

A novel halotolerant xylanase from marine isolate *Bacillus subtilis* cho40: gene cloning and sequencing

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ABSTRACT

Although several xylanases have been studied, only few xylanases from marine micro organisms have been reported. We report here a novel halotolerant xylanase from marine bacterium *Bacillus subtilis* cho40 isolated from Chorao island of mandovi estuary Goa, India. Extracellular xylanase was produced by using agricultural residue such as wheat bran as carbon source under solid-state fermentation (SSF). The optimal pH and temperature of xylanase were reported to be 6.0 and 60°C respectively. Xyn40 was highly salt-tolerant, and showed highest activity at 0.5 M NaCl. Xylanase activity was greatly induced (140%) when preincubated with 0.5M NaCl for 4h. The xylanase gene, xyn40, from marine bacterium *Bacillus subtilis* cho40 was cloned, and expressed in *Escherichia coli*. The xylanase gene was 645 bp long and had a 215 amino acid ORF protein with a molecular mass of 22.9 kDa. It had all features of xylanase enzyme and showed homology to xylanases reported from *B. subtilis*. It differs from the earlier reported xylanase sequences by the presence of more serine residues compared to threonine and also by the presence of polar (hydrophilic) amino acids in higher abundance (61%) than non polar amino acids (39%). The novel xylanase, reported in this study is a halotolerant enzyme from marine isolate and can play a very important role in bioethanol production from marine seaweeds..

Keywords: Xylanase, Halotolerant, Marine, Estuarine, Hemicellulose

Introduction

Xylans are heteropolysaccharides with a backbone chain of 1,4-linked β -D xylopyranose units, and are the main component of hemicelluloses in plant cell walls. Xylanase have immense commercial potential as hemicelluloses are the second most abundant renewable resource, only exceeded by cellulose. Biodegradation of these xylans involves the action of several hydrolytic enzymes including xylanases. Based on the amino acid sequence of catalytic domain, xylanases are mainly categorized into two glycoside hydrolase (GH) families, 10, and 11. However, enzymes with xylanase activity are also found in GH families 5, 7, 8, 16, 26, 43, 52, and 62 [1]. Xylanases within the same GH family generally share a similar three-dimensional structure and a similar catalytic mechanism [2].

The last decade has seen a rise in studies aiming at the isolation of thermophilic and alkalophilic xylanases applicable to pulp and paper processes [3]. Apart from their use in the pulp and paper industry, xylanases have several other applications in the food and feed industries [4-8]. In view of this, there is a need to search for new sources of xylanases, especially with extremophilic properties. Marine xylanases can have some unique properties However there are very few studies on marine xylanases [9-15]. Especially, very little information is available on halophilic and halotolerant xylanases [16-18] while there is only one report on halophilic marine xylanases [19].

A marine isolate identified as *Bacillus subtilis* cho40 produced large amounts of thermophilic and halotolerant enzyme. In this study, our aim was to study the properties of this novel xylanase and to clone and express this xylanase gene in *E. coli* to characterise the molecular sequence. This would not only provide the information about the amino acid sequence of the protein (enzyme) but also help in correlating the structure function relationship of this novel salt tolerant enzyme

Materials and methods

Culture and growth conditions

Bacillus subtilis was isolated from a sediment sample collected from Chorao island of Mandovi estuary, Goa, West coast of India. The culture was grown at room temperature in 15 ml basal salt solution (BSS) containing 5 g of wheat bran as substrate (substrate to moisture ratio 1:3) for 4 days. It was cultivated in a medium containing Basal Salt Solution (BSS). The composition of the BSS medium (w/v) was as follows: NaCl, 30.00 g; KCL, 0.75 g; MgSO₄, 7.00 g; NH₄Cl, 1.00 g; K₂HPO₄ (10%) 7.00 ml; KH₂PO₄(10%) 3.00 ml; trace metal solution , 1.00 ml; distilled water 1000 ml; The pH of the medium was adjusted by using 1N NaOH. Trace metal solution has the following composition: H₃B₃O₃ , 2.85 g; MnCl₂ .7 H₂O, 1.80 g; FeSO₄.7H₂O, 2.49 g; Na -3Tartarate, 1.77 g; CuCl₂, 0.03 g; ZnCl₂ , 0.02 g; CoCl₂, 0.04 g; Na₂MoO₄.2H₂O .0.02 g ; Distilled water, 1000 ml [15].

Preparation of enzyme

Ehrlenmeyer flask (500 ml) containing 10 g of wheat bran and 30 ml of BSS (substrate to moisture ratio 1:3), was sterilized, cooled and inoculated with pre-grown culture of the isolate. The flasks were incubated at room temperature. The culture was harvested in the stationary growth phase, i.e. after 4 days. The content of the flask was suspended in 100 ml ml of 50mM Na-phosphate buffer (pH 6) vortexed thoroughly and centrifuged (10,000 rpm for 10 min, 4 °C). The enzyme was precipitated from the culture supernatant by adding ammonium sulfate to 80% saturation. This was left overnight and the precipitate collected by centrifugation at 10,000 rpm for 10 min. The precipitate obtained was dissolved in phosphate buffer (50 mM, pH 6.0) and dialyzed against the same buffer for 24 h. Dialysis was carried out using cellulose tubing (molecular weight cut-off 13,000 kDa). The crude enzyme extract was further purified by loading onto a anion exchange DEAE Sepharose FF (Sigma–Aldrich Co., USA) column followed by cation exchange CM-Sepharose FF (Sigma–Aldrich Co.) column as per the method described by Khandeparker and Bhosle [14].

