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Improvement in thermostability of metagenomic GH11 endoxylanase (Mxyl) by site-directed mutagenesis and its applicability in paper pulp bleaching process

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Abstract An attempt has been made for enhancing the thermostability of xylanase (Mxyl) retrieved from a compost-soil-based metagenomic library. The analysis of the structure of xylanase by molecular dynamics simulation revealed more structural fluctuations in β-sheets. When the surface of β-sheets was enriched with arginine residues by substituting serine/threonine by site-directed mutagenesis, the enzyme with four arginine substitutions (MxylM4) exhibited enhanced thermostability at 80 °C. The $T_{1/2}$ of MxylM4 at 80 °C, in the presence of birchwood xylan, increased from 130 to 150 min at 80 °C without any alteration in optimum pH and temperature and molecular mass. Improvement in thermostability of MxylM4 was corroborated by increase in T_m by 6 °C over that of Mxyl. The K_m of MxylM4, however, increased from 8.01 ± 0.56 of Mxyl to 12.5 ± 0.32 mg ml^{-1}, suggesting a decrease in the affinity as well as specific enzyme activity. The Mxyl as well as MxylM4 liberated chromophores and lignin-derived compounds from kraft pulp, indicating their applicability in pulp bleaching.

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Introduction

Hemicellulose is an integral part of lignocellulosic materials that accounts for 25–35 % of plant biomass [6, 20, 33]. Xylans, made of β-1,4 linked xylopyranose units, are the main constituents of hemicelluloses. The xylan backbone is linked to various side chains, and thus, make it a heteropolysaccharide. Synergistic action of β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid esterases A is needed for complete hydrolysis of xylan. The enzymes act on xylan in order to liberate xylose and lower xylooligosaccharides from various lignocellulosic residues [2, 43]. Among xylanolytic enzymes, β-1,4-endoxylanase (EC 3.2.1.8) is the most important enzyme that plays a critical role in the hydrolysis of xylan. Based on hydrophobic cluster analysis and multiple sequence alignment, xylanases have been categorized into seven groups [Glyco Hydrolase (GH) family 5, 7, 8, 10, 11, 30 and 43] [6].

Several xylanases are being employed in a variety of applications such as in food/feed to paper/pulp industries on the basis of partial hydrolysis of xylan [16, 28, 38, 47]. The stability of xylanases at higher pH and temperatures is the most critical issue associated with xylanases for pre-bleaching of pulps in the paper and pulp industry. This industry is one of the fastest-growing sectors that requires thermostable and alkalistable xylanases due to the extreme process conditions. Kraft cooking, a process associated with pulping of wood chips, operates at 170 °C in the presence of NaOH and sulfide. Although the temperature goes down to 100 °C at the bleaching step, most of the existing xylanases do not withstand this temperature. Most of the available xylanases are not adequately alkalistable and thermostable for application in paper and pulp industries [25, 47].

The majority of xylanases retrieved either by culture-dependent or culture-independent approaches exhibit their pH and temperature optima in the range of 6.0–8.0 and 40–60 °C, respectively [8, 11, 14, 24, 50]. The xylanase used in this study, retrieved from environmental DNA using metagenomic approach, is not only optimally active at pH 9.0 and 80 °C but also displays high thermostability ($T_{1/2}$ of 2 h at 80 °C) [46].

In this investigation, an attempt has been made to improve the thermostability of the xylanase by substituting ser/thr residues present on the surface with arginine using site-directed mutagenesis. The mutated xylanase (MxylM4) exhibits enhanced thermostability without compromising the catalytic activity.

Materials and methods

Strains and plasmids for DNA manipulations

Recombinant plasmid (*pET28Mxyl*) was used to introduce DNA manipulations, mutations, sequencing, and expression of the xylanase gene. *Escherichia coli* DH5αTM-T1^R cells and *E. coli* BL21(*DE3*) were used for the propagation of the plasmid. Hi-fi Taq polymerase (Kappa) was used for the amplification of the whole plasmid. Mutations were introduced by using the GENEART site-directed mutagenesis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Proposed structure of Mxyl

The preliminary secondary structure of xylanase was predicted using ESpript 2.0. A three-dimensional model of xylanase was proposed using online server ESyPred3D Web Server 1.0 [22], and the PDB file was viewed using PyMol software.

