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ISSN 1330-9862
doi: 10.17113/ftb.55.02.17.4896
original scientific paper

Recombinant Xylanase from *Bacillus tequilensis* BT21: Biochemical Characterisation and Its Application in the Production of Xylobiose from Agricultural Residues

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Received: July 15, 2016
Accepted: February 28, 2017

Summary

Bacterial strain *Bacillus tequilensis* BT21 isolated from marine sediments was found to produce extracellular xylanase. The *xynBT21* gene encoding xylanase enzyme was cloned and expressed in *Escherichia coli*. The gene encoded a protein consisting of 213 amino acid residues with calculated molecular mass of 23.3 kDa. Purified recombinant xylanase had optimum activity at 60 °C and pH=6. The enzyme was highly stable in alkaline pH, at pH=7 it remained 100 % active for 24 h, while its activity increased at pH=8 and 9 during incubation. *B. tequilensis* BT21 xylanase had alkaline pI of 9.4 and belongs to glycosyl hydrolase family 11. The mode of action of XynBT21 on beechwood xylan and xylooligosaccharides was studied. It hydrolysed xylooligosaccharides and beechwood xylan yielding mainly xylobiose (X_2) with a small amount of xylose (X_1), 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 used for the release of xylobiose from wheat bran.

Key words: enzyme, xylanase, alkaline pI, characterisation, *Bacillus tequilensis*, xylobiose

Introduction

Xylan, the most abundant hemicellulose, consists 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 attached. 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*). Regarding the amino acid sequence similarities, xylanases are mostly classified into families 10 and 11 of the glycoside hydrolases. Family GH10 xylanases have high molecular mass (≥30 kDa) and low pI, while GH11 xylanases are normally smaller (≤20 kDa) and have a high pI (*3*).

Xylanases have a range of applications in textile, paper and pulp industries as well as in clarification of fruit juices, aroma production, animal feed, baking industry and production of ethanol. Industrial process conditions are harsh due to extremes of pH, temperature, inhibitors, *etc*. Sufficiently strong enzymes able to withstand such conditions are recommended for these processes. Most of the reported xylanases do not meet such criteria, therefore, enzymes that satisfy these requirements need to be found (*4,5*). The marine environment is highly complex and dynamic with high salinity, high pressure, low or high temperature and unique light conditions, which may explain the significant variations in the enzymes pro-

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duced by marine and terrestrial microorganism. Xylanases from the marine sources can have some remarkable qualities with respect to stability at high temperature and pH, which needs attention. There are a few studies reported on marine xylanases (*6–13*) and it is important to look into more potential xylanases from the marine sources. Recently, the interest in application of endoxylanases in the production of xylooligosaccharides from xylan sources is growing rapidly. Xylobiose stimulates the growth of human intestinal bifidobacteria, which are essential part of sound intestinal microflora. In this study, we report the characterisation of xylanase from a marine bacterium *Bacillus 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-dinitrosalicylic 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, St. Louis, MO, USA. Ethanol and thin layer chromatography plates were purchased from Merck, Darmstadt, Germany, while sulphuric and acetic acids, and sodium chloride from SD Fine-Chem Ltd., Mumbai, India.

Bacterial strains, culture conditions and vectors

Bacillus tequilensis was isolated from sediment samples from 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*). *Escherichia coli* strain JM109 was used as the host for DNA manipulation. The plasmid pCR® 2.1-TOPO® TA cloning vector (Invitrogen, Carlsbad, CA, USA) was employed for cloning and DNA sequencing.

PCR amplification of the 16S rRNA for strain identification

DNA was isolated from cells according to the method described by Khandeparker *et al*. (*13*). The 16S rRNA gene fragment was amplified by a PCR 96 well thermal cycler (Veriti 9902; Applied Biosystems, Foster City, CA, USA) with the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Gene sequence was determined using a Taq Dye Deoxy terminator cycle sequencing kit (PerkinElmer, Foster City, CA, USA) and were analysed with 373A automated DNA sequencer (model 3130xl; Applied Biosystems) (*13*). The obtained sequence was aligned with corresponding sequences of 16S rRNA from the database using BLAST (*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 a previously reported study (*13*). Xylanase gene was amplified using PCR and the amplified gene fragment was then ligated into TOPO TA cloning vector (Thermo Fisher Scientific, San Jose, CA, USA) and transformed into *E. coli* cells. Plasmid was extracted and the xylanase gene was sequenced.

