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Homology Modeling, Docking and Active Site Analysis of Xylanase from *Coprinus cinereus*

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Abstract

Xylanase, an industrially important enzyme has a wide range of applications, especially in the pulp and paper industry. It is responsible for the hydrolysis of linear polysaccharide β-1, 4-xylan into xylose, thus breaking down hemicellulose which plays a major role in paper bleaching. Due to its wide range of applicability at industry level, it is important to understand the structural and functional aspects of xylanase. It is isolated and purified for the first time from *Coprinus cinereus*. The homology modeling approach was adopted to explore the structure of xylanase from *C. cinereus*. The purified xylanase from *C. cinereus* was studied for Peptide mass fingerprinting analysis which showed 54.3% similarity with *Coprinopsis cinerea* okayama 7 # 130 protein Accession gi|169855830 β-1, 4-endo xylanase retrieved from NCBI database. Its 3-D model was generated on the basis of crystal structure of 1XNK-A chain of xylanase from *Chaetomium thermophilum* by employing online server SWISS-MODEL. The template from *C. thermophilum* showed 65% similarity with target sequence. The model was verified and validated on SAVES (Structure Analysis and Verification Server) and PROCHECK programs, respectively. Ramachandran plot obtained through PROCHECK program revealed 99.4% of the total residues were in the allowed region and 0.6% in the generously allowed region and no residues were found to be in the disallowed region. Modeled xylanase overlapping with template 1XNK-A stipulates the amino acid residues Asn35, Tyr68, Arg113, Ser118, Tyr168 and Glu174 that constitute active site of the enzyme. Docking studies of enzyme and substrate revealed the substrate-binding site of modeled xylanase contained six subsites, defined as -3, -2, -1, +1, +2, and +3. Therefore, the present study put forth the three dimensional structure, active site machinery, and enzyme–substrate interactions of *C. cinereus* xylanase.

***Keywords*:** Xylan, *Coprinus cinereus*, Xylanase, Homology modeling, Active site

Xylanase is widespread among actinomycetes (Kansoh and Nagieb, 2004, Rawashdeh *et al*., 2005), fungi (Kitamoto *et al*., 1999, Silva *et al*., 1999, Taneja *et al*., 2002) and bacteria (Baitaillon *et al*., 2000, Gessesse and Mamo, 1998, Wong

et al., 1988). Some of the most important xylanolytic enzyme producers include the *Aspergillus* (Raj and Chandra, 1996, Silva *et al*., 1999), *Trichoderma* (Tan *et al*., 1985), *Streptomycetes* (Keskar *et al*., 1989), *Phanerochaetes* (Dobozi *et al*., 1992) and *Bacillus sp.* (Nakamura *et al*., 1993), which catalyzes the breakdown of the xylan polymer into xylose. Filamentous fungi are particularly interesting; they secrete xylanase in an extracellular medium which results in a higher enzyme level than that of yeast and bacteria. As such, xylanase plays a major role in micro-organisms thriving on plant sources. Additionally, xylanase are present in fungi for the degradation of plant matter into usable nutrients. Xylanases are one of the most widely used enzymes in industries such as the pulp and paper industry, food and feed industry and bio-ethanol industry. The applications include the chlorine-free bleaching of wood pulp prior to the papermaking process; improve the dough's workability, increases the digestibility of silage and fermentative composting (Viikari *et al*., 1992).