Molecular dynamics (MD) simulation

The initial coordinates for the protein Mxyl atoms were taken from the protein data bank file (PDB id: 1HIS) of glycosyl hydrolase family 11. All MD simulations were performed with CHRAMM27 (all atom) force field using the GROMACS 4.5.3 package running on a high-performance Linux cluster computer [12, 40]. The protein atoms were approximately 1.0 Å far from the edge of the water-filled simulation box. Thereafter, energy of the system (having water, neutralizing ions, and protein molecules) was minimized using the steepest descent algorithm followed by system equilibration. The system was equilibrated for 1 ns of MD simulation to equilibrate the positional restraints on the protein with solvent molecules. The final production simulation was, however, done for 10.0 ns with a time step of 1 fs using leap-frog integrator algorithm. The Particle-Mesh Ewald (PME) algorithm was used to model the electrostatic interactions and snapshots were reoriented every 100 ps. The simulation was applied at the optimum temperature of 80 and 85 °C and pressure was controlled with a Berendsen coupling.

Whole-plasmid amplification and sequencing

The recombinant plasmid (*pET28Mxyl*) was isolated from *E. coli* DH5α (*pET28Mxyl*) cells. The construct *pET28Mxyl* was methylated prior to amplification of the whole plasmid using DNA methylase. Four sets of overlapping primers (Dxyl-T68F/R; Dxyl-T69F/R; Dxyl-T90F/R and Dxyl-T92F/R) were designed according to instruction given by GENEART site-directed mutagenesis kit (Invitrogen) to introduce the desired mutations (Table 1). The amplified plasmids having the mutated xylanase gene were transformed in *E. coli* DH5αTM-T1^R-competent cells by heat shock method. Transformed cells (100 μl) were spread on an LB-Kan plate and incubated overnight at 37 °C. The clones were randomly selected and the plasmids were isolated and sequenced for each mutation. For every next mutation, the plasmid of the previous mutation was used to generate other successive mutations. A total of four mutations were generated at the 68th, 90th, 92nd, and 69th positions of *Mxyl* successively. All the substitutions were made to replace ser/thr with arginine. The mutated *Mxyl* genes were sequenced for confirming the introduced amino acid substitutions.

Table 1 Details of primers used in this study

Primers	Mutant	Oligomeric sequences (5′–3′)
Dxyl-T68F	*MxylM1*	TGCAGAGCGGCGGCCGATACCGCTCTCAGTGGAG
Dxyl-T68R		GTATCGGCCGCCGCTCTGCAAGCAGAACGTG
Dxyl-T90F	*MxylM2*	CCCGGCGGACGACGCCGCGTCACCTACTCCGGC
Dxyl-T90R		GCCGGAGTAGGTGACGCGGCGTCGTCCGCCGGG
Dxyl-T92F	*MxylM3*	GGACGACGCCGCGTCCGCTACTCCGGCACGTTC
Dxyl-T92R		GAACGTGCCGGAGTAGCGGACGCGGCGTCGTCC
Dxyl-T69F	*MxylM4*	GGCGGCCGATACCGCCGCCAGTGGAGCAATATC
Dxyl-T69R		GATATTGCTCCACTGGCGGCGGTATCGGCCGCC

Purification of mutated xylanase

Plasmids from the mutants were isolated and transformed into *E. coli* BL21(*DE3*) for the expression of the muteins. The mutated Mxyl from each successive mutation was purified by Ni^{2+}–NTA resin affinity chromatography [46].

Xylanase assay

Xylanase activity was determined by incubating the reaction mixtures at 80 °C (pH 9.0) in a water bath shaker for 10 min at 90 rpm. The reducing sugars liberated were quantitated using DNSA reagent by recording the absorbance at 540 nm in a spectrophotometer (Shimadzu, Japan) using xylose as the standard [27]. One unit (U) of xylanase is defined as the amount of enzyme that liberates 1 μmol of reducing sugars as xylose under the assay conditions.

Determination of thermostability

For determining thermal stability, the purified enzyme was incubated in glycine–NaOH buffer (pH 9.0) for varying time intervals at the desired temperatures, and the activities were determined. The half-life ($T_{1/2}$) values of the enzymes were determined at pH 9.0 and 80 and 90 °C in the presence/absence of substrate (1 % birchwood xylan).

