

Recombinant xylanase from *Bacillus tequilensis* BT21: Biochemical characterization and its application in production of xylobiose from agricultural residues

Rakhee Khandeparker,^{1*} Pankaj Parab¹, Ujwala Amberkar¹

¹National Institute of Oceanography, CSIR, Goa- 403 004, India

* Corresponding author

Dr. Rakhee Khandeparker

E-mail: rakhee@nio.org

Tel.: +91 832 2450540; fax: +91 832 2450606

Abstract

Bacterial strain, *Bacillus tequilensis* BT21 isolated from marine sediments was found to produce extracellular xylanase. *xynBT21* gene encoding xylanase enzyme was cloned and expressed in *Escherichia coli*. The gene encoded a protein consisting of 213 residue polypeptide with calculated molecular mass of 23.3 kDa. Purified recombinant xylanase displayed optimum activity at temperature 60 °C and pH=6. The enzyme was highly stable in alkaline pH. The enzyme was stable at pH=7 and remained 100 % active for 24 h, while the enzyme activity increased at pH=8 and pH=9 over the time of incubation. *B. tequilensis* BT21 xylanase had alkaline pI of 9.4 and belonged to family 11 glycosyl hydrolases. The mode of action of XynBT21 on beechwood xylan as well as xylooligosaccharides was studied. XynBT21 hydrolyzed xylooligosaccharides and beechwood xylan yielding mainly xylobiose (X₂) with a small amount of xylose (X₁), indicating that XynBT21 was probably an endo-acting xylanase. Enzymatic hydrolysis using wheat bran as a substrate revealed that xylanase reported here has the potential to produce xylobiose from wheat bran. Xylooligosaccharides especially xylobiose have strong bifidogenic properties and are increasingly used as a prebiotic. This is the first report that describes this novel xylanase enzyme from marine *B. tequilensis*.BT21 releasing xylobiose from wheat bran.

Keywords: Enzyme. Xylanase. Alkaline pI. Characterization. *Bacillus tequilensis*. Xylobiose

Introduction

Xylan the most abundant hemicellulose consist of β -1, 4-linked xylose residues in the backbone to which o-acetyl, α -L-arabinofuranosyl, D- α -glucuronic and phenolic acid residues are present on the side chains. Endoxylanases degrade β -1,4-xylan randomly, yielding a chain of linear and branched oligosaccharide fragments. Various microorganisms are known to produce endoxylanases (1,2). As per the amino acid sequence similarities, xylanases are mostly classified into families 10 and 11 of the glycoside hydrolases. GH10 xylanases are those having high molecular mass ≥ 30 kDa and with low pI, while GH11 xylanases are normally smaller (≤ 20 kDa) and have a high pI (3).

Xylanases have a range of applications in textile, clarification of fruit juices, aroma, animal feed, baking, production of ethanol as well as in paper and pulp industries. Industrial process conditions are harsh due to extremes of pH, temperature, inhibitors, etc. Sufficiently strong enzymes able to withstand such conditions recommended for such processes. The most of the reported xylanases do not meet such criteria, therefore, enzymes which comply well with the industrial processes needs to look for (4,5). The marine environment is highly complex and dynamic with high salinity, higher pressure, low or high temperature and unique light conditions, these situations may explain to the significant variations in the enzymes produced by marine and terrestrial microorganism. Xylanases from the marine condition can have some remarkable qualities with respect to stability at high temperature and pH which needs attention. There are few studies reported on marine xylanases (6-13) and it is important to look into more potential xylanases from the marine sources. Recently use of endoxylanases for producing xylooligosaccharides from xylan sources is taking lead. Xylobiose has represented its part in invigorating the specific advancement of human intestinal bifidobacteria, which are basic for the upkeep of a sound intestinal microflora. In this study, we report the characterization of xylanase from a marine bacterium *B. tequilensis* BT21 and propose its efficacy in the nutraceutical industry.

Materials and Methods

Chemicals

Xylooligosaccharides were purchased from Megazyme (Bray, Ireland). Xylan (birchwood and beechwood), 3,5-dinitrosalysilic acid, buffers (citrate, phosphate and glycine, tris-HCL), solvents (acetonitrile, ethyl acetate, 2-propanol), protein molecular marker, acrylamide, agarose, sucrose, orcinol, serum albumin, bicinchoninic acid and coomassie brilliant blue were purchased from Sigma-Aldrich, USA. Ethanol and TLC plates were purchased from Merck, Darmstadt, Germany while sulphuric acid and acetic acid, sodium chloride from Sd Fine Chem. Ltd., Mumbai, India.

Bacterial strains, culture conditions and vectors

B. tequilensis was isolated from sediment samples of Chorao island located in Mandovi estuary Goa India. The culture was grown in basal salt solution (BSS) medium at room temperature along with 0.5 % of xylan (13). *E. coli* strain (JM109) was used as the hosts for DNA manipulation. The plasmid pCR®2.1-TOPO® TA cloning vector (Invitrogen, CA, USA) was employed for cloning and DNA sequencing.

PCR amplification of the 16S rRNA for strain identification

DNA was isolated from cells as per method described by Khandeparker *et al.* (13). The 16S rRNA gene fragment was amplified by PCR with the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTACCTTGTTACGACTT-30). The amplification was performed out using a PCR thermal cycler (Veriti, Applied Biosystem, Foster City, CA, USA). Gene sequence was determined using a Taq Dye Deoxy terminator cycle sequence kit (Perkin Elmer, Foster City, CA, USA) and were analyzed with 373A automated DNA sequencer (model 3130xl; Applied Biosystem, Foster City, CA, USA) (13). The sequence obtained was aligned with corresponding sequences of 16S rRNA from the database using BLAST from the website (14).

Molecular cloning, expression and sequencing of xylanase gene

Gene cloning was carried out using standard cloning method (15). The primers *xynF* and *xynR* were obtained from previously reported article (13). Xylanase gene was amplified using PCR and amplified gene fragment was then ligated into TOPO TA cloning vector and transformed into *E. Coli* cells. Plasmid extraction was done and the xylanase gene was sequenced.

Determination of amino acid composition of xylanase gene

Amino acid sequence of xylanase gene of *B. tequilensis* BT21 was determined by translating the gene sequence into the protein sequence using ExpASy server (16) Theoretical molecular mass, isoelectric point (pI) and the total number of positive and negative residues were calculated using the Expasy's ProtParam server.

Enzyme production and fractionation

Recombinant enzyme production was enhanced by incorporating 1 mM isopropyl- β -D thiogalactopyranoside (IPTG) in the growth medium of transformant cells and incubating at 37 °C for 24 h with 140 rpm. Cells were collected by centrifugation (4,000 g for 10 min, 4 °C) using centrifuge 5810R (Eppendorf, Germany) and the pellet was rinsed twice in Tris-HCl (10 mM, pH=8). The obtained pellet after centrifugation was resuspended in 25 % sucrose solution. The suspension was shaken in

presence of 0.5 M EDTA for 10 min at room temperature. This was again centrifuged and the cell pellet was collected. The supernatant obtained in above three steps constitutes for extracellular enzyme fraction (F1). Ice-cold water was vigorously added to the pellet and shaken for 10 mins. Further centrifugation at 9000 rpm for 10 mins, the supernatant was collected which forms periplasmic enzyme fraction (F2). The remaining cell pellet was suspended in 10 mM Tris-HCl buffer (pH=7) and lysed by sonication (sonicator, model-GT-1730QTS, GT Sonic, Guangdong, China). Intracellular enzyme fraction (F3) as supernatant was collected by removing cell pellet by centrifugation (17). For native enzyme cell suspension of *B. tequilensis* BT21 growing on xylan, media was centrifuged and the supernatant containing extracellular xylanase was collected.

