

## Structural Analysis of Xylanase from Marine Thermophilic *Geobacillus stearothermophilus* in Tanjung Api, Poso, Indonesia

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A xylanase gene, *xynA*, has been cloned from thermophilic strain *Geobacillus stearothermophilus*, which was isolated from marine Tanjung Api, Indonesia. The polymerase chain reaction product of 1266 bp of *xynA* gene consisted of 1221 bp open reading frame and encoded 407 amino acids including 30 residues of signal peptide. The sequence exhibited highest identity of 98.7% in the level of amino acid, with an extracellular endo-1,4- $\alpha$ -xylanase from *G. stearothermophilus* T-6 (E-GSX T-6) of the glycoside hydrolase family 10 (GH10). A comparative study between the local strain *G. stearothermophilus* (GSX L) and E-GSX T-6 on homology of amino acid sequence indicated five different amino acids in the gene. They were Threonine/Alanine (T/A), Asparagine/Aspartate (N/D), Lysine/Asparagine (K/N), Isoleucine/Methionine (I/M), Serine/Threonine (S/T) at the position 220, 227, 228, 233, and 245, respectively. Protein structural analysis of those differences suggested that those amino acids may play role in biochemical properties such as enzyme stability, in particular its thermostability.

Key words: enzyme xylanase, *Geobacillus stearothermophilus*, mutation, structural analysis

### INTRODUCTION

High thermal stability of enzymes have attracted considerable research interest due to its widely used in biotechnological and industrial applications such as in food, petroleum, paper, and pulp industries (Haki & Rakshit 2003). Therefore, many attempts have been addressed to improve the enzyme stability either by screening and isolating the enzyme from thermophilic microorganisms or by engineering the enzyme (Khasin *et al.* 1993; Georis *et al.* 2000; Shibuya *et al.* 2000; Sriprang *et al.* 2006; Wu *et al.* 2006).

Site-directed mutagenesis is a powerful tool for the study of protein structure and function. Therefore, it has become one of most widely used strategy for improving enzyme such as thermostability. However, this technique requires some informations such as three-dimensional (3D) structure and amino acid sequence of the enzyme (Georis *et al.* 2000; Sriprang 2006). Comparative study on homology amino acids of the enzymes and their 3D structures from diverse sources of the closely related thermophilics may facilitate in understanding certain amino acid function and its effect to the enzyme characteristics. Development of bioinformatics extensively so far has contributed to predict the structure as well as the function of amino acid on its enzyme characterization (Edwards & Cottage 2003; Arora *et al.* 2009).

Structure-thermostability relationships and the engineering of glycoside hydrolases (GH) have attracted

considerable current interest (Shibuya *et al.* 2000; Kaper *et al.* 2002; Xie *et al.* 2006). The GH 10 of family-10 xylanases perform glycosidic bond hydrolysis through the double displacement mechanism involving two glutamate residues (Harris *et al.* 1994; White *et al.* 1996; Charnock *et al.* 1998). When xylanases are used in biotechnological processes operating at elevated temperatures, a high thermal stability of the enzyme is a desirable property because it reduces costs by extending the life of the biocatalyst. Ideally, the optimum working temperature of the enzyme is the temperature which the biotechnological process operates, also it is required to consider the thermal stabilities of the other components.

The ( $\beta/\alpha$ )<sub>8</sub>-barrel fold, which was found in GH10, and is therefore also known as the TIM-barrel fold, is the most common enzyme fold. Moreover, ( $\beta/\alpha$ )<sub>8</sub>-barrel enzymes cover five of the six enzyme classes defined by the Enzyme Commission, acting as oxidoreductases, transferases, lyases, hydrolases, and isomerases (Nagano *et al.* 2002).

