Expression of a copper activated xylanase in yeast: Differential properties of C-tagged or untagged recombinant xylanases

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Research Article

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Abstract

Background: Endo-xylanase (EC 3.2.1.8), which hydrolyze the backbone structure of β-1,4-xylans, can be used together with laccase (activated by copper) to hydrolyse lignocellulosic and hemicellulosic materials in bioethanol production. A previous study described the cloning of the xylanase gene from *Aspergillus niger* US368 and obtaining the recombinant His-tagged protein using *E. coli* expression system. This recombinant xylanase was activated by copper which is a strong inhibitor for xylanases activities. The activation of the enzyme toward copper is due to the 36 extra amino acids provided by the vector pET28a. However, the expression of this xylanase was relatively low and intracellular that limits its potential industrial application. Hence, we aimed to over-express this interesting enzyme using the *Pichia pastoris* system for use in biofuel production.

Results: A copper activated xylanase produced by *E. coli* BL21 was expressed in *Pichia pastoris* using the pGAPZαB expression vector. Two recombinant xylanase forms were obtained (Non-C-terminal tagged-His-tagged r-XAn11 and C-terminal tagged-His-tagged r-XAn11). The findings revealed that the two recombinant xylanases displayed different behaviors toward the copper. In the presence of 3 mM Cu^{2+}, the relative activity of the Non-C-terminal tagged-His-tagged r-XAn11 was enhanced by about 52%. However, the xylanase activity of the C-terminal tagged one was strongly inhibited by copper. The Non-C-terminal tagged recombinant enzyme was more thermostable, in the presence of 3 mM Cu^{2+}, than the C-terminal tagged one with a half-life of 10 min at 60 °C. 3D models of the two recombinant forms were constructed. The results showed that the created copper site in the Non-C terminal tagged protein was loosed in the C terminal tagged protein due to the high fluctuation and probably new interactions among the C and N-terminal amino acids.

Conclusion: The present work reports the expression and purification of two recombinant xylanases forms (non C-terminal tagged and C-terminal tagged His-tagged r-XAn11).

Background

Hemicellulosic material constitutes around 30–35% of hardwood, 15–30% of graminaceous plants, and 7–12% of gymnosperms [1, 2, 3]. The main heteropolymers of the hemicellulosic component are xylan, mannan, galactans and arabinans. Xylan molecules are mainly constituted by backbones of β-1,4-linked D-xylopyranose units and short side chains, including O-acetyl, α-L-arabinofuranosyl and/or α-D-glucuronyl residues [3, 4]. Xylan polymers are mainly present in the secondary cell wall and covalently linked with lignin phenolic residues and other polysaccharides such as pectins and glucans.

The potential of hemicellulose as a renewable raw material is immense. It is produced and wasted annually in huge amounts and has several likely uses if suitably applied [3]. Efficient conversion of lignocellulosic materials into fuel ethanol has become a world priority for producing environmentally friendly renewable energy at a reasonable price for the transportation sector [5]. In bioethanol production, the biomass is pre-treated using either chemical or enzymatic methods to break bonding and to convert

polysaccharides into monosaccharide [6]. Due to the complexity of the lignocellulosic material, a selection of three or more different enzymes groups is typically required, depending on the intended final product or the degree of hydrolysis required. These three groups of enzymes include cellulose and lignin degrading enzymes, in addition to hemicellulases.

Endo-xylanase (EC 3.2.1.8, endo-1,4-β-xylan 4-xylanohydrolase), belonging to the pentosanases category, is one of the important hemicellulases used for conversion of xylan into xylose and oligo-xylosaccharides [7]. Indeed, endo-xylanase can be used together with laccase (activated by copper) to hydrolyze lignocellulosic and hemicellulosic materials.

In recent years, many kinds of xylanases have been cloned and expressed in heterologous systems [8]. In a previous study conducted in our laboratory, the xylanase gene from *Aspergillus niger* US368 was cloned and expressed using the *E. coli* expression system [9, 10]. The recombinant xylanase (His-tagged r-XAn11) obtained was activated by copper which is a strong inhibitor for the wild xylanase. The behavior of the enzyme toward copper is due to the 36 extra amino acids provided by the vector pET28a. However, the expression of the His-tagged r-XAn11 was relatively low and intracellular that limits its potential industrial application. Hence, in this study, we describe the over-expression and characterization of the His-tagged r-XAn11 in *Pichia pastoris* with great potential in biofuel production. Two recombinant enzymes were purified as C-terminal tagged or Non-C-terminal tagged. Accordingly, the present study was undertaken to further investigate and evaluate the difference observed toward copper between the two recombinant forms (C-terminal tagged or Non-C-terminal tagged) of His-tagged r-XAn11.

