of beneficial role of enzymes, different methodologies are being used for their maximum biosynthesis (Chithra and Muralikrishna, 2008). Sugars like xylose, xylobiose and xylo-oligomers can be prepared by the enzymatic hydrolysis of xylan. The depolymerization action of xylanase results in the conversion of polymeric substances into xylo-oligosaccharides and xylose (Omar *et al.,* 2008). The manipulation of biotechnological techniques has played an important role in the potential utilization of agricultural waste materials (Mohammadi *et al.,* 2006; Okafor *et al.,* 2007). The microbial xylanase synthesis has been studied by several researchers (Suneetha *et al.,* 2011), even then efforts are to make to screen and identify suitable xylanolytic enzymes which are well-suited for the specific purpose (Beg *et. al.,* 2000). Therefore, the present study was designed for the purification of xylanase and its characterization to find the optimum conditions for its maximum functionality.

MATERIALS AND METHODS

Purification

***Ammonium sulphate precipitation*:** The crude enzyme (produced in a previous study: Ahmad *et al.*, 2009) was partially purified from the culture supernatant using ammonium sulphate in a Na-acetate buffer of pH 6.8 (Javed *et al.,* 2009). For the purpose, various ammonium sulphate concentrations, *i.e.* 30, 40, 50, 60, 70 and 80% were used for the precipitation of enzyme. The respective levels were mixed in 500 mL of crude enzyme filtrate and kept at 4°C for two hours with continuous stirring. The precipitates were collected and analyzed for xylanase activity. The optimum xylanase activity at a specific concentration of ammonium sulphate reflected the best concentration to attain maximum enzyme recovery.

***Dialysis*:** After precipitation, the ammonium sulphate present in the enzyme solution was removed by dialyzing against 50 mM Na-acetate buffer of pH 7.0 at 4°C for 24 hours (Carmona *et al*., 1998).

***Gel filtration*:** The dialyzed enzyme sample was further purified using gel filtration technique. The enzyme solution (50.0 mL) was loaded to the gel filtration on Sephadex G-75 column (1.8 cm×70 cm) make Pharmacia, already equilibrated with 50 mM sodium phosphate (pH 7.0). Elusions of 5.0 mL were collected and subjected to xylanase assay procedure. The fractions exhibiting better xylanase activities were separated, pooled and kept at 4°C (Carmona *et al*., 1998).

***Enzyme assay*:** Xylanase activity of the filtrate was measured at 55°C on spectrophotomer (CECIL CE 7200) using 0.6% (w/v) oat spelt xylan (sigma) as a substrate at pH 6.0. Reducing sugars were measured using DNS method (Miller, 1959). Enzyme activity was expressed as IU/mL. One international unit of xylanase (1 IU) corresponds to the amount of enzyme required to release 1 micromole of reducing sugar (xylose) in 1 minute.

***Estimation of protein*:** The method of Bradford (1976) was used for the estimation of protein.

***Characterization*:** Characterization of xylanase for different parameters like temperature (30, 40, 50, 60,70,80,90 and 100°C), pH levels (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) and heat stability at different temperatures (60, 70, 80, 90 and 100°C) for 15 min was performed following the method as described by Coral *et al.* (2002). Various biological buffers including glutamic acid/HCl (pH 2-2.9), Na-acetate/acetic acid (pH 3.2-5.3), MES/KOH (pH 5.6-6.5), MOPS/KOH (pH 6.8-7.4), HEPES/KOH (pH 7.7-8.3), glycine/NaOH (pH 8.6-9.8) and CAPSO/NaOH (pH 10.1-11.0) were used to maintain the pH of reaction mixtures (Nadeem *et al.,* 2009; Riaz *et al.,* 2012). The percentage of original activity retained after heat treatment (100 °C for 15 min) was calculated.

***Molecular mass*:** SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) was applied for determination of molecular mass of the enzyme under study (Carmona *et al*., 1998). The protein marker of 14.5-94 kDa (Fermentas) was used as a reference.