Enzyme xylanase of *Geobacillus stearothermophilus* T-6 has been well characterized from genetic to protein structure level. The enzyme has thermostability under temperature of up to 70 °C, and alkaline-stability of pH 7.0, and thus it has been extensively used in various industries, mainly biobleaching in the pulp and paper industry (Khasin *et al.* 1993; Beg *et al.* 2001). In this study, a 1266-PCR fragment of *xynA* gene encoding enzyme xylanase from thermophilic strain *G. stearothermophilus* which was isolated from marine Tanjung Api, Indonesia, has been cloned and sequenced. Study on the homology of the gene showed that the *xynA* gene shared high identity with

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those of *Geobacillus* sp. WBI (GenBank accession number EU93182) and *G. stearothermophilus* T-6 (GenBank accession number DQ868502) (data not shown), with slightly different in amino acid level. Thus, we are interested to predict the effect of those differences on the structure, which may affect to biochemical properties or enzyme characteristics. Moreover, our aim is preliminary study on the structure enzyme xylanase based on *G. stearothermophilus* T-6 protein structure (protein data bank/PDB file: 1H1Z) and bioinformatics approaches.

## MATERIALS AND METHODS

**Sequence Analysis of Xylanase Gene.** A 1266 bp fragment which was obtained by PCR amplification method with the genomic DNA of the local strain *G. stearothermophilus* from marine Tanjung Api Poso Indonesia, as a template (data not shown), was sequenced by Macrogen Co., Korea. The alignments of DNA and protein sequences for homology study were conducted with BLASTN and BLASTP programs, respectively (<http://www.ncbi.nlm.nih.gov/BLAST/>), and the protein sequences with E value of 0.00 were further analyzed with BioEdit 5.0.9 (BioEdit Sequence Alignment Editor, USA). Moreover, based on homology alignment searches, amino acids sequence with E value of 0.00 used in this study was summarized in Table 1. The composition of amino acids was calculated with ProtParam tool (ExPASy, Switzerland). In addition, signal peptide encoding region was analyzed using SignalP 3.0 Server ([www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)).

**Structural Analysis of Enzyme Xylanase.** The structure of protein was analyzed using Swiss-PDB Viewer 4.0.1 based on the three dimensional (3D) X-ray crystallography structure. Homology model of xylanase from the local strain *G. stearothermophilus* (GSX L) was constructed using Molecular Operating Environment (MOE) software based on 3D structure of extracellular *G. stearothermophilus* T-6 (E-GSX T-6) (PDBID: 1H1Z). The constructed model of GSX L was then used to locate the position of each amino acid which was different. Moreover, the 3D structure of E-GSX T-6, internal GSX T-6 (I-GSX T-6) (PDBID: 1N82), and extracellular xylanase from *Bacillus* sp. NG-27 (E-BX NG-27) (PDBID: 2FGL) were also compared.

## RESULTS

**Sequence Analysis of Xylanase Gene.** Based on sequence analysis, the PCR fragment of 1266 bp of *xynA* from the local strain *G. stearothermophilus* consisted of 1221 bp open reading frame (ORF) and encoded 407 amino acids including 30 residues of signal peptide. The amino acid sequence of GSX L shared highest identity of 98.7% with that of E-GSX T-6 (Figure 1). Moreover, the identity of amino acid sequence of GSX L with those of other xylanases was summarized in Table 2.

**Structural Analysis of Enzyme Xylanase.** To investigate the predicted effect of the different amino acids on the biochemical properties, structure of E-GSX T-6 has been retrieved from PDB file (PDBID: 1H1Z) and performed in Swiss-Pdb Viewer 4.0.1 software (Figure 2). The constructed of homology model of GSX L based on E-GSX T-6 using MOE demonstrated the position of each amino acid which was different. The differences were Threonine/Alanine (T/A) at the position 220 (A220T) which was located at the helix 4, Asparagine/Aspartate (N/D) at the position 227 (D227N) and Lysine/Asparagine (K/N) at the position 228 (N228K) which were located at the loop 4, Isoleucine/Methionine (I/M) at the position 233 (M233I) which was located at the  $\alpha$  strand, and Serine/Threonine (S/T) at the position 245 (T245S) which was located at the helix 5, respectively.