Characterization of mutated xylanases and enzyme kinetics

For determining physical properties of the mutated xylanase, enzymes were assayed at different temperatures (40–100 °C). Similarly, various buffers (pH 3.0–6.0, 0.1 M citrate buffer; 7.0–8.0, 0.1 M citrate buffer and for 9.0–12.0, 0.1 M glycine–NaOH buffer) were used of desired pH for determining the optimum pH. Substrate affinity (K_m) and rate (V_{max}) were calculated by determining the effect of various concentrations of birchwood xylan (1–20 mg ml^{-1}) at 80 °C and drawing a Lineweaver–Burk plot.

Melting temperature (T_m)

The melting temperature of the purified Mxyl and MxylM4 was determined by using circular dichroism. The enzyme was incubated in 20 mM phosphate buffer (pH 8.0) and thermal denaturation was carried out as a function of increasing temperature using a Peltier temperature controller.

Enzymatic treatment of pulp

Ten grams of oven-dried wheat straw pulp was treated with MxylM4 (40 U g^{-1} dry wt. of pulp). The treated pulp was incubated at 80 °C and pH 9.0 in a water bath shaker at 90 rpm. The autoclaved mutated xylanase was used as the control. The samples of varying time interval were collected from the filtrate for analysis. Chromophores and lignin-derived compounds (LDC) were determined by monitoring the absorbance of the filtrate at A_{465} and A_{280}, respectively, and the reducing sugars (mg g^{-1} oven-dried pulp) were quantitated with DNSA reagent [27].

Results and discussion

Proposing the structure of xylanase

The rationale of protein engineering emerged from the studies on a large number of enzymes from mesophiles and comparing their amino acid composition with their thermophilic counterparts [20, 23, 30]. This alignment and comparison of their amino acid sequences in thermal stabilizing domains, catalytic domains, or substrate-binding domains led to the identification of amino acids that are critical in imparting noteworthy properties. These amino acid residues were therefore targeted for alteration in the pursuit of enhancing the useful properties of the engineered proteins [10, 42, 48]. Based on the analysis of available amino acid sequence data of xylanases, specific amino acids have been substituted.

Protein engineering has come to occupy a very significant niche if one wishes to alter the protein of interest with respect to some specific characteristics [31, 39]. Significant improvement has been achieved in enhancing the stability of xylanases, not only at higher temperatures but also at higher pH, using various strategies of protein engineering [4, 53, 54]. Three point mutations at the N-terminus, helix, and cords improved the thermostability of family 11 xylanase of a non-culturable fungus due to the formation of new salt bridges and dense packing of the structure [54]. The preliminary information of the protein is, however, required before proceeding to manipulate any protein [30]. Secondary structure of Mxyl generated by ESpript 2.0 software showed one α-helix (2.79 %), 15 β-sheets (33.24 %), and random coils (63.97 %) (Suppl fig. 1). In addition, the 3D structure showed the highest similarity (74 %) with a GH11 xylanase of an uncultured bacterium (PDB ID: 2VUL). The proposed 3D structure clearly shows the profile of helix and sheets identified from ESpript 2.0 software. The structure has all the signature sequences of GH11 xylanases. The β-jelly shape structure of xylanase revealed fingers, thumb, and catalytic cleft and serine/threonine-rich surfaces. Two catalytically important residues of glutamate were also detected in the catalytic cleft at positions 117 and 209. Similar structures have been reported for GH11 xylanases of *Trichoderma reesei*, *T.*

Fig. 1 a Three-dimensional structure of xylanase (Mxyl) showing a ser/thr-rich surface, and **b** CD spectra analysis showing secondary structure profile
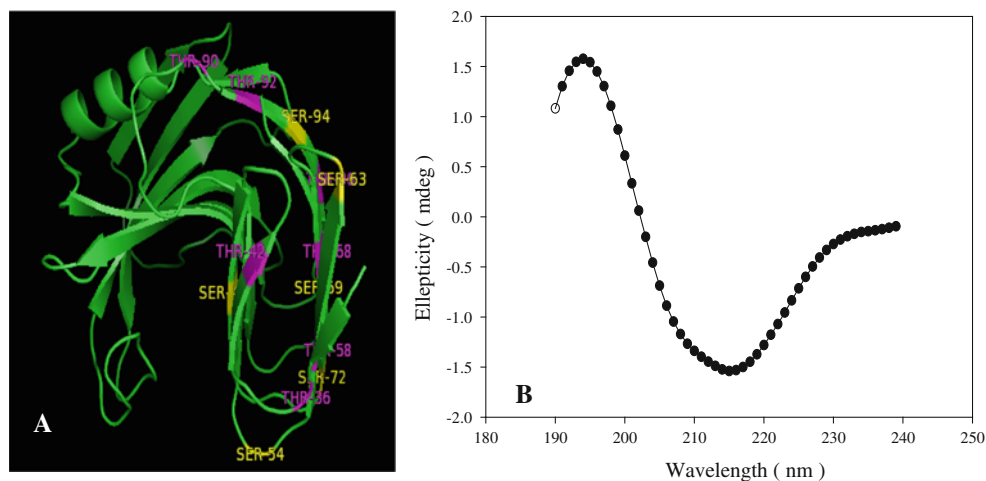
longibrachiatum, Aspergillus niger, Paecilomyces variotii, and *Bacillus circulans* [19, 21, 35, 41, 44]. Serine- and threonine-rich surfaces are the characteristic features of GH11 xylanases [9, 30, 41]. The deduced amino acid sequence of Mxyl revealed a total of 39 serine and 38 threonine residues and most of the residues are present on the surface of the protein (Fig. 1a).