Native and recombinant xylanase purification

Native and recombinant xylanases were precipitated using ammonium sulphate (80 %). The precipitated enzyme was dissolved in phosphate buffer and dialyzed for 24 h (50 mM, pH=6). The crude enzyme was further purified using anion exchange resins followed by cation exchange resins such as DEAE and CM-Sepharose FF (Sigma–Aldrich Co. USA) columns respectively (11). The native enzyme was used only to study kinetic properties otherwise, recombinant enzyme was used unless mentioned.

Enzyme assay and protein estimation

The activity of xylanase enzyme was determined by using 3,5-dinitrosalicylic acid (DNS method) (18). One unit of xylanase activity was calculated as μmol of xylose released per min. Proteins were analyzed by the method of bicinchoninic acid with bovine serum albumin as a standard (19).

Effect of temperature and pH on xylanase enzyme

Optimal temperature of xylanase was studied by analyzing the enzyme activity at different temperatures ranging from 15–75 °C, at pH=6, while the optimal pH of xylanase was analyzed by measuring its activity using different pH buffers of 0.05 molar. Citrate buffer was used for pH range from 4-6, phosphate buffer was employed for pH from 6–8, while glycine–NaOH buffer was used for pH=8 to pH=11. Xylanase activity was measured at 60 °C. Thermal stability of the enzyme was verified by incubating the enzyme at 40 °C, 50 °C and 60 °C for 6 h (pH= 6) using an incubator. pH stability was studied using 100-fold diluted xylanase enzyme using respective buffers (pH ranging from 7 to 9) and incubating it for 24 h at 60 °C. Residual activity was analyzed at regular intervals during incubation.

Isoelectric Focusing (IEF)

Isoelectric Focusing (IEF) was performed using BIO-RAD ReadyPrep 2-D starter kit. IPG strips (SERVA, Heidelberg, Germany, pH range 3-10, 11 cm), were rehydrated for 12-16 h in sample enzyme

(10 µg of protein) and mixed with rehydration buffer provided with the starter kit. PROTEAN IEF chamber (Biorad, Hercules, California, USA) was programmed for a constant temperature of 10 °C and at 50 VH/strip for 20 min at 250 V, with 2 h linear increase to 4,000 V and finally attaining the value of 10,000 VH. Strips were equilibrated in equilibration buffer I and II (provided with the kit) for 10 min each respectively on the rocker. Strips were then sealed on the top of 12 % SDS-PAGE gels (separating gel) using 0.5 % agarose. SDS-PAGE was run on 12 % acrylamide gel at 120 volts for 110 min at 4 °C. Protein molecular mass standard (medium range) was used for electrophoresis in 2nd dimension.

Molecular mass estimation and zymogram analysis

The Molecular mass of xylanase enzyme was assessed by SDS–PAGE (12 %) electrophoresis (20) using medium range molecular mass markers (14.3–97.4 kDa). Protein bands were stained using Coomassie brilliant blue stain. Zymogram was carried out using SDS–PAGE (12 %) electrophoresis as described by Nakamura *et al.* (21). The gel with separated protein bands was thoroughly washed (four times) using 50 mM Phosphate buffer (pH=7.0). Initial 2 washes contain 25 % isopropanol (to get rid of SDS and renature protein). The gel was further incubated at an optimum temperature of the enzyme for 60 min. The gel was stained with 0.1 % (mass/volume) Congo red dye for 30 min and then washed with 1.0 % (mass/volume) NaCl till the spare dye was washed off from the active band. Finally, the gel was flooded in 0.5 % acetic acid, thus changing the background of gel to dark blue, and the activity bands were spotted as clear colourless areas.

Kinetic determinations

Purified native and recombinant xylanase was incubated with birchwood and beechwood as substrates in 50 mM phosphate buffer with pH=7. The rate of xylan hydrolysis was determined at pH=9 and temperature 60 °C. Various concentrations of xylan ranging from 0.5 to 8 mg were used in this study. The kinetic constants K_m and V_{max} were determined using the method of Lineweaver and Burk (22).

Hydrolysis studies

The partially purified periplasmic fraction of xylanase was dissolved in phosphate buffer (pH=7) and used for hydrolysis study. Substrates and enzyme were incubated at 60 °C. Samples were removed at 0, 1, 3, 6, 12 h of intervals. Xylooligosaccharides were also incubated with the enzyme and the samples were picked at different time intervals ranging from 1 h to 3 h. Enzyme-substrate reaction was terminated by putting reaction mixture into boiling water for 5 min (23). The unused polysaccharide was precipitated using isopropanol and centrifugated. The supernatant was collected. Hydrolysis products were identified by thin layer chromatography (TLC). TLC was performed with the mixture of

Acetonitrile: Ethyl acetate: 2-propanol: water as a solvent system. Hydrolysis products were spotted by spraying TLC plates with orcinol spray which is a mixture of ethanol, sulphuric acid and orcinol, and heating plate for 5 min at 150 °C. Similarly, TLC was performed for enzymatic saccharification of wheat bran. Here native enzyme after purification was treated with wheat bran. 1 % substrates (wheat bran) and the enzyme were incubated at 60 °C. Sample (in triplicates) were taken at 24 h of an interval.

Results

Identification of B. tequilensis BT21 by 16S rRNA sequence analysis

The strain BT21 was isolated from mangrove sediment along the bank of Mandovi estuary, on the west coast of India. Biochemical analysis revealed that the isolate belonged to the Gram-positive group, cells are a short rod, motile, oxidase positive and catalase positive. As described in Bergey's Manual of Systematic Bacteriology (24) these characteristics show close similarity to *Bacillus* sp. The amplified 16S rRNA sequence matched to the 16S rDNA sequence in the GeneBank (25), and the results showed 99 % identity with *B. tequilensis* (GenBank accession no. KF054870). Thus, the strain BT21 identified as one strain of *B. tequilensis* (GenBank accession no. KF797798).

Nucleotide sequence analysis of the xylanase gene

The PCR product of total chromosomal DNA acquired from *B. tequilensis* strain BT21, using primers xynF and xynR was ligated with TOPO® TA cloning vector. The recombinant plasmid DNA was introduced into *E. coli* JM109. A complete nucleotide sequence was acquired (GenBank database number KF797799), having 639 bp open reading frame encoding a protein of 23324.6 Da having 213 amino acid residues with pI of 9.44 was identified. The gene sequence is 99 % similar to that of xylanase gene sequence of *B. subtilis* R5 (Accession number AB457186.1). The catalytic domain of xynBT21 (from *B. tequilensis*) showed good homology with xylanase which is classified into family 11 glycosyl hydrolases as per hydrophobic cluster analysis (26).

