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IN VITRO AND *IN SILICO* ANALYSIS OF XYLANASE PRODUCED BY *Bacillus licheniformis*

Ebenezer Jeyakumar*, Rubina Lawrence, Rohit Farmer and Shraddha Sahai

Department of Microbiology and Fermentation Technology, Jacob School of Biotechnology and Bio-Engineering, Sam Higginbottom Institute of Agriculture, Technology and Sciences, Allahabad, Uttar Pradesh – 211 007 (India);
*e-mail: *ebenezerjeyakumar@rediffmail.com, ebenezer_74@yahoo.co.uk*

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ABSTRACT

A potent strain of *Bacillus licheniformis* was isolated among the various xylanolytic bacteria obtained from agricultural fields of Allahabad (India). The strain produced xylanase in a broad temperature (40-70ºC) and pH (5-11) range with optimum production at 50ºC (for 48 hr) and pH 7.0±0.2. The peak enzyme activity was observed when the substrate was supplemented with xylose, ammonium nitrate and tryptone. A single form xylanase with a low molecular weight (42 kDa) was purified to 3.9-fold with 6.7% recovery rate. Further, the homology modeling of xylanase produced by *B. licheniformis* was carried out using a template (PDB ID = 1AXK) having sequence similarity of 90% with the query sequence. The reliability of final model confirmed a 92.8% acceptability of Ramachandran plot statistics when validated through PROCHECK and VERIFY 3D. A similar pocket volume obtained from CastP and Pocket Finder were 253 and 268 Å^3, respectively. The molecular orientation scored the lowest energy level (-6.24 kcal mol^{-1}) suggesting maximum stability of docked complex. The hydrogen bonding and hydrophobic interactions between ligand and active site lining residues suggested their importance in the catalytic activity and hence this xylanase could be used effectively in industrial applications.

Keywords: *Bacillus licheniformis,* docking, homology modelling, xylanase

INTRODUCTION

Plant cells walls, the major reservoir of fixed carbon in nature, has three main polymeric constituents i) cellulose (insoluble fibres of β-1,4-glucan), ii) hemicellulose (non-cellulosic polysaccharides including glucans, arabinans, mannans and xylans), and iii) lignin with a complex polyphenolic structure (Morales *et al*., 1993). Second to cellulose, hemicellulose is the most abundant polysaccharide in plant cell walls which accounts upto 40% of the total carbohydrate fraction, and has immense potential for use as an alternative source of energy. The major cellulose component β-(1,4) xylans are heterogeneous polysaccharides found in cell walls of all land plants and constitutes 15-30% of annual plants. It consists of a linear backbone of β-(1,4) linked xylopyranose units and often has side chains composed of other sugar residues such as arabinose, glucuronic acid, etc. (Kaneko *et al*., 2000). Due to heterogenic nature, the hydrolysis of xylans requires the action of a complex enzyme system, which comprises of endoxylanase, exoxylanase (β-D-xylanxylohydrolase) and β-D-xylosidase. Xylanases, repertoire of hydrolytic enzymes, facilitate the complete hydrolysis of xylopyranosyl linkages of β-(1,4)-xylan to xylo-oligosaccharides and xylose. Xylan can be degraded by either acid or enzymatic hydrolysis. The enzymatic process has the advantage of highly efficient conversion rate, and the mild conditions required are non-corrosive and eco-friendly.

Xylanase is produced by many bacteria and fungi (Marta *et al*., 2000; Polizeli *et al*., 2005) and possesses a range of industrial and environmental applications. Microbial xylanases have important role in the degradation of xylan. *Bacillus* species secrete appreciable quantities of extracellular xylanases (Srinivasan and Meenakshi, 1999). Xylanases have industrial use in paper and pulp making, baking (for improving dough handling and quality of baked products), during extraction of coffee, plant oils, starch, for improvement in nutritional properties of silage and grain, and in combination with pectinase and cellulase for fruit juices clarification. The enzyme is used in textile industry for degumming of plant fiber sources and to enhance fiber quality (Bindu *et al*., 2007; Aysegul *et al*., 2008).

To understand the protein function in greater detail the three dimensional (3D) structural information is required. Using X-ray crystallography, electron microscopy, diffraction and NMR-spectroscopy, the actual 3D structure of a protein is obtained. However, no information is available on the crystal structure of xylanase in *Bacillus licheniformis*. In the absence of experimental data, models based on known 3D structure of homologous proteins are the only reliable method of obtaining structural information. The present study was aimed at to purify and characterize the xylanase enzyme from *Bacillus licheniformis* and to elucidate the three dimensional structure and substrate binding mechanism of the enzyme using computational techniques.

