

Isolation and identification of local *Bacillus* isolates for xylanase biosynthesis

Hassan Ammoneh, Muhanad Harba, Yasser Akeed, Mohanad Al-Halabi, Yasser Bakri*

Department of Molecular Biology and Biotechnology, AECS, Damascus, Syria.

Received: November 2012, Accepted: October 2013.

ABSTRACT

Background and Objectives: *Bacillus* species are attractive industrial organisms due to their rapid growth rates leading to a short fermentation cycle and for their capacity to secrete important enzymes and proteins such as xylanase into the extracellular medium. Considering the industrial importance of xylanase, in this current study, *Bacillus* spp. were isolated from different soils and were screened for their xylanase production.

Materials and Methods: *Bacillus* isolates used in this study were obtained from a national screening program carried out during 2006-2007 in which soil samples that covered areas throughout the interior of Syria were collected. The prepared inoculum from each of *Bacillus* isolates was aliquoted onto xylan agar plates, incubated at 30°C for 72 h and screened for xylanase synthesis.

Results: Xylanolytic isolates were selected depending on the clear zones of xylan hydrolysis. Fifteen isolates having the highest clearing zone were determined and grown in a solid state fermentation. Of the 15 isolates, three bacilli namely SY30A, SY185C and SY190E that showed maximum xylanase production, were identified using the 16S rDNA sequencing method. According to 16S rDNA gene sequence data, the closest phylogenetic neighbor for SY30A was *Bacillus pumilus* and for SY185C and SY190E isolates was *Bacillus subtilis*. Optimal pH and temperature for xylanase activity was 7.0 and 55°C for SY30A and 6.0 and 60°C for SY185C and SY190E, respectively. Under these conditions, the following activities were found to be around 1157 ± 58, 916 ± 46 and 794 ± 39 (U/g) for SY30A, SY185C and SY190E, respectively.

Conclusions: Selected local *Bacillus* isolates were found to be a potential source of xylanase which was proven to be quite suitable for multiple biotechnological applications. These isolates might after extensive optimization steps be an alternative to commercially available strains.

Keywords: *Bacillus* sp, Xylanase, Solid state culture

INTRODUCTION

Xylans, major structural heteropolysaccharides in plants, are α -1,4-linked polymers of xylopyranosyl units with a degree of polymerization ranging from 70 to 200. Depending on their origin, xylans may also contain variable amounts of arabinosyl- and

4-O-methylglucuronic acid residues and acetyl groups (18, 21). Xylanolytic enzymes are a group of enzymes that hydrolyze xylan and arabinoxylan polymers. This group includes endo- α -1,4-xylanase, β -xylosidase, arabinofuranosidase and acetyl xylan esterase (5). Endo- α -1,4-xylanase plays an important role in the animal feed as it increases the body weight gains (14). In pulp and paper industry, xylanases are employed in the prebleaching process to reduce the use of the toxic chlorine chemicals (27). In bread and bakery industry, xylanases are used to increase the dough viscosity, bread volume, and shelf life (19). Other potential applications include the conversion of xylan in wastes from agriculture and food industries

* Corresponding author: Yasser Bakri Ph.D
Address: Department of Molecular Biology and Biotechnology, AECS, P. O. Box 6091, Damascus, Syria.
Tel: +963-11-2132580
Fax: +963-11-6112289
E-mail: ascientific@aec.org.sy

into xylose, and the production of fuel and chemical feedstocks (26).

Many bacteria and fungi have been studied for xylanase production (4, 9, 10). However, *Bacillus* species have been the major workhorse industrial microorganisms for more than a thousand years. *Bacillus* species are attractive industrial organisms for several reasons, including their high growth rates leading to short fermentation cycle times and their capacity to secrete proteins into the extra cellular medium. It is estimated that *Bacillus* spp. enzymes make up about 50% of the total enzyme market (24).

For the production of any industrial enzyme, an inexpensive substrate and an efficient fermentation process are essential for commercial viability. It has been established that solid-state fermentation has several advantages over submerged fermentation, due to a smaller volume of solvent required for product recovery, resulting in higher productivity per unit volume, lower contamination and foaming problems and better exploitation of various agro-residues as substrates (2-7).