Figure 3 showed that the position of A220 at the helix 4 was in opposition to the helix 3, and Figure 4 showed that position of M233 at the strand 5 was in opposition to the helix 5. Amino acid D227 and N228 were located at the loop between the helix 4 and the strand 5, and closed to amino acid R222 was shown in Figure 5. Moreover, Figure 5 also showed that amino acid T245 was surrounded by P242 and E241, and located at the helix 5.

The comparison of 3D structure among E-GSX T-6, I-GSX T-6, and E-BX NG-27 was shown in Figure 6. Interestingly, it was seemed that their structures exhibited high similarity to each other. Therefore, we are going to compare their amino acid compositions.

## DISCUSSION

Xylanase (EC 3.2.1.8) catalyzes the hydrolysis of the main polysaccharide chain ( $\alpha$ -1,4 xylosyl bond) in xylan,

Table 1. Sources of xylanases with E value of 0.00 based on homology alignment searches used in this study

Sources of xylanases	Abbreviations	GenBank accession number	Location
Extracellular <i>G. stearothermophilus</i> T-6	E-GSX T-6	DQ868502	68530..69877
<i>Geobacillus</i> sp. WBI	GSX WBI	EU93182	1..1224
<i>G. thermodenitrificans</i> T-2	GTX T-2	EU599644	1..1224
<i>G. thermodenitrificans</i> NG80-2	GTX NG80-2	NC_009328	1862421..1863644
<i>Geobacillus</i> G11MC16	GX G11MC16	NZ_ABVH01000004	136193..137416
<i>Geobacillus</i> sp. Y412MC61	GX Y412MC61	NC_013411	2735874..2737079
<i>Geobacillus</i> sp. MT-1	GSX MT-1	DQ143882	1..996
<i>G. stearothermophilus</i> T-6 intracellular	I-GSX T-6	DQ868502	48293..49288
<i>Bacillus</i> sp. NG-27	BX NG-27	AF_015445	1351..2568
<i>B. halodurans</i> (C-125)	BHX	NC_002570	1..1191
<i>B. firmus</i>	BFX	AF317713	1..633





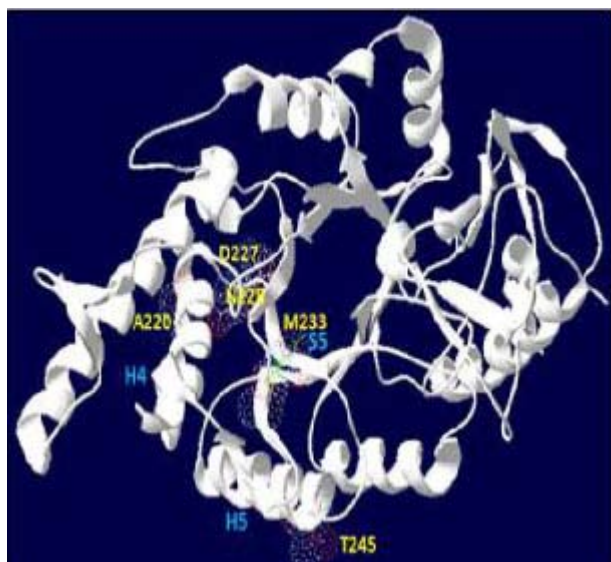


Figure 2. Location of the mutation sites on the three dimensional structure of extracellular enzyme xylanase *G. stearothermophilus* T-6. The differences of amino acid location while the structure helix (H) and strand (S) were shown by blue color.

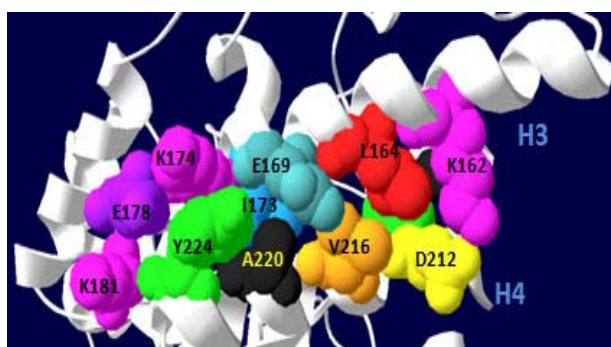


Figure 3. A closer view around the M233 of extracellular xylanase T-6.