Determination of amino acid composition of xylanase gene

Amino acid sequence of *B. tequilensis* BT21 xylanase gene 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 ProtParam server (*16*).

Enzyme production and fractionation

Recombinant enzyme production was enhanced by incorporating 1 mM isopropyl-β-ᴅ-thiogalactopyranoside (IPTG) in the growth medium of transformant cells and incubating at 140 rpm and 37 °C for 24 h. Cells were collected by centrifugation (4000×*g* for 10 min, 4 °C) using centrifuge 5810R (Eppendorf, Hamburg, Germany) and the pellet was rinsed twice in Tris-HCl (10 mM, pH=8). The pellet obtained after centrifugation was resuspended in 25 % sucrose solution. The suspension was shaken with 0.5 M EDTA for 10 min at room temperature. This was again centrifuged and the cell pellet was collected. The supernatant obtained in the above three steps was extracellular enzyme fraction (F1). Ice-cold water was added to the pellet and shaken vigorously for 10 min. After centrifugation at 5976×*g* for 10 mins, the supernatant was collected, forming 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, PR China). Intracellular enzyme fraction (F3) was collected as supernatant by removing the cell pellet by centrifugation (*17*). For native enzyme cell suspension of *B. tequilensis* BT21 grown on xylan, the medium 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 dialysed for 24 h (50 mM, pH=6). The crude enzyme was further purified using anion exchange resins followed by cation exchange resins DEAE and CM Sepharose fast flow (Sigma-Aldrich) columns respectively (*11*). The native enzyme was used only to study the kinetic properties, otherwise recombinant enzyme was used.

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 analysed by the method of bicinchoninic acid with bovine serum albumin as a standard (*19*).

Effect of temperature and pH on xylanase activity

Optimal temperature for xylanase activity was studied by analysing it at different temperatures ranging from 15–75 °C at pH=6, while the optimal pH was analysed by measuring its activity at 60 °C using different pH buffers (0.05 M): citrate buffer for pH=4–6, phosphate buffer for pH=6–8, and glycine-NaOH buffer for pH=8–11. Thermal stability of the enzyme was verified by incubating the enzyme at 40, 50 and 60 °C for 6 h (pH=6) in an incubator (model LSI-125R; Labtop Instrument, Thane, Maharashtra, India). The pH stability was studied using 100-fold diluted xylanase enzyme and respective buffers (pH=7–9) and incubating it for 24 h at 60 °C. Residual activity was analysed at regular intervals during incubation.

Isoelectric focusing

Isoelectric focusing (IEF) was performed using ReadyPrep® 2-D starter kit (Bio-Rad, Hercules, CA, USA). IPG strips (pH range 3–10, 11 cm; Electrophoresis GmbH SERVA, Heidelberg, Germany,) were rehydrated for 12–16 h in the enzyme sample (10 μg of protein) and mixed with rehydration buffer provided with the starter kit. PROTEAN® IEF chamber (Bio-Rad) was programmed at a constant temperature of 10 °C and 50 Vh/strip for 20 min at 250 V, with 2-hour linear increase to 4000 V and finally obtaining the value of 10 000 Vh. Strips were equilibrated on the rocker in equilibration buffers I and II (provided with the kit) for 10 min each, and 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 V and 4 °C for 110 min. Protein molecular mass standard (medium range) was used for electrophoresis in the 2nd dimension.

Molecular mass estimation and zymogram analysis

The xylanase molecular mass was assessed by SDS-PAGE (12 %) electrophoresis (*20*) using medium range molecular mass markers (14.3–97.4 kDa). Protein bands were stained with Coomassie Brilliant Blue stain. Zymogram was obtained 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 two washes contained 25 % isopropanol (to get rid of the SDS and renature the protein). The gel was further incubated at an optimum temperature of the enzyme for 60 min. The gel was stained with 0.1 % (by mass per volume) Congo Red dye for 30 min and then washed with 1.0 % (by mass per volume) NaCl until the remaining dye was washed off from the active band. Finally, the gel was flooded in 0.5 % acetic acid, thus changing the background of the gel to dark blue, and the activity bands were observed as clear colourless areas.

