

Improvement and Immobilization of a new Endo- β -1,4-xylanases KRICT PX1 from *Paenibacillus* sp. HPL-001

Hee Kyung Lim¹, No-Joong Park¹, Young Kyu Hwang^{1,2}, Kee-In Lee^{1,2} and In Taek Hwang^{1,2*}

¹Nanocatalysis Center, Green Chemistry Division, Korea Research Institute of Chemical Technology, Yuseong PO Box 107, Daejeon 305-600, Republic of Korea

²Department of Green Chemistry and Environmental Biotechnology, University of Science and Technology, 217 Gajungro, Yuseong-gu, Daejeon, 305-350, Republic of Korea

Abstract

A new endo-1,4-beta-xylanase gene (FJ380951), KRICT PX1, isolated from *Paenibacillus* sp. HPL-001, was expressed in *E. coli*. Enzyme purification, mutation, immobilization, and molecular simulation were conducted. The P1H1 mutant of one amino acid substitution (168Leu→Gln) showing the strongest xylanase activity was selected among 250 mutant clones. The specific activity of the P1H1 xylanase was 53.3 U/mg protein at 50°C, an approximately five-fold increase compared to that of the original KRICT PX1 xylanase (10.25 U/mg protein) at the same condition of pH 5~10, and enhanced activity from 20.1 to 36.6 U/mg proteins at 60°C. The structural dynamics of the mutant P1H1 became stronger than that of the wild type, which could be eliminated by a helix deformation with the H-bond missing between Leu₁₆₈ and Asp₁₆₄ after mutation. Xylanase could be reused for 5 batches without significant loss of activity after being immobilized on surface functionalized silica-based mesoporous cellular foam. However, xylanase activity declined to 60% at the 10th batch. Further research on practical applications will be necessary for industrial usage.

Keywords: Biocatalyst; Error-Prone PCR; Hemicelluloses; Mutation; Xylanase

Introduction

Endo- β -1,4-xylanases catalyze the hydrolysis of the backbone of xylan to produce xylooligosaccharides, which in turn can be converted to xylose by β -xylosidase [1]. Endo-xylanase (1,4- β -D-xylan xylohydrolase, EC 3.2.1.8) initiates the degradation of xylan into xylose and xylo-oligosaccharides of different sizes [2]. This enzyme also has been widely used in baking and industrial processes such as the clarification of fruit juices, waste treatment, and the bio-bleaching of paper pulp [3]. An increasing number of reports and articles mentioning the isolation of newer microbial species for xylanase production reveal growing interest by the scientific community in this field. Owing to the rising biotechnological importance of xylanases, interest in industrially applicable xylanases has markedly increased. Accordingly, many attempts are being made to isolate new strains and to discover more relevant xylanases [4,5].

Before using xylanase at industrial levels, several criteria have to be fulfilled. Pilot scale processes are generally carried out at high temperatures; therefore bacterial xylanase having broad ranges of pH and temperature stability are preferred in industrial applications [6]. Similarly, xylanases extracted from actinomycetes are also operational over a broad range of reaction parameters [7,8], whereas fungal xylanases are stable under acidic pH conditions, ranging from pH 4 to 6 only [9]. However, some fungal species produce xylanases that are active at highly alkaline pH, but their number is few and they are less efficient in comparison to the acidic fungal xylanases. In order to obtain the maximal effect of xylanase, some of the reaction parameters, such as enzyme dose, retention time, pH, and temperature, should be optimized. Xylan-degrading enzymes of microorganisms are potentially important in various industrial processes for use under high temperatures and a broad range of pH conditions. A large proportion of research goes into finding or creating enzymes with specific properties, but the inherent activity, stability, and enantioselectivity of enzymes are not always suitable for industrial applications; there is consequently a need to engineer natural enzymes [10,11].

Enhancing the characteristics of the enzyme to lend it more suitable characteristics such as thermo-stability, pH optima, or substrate specificity can be accomplished using a number of approaches. The most commonly used random mutagenesis method is error-prone PCR

[12], which introduces random mutations during PCR by reducing the fidelity of DNA polymerase. The fidelity of DNA polymerase can be reduced by adding manganese ions or by biasing the dNTP concentration. The use of a compromised DNA polymerase causes disincorporation of incorrect nucleotides during the PCR reaction, yielding randomly mutated products.

Immobilization of enzymes is an alternative to facilitate downstream processing such as recycling the enzymes as well as enhancing activity, stability, and selectivity. Silica-based mesoporous materials are attractive supports for enzyme immobilization because they have large surface areas and high pore volumes, with well-defined pore sizes. They also offer the possibility of enzyme immobilization at mild conditions. Among mesoporous materials used as enzyme supports; mesocellular foams (MCF) have shown better performance in terms of retaining activity and mass transfer feasibility [13].

We have recently discovered a new xylanase, KRICT PX1, from a strain of *Paenibacillus* sp. HPL-001 (Korean Collection for Type Culture: KCTC 11365BP) and expressed in *Escherichia coli*. Also, some of the biochemical properties of the purified enzyme were reported [5]. In the present study, we immobilized the enzyme on silica-based Mesoporous Cellular Foam (MCF) and enhanced the thermo-stability of the enzyme to make it more suitable for industrial needs using error-prone PCR.

Materials and Methods

Isolation and mutation of KRICT PX1 gene

Xylanase KRICT PX1 has been isolated from a *Paenibacillus* sp.