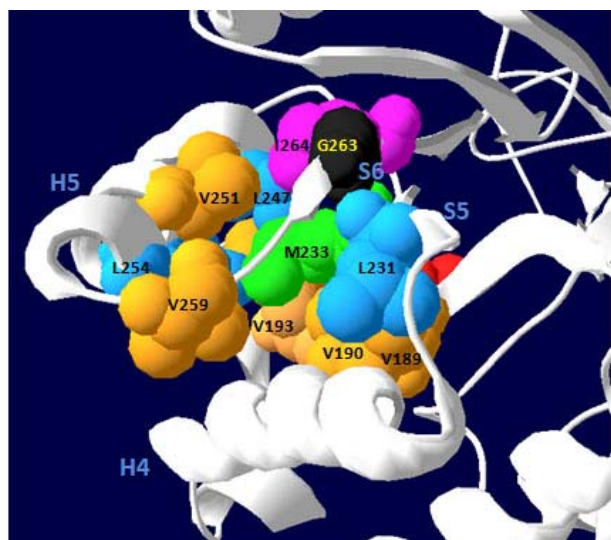


Figure 4. A closer view around the A220 of extracellular xylanase T-6.

As shown in Figure 1, the amino acid sequence of (GSX L) showed extensive homology to endo-1,4  $\alpha$ -

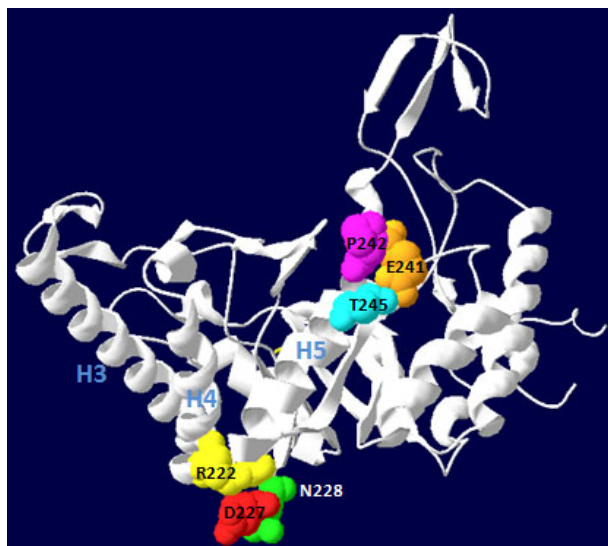


Figure 5. A closer view around the D227, N228, and T245 of extracellular xylanase T-6.



Figure 6. Comparison of three-dimensional (3D) structure among extracellular xylanase T-6, intracellular xylanase T-6, and extracellular NG-27.

xylanase belonging to GH10 family. Catalytic amino acid residues have been identified as two glutamate residues which conserved in GH10 xylanases (Collins *et al.* 2005). We noticed that catalytic glutamates at the position of 192 and 299 were conserved. Extracellular GSX T-6 and the GSX L have a signal peptide encoding region, while I-GSX T-6 does not. Signal peptide analysis showed that signal peptide encoding region of the sequence number 1 to 30. This was consistent with the average length of gram positive bacteria which is around 29 to 31 amino acids as reported by Von Heijne and Abrahamsen (1989). Interestingly, there were clearly fixed different pattern of signal peptide among gram-positive bacteria (Figure 1). Considering that signal peptide encoding region in *Escherichia coli* is 21 to 24 amino acid and the fact that differences in signal peptide will affect into secretion efficiency, therefore investigation on signal peptide effect on xylanase secretion will be taken into account.