Kinetic determinations

Purified native and recombinant xylanases were incubated with birchwood and beechwood as substrates in 50 mM phosphate buffer at pH=7. The rate of xylan hydrolysis was determined at pH=9 and 60 °C. Various masses 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 Lineweaver and Burk method (*22*).

Hydrolysis studies

The partially purified periplasmic xylanase fraction was dissolved in phosphate buffer (pH=7) and used for hydrolysis study. Substrates and the enzyme were incubated at 60 °C. Samples were collected at intervals of 0, 1, 3, 6, 12 h. Xylooligosaccharides were also incubated with the enzyme and the samples were collected at different time intervals ranging from 1 to 3 h. Enzyme-substrate reaction was terminated by putting the reaction mixture into the boiling water for 5 min (*23*). The unused polysaccharide was precipitated using isopropanol and centrifuged. The supernatant was collected. Hydrolysis products were identified by thin layer chromatography (TLC) with the mixture of acetonitrile/ethyl acetate/2-propanol/water as a solvent system, and then spotted by spraying the TLC plates with orcinol spray, a mixture of ethanol, sulphuric acid and orcinol, and heating them for 5 min at 150 °C. Similarly, hydrolytic products obtained by enzymatic saccharification of wheat bran were analysed by TLC. Here xylanase enzyme was incubated with wheat bran (1 %) used as substrate at 60 °C. Samples were taken in triplicates in 24-hour intervals.

Results

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

Biochemical analysis of *B. tequilensis* BT21 isolate revealed that it belonged to the Gram-positive group, its cells are short motile rods, 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 GenBank (*25*), and the results showed 99 % identity with *Bacillus tequilensis* (GenBank accession no. KF054870). Thus, the strain BT21 identified as a 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 (Invitrogen). The recombinant plasmid DNA was introduced into *Escherichia coli* JM109. A complete nucleotide sequence was acquired (GenBank accession no. KF797799), with a 639-bp open reading frame encoding a protein of 23 324.6 Da having 213 amino acid residues with pI=9.44. The gene sequence is 99 % similar to that of xylanase gene sequence of *B. subtilis* R5 (GenBank accession no. AB 457186.1). The catalytic domain of *xynBT21* (from *B. tequilensis*) showed good homology with xylanase that is classified into family 11 glycosyl hydrolases according to hydrophobic cluster analysis (*26*).

Amino acid composition of recombinant xylanase

Recombinant xylanase from *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 pyrrolysine (Table 1). Highly conserved Glu104 and Glu196, which are crucial for the catalytic activity of family 11 glycosyl

Table 1. Amino acid composition of xylanase from *Bacillus 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
Methionine	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 (aspargine+glutamic acid) is 9
Total number of positively charged residues (arginine+lysine) is 16

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 was lower (asparagine+glutamic acid=9), than of positively charged residues (arginine+lysine= 16). The enzyme had 41 % polar amino acids and 59 % nonpolar (hydrophilic) amino acids.

Characterisation of xylanase from B. tequilensis BT21

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

Kinetic parameters

The K_m and v_{max} values of native and recombinant xylanase were obtained from Lineweaver-Burke plot. The K_m of native and recombinant xylanases on birchwood xylan was 11.1 and 3.3 g/L, while v_{max} was 2222 and 3125 μmol/(mg·min) respectively. The K_m of native and recombinant xylanases on beechwood xylan was 16.6 and 5.0 g/L while v_{max} was 2500 and 4347 μmol/(mg·min) respectively.

Molecular mass estimation, zymogram analysis and isoelectric focusing

Molecular mass of recombinant xylanase enzyme deduced from amino acid sequence was 23 324.6 Da, which is in full agreement with the results of the zymographic analysis (*7*). The molecular mass of recombinant xylanase obtained by zymogram analysis is approx. 23 kDa (Fig. 5). 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 a molecular mass of 22 to 23 kDa (Fig. 5).

```
B. tequilensis BT21   MLKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN   60
B. subtilis 168       MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN
B. subtilis R5        MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN
B. circulans          MFKFKKNFLVGLSAALMRIILFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN

Query 61              TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG   120
B. subtilis 168       TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG
B. subtilis R5        TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG
B. circulans          TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWPRSPLIEYYVVDSWGTYRPTG
                                                                ♦ Glu 104
Query 121             TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQTKRPTGSNATITFSNHVNA   180
B. subtilis 168       TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFSNHVNA
B. subtilis R5        TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQTKRPTGSNATITFSNHVNA
B. circulans          TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFTNHVNA

Query 181             WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW   213
B. subtilis 168       WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW
B. subtilis R5        WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW
B. circulans          WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW
                                          Glu 197
```