Validation of the model

The stereochemical properties and quality of the model were validated by Ramachandran's plot using RAMPAGE server as in other xylanases [26, 37]. The conformation of the main chain atoms in a protein is determined by one pair of phi and psi angles for each amino acid. In the Ramachandran's plot obtained after the structure validation, each dot represents an observed pair of angles in a protein. According to this plot, 98 % of residues are in the favored regions, 2.0 % in the allowed regions and 0 % in outlier regions. In addition, far-UV CD analysis of Mxyl showed 4.57 % α-helix and 46.99 % β-sheets and this is very proximate to the proposed secondary and tertiary structures that further validate the integrity of the structure (Fig. 1b).

Molecular dynamics (MD) simulation

Thermal fluctuation analysis has been studied to understand the role of unstable residues for improving thermostability of xylanase [15]. Interestingly, molecular dynamics revealed the crucial significance of the role of N terminal region, alpha helix and palm for stabilizing xylanases at higher temperatures [34]. A comparative study of native and mutated xylanases suggested that root mean square deviation (RMSD) of Mxyl for 10-ns MD simulation at 80 and 85 °C showed differences in fluctuation profile. At higher temperature (85 °C), structure fluctuations clearly indicated the disturbance of signature

confirmation (β-jelly) of the xylanase (Suppl fig. 2A and B). There were more fluctuations in the β-sheet structure, while there is a need for detailed investigation on the reasons for this difference in the behavior of the fluctuations in the β-sheet region as compared to the other region of the enzyme. Any attempt to design thermostable xylanase should include mutations that could retain β-sheet fluctuation of metagenomic xylanase at a similar level as that of fluctuations at the optimum temperature.

Site-directed mutagenesis

Site-directed mutagenesis is a rational way to alter the properties of proteins [30, 54]. Attempts have successfully been made to improve the thermostability of xylanases using this approach [18, 51, 52]. Thermostability of GH11 xylanase of *Thermomyces lanuginosus* was significantly enhanced due to the introduction of disulfide bridges at the N-terminus region [51]. On extensive comparisons of mesophilic and thermophilic xylanases, it has been found that thermophilic xylanases have, on average, higher arginine content on the surface of xylanase and are more stable than mesophilic proteins at elevated temperatures [3, 29, 30, 41]. Protein engineering strategies that include domain-swapping of mesophilic xylanase with hyperthermophilic glucanase [23] and enhanced combinatorial library method [13] have successfully been attempted to enhance the thermostability of xylanases. Furthermore, threonine/serine residues have been selected for substitution because these are present on the surface and far from the catalytic groove, and therefore such substitutions will not alter the conformation or the catalytic behavior of the xylanase [30, 45]. The mutation of ser/thr amino acid residues on the surface neither altered the enzyme catalysis nor caused conformational change in the structure [41]. An attempt has therefore been made to introduce mutations on the surface of Mxyl using site-directed