RESULTS AND DISCUSSION

***Purification*:** Table 1 elaborates the summary of results when xylanase was subjected to different purification steps. The crude enzyme was found to have total xylanase activity as 7700 IU, total protein 184 mg and the specific activity 41.85 IU/mg protein. Initially, the crude filtrate containing xylanase was subjected to different ammonium sulfate concentrations (30, 40, 50, 60, 70 and 80%) for precipitation. It was observed that 70% concentration of $(NH_4)_2SO_4$ showed better performance for the enzyme precipitation. This step purified the crude extract resulting 6052 IU activity, 78.3 mg total protein, 77.29 IU/mg specific activity, 78.60% purification yield and the purification fold was 1.85. After ammonium sulphate precipitation the enzyme was loaded on the column packed with sephadex G-75 and different fractions of 5 mL were collected and assayed for enzyme activity.

Each of the purification step exhibited increased specific xylanase activity and the maximum *i.e.* 613.13 IU/mg was calculated after gel filtration through Sephadex G-75 followed by 77.29 IU/mg after ammonium sulphate precipitation while minimum specific activity 41.85 IU/mg was calculated in case of crude enzyme.

Likewise, there was a decrease in the total protein contents from 184 mg (crude extract) to 4.8 mg (after gel chromatography). The decrease in protein content indicated the separation of protein impurities from the target enzyme fraction (xylanase). Each step of the process increased the purification fold; 1.85 folds purification was calculated after ammonium sulphate precipitation while the maximum purification was observed after gel filtration (7.93 fold). Similarly, there was a decrease in enzyme yield after each step carried out during the purification process. The enzyme yield reduced to 78.60% after ammonium sulphate precipitation that reached to its minimum level *i.e.* 48.63 %, after gel filtration. The results showed that every step in enzyme purification resulted in the separation of unwanted fractions of protein as manifested by the increased specific activity of purified enzyme. The findings of present study regarding purification of xylanase are in corroboration with the results of Carmona *et al.* (1998).

Table 1. Purification of xylanase produced form *Aspergillus niger* under submerged fermentation

Step	Total Protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Yield (%)	Purification fold
Crude Extract	184	7700	41.85	100	1.00
$(NH_4)_2SO_4$	78.3	6052	77.29	78.60	1.85
Sephadex G -75	4.8	2943	613.13	48.63	7.93

***Characterization*:** After purification, xylanase was subjected to characterization to find out the optimum conditions for its maximum activity. Data regarding characterization of xylanase would be a guide line for its application. For the purpose, xylanase produced in the study was characterized for temperature, pH, heat stability and molecular mass.

***Effect of temperature on xylanase activity*:** Fig.1 depicts the effect of different temperatures on relative activity of xylanase. It is obvious, when enzyme assay was performed at various temperatures; the xylanase activity increased with rise in temperature up to 60°C and exhibited maximum activity. However, further increase in temperature caused a decrease in activity. Minimum activity was observed at 100°C; the highest temperature investigated in present study. As enzymes are biological entities and their activity is temperature dependent; that is why up to certain temperature the enzyme exhibited increased activity while a further rise in temperature caused reduction in xylanase activity.

The results of current work are in close conformity to the findings of Kavita *et al.* (2002) who reported that the purified xylanase from *Aspergillus nidulans KK-99* exhibited the highest activity at 55°C. Likewise, Carmona *et al.* (1998), and Uhlig (1998), also documented optimum activity of xylanase at 55°C; however, Damasco *et al.* (2000) reported 75°C as optimum temperature.

