KATHMANDU UNIVERSITY JOURNAL OF SCIENCE, ENGINEERING AND TECHNOLOGY
VOL. 5, No. II, SEPTEMBER, 2009, pp 137- 148.

PARTIAL PURIFICATION AND CHARACTERIZATION OF *BACILLUS PUMILUS* XYLANASE FROM SOIL SOURCE

R. Monisha., M.V. Uma*, V. Krishna Murthy

Department of Biotechnology, PES Institute of Technology
Bangalore-560 085, Karnataka, India.
* Corresponding author: umamv@pes.edu

Received 15 July, 2009; Revised 16 August, 2009

ABSTRACT
Hydrolysis of xylan, the chief type of hemicellulose is achieved by endo-1, 4-β-xylanase and β-xylosidase among other such enzyme complex. These enzymes are mainly produced by fungi, bacteria, etc. Xylanase finds applications in animal feed, manufacture of bread, beverages, textiles, bleaching cellulose pulp, ethanol and xylitol production. Xylanase depolymerizes xylan molecules into xylose units, a primary carbon source for bacteria and fungi. In this study, we obtained a bacterial isolate exhibiting good extracellular xylanase activity by screening several soil samples. The bacterium identified was *Bacillus pumilus.* The isolate produced xylanase demonstrating maximal activity at 35^0C and at pH 7.0. Crude extract fractionated by ammonium sulphate precipitation had a specific activity of 0.69 μM min^{-1} mg^{-1}. Enzyme kinetics and properties were studied by DNS assay method. Xylanase had K_m of 4.0 mg ml^{-1} and V_{max} of 0.068×10^{-4} mM min^{-1} mg^{-1}. Molecular weight of xylanase was 19 kDa as determined on SDS PAGE. Enzyme was partially purified to a fold of 3.79 and a yield of 66% suggesting the method with fine-tuning could be suitable for scale-up process.

Key words: Industrial enzyme, biobleaching, *Bacillus pumilus*, xylan, environmental application

INTRODUCTION
Microbial xylanases have important applications in the degradation of xylan. Substrate xylan, a biopolymer comprising of D-xylose monomers linked through β-1,4-glycosyl bond, is found abundantly in lignocellulosic biomass. Xylanase is produced by many bacteria and fungi (Marta *et al.,* 2000; Polizeli *et al.,* 2005) and possesses a range of industrial and environmental applications. It can be classified as endo- and exo-xylanases (Min-Jen *et al.*, 2002). Exo-xylanase (ß-D-xylopyranosidase) is sometimes referred to as extracellular xylanase. *Bacillus* species secrete appreciable levels of extracellular xylanases (Srinivasan and Meenakshi, 1999). Industries that have demand for xylanase are paper and pulp, baking for improving dough handling and quality of baked products, during extraction of coffee, plant oils, starch, for improvement of nutritional properties of silage and grain, and in combination with pectinase and cellulase for clarification of fruit juices. The enzyme finds applications in textile industry for degumming of plant fiber sources as well as to enhance fiber quality (Bindu *et al.*, 2007; Aysegul

et al., 2008; Vasimon *et al.*, 2007). Xylanase gene has also been cloned to study the recombinant protein in different hosts like *Escherichia coli* (Ping *et al.*, 2006).

The present study has an objective of partially purifying and characterizing the enzyme obtained from soil bacterial isolate producing xylanase. We also intended to look for a favorable property in terms of activity over a broad range of pH. Xylanase activity in alkaline pH is particularly desirable in operation of biobleaching of pulp. Since chlorine has associated toxicity upon exposure to both humans and environment, partial or full replacement of chlorine as commercial bleaching agent by xylanase, an industrially important enzyme, may be a possibility.