Fig. 1. Alignment of the amino acid sequence of xylanase gene from *Bacillus tequilensis* BT21 (AHN14743.1) with xylanases from other *Bacillus* sp. strains: *B. subtilis* 168 (AOA11206.1), *B. subtilis* R5 (AB457186.1) and *B. circulans* (AAM08360.1). The Glu residues corresponding to our xylanase Glu-104 and Glu-196, essential to the catalytic activity, are marked with diamond and triangle, respectively

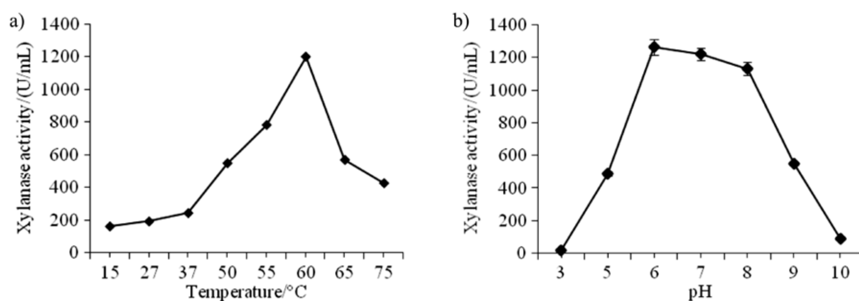

Fig. 2. The effect of: a) temperature (at pH=6), and b) pH (at 60 °C) on the activity of xylanase from *Bacillus tequilensis* BT21

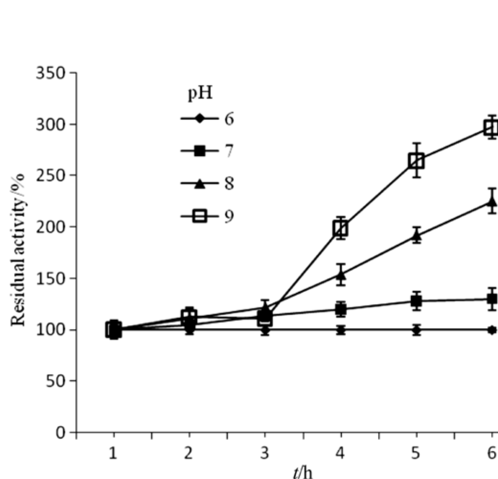

Fig. 3. Effect of pH on xylanase stability. The enzyme was diluted with phosphate buffers of various pH values and incubated at room temperature for t=24 h. Residual activity was essayed at pH=6 and 60 °C

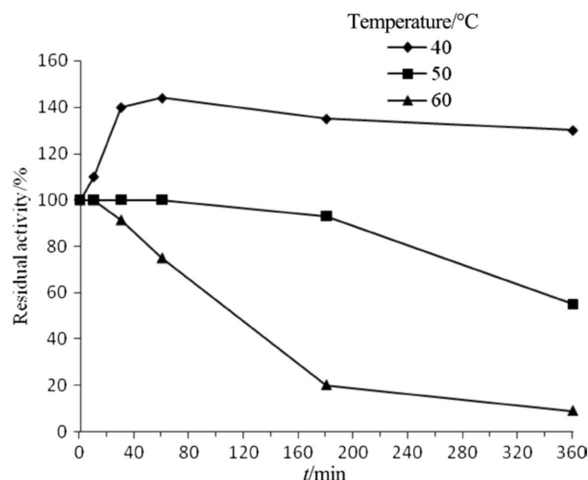

Fig. 4. Effect of temperature on xylanase stability. The pure enzyme was incubated in phosphate buffer (pH=6) at 40, 50 and 60 °C for different intervals and residual activity was determined