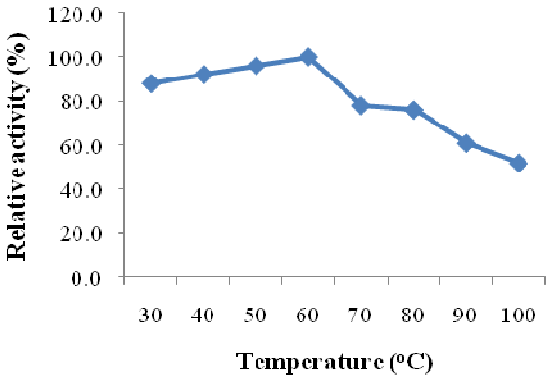

Figure 1. Xylanase activity at pH 6.0 under different temperatures

***Effect of pH on xylanase activity*:** The enzyme activity under different pH levels (Fig. 2) indicated that the enzyme exhibited minimum relative activity at pH 3.0 (19.0 IU/mL). The graphical representation shows that with increase in pH, the activity of the enzyme varied randomly up to pH 5.5, where the enzyme activity was 24.0 IU/mL. When the pH was raised above 5.5, a gradual increase in enzyme activity was observed and at pH 7.5 the enzyme exhibited maximum activity. However, further increase in pH showed a gradual decrease in xylanase activity.

The results of current work are in line with the findings of Kavita *et al.* (2002); that xylanase exhibits maximum activity in the pH range of 4.0 to 9.5. Likewise, Uhlig (1998) calculated maximum xylanase activity at pH levels 6.0 to 7.0. The results are also in close conformity with the findings of Huang *et al.* (1991) who described the pH 5.5 and temperature 60 °C, as optimum for the highest xylanase activities. Later, Goulart *et al.* (2005) cultivated *Rhizopus stolonifer* on wheat bran to produce cellulase free xylanase. The purified xylanase exhibited optimum pH and temperature as 6.0 and 45°C, respectively.

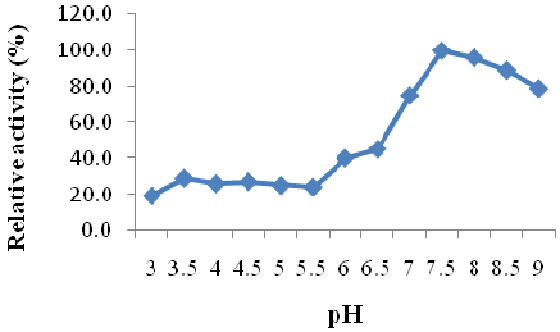

Figure 2. Xylanase activity at 60°C under different pH levels

***Effect of heat treatment on xylanase activity*:** Heat stability of enzyme is a basic parameter regarding its utilization in various processes. So, when the activity of xylanase was calculated at different temperatures, it showed maximum activity at 60°C (Fig. 3) and an increase in temperature showed negative effect on enzyme performance. When temperature was 70°C, enzyme exhibited 78% activity that infers it lost 22% of the total activity. However, when enzyme activity was calculated at 100°C, it exhibited only 52% of the total activity. The decrease in heat stability is possibly due to enzyme denaturation at higher temperatures. The findings of present study are supported by the results found by Christakopoulos *et al.* (1996) who produced xylanase from the fungus *Fusarium oxysporum F3* that was stable at a temperature of 55°C.

***Molecular mass*:** The results of electrophoresis (SDS-PAGE) revealed the molecular mass of protein band as 30 kDa (Fig. 4)

The findings of present study are synchronized with the results of Coral *et al.* (2002) who determined the molecular mass of xylanase produced by an *Aspergillus niger* strain as 36 kDa. Likewise, Sardar *et al.* (2000) purified xylanase, subjected to SDS-PAGE and reported its molecular mass as 24 kDa. The findings of present study are also in close association with the results of Camacho and Aguillar (2003) who reported the molecular mass of xylanase from *Aspergillus sp* as 22 kDa.