Materials And Methods

Expression of the His-tagged r-XAn11 gene in *P. pastoris*

In order to express the gene encoding the His-tagged r-XAn11 in *Pichia pastoris*, the *His-taggedr-XAn11* gene was amplified using the recombinant plasmid pET28a-XAn11 [10] with primers Xyl-his-tag- PstI (AGC TGC AGA AAT GGG CAG CAG CCA TCA TCA TCA TCA T) and Xyl- XbaI (GCT CTA GAG CAG TGG AG ATC GTG ACA CTG GC) or Xyl-NotI (TTG CGG CCG CAA TTA AGT GGA GAT CGT GAC ACT GGC) supplemented with *Pst*I and *Xba*I or *Not*I restriction sites (underlined).

The thermal profiles involve 30 cycles of denaturation at 94°C for 30 s, primer annealing at 65°C for 60 s and extension at 72°C for 90 s, with a final extension at 72°C for 10 min. The PCR products were purified and ligated into pCR®-Blunt vectors and the resulting plasmids were transformed into *E. coli* Top10. The transformants were screened on LB agar medium supplemented with 50 µg mL^{-1} of kanamycin.

The recombinant plasmids were digested with *Pst*I and *Xba*I or *Pst*I and *Not*I and ligated into pGAPZαB vectors predigested with the same restriction enzymes. The resulting plasmids, pGAPZαB-*His-taggedr-XAn11,* were transformed into *Pichia pastoris* SMD1168H strain (*pep4*, Mut^{+}). Electrocompetent *P. pastoris* cells were prepared using standard methods [15]. The recombinant yeast clones were selected on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol and 1.5%

agar) containing 100 µg mL^{-1} zeocin. Selected positive colonies were cultured in 5 mL of YPD medium (1% yeast extract, 2% peptone and 2% dextrose) with zeocin. These cell cultures were further used to inoculate 250 mL Erlenmeyer flasks containing 50 mL YPD medium with zeocin, at 30°C for 72 h under shaking at 100 rpm. All yeast cultures had an initial optical density A_{600nm} of 0.2. The most efficient xylanase secreting clones were selected for larger cultures performed in a 1 L baffled Erlenmeyer flask containing 200 mL YPD medium and were stopped after 72 h. Culture supernatants obtained after centrifugation (4500 rpm, 10 min, 4 °C) were dialyzed against 1×Phosphate Buffer by frontal flow ultrafiltration on a 10 kDa membrane (Millipore, USA). The concentrated enzymes were applied to 1 mL HisTrap Chelating Ni-affinity columns (Amersham Pharmacia Biotech, USA) equilibrated with 1×Phosphate Buffer containing 20 mM of imidazole. The adsorbed proteins were eluted using a linear gradient of imidazole (50 mM-200 mM). The fractions containing the xylanase activity were pooled and concentrated. Protein purity was checked using SDS-PAGE electrophoresis [16]. Protein bands were visualized by Coomassie brilliant blue R-250 (Bio-Rad) staining.

Protein concentration was measured according to Bradford's method with Bovine Serum Albumin as standard,[17].

Assay of xylanolytic activity

0.5 mL of the enzyme solution, diluted in citrate buffer (0.1 M, pH 5), was incubated for 20 min 50 °C with 0.5 mL of 1% soluble beechwood xylan (Sigma-Aldrich Co., St. Louis, MO, USA). The amount of reducing sugars released was determined by the dinitrosalicylic acid (DNS) method [18], using D-xylose as standard.

One unit of xylanase activity was defined as the amount of enzyme which produces one micromole of xylose equivalents per minute.

Effect of metal ions and EDTA on recombinant xylanase activity

Xylanase activities were measured under optimal conditions in the presence of EDTA or several metallic ions (Co^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+} and Mn^{2+}) at 5 mM.

Effects of pH and temperature on recombinant xylanase

The effect of pH on the recombinant xylanases activities was determined at 50 °C using the different buffer solutions at 100 mM. Buffers used were citrate buffer (pH 3 - 5.5), phosphate (pH 5.5 - 8), Tris-HCl (pH 8 - 9) and glycine-NaOH (9 - 10).

The optimum temperatures for the recombinant xylanases activities were determined by carrying out the enzymes assays at different temperatures (40 - 60 °C) at pH 5. The thermal stabilities of the recombinant enzymes were determined by incubating the pure enzymes at different temperatures (55 °C and 60 °C) at pH 5. Aliquots were drawn at regular time intervals and immediately cooled in ice-cold water. The residual activity was determined, after centrifugation, under standard assay conditions.

