

Deletion Analysis of a Xylanase Gene from *Fibrobacter succinogenes* S 85 and Its Expression in *Escherichia coli* HB 101

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ABSTRAK

Analisis Delesi Gen Xilanase Asal *Fibrobacter succinogenes* S 85 dan Ekspresinya pada *Escherichia coli* HB 101. Plasmid pBX6 dengan sisipan DNA berukuran 3 Kb asal DNA genomik *Fibrobacter succinogenes* S 85, menyandi aktifitas xilanolitik (mampu mendegradasi substrat xilan) bila ditumbuhkan dalam vektor *E. coli* HB 101. Tujuan penelitian ini adalah untuk mengidentifikasi lokasi fragmen DNA yang berperan dalam menentukan aktifitas pBX6 menggunakan analisis delesi. Suatu seri analisis delesi terhadap pBX6 telah dilakukan dengan menghilangkan beberapa fragmen DNA dengan cara kloning hasil restriksi enzim maupun PCR. Satu fragmen delesi asal produk PCR berukuran 0.7 Kb di sub kloning menggunakan *cloning kit pCR TOPO* dan aktifitas xilanolitiknya ditapis lebih lanjut dengan menumbuhkan dalam media agar *oat spelt xylan-Remazol brilliant blue* (RBB) ditambah antibiotik ampisilin (50 µg/ml). Transforman positif selanjutnya diekstraksi dan dikarakterisasi. Hasil penelitian menunjukkan klon yang telah terpotong kehilangan sebagian besar aktifitas xilanolitiknya dibanding klon asal, bila diuji pada kondisi yang sama untuk menghidrolisis substrat xilan. Klon plasmid rekombinan yang telah terpotong menunjukkan aktifitas spesifik sebesar $41,29 \pm 0,025$ U/mg.

Key word: Delesi, *F. succinogenes* S 85; *E. coli* HB 101, xilanase

INTRODUCTION

In recent years there has been renewed interest in the utilization of plant materials as a source of chemicals and other end product. The main component of plant materials with potential source is polysaccharides that consist of cellulose (40-60%), hemicellulose (20-30%) and lignin (15-30%) (Bastawde 1992). Xylan and cellulose are very difficult to solubilize due to owing the α 1-4 glucosidic linkage. There are two

enzymes involved in the hydrolysis of the xylan main chain i.e., endo β 1, 4-xylanase (EC 3.2.1.8) and β xylosidase (EC 3.2.1.37). Xylanase can act as a strong elicitor of defence molecules in plants (Esteban *et al.* 1983). In addition, xylanases catalyzes the hydrolysis of xylan into xylooligosaccharides and xylose which are useful for other uses.

Several genes coding for the xylanase enzyme have been isolated and characterized along with their translated products from microorganisms such as

fungi and bacteria (Wong *et al.* 1986). Among the rumen bacteria, a strain of *Fibrobacter succinogenes* was reported possess a complement of fibrolytic enzyme that extensively degrades plant cell wall polymers (Forsberg & Cheng 1992).

The molecular mechanism underlying the regulation of this gene needs to be elucidated. Previously, the xylanase gene from *F. succinogenes* S 85 has been cloned into the plasmid vector pUC 19 (Sipat *et al.* 1987). The recombinant plasmid namely pBX6 containing about 3 Kb of insert DNA fragment showed xylanase activity when transformed into *E. coli* HB 101. However, characteristics of this cloned that contain xylanase gene has not yet been determined thoroughly. There is few report of the xylanase activity present in the putative insert gene from *F. succinogenes* S 85. Soong (1996) reported that about 1.6 Kb fraction of xylanase from pBX6 has been shown to be involved in active site of xylanase. Multiple genes have been observed produced by *F. succinogenes* S 85 which differed in substrate specificity (Matte & Forsberg 1992). Examination of xylanase multiplicity in *F. succinogenes* S 85 suggests that the bacteria possess 4 distinct xylanases differing in molecular weight and isoelectric point (pI) (Paradis *et al.* 1993).

Sequence analysis can extract structural and functional information from protein sequences as long as homologies can be reliably traced. With DNA sequence now available for a number of *F. succinogenes* genes, it is increasingly becoming evident that there

is shared modular structure for some of its xylanase that differs from other xylanolytic microorganisms.