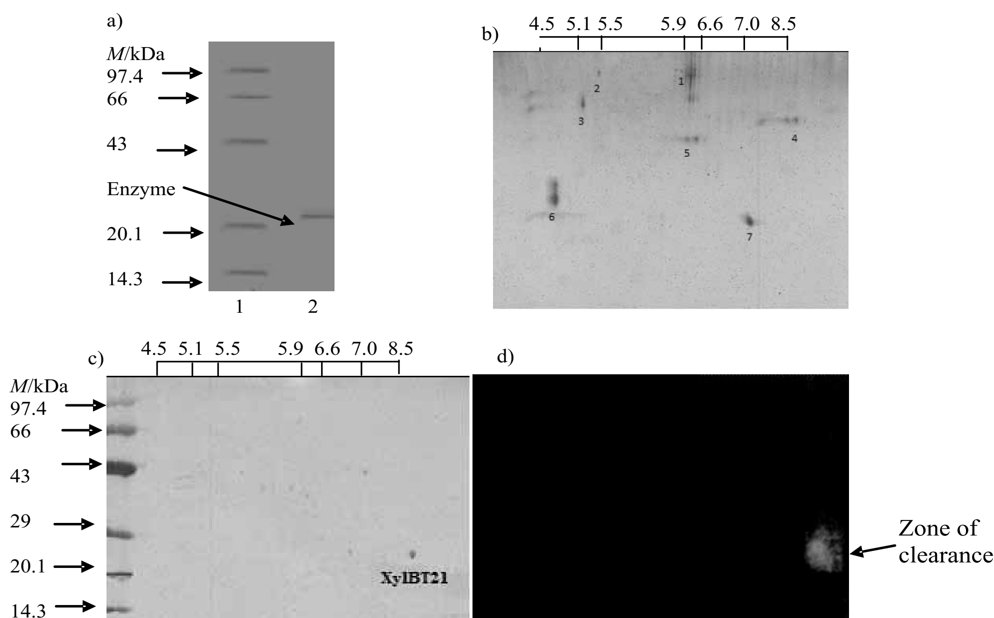

Fig. 5. SDS-PAGE analysis of recombinant xylanases XynBT21: a) lane 1: molecular markers in kDa, lane 2: pure xylanase enzyme. Isoelectric focusing of: b) pI standards, c) enzyme sample, and d) zymogram analysis of recombinant xylanases from *Bacillus tequilensis* BT12 grown on 1 % birchwood xylan and separated by two-dimensional 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. The position of XynBT21 in native PAGE is indicated

Enzymatic hydrolysis of xylan, xylooligosaccharides and wheat bran

The mechanism by which recombinant xylanase acts on xylan was followed by allowing it to react with beechwood xylan and different xylooligosaccharides. Beechwood hydrolysis released the xylooligosaccharides of higher molecular mass during initial stages (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).

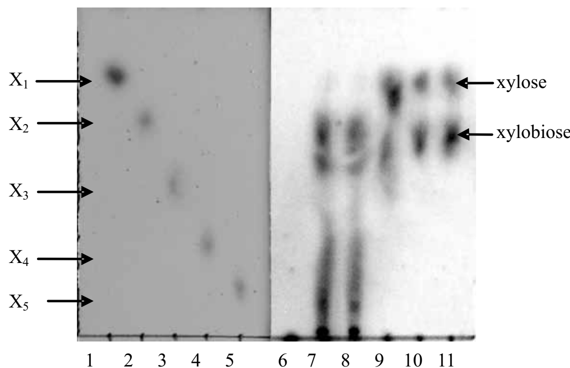

Fig. 6. Time course of the hydrolysis of beechwood xylan by the recombinant xylanase. Beechwood xylan was incubated at 60 °C with 20 U/mL of XynBT21 and analysed by thin-layer chromatography. Lanes 1 to 5 contain standards: xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4) and xylopentaose (X_5), respectively. Lanes 6 to 11: samples were taken at time 0, 0.5, 1, 1, 3, 12 h. The reaction was carried out at 60 °C

To understand the hydrolysis pattern more clearly, xylooligosaccharides (X_2–X_5) were treated with XynBT21 at time interims (t_1=1 h, t_2=3 h). Observations after 1 h of incubation revealed that there was no breakdown of xylobiose (X_2), while xylotriose (X_3) was partially degraded to xylose (X_1) and xylobiose (X_2), xylotetraose (X_4) released xylobiose (X_2), while xylopentaose (X_5) was partially hydrolysed to give xylose (X_1), xylobiose (X_2) and xylotriose (X_3). After 3 h of incubation, xylotriose, xylotetraose and xylopentaose produced xylose (X_1) and xylobiose (X_2), while xylobiose was not cleaved at all (Fig. 7).