MATERIALS AND METHODS

Isolation and screening: Soil samples obtained from the agricultural fields and riverbanks of Allahabad region were serially diluted to 10^{-2}, plated on tryptone yeast extract medium and incubated at 50$\pm$1ºC for 24-48 hr. The thermophilic isolates were screened on Congo red xylan agar plates for xylanase production. The best potential strain showing maximum xylanolytic activity was selected on the basis of large clear zone formation. Xylanolytic bacteria were identified and characterized by morpho-biochemical tests using *Bergey's Manual of Systematic Bacteriology* (Sneath *et al*., 1996).

Xylanase production and assay: Xylanase production was performed in standard media given by Gessesse and Gashe (1997). The standard broth media (100 ml) was inoculated with 2 ml culture of isolated bacterial strain and incubated at 50$\pm$1ºC for 24-48 hr. The broth was then centrifuged at 10,000 rpm for 20 min. at 4ºC. The cell-free supernatant was used as crude enzyme preparation for enzyme assay. The xylanase assay was performed by measuring the release of reducing sugar from oatspelt xylan following the DNS method (Miller, 1959).

Optimization of production parameters: The production parameters such as incubation time (24-96 hr), temperature (40-70ºC), pH requirement (5-11), carbon source (1% xylose, arabinose, glucose, sucrose and galactose), nitrogen source [NH_4Cl, $NaNO_3$, $(NH_4)_2SO_4$, yeast extract, peptone and tryptone] were assessed in laboratory to determine the optimal production conditions of xylanase. The kinetic constants K_m and V_{max} were estimated following Lineweaver and Burk method (Bruno *et al*., 1994).

Production of xylanase under optimized conditions: The standardized media was prepared according as per optimal production conditions. The fermented broth media, incubated at optimized conditions, was subjected to the extraction of enzyme. The cells were removed by centrifugation at 10,000 rpm for 20 min. at 4ºC. The supernatant was used for further purification and enzyme assay determined using standard assay method (Miller, 1959).

Acetone precipitation method: The protein contents, including enzymes, were subject to organic precipitation, exploiting the hydrophobic interaction properties of proteins (Reis *et al*., 2003). To 300 ml crude extract, 700 ml chilled acetone (4ºC) was added slowly over a period of 30 min. while stirring the mixture to achieve a 70% saturation, and the mixture was kept at 4ºC overnight. The supernatant was then decanted carefully and the remaining supernatant along with the precipitated material spun at 4500 rpm at 4ºC. The supernatant obtained was removed carefully and precipitate dissolved in 4 ml

0.05 M Tris-HCl buffer (pH 7.4). Xylanase assay was performed with this solution to check the enzyme activity followed by SDS-PAGE.

Gel filtration chromatography: For the purification of xylanase the column (2.6 x 100 cm, bed height 80 cm) was packed with sephadex G-100 swollen in 0.1 M Tris-HCl buffer [pH 7.4] at 4ºC overnight. The packed column was equilibrated with this buffer for 12 hr at a flow rate of 1 ml min^{-1}. The sample was loaded carefully onto the column to form an unmixed layer of protein sample at the interface. The column was developed in the same buffer at a flow rate of 1 ml $min^{-1.}$ The detector was set at 280 nm and absorption unit full scale (AUFS) of 2.0. The effluent from detector was directed to a fraction collector that was programmed to collect 5.0 ml fractions per tube. The event marker from the detector was connected to a recorder that recorded absorption simultaneously with the elution. The peak fractions were tested for the presence of xylanase activity. Positive fractions were pooled and protein content and xylanase activity assayed.

Protein estimation: The protein content of crude extract, solubilized acetone precipitate and column fractions, were estimated by bicinchoninic acid method with bovine serum albumin as standard (Smith *et al.,* 1985).