***Corresponding author:** In Taek Hwang, Department of Green Chemistry and Environmental Biotechnology, University of Science and Technology, 217 Gajungro, Yuseong-gu, Daejeon, 305-350, Republic of Korea, Tel: 82-42-862-7392; E-mail: ithwang@kRICT.re.kr

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HPL-001 (KCTC11365BP) and the sequences of nucleotides or deduced amino acids were analyzed with CLC Free Workbench, Ver. 3.2.1 (CLC bio A/S, www.clcbio.com). Related sequences were obtained from database searches (SwissPort and GenBank). The genome sequence of KRICT PX1 was submitted to GenBank, and assigned as Accession Number FJ380951. The xylanase KRICT PX1 gene consisting of 996 bp encoding a protein of 332 amino acids (38.1 kDa) has been cloned and expressed in *Escherichia coli*. The xylanase KRICT PX1 was purified with an immobilized glutathione column and cleaved with Factor Xa before elution through a benzamidine column.

Site-directed mutagenesis was performed on KRICT PX1 using an Error-prone PCR kit from Clontech according to the protocol recommended by the manufacturer and using the following primers: PX1M FP-*Bam*HI (forward), AGGGATCCCATGAGGTTGCG, PX1M RP-*Hind*III (reverse), CCAAGCTTGGCCTAAAGAGTCAAG. The PCR conditions used were as follows: 1 min at 95°C, 25 cycles (30 sec at 94°C, 30 sec at 55°C, 1 min at 68°C), and 5 min at 68°C. Products were digested with *Bam*HI and *Hind*III and cloned into the pSTV28 plasmid vector (Takara Bio Inc.). The obtained plasmid library was used to transform competent *E. coli* JM109 (Promega).

Selection and Expression of the KRICT PX1 Mutant Gene in *E. coli*

E. coli JM109 transformants were picked with a sterile tooth pick and re-suspended in separate wells of a 96-well flat-bottom block containing 200 μ l of LB broth (Difco) and ampicillin in each well. After growing the cells for 24 h at 37°C, the cells were collected by centrifugation and then suspended in 200 μ l of 1 \times phosphate-buffered saline (Sigma-Aldrich). Lysis was directly performed with a 96 pin probe-attached sonic disrupter (Sonic vibra-cells, Sonics & Materials). Activity was determined by the DNS method [14]. An aliquot of 50 μ l of lysate from each well was mixed in 50 μ l of 2% birch wood xylan (Fluka). After incubation at 50°C for 30 min, two volumes of stop DNA solution (1% (w/v) DNS, 30% (w/v) potassium sodium tartarate, and 0.4 N NaOH) were added. The mixture was boiled at 99°C for 10 min, and activity was determined from the increase of absorbance at 540 nm on a Bench Mark Plus plate reader (Bio-rad).

The four xylanase-active clones were selected from the library screening, and the nucleotide sequence of each insert was determined by automated sequencing under BigDye™ terminator cycling conditions. The reacted product was purified using ethanol precipitation and analyzed with an Automatic Sequencer 3730 \times 1 (Applied Biosystems, Weiterstadt, Germany). Nucleotide and deduced amino acid sequences of selected KRICT PX1 mutant clones were analyzed with KRICT PX1 using the ClustalW2 tool (<http://www.ebi.ac.uk/>). The KRICT PX1 mutant gene was inserted into the pIVEXGST fusion vector and transformed into *E. coli* BL21 (Roche Applied Science) to produce a recombinant fusion protein.

Purification and analysis of recombinant enzyme

The transformed *E. coli* with GST-fused xylanase were grown overnight in 100 ml of LB medium containing ampicillin (100 μ g/ml) at 37°C and 200 rpm in a shaking incubator. After 4 h, the cultures were induced with 1 mM IPTG and incubated under the same conditions for 4 h longer. The induced bacteria were collected by centrifugation and suspended in 10 ml of ice-cold 1X phosphate-buffered saline (Sigma-Aldrich), and this process was performed three times. The cells from the final washing process were resuspended in the lysis buffer (pH 7.0,

200 mM Tris-HCl, 10 mM NaCl, 10 mM β -mercaptoethanol, 1 mM

EDTA), and treated with a sonic disruptor (CosmoBio Co., LTD). After cell disruption, the lysate was centrifuged at 10,000 g for 20 min at 4°C and the supernatant was eluted through a GST binding resin column (Novagen, Madison WI, USA). The column was previously equilibrated with a washing buffer (pH 7.0, 50 mM Tris-HCl, 15 mM NaCl), and the lysate was then applied to the equilibrated column with a flow rate at 10-15 cm/h. After washing the GST resin with 20 bed volumes of cold washing buffer, the fusion protein was eluted and fractionated with 10 bed volumes of freshly prepared elution buffer (pH 7.0, 20 mM Glutathione, 50 mM Tris-HCl, 15 mM NaCl). All fractions were examined with Bradford's protein determination, a PAGE analysis, and a DNS assay. Active fractions were pooled and treated with Restriction Protease Factor Xa (Roche Applied Science), and eluted through a *p*-aminobenzamidine-agarose column (Sigma- Aldrich) according to the manufacturer's instruction. The elute was precipitated with 70% ammonium sulfate, solubilized in phosphate-buffered saline, and dialyzed to concentrate at 4°C with a dialysis membrane (Spectra/Por CE, MWCO 10,000), and then the protein content was determined prior to being stored at -20°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide was performed by the method reported by Laemmli [15]. The protein fractions were boiled for 3 min and applied to the gel. Proteins were visualized by Coomassie brilliant blue R 250 staining. The protein concentration was determined by a Bradford [16] assay using bovine serum albumin as a standard.