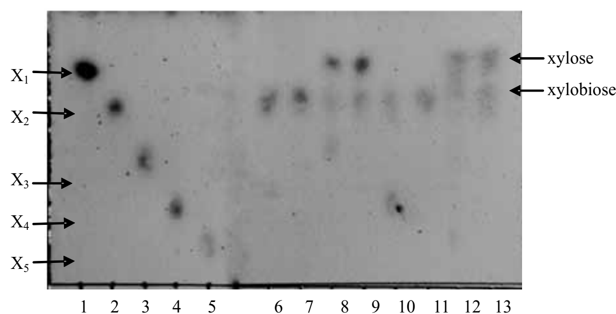

Fig. 7. Time course of xylooligosaccharide hydrolysis by the recombinant xylanase. Xylooligosaccharides were incubated at 60 °C with 20 U/mL of XynBT21 at different time intervals (t_1=1 h, t_2=3 h) and analysed by thin-layer chromatography. Lanes 1 to 5 contain standards: xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4) and xylopentaose (X_5), respectively. Hydrolysis of xylobiose at t_1 (lane 6) and t_2 (lane 7), xylotriose at t_1 (lane 8) and t_2 (lane 9), xylotetrose at t_1 (lane 10) and t_2 (lane 11), xylopentose at t_1 (lane 12) and t_2 (lane 13) was determined

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

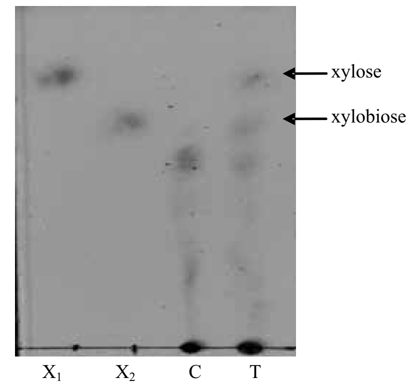

Fig. 8. Time course of wheat bran hydrolysis by the recombinant xylanase. Wheat bran was incubated at 60 °C with 20 U/mL of XynBT21 for 24 h and analysed by thin-layer chromatography. Xylose (X_1) and xylobiose (X_2) are standards, while C is control (sample without enzyme fraction) and T is the test sample with active enzyme

Discussion

A potential xylanolytic *B. tequilensis* strain BT21 isolated from mangrove area is reported. Genus *Bacillus* is known to produce industrially valuable xylanases, a number of which are reported (*13,16,19,27–29*). The catalytic domain of *xynBT21* (from *B. tequilensis*) showed high homology with xylanases belonging to glycosyl hydrolase family 11 according to the hydrophobic cluster analysis (*27*). There is a widespread occurrence of xylanase family 11 in *Bacillus* sp. (*13,27–29*). The amino acid sequence of the *B. tequilensis* BT21 xylanase was compared with other reported xylanases in the NCBI database with the help of BLAST search program (*14*). It showed 99 % similarity with xylanase from *Bacillus subtilis* R5 (AB457186.1), which belongs to GH family 11, but it was 98 % similar to *Bacillus subtilis* 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 position 2 replaced F (phenylalanine), while when compared with amino acid 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 influence the structure of the protein complex because of the changes in binding affinity of the amino acid. More than half of monogenic diseases are caused by single mutations, in which amino acid substitution causes changes in protein stability (*30*). The optimum temperature for *B. tequilensis* BT21 xylanase activity was 60 °C while for *B. subtilis* R5 xylanase it was 40 °C. Although that of *B. subti-*