2.3. Enzyme assay

Xylanase activity was examined by the 3,5-dinitrosalicylic acid (DNS) method [20] by measuring the amount of reducing sugars liberated from birch-wood xylan which was solubilized in 50 mM sodium phosphate buffer at pH 6.0 The reaction mixture was incubated for 10 min at 60 °C. The incubation was followed by the addition of DNS solution. The samples were boiled for 10 min, Roschell salt was added for colour stabilization, cooled, and the absorbance was measured at 510 nm. The xylanase activity was calculated from the calibration curve constructed by using D-xylose as a standard. Two control experiments included in the study were: i) reaction mixture without enzyme and ii) reaction mixture without birch-wood xylan.

Bacterial DNA extraction

Bacterial genomic DNA was isolated using a modification of existing techniques [21]. Briefly the technique entailed the following: 5ml of mid- to late log phase bacteria were collected by centrifugation and resuspended in a small volume of lysis buffer (0.05M Tris HCL pH 8.0, 0.01M EDTA). SDS was added to this solution at a final concentration of 0.5%, mixed gently and incubated for five minutes at room temperature. The solution was sequentially extracted with equal volumes of TE (pH 8.0) saturated phenol, phenol-chloroform-isoamyl alcohol and chloroform. DNA was precipitated by adding 1/10 volume of 1M NaCl and 2.2 volumes of 100% ethanol and incubated for one hr. at 20 °C. The precipitated DNA, after centrifugation was rinsed three times with 70% ethanol, dried and resuspended in sterile distilled water.

PCR amplification of the 16S rDNA and sequence determination

The 16S rDNA gene fragments were amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-30) [20]. The amplification was carried out in 25 µl of reaction mixture containing DNA template, PCR buffer, dNTP (10 mM), MgCl₂ (25 mM), the primers 27F (10 ppm) and 1492R (10 ppm) respectively, Taq polymerase (Sigma) (2U) and autoclaved Milli-Q water (Millipore). The amplification was carried out for 35 cycles of 94 °C, for 45 s, 60 °C for 45 s and 72 °C for 45 s, 72 °C for 7 min with initial 05-min denaturation at 94 °C using a PCR-Express thermal cycler (Hybaid). The DNA sequences of the PCR products were determined using a Taq Dye Deoxy terminator cycle sequence kit (Perkin–Elmer) using the protocol recommended by the supplier. Sequencing reaction products were analyzed with a model 373A automated DNA sequencer (Applied Biosystems). Databases (GenBank) were searched for sequences similarity analysis of the 16S rDNA sequence obtained.

Primer design, amplification and cloning

According to the published sequence of xylanase gene from *B. subtilis* by Huang et.al. [23], oligonucleotide primers were designed and synthesized to check for the amplification of xylanase gene from genomic DNA of *B. subtilis* cho40. The PCR product was then subjected to cloning in pGMT vector.

Primers derived from article [24] were as follows:

xynF: 50> ACGAATTCCATGTTTAAGTTTAAAAGAATTTCTTAGTT
<30 (Positions 1 to 30 of the xylanase gene).

xynR: 50> GAGGATCCTTACCACACTGTTACGTTAGAACTTCCACT
<30 (Positions 613 to 642, antisense).

The PCR using specific primers for xylanase gene resulted in the amplification of DNA fragment around 650 bp .The amplification was carried out for 35 cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 45 s, 72 °C for 7 min with initial 05-min denaturation at 94 °C using a PCR-Express thermal cycler (Hybaid). The PCR amplified DNA was then ligated in pGMT vector and subjected to transformation in *E.Coli*. JM 109 cells. For screening of recombinants, each clone was picked from LB agar plate containing 50µg/ml of ampicillin, 0.1 M isopropyl-β-D-thiogalactopyranoside (IPTG) and 20 mg/ml of 5-bromo-4-chloro-3-indoly- β -D-galactopyranoside (XGal) .

Electrophoretic analyses

PCR amplified xylanase gene of *B. subtilis* cho40 was loaded onto 1% agarose gel electrophoresis followed by staining with ethidium bromide and visualizing under short wave ultra violet light.

Plate assay for screening recombinants

The recombinants containing desired PCR product inserts were screened for the expression of xylanase activity by the Congo-red assay described by Teather and Wood [25]. The recombinant *E. coli* clones containing the recombinants were overlaid with 0.5% birchxylan dissolved in solid LB and then incubated at 37 °C overnight. Following incubation, the plates were stained with 1% Congo-red for 30 min and destained with 1 M NaCl. Positive clones were identified by a zone of clearance around xylanase-expressing clones truncated.