Xylanase activity was measured according to a method slightly modified from Saha [17] using 50 μ l of a 1% (w/v) solution of birch wood xylan (Fluka) and 200 mM each pH buffer (50 mM citric buffer for pH 2-6.5, phosphate buffer for pH 7-9, and glycine buffer for pH 9.5-12) incubated with 30 μ l of an appropriately diluted enzyme (3.3 mg/ml) for 20 min at different temperatures and pHs. The activity of the xylanase according to the different substrates was assessed at the same conditions of 1% xylan from beech wood, birch wood, and oat spelt less than 50°C at pH 7.0. The released reducing sugars were assayed using the DNS method [14]. One unit of xylanase activity was defined as the amount of enzyme that liberated 1 μ mol of xylose equivalents per minute under the assay conditions. The optimal temperature and pH conditions for the xylanase activity of recombinant KRICT PX1 and KRICT P1H1 protein were examined in 96-well micro plates with a DNS assay at various temperatures (50°C, 60°C) and pH conditions (ranging from pH 5 to 10). The effect of the birch wood xylan concentration on the xylanase activity was evaluated under optimal assay conditions. A diluted enzyme solution (100 μ g protein in 30 μ l) was incubated with 0.5 ml of various concentrations (0-10 mg/ml) of xylan in 50mM citrate buffer (pH 5.5) and glycine buffer (pH 9.5) at 50°C for 20 min. Xylanase activity was measured with a DNS assay at 540 nm, as described above. The kinetic parameters (Michaelis-Menten constant, K_m , and maximal reaction velocity, V_{max}) were estimated by a linear regression from double-reciprocal plots according to Lineweaver and Burk [18]. The effects of metallic ions and other chemicals on the xylanase activity of KRICT PX1 and KRICT P1H1 proteins were studied as described above at pH 7 with addition of 1 mM NaCl, LiCl, KCl, NH_4 Cl, $CaCl_2$, $MgCl_2$, $MnCl_2$, $CuSO_4$, $ZnSO_4$, $FeCl_3$, $CsCl_2$, ethylenediamine tetra-acetic acid (EDTA), 2-mercaptoethanol (2-ME), dithiothreitol (DTT), phenylmethane sulphonyl fluoride (PMSF), acetate, and furfural, respectively.

Structure modeling and molecular dynamics simulation analysis

The three-dimensional structures of xylanases KRICT PX1 and

KRICT P1H1 were modeled using the Discovery Studio version 3.0 program at the ExPASy server. The RCSB Protein Data Bank entries for these proteins are 2q8x and 2fgl. The modeled structures were visualized and analyzed using Swiss-Pdb Viewer version 3.51. The Figures were created with MOLSCRIPT version 2.1. A molecular dynamics simulation analysis was conducted with the calculation protocol of minimization 2,000 step (steepest descent, fixed backbone constraint, 1st stage only), minimization 2,000 step (steepest descent), heating 100,000 step (0 \rightarrow 300K), equilibration 500,000 step (300K), production 400,000 step (300K, NVT, 200 structures), and implicit solvent (distance dependent: dielectric constant 1).

Synthesis of Mesoporous Cellulose Foam (MCF) and enzyme immobilization

MCF was prepared by a hydrothermal method that has been reported elsewhere [19]. MCF synthesis: 1.62 g (0.279 mmol) of Pluronic P123 (triblock copolymer, chemical formula:

$\text{HO}(\text{CH}_2\text{CH}_2\text{O})_{20}(\text{CH}_2\text{CH}(\text{CH}_3)\text{O})_{70}(\text{CH}_2\text{CH}_2\text{O})_{20}\text{H}$, molecular weight: 5,800) was transferred into a 100 ml volume of a polypropylene bottle and dissolved in a mixture of 33.33 g (1,852 mmol) of deionized water containing 0.8 g (13.32 mmol) of acetic acid. After clearly dissolved, the solution was heated to 60°C and kept for 1 hr in an oil bath. Another solution was prepared with 2.67 g (11 mmol) of sodium

silicate as a silica source in 33.33 g of water. The molar composition of the final solution was $\text{Na}_2\text{SiO}_3\text{:P123:H}_2\text{O:acetic acid}=1\text{:}0.025\text{:}336.4\text{:}121$. The latter solution was dropped into the solution, and then aged at 60°C for 1h and reacted at 100°C for 12 h. The bottle was subsequently cooled to room temperature. A white precipitated product was filtered with water and ethanol. The final product was obtained by heat treatment at 550°C for 6 hrs. The surface area (Braunauer Emmett Teller, BET) of MCF and a Transmission Electron Microscope (TEM) image of MCF are presented in Figure 1.

Amine grafted MCF: Amine grafted MCF was prepared by post grafting with 3- aminopropyl triethoxysilane as an amine source. 1 g of MCF dried at 200°C for 12 h was added to 80 ml toluene under N_2 gas with a flow rate of 5 cc/min. 1.11 g (5 mmol) of (3- aminopropyl) triethoxysilane was slowly dropped to the solution. After mixing for 5 min, the mixture was heated to 110°C and maintained at that temperature for 24 hrs under reflux condition. Final product was recovered by filtration with water and ethanol.

