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In Silico Study of *Bacillus brevis* Xylanase - Structure Prediction and Comparative Analysis with Other Bacterial and Fungal Xylanase

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Abstract

The most important building block of hemicelluloses is xylan. It is broken down into xylose oligomer residues by Xylanase - an enzyme, produced by most organisms, to utilize xylose as primary source of carbon. The Xylanase produced are classified into families, viz 5, 8, 10, 11 and 43 - of Glycoside Hydrolases (GH). Xylanase from family GH 11 are monospecific, they consist solely of Xylanase activity, exclusively active on D-xylose containing substrates. They are inactive on aryl cellobiosides. The fungal Xylanase are produced in higher concentrations, as compared to bacterial Xylanase, but have limited use in pulp bleaching, as they affect the viscosity and strength of the product. In the present study, we have worked upon the Xylanase of *Bacillus brevis*, which is fulfilling all the required quality needed to be a commercial Xylanase, and thus is used by many industries. The enzyme, when studied after modelling, provided similar structural configuration with high stability. When compared with other bacterial and fungal Xylanase structures, it provided better potential to 'activity enhancement' and '*in silico* handling'.

Keywords: Endo-beta-1, 4-D-Xylanase; Glycoside hydrolases; Enzyme modelling; Stability

Background

Hemicellulose is one of the most important polysaccharide found in the cell wall of the woody plants. It is made up of various building blocks, which are heteropolysaccharides found along with cellulose constituting about 20-30% of the wood dry weight [1]. It is the second most abundant polysaccharide after cellulose [2]. Xylan is built from homopolymeric backbone chain of 1, 4-linked β-$_D$-xylopyranose units, including short chains of *O-acetyl*, α-$_L$-arabinofuranosyl and $_D$-glucuronyl or *O*-methyl-$_D$-glucuronyl residues [3]. Complete degradation of xylan requires a concerted and synergistic function of several enzymes - including endo-beta-1, 4-$_D$-Xylanase (EC 3.2.1.8). Xylanase break down the xylan into oligoxylose residues, which are utilized by microbes as primary source of carbon.

Different types of Xylanase have been grouped under the category of Glycoside Hydrolases (GH), which are further classified into various families. These families are classified on the basis of similarities in their amino acid sequences and hydrophobic cluster analysis. Xylanase are classified into many families like 5, 8, 10, 11 and 43 of Glycoside Hydrolases [4]. Xylanase are also classified into two groups, based on their molecular weight and pI. One group has low molecular weight <30 kDa and basic pI, while the other group has higher molecular weight >30 kDa and acidic pI [5]. Xylanase from family 10 (GH10) and family 11 (GH11) of Glycoside Hydrolases are the major and best-studied Xylanase.

GH10 Xylanase generally has higher molecular mass and lower isoelectric point than GH11 Xylanase. It has also been studied and found, that Xylanase of GH10 are mainly involved in hydrolysis of 1,4-beta-$_D$-xylosidic linkages in xylan [4]. Xylanase from family 11 are monospecific, they consist solely of Xylanase activity, exclusively active on D-xylose containing substrates. They are also inactive on aryl cellobiosides [4,6].

Xylanase are produced by diverse species of micro-organisms and have been studied mostly from bacteria, fungi and yeast [7]. The fungi produce high level of Xylanase with high stability and high optimum temperature, but most of them possess residual cellulase activity, which may be due to presence of some amount of hemicellulose in the cellulosic substrates though, selective production of xylanase may be possible using only xylan as the carbon source [8,9]. Bacterial Xylanase are generally free form cellulose activity [10] but their level of production is low as compared with fungal xylanase and most of the industries demand xylanase which is free from cellulose activity. Therefore bacterial Xylanase specially, *Bacillus* sp. has been studied in more detail [11,12].

For making bleaching process eco-friendly and reducing needs for toxic chlorinated and other bleaching compounds, paper and pulp industries are using xylanase as a bio-bleaching agent, which also reduces the kappa number [13]. For bio-bleaching the kraft pulp, industry requires a thermostable and alkali stable cellulase free Xylanase enzyme - having optimum activity at high temperature and pH 6-10 [14-16]. Due to presence of residual cellulase activity fungal xylanase has limited commercial application in paper and pulp industry.

Along with paper and pulp industries, Xylanase have also been used for clarifying fruit juices/wines, - thus enhancing the nutritional value of animal feed, production of bread and for the extraction of coffee [17].