MATERIALS AND METHODS

Bacterial isolate and culture conditions

The bacterium was sourced from soil samples collected from country-side (Yelachenahalli, Bangalore, India); soil specimen from agricultural, garbage, underground, garden and uncultivated lands were collected and screened to obtain bacterial isolate producing xylanase. The medium used to screen these samples contained Berg's mineral salts like 0.3% $NaNO_3$, 0.05% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.002% $MnSO_4.H_2O$, 0.002% $FeSO_4.H_2O$, 0.002% $CaCl_2.2H_2O$ with agar (Min-Jen *et al.*, 2002). Besides, 0.1% yeast extract and 0.5% birchwood xylan as a source of carbon was added to the medium so that the colonies selected have the ability to grow in xylan, by virtue of xylanase production. The pH was adjusted to 7.0, using 1% Na_2CO_3. About 48 random colonies later to be tested for extracellular xylanase activity were allowed to grow on Berg's mineral salt agar at pH 9.0 with an intention to obtain isolates producing xylanase that remained active at alkaline pH. Under these conditions, only 14 colonies grew which were inoculated to Berg's mineral salts broth at pH 9.0, incubated at 37^0C for 24 h. The supernatant comprising crude enzyme was subjected to the estimation of xylanase activity by DNS method. All chemicals used were procured from Qualigens.

Enzyme assay by DNS method

Xylanase activity was determined by measuring the increase in concentration of reducing sugars formed by enzymatic hydrolysis of birchwood xylan. The assay mixture consisted of 10 µl of crude enzyme sourced from cell free supernatant containing 100 µg total protein and 200 µl of 0.5% xylan in 100 mM Tris-HCl buffer which established a pH of 7.0. The mixture was incubated at 37^0C for 40 min and the reaction was arrested by addition of 1 ml DNS reagent. The reducing sugar generated was quantified at A_{540}, by addition of 7 ml of water and using D-xylose as a standard by spectro-colorimetric estimation (Marta *et al.*, 2000). One unit of enzyme activity was defined as 1 µM of xylose equivalent produced per minute under the assay conditions. Xylan without enzyme was a control (reagent blank) to eliminate the possibility of substrate having any reducing sugar residues. The bacterial isolate that demonstrated highest activity was selected for further study and was identified as *Bacillus pumilus* based on 16S rDNA. The task of identification was outsourced to M/s. Bangalore GeNei, a professional company in Bangalore, India.

Extraction, protein estimation and partial purification of xylanase

The culture of *B. pumilus* was inoculated in 1 L of Berg's mineral salts broth at pH 9.0, incubated at 37^0C for 24 h with shaking at 40 rpm. Broth was centrifuged at 12,000 rpm for 10 min at 4^0C, supernatant was collected and maintained at 4^0C. The protein concentration was measured by Lowry's method (Bruno *et al.*, 1994). Bovine serum albumin was used as a standard. Xylanase was partially purified from 1L of culture supernatant. Ammonium sulphate was added to cell-free supernatant to bring to 75% saturation and precipitation was carried overnight at 4^0C (however precipitation was tried with 30, 40, 50, 60 and 70% saturation with ammonium sulphate. There was no activity found in the supernatant with 75% saturation). The supernatant was discarded after centrifugation at 12,000 rpm for 20 min at 4^0C and the pellet was dissolved in a total of 60 ml of 100 mM Tris-HCl buffer (pH 7.0). The pellet collected was dialyzed against the same buffer at 4^0C for 48 h, with three changes at every 12 h interval. This partially purified enzyme was used for all other analyses.

Qualitative assay of xylanase

The ability of xylanase to degrade xylan on solid medium was determined at various pH levels. A capillary transfer method was developed for qualitative assay of xylanase (Paper accepted in The Research Journal of Biotechnology, India).

Characterization

The activity of xylanase was estimated at varying temperature, pH and substrate levels and molecular weight of the enzyme determined.

Effect of temperature

Xylan (200 µl of 0.5% in 100 mM Tris-HCl, pH 7.0) was incubated with diluted enzyme (xylanase 0.11 U (5 µl) with 95 µl of 100 mM Tris-HCl, pH 7.0), at 4, 15, 25, 35, 45, 55, 65 and 75^0C for 40 min. The reaction was arrested by adding 1 ml DNS and assayed as described previously.

Effect of pH

The pH of reaction mixtures was adjusted with 100 mM of the following buffer solutions: acetate buffer (pH 5.0 and 5.5), phosphate buffer (pH 6.0 and 6.5), Tris-HCl buffer (pH 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5) and glycine-sodium hydroxide buffer (pH 10.0, 10.5, 11.0 and 11.5). 200 µl of 0.5% xylan in respective buffers was incubated with diluted enzyme (xylanase 0.11 U (5 µl) along with 95 µl of 100 mM Tris-HCl, pH 7.0), incubated at 37^0C for 40 min and subsequently assayed.