Amino acid composition of recombinant xylanase

Recombinant xylanase of *B. tequilensis* showed a relatively, high percentage of glycine (12.2 %), threonine (12.2 %) followed by serine (10.8 %) and asparagine (8.9 %), while there were no traces of cystine and pyrrolisine (Table. 1). Highly conserved Glu104 and Glu196 which are crucial for the catalytic activity of family 11 glycosyl hydrolases, were found in two conserved regions (Fig. 1). The *B. tequilensis* BT21 xylanase contained double the number of acidic amino acids compared to basic amino acid, also the total number of negatively charged residues in the enzyme were less (Asparagine +

Glutamic acid= 9), compared to positively charged residues (Arginine + Lysine =16). The enzyme had 41 % polar amino acids while 59 % nonpolar (hydrophilic) amino acids

Characterization of xylanase from B. tequilensis BT21

Xylanase enzyme had optimal pH of 6.0 while the enzyme had the optimal temperature of 60 °C at pH=6 (Fig. 2). At pH=6 and at pH=7 enzyme retained 100 % of activity when incubated for 24 h, while at pH=8 and pH=9 enzyme showed an increase in activity over the period of incubation (Fig. 3). At 40 °C, enzyme activity increased with the incubation time, while at 50 °C enzymes remained stable for almost 1 h. At 60 °C, enzyme showed 100 % activity up to 20 mins and remained active for 2 h retaining 75 % activity (Fig. 4).

Kinetic parameters

The K_m and v_{max} value of native and recombinant xylanase was obtained from Lineweaver-Burke plot. The K_m of native and recombinant xylanase for birchwood xylan was 11.1 (mg/mL) and 3.3 (mg/mL) while v_{max} was 2222 $\mu\text{mol}/(\text{min}\cdot\text{mg})$ and 3125 $\mu\text{mol}/(\text{min}\cdot\text{mg})$ respectively. K_m of native and recombinant xylanase for beechwood xylan was 16.6 (mg/mL) and 5.0 (mg/mL) while v_{max} was 2500 $\mu\text{mol}/(\text{min}\cdot\text{mg})$ and 4347 $\mu\text{mol}/(\text{min}\cdot\text{mg})$ respectively.

Molecular mass estimation and zymogram analysis

Deduce molecular mass of recombinant xylanase enzyme as per amino acid sequence was of 23324.6 Da which is in full agreement with results from the zymographic analysis (7). The molecular mass of recombinant xylanase from zymogram analysis is ~23 kDa (Fig.5).

Isoelectric Focusing (IEF)

Isoelectric focusing of the enzyme run under denaturing and non-denaturing conditions gave a band at pI value above 9. SDS-PAGE zymograms showed a single clearance zone coinciding with molecular mass 22 to 23 kDa (Fig. 5).

Enzymatic hydrolysis of xylan, xylooligosaccharides and wheat bran

The mechanism by which recombinant xylanase act on xylan was followed by allowing it to react with beechwood xylan as well as different xylooligosaccharide. Beechwood hydrolysis showed the release of xylooligosaccharides of higher molecular mass during initial stages of hydrolysis (T_1 -30 min, T_2 -1 h). At the end of 3 h (T_3), 6 h (T_4) and finally at 12 h (T_5) xylooligosaccharides were completely broken to xylobiose and xylose (Fig. 6).

To understand the hydrolysis pattern, more clearly xylooligosaccharides (X₂-X₅) were treated with XynBT21 at time interims (T₁-1 h, T₂-3 h). Observations after 1 h of incubation revealed that there was no breakdown of xylobiose (X₂), While xylotriose (X₃) was partially degraded to xylose (X₁) and xylobiose (X₂), xyloetraose (X₄) showed release of xylobiose (X₂), while xylopentaose (X₅) was partially hydrolyzed to give xylose (X₁), xylobiose (X₂), xylotriose (X₃). After 3 h incubation xylotriose, xyloetraose and xylopentaose showed xylose (X₁) and xylobiose (X₂) as hydrolysis product, while xylobiose was not at all cleaved (Fig. 7).

Hydrolysis products of wheat bran were also analyzed by TLC. By the end of 24 h, wheat bran released xylobiose and xylose when treated with partially purified native xylanase enzyme (Fig. 8).

Discussion

We report a potential xylanolytic *B. tequilensis* strain BT21 isolated from mangrove area. Genus *Bacillus* is known to produce industrially valuable xylanases, a number of them are reported (13,16,19,27-29). The catalytic domain of *xynBT21* (from *B. tequilensis*) showed high homology with xylanases belonging to family 11 glycosyl hydrolases as per the hydrophobic cluster analysis (27). There is a widespread occurrence of family 11 xylanases in *Bacillus* sp. (13, 27-29). The amino acid sequence of the *B. tequilensis* strain BT21 xylanase was compared with other reported xylanases in the NCBI database with the help of BLAST search program, it showed 99 % similarity with xylanase from *Bacillus subtilis* str. R5 (AB457186.1) which belongs to GH family 11, while it was 98 % similar to *Bacillus subtilis* str. 168 (AOA11206.1). It also had high amino acid sequence identity (98 %) with xylanase from *Bacillus circulans* (X07723.1). Sequence comparison of *B. tequilensis* with *B. subtilis* showed that only one amino acid L (Lysine) at positions 2 replaced F (Phenylalanine), while when compared with amino acids sequence of *B. circulans*, L, S (Serine), S and T (Threonine) replaced F, R (Arginine), I (Isoleucine) and S respectively (Fig. 2). With the difference in one single amino acid in xylanase gene of the *B. tequilensis* strain BT21 and *Bacillus subtilis*, enzymes showed diverse properties. Single amino acid mutations are accounted for influencing the structure of the protein complex because of changes in binding affinity of amino acid. More than half of monogenic diseases cause by single mutations, in which amino acid substitution cause changes in protein stability (30)Xylanase of *B. tequilensis* BT21 showed the optimum temperature of 60 °C while *B. subtilis* str. R5 xylanase had temperature optima of 40 °C, although *B. subtilis* str.168 xylanase showed an optimum temperature of 65 °C, the stability of XynBT21 was better compared to *B. subtilis* str.168 xylanase. *B. tequilensis* BT21 xylanase had pH optima of 6.0 and temperature optima of 60 °C. Xylanase from *Bacillus subtilis* cho40 reported previously by Khandeparker *et al.* (13) also showed the same pH and temperature optima but *Bacillus subtilis* xylanase showed drastic loss of activity after it reached optimum pH of 6.0 while *B.*

tequilensis BT21 xylanase remained active over a range of pH (from pH=6 to pH=8) without much change in activity. Also, xylanase had excellent pH stability such that instead of a drop in enzyme activity, activity increased with the incubation time. *Arthrobacter* sp. reported by Khandeparker and Bhosle (12) showed similar effect while studying the pH effect on xylanase enzyme. Xylanase enzyme from *Bacillus pumilus* SV-85S also showed the increase in residual activity of xylanase after 1 hour of incubation at alkaline pH (31). Bai *et al.* (32) reported high molecular mass xylanase (42.5 kDa) from *Alicyclobacillus* sp. retaining 80 % of enzyme activity after incubation at pH= 2.6 to 12.0 for 1 h at 37 °C. Xylanase isolated from *S. actuosus* reported to retain 80 % of its activity in the pH range of 5–8 when incubated for 30 min (33). The stability of a protein is controlled by pH mainly by altering the net charge on the protein. The protein stability is controlled by pH mainly by changing the net charge on the protein. Denaturation of many proteins takes place at extreme pH due to destabilizing repulsive interactions which are present between similar charges in the native protein. The behavior of a given protein at low or high pH depends mainly on stabilizing and destabilizing forces, which are sensitive to the environment (34). If we compare pH stability of earlier reported xylanases, pH stability of *B. tequilensis* BT21 is exceptionally superior.