Kinetics parameters

The kinetic parameters of the two recombinant xylanases were studied by determining the hydrolysis rates of beechwood xylan used at various concentrations ranging from 0 to 2 mg mL^{-1}. Assays were performed for 5 min at 50°C. The maximum velocity (V_{max})and the Michaelis-Menten constant(K_m) values were determined from the Lineweaver-Burk curves.

Homology modeling

The molecular modeling of the Non-C-terminal tagged-His-tagged r-XAn11 and the C-terminal tagged-His-tagged r-XAn11 were performed with the SWISS-MODEL server (http://www.expasy.org/swissmod/) using the crystal structure of Xyn1 from *A. niger* (PDB accession code 1UKR) as a template with which the XAn11 possess 93.48% of sequence identity. The C and N terminal His-tag were generated using Swiss-Pdb Viewer 4.0.1 [19]. The constructed models were subjected to energy minimization using GROMACS software [20] and single point energy computation. The PyMol Molecular Graphics System (DeLano Scientific, SanCarlos, CA. http://www.pymol.org) was used to visualize and analyze the generated model structures.

Prediction of protein flexibility of Non-C-terminal tagged-His-tagged r-XAn11 and the C-terminal tagged-His-tagged r-XAn11

The structural flexibility simulations of the two enzymes were presented as Root Mean Square Fluctuation plots and were performed by the CABS-FLEX tool. For each simulation, 10 clustered structure models were obtained and analyzed through residue fluctuation and dynamic movements. CABS-flex is based on the CABS model, a well-established coarse-grained protein modeling tool [21].

Results

Gene expression of His-tagged r-XAn11 in Pichia pastoris

The xylanase encoding gene from *Aspergillus niger* US368 was cloned and expressed in *E. coli* BL21 [9, 10]. The activity of the recombinant xylanase (His-tagged r-XAn11) is enhanced by copper which is a strong inhibitor for the wild xylanase. The activation of the enzyme by copper is due to the 36 extra amino acids provided by the vector pET28a [10].

In order to express the copper activated xylanase encoding gene from *E. coli* BL21in *Pichia pastoris* SMD1168H, the *His-tagged r-XAn11* gene was amplified using the sequence-specific PCR primers as described in Materials and methods. Two forms of the gene were obtained. The first one was amplified using the primers Xyl-his-tag-PstI and Xyl-XbaI (without a stop codon) and the encoded protein will be tagged on the NH_2 and COOH terminal sides. The second form of the gene was amplified using the primers Xyl-his-tag-PstI and Xyl-NotI (which contains a stop codon) and the protein will be tagged only on the NH_2 terminal side. Each form of gene obtained was fused in-frame with the α-factor secretion signal

of the pGAPZαB plasmid. The resulting plasmids were linearized and transformed into *Pichia pastoris* SMD1168H. The highest-expressing recombinant *P. pastoris* clones, named Non-C-terminal GX and C-terminal GX, reached 32U mL^{-1} and 22 U mL^{-1} of xylanase activity, respectively.

Purification of the Non-C-terminal and C-terminal tagged recombinant xylanases

The two recombinant clones Non-C-terminal GX and C-terminal GX were grown in 200 mL YPD mediums at 30°C with constant shaking at 100 rpm for 72 h.

The culture supernatants were concentrated and then loaded into Ni-NTA affinity chromatography columns. Fractions containing xylanase activities were pooled, concentrated and analyzed by SDS-PAGE. Our results showed the presence of clear and unique bands with molecular weights of about 25 kDa and 28 kDa for Non-C-terminal tagged-His-tagged r-XAn11 and C-terminal tagged-His-tagged r-XAn11, respectively (Fig. 1).

The specific activity of the Non-C-terminal tagged-His-tagged r-XAn11 reached 450.48 U mg^{-1} using beechwood xylan as substrate. However, the specific activity of the C-terminal tagged-His-tagged r-XAn11 was about 309.58 U mg^{-1}.

Effects of metal ions

The effect of bivalent metallic ions on purified recombinant xylanases activities was carried out at a concentration of 5 mM using beechwood xylan as substrate under standard conditions (Table 1). The effects of bivalent metallic ions on Non-C-terminal tagged-His-tagged r-XAn11 and C-terminal tagged-His-tagged r-XAn11 are almost the same. However, in the presence of 5 mM Cu^{2+}, the relative activity of the Non-C-terminal tagged-His-tagged r-XAn11 was enhanced by about 38% while the xylanase activity of the C-terminal tagged was strongly inhibited by copper.