Considering the industrial importance of xylanase, in the present study, *Bacillus* spp. were isolated from different soils and screened for xylanase production in a solid state fermentation process. The investigation led to the identification of three high producing xylanase isolates, *B. pumilus* SY30A and *B. subtilis* SY185C and SY190E.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Bacillus* isolates analyzed in this study were obtained from a national screening programme carried out in 2006 and 2007 from soil samples distributed throughout the interior of Syria (1). Isolates were routinely cultured on nutrient agar plates (NA). Plates were incubated at 30°C until bacterial colonies developed, kept at 4°C and sub-cultured every fifteenth day. Subsequently, selected isolates were maintained in 20% glycerol at -80°C.

Inoculum preparation. Five milliliter of medium containing nutrient broth was transferred to a 50 ml tube and sterilized in an autoclave at 121°C for 20 min. After cooling, a loopful of bacterial culture was aseptically transferred and rotated overnight at 200 rpm and 30°C. 1% of this culture was used

to inoculate 20 ml of the same medium in 100 ml flask and incubated in an orbital shaker at 30°C until the optical density at 600 nm (OD_{600}) reached 0.15 (cell density about 2×10^8 colony-forming unit (CFU)/ml).

Screening of xylanase producing bacteria. Twenty five microliter of the prepared inoculum for each *Bacillus* isolate was aliquoted onto xylan agar plates, incubated at 30°C for 72 h and screened for xylanase synthesis. Positive xylanolytic isolates were decided according to the clear zones of hydrolysis on the xylan. Fifteen isolates having the highest clearing zone were selected and grown in solid state fermentation. The amount of xylanase produced by each isolate was determined from the extract culture filtrate. Subsequently, the selected isolates were grown on NA plates at 30°C and maintained at $4 \pm 1^\circ\text{C}$. Isolates were routinely subcultured every two weeks on NA plates.

Solid state fermentation. Enzyme production was checked for selected *Bacillus* isolates in solid state fermentation using wheat bran procured from local market. Enzyme production was carried out in 100 ml Erlenmeyer flasks containing 5 g-of wheat bran and nutrients plus distilled water to adjust the moisture to 60%. The fermentation medium consisted of (g/L): K_2HPO_4 , 1; NaCl, 3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3; and yeast extract, 3 and peptone 5, as nitrogen source. 1 ml of the prepared inoculum for each *Bacillus* isolate was transferred into the solid medium and placed in an incubator. After 3 days of cultivation flasks were removed and the enzyme was extracted by the addition of 25 mL distilled water containing 0.1% Triton X 100. Flasks contents were stirred for 1.5 h on a magnetic stirrer and the clear supernatant was obtained by centrifugation at $9800 \times g$ for 15 min and used as enzyme source.

Xylanase assay. Xylanase activity was determined as described by Bailey et al. (3) using 1% birch wood xylan as substrate. Xylan solution and the enzyme at an appropriate dilution were incubated at 55°C for 5 minutes and the reducing sugars were determined by the dinitrosalicylic acid (DNS) procedure with xylose as a standard (15). The released xylose was measured spectrophotometrically at 540 nm. One unit (U) of enzyme activity is defined as the amount of enzyme releasing 1 μmol xylose per ml per minute under the

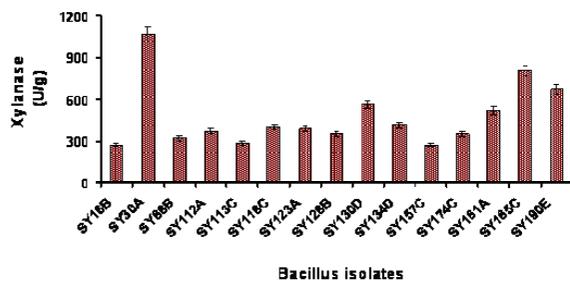


Fig. 1. Extracellular xylanase production from *Bacillus* isolates.

described assay conditions.

Effect of temperature on xylanase activity and stability. Enzyme activity was evaluated by measuring the xylanase activity at different temperatures ranging from 40 to 75°C in 0.1 M sodium phosphate buffer (pH 7.0) and 1% soluble birchwoodxylan. To evaluate thermal stability, the remaining xylanase activity was determined after preincubation of the crude enzyme preparations at 30-90°C in buffers of optimal activity, without substrate, for 1 h.