Kinetic studies performed on *B. tequilensis* BT21 revealed that K_m of recombinant enzyme was much lower compared to native enzyme, which clearly shows that recombinant enzyme had better affinity than native enzyme. K_m values of recombinant xylanase enzyme reported by *B. tequilensis* BT21 were 3.3 and 5.0 mg/mL for birchwood xylan and beechwood xylan respectively, this indicates that birchwood xylan was more efficiently utilized compared to Beechwood xylan. K_m of the recombinant xylanase II by *Trichoderma reesei* reported was 13.8 mg/mL using birchwood xylan as the substrate, while *Bacillus alcalophilus* xylanase enzyme showed K_m of 4.9 and 4.5 mg/mL using beechwood xylan and birchwood xylan respectively.

IEF analysis and zymograms showed a sole and prominent activity band in the alkaline pH range indicating BT21 xylanase has a pI of 9.0 or higher with a molecular mass around 23 kDa. *Bacillus* sp. (NCL 87-6-10) produces two xylanases, A and C. Xylanase A with the molecular mass of 44 kDa and a pI of 5.3, while xylanase C with 25 kDa with a pI of 8.9 (35). Similarly, there are reports on *B. circulans* and *B. polymyxa* producing high and low molecular mass xylanases with acidic and basic pI respectively (20,35). Proteins with acidic isoelectric points degraded faster compared to those with neutral or basic isoelectric points (36), this might be because of extraordinary chemical properties of the acidic or basic polypeptides. As a result, xylanase from *B. tequilensis* BT21 which is having basic pI and high pH stability finds many advantages. Although there is an exception to this behaviour as there are some cases where an acidic protein with acidic pI degrades extremely slowly (37).

Xylanase from *B. tequilensis* BT21 predominantly releases xylobiose from the xylan backbone suggesting the endo-acting nature of XynBT21. At the first stage, the mixture of oligomers was also detected, this might be because of random hydrolysis of xylan. Hydrolysis products similar to XynBT21 have been reported, for XynB of *Thermotoga maritime*, XynC from *Clostridium stercorarium*, and an endoxylanase from *Bacillus* sp (38-40). Endoxylanase from *Streptomyces* sp. S27 have also been reported to release xylobiose (>75%), as a hydrolysis product of xylan by XynBS27 (41), while xylanase from *Streptomyces thermocyaneoviolaceus* is reported to release diverse xylooligosaccharides (X₁ to X₅) from birchwood xylan (42).

XynBT21 with an ability to hydrolyze polymeric substrates, beechwood xylan into dimer and monomer may find helpful application in saccharifying xylan-rich materials. A lignocellulosic material wheat bran which is a by-product of conventional wheat milling is available in large quantities (43) and a good source for xylooligosaccharide production. Enzymatic hydrolysis by xylanase from *B. tequilensis* BT21 on wheat bran showed a release of xylobiose and xylose. Koga *et al.* (44) reported uses of xylobiose in cosmetics, drugs or quasi-drugs, hair-care products and detergents. There are reports of xylobiose being used as prebiotics (45). Xylooligosaccharides deduced from wheat bran improve blood lipid metabolism and antioxidant status in rats which feed on the high-fat diet, suggesting that wheat bran xylooligosaccharides might be useful in shielding humans against high-fat diet induced by oxidative stress (46). Accordingly, streamlining the condition such as enzyme dose and pre-treatment strategies, large-scale generation of xylobiose from wheat bran can be initiated from xylanase enzyme of *B. tequilensis* BT21.

Conclusion

The recombinant xylanase is an “alkaline pI-low molecular mass enzyme”, which belongs to the group of family 11 glycanases. XynBT21 has excellent pH stability and also has the ability to produce xylobiose from agricultural residues. Looking into the growing interest of xylobiose in nutraceutical industries we look forward using this novel enzyme for industrial purpose.

Acknowledgements

Authors thank The Director, of the institute for his help and encouragements. We are obliged for the support provided during the entire work by Head BOD Dr. Ramaiah. Authors also thank Ram Murti Meena for his help in gene sequencing. Financial supports from the CSIR funds under PSC0206 are gratefully acknowledged. This is National Institute of Oceanography (NIO) contribution number -----.

References

1. Bhat MK, Hazlewood GP. Enzymology and other characteristics of cellulases and xylanases. In: by Bedford M, Partridge G, editors. Enzymes in farm animal nutrition. UK: CABI Publishing; 2001. pp.11–57.
2. Kormelink FJM, Searle-Van LMJF, Wood TM, Voragen AGJ. Purification and characterization of three endo-(14)- β -xylanases and one β -xylosidase from *Aspergillus awamori*. J Biotechnol. 1993;27:249–265.
[https://doi.org/10.1016/0168-1656\(93\)90089-6](https://doi.org/10.1016/0168-1656(93)90089-6)
3. Watanabe M, Inoue H, Inoue B, Yoshimi M, Fujii T, Ishikawa K. Xylanase (GH11) from *Acremonium cellulolyticus*: homologous expression and characterization. AMB Express. 2014;4:27.
<https://doi.org/10.1186/s13568-014-0027-x>
4. Sharma P, Bajaj BK. Production and partial characterization of alkali-tolerant xylanase from an alkalophilic *Streptomyces* sp CD3. J Sci Ind Res. 2005; 64:688-697.
5. Bocchini DA, Gomes E, DaSilva R. Xylanase Production by *Bacillus circulans* D1 Using Maltose as Carbon Source. Appl Biochem Biotech. 2008;146:29-37.
<https://doi.org/10.1007/s12010-007-8051-9>
6. Khasin A, Alchanati I, Shoham Y. Purification and characterization of a thermostable xylanase from *Bacillus stearothermophilus* T-6. Appl Environ Microbiol. 1993;59:1725-1730.
7. Winterhalter C, Heinrich P, Candussio A, Wich G, Liebl W. Identification of a novel cellulose-binding domain within the multi domain 120 kDa xylanase XynA of the hyperthermophilic bacterium *Thermotoga maritime*. Mol Microbiol. 1995;15:431-44.
<https://doi.org/10.1111/j.1365-2958.1995.tb02257.x>
8. Keen NT, Boyd MC, Henrissat B. Cloning and characterization of a xylanase gene from corn strains of *Erwinia chrysanthemi*. Mol Plant-Microb Interact. 1996;9:651–657.
9. Araki T, Hashikawa S, Morishita T. Cloning Sequencing and Expression in *Escherichia coli* of the New Gene Encoding β -1 3-Xylanase from a Marine Bacterium *Vibrio* sp Strain XY-214. App Environ Microbiol. 2000;66:1741-1743.
10. Crawford AC, Richardson RN, Mather PB. Comparative study of cellulase and xylanase activity in freshwater crayfish and marine prawns. Aquacult Res. 2005;36: 586-592.
<https://doi.org/10.1111/j.1365-2109.2005.01259.x>
11. Khandeparkar R, Bhosle N. Purification and characterization of thermoalkalophilic xylanase isolated from the *Enterobacter* sp. MTCC 5112. Res Microbiol. 2006a;157: 315-325.
<https://doi.org/10.1016/j.resmic.2005.12.001>