Table 1
Effects of 5 mM of metal ions and EDTA on xylanase activity. The relative activities were measured at optimum of pH and temperature and enzyme activities without metal ions were taken as 100%.

Metal ions (5 mM)	Relative activity of Non-C-terminal tagged-His-tagged r-XAn11 (%)	Relative activity of C-terminal tagged-His-tagged r-XAn11 (%)
None	**100**	**100**
EDTA	104.5	101.8
Mg^{2+}	109.6	98.7
Cu^{2+}	138.4	46.4
Zn^{2+}	116.3	97.2
Fe^{2+}	103	98.4
Co^{2+}	87.2	65.3
Ca^{2+}	123.4	88.4
Mn^{2+}	44.8	44.6

The activity of the Non-C-terminal tagged-His-tagged r-XAn11was investigated at different concentrations of Cu^{2+} (from 1 to 8 mM) using beechwood xylan as substrate. The results showed that the Non-C-terminal tagged-His-tagged r-XAn11 reach a maximum to be 152% when it was measured in the presence of 3 mM of Cu^{2+}.

Optimum pH, temperature and stability

Enzyme assays revealed that the highest activities of the recombinant xylanases were obtained at 50°C and pH 5.

The thermostability study of the different enzymes is shown in Fig. 2. The recombinant enzymes had similar thermal stability and were noted to be unstable since they were inactivated after 15 min of incubation at 55°C (Fig. 2A and B). However, in the presence of 3 mM Cu^{2+}, the Non-C-terminal tagged recombinant enzyme was more thermostable with a half-life of 10 min at 60°C (Fig. 2A).

Kinetic parameters

The Michaelis-Menten constants were determined for the two recombinant xylanases (Table 2). Results showed that the Non-C-terminal tagged-His-tagged r-XAn11 presents almost the same kinetic parameters compared with the recombinant xylanase produced by *E. coli* [10]. However, the comparison between the kinetic parameters of the two recombinant xylanases, the object of this study, showed differences. In fact,

the Non-C-terminal tagged-His-tagged r-XAn11 displayed the highest catalytic efficiency, namely 129.32 mL mg^{-1} s^{-1}. However, the catalytic efficiency of the C-terminal tagged-His-tagged r-XAn11 was approximately 4 folds lower (35.24 mL mg^{-1} s^{-1}).

Table 2
Kinetic parameters of purified recombinant xylanases on beechwood xylan as substrate.

	V_{max} (U mg^{-1})	K_m (mg mL^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (mL mg^{-1} s^{-1})
Non-C-terminal tagged-His-tagged r-XAn11	514,8	2,72	351.75	129.32
C-terminal tagged-His-tagged r-XAn11	231.64	4,1	144.47	35.24

The Non-C-terminal tagged-His-tagged r-XAn11 presents the highest affinity towards the beechwood xylan with a K_m value of 2.72 mg mL^{-1} compared to the C-terminal tagged-His-tagged r-XAn11 (K_m value of 4.1 mg mL^{-1}).

Molecular modeling analysis

The 3D model of the C-terminal tagged-His-tagged r-XAn11 was generated and according to PDB file, the β-factors for the C-terminal region were important. Thus, a prediction of local protein flexibility from the sequence was performed for the two compared proteins (Fig. 3).

The inspection of all possible conformations proposed by CABSflex showed that the C-terminal end could, in some conformation, interact with the catalytic site (Fig. 4).

Discussion

The copper activated xylanase produced by *E. coli* BL21 [10] was expressed in *Pichia pastoris* using the pGAPZαB expression vector. Two recombinant xylanases forms were purified (Non-C-terminal tagged-His-tagged r-XAn11 and C-terminal tagged-His-tagged r-XAn1). The specific activity of the Non-C-terminal tagged-His-tagged r-XAn11 is almost the same of the specific activity of the recombinant His-tagged r-XAn11 produced by *E. coli* [10]. However, the decrease in the specific activity of the C-terminal tagged-His-tagged r-XAn11could be due to the addition of the His-tag on the C-terminal side. Several studies on different enzymes confirm that the addition of the His-tag leads to a decrease in the specific activity of the enzyme [11, 12].

The effect of copper on the purified recombinant xylanases activities was carried out at a concentration of 5 mM. The results showed that the Non-C-terminal tagged-His-tagged r-XAn11 has the same behavior towards copper as the His-tagged r-XAn11 produced by *E. coli* [10]. However, the activity of the C-terminal tagged xylanse was strongly inhibited by copper.