In the present study, we have done *in silico* genomic and proteomic study of *B. brevis* Xylanase. The *B. brevis* Xylanase has 213 amino acid residues. This enzyme possesses two domains, 1) the signal peptide which signals the transfer of the enzyme outside the cell and 2) the main

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glycoside hydrolases domain acting on the xylose moieties [18]. The enzyme is modelled and studied *in silico* to analyse its structure and the stability with comparison with other known structures of xylanase, so that in future we can incorporate the possible changes in the xylanase of *B. brevis* to make it more suitable for its industrial applications.

Materials and Methods

The xylanase gene from *Bacillus brevis* was isolated and successfully cloned in *E. coli* BL 21 for heterologous expression of Xylanase. Sequencing of the cloned gene was done at DUSC, Department of Biochemistry. The sequence was thus converted into six open reading frames, and each frame was translated into specific amino acid sequence, using Star ORF and ORF Finder from NCBI. All the translated specific amino acid sequences were analysed. The translated protein sequences were checked for sequences in the databases. BLAST, performed on the protein sequences, provided the functionality and similarity of the protein with already identified Xylanase enzyme sequences, which was extracted from various organisms, including mostly *Bacillus* sp. [19]. After checking the functionality, the structurally similar sequences were looked for using PDB BLAST. The closely related and identical sequences, whose structure was available in PDB, were selected for building the putative structure of the Xylanase from *B. brevis.* The structures and sequences were also checked for the conserved domains present to verify the functionality of the protein. This was performed using the CD Search at NCBI [20]. The sequence, when verified, was recognized to have two domains, and the signal peptide sequence was recognized. The signal peptide sequence was identified using the SignalP 4.1 Server [21]. The sequences of the nearly identical Xylanase, whose structure was available in PDB, as received from the PDB BLAST result, were used to predict the structure of our target *B. brevis* Xylanase. For the structure prediction MODELLER 9.11 was used [22]. Along with MODELLER, Schrodinger–PRIME and Swiss Model Workspace were used side by side, for verification of predicted structures. The homology modelling concept was used, and structures used for the putative structure prediction were 1XXN_A from *B. subtilis*, 2QZ3_A from *B. subtilis,* 3LB9_A from *B. circulans* and 1HV1_A also from *B. circulans.* The structure was verified using the SAVES server, the Structural Analysis and Verification Server, providing the Ramachandran plot, verify_3D and Errat results [23-25]. The predicted structure was compared with that of fungal Xylanase, the *A. niger* Xylanase 2QZ2_A 183 residue structure and the P55329 as the Uniprot ID. The comparison was also conducted at the active site level, using the active site prediction tool i.e. ACTIVE SITE PREDICTION SERVER [26]. The comparison of structure was also performed for the Xylanase produced by fungal and bacterial Xylanase, along with that of our query Xylanase of *B. brevis,* using the PDBeFold the structural alignment tool [27].

Result and Discussion

Sequence analysis

The Xylanase from *B. brevis* was found to be 213 amino acids long, as translated from the ORF Finder from NCBI. The +1 frame gave the best result of 213 amino acids length - derived from the 642 base pair size of the coding region, taken from the contig 4,7,3,2 and 9 as numbered, of size 921 base pair. The nucleotide sequence, when translated, produced 213 amino acids protein sequence, having only one conserved domain of GH11, available in Pfam with the ID 00457. All the members of the family GH 11 possess this domain, which is specifically involved in beta-1, 4-D glycosidic bond breakage. The other important region that was found in this sequence, was the signal peptide coded by the sequence having initial 28 amino acids, and involved in transporting the enzyme outside the cell as predicted by SignalP 4.1, as depicted in Figure 1.

Though all the Contig were identical, the nucleotide sequences were having differences in the 5′UTR and 3′UTR region which can be clearly seen in the multiple sequence alignment of all in Figure 2. The Contig 9 gave different results, due to differences with absence of 3 C-terminal amino acid, as the residues Threonine, Tryptophan and Valine were missing. In Contig 3 the Glycine residue at 131st position was missing, visible in Figures 3 and 4.

Structure prediction

The PDB BLAST performed gave the structurally similar sequences for predicting the structure of *B. brevis* Xylanase. Structure, using MODELLER 9.11., was predicted for all translated contig, the contig 4, 2 and 7 gave identical structure as the translated protein sequence was also identical (Table 1).