Aldehyde grafted MCF: 0.5 g amine functionalized MCF was added to 50 ml water and then 0.2 g (2 mmol) of glutaraldehyde was injected to the mixture. After stirring at room temperature for 5min, the mixture was heated to 60°C and maintained at that temperature for 24 hrs. The final product was filtered with water and ethanol and FT-IR spectrum was presented at Figure 2. Enzyme immobilization was conducted at pH 7, MCF or alkyl MCF (2 mg) was added to 4 ml

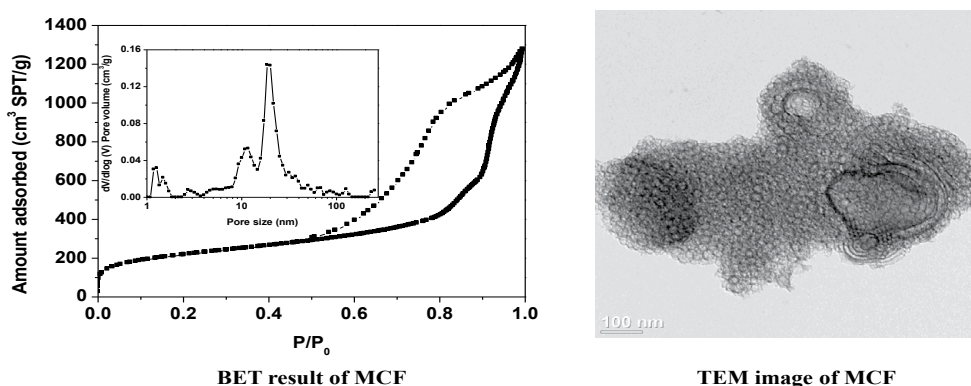


Figure 1: Surface area (BET) and Transmission Electron Microscope image (TEM) of Mesocellular Foams (MCF).

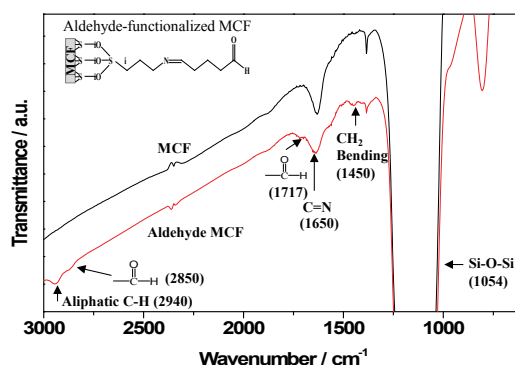


Figure 2: Aldehyde-functionalized MCF was confirmed by Fourier Transform Infrared Spectroscopy (FT-IR). Black line: standard MCF, Red line: aldehyde-functionalized MCF.

buffer solution (50 mM of Tris-HCl, pH 7.0) containing xylanase (0.5 mg/ml) in a 15-ml micro tube. The micro tube was shaken at 85 strokes per minute using a shaking incubator model SI-300 overnight at 20°C. The suspension was centrifuged at 2,000 rpm for 2 min and then the supernatant was taken from each micro tube. The solid (the MCF or alkyl-MCF with immobilized xylanase) was washed twice with 4 ml of buffer, centrifuged and the liquid removed. The supernatants were collected and checked for residual protein concentration according to the Bradford protein assay using bovine serum albumin as standard. The adsorption ratio (%) of xylanase protein on the supports was defined as $(m_i - \Sigma mf) / m_i$, where m_i and Σmf are the masses of xylanase protein of initial xylanase solution and supernatants, respectively. The xylanase protein loading on the supports was defined as $(m_i - \Sigma mf) / W_{support}$, where m_i and Σmf are the masses of xylanase protein of in the initial xylanase solution and supernatants, respectively. $W_{support}$ indicates the weight of the support. Repeated batch reaction was conducted with xylanase immobilized on MCF using the same reaction mixture as for batch mode reaction of xylanase activity assay after wash enzyme and filtering with membrane filter (ϕ 100 μ m).

Results and Discussion

Enzyme improvement with error-prone PCR

We screened a random mutation library generated by error prone PCR of the xylanase gene *KRICT-PX1* for enhanced thermo-stability and to make it more suitable for industrial needs. A DNS assay in a 96-well micro-plate was used to select mutants with enhanced xylanase activity. Several mutants showed xylanase activity at pH 9.0. From the

results, we identified 6 transformants that expressed a library of 250 random mutants. Table 1 lists the top six mutants selected from the pH 9.0 conditions, together with their DNA and amino acid changes, and activity at 50°C and 60°C. DNA and amino acid sequence analyses showed that the mutant of P1F10 had weak xylanase activity and a DNA change of $_{45}$ Cytosine to Thymine without an amino acid change. The mutant of the PIC11 clone had weak xylanase activity and a DNA sequence change of $_{841}$ Adenine to Thymine with a $_{281}$ Threonine to Serine amino acid change. Mutant P1H1 showed three DNA sequences ($_{183}$ Thymine to Adenine, $_{503}$ Thymine to Adenine, $_{534}$ Thymine to Cytosine) and one amino acid ($_{168}$ Leucine to Glycine) changes with remarkable improvement of xylanase activity. Mutant P2A2 showed two DNA ($_{386}$ Cytosine to Thymine, $_{177}$ Adenine to Guanine) and one amino acid ($_{123}$ Alanine to Valine) change with moderate xylanase activity. P2C1 showed only one DNA ($_{102}$ Cytosine to Thymine) change without an amino acid mutation and weak xylanase activity. P2D3 showed one DNA ($_{800}$ Guanine to Adenine) and one amino acid ($_{267}$ Glycine to Serine) change. Among the 250 mutant clones, the P1H1 clone was selected; it showed the greatest xylanase activity, a maximum of 53.3 U/mg protein at pH 7.0 and 50°C, which was approximately a 5-fold increase compared to the original KRICT PX1 xylanase activity of 10.25 at the same conditions. Also, the xylanase activity of P1H1 was enhanced from 20.1 to 36.6 U/mg proteins at pH 7.0 and 60°C (Figure 3). A molecular dynamics simulation study showed that the structural dynamics of the mutant P1H1 became stronger than that of the wild type, which could be eliminated by a helix deformation with the H-bond missing between $_{168}$ Leu and $_{164}$ Asp after mutation (Figure 4).