In our previous work, the 3D model of the native xylanase and the N-terminal tagged one were generated and deeply compared [10]. Results showed the probable interaction between some catalytic site residues and the N-terminal tag creating a well-coordinated copper ion site activating the enzyme.

In this study, a prediction of local protein flexibility was performed for the two compared C-terminal and Non-C-terminal tagged enzymes, and results are presented in Fig. 3. The latter confirms that the added 23 amino acids to the C-terminal side were very flexible reaching an RMSF of almost 7 Å which is very important indicating the instability of this tag and the possibility for it to adapt various conformations in each folded enzyme. For the majority of generated CABSflex models, the C terminal tag could adopt a large unstable loop that interacts with the long N-terminal tag (32 amino acids). Compared to the only N-terminal tagged enzyme, the copper ion site would be eventually loosed and thus no longer activate the enzyme as demonstrated in Table 1.

The comparison between the kinetic parameters of the two recombinant xylanases showed that the catalytic efficiency of the Non-C-terminal tagged-His-tagged r-XAn11 was approximately 4 folds than the catalytic efficiency of the C-terminal tagged-His-tagged r-XAn11. The Non-C-terminal tagged-His-tagged r-XAn11 presents the also highest affinity towards the beechwood xylan compared to the C-terminal tagged-His-tagged r-XAn11. The investigation of all possible conformations proposed by CABSflex showed that, in some conformation, the C-terminal end could interact with the catalytic site and strongly hamper the substrate to go directly into the catalytic pocket. This observation could explain in part the decrease in the substrate affinity and the decrease of the catalytic efficiency as well since some expressed proteins would be inactive. In this context, as demonstrated in Fig. 3A, the N-terminal tag, when present alone, will be more stable and less flexible compared to the one in the two-sided tagged enzyme (Fig. 3B).

The optimum temperature and pH for the recombinant xylanases are almost the same as the wild xylanase produced by *Aspergillus niger* US368 [9] or the recombinant one produced by *E. coli* [10].

The recombinant enzymes had similar thermal stability and were noted to be unstable. However, in the presence of 3 mM Cu^{2+}, the Non-C-terminal tagged recombinant enzyme was more thermostable than the C-terminal tagged one. All structural findings corroborate the better thermal stability of the one-sided tagged enzyme since it is well known that higher thermal agitation especially at the enzyme extremities, would strongly affect the enzyme stability [12, 13, 14].

Conclusion

This work was undertaken to gain insights and understanding of the overall differences in the biochemical and kinetic properties displayed by the two recombinant xylanases forms (Non-C-terminal tagged His-tagged r-XAn11 and C-terminal tagged His-tagged r-XAn11). In fact, the activity of the Non-C-terminal tagged-His-tagged r-XAn11 was enhanced by copper while the activity of the C-terminal tagged was strongly inhibited. The Non-C-terminal tagged recombinant enzyme was more thermostable, in the

presence of copper, than the C-terminal tagged one. The findings also revealed that the Non-C-terminal tagged-His-tagged r-XAn11 presents a high affinity towards the beechwood xylan compared to the C-terminal tagged-His-tagged r-XAn11. 3D models showed that the created copper site in the Non-C terminal tagged protein was loosed which explain the differences in enzymes' proprieties.

Declarations

Authors' contributions

AHS supervised the experimental workJSL. FE designed this study, performed the experiments, analyzed the data, and drafted the manuscript. HBH performed the 3 D modelling. All authors read and approved the fnal manuscript.

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Availability of data and materials

The data sets used and/or analysed during the current study are available from the corresponding authors on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

Not applicable.

References

1. Whistler RL, Richard EL. Hemicelluloses. In: Pigman W, Horton D, editors. The Carbohydrates, New York: Academic Press; 1980. p. 447-469.
2. Wong KKY, Tan LUL, Saddler JN. Multiplicity of β -1,4-xylanase in microorganisms: Functions and applications. Microbiol Rev. 1988; (52): 305-317.