Effect of temperature and pH on xylanase activity

The optimal temperature for the xylanase enzyme was obtained by assaying the enzyme activity at different temperatures (28–110 °C). At each temperature, the enzyme along with the substrate (2.5 mg) was incubated. The optimal pH for the xylanase enzyme was determined at various pHs. The pH range used varied from 4 to 11. Three different buffers (0.05 M) were used. Citrate buffer (0.05 M) was used for pH 4–6. Phosphate buffer (0.05 M) was used for pH 6–8 and glycine–NaOH buffer (0.05 M) was used for pH from 8 to 11. xylanase activity was measured at 60 °C. Each experiment was performed in triplicates until mentioned

Effect of metal ions on xylanase activity

Enzyme was respectively incubated with 1mM solution of Ca^{+2} , Mg^{+2} , Fe^{+2} , Cu^{+2} , Mn^{+2} , CO^{+2} and Zn^{+2} for 1 h at room temperature (28 ± 2 °C). Residual activity was measured using enzyme assay described above.

Effect of Sodium chloride on xylanase activity

To investigate the effect of NaCl on the xylanase activity of *Bacillus subtilis* cho40, 0.1–3.0 M NaCl was added to the reaction mixture, and then the xylanase activity was assayed at pH 6.0, at 60 °C at different time intervals from 0 hrs to 8 hrs.

Results

Culture and growth conditions

The culture producing extracellular xylanase was isolated locally from a sediment sample along the coast of Mandovi estuary, west coast of India. The bacterial isolate showed good growth at 28 ± 2 °C in BSS medium of pH 6 supplemented with 0.5% birchwood xylan. Its ability to produce xylanase enzyme was further confirmed when it formed orange digestion halos on birchwood xylan plates, when treated with Congo red and washed with 1M NaCl.

Identification of Strain cho40 by 16S rDNA sequence

The strain cho40 was isolated locally from sediment sample collected from the mangrove area of the Mandovi estuary, the west coast of India. The isolate was grown in BSS medium pH 7 at room temperature. The isolate was Gram-positive, short rod, motile, oxidase and catalase positive. These characteristics of the culture are similar to those described for *Bacillus* sp. in the *Bergey's Manual of Systematic Bacteriology* [26]. The amplified 16S rDNA sequence was compared to the 16S rDNA sequence in the GenBank database and the results indicated an identity of 99.2% with *B. subtilis* (GenBank accession no. AB065370). Therefore, the strain cho40 was considered as one strain of *B. subtilis* (GenBank accession no. HM159998).

Isolation of a positive clone, with a xylan hydrolytic activity

The PCR product was obtained using total chromosomal DNA of *B. subtilis* strain cho40 as a PCR template, and primers xynF and xynR (see Section 2). The PCR product was about 645 bp, and no other non specific bands were found (Fig. 1). The PCR product was ligated with pGEM-T Easy Vector (Promega), and the recombinant plasmid were transferred into *E. coli* JM109., Positive clones, screened by ampicillin, IPTG and X-Gal and detected by the presence of clear zones around their colonies suggesting xylan hydrolysis, were obtained (Fig. 2). One recombinant plasmid, pGEM-T-XY, containing a 645 bp fragment insert was selected for further characterization.

Identification and DNA sequence analysis of the xylanase gene

The complete nucleotide sequence of the insert in pGEM-T-XY was determined. An open reading frame of 645 bp (the xylanase gene) encoding a protein composed of 215 amino acid residues was identified. The molecular mass and pI of the protein were 22,907 Da and pH 6.1, respectively.

The xylanase gene sequence of *B. subtilis* cho40 (GenBank accession no. HM159998) is 94% identical to the *Bacillus* xylanase gene sequence in the DDBJ/EMBL/GenBank nucleotide sequence

databases under the accession no. AL009126. The catalytic domain of Xyl40 (from *B. subtilis* cho40) showed extensive homology with xylanase assigned to the family 11 glycosyl hydrolases based on hydrophobic cluster analysis [27].

3.6. Amino acid composition

The amino acid composition of the purified xylanase is shown in Table 1 In *B. subtilis* cho40 xylanases, relatively high abundance of threonine (15.1%), glycine (12.3%) and asparagine (9%) was found. Histidine content was the lowest (0.9%) while there was no cystine. Two highly conserved residues, Glu104 and Glu196, which are considered to be crucial for the catalytic activity of family 11 glycosyl hydrolases, were found in two conserved regions (Fig. 3, Fig. 4). Xyn40 showed high amino acid sequence identity to some of the *B. subtilis* xylanases reported in databases, such as the strain EMBL_Q59254 (93%), EMBL_Q8RMN9 (93%), EMBL_Q45VU1 (94%), EMBL_C6F1T5, 5. EMBL_C7F433 (93%), EMBL- BAH28803.1(94%).