The predicted structure was refined in Schrodinger and visualized

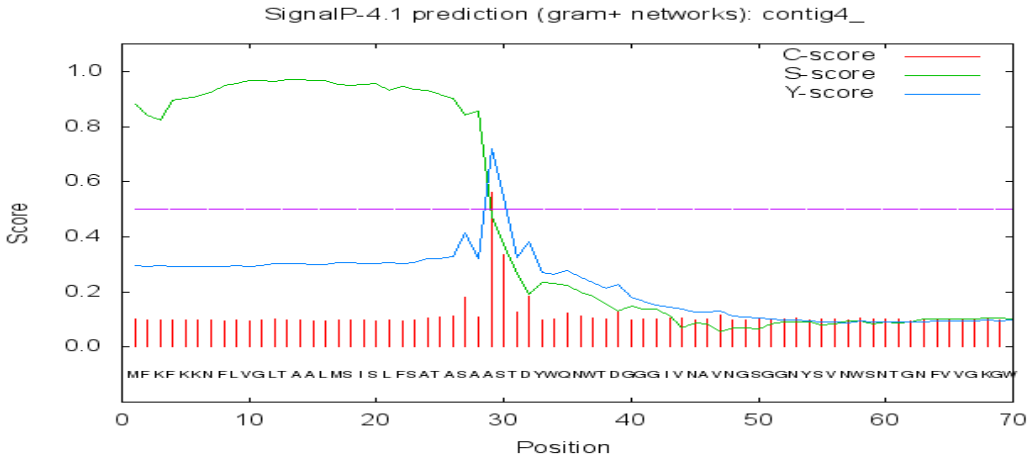

Figure 1: The graph represents the probability of initial 28 amino acids just before Alanine to be a part of signal peptide Cleavage site between pos. 28 and 29: ASA-AS D=0.801 D-cutoff=0.450.

Figure 2: Multiple sequence alignment result of all Contig aligned, showing differences in 5′and 3′ UTRs.

Figure 3: Multiple sequence alignment of the translated Contig 99% identical with only one Glycine residue at 131st position in Contig 3 missing highlighted in yellow colour.

Organism Name	PDB ID	Percentage Identity
Bacillus subtilis	1XXN_A	86%
Bacillus subtilis	3QZ3_A	86%
Bacillus circulans	3LB9_A	95%
Bacillus circulans	1HV1_A	86%

Table 1: The structures selected for the structure prediction by homology modelling of the query xylanase sequence from *Bacillus brevis,* using MODELLER 9.11 and Schrodinger-PRIME.

in PyMol. The predicted structure showed 12 beta sheets/strands, and only 1 alpha helix. The predicted active site was at 106th amino acid - the Glutamic acid acting as the nucleophile, and 200th amino acid (also a Glutamic acid) as proton donor. The Aspartic acid is placed exactly at the tip of the thumb, to interact with the substrate to catch hold of it and carry on the required activity.

Structure validation

The SAVES server gave qualified results considering the predicted structure. The Ramachandran plot gave values ranging 85.6% to 86.1% of the amino acids, which were found in the allowable region. The

Contig No.	Procheck	Verify 3D	Errat
Contig 4	86.1%	100%	94.350%
Contig 2	86.4%	100%	94.350%
Contig 3	86.1%	100%	94.22%
Contig 7	86.1%	100%	94.350%
Contig 9	85.8%	100%	93.642%

Table 2: Represents the Structure analysis and verification scores which clearly specify that Contig 4, 2 and 7 are exactly the same while Contig 9 and 3 have some variations.

Verify_3D gave results of having 100% of the residues averaged under 3D-1D score >0.2, specific for crystal structure comparison. The scores verified that the structure predicted was stable, of high quality and appropriate for further in-silico analysis (Table 2).

Comparative analysis

The structures, when superimposed, gave the above results of exactly identical structure. Structure alignment was performed using Pymol. The detailed analysis of the structures provided the facts, which clearly depicted active Xylanase. It had the required characteristics of

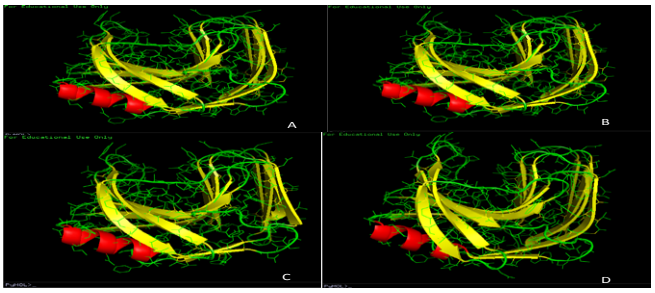

Figure 4: Structures predicted using MODELLER9.11, Swiss Model and Schrodinger-PRIME visualized using Pymol software licensed for academic purpose, showing the cartoon structure. (A) Contig 4 and 7. (B) Contig 2. (C) Contig 9. (D) Contig 3.