3. Dhiman SS, Sharma J, Battan B. Industrial applications and future prospects of microbial xylanases: A review. BioResources. 2008; (3): 1377-1402.
4. Bastawde KB. Xylan structure microbial xylanases, and their mode of action. World J Microbiol Biotechnol. 1992; (8): 353-368.
5. Hill J, Nelson E, Tilman D, Polasky S, Tiffany D. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. PNAS. 2006; (103): 11206-11210.
6. Garg S. Xylanase: Applications in Biofuel Production. Curr Metabolomics 2016, 4: 23-37.
7. Sharma M, Kumar A. Xylanase: An Overview. Br. Biotechnol J. 2013; (3): 1-28.
8. Zhou C, Wang Y, Wu M, Wang W, Li D. Heterologous Expression of Xylanase II from *Aspergillus usamii* in *Pichia pastoris*. Food Technol Biotech. 2009; (47): 90-95.
9. Hmida-Sayari A, Taktek S, Elgharbi F, Bejar S. Biochemical characterization, cloning and molecular modeling of a detergent and organic solvent-stable family 11 xylanase from the newly isolated *Aspergillus niger* US368 strain. Process Biochem. 2012; (47): 1839-1847.
10. Elgharbi F, Ben Hlima H, Farhat-Khemakhem A, Ayadi-Zouari D, Bejar S, Hmida-Sayari A. Expression of *A. niger* US368 xylanase in *E. coli*: Purification, characterization and copper activation. Int J Biol Macromol. 2015; (74): 263-270.
11. Horchani H, Ouertani S, Gargouri Y, Sayari A. The N-terminal His-tag and the recombination process affect the biochemical properties of *Staphylococcus aureus* lipase produced in *Escherichia coli*. J Mol Catal B: Enzym. 2009; (61): 194-201.
12. Ben Hlima H, Ayadi D, Aghajari N, Bejar S. Differential properties of native and tagged or untagged recombinant glucose isomerases of *Streptomyces* sp. SK and possible implication of the glycosylation. J Mol Catal B: Enzym. 2013; (94): 82- 87.
13. Daniel RM, Dines M, Petach HH. The denaturation and degradation of stable enzymes at high temperatures. Biochem J. 1996; (317): 1-11
14. Bhardwaj A, Mahanta P, Ramakumar S, Ghosh A, Leelavathi S, Reddy VS. Emerging role of N-and C-terminal interactions in stabilizing $(\beta/\alpha)_8$ fold with special emphasis on family 10 xylanases. Comput Struct Biotechnol J. 2012; (2): e201209014.
15. Cregg JM, Russell KA. Transformation. Methods Mol Biol 1998; 103: 27-39.
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; (227): 680-685.
17. Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-254.
18. Miller GL. Use of dinitrosalycilic acid reagent for determination of reducing sugar. Anal Chem 1959; (31): 426-428.
19. Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis. 1997; (18): 2714-2723.

20. Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJ. GROMACS: fast, flexible, and free. J Comput Chem. 2005; (26): 1701-1718.
21. Kmiecik S, Gront D, Kolinski M, Wieteska L, Dawid AE, Kolinski A. Coarse-Grained Protein Models and Their Applications. Chem Rev. 2016; (116): 7898-7936.

Figures

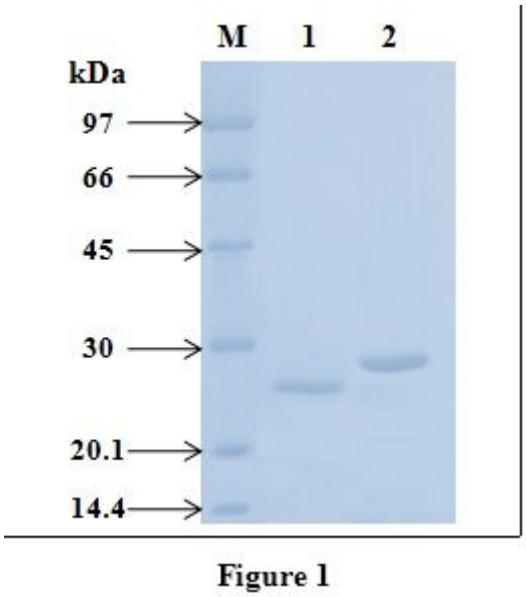

Figure 1

12% SDS-PAGE of the recombinant xylanases. Lanes are designated as follows: lane M, molecular mass standard proteins (LMW); lane 1, the profile of Non-C-terminal tagged-His-tagged r-XAn1; lane 2, the profile of C-terminal tagged-His-tagged r-XAn11.