Xylanase breaks down hemicellulose mainly xylan into xylose. Hemicellulosic polysaccharides are complex molecules that associate with cellulose microfibrils, providing a cross-linked matrix (Lerouxel *et al*., 2006). Xyloglucan and Arabinoxylan are two of the most abundant hemicelluloses. Xyloglucan has a similar backbone to cellulose, but is decorated with xylose branches on three out of four glucose residues and can also be serially appended with galactose and fucose residues. However, arabinoxylan consists of a β-D-xylan (1, 4-linked) backbone with arabinose branches (Cosgrove, 2005). Xylan is the second most abundant polysaccharide found in nature after cellulose. It is a polymer consisting mainly of β-D-xylopyranoside residues linked by β-1, 4-glycosidic bonds. Due to xylan's heterogeneity and complexity, three main groups of glycoside hydrolases are involved in its multi-step degradation. Xylanases (β-1, 4-D-xylan hydrolase, E.C. 3.2.1.8) hydrolyse internal β-1, 4 xylosidic bonds of the xylan backbone, α- Glucuronidases (E.C. 3.2.1.55) breaks apart glucuronic acids attached to the xylan molecule and β-xylosidases (β-1, 4-D-xylan xylohydrolases, E.C. 3.2.1.37) complete the hydrolysis of xylan into free xylose (Collins *et al*., 2005). These glycoside hydrolases show potential applications in different industrial fields (Beg *et al*., 2001). The study of microbial xylanase production has been extensively reviewed by several groups (Bastawde, 1992, Biely, 1985, Haltrich *et al*., 1996, Sunna, and Antranikian, 1997, Thomson, 1993, Wong *et al*., 1988).

Xylanase enzyme when used for pulp treatment should not accompany any cellulolytic activity as it adversely affects the quality of the paper pulp. Some of the initial approaches to overcome cellulase activity in xylanase preparation included treatment with mercurial compounds to selectively inhibit cellulase or cloning and selective expression of xylanase genes in heterologous host systems. The current best practice is to screen for naturally occurring microbial strains that are capable of secreting cellulase-free xylanases under optimized fermentation

conditions. A summary of such organisms and their enzymatic characteristics presented by Srinivasan and Rele (1992) provide information and data on the efficacy of xylanase enzymes in pulp biotechnology.

In order to illuminate the structural and functional aspects of xylanase, the three dimensional protein structures provide valuable insights into the molecular basis, allowing an effective design of experiments, such as site-directed mutagenesis and the structure based design of specific inhibitors. Homology modeling of purified xylanase from *C. cinereus* was performed by SWISS-MODEL, fully automated protein structure homology-modeling server, based on expert system dedicated to homology modeling of protein 3D structures (Schwede *et al.,* 2003). As X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR), are still a time-consuming process without guaranteed success. The SWISS-MODEL workspace integrates programs and databases required for homology modeling in an easy-to-use web-based modeling workbench. It allows the user to construct the comparative protein models from a computer with web connection without the need of downloading and installing large program packages and databases (Arnold *et al*., 2006).

On the basis of the amino acid sequence similarities, the xylanases are generally classified into glycosyl hydrolase family 10 and 11 (Henrissat, 1991, Henrissat and Bairoch, 1993) and a few of the xylanases belongs to family 5, 7, 8 and 43 (Collins *et al*., 2002, Larson *et al*., 2003, Parkkinen *et al*., 2004) (http://afmb.cnrs-mrs.fr/CAZY/). The three dimensional structure of family 11 xylanases have one single catalytic domain with a β-jelly roll fold conformation (Törrönen *et al*., 1994). For understanding the substrate specificity and catalytic mechanism of the enzymes, the study of substrate binding sites is necessary (Sidhu *et al*., 1999). The substrate binding sites of glycosyl hydrolases often comprise aromatic residues, mostly Tyrosine and Tryptophan, which can pack against the sugar units as well as the amino acid residues that form hydrogen bonds with the hydroxyl group of substrate (Vyas, 1991).

Materials and Methods

Isolation of fungus *C. cinereus* from the environment

The fungal strain was isolated using enrichment method from a decaying wood sample collected from the vicinity of Forest Research Institute (FRI), Dehradun, India. The fungus was identified based on the NCBI-BLAST search of the ITS/5.8S-rRNA gene sequence data at MTCC and Gene bank, Institute of Microbial Technology, Chandigarh, India. The research began with purified xylanase which was studied for Peptide mass fingerprinting analysis for the confirmation of xylanase (CIF, JNU, New Delhi).