Molecular weight determination: The molecular weight of xylanase was determined from SDS-PAGE profiling of xylanase obtained from different stages of purification (Laemmli, 1970). The crude extract, acetone precipitated material, peak fractions of gel filtration step and molecular weight markers (SDS-VII Dalton Marker, Sigma) were, respectively, mixed with sample buffer and boiled for 5 min. at 95ºC and then spun down at 13,000 rpm for 10 min. The casted gel in electrode assembly and buffer tank was filled with chilled buffer (1 X electrode, stored at 4ºC). The samples and markers were loaded with the help of micro-syringe in wells, and electrophoresis performed at 200 V, 60 mA for 45 min. After the separation was over the gel was thoroughly washed at least 3-4 times with Milli Q water, stained with coomassie brilliant blue R and then destained.

Template search and sequence alignment: The protein sequence of *B. licheniformis* was retrieved from Uniprot database (accession ID A5H0S3) in FASTA format. The template structure for homology modelling was selected with the help of BLAST (Altschul *et al*., 1990) search against PDB database. Based on maximum identity with high score and lower e-value, 1,4-β-xylanase from *B. subtilis* (wtXYN) at 2.10 Å resolution (PDB code: 1AXK) was used as template. Sequence identity was 90% over the length of 180 residues starting from 32^{nd} amino acid till the last of query sequence.

Three dimensional structure generation and validation: The academic version of MODELLER 9 v.6 (*http://www.salilab.org/modeller*) was used for 3D structure generation based on the information obtained from sequence alignment (Eswar *et al*., 2006). The homology modelling method is based on the assumption that the structure of unknown protein will be similar to the known structure of some reference proteins. Out of the five models generated by MODELLER, the one with best G-score of PROCHECK (Laskowski *et al*., 1993) and with best VERIFY3D profile (Lüthy *et al*., 1992) was selected as final model. The structural super-imposition of C^{α} trace of the model on template structure was performed using combinatorial extension (CE: *http://cl.sdsc.edu/ce.html*). Different servers (DAS, HMMTOP, TMHMM and TMPRED) were used (Hofmann and Stoffel, 1993; Cserzo *et al*., 1997; Tusnády and Simon, 1998; Krogh *et al*., 2001) to predict transmembrane helices in *B. licheniformis* protein sequence and PSORT web server was used to predict protein subcellular localization.

Determination of active site: The active site of enzyme was determined and compared using CastP (*http://sts. bioengr.uic.edu/castp/*) (Dundas *et al*., 2006), PocketFinder (*http://www.modelling.leeds. ac. uk /pocketfinder*) web servers and PASS (Brady and Stouten, 2000). Apart from the convention that the largest pocket is considered to be the right one, the structural homologues available in PDB database already complexed with the ligand were studied to correctly locate the position of active site.

Molecular docking: To understand the interaction between active site lining residue of enzyme and its natural substrate, β-(1,4)-xylan was docked into the enzyme using AUTODOCK 4.0. The structure of β-(1,4)-xylan was drawn and 3D optimized using CHEMSKETCH 10.0. Out of ten docking solutions the best docking solution was selected based on energy score. The interaction between active site lining residues and docked substrate was studied using LIGPLOT software in terms of hydrogen bonds and hydrophobic contacts. The active site lining residues were also traced around 5Å radius of the docked ligand. The structures were drawn using Pymol Molecular Graphics system and Jmol web applet.

Statistical analysis: The optimization of growth parameters such as temperature and incubation time, effect of carbon and nitrogen sources on xylanase production was analyzed using two way analysis of variance technique (Paulson, 2008). The effect of pH on production was assessed using Linear Regression and co-relation coefficient followed by test of significance at 5% level.

RESULTS AND DISCUSSION

Incidence of xylanolytic bacteria: Of the twenty thermophilic bacterial isolates obtained from thirty soil samples 12 (40%) isolates possessed xylanolytic activity (Table 1). Among the 12 xylanolytic bacteria 6 were identified as *Bacillus licheniformis* followed by 4 as *B. cereus* and 2 as *B. subtilis* (Table 2). On the basis of maximum zone produced on Congo red xylan agar, *Bacillus licheniformis* was used for further studies.