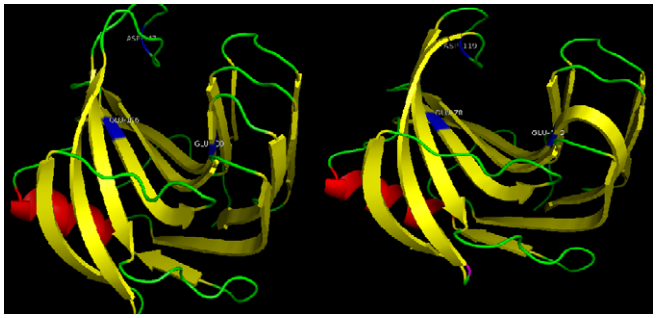

Figure 5: The predicted active sites labelled and colored blue in PyMol, are the Glutamic acid, as 106 and 200, when taking initial amino acid count as 29 and at position 78 and 172 when counted initial residue as 1. The labelled Aspartic acid at the thumb loop at position 146/119 plays important role in substrate binding.

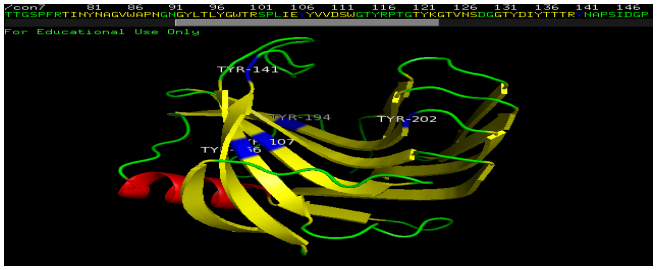

Figure 6: The labelled Tyrosine are placed accurately at the specified positions which is important for the xylan binding domain.

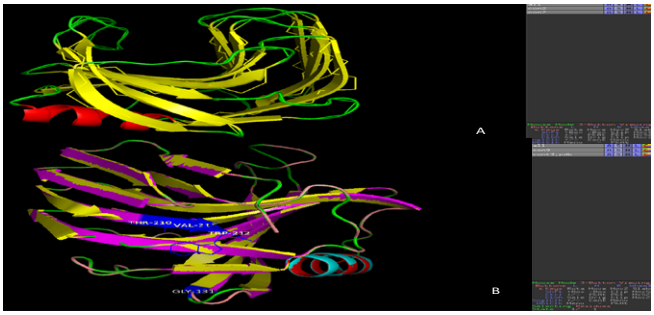

Figure 7: The superimposition of the structure. (A) Contig 7 and 2 superimposed. (B) Shows structure-structure alignment of Contig 3 and 9, the blue colour residues are the non-identical residues which did not superimpose.

##	Scoring			RMSD	N_{align}	N_g	$\%_{seq}$	Query			
	Q	P	Z					$\%_{sse}$	Match	$\%_{sse}$	N_{res}
1	0.97	36.5	18.1	0.49	185	0	96	100	1bcx:A	100	185

##	Scoring			RMSD	N_{align}	N_g	$\%_{seq}$	Query			
	Q	P	Z					$\%_{sse}$	Match	$\%_{sse}$	N_{res}
1	0.72	17.9	12.6	1.34	171	8	43	93	2qz2:A	81	183

Figure 8: Structure-Structure alignment of the xylanase of *B. brevis* with *B. circulans* and *A. niger.*