Clone ID*	DNA change**	Amino acid change***	Activity****		
			pH 6.0, 50°C	pH 9.0, 50°C	pH 9.0, 60°C
P1F10	45 C → T	None	★★	★	-
P1C11	841 A → T	281 Thr → Ser	★	★★	★
P1H1	183 T → A	None	★★★★★	★★★★★	★★★★★
	503 T → A	168 Leu → Gln			
	534 T → C	None			
P2A2	386 C → T	123 Ala → Val	★★	★★★	★★
	177 A → G	None			
P2C1	102 C → T	None	★	★	-
P2D3	800 G → A	267 Gly → Ser	★★	★★★	★

*Clone ID means clone position among mutant library in 96-well plate.

**Position and base in DNA sequence of xylanase kricht PX1; Adenin (A), Timin (T), Sitosin (C), Guanin (G).

***Position and amino acid in natural xylanase and mutant; Threonine (Thr), Leucine (Leu), Serine (Ser), Glutamine (Gln), Alanine (Ala), Valine (Val), Glycine (Gly).

****The activity was arbitrarily expressed with results from 96-well micro-plate DNS assay.

Table 1: Changes of DNA, amino acid, and activity in selected mutants.

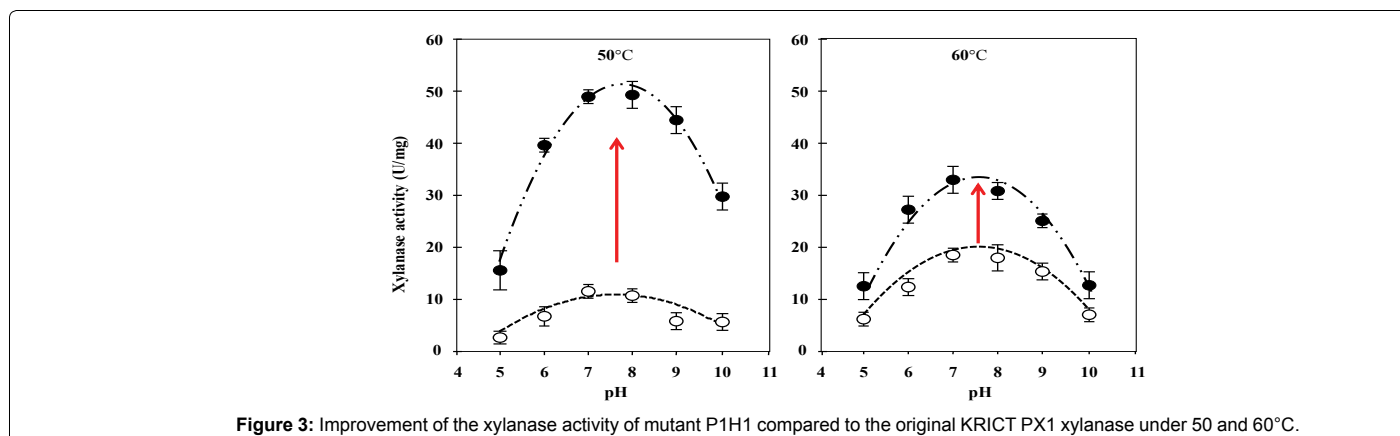
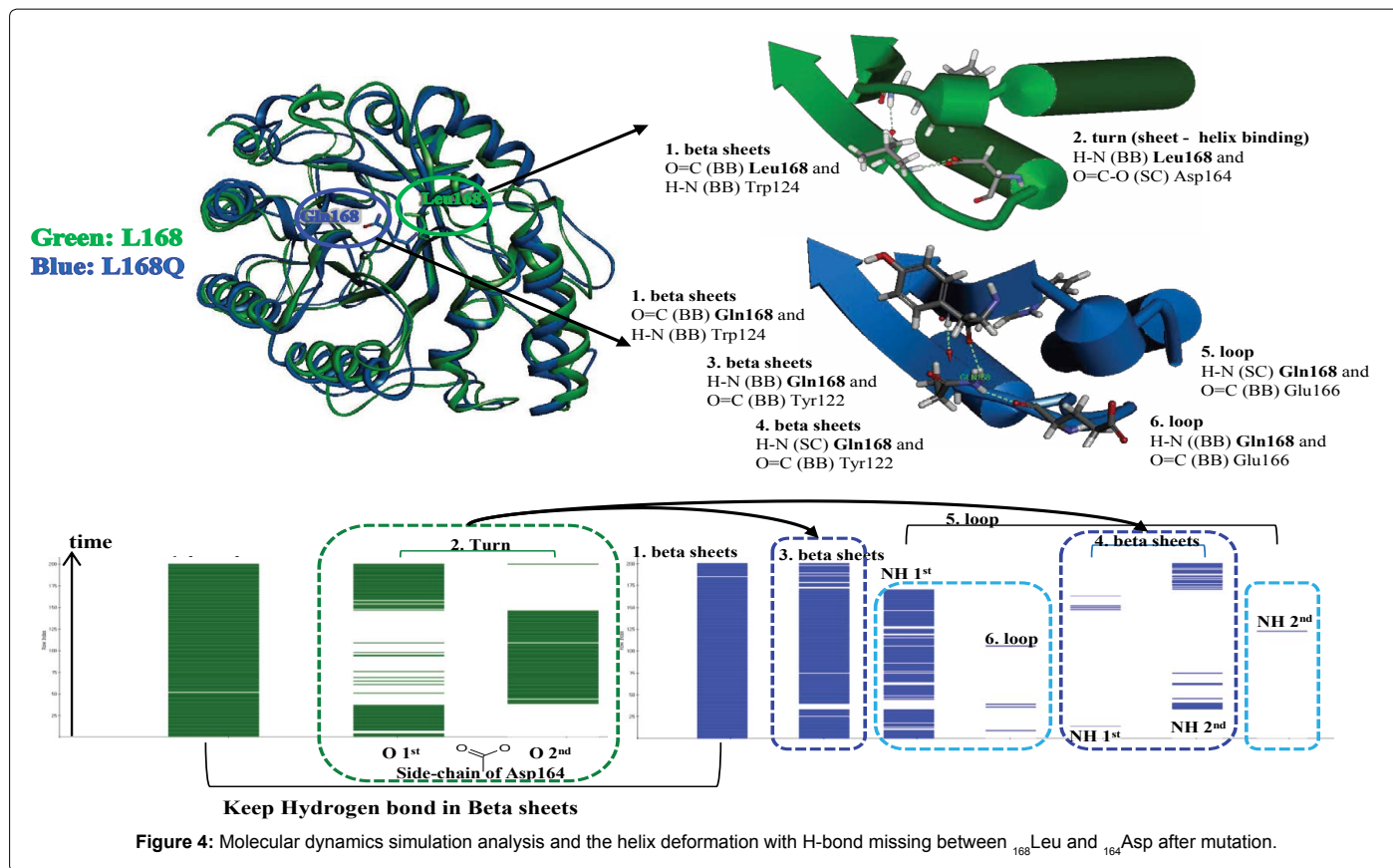
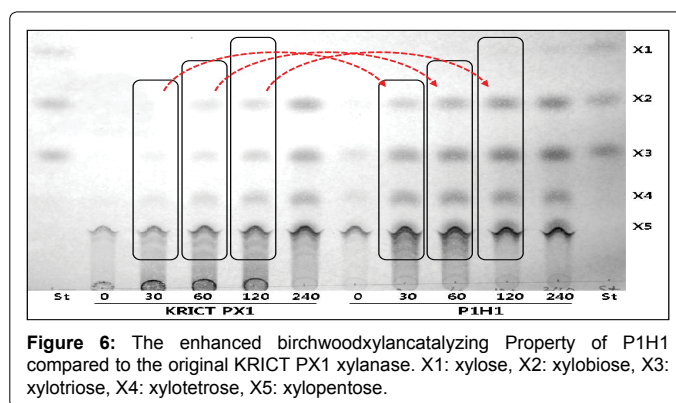
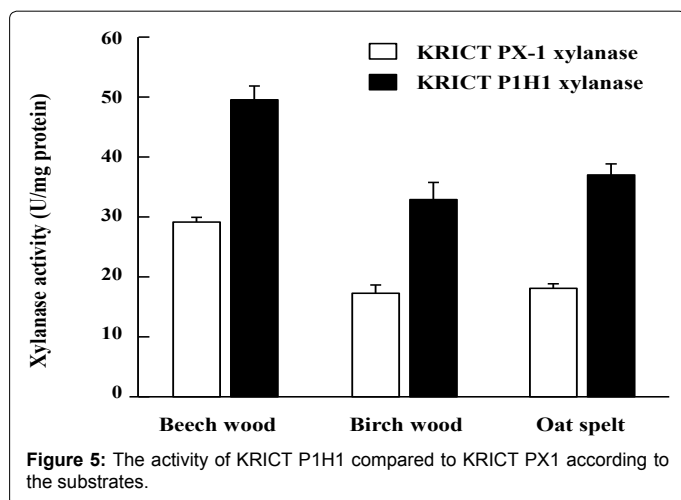


Figure 3: Improvement of the xylanase activity of mutant P1H1 compared to the original KRICT PX1 xylanase under 50 and 60°C.



There is a need to engineer natural enzymes [10,11] because the inherent activity, stability, and enantioselectivity of existing enzymes are not always suitable for industrial applications. The most commonly used random mutagenesis method is error-prone PCR [12], which introduces random mutations during PCR by reducing the fidelity of DNA polymerase (Figure 5). The use of compromised DNA polymerase causes misincorporation of incorrect nucleotides during the PCR reaction, yielding randomly mutated products. Determining which mutation positions are critical in gaining the ability to enhance practical applications. No dramatic enhancement of activity was found for xylan



hydrolysis among the 250 mutations. However, one of the 6 selected mutants, P1H1, showed good thermostability and enhanced xylanase activity. Nevertheless, further research on practical applications will be necessary for industrial usage. The activity of mutant P1H1 was improved roughly two-fold relative to that of KRICT PX1 when compared to different substrates, that is, beech wood, birch wood, and oat spelt xylan by these two xylanases revealed that the hydrolysis of birchwood xylan by P1H1 xylanase was significantly greater than that of KRICT PX1, and xylobiose was the major product with smaller amounts of xylotriose and xylose (Figure 6).