On sequence comparison it can be seen that the amino acids T, G, T, A, and N at positions 2, 21, 24, 93, and 141 (*B. subtilis* gene numbering) were replaced by S, S,I, S, and R respectively in strains EMBL Q45VU1, EMBL C6F1T5, EMBL_C7F433, EMBL- BAH28803.1 sequences (Fig:3). Analysis of the amino acid sequence of *B. subtilis* cho40 xylanase with other xylanases from *Bacillus sp.*, showed similarities to a lesser extent namely, *Bacillus stearothermophilus* showed 79% identity while *Bacillus halodurans* showed 71 % identity with major differences in the amino acids at N terminal end (Fig. 4).

Characterization of Native xylanase from *B. subtilis* cho40

Partially purified xylanase obtained from *B. subtilis* cho40 was used for the analysis. Under the standard conditions, the optimal pH of the xylanase was 6.0. The optimal temperature for enzyme activity at pH 6.0 was 60 °C (Figs. 5 and 6). Xylanase activity was assayed in the presence and absence of metal ion and metal chelator (EDTA). Fe^{+3} , Cu^{+2} and EDTA inhibited the activity of the xylanase enzyme, while Ca^{+2} , Zn^{+2} and Co^{+2} enhanced the xylanase activity (Table 2).

The effect of NaCl concentration on xylanase activity was studied (Fig. 7). The enzyme showed activity in the presence of 0M to 3M NaCl. Preincubation of enzyme with NaCl resulted in increase in enzyme activity. Activity of enzyme at 0 h remained almost constant at different concentrations of NaCl. With the increase in the incubation time the enzyme showed increase in activity and this activation differed with the varying concentration of NaCl. After one hour of incubation with NaCl enzyme activity increased till 0.5 M NaCl concentration and then remained almost steady. After 2 h and 4 h of incubation the enzyme showed strong increase in activity at 0.5M NaCl concentration. The activity decreased with further increase in NaCl concentration but was still higher than the enzyme

without NaCl. After 8 h the activity decreased and at higher NaCl concentration it was almost equal to control without any addition of NaCl (Fig.7).

Discussion

Several recombinant xylanases from bacteria, Yeast and fungi have been cloned and expressed in *E. coli* by construction of genomic libraries followed by expression cloning. In our study, a highly xylanolytic *B. subtilis* strain Cho40 was isolated from mangrove area. *Bacillus* species are known to produce and secrete a variety of hydrolytic enzymes and thus can utilize various complex carbohydrates in their natural habitats such as soil. Eustarine and marine habitats provide a unique ecosystem which can often lead to isolation of biomolecules and enzymes with novel properties.

In this report, the primers were designed and a xylanase gene was cloned from the *B. subtilis* strain cho40 by PCR amplification. When the deduced amino acid sequence of the *B. subtilis* strain cho40 xylanase (Xyl40) was compared with the sequences of other xylanases in the NCBI database using the BLAST search program, it showed high homology with xylanases from *Bacillus* strains belonging to GH family 11 such as *Bacillus subtilis* 168 (GenBank Accession No. AL009126.3 (identity 95%), *Bacillus megaterium* CX3 (GenBank Accession No GQ228845.1) (identity 95%), *Bacillus pumilus* (GenBank Accession No DQ100304.1) (identity 95%), *Bacillus circulans* (GenBank Accession No X07723.1) (identity 95%), *Bacillus amyloliquefaciens* 5582 (GenBank Accession No EU980327.1) (identity 94%),

In *Bacillus* sp. xylanases with relatively high concentration of Asx-Glx [28-30] or Asx-Gly [31-32] have been found. *B. subtilis* cho40 falls into the second category and possesses higher Asx-Gly residues. Significant amounts of the hydroxyl amino acids, threonine and serine (especially the former), were seen to be present. They are presumed to provide flexibility in the structure of polysaccharide hydrolases [33]. As it has been reported for microbial xylanases *B. subtilis* cho40 xylanase also showed very low methionine content which is normally the case with microbial xylanases [29], while no cysteine was detected. Breccia et al. [34] reported similar amino acid composition of a thermostable xylanase from *Bacillus amyloliquefaciens* with the exception that asparagine was the most abundant whereas in the present study threonine was abundant. Khasin et al. [29] reported high concentration of Lysine (10.8%) residues in xylanase gene from *Bacillus stearothermophilus* while we have reported only 2.4% of lysine, Moreover threonine content in *Bacillus stearothermophilus* is very low (2.9%) compared to our data which is having more of threonine (15.1%) .

When we analysed the amino acid sequene of *B. subtilis* cho40 xylanase it was noticeable that in xyl40 xylanase serine is replaced by threonine at 2 and 24 position when compared to other *B.*

subtilis strains. It is reported that in Ntn family member, glycosylasparaginase, the replacement of the catalytic threonine by serine reduces its activity by more than 10-fold [35]. Also, due to additional methyl group present in threonine it influences the hydrolysis of ester, where the N-terminal serine is inherent to cleave peptide bonds sufficiently [36]. So taking these observations into account it is likely that the replacement of serine by threonine may be partly responsible for difference in salt tolerance of xyl40.