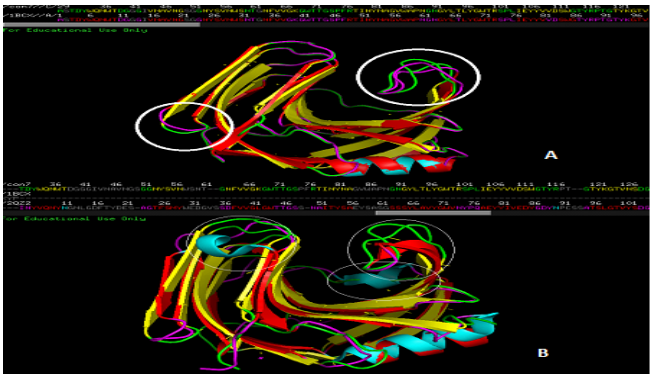

Figure 9: The superimposed structures A: has *Bacillus brevis* xylanase aligned with *B. circulans* which is nearly identical with the circled area showing difference. B: has *Bacillus brevis* xylanase aligned with *A. niger* xylanase which is different at various area having helices at two more places not found in *B. brevis* xylanase.

having the active proton donors, and binding catalytic sites at the most probable locations as visible in the Figures 5 and 6. The positioning of Tyrosine and Aspartic acid, in the palm and the thumb area, clearly showed the enzyme ready for the binding with the substrate and catalytic activity. The alignment of structure shown in Figure 7 labelled Threonine, tryptophan and Valine at C-terminal end, while Glycine in between as non-superimposed amino acid in Contig 9. The structural comparison, with other Xylanase enzyme structure belonging to bacteria and fungi already reported, clarified the result. The structure of *B. circulans* and *B. subtilis* Xylanase also had mainly beta sheet structure and had only 1 alpha helix, which was similar to the structure predicted for our Xylanase of *B. brevis*. The structural comparison of *B. circulans* 1BCX_A and that of our Xylanase, gave 100% similarity in the secondary structure elements similar in both, with 96% of the sequence identity, as predicted by PDBeFOLD. In case of fungal Xylanase, the *A. niger* 2QZ2_A and our Xylanase, it was having 93% similar secondary structure elements, while the sequence identity was only 43% in the structure-structure alignment in PDBeFOLD, as shown in the Figure 8. The Figure 9 clearly specifies the structural differences between bacterial and fungal Xylanase. The structure of bacterial Xylanase of *B. brevis* and *B. circulans* is completely identical with minute differences at the loop areas. The fungal Xylanase of *A. niger* was seen to have the alpha helices, as compared to bacterial Xylanase with one small helix. Not just the helical region, but also various loop regions were different, though both belonged to class GH 11. The one thing, which was found to be similar in all the Xylanase, was that the nucleophile and proton donor is always the Glutamic acid, though the position can change.

These active sites were present in the beta sheets, generally in the beginning or at the end of the beta sheet.

The need of the hour for pulp and paper industry is a stable and pure Xylanase, cellulase-free enzyme, easy to handle, with higher activity at higher temperature ranging from 50-80°C and pH 6-10. The studied bacterial Xylanase is not only pure but also stable at alkaline pH and higher temperature, without any cellulase activity, which is the most specific requirement of pulp and paper industry.

Conclusion

The Xylanase have numerous important industrial application, thus its production and purity is of utmost importance. The fungal Xylanase, though produced in higher quantity, have not been purified with sole Xylanase activity. Thus the bacterial Xylanase is free from cellulase activity and are the need of the day. Due to specific Xylanase activity, bacterial xylanases have great industrial applications. Among them *B. brevis* is one of the most promising candidate which produces endo-1, 4-beta Xylanase. The small sequence and sequence identity, with predicted structure, provides a platform for in silico enhancement of the activity of the enzyme. The predicted structure falls in the same frame, as of the known Xylanase of *B. circulans, B. subtilis*, whose structures have been crystallized. The active site was located as the energy rich proton donor, the Glutamic acid being at both the positions 106 and 200 in the structure. The catalytic site involved the Tyrosine and Aspartic acid, located exactly in the thumb flexible region, involved in the activity – hence known as xylan binding residues.

The predicted structure of *B. brevis* Xylanase belonged clearly to GH11 family, with a well packed structure mainly constituted of β-pleated sheets. The palm and thumb loops were clearly depicted, which was a cleft where the substrate came and bound to lead to the required product. The compact and pleated structure may be one of the reasons for the thermostability of the enzyme, which can be further enhanced by increasing the electrostatic interactions and disulphide S-S, bridges, which is one of the most important features of the enzyme to be applicable in paper and pulp industry. The fungal Xylanase being bigger in size and acidic pH as the stability medium-*B. brevis* Xylanase with apt size and alkaline pH stability is the most promising enzyme to work upon for industry purpose.

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