12. Khandeparkar R, Bhosle N. Isolation purification and characterization of the xylanase produced by *Arthrobacter* sp MTCC 5214 when grown in solid-state fermentation. *Enz Microbial Technol.* 2006b; 39:732-742.
<https://doi.org/10.1016/j.enzmictec.2005.12.008>
13. Khandeparker R, Verma P, Deobagkar DA. Novel halotolerant xylanase from marine isolate *Bacillus subtilis* cho40: gene cloning and sequencing. *New Biotech.* 2011;28:814-821.
<https://doi.org/10.1016/j.nbt.2011.08.001>
14. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997;25:3389–402.
<https://doi.org/10.1093/nar/25.17.3389>
15. Sambrook JEF, Fritsch MT. *Molecular Cloning: A Laboratory Manual.* New York: Cold Spring Harbor Press; 1989.
16. Artimo P, Jonnalagedda M, Arnold K, Baratin D, Csardi G, de Castro E, et al. ExPASy: SIB bioinformatics resource portal. *Nucleic Acids Res.* 2012;41:W597–W603.
<https://doi.org/10.1093/nar/gks400>.
17. Cornelis P, Digneffe C, Willemot K. Cloning and expression of a *Bacillus coagulans* amylase gene in *Escherichia coli*. *Mol Gen Gene.* 1982;186 (4): 507-511.
<https://doi.org/10.1007/BF00337957>
18. Miller GL. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal Chem.* 1959;31:426-428.
<https://doi.org/10.1021/ac60147a030>
19. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem.* 1985;150:76–85.
20. Esteban R, Villanueva JR, Villa TG. β -D-Xylanases of *Bacillus circulans* WL-12. *Can J Microbiol.* 1982;28:733-739.
<https://doi.org/10.1139/m82-112>
21. Nakamura S, Wakabayashi K, Nakai R, Aono R, Horikoshi K. Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp strain 41 M-1. *Appl Environ Microbiol.* 1993;59:2311-2316.
22. Lineweaver H, Burk D. The determination of enzyme dissociation constants. *J Am Chem Soc.* 1934;56:658-66.
<https://doi.org/10.1021/ja01318a036>

23. Takahashi Y, Kawabata H, Murakami S. Analysis of functional xylanases in xylan 27 degradation by *Aspergillus niger* e-1 and characterization of the gh family 10 xylanase 28 xynvii. SpringerPlus. 2013;2:1-11.
- <https://doi.org/10.1186/2193-1801-2-447>
24. Sneath PHA. In: WM Hensyl, editor. Bergey's Manual of Systematic Bacteriology ninth ed. Philadelphia PA: Williams & Wilkins; 1994.
25. Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. GenBank. Nucleic Acids Res. 2013;41 (Database issue):D36-D42.
- <https://doi.org/10.1093/nar/gku1216>
26. Gilkes NR, Henrissat B, Kilburn DG, Miller RCJ, Warren RAJ. Domains in microbial β -1,4-glycanases: Sequence conservation function and enzyme families. Microbiol Rev. 1991;55:303-315.
27. Gallardo O, Diaz P, Pastor FIJ. Cloning and Characterization of Xylanase A from the Strain *Bacillus* sp BP-7: Comparison with Alkaline pI-Low Molecular Weight Xylanases of Family 11. Curr Microbiol. 2004;48:276-279.
- <https://doi.org/10.1007/s00284-003-4196-0>
28. Baek LCU, Chung SG, Cho YR, Kim JHI. Cloning of a Family 11 Xylanase Gene from *Bacillus amyloliquefaciens* CH51 Isolated from *Cheonggukjang*. J Ind Microbiol. 2012;52:695-700.
- <https://doi.org/10.1007/s12088-012-0260-4>
29. Goswami GK, Krishnamohan M, Nain V, Aggarwal C, Ramesh B. Cloning and heterologous expression of cellulose free thermostable xylanase from *Bacillus brevis*. SpringerPlus. 2014;3:20.
- <https://doi.org/10.1186/2193-1801-3-20>
30. Teng S, Srivastava AK, Schwartz CE, Alexov E, Wang L. Structural assessment of the effects of Amino Acid Substitutions on protein stability and protein-protein interaction. Int J Comput Biol Drug Des. 2010;3:334-349.
31. Nagar S, Gupta VK, Kumar D, Kumar L, Kuhad, RC. Production and optimization of cellulase-free, alkali-stable xylanase by *Bacillus pumilus* SV-85S in submerged fermentation. J Ind Microbiol Biotech. 2010;37:71-83.
32. Bai Y, Wang J, Zhang Z, Yang P, Shi P, Luo H, Meng K, Huang H, Yao BA. New xylanase from thermoacidophilic *Alicyclobacillus* sp A4 with broad-range pH activity and pH stability. J Ind Microbiol Biotech. 2010;37: 187-194.
- <https://doi.org/10.1007/s10295-009-0662-4>
33. Wang SL, Yen YH, Shih IL, Chang AC, Chang WT, Wu WC, Chai YD Production of xylanases from rice bran by *Streptomyces actuosus* A-151. Enz Microbial Technol. 2003;33:917-925.

[https://doi.org/10.1016/S0141-0229\(03\)00246-1](https://doi.org/10.1016/S0141-0229(03)00246-1)

34. Prajapati S, Bhakuni V, Babu KR, Jain SK. Alkaline unfolding and salt-induced folding of bovine liver catalase at high pH. *Eur J Biochem.* 1998;255:178-184.

<https://doi.org/10.1046/j.1432-1327.1998.2550178.x>

35. Balakrishnan H, Kamal KB, Dutta-Choudhury M, Rele MV. Characterization of Alkaline Thermoactive Cellulase-free Xylanases from Alkalophilic *Bacillus* (NCL 87-6-10). *J Biochem Mol Biol Biophys.* 2002;6:325-334.

<https://doi.org/10.1080/1025814021000003229>

36. Wang CC, Touster OS. Turnover studies on proteins of rat liver lysosomes. *J Biolog Chem.* 1975 ;250:4896-902.

37. Yang RC, MacKenzie CR, Bilous D, Narang SA. Identification of two distinct *Bacillus circulans* xylanases by molecular cloning of the genes and expression in *Escherichia coli*. *Appl Environ Microbiol.* 1989;55:568-572.

38. Dice JF, Goldberg AL. Relationship between in vivo degradative rates and isoelectric points of proteins. *PNAS* 1975;72:10-12.

39. Jiang ZQ, Denga W, Zhua YP. The recombinant xylanase B of *Thermotoga maritima* is highly xylan specific and produces exclusively xylobiose from xylans a unique character for industrial applications. *J Mol Catal B Enz.* 2004;27:207-213.

<https://doi.org/10.1016/j.molcatb.2003.11.012>

40. Jeong KJ, Park IY, Kim MS, Kim SC. High level expression of an endoxylanase gene from bacillus sp. in *Bacillus subtilis* DB 104 for the production of xylobiose from xylan. *Appl Microbiol Biotechnol.* 1998;50:113–118.

<https://doi.org/10.1007/s002530051264>

41. Li N, Shi P, Yang P, Wang Y, Luo H, Bai Y, Zhou Z, Yao B. Cloning, expression, and characterization of a new *Streptomyces* sp. S27 xylanase for which xylobiose is the main hydrolysis product. *Appl biochem biotechnol.* 2009;159:521-31.

<https://doi.org/10.1007/s12010-008-8411-0>

42. Shin JH, Choi JH, Lee OS. Thermostable xylanase from *Streptomyces thermocyaneoviolaceus* for optimal production of xylooligosaccharides. *Biotechnol Bioprocess Eng.* 2009;14:391-9.

<https://doi.org/10.1007/s12257-008-0220-3>

43. Amrein TM, Granicher P, Arrigoni E. In vitro digestibility and colonic fermentability of aleurone isolated from wheat bran. *Lebensm Wiss Technol.* 2003;36:451–460.
[https://doi.org/10.1016/S0023-6438\(03\)00036-7](https://doi.org/10.1016/S0023-6438(03)00036-7)

44. Koga K, Kobayashi T, Fujikawa S, Sawada M. A xylobiose composition for reducing the roughness and dryness of human skin and hair. US Patent 5660838. 1997.