One of the exciting applications of xylanases is the production of xylo-oligosaccharides. Xylo-oligosaccharides have stimulatory effects on the selective growth of human intestinal *bifidobacteria* and are frequently defined as probiotics. Xylo-oligosaccharides (xylobiose,

xylotriose, xylotetraose, etc.) prepared from various sources of xylans such as wheat bran, birchwood, or corncob can be utilized selectively by the beneficial intestinal microflora, viz. *bifidiobacteria*, are thus expected to be used as a valuable food additive [20]. Further studies on the production of xylo-oligosaccharides from economical agricultural residues such as wheat bran and rice bran are currently underway.

Another change in the characteristics of the P1H1 mutant was that the effects of additives on the xylanase activity were weakened by CaCl₂, MgCl₂, MnCl₂, and FeCl₃ (Table 2). Xylanase activity of KRICT PX1 was completely inhibited by 1 mM CaCl₂, MgCl₂, MnCl₂, and FeCl₃ whereas

Additives	Relative activity (%) at 1 mM	
	KRICT P1H1	KRICT PX1
None	100	100
NaCl	100	100
LiCl	100	100
KCl	100	100
NH ₄ Cl	100	100
CaCl ₂	100	0
MgCl ₂	100	1
MnCl ₂	83	3
CsCl ₂	99	100
CuSO ₄	21	8
ZnSO ₄	30	2
FeCl ₃	79	6
EDTA	54	100
2-ME	100	100
DTT	100	100
PMSF	100	100
Acetate	72	100
Furfural	100	100

Table 2: The improved activity of P1H1 compared to the original KRICT PX1 xylanase effective of additives.

these metal chloride ions did not have any dramatic effects on the P1H1 xylanase. Acetate and furfural are the major residual components of biomass pretreatment involving enzyme deactivation or inhibition. However, P1H1 xylanase was safe up to 1 mM of acetate, furfural, phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT), and 2-mercaptoethanol (ME), respectively.

Enzyme immobilization

To investigate the potential of surface functionalized MCF spheres for immobilization with a new xylanase KRICT PX1 from *Paenibacillus* sp. HPL-001, enzymes functionalized with on the three supports regardless of surface functionalization, but the residual xylanase activity was higher in the case of immobilization on MCF-C₃-NH₂ and MCF-C₃-RC(=O)H than standard MCF. However, the residual enzyme activity was decreased to 70, 80, and 50% of the original activity after immobilization on MCF-C₃-NH₂, MCF-C₃-RC(=O)H, and standard MCF, respectively (Figure 7A). This might be due to the decreasing opportunity of contact between the enzyme and the substrate, because the immobilized body became bigger, and pores could impede enzyme action.

Among these results, MCF-C₃-NH₂ immobilized xylanase was conducted repeat-batch reaction as using the same reaction mixture of xylanase activity assay after washing the enzyme and filtering with a membrane filter (ø 100 μm). The residual activity was decreased with the number of recycling steps; however, the immobilized enzyme showed only a slight reduction of residual activity after 6 cycles. At the 10th batch reaction, about 40% of the original activity was lost. This loss of specific activity suggests a diffusion limitation on the substrate or product flux due to the association of the enzyme within the pores of the carriers (Figure 7B).

Similar to our work, Mukesh and Ramesh [20] reported that the xylanase from *Bacillus pumilus* strain MK001 showed the maximum xylanase immobilization efficiency on different matrices of entrapment