Table 1 Incidence of xylanolytic bacteria

Total soil samples	30	
No. of thermophilic isolates	20	
Xylanolytic bacteria	12	(40.0%)
Bacillus subtilis	2	(16.6%)
Bacillus cereus	4	(33.3%)
Bacillus licheniformis	6	(50.0%)

Optimization of production parameters: The enzyme activity for *Bacillus licheniformis* was studied on the basis of temperature, incubation time, pH, carbon source and nitrogen source. Among the temperature and time combinations studied, maximum xylanase activity was observed at 48 hr. A significant increase ($P < 0.01$) in the enzyme production from 24 to 48 h was observed in all the temperature treatments (Fig. 1). After 48 hr incubation, a decreasing trend in enzyme activity towards 96 hr was observed. The optimum time-temperature combination was 50°C and 48 hr. The reduction in xylanase yield after this time was due to the depletion of available nutrients. The optimum time-temperature combination for xylanase production is highly in agreement with Nakamura *et al.* (1993), Kulkarni and Rao (1996), Liu *et al.* (1998) and Gupta *et al.* (2000). Xylanase was produced in wide range of temperature viz., 40 to 70°C in the present study which is in agreement with Cordeiro *et al.* (2002) and Heck *et al.* (2005). Peak enzyme activity was observed at optimum temperature which suggests that the thermostable enzyme from *B. licheniformis* is advantageous as it enhances reaction speed so contributes to increased technical and economic variability of the process (Silva *et al.*, 2005).

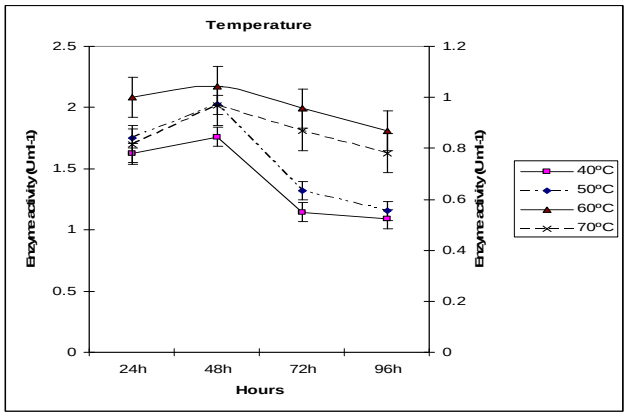


Fig. 1: Effect of incubation time and temperature on on xylanase production

A positive correlation ($r = 0.19553$) of pH on xylanase production was observed in the present study. With the broad range of pH variation studied (5-11) for xylanase activity, the optimum pH was observed at 7.0. An increase in enzyme production was noticed from pH 5 towards 7 and a gradual

decrease towards pH 11 (Fig. 2). The values of K_m and V_{max} were found to be 0.0208 and 0.6944 Uml^{-1}. Archana and Satyanarayana (1997) reported an optimum xylanase at pH 7.0 by *Bacillus licheniformis* which is in agreement with the present findings. High enzyme activity at neutral to alkaline pH (7-11) is a very desirable property to use xylanases in selective hydrolysis of the hemicellulose components in paper and pulp industries for biobleaching purposes.

Among the various carbon sources such as xylose, arabinose, glucose, sucrose and galactose (1%) supplemented in the production media, xylose showed significant effect on xylanase production (Fig. 3). The present observation is in agreement with the findings of Gessesse and Gashe (1997). It appears that low molecular mass fragments of xylan play a key role in the regulation of xylase biosynthesis. These fragments include xylose, xylobiose, xylo-oligosaccharides, heterodisaccharides of xylose, glucose and their positional isomers (Thompson, 1993). The decrease in enzyme production with other carbon sources may be attributed to catabolite repression, likewise described for other xylanolytic microorganisms (Kadowaki *et al.,* 1997).

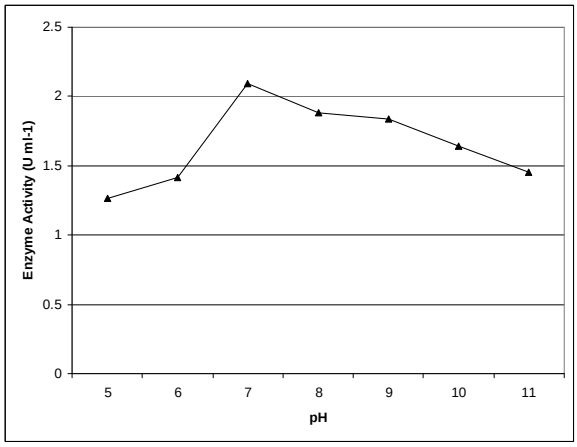


Fig. 2: Effect of pH on xylanase production

Different organic and inorganic nitrogen sources supplemented in media showed significant effect ($P < 0.01$) on xylanase production (Fig. 4). Maximum activity was noticed in case of tryptone (amongst organic) and ammonium nitrate (amongst inorganic). These findings are in agreement with Svarachorn, (1999), Szendefy *et al.* (2006) and Duarte *et al.* (2006). The fact that maximum growth of *B. licheniformis* occurs at highest nitrogen content (tryptone) also exemplifies the role of easily fermentable carbon compounds in the medium. The production of primary metabolite by microorganisms is highly influenced by their growth, which is determined by the availability of nutrients in medium. Therefore, it is expected that improvement in nutritional value of medium supplemented with organic and inorganic nitrogen sources will also improve the growth of *B. licheniformis* and subsequently enhance xylanase production.