Effect of substrate concentration

Rate of xylan hydrolysis was determined by incubating substrate at various concentrations (2, 4, 6, 8, 10 and 12 mg ml^{-1}) with 22.2 U (1 ml) of partially purified xylanase. The enzyme incubated without xylan served as control. The kinetic constants k_m and V_{max} were estimated following Lineweaver and Burk method (Bruno *et al.*, 1994).

Molecular weight determination by SDS-PAGE

SDS-PAGE of partially purified xylanase was performed in a 10% acrylamide gel (Sambrook *et al.*, 2001). Coomassie brilliant blue R-250 was used to stain the gel. The protein molecular weight standard used was of medium range containing phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soyabean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa), (Bangalore GeNei, India).

RESULTS AND DISCUSSION

The source of our isolate *B. pumilus*, is agricultural land soil. Photomicrograph of Gram positive cells is shown in Figure 1. Extracellular xylanase was obtained from the cell-free culture supernatant of *B. pumilus* grown on Berg's mineral salt medium containing pure xylan as substrate. Media containing wheat bran, wheat straw, rice husk, saw dust, bran of maize and gram, groundnut as xylan source have been reported (Ashwani *et al.*, 2008; Anuradha *et al.*, 2007). The ammonium sulphate ranging from 60% to 70% has been reported for the purpose of precipitation of xylanase. In the present study, xylanase was fractionated with 75% ammonium sulphate and the summary of purification of xylanase is presented in Table 1.

Xylanase obtained from *B. pumilus* ASH by fermentation, without purification was used for commercial application (Marta *et al.*, 2000; Bindu *et al.*, 2007); however, purification would enhance the extent of its efficacy as a biobleaching agent and hence purity is warranted. A recovery rate of 66% with 3.79 fold purification was achieved with the present process. A similar study reports a recovery rate of 78.9% xylanase from a fungus *Paecilomyces themophila* upon partial purification using 20-50% ammonium sulphate saturation (Lite *et al.*, 2006). However 1.25 fold purity was achievable with this process compared to a 3.79 fold purification achieved by our process. Thus although recovery rate was relatively lesser the purity achievement was substantial by the present method. It is relevant to indicate that xylanase finds industrial application even at a lesser purity level and such instances have been reported.

The relationship of purity, molecular weight of the enzyme obtained and the ammonium sulphate saturation revealed interesting findings. A study from Taiwan (Min-Jen *et al.*, 2002) indicates that at 65% ammonium sulphate saturation, the enzyme obtained had a molecular weight of 23 kDa and a 2.1 fold purity was achieved. Comparing to this study, the enzyme obtained by present method had a molecular weight of 19 kDa with a 3.79 fold purity by using 75% ammonium sulphate saturation. Interestingly the Taiwan study also reports 19.5 fold purity with an enzyme molecular weight of 45 kDa by usage of the same 65% ammonium sulphate

saturation. This indicates that the rate of purity is not much dependent upon the percent saturation of ammonium sulphate used but possibly dependent upon the molecular weight of the enzyme obtained from the process.

The specific activity increased after fractionation with ammonium sulphate, from 0.182 U mg^{-1} associated with crude xylanase to 0.69 U mg^{-1} with respect to partially purified xylanase. The molecular weight of xylanase also appears to have bearing on its activity. Studies conducted on xylanases derived from *B. firmus* depict an increase in specific activity from 1.75 U mg^{-1} to 34.08 U mg^{-1} with 45 kDa enzyme and to 3.67 U mg^{-1} with 23 kDa (Min-Jen *et al.*, 2002). Specific activity increased from 517.3 U mg^{-1} to 645.2 U mg^{-1} during purification of *P. themophila* xylanase (Lite *et al.*, 2006). Oat spelt xylan too was used by us to obtain extracellular xylanase, however purification extent was not significant.