The target protein sequence and alignment with template sequence

As part of ongoing effort to study the structure and catalytic site analysis of xylanase, the peptide sequence obtained by Peptide mass fingerprinting analysis was matched against the NCBI database and the FASTA protein sequence database (Mascot search). Then the Blastp (Protein BLAST) was performed to study the homology among the various xylanases. As a result the sequence coverage of the peptide against the *Coprinopsis cinerea* okayama 7 # 130 protein Accession gi|169855830 β-1, 4-endoxylanase reached 54.3%, which belongs to the glycosyl hydrolase11 family and was used for further studies.

The protein sequence of xylanase from *C. cinereus* (Stajich *et al*., 2010) was retrieved from the NCBI protein sequence data base with accession number XP_001834579 (http://www.ncbi.nlm.nih.gov/protein). The signal sequence was not considered during this study because in eukaryotic cell signal sequence is cleaved after it is translocated to its specific target site for its further modifications. According to the RCSB Protein Data Bank the X-ray crystal structure of xylanase from *C. cinereus* is not publically available. So homology modeling approach was adopted for structural and functional study of catalytic site. The xylanase from *C. cinereus* consist of 186 amino acids. The PSI- BLAST program conducted on so obtained amino acid sequences revealed about the xylanase related three dimensional structures as a template (Altschul *et al*., 1997). The most suitable high-resolution X-ray crystallography structure of the xylanase 1XNK-A from *Chaetomium thermophilum* was selected as the template protein (Jänis *et al*., 2005). The multiple sequence alignment of target and template protein sequence was performed by ClustalW, a general purpose multiple sequence alignment program for DNA and proteins.

Xylanase homology modeling

Xylanase homology modeling was performed with the help of SWISS-MODEL a homology modeling server (Bordoli *et al*., 2009). After aligning, the target and template (1XNK-A) sequences were used as input in SWISS-MODEL alignment mode. SWISS-MODEL derives the restraints automatically from related known structure provided as template. The validated model was chosen for further studies and refinements.

Model optimization and evaluation

It is essential to minimize the energy for regularization of local bond, angle of geometry and to relax close contacts in the geometric chain because the modeled structure produces unfavorable bond length, bond angles, and torsion angles. The final 3D model of xylanase was verified using the Structural Analysis and Verification Server (SAVES) which has various programs such as PROCHECK,

WHAT CHECK, and VERIFY 3D (http://nihserver.mbi.ucla.edu/SAVES). The stereochemical quality and overall structural geometry of the xylanase model were confirmed by the PROCHECK program (Laskowski *et al*., 1994), however, the stereo- chemical parameters were ensured by the WHAT CHECK program (Hooft *et al*., 1996, Vriend, 1990) The compatibility of an atomic model (3D) with its own amino acid sequence (1D) was done by assigning a structural class on the basis of its location, environment (alpha, beta, loop, polar, non-polar, etc.), and results comparison with excellent database structures were determined by the VERIFY 3D program (Bowie, *et al*., 1991).

Active site analysis

The catalytic activity of an enzyme is performed by a small number of highly conserved residues within the active site and it remains conserved among distantly related enzymes. Identification of the active site includes superimposition of the three dimensional model with a template which provides integrity to the homology model and helps in identification and location of conserved active site residues. The information about the active site was obtained for template structure from Catalytics Site Atlas (CSA) database of European Bioinformatics Institute (http:// www.ebi.ac.uk/thorntonsrv/ databases/CSA/) (Porter *et al*., 2004).

Docking study of modeled xylanase by PatchDock

The docking study was performed by using Patchdock, a geometry-based molecular docking algorithm (Duhovny *et al*., 2002). It is aimed at finding docking transformations that yield good molecular shape complementarity. Such transformations, when applied, induce both wide interface areas and small amounts of steric clashes. A wide interface is ensured to include several matched local features of the docked molecules that have complementary characteristics (Schneidman-Duhovny *et al.,* 2005). PatchDock has different sets of parameters, optimized for different types of complexes. In this study, Xyloheptaose (X_7) (Xyloheptaose was selected primarily because xylanase binds with the polymer of xylose sugar and secondarily we wanted to gather the complete information regarding the interaction between the active site of enzyme and specific residues of the different monomers of the polymer.) was used for docking study with xylanase in Protein-small ligand complex type optional field with the 4Å default value of clustering Root mean square deviation (RMSD).