The recombinant plasmid pBX6 was previously determined with several different restriction enzymes which showed single restriction sites of *Eco* RI, *Kpn* I, *Apa* I, *Sac* I, *Eco* RV and *Pst* I (Soong 1996; Suryadi *et al.* 2003). The recombinant molecule grown in *E. coli* HB 101 secreted functional xylanase in the presence of xylan.

To study the role of the domain which constitutes xylanase, modified gene encoding various deletion derivatives cloned from pBX6 were constructed. This work was aimed to analyze plasmid recombinant properties and deletion analysis of the xylanase gene from *F. succinogenes* S 85 identifying the region required for the activity of xylanase. The data obtained may be useful in understanding expression of xylanase gene as well as protein engineering study.

MATERIALS AND METHODS

Source of Bacteria and Culture Condition

Recombinant plasmid pBX6 was previously cloned from 3 Kb genomic DNA of *F. succinogenes* S 85 containing a putative xylanase gene into the vector pUC19 and expressed xylanase activity when grown in *E. coli* HB 101 (Sipat *et al.* 1987). The bacteria were maintained on Yeast Tryptone (YT) solid agars medi-um (1.5% agar, 1% yeast extract, 0.5% NaCl, 1.6% tryptone,

pH 7.2) supplemented with antibiotic ampicillin (50 µg/ml).

Deletion Analysis of Xylanolytic pBX6, Sub cloning and Agarose Electrophoresis

Primer pairs for PCR used in this study were constructed previously from pBX6 based upon sequencing data which showed 2 open reading frames (ORFs) (Suryadi *et al.* 2003) (Table 1). The 3 Kb *Eco* RI- *Pst* I fragment

containing full length encoded polypeptide and a regulatory sequence was sub cloned in both orientations. Deletion strategy of pBX6 by PCR approach was outlined in Fig 1. PCR reaction mixtures were performed in a PCR machine (Hybaid) using PCR 30 cycles with the condition as follow: initial denaturation at 94°C for 3 min, denaturation at 94°C for 1 min, annealing varied from 60-68°C (depending the Tm of primer) for 1 min,

Table 1. Oligonucleotide primers used in deletion analysis of pBX6

Primers /Direction	Sequences (5' to 3')
F1/F	GCACCAGTACGACTTATCACC
Fa-36/F	CGAACCTCCGCATGAATTAC
Fa-37/F	GAGAATAAAGCGTCGGCCAAGG
FoY/F	CTTACTGCGCTCTGGAACC
Fa-38/F	AGAAGGCGATTACCGCCTGG
F1Y/F	GAGAACTCTTGAGCGTATTG
F2Y/R	AGCTTCGTTACCACGTCGA
F3Y/R	GGAGTTGCGAAGGCCGCACG
RoY/R	CAGTTATAGGCAGCATCGCA

Remark: F=forward, R=reverse. Primers were synthesized by Operon Tech.(USA)

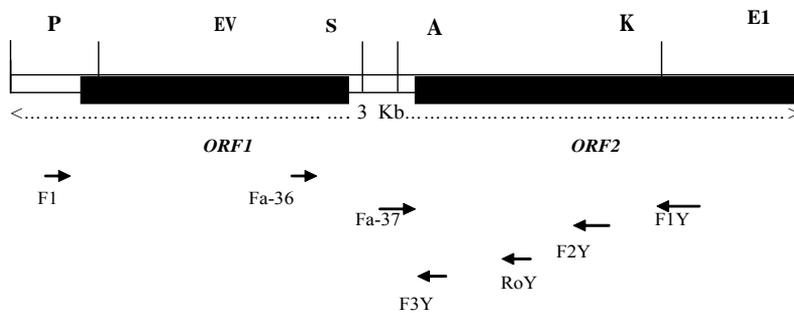


Figure. 1. Deletion strategy of pBX6 by PCR approaches ORF= open reading frames. An arrow indicated primers constructed in both ORFs (F1, Fa36, Fa37, F1Y, F2Y, RoY, F3Y primers constructed to delete region of ORF1 and ORF2). P=*Pst*I, E1=*Eco*RI, EV = *Eco*RV, S = *Sac* I, A = *Apa* I, K = *Kpn* I

→ = Forward primer, ← = Reverse primer.

extension at 72°C for 1 min and final extension at 72°C for 5 min.