The characterization of xylanase from bacterial and fungal sources generally occurs as a lower molecular weight protein although higher molecular weights have also been reported. In the present case, the protein band of partially purified xylanase on SDS-PAGE gel had a molecular mass of 19 kDa shown in Figure 2. The enzyme from a fungus *Plectosphaerella cucumerina* had a molecular weight of 19 kDa which compares well with our result (Gui *et al*., 2007). Xylanase from *Aspergillus terreus*, UL 4209 strain isolated from soil had 22 kDa (Silas *et al.*, 2008) and that obtained from alkaliphilic *Bacillus sp*. strain K-8 was 24 kDa (Chakrit *et al.*, 2006). Xylanase produced by *B. circulans* is reported to be 38 kDa (Júlio *et al.*, 2006). A second xylanase from *B. firmus* had a molecular weight of 45 kDa (Min-Jen *et al.*, 2002). Thus xylanase obtained from varied sources do have different molecular masses.

The activity of partially purified xylanase was found to be maximal at 35^0C as indicated in Figure 3, a finding similar to *A. terreus* UL 4209 that showed optimum activity at 35^0C (Silas *et al.*, 2008). Xylanase from a thermoalkaliphilic bacterium showed optimum activity at 50^0C (Sapre *et al.*, 2005). Optimum activity of xylanase obtained from both *B. circulans* and *B. amyloliquefaciens* was at 50^0C (Júlio *et al.*, 2006; Javier *et al.*, 1998). The industrial importance of an enzyme will be more if the temperature input for its optimal activity is less.

The optimum pH activity of xylanase was at pH 7.0 as seen in Figure 4. Incidence of wide range of activity of xylanase, specially at alkaline pH is advantageous in application of the enzyme in biobleaching of kraft pulps. The optimum pH activity at 6.8-7.0 of xylanase reported from *B. amyloliquefaciens* (Javier *et al.*, 1998) is in congruence with our finding. Xylanase from a fungal source *A. terreus* UL 4209 showed maximum pH activity at 6.0 (Silas *et al.*, 2008). Thermostable alkaline xylanase from a *Bacillus sp.* showed three optimum peaks for pH 6.5, 8.5, 10.5 (Sapre *et al.*, 2005). A wide range of pH activity from 5.0-8.0 was observed in *B. circulans* BL53 upon solid state cultivation (Júlio *et al.*, 2006). The persistence of activity in a large range of pH is a desirable quality of an industrial enzyme.

Larger the velocity higher will be the amount of substrate binding which is a desirable quality for an enzyme. The substrate concentration K_m was found to be 4 mg ml^{-1} and the velocity reflected as V_{max} of partially purified xylanase was 0.068 × 10^{-4} mM min^{-1} mg^{-1}, as determined by Lineweaver Burk plot is depicted in Figure 5. Xylanase obtained from a fungus *Plectosphaerella cucumerina* had a K_m and V_{max} value of 2.06 mg ml^{-1} and 0.49 mM min^{-1} mg^{-1} respectively which was comparatively lesser than the present study (Gui *et al.*, 2007). *Streptomyces cyaneum* SN32 xylanase had a K_m and V_{max} value of 11.1 mg ml^{-1} and 45.45 µM min^{-1} mg^{-1} respectively (Suchita *et al.*, 2008). Low affinity of enzyme towards its substrate is indicated by high K_m; we have been able to get xylanase with higher affinity (nearly three times) to substrate compared to this report.

CONCLUSION

We have obtained a xylanase producing bacterial isolate from rather by a simpler process of precipitating using ammonium sulphate. The identified bacterium was *B. pumilus* sourced from agricultural soil, cultured in a medium containing birchwood xylan as substrate. A broad range of activity, from acidic to alkaline was noted as clear zone of hydrolysis of the substrate. This property of the enzyme could be of potential application in pulp industry. There is a scope to enhance enzyme yield, quality and efficacy by innovation so that scale-up efforts could be accomplished.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude and appreciation to the PES Institute of Technology, Bangalore, India, for financial support of this research work. The support and encouragement of Ms. Renuka Sudarshan and other colleagues is gratefully acknowledged.

REFERENCES

1. Anuradha, P., Vijayalakshmi, K., Prasanna, N. D. and Sridevi, K. 2007. Production and properties of alkaline xylanases from *Bacillus sp*. isolated from sugarcane fields. Current Science. 92 (9): 1283-1286.