45. Achary A, Prapulla SG. Xylooligosaccharides (XOS) as an emerging prebiotic: microbial synthesis utilization structural characterization bioactive properties and applications. *Comp Rev Food Sci Food Safety*. 2011;10: 2-16.

<https://doi.org/10.1111/j.1541-4337.2010.00135.x>

46. Wang J, Cao Y, Wang C. Wheat bran xylooligosaccharides improve blood lipid metabolism and antioxidant status in rats fed a high-fat diet. *Carbohydr Poly*. 2011;86:1192-1197.

<https://doi.org/10.1016/j.carbpol.2011.06.014>

Table 1. Deduced amino acid composition of xylanase enzyme from *B. tequilensis* BT21

| Amino acid | w/% |
|-------------------|------------|
| Alanine | 6.6 |
| Arginine | 3.3 |
| Asparagine | 8.9 |
| Aspartic acid | 3.3 |
| Cystine | 0.0 |
| Glutamine | 2.3 |
| Glutamic acid | 0.9 |
| Glycine | 12.2 |
| Histidine | 0.9 |
| Isolucine | 3.3 |
| Leusine | 4.2 |
| Lysine | 3.8 |
| Methioninev | 1.9 |
| Phenylalanine | 3.3 |
| Proline | 2.8 |
| Serine | 10.2 |
| Threonine | 12.2 |
| Trptophan | 5.2 |
| Tryosine | 7.0 |
| Valine | 7.0 |
| Pyrrolysine | 0.0 |

Total number of negatively charged residues (Asparagine + Glutamic acid) is 9

Total number of positively charged residues (Arginine + Lysine) is 16

Legends of figures:

Fig. 1. Sequence alignment of the deduced amino acid sequence of xylanase gene from *B. tequilensis* BT21 (AHN14743.1) with other *Bacillus* sp. strains xylanases: 1. *Bacillus subtilis* str. 168 (AOA11206.1) 2. *Bacillus subtilis* strain R5 (AB457186.1) 3. *Bacillus circulans* (AAM08360.1). The Glu residues corresponding to our xylanase Glu-104 and Glu-196, essential to the catalytic activity are marked by (◆) and (▲) respectively.

Fig. 2. a) The effect of temperature and b) pH at pH=6 and temperature 60 °C respectively on xylanase activity of *B. tequilensis* BT21.

Fig. 3. Effect of pH on xylanase stability. The enzyme was diluted with buffers of varying pH values and incubated at room temperature over a period of 24 h. Residual activity was assayed at pH=6 and 60 °C. Buffer used was phosphate.

Fig. 4. Effect of temperature on xylanase stability. The pure enzyme was incubated in phosphate buffer (pH= 6) at (◆) 40 °C, (■) 50 °C and (▲) 60 °C for different intervals and residual activity was determined.

Fig. 5. a) SDS-PAGE analysis of recombinant xylanases XynBT21. Lane 1: molecular markers in kDa; lane 2: pure xylanase enzyme. Isoelectric focusing of b) pI standards c) enzyme sample and d) zymogram analysis of recombinant xylanases from *Bacillus tequilensis* BT12 grown with 1 % birchwood xylan by two-dimension gel electrophoresis. Proteins (10 µg) were first separated in 6 % native PAGE, the lane was stripped and then run in 8 % SDS-PAGE. Xylanase activity was examined by Congo red. Positions of XynBT21 in native PAGE are indicated.

Fig. 6. Time course of hydrolysis of the beechwood xylan by the recombinant xylanase. Beechwood xylan was incubated at 60 °C with 20 U/ml of XynBT21 and analysed by thin-layer chromatography. Lane 1 to lane 5 contains standards: xylose (X₁), xylobiose (X₂), xylotriose (X₃), xylotetraose (X₄), xylopentaose (X₅) respectively. Samples were taken at time 0 (lane 6), 30 min (lane 7), 60 min (lane 8), 1 h (lane 9), 3 h (lane 10), 12 h (lane 11). T₄-6 h, T₅-12 h). The reaction was carried out at 60 °C.

Fig. 7. Time course of hydrolysis of xylooligosaccharides by the recombinant xylanase. Xylooligosaccharides were incubated at 60 °C with 20 U/ml of XynBT21 at different time intervals (T₁= 1 h, T₂=3 h) and analysed by thin-layer chromatography. Lane 1 to lane 5 contains standards: xylose (X₁), xylobiose (X₂), xylotriose (X₃), xylotetraose (X₄), xylopentaose (X₅) respectively. Hydrolysis of xylobiose at time T₁ (lane 6) and T₂ (lane 7), xylotriose at time T₁ (lane 8) and T₂ (lane 9), xylotetraose at time T₁ (lane 10) and T₂ (lane 11), xylopentaose at time T₁ (lane 12) and T₂ (lane 13) was determined.

Fig. 8. Time course of hydrolysis of wheat bran by the recombinant xylanase. Wheat bran was incubated at 60 °C with 20 U/ml of XynBT21 for 24 h and analysed by thin-layer chromatography. Xylose (X₁) and xylobiose (X₂) are standards while C is control (sample without enzyme fraction) and T is test (sample with active enzyme).

| | | |
|----------------------------|--|-----|
| <i>B. tequilensis</i> BT21 | MLKF K KNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN | 60 |
| <i>B. subtilis</i> 168 | MFKF K KNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN | |
| <i>B. subtilis</i> R5 | MFKF K KNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN | |
| <i>B. circulans</i> | MFKF K KNFLVGLSAALMRIILFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN | |
| Query 61 | TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLI E YYVVD S WGT Y RPTG | 120 |
| <i>B. subtilis</i> 168 | TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLI E YYVVD S WGT Y RPTG | |
| <i>B. subtilis</i> R5 | TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLI E YYVVD S WGT Y RPTG | |
| <i>B. circulans</i> | TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGW P RSPLI E YYVVD S WGT Y RPTG | |
| | ◆ Glu 104 | |
| Query 121 | TYKGTVKSDGGTYDIYTTTRYNAPSIDGDR T TFTQYWSVRQTKRPTGSNATITFSNHVNA | 180 |
| <i>B. subtilis</i> 168 | TYKGTVKSDGGTYDIYTTTRYNAPSIDGDR T TFTQYWSVRQSKRPTGSNATITFSNHVNA | |
| <i>B. subtilis</i> R5 | TYKGTVKSDGGTYDIYTTTRYNAPSIDGDR T TFTQYWSVRQTKRPTGSNATITFSNHVNA | |
| <i>B. circulans</i> | TYKGTVKSDGGTYDIYTTTRYNAPSIDGDR T TFTQYWSVRQSKRPTGSNATITFTNHVNA | |
| Query 181 | WKSHGMNLGSNWAYQVMATE E GYQSSGSSNVTW | 213 |
| <i>B. subtilis</i> 168 | WKSHGMNLGSNWAYQVMATE E GYQSSGSSNVTW | |
| <i>B. subtilis</i> R5 | WKSHGMNLGSNWAYQVMATE E GYQSSGSSNVTW | |
| <i>B. circulans</i> | WKSHGMNLGSNWAYQVMATE E GYQSSGSSNVTW | |
| | ▲ Glu 197 | |

Fig. 1. Sequence alignment of the deduced amino acid sequence of xylanase gene from *B. tequilensis* BT21 (AHN14743.1) with other *Bacillus* sp. strains xylanases: 1. *Bacillus subtilis* str. 168 (AOA11206.1) 2. *Bacillus subtilis* strain R5 (AB457186.1) 3. *Bacillus circulans* (AAM08360.1). The Glu residues corresponding to our xylanase Glu-104 and Glu-196, essential to the catalytic activity are marked by (◆) and (▲) respectively.