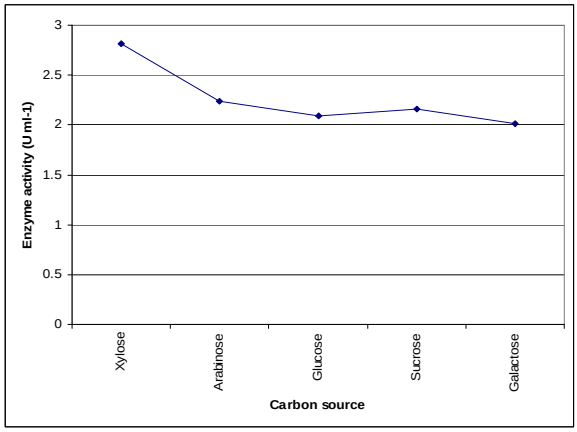


Fig. 3: Effect of C source on xylanase production

Purification of xylanase: The xylanase was purified to 1.8-fold with approximately 9.2% recovery. The enzyme after subjection to gel filtration column yielded highest specific activity of 26.01 U mg^{-1}. The xylanase was finally purified to 3.9-fold with 6.7% recovery (Table 2). The low yield of xylanase obtained is probably due to the difficulty in elimination of xylooligosaccharides produced by the strain that is found to be strongly associated with

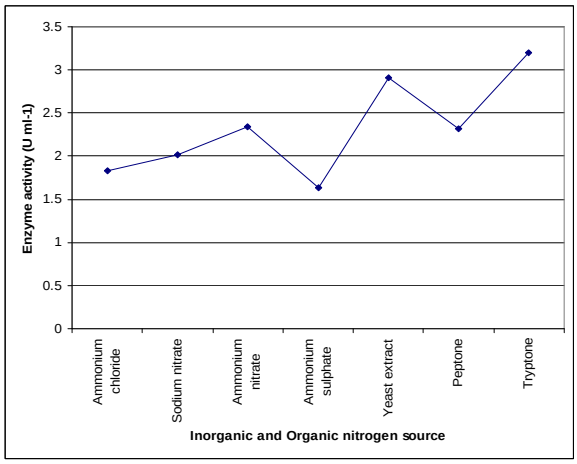


Fig. 4: Effect of N sources on xylanase production

xylan hydrolysis. Gupta *et al.* (2000) purified xylanase with only 5% recovery yielding a specific activity of 2.74 U mg^{-1} protein which is very less as compared to the present findings. The elution profile of xylanase obtained in present study revealed that fractions (58-74) exhibited xylanolytic activity indicating that the enzyme is detected in all the fractions obtained. A single peak in profile illustrates the fact that there is no other isoform of xylanase (isozyme) produced by the organism in substrate (Fig. 5). Further, being a single form of xylanase suggests that a specific target can be obtained with this form of xylanase. In contrast, Wong *et al.* (1988), Elegir *et al.* (1994) and Flint *et al.* (1994) observed multiple forms of xylanase produced by *B. licheniformis*.

Table 2: Purification of xylanase from *Bacillus licheniformis*

Purification stage	Total activity (U ml^{-1})	Total protein conc. (mg ml^{-1})	Specific activity (U mg^{-1})	Purification fold	Yield (%)
Crude extract	1112.10	168	6.62	1.0	100
Acetone precipitation	102.69	8.56	11.99	1.8	9.2
Gel filtration	74.93	2.88	26.01	3.9	6.7