Effect of pH on xylanase activity and stability. The optimal pH for enzyme activity was determined by changing the assay reaction mixture pH using the following buffers (0.1 M): sodium acetate (pH 5.0), sodium phosphate (pH 6.0–7.0), Tris–HCl (pH 8), glycine–NaOH buffer (pH 9–10) and 1% soluble birchwood as a substrate. To evaluate the stability of the enzyme at each pH, xylanase activity was measured after incubation the enzyme solution in pH buffers ranging from 4.0 to 10.0 at 25°C for 24 hours. Residual activity was determined under optimal assay conditions for each isolate.

Polymerase chain reaction (PCR) amplification and 16S rDNA sequencing. Two primers BacF (5'-GTGCCTAATACATGCAAGTC-3') and BcaR (5'-CTTTACGCCAATAATTCC-3') flanking a highly variable sequence region of 545 bp towards the 5' end of the 16S rDNA region were used (16). Genomic DNA was extracted and purified using DNA extraction kit according to the manufacturer's recommendations (BIOTOOLS, Cat. NO. 21.002). PCR mixtures were prepared using 10–20 ng of template DNA, 0.4 μM of each primer, 1U of *Taq* DNA polymerase (Promega), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Promega), 2 mM MgSO₄, and 3% dimethyl sulfoxide (DMSO). Amplification

was done in a Bio-Rad T gradient thermocycler under the following conditions: a 5 min denaturation step at 95°C, followed by 30 amplification cycles (1 min at 95°C, 1 min at 56°C and 1 min at 72°C) and an extra extension step of 10 min at 72°C. PCR products were separated on a 1% agarose gel to which ethidium bromide was added and photographed under UV light. Amplification products were purified using QIAquick Gel Extraction kit (QIAGEN, Cat. No. 28704) and sequenced on both strands using an ABI 310 sequencer machine (Department of Molecular Biology and Biotechnology, AECS). The sequences were subjected to a BLAST search against the full EMBL/GenBank database available at NCBI public database (<http://www.ncbi.nlm.nih.gov>).

RESULTS AND DISCUSSION

Screening of xylanase producing Bacilli. Five hundred and twenty-five bacterial isolates from 200 soil samples collected from different areas of Syria including cereal fields, olive fields, forests, desert and gardens, were evaluated for xylanase production. Fifteen of these isolates with a high xylanase production level were determined (Fig. 1) and thus three isolates SY30A, SY185C and SY190E were selected for further studies.

Identification of the selected isolates. DNA sequence analysis methods are an objective, reproducible, and rapid means of species identification, therefore, they have been widely utilized (13). SY30A, SY185C and SY190E Identification was performed using the 16S DNA gene sequences. The nucleotide BLAST similarity search analysis, based on the 16S DNA gene sequence revealed that these isolates belong to the genus *Bacillus*. The closest phylogenetic neighbors according to the 16S DNA gene sequence data for SY30A was *Bacillus pumilus* and for the SY185C and SY190E isolates were *Bacillus subtilis* with a 100 % homology.

Effect of temperature on xylanase activity and stability. The effect of temperature on xylanase activity against birch wood xylan at pH 6.0 was examined in the temperature range of 40-75°C. Xylanases produced by SY30A exhibited maximum activity at 55°C compared with 60°C for the other two isolates, SY185C and SY190E (Fig. 2). Under these conditions, enzyme activity were 1013, 854 and 731

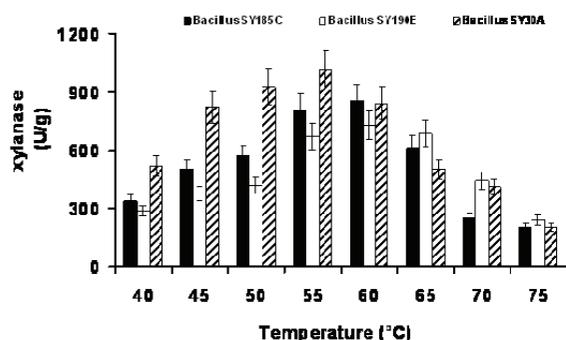


Fig. 2. Effect of temperature on the xylanase activity of three *Bacillus* strains.