Results and Discussion

Xylanase homology modeling

C. cinereus was isolated from a decaying wood sample and identified based on the NCBI- BLAST search of the ITS/5.8S rRNA gene sequence data at microbial type culture collection (MTCC) and gene bank. Xylanase was extracted and purified

from *C. cinereus*. The purified xylanase was further studied for confirmation of the protein by Peptide mass fingerprinting techniques. Homology template search for *C. cinereus* xylanase resulted into a large number of sequences by running PSI-BLAST against Protein data base (PDB) database. The target sequence showed high identity (65%) with 1XNK chain A of xylanase family 11 from *C. thermophilum*. 1XNK-A (*C. thermophilum*), 1XYP (*Trichodrema reesei*), and 1IGO (*Bacillus subtilis*) were used in multiple sequence alignment performed by clustalW as described in Figure 1. The large sequence similarity of *C. cinereus* xylanase with *C. thermophilum* strongly supports that these two enzymes have evolved from a common ancestor, particularly glycosyl hydrolases family 11.

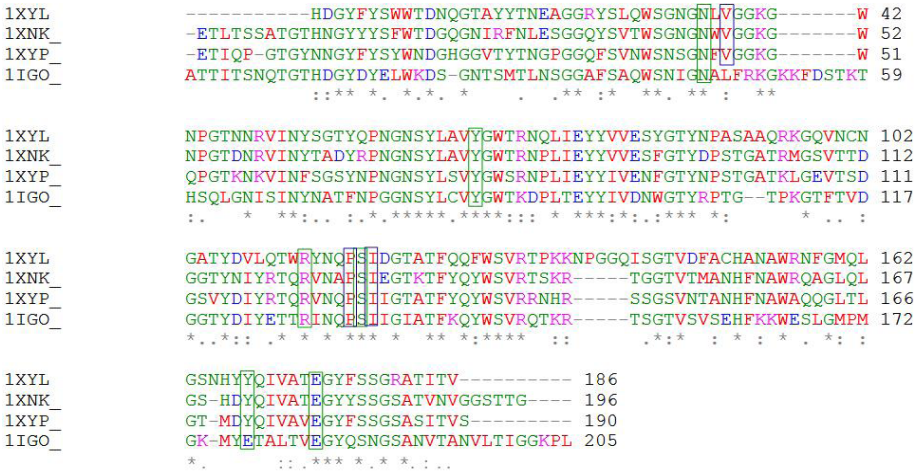

Fig. 1. Multiple sequence alignment of target (1XYL) and templates C. thermophilum (PDB ID: 1XNK-A), T. reesei (PDB ID: 1XYP) and B. subtilis (PDB ID: 1IGO). The important residues from active site point of view are highlighted with green rectangles and hydrophobic residues with blue rectangles. The residual variations in active site are highlighted with red rectangles

Xylanase structure

Due to the availability of the X-ray crystallographic structure of 1XNK-A from *C. thermophilum*, it was selected as a template for homology modeling of xylanase from *C. cinereus*. After the homology modeling with the help of online server SWISS-MODEL, Ramachandran plot drawn through PROCHECK program validated the model with 85.1% of total residues confined in the core region and 14.3% in additional allowed region while 0.6% residues in generously allowed region and no residue in the disallowed region (Figure 2). This confirms that the protein back bone dihedral angles phi (φ) and psi (ψ) occupied reasonably accurate positions in the 3-D model. The 3-D model structure of xylanase of *C.*

cinereus was also compared against protein database (PDB) using DaliLiteV3.1 server (Hasegawa and Holm, 2009) which illustrated RMSD 0.3, identity 66% and Z score value 35.2 with template1XNK-A. The 186 amino acid residues of *C. cinereus* xylanase were folded into a domain (β-jelly roll) structure comprising two parallel β-sheets and a single α-helix, like other enzymes particularly in glycoside hydrolases family 11 (http://www.cazy.org/GH11.html) in which the substrate-binding groove was formed by the concave face of one of the β-sheets. The homology modeled structure of xylanase is shown in Figure 3.