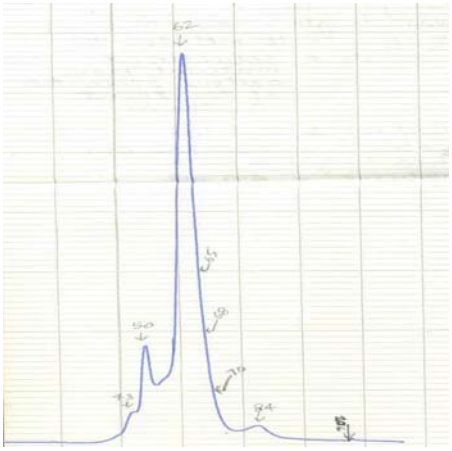
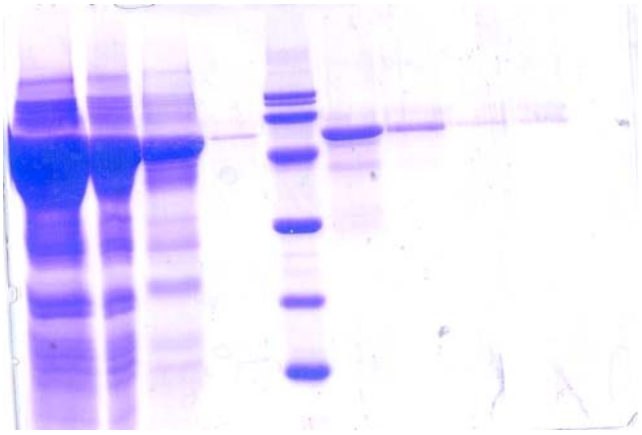


Fig. 5: Elution profile of xylanase
Flow rate 1 ml min^{-1};
Chait speed, 0.1 mm min^{-1};
Fraction size, 5.0 ml;
AUFS, 1.0;
Range, 10 mV;
Wavelength 280 nm;
Buffer 0.1 m Tris-HCl;
Bed volume 400 ml;
Column, 2.6 x 100 cm;
Bed height, 80 cm;
Volume loaded, 4 ml



67 kDa
45 kDa
36 kDa
29 kDa
21 kDa
14.4kDa

Lane 1: Crude Extract
Lane 2: Acetone preceipitate
Lane 3: 1: 4 dilution of acetone ppt. material
Lane 4: Fraction: 50
Lane 5: Mol. Wt markers
Lane 6: Fraction 62
Lane 7: Fraction 65
Lane 8: Fraction 68
Lane 9: Fraction 70
Lane 10: Fraction 84

Lane 1 2 3 4 5 6 7 8 9 10

Fig. 6: SDS-PAGE analysis of purified xylanase

Molecular weight of xylanase: The analysis of xylanase by SDS-PAGE revealed a single band in all the fractions (Fig. 6). Among the pooled fraction from gel filtration column, fraction 62 was estimated

to have molecular weight of about 42 kDa. The enzyme migrated in a single band on the gel. The molecular weight obtained for xylanase lied within the range detected for xylanases belonging to family G/11 (Salles *et al.,* 2000). Low molecular weight xylanase have the capability to penetrate fibre wall structure and modify more efficiently the pulp properties thus making it suitable for biobleaching in paper industries.

Model building: Xylanase obtained from *B. licheniformis* is a 213 amino acid long enzyme molecule with maximum sequence identity of 90% with any of the structures available within PDB database using BLAST similarity search method. The PDB deposited structure IAXK chain A was selected as template structure for comparative modeling of *B. licheniformis* xylanase with sequence identity of 90% over the length of 182 residues starting from 32^{nd} amino acid till the last of query sequence. The structure of enzyme is a single-domain jellyroll (Chelvanayagam *et al.,* 1992), which belongs to the domain folds (superfolds) that most frequently are found in globular proteins (Stirk *et al.,* 1992). It is characterized by two strictly antiparallel β-sheets forming a distorted β-barrel (Fig. 7a).

Protein structure validation: The hypothetical protein models generated were analyzed online by submitting to NIH MBI Laboratory for Structural Genomics and Proteomics' SAVES server. Validity reports generated by PROCHECK and Verfiy_3D judged accuracy of protein models. A comparison of results obtained from above mentioned validation tools, revealed that one of the models generated by MODELLER is more acceptable in comparison to others. The Phi/Psi angles of amino acids that determine the secondary structural property of hypothetical protein were computed and represented as Ramachandran plot having plot statistics 92.8% residues in most favoured region, 7.2% residues in additionally allowed region and none in disallowed regions (Fig. 7b). The compatibility of atomic model (3D) with its own amino acid sequence (1D) computed by Verify_3D reported 100% of residues having an average 3D-1D score >0.2. The structural superimposition of C^{α} trace of model on template structure 1AXK resulted in RMSD value and Z-score 0.2 A and 7.3, respectively, showing family level similarity. The xylanase sequence was analyzed using different transmembrane prediction algorithms-DAS, HMMTOP, TMHMM, TMPRED to predict the transmembrane helices in protein. In transmembrane region 29 amino acids were identified by prediction servers in N terminal suggesting to be signal peptide region.