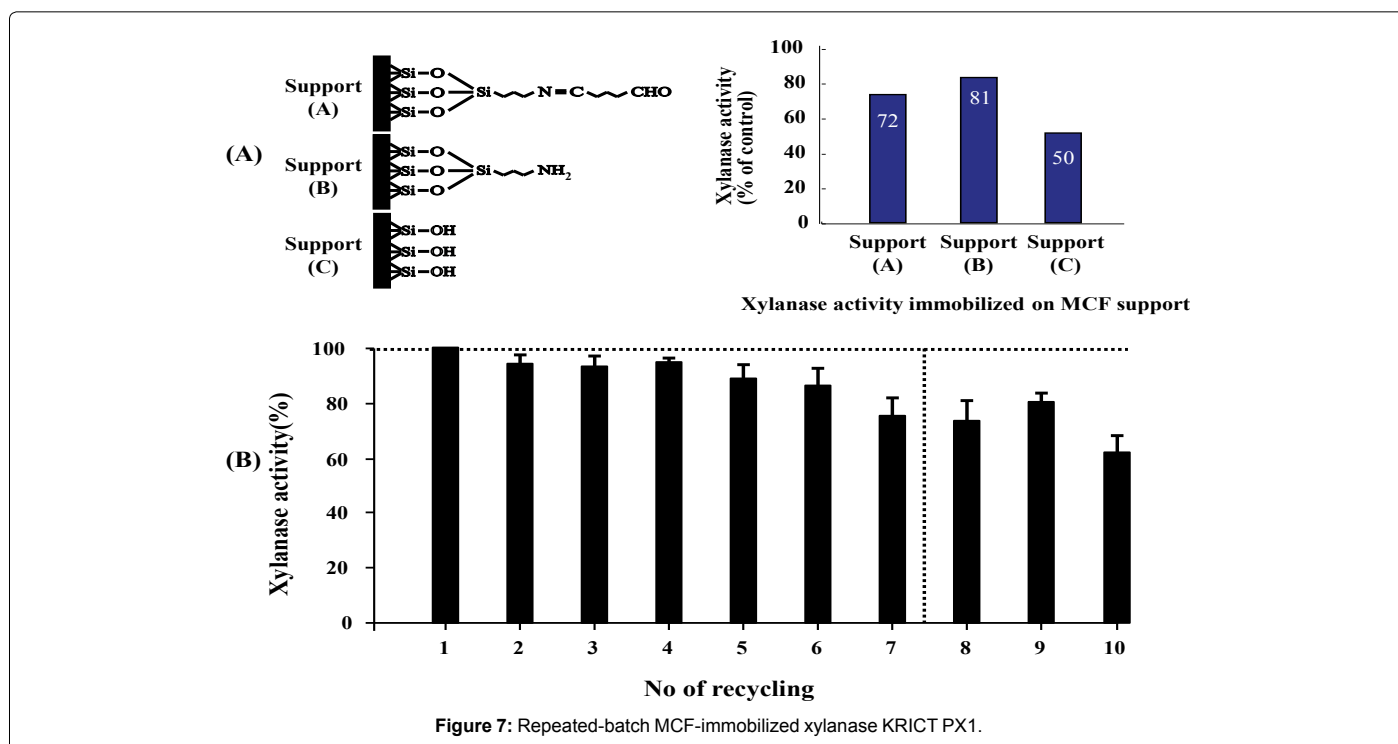


Figure 7: Repeated-batch MCF-immobilized xylanase KRICT PX1.

using gelatin (40%, GE), physical adsorption on chitin (35%, CH), ionic binding with Q-sepharose (45%, Q-S), and covalent binding with HP-20 beads (42%, HP-20). The reason for low immobilization efficiency may be the crowding of other proteins on the support with a direct effect on the accessibility of enzyme molecules to the adsorption material. MCF has high mechanical stability and rigidity, regenerability, and ease of preparation in different geometrical configurations, providing the system with permeability and suitable surface area for a given biotransformation and reducing the system cost.

Xylanases are an important group of carbohydrases, with a worldwide market of approximately 200 million dollars, and are being routinely used in various industrial processes such as animal feed digestion, waste treatment, energy generation, production of chemicals, and paper manufacturing. One of the exciting applications of xylanases is the production of xylo-oligosaccharides. They also show remarkable potential for utilization in pharmaceuticals, in feed formulations, in agricultural applications, and as food additives. In industrial applications, the free forms of enzymes pose difficulties, e.g., instability of enzyme structure and sensitivity under harsh process conditions, non-recovery of the active form of the enzyme from the reaction mixture for reuse, and contamination of the final product [21]. The strategies used for improving the stability of proteins include the use of additives, introduction of disulfide bonds [22], site-specific mutagenesis [23], and chemical modification or cross-linking [24]. However, in all these strategies, the yield and the reusability of free enzymes as industrial catalysts are quite limited. Increased attention therefore has been paid to immobilization techniques, which offer advantages over free enzymes in terms of choice of batch or continuous processes, rapid termination of reactions, controlled product formation, ease of enzyme recovery from the reaction mixture, and adaptability to various engineering designs. The extent of stabilization depends on the enzyme structure, the immobilization method, and the type of support [21]. During the last decade, numerous supports for xylanase immobilization have been investigated [25-30].

The reuse of an immobilized enzyme is an important factor considering cost effectiveness for commercial applications. A continuous assay of residual enzyme activity of xylanase immobilized on surface functionalized-MCF was performed to determine the retention of xylanase activity by each support over 10 enzyme reaction cycles. Xylanase immobilized on surface functionalized-MCF showed better retention and retained up to 70% activity after seven cycles.

Conclusion

A mutation library was produced by error-prone PCR of the xylanase gene *KRICT-PX1*. A DNS assay in a 96-well micro-plate used to select mutants with enhanced xylanase activity from mutant clones. P1H1 clone was selected on the basis of it having the highest xylanase activity of 53.5 U/mg protein at pH 7 and 50°C, which is approximately a five-fold increase compared to that of the original KRICT-PX1 xylanase with activity of 10.25 U/mg protein at the same conditions. The TLC analysis of the products catalyzed by two xylanases revealed faster degradation of birchwood xylan and larger production of small xylo-oligomers by P1H1. DNA sequencing of the P1H1 clone showed that it had one amino acid substitution (168Gly \rightarrow Gln) caused by a point mutation of 503rd thymine to adenine. Further research on the biochemical properties and a structural analysis of this point-mutated xylanase should be carried out to shed light on the catalysis mechanism of xylanase. The overall performance of the immobilized xylanase in terms of catalytic activity, thermal and pH stability reuse, and xylo-oligosaccharide production

is more promising than that of the free enzyme. The higher efficiency observed for xylanase immobilized on cost effective supports such as MCF favors its commercial utilization for various industrial processes such as production of xylo-oligosaccharides, animal feed digestion, pharmaceuticals, waste treatment, energy generation, production of chemicals, and paper manufacturing, as well as in agricultural applications and as food additives.

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