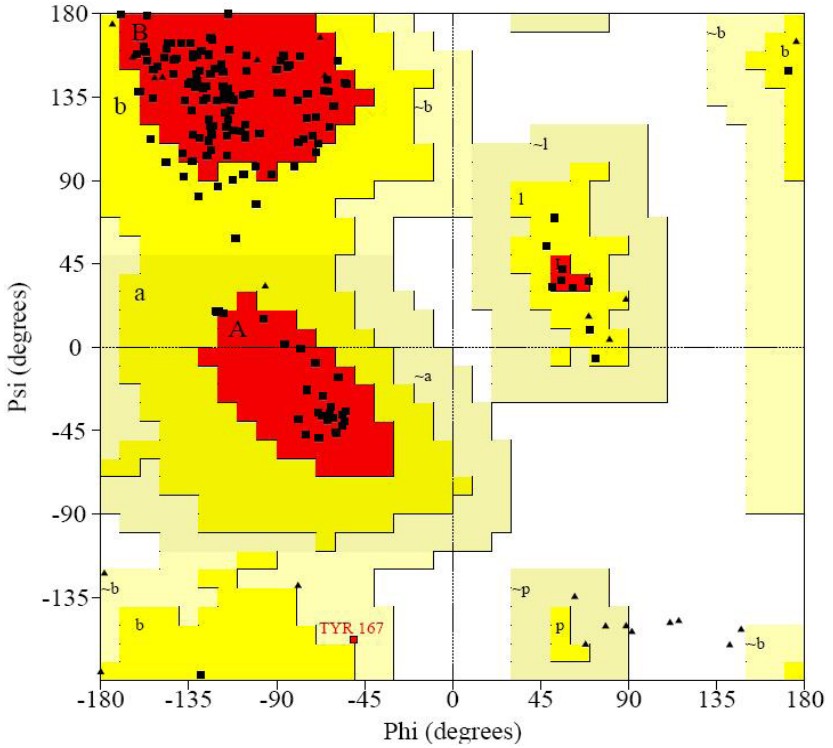

Fig. 2. Ramachandran plot of modeled xylanase obtained by PROCHECK validation package.

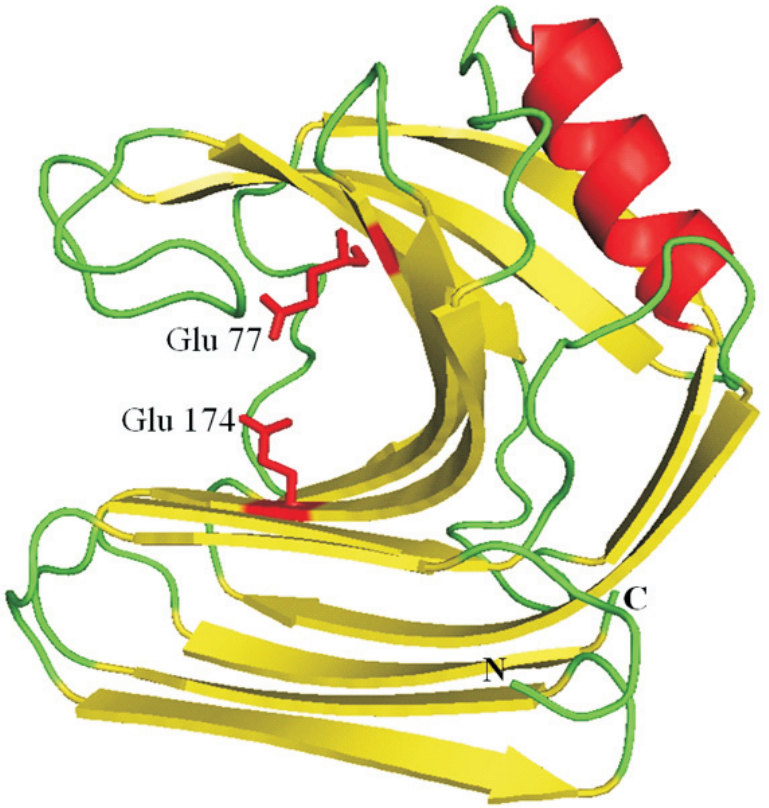

Fig. 3. Ribbon diagram of homology-modeled structure of xylanase from Coprinus cinereus. A single domain (β-jelly roll) structure comprising two parallel β-sheets and a single α-helix, like other enzymes of glycoside hydrolases family 11.