Elution of DNA fragment was resolved using 1% (w/v) low melting point agarose following electrophoresis condition in 1x Tris acetate-EDTA (TAE) buffer and running condition at 100 V (150 mA) for 1 h. The gel was visualized using Polaroid film 667. The DNA bands showing desired fragment was cut from the gel using sharp razor blade. It was further purified using *DNA Gene Clean Kit* following the instruction from the manufacture Bio 101 Inc, (USA). Gels slice containing the DNA fragment was put on 1.5 ml eppendorf tube which was added by 2.5 volume of NaI buffer solution. The tubes was melted at 55-65°C on water bath for 5 min until the whole agarose melted and then it was diluted with one volume of DNA binding matrix solution before spin down at 15,600g for 1 min. The pellet was resuspended in 100 ml TE buffer containing RNase (10 µg/ml).

The PCR product that has been eluted from gel was used as an insert of DNA fragment for sub cloning, either into pBR322 vector or pCR 21 TOPO (Invitrogen, USA). Both insert and vector were ligated and transformed into competent cells of *E. coli* TOP 10 and further ligation mixture was used to transform *E. coli* following the protocol described by the company.

Purified fragment that obtained from PCR product was concentrated by addition 1/10 volume of 3 M sodium acetate pH 4.8 and 2.5 volume of 80% isopropanol followed by centrifugation at full speed (15,600g) for 15 min, and then

the pellet was resuspended in 20 ml sterile distilled water. Twenty nanograms of pCR^R 2.1-TOPO vector and 2 ml of PCR fragment was ligated at room temperature for 30 min in a final volume of 6 ml containing 2 ml of buffer solution and sterile water. Competent cells were prepared according to the method as described by Sambrook *et al.* (1989). One colony of *E. coli* TOP 10 was inoculated into 10 ml of Luria broth (LB) medium using a 50 ml Falcon tube and shaken vigorously (200 rpm) at 37°C for overnight, then 300 ml of overnight culture was sub cultured into freshly prepared 50 ml of LB medium and incubated at 37°C for 2 h with orbital shaking (200 rpm). Bacterial density about 5×10^7 cell/ml was checked by OD₆₀₀ equal to 0.5, and then cells were centrifuged (7,800g) at 4°C for 5 min. The pellet was resuspended in 6 ml cold 0.1 M MgCl₂ and again centrifuged (7,800 g) for 5 min. It was resuspended in 6 ml cold 0.1 M CaCl₂ incubated on ice for 30 min, and it was centrifuged as before for 5 min and finally 5 ml cold 0.1 M CaCl₂ was added to the tube. Two hundred ml aliquots of competent cells were transferred into sterile microfuge tube and chilled on ice.

The ligation mixture of 2 ml TOPO cloning reaction was added to 200 ml competent cell, mixed and incubated on ice for 5 to 30 min, and then it was heat shocked by placing the tube at 42°C for 30 seconds in circulating water bath. The ligation mixture was rapidly chilled on ice for 5 min then 250 ml LB medium was added to the reaction mixture which incubated at 37°C for 1 h on water bath

with gently shaking (100 rpm). The reaction mixture was spread out on LB plates containing ampicillin (50 mg/ml) and X-gal (40 µg/ml) followed by incubation at 37°C for 12-16 h.

Expression of Truncated Cloned in *E. coli* HB 101, and Plasmid Extraction

Screening transformant was done on LB plates or RBB-xylan agar containing ampicillin (50 µg/ml) (Biely *et al.* 1985). Positive colony (showing xylanase activity on RBB- xylan agar) was extracted for its plasmid identity by restriction enzyme. Plasmid DNA extraction was done in small scale plasmid isolation (minipreps) using alkaline lysis method (Birnboim & Dolly 1979). The pellet was resuspended in 50 ml of TE buffer containing RNase (10 µg/ml) and it was compared with 1 DNA/*Hind* III as molecular marker.