2. Ashwani, S., Neelam, G., Jitender, S., Kalika, K., Ramesh, C.K. and Vijay, K. G. 2008. Optimization of xylanase production using inexpensive agro-residues by alkalophilic *Bacillus subtilis* ASH in solid-state fermentation. World Journal of Microbiology and Biotechnology. 24 (5): 633-640.

3. Aysegul, E. Y., Feride, I.S. and Mehmet, H. 2008. Isolation of Endophytic and Xylanolytic *Bacillus pumilus* strains from *Zea mays*. Brazilian Archives of Biology and Technology. 14 (4): 374-380.

4. Bindu, B., Jitender, S., Saurabh, S. D. and Ramesh, C. K. 2007. Enhanced production of cellulose- free thermostable xylanase by *Bacillus pumilus* ASH and its potential application in paper industry. Enzyme and Microbial Technology. 41: 733-739.

5. Bruno, K. K., Tohru, S., Hiroyuki, H., Keiichi, K. and Kazuhiro, T. 1994. Purification and Characterization of Aeromonas caviae ME-1 Xylanase V, Which Produces Exclusively Xylobiose from Xylan. Applied and Environmental Microbiology. 60 (2): 531-535.

6. Chakrit, T., Khin, L. K. and Khanok, R. 2006. Purification of xylanase from alkaliphilic *Bacillus sp*. K-8 by using corn husk column. Process Biochemistry. 41 (12): 2441-2445.

7. Gui, M. Z., Jun, H., Guang, R. H., Li, X. M. and Xian, E. Z. 2007. Molecular cloning and heterologous expression of a new xylanase gene from *Plectosphaerella cucumerina*. Applied Micrbiology and Biotechnology. 74: 339-346.

8. Javier, D. B., Faustino, S., Mario, D. B., Guillermo, R. C.. and Rajni, H. K. 1998. Purification and characterization of a thermostable xylanase from *Bacillus amyloliquefaciens.* Enzyme and Microbial Technology. 22 (1): 42-49.

9. Jeyagowri, K., Alfredo, J. A., Gulelat, D. H., Sudip, K. R. 2008. Production, purification and characterization of *Bacillus* sp. *GRE7* xylanase and its application in eucalyptus Kraft pulp biobleaching. World Journal of Microbiology and Biotechnology. 24 (5): 605-612.

10. Júlio, X. H., Luís, H. B. S., Plinho, F. H., Marco, A. Z. A . (2006). Purification and properties of a xylanase produced by *Bacillus circulans* BL53 on solid-state cultivation. Biochemical Engineering Journal. 32 (3): 179-184.

11. Lite, L., Hongmei, T., Yongqiang, C., Zhengqiang, J., Shaoqing, Y. 2006. Purification and characterization of a thermostable cellulose free xylanase fron the newly isolated *Paecilomyces themophila.* Enzyme and Microbial Technology. (38): 780-787.

12. Marta, C. T. D., Ana, C. A. P., Edilberto, P. P., Alexandre, N. P. and Telma, T. F. 2000. Characterization of alkaline xylanases from *Bacillus pumilus*. Brazilian Journal of Microbiology. 31: 90-94.

13. Min-Jen, T., Mee-Nagan, Y., Khanok, R., Khin, L. K. and Shui-Tein, C. 2002. Purfication and Characterization of two cellulose free xylanases fron an alkaliphilic *Bacillus firmus*. Enzyme and Microbial Technology. 30: 590-595.

14. Ping, L., Ming-Guang, F., Wei-Fen, L. and Chun-Xia, H., 2006. Construction and characterization of a bifunctional fusion enzyme of *Bacillus*-sourced β-glucanase and xylanase expressed in *Escherichia coli*. FEMS Microbiology Letters. 261: 224-230.

15. Polizeli, M. L. T. M., Rizzatti, A. C. S., Monti, R., Terenzi, H. F., Jorge, J. A. and Amorim, D. S. 2005. Xylanases from fungi: properties and industrial applications. Applied Microbiology and Biotechnology. 67 (5): 577-91.

16. Sambrook, Joseph, R. and David, W. 2001. Molecular cloning, A Laboratory Manual, 3rd. Cold Spring Harbor Laboratory Press, New York, A8.32.