MxylM1

MT A S L R K I A F T S K S V A A A I V GMA A L Y V S P A D A Q C L T N N Q T G T H D G Y Y Y S F WK D S G N V T F
C L Q S G G R Y **R** S Q W S N I N N W V G G K G W N P G G R R T V T Y S G T F N P N G N A Y L T L Y GW T T N P L V E
Y Y I V D N W G T Y R P P G G Q G YMG T V N S D G G T Y D I Y R T Q R V N A P C I T G N N C T F W Q Y W S V R Q Q
R R T G G T I T T G N H F D A W A S L GMN L G Q H N YMV M A T E G Y Q S S G S S D I T V G G T S S G G S S S G G S S S
S S S S S S S S G G G G S K T I V V R A R G S T G G E Q I S L R V N N Q T V Q N W T L G T GMQ N Y T A T T N L S G G I T
V H F T N D N G A R D V Q V D Y I Q V N G Q I R Q S E Q Q S Y N T G L Y A N G R C G G G G Y S E WM H C N G A I G Y
GN T P

MxylM2

MT A S L R K I A F T S K S V A A A I V GMA A L Y V S P A D A Q C L T N N Q T G T H D G Y Y Y S F WK D S G N V T F
C L Q S G G R Y **R** S Q W S N I N N W V G G K G W N P G G R R **R** V T Y S G T F N P N G N A Y L T L Y GW T T N P L V E
Y Y I V D N W G T Y R P P G G Q G YMG T V N S D G G T Y D I Y R T Q R V N A P C I T G N N C T F W Q Y W S V R Q Q
R R T G G T I T T G N H F D A W A S L GMN L G Q H N YMV M A T E G Y Q S S G S S D I T V G G T S S G G S S S G G S S S
S S S S S S S S G G G G S K T I V V R A R G S T G G E Q I S L R V N N Q T V Q N W T L G T GMQ N Y T A T T N L S G G I T
V H F T N D N G A R D V Q V D Y I Q V N G Q I R Q S E Q Q S Y N T G L Y A N G R C G G G G Y S E WM H C N G A I G Y
GN T P

MxylM3

MT A S L R K I A F T S K S V A A A I V GMA A L Y V S P A D A Q C L T N N Q T G T H D G Y Y Y S F WK D S G N V T F
C L Q S G G R Y **R** S Q W S N I N N W V G G K G W N P G G R R **R** V **R** Y S G T F N P N G N A Y L T L Y GW T T N P L V
E Y Y I V D N W G T Y R P P G G Q G YMG T V N S D G G T Y D I Y R T Q R V N A P C I T G N N C T F W Q Y W S V R Q
Q R R T G G T I T T G N H F D A W A S L GMN L G Q H N YMV M A T E G Y Q S S G S S D I T V G G T S S G G S S S G G S
S S S S S S S S S G G G G S K T I V V R A R G S T G G E Q I S L R V N N Q T V Q N W T L G T GMQ N Y T A T T N L S G G
I T V H F T N D N G A R D V Q V D Y I Q V N G Q I R Q S E Q Q S Y N T G L Y A N G R C G G G G Y S E WM H C N G A I G
Y GN T P

MxylM4

MT A S L R K I A F T S K S V A A A I V GMA A L Y V S P A D A Q C L T N N Q T G T H D G Y Y Y S F WK D S G N V T F
C L Q S G G R Y **R** **R** Q W S N I N N W V G G K G W N P G G R R **R** V **R** Y S G T F N P N G N A Y L T L Y GW T T N P L V
E Y Y I V D N W G T Y R P P G G Q G YMG T V N S D G G T Y D I Y R T Q R V N A P C I T G N N C T F W Q Y W S V R Q
Q R R T G G T I T T G N H F D A W A S L GMN L G Q H N YMV M A T E G Y Q S S G S S D I T V G G T S S G G S S S G G S
S S S S S S S S S G G G G S K T I V V R A R G S T G G E Q I S L R V N N Q T V Q N W T L G T GMQ N Y T A T T N L S G G
I T V H F T N D N G A R D V Q V D Y I Q V N G Q I R Q S E Q Q S Y N T G L Y A N G R C G G G G Y S E WM H C N G A I G
Y GN T P

Fig. 2 Deduced amino acid sequences of mutated xylanases showing substitutions (*bold* and *highlighted*)

mutagenesis in order to determine whether this enhances the thermostability.

Generation of mutant xylanases and purification

The plasmids with mutated xylanase genes were amplified (Suppl fig. 3) and four mutants were generated having 68th, 90th, 92nd, and 69th substitutions (Fig. 2; Suppl fig. 4) (Table 1). Following the transformation of the plasmids harboring the mutations into *E. coli* BL21(*DE3*) and expression, the muteins have been purified to homogeneity (Fig. 3a, b). The mutated xylanases displayed xylanase activity on birchwood xylan agar plates (Fig. 3c).