SDS-PAGE and Analysis of Xylanolytic Activity

Analytical sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12.5% separating gel containing resolving gel (1.5 M Tris-HCl buffer pH 8.8; 10% SDS) and the stacking gel (0.5 M Tris-HCl buffer pH 5; 10% SDS). Electrophoresis was performed in a vertical slab gel unit SE 600 (Hoeffer-USA) using Trisglycine buffer. The zymogram analysis was performed to the derivative recombinant clone. For additional comparison, subcellular fractions of pBX6 were also included in zymogram test. To this purpose, the supernatant (culture filtrate) was extracted by several centrifugation steps following protocol of Cornelis *et al.*

(1982). The extracellular xylanase fraction was determined in total activity found in culture fluid, the two washes and the supernatant of the EDTA treatment. The periplasmic xylanase was obtained after ice cold water treatment; whilst the intracellular xylanase (cytosol) was the supernatant obtained after the sonication step. The gel was run at 2 mA for at least 2 h at room temperature (Hames 1981). Staining of enzyme was done using Coomassie blue R 250, and further the electrophoregram was compared with Mid Range (MR) protein molecular weight standard (Biorad) containing various size of proteins: 97 kDa, 66 kDa, 45 kDa, 31 kDa, 21 kDa and 14 kDa, respectively .

Xylanase activity was determined using Remazol brilliant blue (RBB)-xylan agar plate medium supplemented with 50 mg/ml ampicillin (Biely *et al.* 1985). Positive xylanase plaques were observed after overnight incubation at 37°C by measuring distance of the clearance colony zone that showed hydrolysis of xylan substrate. Clearance zone > 5mm was categorized as strong xylanase activity, whilst less than 5 mm was categorized as weak activity (Suryadi *unpublished report*). The quantitative enzyme activity was determined by pre incubating approximately 25 µl of crude enzyme with 2 g oat spelt xylan in 225 µl of Mc-Ilvaine buffer (0.1 M citric acid and 0.2 M Na₂HPO₄, pH 7.2), measured at 37°C for 30 min. Reduction viscosity of the solution (sugar released) was measured by Somogyi-Nelson's method (Whitehead *et al.* 1991). Protein concentration was measured by the

method of Lowry (Lowry *et al.* 1951) using bovine serum albumin (BSA) (Sigma, USA) as the standard. In control assay, the enzyme had been inactivated by heating in boiling water bath for 30 min. Specific activity was defined as units per mg (U/mg) of protein (Whitehead *et al.* 1991). One unit of enzyme was defined as the amount xylanase activity corresponding to 1 mmol of reducing sugar released per minute using D-xyllose as a standard.

RESULTS

Deletion of pBX6 and Expression of the Truncated Cloned

Original plasmid pBX6 had 5.6 Kb in size. Double digestion to the plasmid with *Eco* RI and *Sac* I gave 2 fragments which showed fragment size at 1.4 Kb and 4.2 Kb, whereas double digestion with *Kpn* I and *Eco* RV produced band at 2.2 Kb and 3.4 Kb (*data not shown*).

The results of deletion derivatives of xylanase encoded by the modified gene are summarized in Fig. 2 (A, B, C). This functional analysis of the xylanase region was obtained by a serial deletion using primer pair's combinations were as follows: *ORF1*: F1/F1Y; F1/F2Y; F1/F3Y; F1/RoY; Fa 36/F1Y, Fa36/F2Y and for *ORF2*: Fa37/F1Y; Fa37/F2Y; Fa37/RoY.

The result of PCR amplification product revealed the expected yield, which showed amplification product of the size ranging from 0.5 Kb to 2.4 Kb (Fig. 2A). After selection of single copy, transformants were grown in medium. The single PCR product showing the

desired size of DNA fragment up to 0.5 Kb that was analyzed by sub cloning into pBR322 vector resulted zero growth of recombinant colony (*data not shown*).

The sub cloning PCR product of 700 bp fragment in *ORF2* (Fa37/F2Y) into pCR21 TOPO has been successfully obtained a transformant colony (*data not shown*). Using RBB – oat spelt xylan plate assay, similar xylanase activity was detected of one transformant. Test of PCR amplification using similar primer pairs (Fa37/F2Y) to the derivative plasmid recombinant culture showed a similar size about 0.7 Kb PCR products (Fig. 3).

Based on zymogram analysis to the enzyme property of truncated clone of pBX6, it was estimated from the standard curve (log MW vs relative mobility, Rf) that at least two major bands were observed having the size ranging approximately from 31 kDa to 42 kDa (Fig. 4). The extracellular and periplasmic fractions of pBX6 enzyme contain almost similar zymogram pattern; whilst cytosol had several protein bands.