17. Sapre, M., Jha, H. and Patil, M. 2005. Purification and characterization of a thermoalkalophilic xylanase from *Bacillus sp*. World Journal of Microbiology and Biotechnology. 21 (5): 649-654**.**

18. Silas, B.C., Busiswa, G., Ignatious, N., Elbert, J. V. R., Andrew, C. and Emil, K.A. 2008. Production, purification and characterization of celullase-free xylanase from *Aspergillus terreus* UL 4209. African Journal of Biotechnology. 7 (21): 3939-3948.

19. Srinivasan, M. C. and Meenakshi, V. R. 1999. Microbial xylanase for paper industry. Current Science. 77 (1): 137-142.

20. Suchita. N., Mukesh, K. and Ramesh, C. K. 2008. Purification and characterization of extracellular xylanase from *Streptomyces cyaneum* SN32. Bioresource Technology. 99 (5): 1252-1258.

21. Vasimon, R., Rutachandaporn, S., Nakul, R., Pacawadee, T., Duriya, C., Sutipa, T., Kusol, P., Lily, E. 2007. Cloning, expression, characterization, and high cell-density production of recombinant endo-1,4-β-xylanases from *Aspergillus niger* in *Pichia pastoris.* Enzyme and Microbial Technology. 41: 19-25.

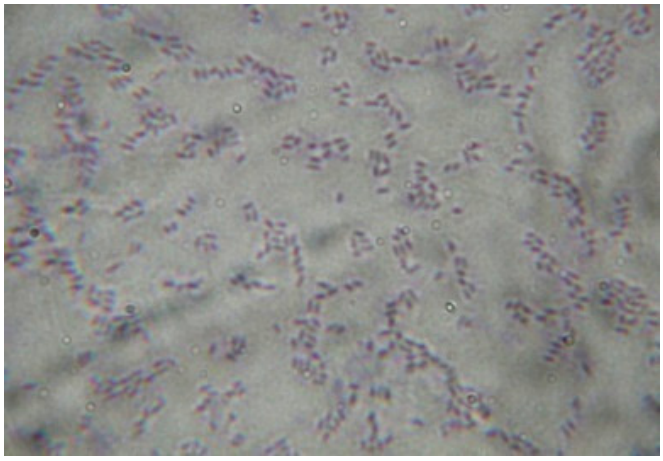

Figure 1: Photomicrograph of Gram stained *Bacillus pumilus* cells

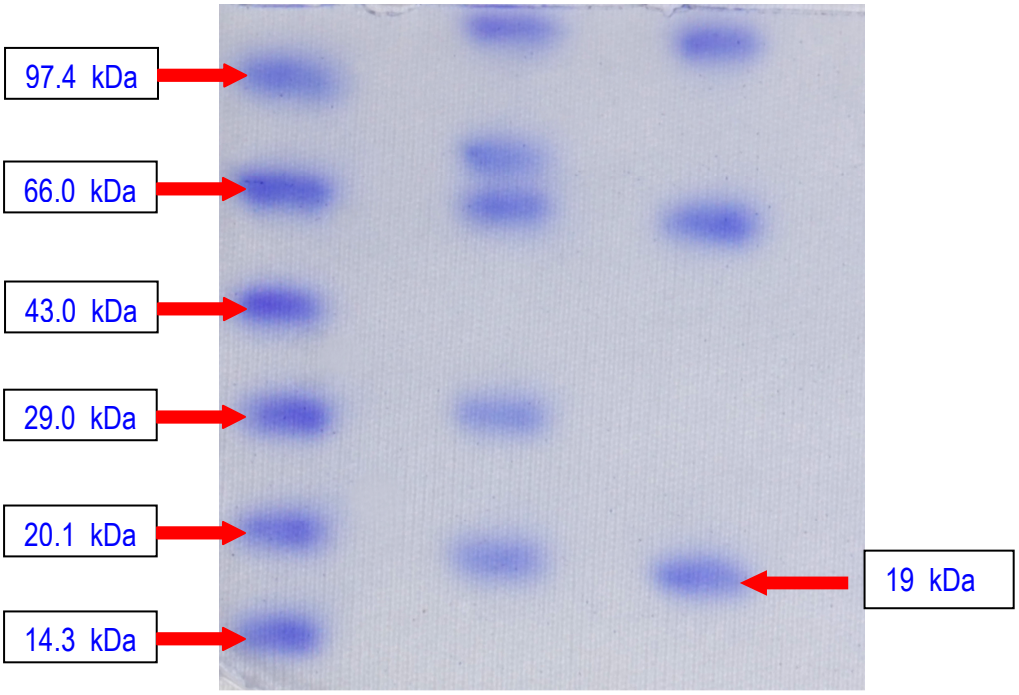

Figure 2: (a) Medium range protein molecular weight marker, (b) Crude xylanase, (c) Partially purified xylanase