Active site analysis

The Information about the active site was obtained through superimposing 3-D model structure of the target enzyme with that of template protein 1XNK-A from *C. thermophilum*, which provided accuracy of homology between the structures, and also helped in positioning the conserved active site residues. Information related to active site of the template structure was obtained from Catalytic Site Atlas (CSA) data base of European Bioinformatics Institute (http://www.ebi.ac.uk/thornton-srv/databases/CSA/) and by overlapping with target protein (Figure 4).

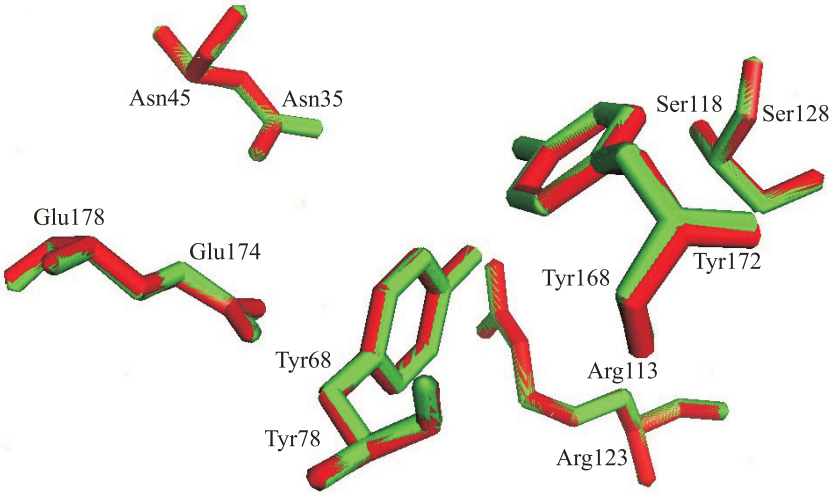

Fig. 4. Superimposition of active site residues of modeled xylanase and template1XNK-A. Green and red color sticks represents modeled and template proteins, respectively.

Docking of substrate into the active site

For the interaction study, the most important requirement was the proper orientation and conformation of substrate (xyloheptaose, X_7) which is to be fitted into the modeled xylanase active site appropriately and form enzyme substrate complex. Therefore, optimal interactions and the best Patchdock score were used as criteria to describe the best conformation (Figure 5) among the 30 conformations, generated by Patchdock server. The findings of this docking study were in accordance with earlier results (Esteves *et al*., 2004, Jommuengbout *et al*. 2009).

The hydrogen bonding and stacking interaction are very important aspects to study subsites in substrate binding sites of xylanases because both are actually involved in the binding mechanism between xylanases and xylose units of xylans before the enzymatic hydrolysis reaction. They are also aimed to determine the potential amino acid residues that formed bond with xylose unit at individual subsite (-3 to +3) of modeled xylanase. Due to the modeled xylanase consisting of six subsites, the modeled Xylanase-X_7 complex was represented.

The neighboring amino acid which possibly form hydrogen bonds and stacking interactions with the bound X_7 at individual subsites are shown in Figure 5. Most of the aromatic residues in each subsite potentially formed both hydrogen bond and stacking interaction, except Tyr87, Tyr79 and Tyr167 at subsite -3, -1 and +3 respectively, which formed only stacking interaction.