The crude xylanase enzyme of truncated recombinant cloned grown in *E. coli* HB 101 showed specific activity (s.a) of 41.29 ± 0.025 U/mg. This xylanase activity was slightly lower than that of control original plasmid pBX6 (s.a 120 ± 0.004 U/mg).

DISCUSSION

The characteristic enzyme produced by pBX6 showed that molecular mass (Mr) of the enzyme was previously reported containing approximately 60 kDa or approximately has a nucleotide size of

Deletion Analysis of a Xylanase

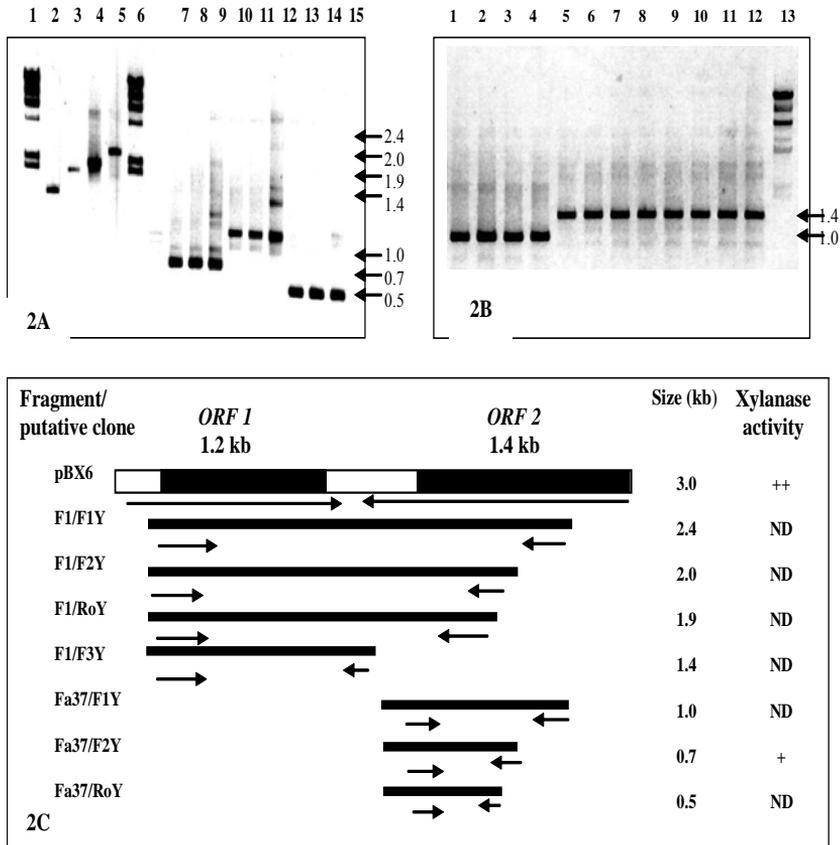


Fig. 2A. Deletion analysis of plasmid recombinant (pBX6) by PCR amplification. Size of the fragment yielded from primer pair's combination. Lane 1/6= MW marker (ϕ DNA/*Hind*III), 2=F1/F2Y, 3=F1/RoY, 4=F1/F2Y, 5=F1/F1Y, 7-9=Fa37/F2Y, 10-12=Fa37/F1Y, 13-15=Fa37/RoY.

Fig. 2B. 13= MW (ϕ DNA/*Hind* III), 1-4=Fa36/F2Y; 5-12= Fa36/F1Y.

Fig. 2C. Deletion by PCR amplification using primer pairs combination and their expression of xylanase activity on RBB xylan agar plates. The PCR product size was 0.5 Kb, 0.7 Kb, 1.0 Kb, 1.4 Kb, 1.9 Kb, 2 Kb, 2.4 Kb, 3.0 Kb. Arrows indicated direction of primers, ND= not detected. ++= strong xylanase activity (> 5 mm clearance zone); += weak xylanolytic activity (less than 5 mm clearance zone).

1.8 Kb gene function (Soong 1996). However, Sipat *et al.* (1987) had estimated size of 1.2 Kb of the 3.0 Kb DNA fragment from *F. succinogenes* S 85 showed xylanase gene function. This

means that the encoded polypeptide could be sequentially reduced in size to remove unnecessary region.