lis 168 xylanase was 65 °C, the stability of XynBT21 was better than of *B. subtilis* 168 xylanase. *B. tequilensis* BT21 xylanase had pH optimum of 6.0 and temperature optimum of 60 °C. Xylanase from *Bacillus subtilis* cho40 reported previously by Khandeparker *et al.* (*13*) also had the same pH and temperature optima, but *B. subtilis* cho40 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 8) and its activity increased with the incubation time. *Arthrobacter* sp. reported by Khandeparker and Bhosle (*12*) showed similar properties when studying the pH effect on xylanase enzyme. The residual activity of xylanase from *Bacillus pumilus* SV-85S also increased after 1 h of incubation at alkaline pH (*31*). Bai *et al.* (*32*) reported that high molecular mass xylanase (42.5 kDa) from *Alicyclobacillu*s sp. retained 80 % of enzyme activity after incubation at pH=2.6 to 12.0 for 1 h at 37 °C. Xylanase isolated from *Streptomyces actuosus* was reported to retain 80 % of its activity in the pH range of 5–8 when incubated for 30 min (*33*). The protein stability is controlled by the pH mainly by changing the net charge of the protein. Denaturation of many proteins takes place at extreme pH due to destabilising repulsive interactions that are present between similar charges in the native protein. The behaviour of a given protein at low or high pH depends mainly on stabilising and destabilising forces, which are sensitive to the environment (*34*). If we compare the 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 the recombinant enzyme was much lower than of the native enzyme, which clearly shows that the recombinant enzyme had better affinity than the native enzyme. K_m values of recombinant xylanase enzyme reported in *B. tequilensis* BT21 were 3.3 and 5.0 g/L on birchwood xylan and beechwood xylan, respectively, which indicates that birchwood xylan was more efficiently utilised than beechwood xylan. K_m of the recombinant xylanase II by *Trichoderma reesei* was 13.8 g/L using birchwood xylan as the substrate, while *Bacillus alcalophilus* xylanase enzyme showed K_m of 4.9 and 4.5 g/L using beechwood xylan and birchwood xylan, respectively.

IEF analysis and zymograms showed a sole and prominent activity band in the alkaline pH range indicating that BT21 xylanase had a pI of 9.0 or higher with a molecular mass of around 23 kDa. *Bacillus* sp. (NCL 87-6-10) produces two xylanases, A, with the molecular mass of 44 kDa and pI=5.3, and C, with 25 kDa and pI=8.9 (*35*). Similarly, there are reports that *B. circulans* and *B. polymyxa* produce high- and low-molecular mass xylanases with acidic and basic pI respectively (*20,35*). Proteins with acidic isoelectric points degraded faster than those with neutral or basic isoelectric points (*36*), which might be because of chemical properties of the acidic or basic polypeptides. As a result, xylanase from *B. tequilensis* BT21, with basic pI and high pH stability, has many advantages, although there is an exception to this behaviour as when 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. In the first stage, the mixture of oligomers was also detected, which might be because of random hydrolysis of xylan. Hydrolysis products similar to XynBT21 have been reported such as XynB from *Thermotoga maritima*, XynC from *Clostridium stercorarium*, and an endoxylanase from *Bacillus* sp. (*38–40*). Endoxylanase from *Streptomyces* sp. S27 has 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 (X_1 to X_5) from birchwood xylan (*42*).

XynBT21 with an ability to hydrolyse polymeric substrates such as 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 of raw material for xylooligosaccharide production. Enzymatic hydrolysis by xylanase from *B. tequilensis* BT21 on wheat bran released 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 used as prebiotics (*45*). Xylooligosaccharides obtained on wheat bran improve blood lipid metabolism and antioxidant status in rats that feed on the high-fat diet, suggesting that wheat bran xylooligosaccharides might be useful in protecting humans against high-fat diet-induced oxidative stress (*46*). Accordingly, streamlining the conditions such as enzyme dose and pre-treatment strategies, large-scale production of xylobiose from wheat bran can be achieved with *B. tequilensis* BT21 xylanase.

Conclusion

The recombinant xylanase from *Bacillus tequilensis* BT21 is a low-molecular-mass enzyme with alkaline pI, that belongs to the group of glycanase family 11. XynBT21 has excellent pH stability and also the ability to produce xylobiose from agricultural residues. With the growing interest in the application of xylobiose in nutraceutical industries, we look forward to using this novel enzyme for industrial purposes.

Acknowledgements

Authors thank the Director of the Institute for his help and encouragements. We are grateful for the support provided during the entire work by the head of Biological Oceanography Department, Dr. Ramaiah. Authors also thank Ram Murti Meena for his help in gene sequencing. Financial support from the CSIR fund under no. PSC0206 is gratefully acknowledged. This is National Institute of Oceanography (NIO) contribution number 7745.

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