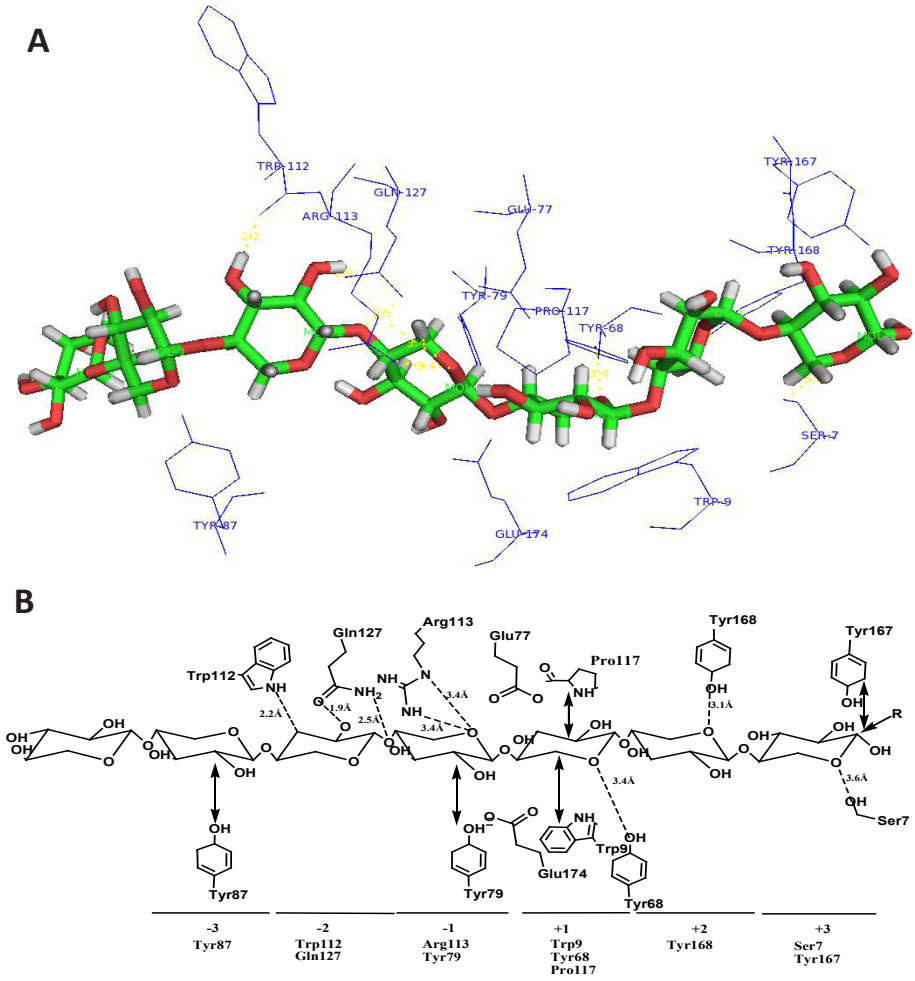

Fig. 5. The binding interaction of modeled xylanase and Xyloheptaose.

A-Ligand–protein interactions as obtained by docking studies. Blue color sticks represents the active site residues where as Xyloheptaose is represented by multicolored sticks. **B**-Schematic representation of Xylanase-X7 complex showed possible hydrogen bond (---) and stacking interaction (↔) in the individual subsites. R symbol indicate the reducing end.

The overall structure of xylanase from *C. cinereus* was found to be almost similar to that of 1XNK-A from *C. thermophilum* as revealed by superimposing 3-D structures and DaliLite V3.1 server. These features conclude that the two xylanases of different origins have evolved from a common ancestor and play a pivotal role in xylan degrading mechanism. The overall structure of xylanase resembled a closed right hand. The most predominant feature of the structure was

a long open cleft containing the active site. In active site, two conserved glutamic acid residues (Glu77 and Glu174), located on either side of the cleft, play a pivotal role in catalysis of the degradation of xylan (Miao *et al.,* 1994). The xylanase structure was further investigated to study the substrate binding site. Active site was represented by amino acid residues Asn35, Tyr68, Arg113, Ser118, Tyr168 and Glu174 corresponding to Asn45, Tyr78, Arg123, Ser128, Tyr172 and Glu178 of template protein. Thus, amino acids involved in active site formation were found to be highly conserved among xylanases (Figure 1 and 4). Active site analysis also showed the presence of a hydrophobic pocket in the target protein constituted by residues Val37, Tyr68, Pro117, Ile119 and Tyr168. Amino acids associated in hydrophobic pocket constitution were also observed to be strictly conserved among xylanases. The catalytic nucleophile Glu174 in target protein was corresponding to the catalytic nucleophile Glu178 in template protein whereas the second catalytic residue Gln77 was corresponding to Gln87 of template protein.