Characterization of mutated xylanases and enzyme kinetics

MxylM1 and MxylM2 showed a very similar profile of thermostability to Mxyl (data not shown). The xylanase MxylM3 exhibited a slight improvement in thermostability (data not shown). MxylM4 showed significantly higher thermostability at 80 and 90 °C (Fig. 4a), and thus was used in further studies. Thermostability of MxylM4 was improved by 10 min with $T_{1/2}$ of 150 min at 80 °C in comparison with 130 min of the parental xylanase (Mxyl)

at 80 °C in the presence of xylan. Similarly, thermostability was enhanced by 5 min at 90 °C (Table 2). There was, however, no significant enhancement in thermostability in the absence of substrates as reported in *A. niger* [41]. Thermostability of the mutated xylanase of *A. niger* with five arginine residues exhibited a 20-fold increase in thermostability without compromising the specific enzyme activity [41]. There was a decline in specific enzyme activity of MxylM4 (Table 3). The affinity towards birchwood xylan decreased as indicated by the increase in K_m; this could be partly responsible for the decrease in specific enzyme activity of MxylM4 over Mxyl. The lowered diffusion rate of arginine-rich xylanase may play a significant role in lowering the affinity towards substrates, since high molecular weight xylanases exhibit high K_m values as compared to those with low molecular weight [39, 41]. There was no observable change in optimum pH, temperature, or molecular mass of the mutant xylanases as compared to Mxyl (Table 3). These substitutions did not alter the conformation or the catalytic behavior of the xylanase. The higher stability of xylanase may be due to bulky and basic arginine that provides a positive charge at the surface and allows charge-to-charge interactions for the formation of multiple non-covalent interactions. Moreover, arginine guanidino moiety has a high pKa value of 12, and