Active site of xylanase: The active site determined by CastP, PocketFinder and PASS programmes under default settings resulted in various locations of all possible pockets in enzyme structure. Apart from the convention that the largest pocket is considered to be the right one, the structural homologues available in PDB databases already complexed with the ligand are studied to position the exact location of active site. Thus out of many pockets found by the programmes similar pockets was selected as the plausible active site. The pocket volume found by CastP and Pocket Finder are 253 and 268 $Å^3$, respectively, which are nearly the same (Fig. 8a). The cavity lining residues were also similar in pocket found by both the programmes. The co-ordinates of centre of mass of active site found by PASS programme was selected as the grid centre to generate grid maps by AUTOGRID programme for AUTODOCK (*http://autodock.scripps.edu)*.

***Molecular docking of xylanase*:** The interaction between active site lining residues and substrates plays an important role in any catalytic reaction. Such interactions can be studied by calculating the number of hydrogen bonds and hydrophobic interactions between substrate molecules and enzyme residues within the active site. Fig. 8a shows β-(1,4)-xylan molecule docked in active site of enzyme. This molecular orientation scored lowest energy of -6.24 kcal mol^{-1} among the other ten docked complexes thereby suggesting its maximum stability (Fig. 8b). The docked complex molecule was then subjected to LIGPLOT software to calculate hydrogen bonding and hydrophobic interactions within the active site with substrate molecule. ASN 10, TRP 11, TYR 71 and TYR 168 contribute to the hydrogen bonding and PRO 9 and PRO 118 form hydrophobic interactions thus suggesting their role in catalytic