The xylanase enzyme from *B. subtilis* cho40 had a pH optimum of 6.0 and a temperature optimum of 60 °C. Enzyme activity was inhibited by Fe^{+3} , Cu^{+2} and EDTA. In previous reports, it is seen that Cu^{+2} strongly inhibit the activity of several xylanases [37-41]. While Guo et al. [19] reported almost no effect of copper on xylanase. EDTA was reported to have inhibitory effect on some xylanases, e.g., xylanases from *Aspergillus niger* [42], and *Aspergillus giganteus* [39] and have no effect on some other xylanases [43-45]. In some other xylanases, Fe^{+3} has been found to have stimulating effect on xylanase [39], while, Guo et al. [19] reported strong inhibitory effect of Fe^{+3} on halophilic xylanase. We report that divalent cation Ca^{+2} , Co^{+2} and Zn^{+2} enhanced xylanase activity. Xylanases from marine bacteria *Enterobacter* sp and *Arthrobacter* sp. have also been shown enhancement of enzyme activity in the presence of Ca^{+2} , Co^{+2} and Zn^{+2} [14,15], Guo et al. [19] reported that Co^{+2} and Zn^{+2} to inhibited the xylanase activity, while Ca^{+2} to stimulated this activity.

The xylanase from marine microorganisms may have some unique characters such as increased salt tolerance. In view of this, xyl40 was subjected to different concentrations of NaCl (from 0.5 M to 3 M of NaCl), In presence of salt the xylanase activity increased at all the concentrations almost equally but with the increase in the contact time of enzyme with NaCl, enzyme activity increased drastically. The observation that exposure to 0.5 M of NaCl led to increase in enzyme activity by 40%, is unique. It is that the 0.5 M concentration of NaCl corresponds to ~ 25 ppt of NaCl which is normally present in sea water. It has been reported that in the halobacterial proteins the amount of acidic amino acids are more then the basic amino acids [46-47]. Additionally, the halophilic proteins seem to be deficient in hydrophobic amino acids. The observed excess of acidic amino acids and deficiency of hydrophobic amino acids results in structural instability, which may be stabilised with the exposure to increasing NaCl concentration. The *Bacillus subtilis* cho40 xylanase possesses same ratio of acidic and basic amino acids while there is an excess of polar (hydrophilic) amino acids (61%) over the non polar amino acids (39%).

Halotolerent enzymes need high concentration of salt to function while at low salt concentration they loose their functional properties. In case of xyl40, the enzyme activity was observed in all concentrations of NaCl. This may be due to the fact that *B. subtilis* cho40 is estuarine organism and thus may have adapted to salt fluctuations. It not only shows high tolerance to activity at high salt

concentrations, but also shows a wide tolerance to changes in salt concentrations. Although there is a report on halophilic xylanase which showed maximum xylanase activity at 20% NaCl concentration, there is no further information on enzyme characterization or any molecular aspect of this enzyme [48]. *Glaeociccola mesophila* KMM 241 has been reported to secrete cold active and salt tolerant xylanase belonging to GH10 family. It showed highest activity at 0.5M NaCl while retaining 90 % activity in 2.5M NaCl. Xylanase from a marine *Bacillus pumilus* has been reported to show enhanced xylanase activity (160 ~ 120%) in 2.5 to 7.5% NaCl with 87 and 73% retention of activity in 10 and 15% of NaCl, but there are no report on the stability of this enzyme with respect to NaCL concentration [49]. Recent studies have shown that exozymes, produced by halophilic microorganisms, exhibit some unique structural and biochemical characteristics[50-52]. Halophiles are not only interesting from the basic scientific view point, they may also be of potential interest in many industrial applications and saline waste water treatment [4].

The xylanase reported in this study is different and it may be described as halotolerent. It therefore will find applications in the processing of sea food and saline food. Production of bioethanol from seaweeds is considered a new idea which can be evaluated in terms of sustainability and environmental conservation. Seaweeds live in saline seawater and grow hydroponically. In other words, cultivation of seaweeds does not compete for resources with conventional agriculture. This makes a huge difference with other terrestrial crops which in fact exploit natural resources such as soil and fresh water [53].

It should be noted that the commercially available enzymes are too expensive and much more development is needed on formulating xylanases. Halotolerent xylanase reported here from *Bacillus* sp. cho 40 can play a very important role in bioethanol production from marine seaweeds by optimization of enzyme dose as well as time and pre-treatment strategies, which is our further plan of work.

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Table1

% Composition of amino acid from amino acid sequence of xylanase enzyme from *Bacillus subtilis* Cho40

Amino acid	composition
Alanine (Ala) (A)	15 7.1%
Arginine (Arg) (R)	6 2.8%
Asparagine (Asn) (N)	19 9.0%
Aspartic acid (Asp) (D)	7 3.3%
Cystine (Cys) (C)	0 0.0%
Glutamine (Gln) (Q)	5 2.4%
Glutamic acid (Glu) (E)	5 2.4%
Glycine (Gly) (G)	26 12.3%
Histidine (His) (H)	2 0.9%
Isoleucine (Ile) (I)	6 2.8%
Leucine (Leu) (L)	7 3.3%
Lysine (Lys) (K)	5 2.4%
Methionine (Met) (M)	3 1.4%
Phenylalanine (Phe) (F)	5 2.4%
Proline (Pro) (P)	7 3.3%
Serine (Ser) (S)	21 9.9%
Threonine (Thr) (T)	32 15.1%
Tryptophan (Trp) (W)	11 5.2%
Tyrosine (Tyr) (Y)	15 7.1%
Valine (Val) (V)	14 6.6%
Pyrrolysine (Pyl) (O)	1 0.5%

Table 2

Effect of metal ion on the activity of the xylanase enzyme produced by *B. subtilis* cho40.