Fig. 3 Analysis of mutated xylanase using SDS-PAGE (15 % polyacrylamide gel). **a** Mutated xylanases were eluted using different concentrations of imidazole (100, 150, 200, 250, 300, 350, 400, 450, 500, and 550 mM). **b** Mutated xylanase from respective mutants showed molecular mass of ∼40 kDa on staining with Coomassie Brilliant Blue R-250. **c** Xylan-agar plate showing zone of hydrolysis from various mutated xylanases
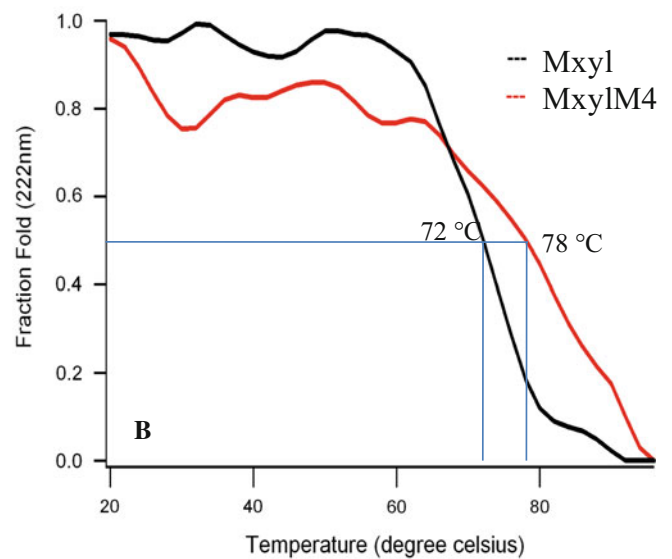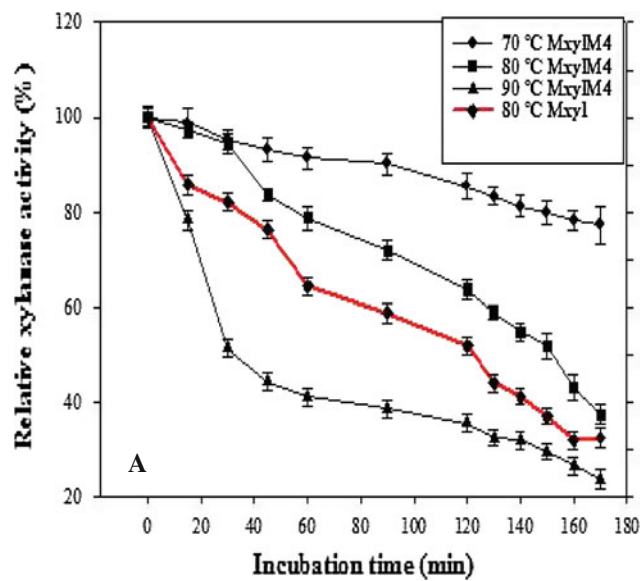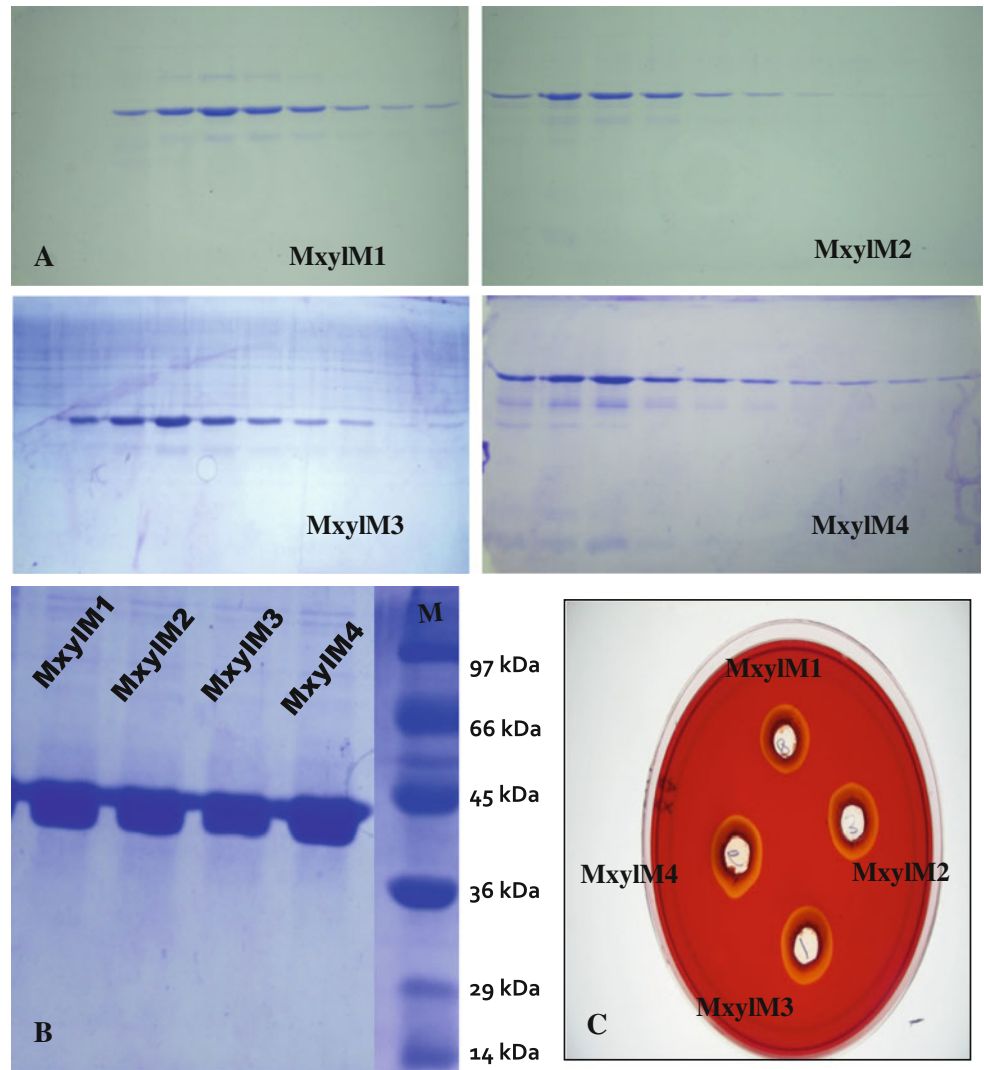
Fig. 4 a Thermostability of MxylM4 at different temperatures. **b** Melting temperature of Mxyl and MxylM4

therefore, it shows low dissociative behavior towards chemicals and the solution. Consequently it increases the hydrophobicity towards aqueous environment, and thus, provides more compactness to protein that is considered responsible for enhancing the thermostability at extreme temperatures; the higher pKa value of arginine upholds a net-positive charge at elevated temperatures [1, 41, 45, 49]. An increase in the thermostability of *T. reesei* and *A. niger* xylanase has been reported when five arginine residues were engineered into the ser/thr surface [41, 45]. Enhancement of the melting temperature of MxylM4 by 6 °C over Mxyl further confirmed the improvement in integrity of the mutant xylanase at elevated temperatures (80–90 °C) (Fig. 4b). An increase in denaturation temperature transition midpoints has also been reported from the mutant xylanase variants of *Bacillus subtilis* and XYL7746 [32, 36].