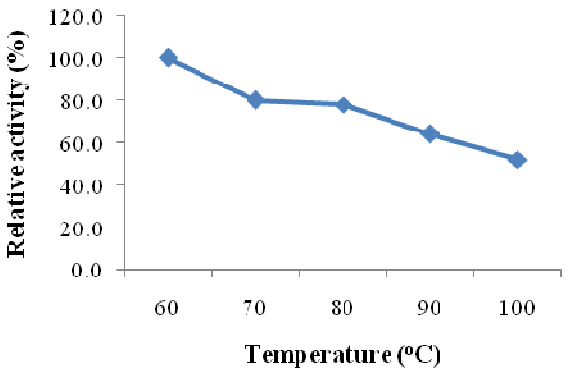

Figure 3. Xylanase heat stability at pH 6.0 under different temperatures

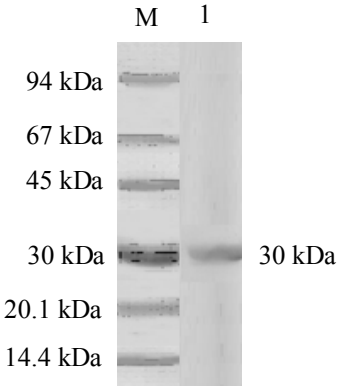

Figure 4. SDS-PAGE of purified xylanase from *A. niger*. Lane M, protein standard marker; lane 1, purified enzyme

***Conclusion*:** The study gives the insight; with each purification step the specific activity of xylanase went on increasing and the enzyme was purified to a purification fold of 7.93 with Sephadex gel filtration and the molecular mass of enzyme was found as 30 kDa. The stability of xylanase under a wide range of pH and at varied temperature shows its potential for utilization in different processes of food, feed and paper manufacturing.

***Acknowledgement*:** The authors are thankful to the National Institute of Food Science and Technology (NIFSAT), University of Agriculture Faisalabad for providing facilities for the successful completion of this project.

REFERENCES

Ahmad, Z., M.S. Butt, F.M. Anjum and M. Asgher. 2009. Effect of wheat bran concentration on xylanase biosynthesis by *Aspergillus niger*. Int. J. Agric. Biol. 11: 571-576.

Ahmad, Z., M.S. Butt, A. Ahmed, M. Riaz, S.M. Sabir, U. Farooq and F. Rehman. 2012. Effect of *Aspergillus niger* xylanase on dough characteristics and bread quality attributes. J. Food Sci. Technol. DOI-10.1007/s13197-012-0734-8.

Beg, Q.K., B. Bhushan, M. Kapoor and G.S. Hoondal. 2000. Enhanced production of a thermostable xylanase from *Streptomyces sp*. QG-11-3 and its application in biobleaching of eucalyptus Kraft pulp. Enzyme and Microbial Technol. 27:459-466.

Bhosale, H.J., S.R. Sukalkar, S.M.Z. Uzma and T.A.Kadam. 2011. Production of xylanase by *Streptomyces rameus* grown on agricultural wastes. Biotechnol. Bioinf. Bioeng. 1:505-512.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-57.

Camacho, N.A. and G.O. Aguilar. 2003. Production, purification and characterization of low molecular mass xylanase from *Aspergillus* sp. and its application in baking. Appl. Biochem. Biotechnol. 104:159-172.

Carmona, E.C., R.B.B. Marcia, A.P.K. Aline and A.J. Jao. 1998. Purification and biochemical characterization of an endoxylanase from *Aspergillus versicolor*. FEMS Microbiol. Letters 166:311-315.

Chithra, M. and G. Muralikrishna. 2008. An improved method for obtaining xylanase from finger millet (*Eleusine coracana* var. Indaf-15) malt. J. Food Sci. Technol. 45:166-169.

Christakopoulos, P., W. Nerinckx, D. Kekos, B. Macris and M. Claeyssens.1996. Purification and characterization of two low molecular mass alkaline xylanases from *Fusarium oxysporum* F3. J. Biotechnol. 51:181-189.

Coral, G., B. Arikan, M.N. Unaldi and H. Guvenmez. 2002. Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 wild-type strain. Turk. J. Biol. 26:209-213.

Damasco, M.C.T., C.M.M.C. Andrade and N. Pereira. 2000. Use of corn comb for endoxylanase production by thermophilic fungus *Thermomyces lanuginosus* IOC - 4145. Appl. Biochem. Biotechnol. 84(86):821-834.

Goulart, A.J., E.C. Carmona and R. Monti. 2005. Partial purification and properties of cellulose-free alkaline xylanase produced by *Rhizopus stolonifer* in solid state fermentation. Braz. Archives Biol. Technol. 48:327-333.