Metal ions (1 mM)	Relative activity (%)
None	100
Ca ⁺²	140
Mg ⁺²	90
Zn ⁺²	120
Fe ⁺³	89
Cu ⁺²	91
Co ⁺²	112
Mn ⁺²	98
EDTA	82

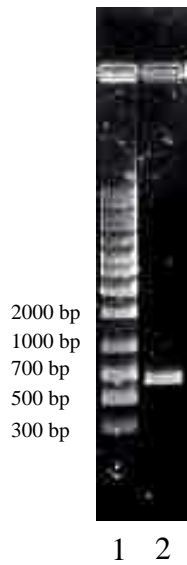


Fig. 1. Agarose gel electrophoresis of PCR product by amplification of xylanase gene of *B. subtilis* cho40 by using xylanase specific primers (1) Marker (MBI) and (2) PCR product

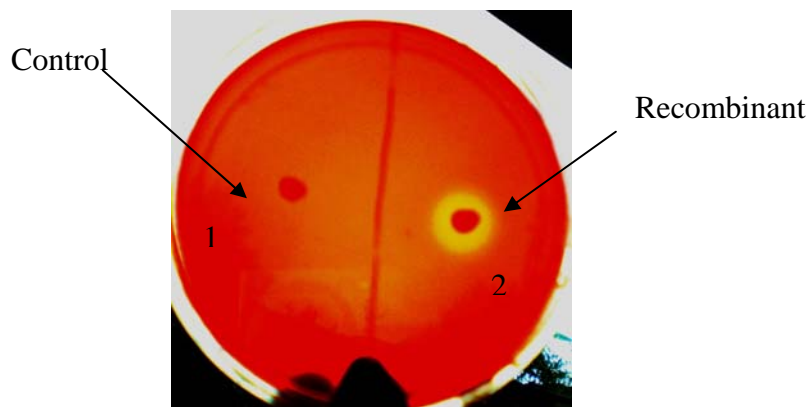


Fig. 2. Clearing haloes around recombinant colonies by Congo-red plate assay utilizing an overlay of birchwood xylan. (1) *E. coli* JM109 with pGemT (control) and (2) recombinant colonies


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Query: 1   LTAALMETSISLFSATASAAGTDYWQNWTDGGGTVNAVNGSGGNYSVNWSNTGNFVVGKG 60
Sbjc1: 12 LSAALM--SISLFSATASAAS TDYWQNWTDGGGI VNAVNGSGGNYSVNWSNTGNFVVGKG 69
Sbjc2: 12 LSAALM--SISLFSATASAAS TDYWQNWTDGGGI VNAVNGSGGNYSVNWSNTGNFVVGKG 69
Sbjc3: 12 LSAALM--SISLFSATASAAS TDYWQNWTDGGGI VNAVNGSGGNYSVNWSNTGNFVVGKG 69
Sbjc4: 12 LSAALM--SISLFSATASAAS TDYWQNWTDGGGI VNAVNGSGGNYSVNWSNTGNFVVGKG 69
Sbjc5: 12 LTAALM--SISLFSASASAAGTDYWQNWTDGGGTVNAVNGSGGNYSVNWSNTGNFVVGKG 69
Sbjc6: 12 LSAALM--SISLFSATASAAS TDYWQNWTDGGGI VNAVNGSGGNYSVNWSNTGNFVVGKG 69

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Query: 61   WTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRAPLIEYYVDSWGTYRPTGTYKGTVKSD 120
Sbjc1: 70   WTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVDSWGTYRPTGTYKGTVKSD 129
Sbjc2: 70   WTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVDSWGTYRPTGTYKGTVKSD 129
Sbjc3: 70   WTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVDSWGTYRPTGTYKGTVKSD 129
Sbjc4: 70   WTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVDSWGTYRPTGTYKGTVKSD 129
Sbjc5: 70   WTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVDSWGTYRPTGTYKGTVNSD 129
Sbjc6: 70   WTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVDSWGTYRPTGTYKGTVKSD 129