Enzymatic treatment of pulp

The treatment of pulp using Mxyl/MxylM4 xylanase clearly indicated the release of lignin-derived compounds (LDC) and chromophores, confirming the applicability of MxylM4 at elevated temperature under alkaline conditions. Paper and pulp industries need such enzymes in pre-bleaching of pulps for mitigating the chlorine requirement. An increase in the absorbance at 280 and 465 nm and release of reducing sugars indicated the hydrolytic action of xylanase on pulp fibers and its applicability in biobleaching of pulps. MxylM4 displayed better and longer effects on the hot and alkaline pulp samples (Table 4). Similar effects of xylanases from *B. licheniformis* [7], *B. pumilus* [17], and *B. coagulans* [5] have been reported. To the best of our knowledge, this is the first report on metagenomic xylanase that has been reported to have potential application in pulp bleaching as most of the xylanases retrieved from environmental metagenomes do not withstand extreme conditions.

Conclusions

Improvement in thermostability at 80 and 90 °C and increase in T_m of MxylM4 has been achieved by

Table 2 Comparison of thermostabilities of MxylM4 with Mxyl

Enzyme	Without substrate ($T_{1/2}$ min)		With birchwood xylan ($T_{1/2}$ min)	
	80 °C	90 °C	80 °C	90 °C
Mxyl	120	15	130	25
MxylM4	125	20	150	30

Table 3 Comparison of properties of MxylM4 with the parental xylanase (Mxyl)

Properties	Mxyl	MxylM4 (mutein)
Sp. enzyme activity	250 ± 2.79 U mg^{-1}	186 ± 2.14 U mg^{-1}
Temperature optimum	80 °C	80 °C
pH optimum	9.0	9.0
Molecular weight	∼40 kDa	∼40 kDa
K_m	8.01 ± 0.56 mg ml^{-1}	12.5 ± 0.32 mg ml^{-1}
V_{max}	333.06 ± 2.35 μmol mg^{-1} min^{-1}	294 ± 1.95 μmol mg^{-1} min^{-1}
K_{cat}	2,220 ± 10.24 s^{-1}	1,781 ± 8.35 s^{-1}
$T_{1/2}$ (80 °C)	130 min	150 min
$T_{1/2}$ (90 °C)	25 min	30 min

Table 4 Effect of Mxyl and MxylM4 in biobleaching of kraft pulp

Incubation time (h)	Chromophores (A_{465})		LDC (A_{280})		Reducing sugars released (mg g^{-1} oven-dried pulp)	
	Mxyl	MxylM4	Mxyl	MxylM4	Mxyl	MxylM4
0	0.003 ± 0.011	0.003 ± 0.011	0.09 ± 0.002	0.07 ± 0.002	02.12 ± 0.23	02.12 ± 0.14
2	0.059 ± 0.015	0.065 ± 0.015	0.123 ± 0.003	0.131 ± 0.003	16.83 ± 2.32	17.34 ± 1.49
4	0.074 ± 0.012	0.079 ± 0.012	0.201 ± 0.004	0.215 ± 0.004	20.12 ± 1.43	22.19 ± 1.42
5	0.089 ± 0.014	0.094 ± 0.014	0.215 ± 0.005	0.223 ± 0.005	21.32 ± 2.51	23.23 ± 2.11
8	0.099 ± 0.012	0.110 ± 0.012	0.224 ± 0.003	0.226 ± 0.003	22.30 ± 1.62	27.10 ± 1.57
10	0.112 ± 0.016	0.124 ± 0.016	0.226 ± 0.002	0.232 ± 0.002	22.13 ± 1.42	28.13 ± 2.01
12	0.116 ± 0.016	0.142 ± 0.016	0.234 ± 0.001	0.245 ± 0.003	23.12 ± 2.37	27.96 ± 2.42

engineering four arginines on the surface of Mxyl. The enhancement in the liberation of chromophores and lignin derived compounds from kraft pulp on treatment with MxylM4 confirms its applicability in pulp bleaching.

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