(U/g) for SY30A, SY185C and SY190E, respectively. Similar temperature optima have been reported by many other workers for xylanase production from varied sources. Sá-Pereira et al. (20) reported that optimal xylanase activity of a *B. subtilis* strain was at 60°C on phosphate buffer, at pH 6.0.

Additionally, xylanase from a thermo alkaliphilic bacterium showed optimum activity at 50°C (23). The highest activity of xylanase obtained from both *B. circulans* and *B.amyloliquefaciens* was at 50.0°C (11, 12). Sanghi et al. (22) found that xylanase produced from *Bacillus subtilis* ASH gave the best activity at 55°C

Enzyme stability is the most important factor in studying enzyme characteristics. Thus, thermal stability tests were carried out by pre-incubating xylanase for 60 min in a temperature range of 30 to 90°C (Fig. 3). Our results showed that there was no significant decrease in xylanase activity during 60 min incubation at 30–50°C, while at 50°C the residual xylanases activities were 93.4, 90.2 and 89.6% for SY30A, SY185C and SY190E, respectively. The enzyme was sensitive at 70°C, retaining 17.1% activity for SY30A, while retaining 58 and 70.8% activity for SY185C and SY190E, respectively.

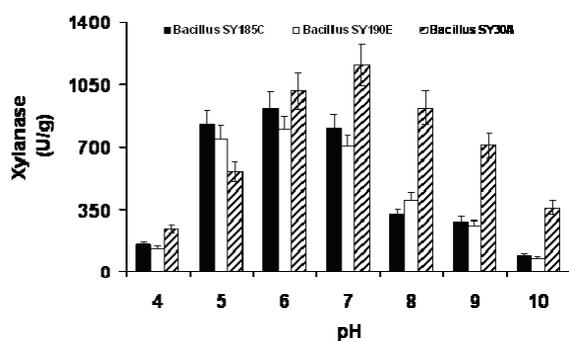


Fig. 4. Effect of pH on the xylanase activity of three *Bacillus* strains.

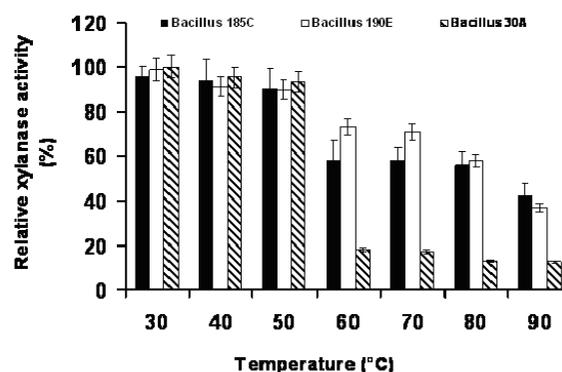


Fig. 3. Effect of temperature on the xylanase stability of three *Bacillus* strains.

activities for SY185C and SY190E, respectively. At higher temperature values xylanase stability gradually was found to decline. Thermal stability of xylanase is an important property due to its potential applications in several industrial processes. The industrial importance of an enzyme will be more when the effect of temperature input on its optimal activity is less. Strains isolated by us could be a good source for biotechnological applications.

Effect of pH on xylanase activity and stability.

Enzyme activity is markedly affected by pH because substrate binding and catalysis are often dependent on charge distribution on both, substrate and, in particular enzyme molecules (25). A pH range from 4 to 10 was used to study the effect of pH on xylanase activity and the results are given in (Fig. 4). Optimum pH was found to be 7 for SY30A and 6 for both SY185C and SY190E. Enzyme activity at pH 8 and 9 was 920 and 711 U/g for SY30A, 325 and 286 U/g for SY185C and 405 and 265 U/g for SY190E, respectively. Xylanase activity was shown to decrease at pH 10. These clearly indicates that SY30A produced enzyme is more suitable for any application in the pH range of 6.0-9.0 and 5-8 for the SY185C and SY190E enzymes.