Amino acid sequence of GSX has a fixed pattern with those of *G. thermodenitrificans* (GTXs) and of *B. halodurans* (BHX) (Figure 1). Based on the pattern, it was also seemed that GSX L may belong to GSX, whereas

xylanase *Geobacillus* G11MC16 (GX G11MC16) may belong to GTX. It was speculated that the fixed pattern may play an important role on the different characteristics between GSX and GTXs. Unfortunately, characterization of GTXs has not been yet reported, thus comparison of both xylanases characteristics cannot be investigated further.

The deduced amino acid sequence of GSX L in this study, exhibited high identity with that of E-GSX T-6, *Geobacillus* sp. WBI (GX WBI) and GTX (Figure 1 & Table 2). Moreover, the sequence with a signal peptide shared high identity with GSX T-6, while the mature protein without signal peptide shared the highest identity with GX WBI. On the other hand, there were five different amino acids between GSX L and E-GSX T-6: T/A, N/D, K/N, I/M, S/T at the position of 220, 227, 228, 233, and 245, respectively. Threonine and serine residues belong to polar uncharged amino acid while alanine and methionine residues belong to non polar amino acid. Asparagine residue is belong to polar uncharged amino acid while aspartate residue and lysine residue belong to negatively and positively charged amino acid, respectively (Rashidi & Buehler 2000). As shown in Figure 1, the location of those amino acids differences were at conserved regions among GSXs. It was suggested that those differences will affect into biochemical properties of the enzymes, and therefore it is interesting to compare the biochemical properties between GSX L and well known characterized E-GSX T-6.

Position of A220 at the helix 4 was in opposition to the helix 3, and interestingly it was found on the protein surface (Figure 3). In general, amino acid which is on the surface protein tends to be hydrophilic in relation to protein solubility. However, it was also found that amino acids which most belong to non polar aliphatic group, such A221, I173, V177, V189, G225, and G226, except Y187 of aromatic group, were encompassed A220. Alanine, isoleucine, valine, and glycine are widely accepted as relatively hydrophobic due to their non polar side chains, including tyrosine which its aromatic side chain is relatively non polar contributing to its hydrophobic characteristic (Rashidi & Buehler 2000). Thus, interaction among those amino acids resulted in hydrophobic area formation and A220 was likely to have function in keeping the structure hydrophobicity rather than increasing protein solubility. Mutation of A220T resulting in replacement of alanine into threonine which belong to polar uncharged group and is hydrophilic, may lead to reduce hydrophobicity, then structure instability of the enzyme.

Our investigation on the structure around M233 (Figure 4) found that M233 was surrounded by amino acids I214 and F218 at the helix 4, L250, V251, and L254 at the helix 5, V259, V189, V190, and V193 at the strand 4 where active site of E192 was present, and I264 at the strand 6. Except F218 which belongs to aromatic amino acid group and is quite hydrophobic, most those amino acids belong to non polar aliphatic group and are hydrophobic (Rashidi &

Buehler 2000). Hence, the region was kept hydrophobically that may avoid enzymatic degradation. Therefore, mutation of M233I which substituted methionine to isoleucine was seemed not affect much since both of them are hydrophobic. The presence of the sulphur on the side chain of methionine that may contribute or add to its hydrophobic nature, however, was still unclear.

Interaction of amino acids around M233 and A220 rendered hydrophobic area formation. These intermolecular hydrophobic contacts induce aggregation at high protein concentrations and may contribute to the thermostability of the xylanase. Similar phenomenon also found in xylanase of GH11 family such as in *B. subtilis* xylanase which hydrophobic environment around the hydrophobic nature of side chain accounted for enhancing stability (Miyazaki *et al.* 2006), and in *Bacillus* D3 thermostable xylanase which the surface aromatic side chains form hydrophobic clusters between molecules contributed to the thermostability (Harris *et al.* 1997). Moreover, the hydrophobic interaction at the surface of a protein has also been reported (Van den Burg *et al.* 1994; Funahashi *et al.* 2000) to stabilize the protein.