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◆ Glu 104

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Query: 121  GGTYDIYTTTRYNAPSIDGDNRTTFTQYWSVRQTKRPTGSNATITFSNHVNAWKSHGMETN 180
Sbjc1: 130  GGTYDIYTTTRYNAPSIDGDRRTTFTQYWSVRQTKRPTGSNATITFSNHVNAWKSHGM--N 187
Sbjc2: 130  GGTYDIYTTTRYNAPSIDGDRRTTFTQYCSVRQTKRPTGSNATITFSNHVDNAWKSHGM--N 187
Sbjc3: 130  GGTYDIYTTTRYNAPSIDGDRRTTFTQYWSVRQSKRPTGSNATITFSNHVNAWKSHGM--N 187
Sbjc4: 130  GGTYDIYTTTRYNAPSIDGDRRTTFTQYWSVRQTKRPTGSNATITFSNHVNAWKSHGM--N 187
Sbjc5: 130  GGTYDIYTTTRYNAPSIDGDNRTTFTQYWSVRQTKRPTGINAATITFSNHVNAWKSHGM--N 187
Sbjc6: 130  GGTYDIYTTTRYNAPSIDGDRRTTFTQYWSVRQTKRPTGSNATITFSNHVNAWKSHGM--N 187

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Query: 181  LGSNWAYQVMETATEGYQSSGSSNVTVW 208
Sbjc1: 188  LGSNWAYQVM--ATEGYQSSGSSNVTVW 213
Sbjc2: 188  LGSNWAYQVM--ATEGYQSSGSSNVTVW 213
Sbjc3: 188  LGSNWAYQVM--ATEGYQSSGSSNVTVW 213
Sbjc4: 188  LGSNWAYQVL--ATEGYQSSGSSNVTVW 213
Sbjc5: 188  LGSNWSYQVL--ATEGYQSSGSSNVTVW 213
Sbjc6: 188  LGSNWAYQVM--ATEGYQSSGSSNVTVW 213

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▲ Glu 196

Fig. 3. Sequence alignment of the deduced amino acid of *B. subtilis* Cho40 xylanase with other *B. subtilis* strain xylanases: 1 EMBL-Q59254 2. EMBL Q8RMN9, 3. EMBL Q45VU1, 4. EMBL C6F1T5, 5. EMBL_C7F433, 6 .EMBL- BAH28803.1The Glu residues corresponding to our xylanase Glu-104 and Glu-196, essential to the catalytic activity are marked by (◆) and (▲) respectively .

Query 1	LTAALMETSISLFSATASAAGTDYWQNWDGGGTVNAVNGSGGNYSVNWSNTGNFVVGKG	60
Ba.Stea12	LTASM---SFGLFGATSSAA-TDYWQYWDGGGMVNAVNGPGGNYSVTWQNTGNFVVGKG	67
Ba. sub12	LSAALM--SISLFPATASAASTDYWQNWDGGGGI VNAVNGSGGNYSVNWSNTGNFVVGKG	69
Ba. Hol8	VLTVVIAATISFCLSAVPA SANTYWQYWDGGGTVNAV TNGPGGNYSVTW RDTGNFVVGKG	67
Query 61	WTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRAPLIEYYVVDSWGTYRPTGTYKGTVKSD	120
Ba.Stea68	WTVGSPNRVINYNAGIWEPSGNGYLTLYGWTRNALIEYYVVDSWGTYRPTGNYKGTVNSD	127
Ba. sub70	WTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSP LIEYYVVDSWGTYRPTGTYKGTVKSD	129
Ba. Hol 68	WEIGSPNRTIHYNAGVWEPSGNGYLTLYGWTRNQLIEYYVVDNNGTYRPTGTHRGTVVSD	127
	♦ Glu 104	
Query 121	GGTYDIYTTTRYNAPSIDGDNTTFTQYWSVRQTKRPTGSNATITFNSHVNNAWKSHGMETN	180
Ba.Stea128	GGTYDIYTTMRYNAPSIDGTQ-TFQQFWSVRQSKRPTGSNVSITFNSHVNNAWRSKGM--N	184
Ba. sub 130	GGTYDIYTTTRYNAPSIDGDR TTFQYWSVRQSKRPTGSNATITFNSHVNNAWKSHGM--N	187
Ba. Hol128	GGTYDIYTTMRYNAPSIDGTQ-TFQQFWSVRQSKRPTGNNVSI TFSNHVNNAWRNAGM--N	184
Query 181	LGSNWAYQVMETATEGYQSSGSSNVTVW	208
Ba.Stea185	LGSSWAYQV L--ATEGYQSSGRSNVTVW	210
Ba. sub188	LGSNWAYQVM--ATEGYQSSGSSNVTVW	213
Ba.Hol 185	LGSSWSYQV L--ATEGYQSSGRSNVTVW	210
	▲ Glu 196	

Fig. 4. Sequence alignment of the deduced amino acid of *B. subtilis* Cho40 xylanase with other *Bacillus* sp. strains xylanases: 1. Ba.Stea., *Bacillus stearothermophilus* (endo-beta-1,4-xylanase) 2. Ba. Sub., *Bacillus subtilis* 3. Ba. Hol :*Bacillus halodurans* (endo-1,4-beta-xylanhydrolas) The Glu residues corresponding to our xylanase Glu-104 and Glu-196, essential to the catalytic activity are marked by (♦) and (▲) respectively