Up to date, xylanases produced by most alkaliphiles are reported to have their optimum pH around neutrality. Nakamura et al. (17) reported the first alkaline xylanases produced by *Bacillus* sp. strain 41 M-1, which had an optimum pH at 9.0. Yang et al. (28) isolated an alkaliphilic *Bacillus* sp. VI-4 from a hard wood kraft pulp, which produced xylanase having an optimum pH of 6-8.5. Similarly, the optimum pH activity at 6.8-7.0 of xylanase reported from *B. amyloliquefaciens* (11). Thermostable alkaline xylanase from a *Bacillus* sp. showed three

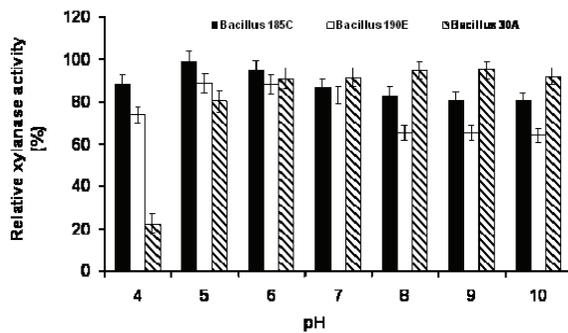


Fig. 5. Effect of pH on the xylanase stability of three *Bacillus* strains.

optimum peaks for pH 6.5, 8.5 and 10.5 (23). A wide range of pH activity from 5.0-8.0 was observed in *B. circulans* BL53 upon solid state cultivation (12). The activity persistence in a large range of pH is a desirable quality of an industrial enzyme. When pH stability was measured at values between 4 and 10, the xylanase stability was over a broad neutral to alkaline pH range (6-10) and retained more than 75% of its activity after 24 h of incubation at room temperature (Fig. 5). Stability at extreme pH values may be due to charged amino acid residues. The enzymes stable in alkaline conditions were characterized by a decreased number of acidic residues and an increased number of arginines (8).

CONCLUSION

Results obtained in the current study demonstrate that the local *Bacillus* isolates are a potential source of xylanase production. The temperature properties for enzyme activity and stability make the enzyme from some of the tested isolates a quite suitable for biotechnological applications. Therefore, these isolates could be alternative of the commercial strains. However, the process of xylanase production from the new local *Bacillus* isolates may be commercialized after further optimization for enhanced enzyme production.

ACKNOWLEDGEMENT

The authors thank the Director General of AECS and the Head of the Molecular Biology and Biotechnology Department for their continuous support throughout this work.

REFERENCES

1. Ammoun H, Hraha M, Idris E, Makee H. Isolation and characterization of native *Bacillus thuringiensis* isolates from Syrian soil and testing their insecticidal activities against some insect pests. *Turk J Agric For* 2011; 35: 421-431.
2. Archana A, Satyanarayana T. Xylanase production by thermophilic *Bacillus licheniformis* A 99 in solid state fermentation. *Enzyme and Microb Technol.* 1997; 21: 12-17.
3. Bailey MJ, Biely P, Poutanen K. Interlaboratory testing of methods for assay of xylanase activity. *J Biotechnol* 1992; 23: 257-270.
4. Bakri Y, Jawhar M, Arabi MIE. Improvement of Xylanase Production by *Cochliobolussativus* in Solid State Fermentation. *Brazi J Microbiol* 2008; 39: 238-240.
5. Biely, P(1993). Biochemical aspects of the production of microbial hemicellulases. pp. 29-51. In: Coughlan MP, and Hazlewood GP (eds) Hemicellulose and Hemicellulases. Portland Press, London.
6. Duarte MCT, Pellegrino ACA, Portugal EP, Ponezi AN, FrancoTT. Characterization of alkaline xylanases from *Bacillus pumilus*. *Brazi J Microbiol* 2000; 31: 90-94.
7. Gupta U, Kar, R. Xylanase Production by a Thermotolerant *Bacillus* Species under Solid-state and Submerged Fermentation. *Braz. Arch. Biol. Techn.* 2009; 52: 1363-1371.
8. Hakulinen N, Turunen O, Jänis J, Leisola M, Rouvinen J. Three-dimensional structures of thermophilic β -1,4-xylanases from *Chaetomium thermophilum* and *Nonomuraea flexuosa* comparison of twelve xylanases in relation to their thermal stability. *Eur J Biochem* 2003; 270: 1399-1412.
9. Heck JX, Hertz PF, Ayab MAZ. Cellulase and xylanase production by isolated amazon *Bacillus* strains using soybean industrial residue based solid state cultivation. *Brazi J Microbiol* 2002; 33: 213-218.
10. Kinegam S, Tanasupawat S, Akaracharanya A. Screening and identification of xylanase-producing bacteria from Thai soils. *J Gen Appl Microbiol* 2007; 53: 57-65.
11. Javier DB, Faustino S, Mario, D.B, Guillermo RC, Rajni HK. Purification and characterization of a thermostable xylanase from *Bacillus amylolique faciens*. *Enzyme Microb Technol* 1998; 22: 42-49.
12. Júlio XH, Luís HBS, Plinho FH, Marco AZA. Purification and properties of a xylanase produced by *Bacillus circulans* BL53 on solid-state cultivation. *Biochem Eng J.* 2006; 32: 179-184.
13. Li Y, Liu Z, Cui F, Xu Y, Zhao H. Production of xylanase from a newly isolated *Penicillium* sp. ZH-30. *World J Microbiol Biotechnol* 2007; 23: 837-843.
14. Medel P, Baucells F, Gracia MI, De-Blas C, Mateos GG. Processing of barley and enzyme supplementation in diets for young pigs. *Anim Feed Sci Tech* 2002; 95: 113-122.
15. GL Miller. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal Chem* 1959; 31: 426-428.
16. Nair JR, Singh G, Sekar V. Isolation and characterization