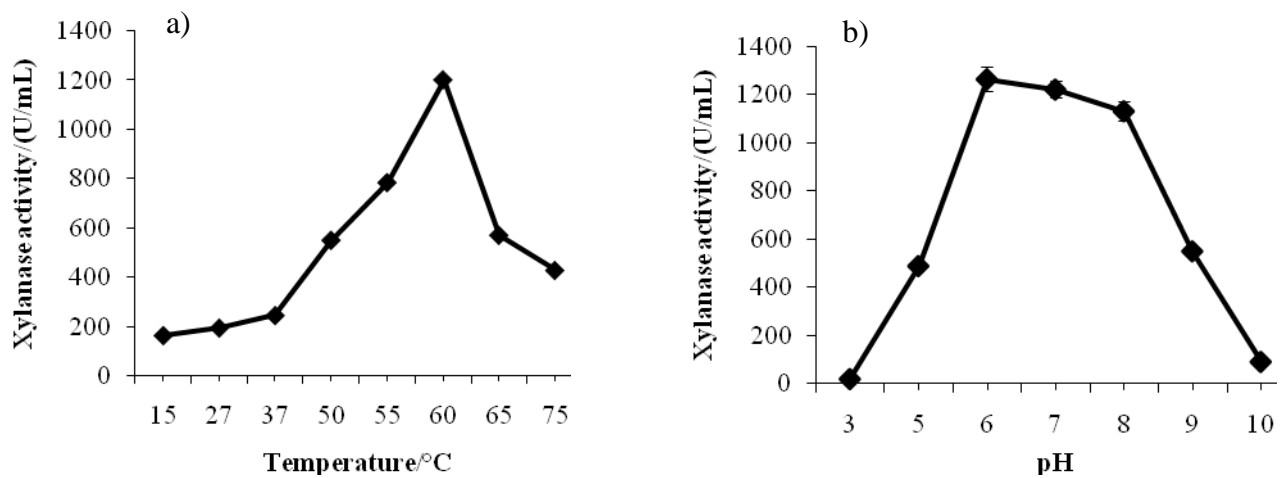


Fig. 2. a)The effect of temperature and b) pH at pH=6 and temperature 60 °C respectively on xylanase activity of *B. tequilensis* BT21.

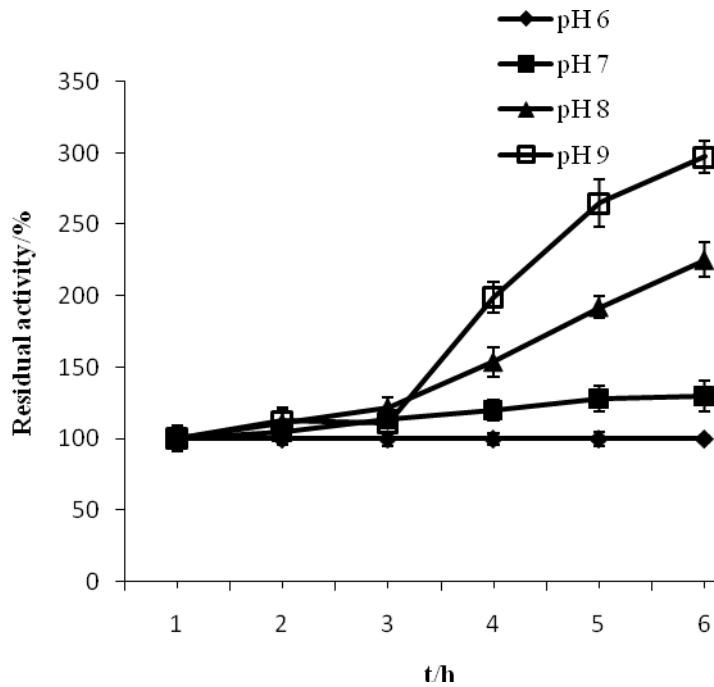


Fig. 3. Effect of pH on xylanase stability. The enzyme was diluted with buffers of varying pH values and incubated at room temperature over a period of 24 h. Residual activity was assayed at pH = 6 and 60 °C. Buffer used was phosphate.

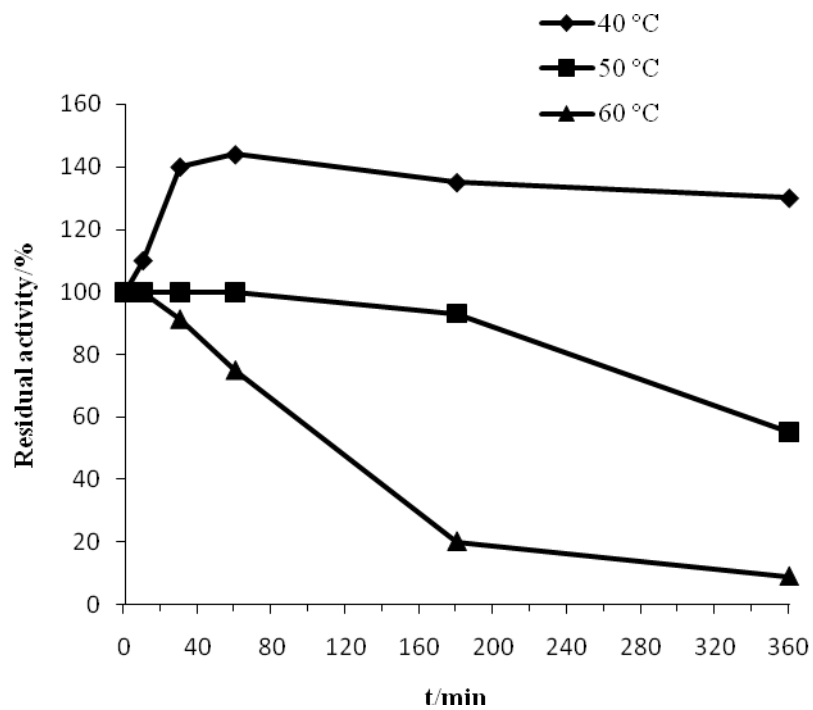


Fig.4. Effect of temperature on xylanase stability. The pure enzyme was incubated in phosphate buffer (pH= 6) at (♦) 40 °C, (■) 50 °C and (▲) 60 °C for different intervals and residual activity was determined.