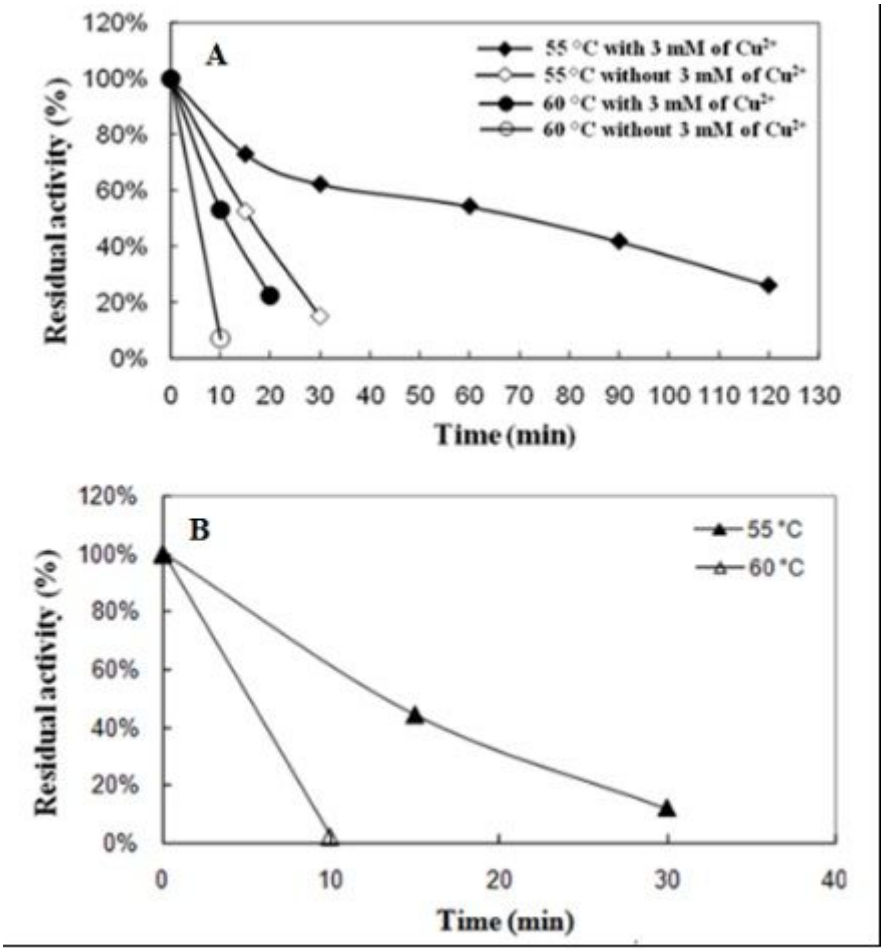

Figure 2

Figure 2

(A) Thermostability of the Non-C-terminal tagged-His-tagged r-XAn1 at pH 5.0 and temperatures of 55 °C in presence of 3 mM of Cu^{2+}(t), 55 °C in the absence of Cu^{2+} (◊), 60 °C in presence of 3 mM of Cu^{2+} (●) and 60 °C in the absence of Cu^{2+} (●).The enzyme without incubation was defined as 100% relative activity. **(B)** Thermostability of the C-terminal tagged-His-tagged r-XAn1 at 55 °C (▲) and 60 °C (Δ)