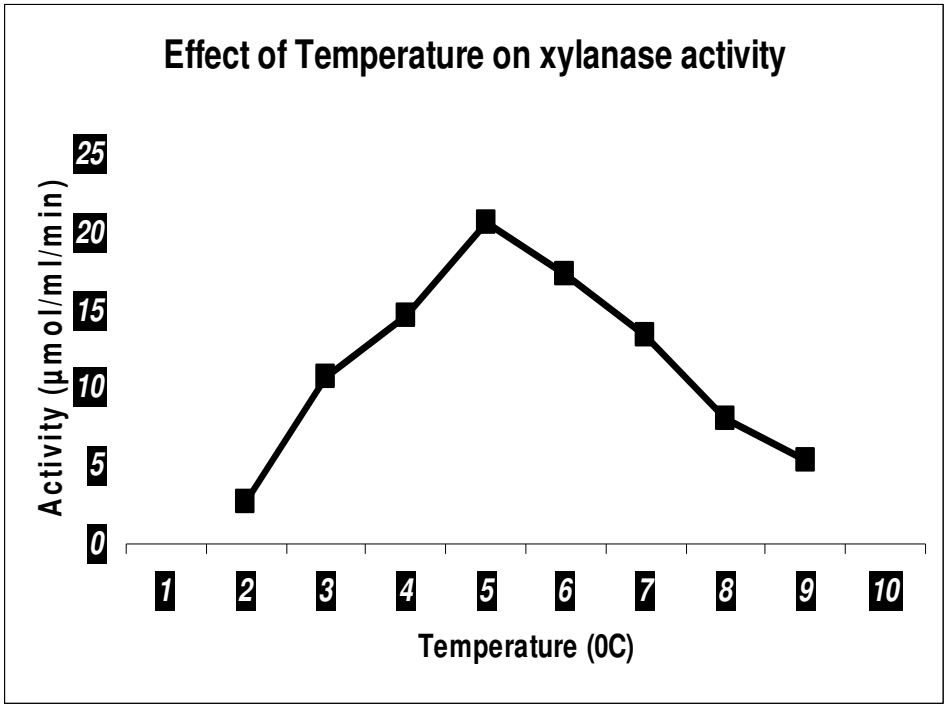

Figure 3: Effect of temperature on Xylanase

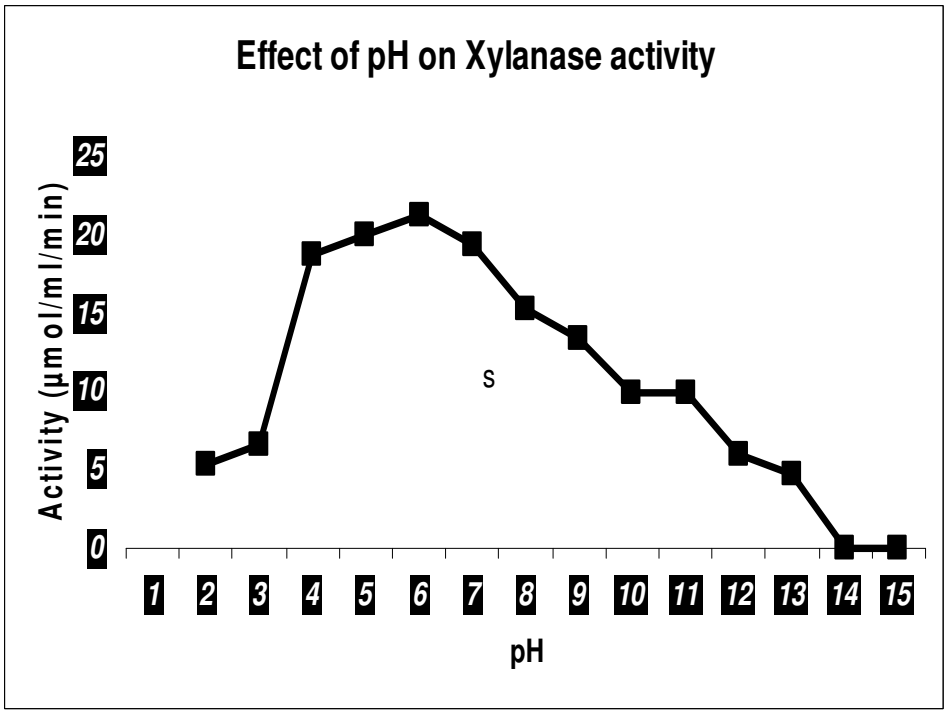

Figure 4: Effect of pH on Xylanase

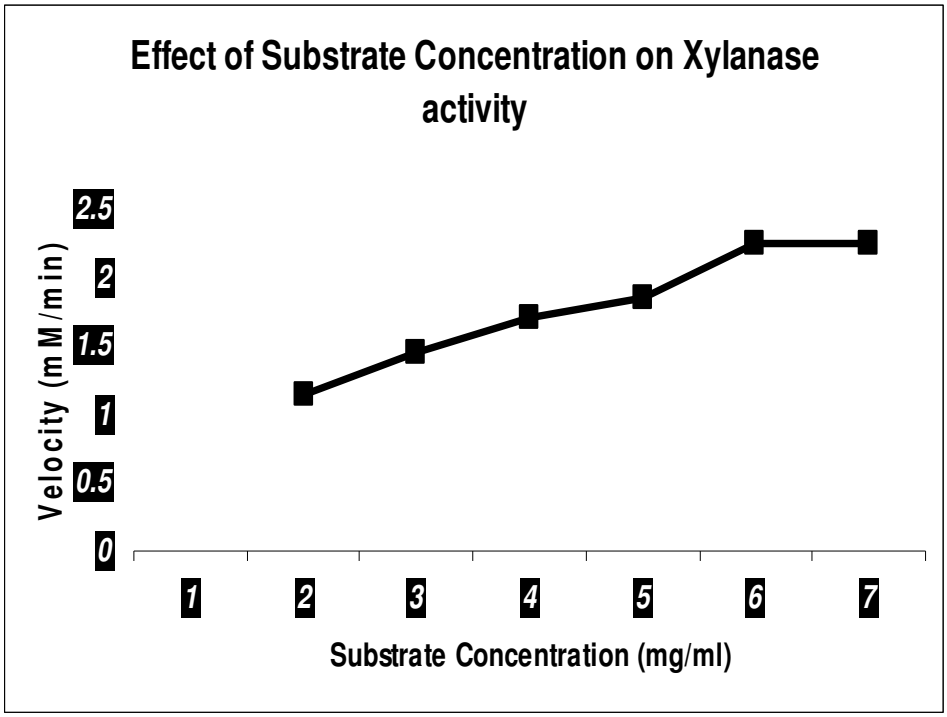

Figure 5: Effect of Substrate Concentration on Xylanase

Table 1: Purification of xylanase from *B. pumilus*

Quality level of enzyme	Volume of extract (ml)	Total enzyme activity ($\mu M\ min^{-1}$)	Total protein (mg)	Specific activity of enzyme ($\mu M\ min^{-1}\ mg^{-1}$)	Fold purification	Yield %
Crude	1000	1998.26	10960	0.182	1.00	100
Partially Purified	60	1332.17	1920	0.69	3.79	66

Abbreviations:

DNS	: Dinitro salicylic acid
SDS PAGE	: Sodium dodecylsulphate-polyacrylamide gel electrophoresis
$NaNO_3$	: Sodium Nitrate
K_2HPO_4	: Di potassium hydrogen phosphate
$MgSO_4.7H_2O$	: Magnesium sulphate
$MnSO_4.H_2O$	: Manganese sulphate
$FeSO_4.H_2O$	: Ferrous sulphate
$CaCl_2.2H_2O$	: Calcium chloride
Na_2CO_3	: Sodium nitrate
HCl	: Hydrochloric acid
NaCl	: Sodium chloride