- of a novel *Bacillus* strain from coffee phyllosphere showing antifungal activity. *J Appl Microbiol* 2002; 93: 772-780.
17. Nakamura S, Wakabayashi K, Nakai R, Horikoshi K. Production of alkaline xylanase by a newly isolated alkaliphilic *Bacillus* sp. strain 41M-1. *World J Microbiol Biotechnol* 1993; 3: 221-224.
 18. Poutanen K, Rättö M, Puls J, Viikari L. Evaluation of different microbial xylanolytic systems. *J Biotechnol* 1987; 6: 49-60.
 19. Romanowska I, Polak J, Jonowska K, Bielecki S. The application of fungal endoxylanase in bread-making. *Comm Agr Appl Biol Sci* 2003; 68: 317-320.
 20. Sa'-Pereira P, Costa-Ferreira M, Aires-Barros MR. Enzymatic properties of a neutral endo-1,3(4)- β -xylanase Xyl II from *Bacillus subtilis*. *J Biotechnol* 2002; 94: 265-275.
 21. Saha BC. Hemicellulose bioconversion. *J Ind Microbiol Biotechnol* 2003; 30: 279-291.
 22. Sanghi A, Garg N, Gupta VK, Mittal A, Kuhad RC. One step purification and characterization of cellulase free xylanase produced by alkaliphilic *Bacillus subtilis* ASH. *Braz J Microbiol* 2010; 41: 467-476.
 23. Sapre M, Jha H, Patil M. Purification and characterization of a thermoalkalophilic xylanase from *Bacillus* sp. *World J Microbiol Biotechnol* 2005; 21: 649-654.
 24. Schallmey M, Singh A, Ward OP. Developments in the use of *Bacillus* species for industrial production. *Can J Microbiol* 2004; 50: 1-17.
 25. Shah AR, Madamwar D. Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterization. *Process Biochem* 2005; 40: 1763-1771.
 26. Sunna A, Antranikian G. Xylanolytic enzymes from fungi and bacteria. *Crit Rev Biotechnol* 1997; 17: 39-67.
 27. Wong K.K.Y., James C.S., Campion S.H. Xylanase pre- and post-treatments of bleached pulps decrease absorption coefficient. *J Pulp Pap Sci* 2000; 26: 377-383.
 28. Yang VW, Zhuang Z, Elegir G, Jeffries TW. Alkaline active xylanase produced by an alkaliphilic *Bacillus* sp. isolated from kraft pulp. *J Ind Microbiol* 1995; 15: 434-441.