Haq, I., A. Khan, W.A. Butt, S. Ali and M.A. Qadeer. 2002. Effect of carbon and nitrogen sources on xylanase production by mutant strain of *Aspergillus niger* GCBMX-45. J. Biol. Sci. 2:143-144.

Huang L., T.H. Hseu and T.T. Wey. 1991. Purification and characterization of an endoxylanase from *Trichoderma koningii* G-39. Biochem. J. 278:329-333.

Javed, M.R., M.H. Rashid, H.U. Nadeem, M. Riaz and R. Perveen. 2009. Catalytic and thermodynamic characterization of endoglucanase (CMCase) from *Aspergillus oryzae* cmc-1. Appl. Biochem. Biotech. 157:483-497.

Javed, M.R., M.H. Rashid, Z. Mukhtar, M. Riaz, H.U. Nadeem, T. Huma and N. Ashiq. 2011. Kinetics and thermodynamics of high level β-glucosidase production by mutant derivative *Aspergillus niger* under submerged fermentation conditions. African J. Microbiol. Res. 5:2528-2538.

Kavita, T., S. Gupta and R.C. Kuhad. 2002. Properties and application of a partially purified alkaline xylanase from an alkalophilic fungus *Aspergillus nidulans* KK-99. Bioresource Technol. 85:39-42.

Leisola, M., J. Jokela, O. Pastinen, O. Turunen and H. Schoemaker. 2002. Industrial use of enzymes. Encyclopedia of Life Support Systems (EOLSS), EOLSS Publishers Co., Oxford, UK.

Miller, G.L. 1959. Use of dinitrosalcyclic acid reagent for the determination of reducing sugars. J. Anal. Chem. 31:426-429.

Mohammadi, I.M. 2006. Agricultural waste management extension education (AWMEE.): The ultimate need for intellectual productivity. Amer. J. Environ. Sci. 2:10-14.

Nadeem, H., M.H. Rashid, M. Riaz, B. Asma, M. R. Javed and R. Perven. 2009. Invertase from hyper producer strain of *Aspergillus niger*: physiochemical properties, thermodynamics and active site residues heat of ionization. Protein Pept. Lett. 16:1098-1105.

Okafor, U.A., V.I. Okochi, B.M. Onyegeme-okerenta and S. Nwodo-Chinedu. 2007. Xylanase production by *Aspergillus niger* ANL 301 using agro – wastes. Afr. J. Biotechnol. 6:1710-1714.

Omar, A.W., M.H. Khataibeh and K. Abu-Alruz. 2008. The use of xylanases from different microbial origin in bread making and their effects on bread quality. J. Appl. Sci. 8:672-676.

Riaz, M., M.H. Rashid, L. Sawyer, S. Akhtar, M.R. Javed, H.U. Nadeem and M. Wear. 2012. Physiochemical properties and kinetics of glucoamylase produced from deoxy-D-glucose resistant mutant of *Aspergillus niger* for soluble starch hydrolysis. Food Chem. 130:24-30.

Sardar, M., I. Roy and M.N. Gupta. 2000. Simultaneous purification and immobilization of *Aspergillus niger* xylanase on the reversibly soluble polymer Eudragit ™ L-100. Enzyme Microbial. Technol. 27:672-679.

Suneetha, V., K. Ram, M. Bishwambhar and C. Pravesh. 2011. An overview of screening and tentative optimization of microbial xylanase from soil samples collected from chittoor paper industry. Ind. J. Fundamental and Appl. Life Sci. 1:173-177.

Uhlig, H. 1998. Industrial enzymes and their application*s*. John Wiley and Sons, Inc. New York., pp.435.

Walk, C.L., A.J. Cowieson**,** J. Remus, C. Novak and A. McElroy. 2011. Effects of dietary enzymes on performance, goblet cells and apparent ileal amino acid digestibility of broilers exposed to a live coccidian oocyst vaccine. Poult. Sci. 90:91-98.