For hydrogen bonds in non-reducing end direction, the xylose moiety at subsite -1 formed hydrogen bonds between its O5 position to the side chains of Arg113. The hydroxyl groups of xylose moiety at subsite -2 formed hydrogen bond with side chain of Gln127 at OH2 and Trp112 at OH3. At subsite -3, hydrogen bond was not found, only Tyr87 formed stacking interaction to xylose moiety. At reducing end, the xylose unit formed hydrogen bond at O5 position to the side chain of Tyr68, while Trp9 and Pro117 formed stacking interaction at subsite +1. The side chain of Tyr168 and Ser7 formed hydrogen bonds at O5 position of the xylose unit at subsite +2 and subsite +3. The potential residues in individual subsite of modeled xylanase were compared to other family 11 xylanases whose binding sites have been studied (Table 1).

Arg113 at the subsite -1 of modeled xylanase resembled the same subsite of X-ray complex structure of XlnA from *B. circulans* (Wakarchuk *et al.,* 1994) and Xyl11 from *B. agaradhaerences* (Sabini *et al.,* 1999) and also with the subsite of computational structure of Xyn11A from *B. firmus* K-1(Jommuengbout *et al.,* 2009). As Tyr80 of Xyn11A from *B. firmus* K-1 and Tyr80 of XlnA from *B. circulans* (Wakarchuk *et al.,* 1994) are involved in the substrate binding similarly Tyr79 of modeled xylanase showed extremely critical role in substrate binding at the same subsite -1. Tyr68, at subsite +1, of modeled xylanase corresponded to the Xyn11A from *B. firmus* K-1(Jommuengbout *et al*., 2009). Tyr168, at subsite+2, of modeled xylanase resembled the same subsite of X-ray complex/computational structure of (Tyr179) XYNII from *T. ressei* (Törrönen and Rouvinen, 1995). Tyr167 forming stacking interaction with xylose, at subsite +3, corresponded to the Tyr173 of Xyn11A from *B. firmus* K-1(Jommuengbout *et al*., 2009). On the other hand, subsite +1 (Pro117 and Trp9), subsite +3 (Ser7), subsite -3 (Tyr87) and subsite -2 (Gln127 and Trp112) of modeled xylanase were not similar to the substrate binding sites of family 11 xylanases (Table 1).

Table 1. Comparison of the amino acid residues in each subsite of modeled xylanase and other family 11 xylanases

S. No.	**Family 11 xylanases**	Strategy	Amino acid residues in individual subsite						References
			-3	-2	-1	+1	+2	+3	
1.	Modeled xylanase from *C. cinereus*	Computational method	Y87	W112, Q127	R113, Y79	W9, Y68, P117	Y168	S7, Y167	This study
2.	Xyn11 from *B.firmus* K-1	Computational method	Y165	Q7, W9, Y69	Y80, R112, P116	Y65, Q126	Y88	Y173	Jommuengbout *et al.*, 2009
3.	XlnA from *B. circulans*	X-Ray crystallography		W9, Y69, Y166	Y80, R112, P116				Warkarchuck *et al.*, 1994
4.	Xyl11 from *B. agaradhaerens*	X-Ray crystallography		E17, W19, R49, Y85	Y85, R129, P133				Sabini *et al.*, 1999
5.	XYNII from *T. reesei*	X-Ray crystallography / Computational method		W18	-	-	Y179	Y96	Törrönen and Rouvinen, 1995

Conclusion

Thus the substrate binding site of modeled xylanase consisted mostly of tyrosine and tryptophan, which pack the xylose units and the amino acid residues that form hydrogen bonds with the substrate. Thus, the difference in the amino acid composition in each subsite showed that various family 11 xylanases have distinct substrate binding sites.

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