Amino acid residues at the position of 222 were conserved in all bacterial xylanases (Figure 1), while that of at the position of 227 was asparagine (N) in GX WBI and GTX, and it was glutamate (E) in BX NG-27, BHX, and BFX. Interestingly, amino acid residue at the position 227 in GSX T-6 was aspartate (D). It was seemed that strongly ionic binding may be performed between D227 (negatively charged) and R222 (positively charged) rather than N227 and R222 (Figure 5). This probably plays a role in the enzyme stability. The thermostability of xylanase from thermophilic fungus was ascribed to ion-pair interactions throughout the protein, has been reported (Gruber *et al.* 1998; Ihsanawati *et al.* 2005). Thus, replacement of D227 with N227 may not affect into structural change since N227 has polar uncharged side chain. Hence, it did not favor thermostability, but the problem may come from amino acid residue beside N227, the lysine residue at the position 228 (K228), which is a positively charge amino acid.

Amino acid T245 which belongs to polar uncharged group was surrounded by polar uncharged P242 and negatively charged (acidic) E241, and located at the helix 5 (Figure 5). Therefore, it was thought that binding between T245, P242, and E241 may allow the bond to be turned and formed a loop. In this point of view, replacement of T245 into S245 may not give any effect since both threonine and serine belong to polar uncharged group. Moreover, due to its location at the surface protein, both threonine and serine may affect on solubility of protein.

Three-dimensional structure performed a TIM barrel structure as characteristic of GH10 xylanases (Collins *et al.* 2005). The 3D structures among E-GSX T-6, I-GSX T-6, and BX NG-27 (Figure 6) showed that those enzymes were seemed exhibit high similarity. However, amino acid sequence alignment exhibited low identity of E-GSX T-6 with those of BX NG-27 and I-GSX T-6 as 56.3 and 35.4% respectively. Moreover, amino acid composition showed



that both E-GSX T-6 and BX NG-27 did not consist of cystein residue, except for I-GSX T-6. Therefore, the high thermal stability and alkaline stability may supported by their structural perform rather than cystein binding. Due to xylanase belong to GH10 which has the most common enzyme fold of TIM-barrel fold, the xylanase will be a good sample to be studied on the relationship between structure and their properties, especially high thermal stability.

Moreover, study on 3D structure of those enzymes and their biochemical properties also showed that there were structurally differences among E-GSX T-6, I-GSX T-6, and BX NG-27. Regarding to the general mapping of GH 10, xylanase is divided by 3 domains that are Thermo Stable Domain (TSD), GH 10 domain, and following by Cellulose Binding Domain (CBD) (Blanco *et al.* 1999). Analysis of 3D structure showed that there were a significantly different between E-GSX T-6 and others two enzymes on the CBD region where in E-GSX T-6, I-GSX T-6 and BX NG-27 were found 3 strands, 2 loops and 1 helix followed by 1 loop, respectively. Those differences may related to the xylose binding ability. However, this assumption should be evaluated and thus further investigation on protein binding capacity is required.

In conclusion, our GSX L has high similarity with well characterized E-GSX T-6, and therefore we were able to compare its 3D structure with others well characterized I-GSX T6, and BX NG-27. The GSX L has predicted to have mainly 3 domains, that are TSD, GH10 domain, and CBD, where CBD has different pattern with those of BX NG-27 and did not present in I-GSX T-6. Therefore, in our opinion it will be interesting study to investigate the role of their structure i.e. strand helix and loop on binding ability. We were also able to utilize the 3D structure data of E-GSX T-6 to predict the possibility role of differences amino acids present in GSX L. Combination between 3D structure and bioproperties of the enzyme can be used to predict the essential amino acids which play an important role in enzyme thermostability or pH stability, and the same time to design rational mutation of the enzyme. However, to prove the theory, the GSX L is required to be expressed. The expression system is under construction.

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