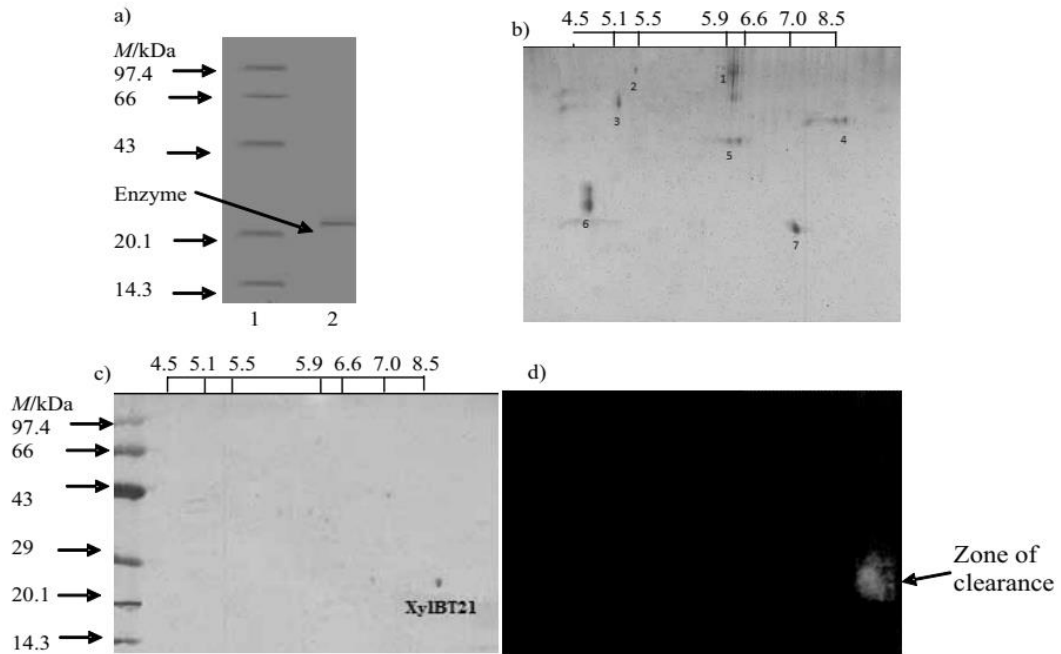


Fig.5. a) SDS-PAGE analysis of recombinant xylanases XynBT21. Lane 1: molecular markers in kDa; lane 2: pure xylanase enzyme. Isoelectric focusing of b) pI standards c) enzyme sample and d) zymogram analysis of recombinant xylanases from *Bacillus tequilensis* BT12 grown with 1 % birchwood xylan by two-dimension gel electrophoresis. Proteins (10 μ g) were first separated in 6 % native PAGE, the lane was stripped and then run in 8 % SDS-PAGE. Xylanase activity was examined by congo red. Positions of XynBT21 in native PAGE are indicated.

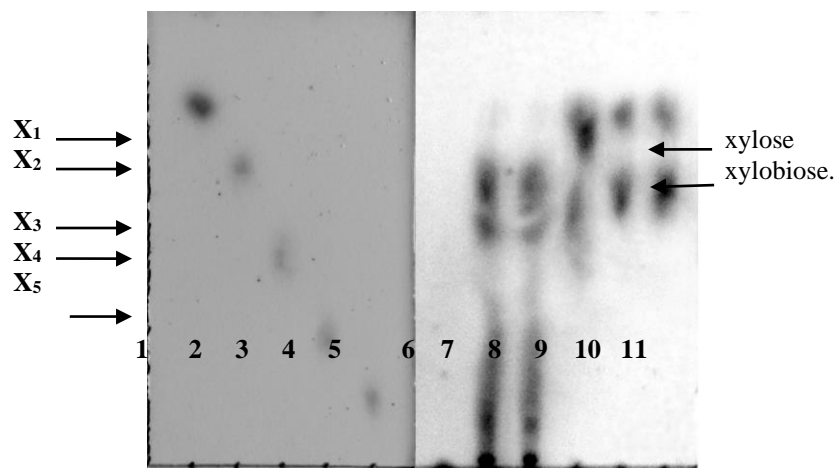


Fig.6. Time course of hydrolysis of the beechwood xylan by the recombinant xylanase. Beechwood xylan was incubated at 60 °C with 20 U/ml of XynBT21 and analysed by thin-layer chromatography. Lane 1 to lane 5 contains standards: xylose (X₁), xylobiose (X₂), xylotriose (X₃), xylotetraose (X₄), xylopentaose (X₅) respectively. Samples were taken at time 0 (lane 6), 30 min (lane 7), 60 min (lane 8), 1 h (lane 9), 3 h (lane 10), 12 h (lane 11). T₄-6 h, T₅-12 h). The reaction was carried out at 60 °C.

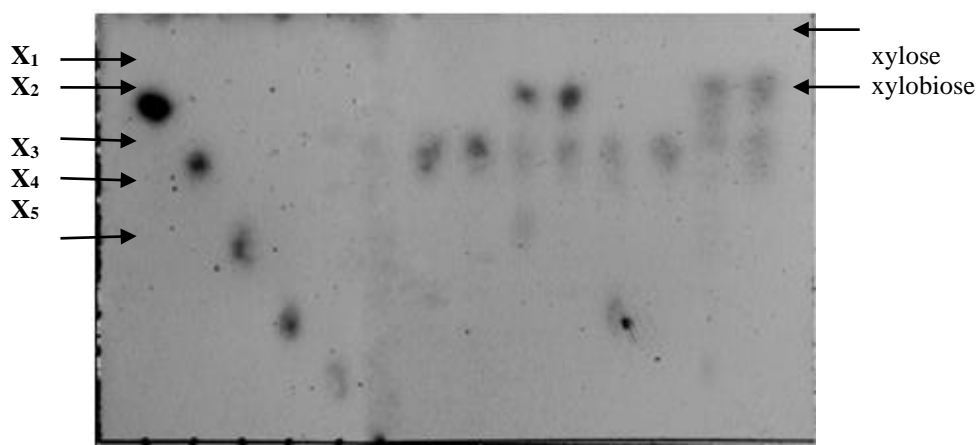


Fig.7. Time course of hydrolysis of xylooligosaccharides by the recombinant xylanase. Xylooligosaccharides were incubated at 60 °C with 20 U/ml of XynBT21 at different time intervals (T₁= 1 h, T₂=3 h) and analysed by thin-layer chromatography. Lane 1 to lane 5 contains standards: xylose (X₁), xylobiose (X₂), xylotriose (X₃), xylotetraose (X₄), xylopentaose (X₅) respectively. Hydrolysis of xylobiose at time T₁ (lane 6) and T₂ (lane 7), xylotriose at time T₁ (lane 8) and T₂ (lane 9), xylooligosaccharides at time T₁ (lane 10) and T₂ (lane 11), xylopentaose at time T₁ (lane 12) and T₂ (lane 13) was determined.

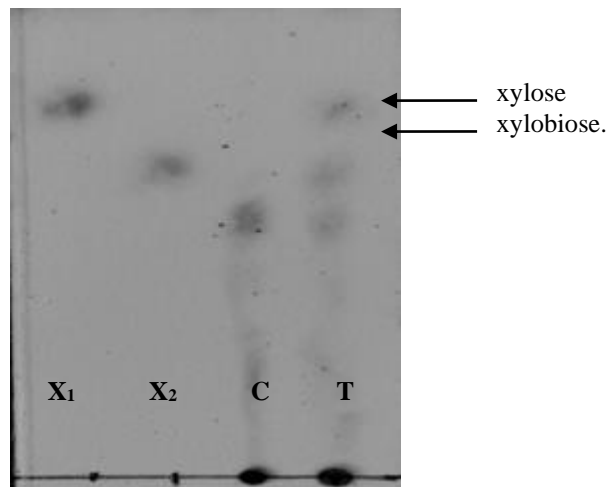


Fig.8. Time course of hydrolysis of wheat bran by the recombinant xylanase. Wheat bran was incubated at 60 °C with 20 U/ml of XynBT21 for 24 h and analysed by thin-layer chromatography. Xylose (X₁) and xylobiose (X₂) are standards while C is control (sample without enzyme fraction) and T is test (sample with active enzyme).