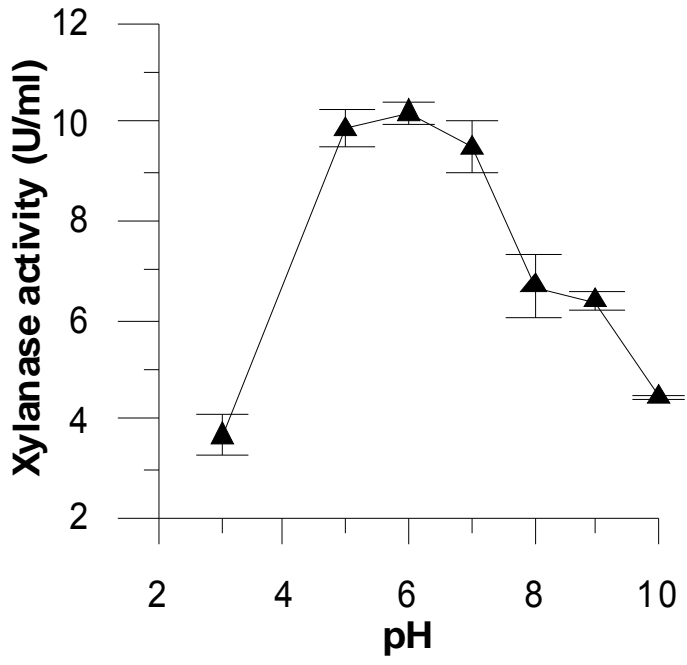


Fig. 5. The effect of pH on the xylanase activity. Enzyme activity was assayed at 50 °C

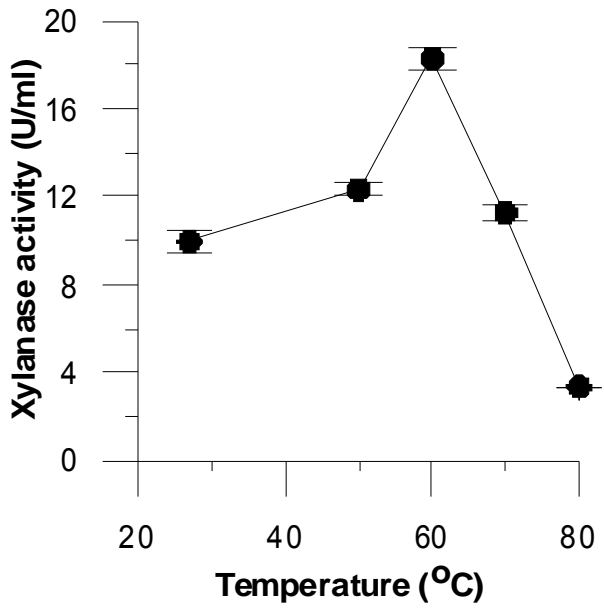


Fig. 6. Effect of temperature on the activity of xylanase. Enzyme activity was assayed in phosphate buffer pH 8 at different temperature

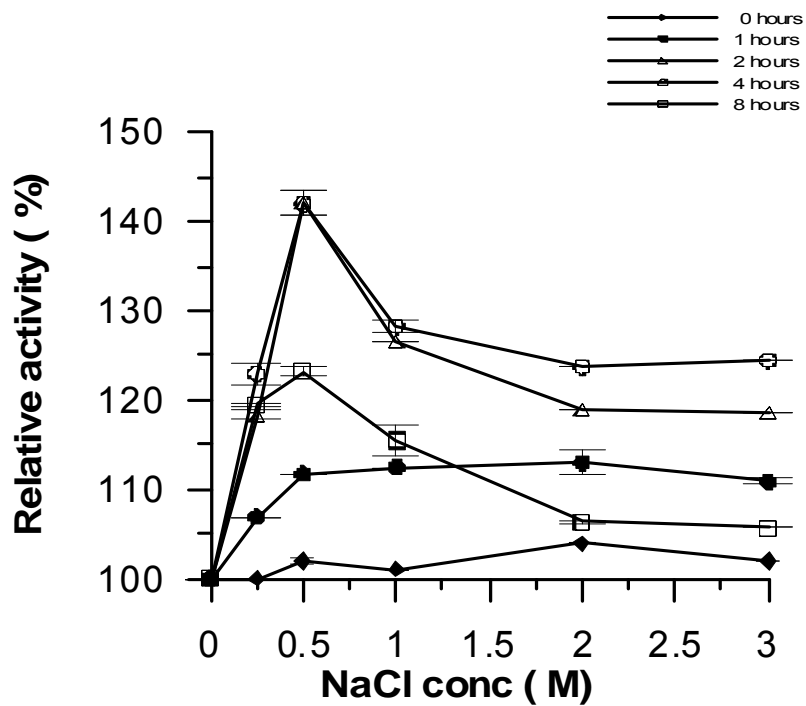


Fig. 7. Effects of NaCl concentrations on the xylanase activity of the *B. subtilis* cho40. The assay was carried out at 50°C in (a) 50mM Tris-hydrochloride buffer (pH 6.0) containing various concentrations of NaCl for 10 min. The xylanase activity taken as 100% in each assay at 0 hours