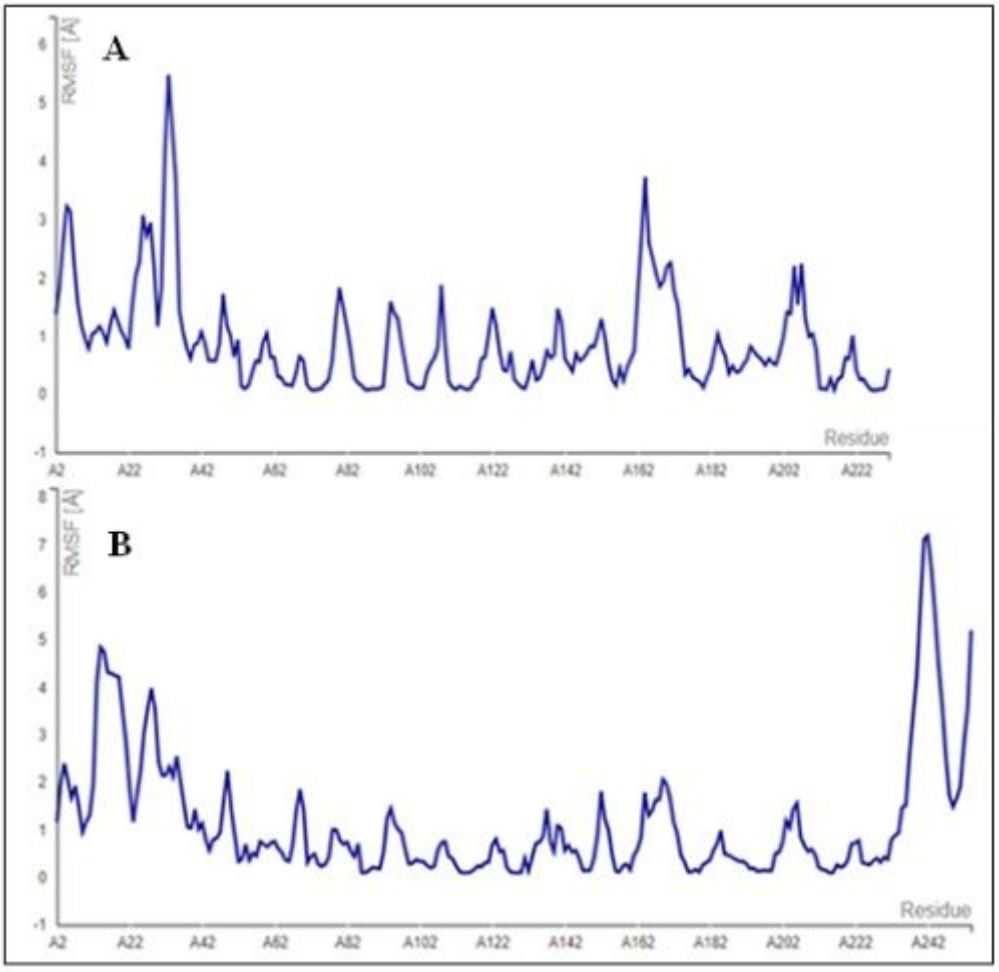

Figure 3

Figure 3

RMS fluctuation prediction deduced from local protein flexibility from its sequence performed by CABSflex tool for Non-C-terminal tagged-His-tagged r-XAn11 **(A)** and C-terminal tagged-His-tagged r-XAn11 **(B)**.

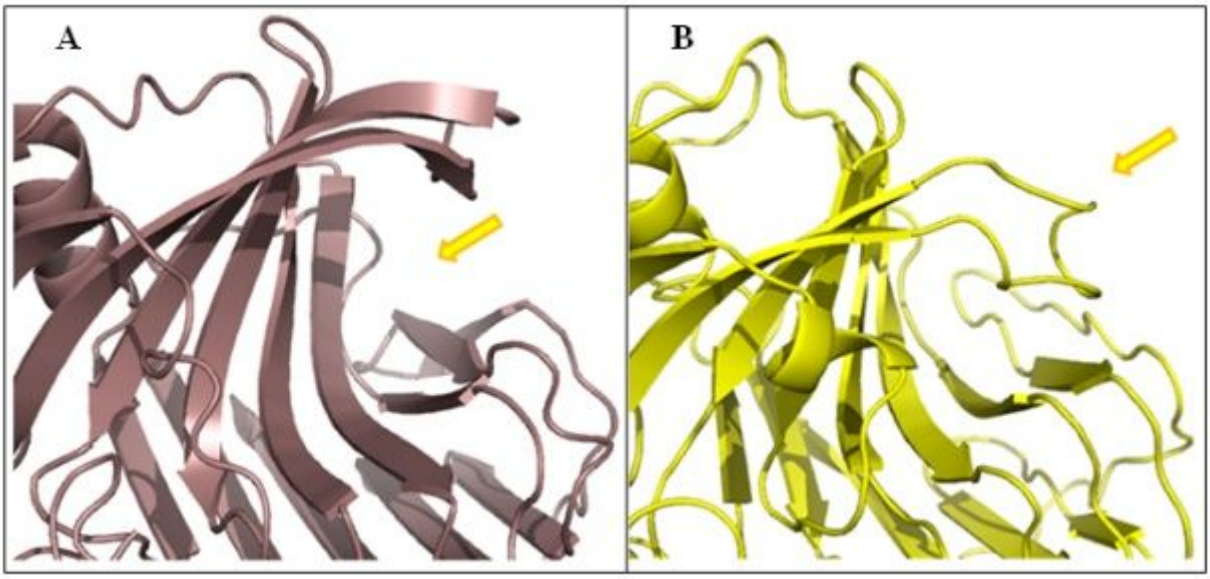

Figure 4

Figure 4

Close up view of the catalytic pocket entrance for Non-C-terminal tagged-His-tagged r-XAn11 **(A)** and C-terminal tagged-His-tagged r-XAn11 **(B)**. The yellow arrow shows the absence and the presence of a large loop hampering substrate entry for Non-C-terminal and C-terminal tagged enzyme, respectively.