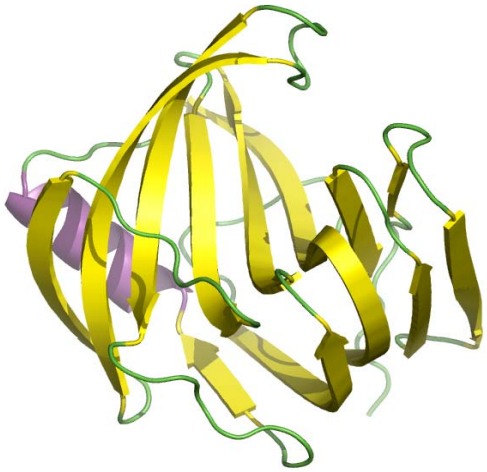


Fig. 7a: Cartoon drawing of final modeled structure

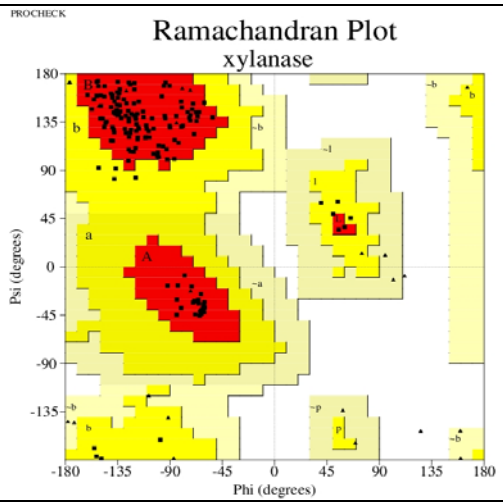


Fig. 7b: Ramachandran plot for final structure

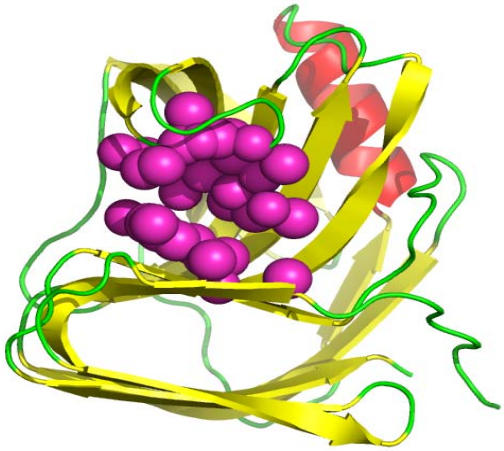


Fig. 8a: Space filled representation of active site predicted by CastP

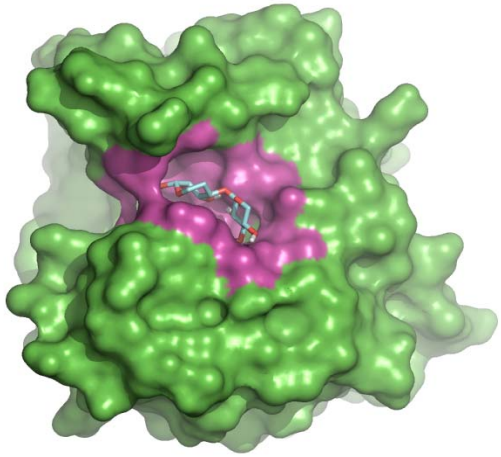


Fig. 8b: Surface representation of xylanase docked with β-(1,4)-xylan with energy score -6.24 k cal mol^{-1}

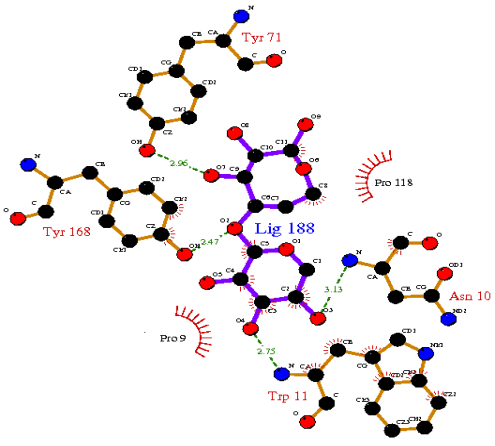


Fig. 9a: Ligplot of interaction of β-(1,4)-xylan with xylanase

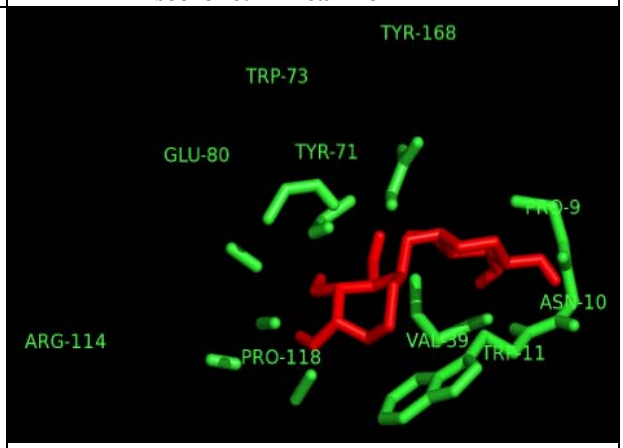


Fig. 9b: Active site binding residues around 5Å radius of Docked ligand

activity of the enzyme as represented in Fig. 9a. PRO 9, ASN 10, TRP 11, VAL 39, TYR 71, TRP 73, GLU 80, ARG 114 and PRO 118 were the active site lining residues around 5Å radius of Docked ligand obtained from Pymol (Fig. 9b). The presence of glutamic acid in the xylanase structure observed in present study was also predicted by Uzuner *et al.* (2010). Similarly, Shi *et al.* (2010) demonstrated that presence of glutamic acid in xylanase is responsible for the catalytic activity of xylanase.

It is well known that the stacking of aromatic compounds and hydrogen bonding are two major kinds of interaction between an enzyme and its substrate (Krengel and Dijkstra, 1996). Very likely the substrate stabilizing effect on the xylanase obtained in the present study may be attributed to hydrogen bonds between the residues highlighted in the ligplot analysis and the substrate molecule. The top scoring ligand was found to be lying deep into the binding cavity exhibiting all major interactions such as hydrogen bonding, vander Waals (VDW) and hydrophobic interactions.

Conclusion: The xylanase produced from *Bacillus licheniformis* in the present study was observed to be active at a wide range of pH, temperature, carbon and nitrogen sources. Further, the lower molecular weight xylanse suggests a high penetrative capability in the substrate. Moreover, the molecular docking study emphasizes that the amino acid residues ASN 10, TRP 11, TYR 71, GLU 80 and PRO 118 to be the key catalytic residues of the functional enzyme. With such potent capabilities the xylanase produced by *Bacillus licheniformis* could be effectively used for biobleaching in paper industry by replacing other environmentally hazardous methods.

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