The expression of xylanase gene of *F. succinogenes* S 85 in *E. coli* was

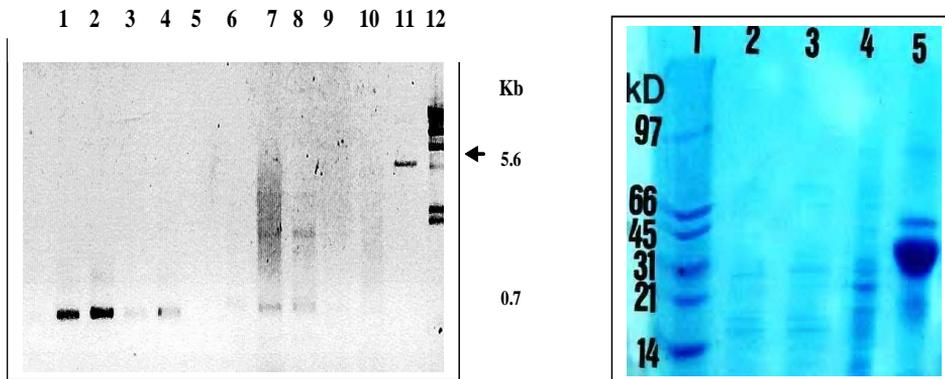


Fig. 3. PCR confirmation of derivative recombinant colony (transformant) by PCR using primer pairs Fa37/F2Y showing size at 0.7 Kb. 1-8= random sample of derivatives recombinant culture, 9-10=water (control), 11= original plasmid (pBX6) (5.6 Kb); lane 12 = molecular marker (ϕ DNA/*Hind* III).

Fig. 4. Zymogram analysis to the derivative recombinant clone pBX6. Lane 1= MW = Mid Range molecular weight marker (Biorad, USA), 2 = extracellular of pBX6; 3 = periplasmic fraction of pBX6, 4= fraction of cytosol of pBX6=, 5 = truncated derivative cloned pBX6 culture

considered to be under the control of its own regulatory sequences (Paradis *et al.* 1993) hence, to facilitate the location of the enzymatically functional domains polypeptide could be reduced in size by a series of deletion at both the 5' and 3' ends of the insert either by restriction analysis or PCR amplification.

DNA fragment up to 1.0 Kb was previously deleted from original plasmid pBX6 by restriction analysis, cloned and tested for its transformant; however after random colony was picked up and check for xylanase activity it showed no activity present in cloned. This might be due to lack of plasmid expression when it was grown in *E.coli* HB 101 (Sipat *pers. comm*). Similar result was obtained from preliminary study using restriction analysis upon deletion fragments of *Eco*

RI – *Sac* I (1.4 Kb) and *Kpn* I – *Eco* RV (2.2 Kb). The fragment containing size of 1.4 Kb and 2.2 Kb that was cloned into pGEM 3Z vector and grown on 2 YT + RBB-oat spelt xylan medium, showed no xylanase activity. This was probably the gene coding for xylanase activity was not in the same reading frame (Suryadi, *unpublished report*). Study conducted by Noorhayati (1994) reported that restriction upon deletion sites of *Kpn* I – *Sac* I (0.9 Kb) and *Pst* I – *Sac* I (1.6 Kb) showed no xylanase activity was observed after transformation into the host *E. coli* HB 101. Another deletion report of fragment DNA insert was carried out to the *Pst* I – *Eco* RV (0.3 Kb) fragment and *Eco* RI – *Kpn* I fragment (0.5 Kb). All transformants obtained did not produce any

clearing zones on RBB-xylan (Zhui 1995). This phenomenon thought to be the promoter region has been deleted out from the insert, hence to further deleted unnecessary nucleotide base region, stable sub cloning of the pBX6 into the vector was screened by PCR

The PCR cloning system used in this study (pCR21 TOPO-Invitrogen, USA) was fastest and most efficient method for sub cloning PCR product. The 3' end T-overhang in the vector can be ligated directly with PCR product which has 3' A-overhangs in each end.

Following gene deletion of PCR product using the PCR cloning system, examination of xylanase expression of truncated proteins was carried out. The result obtained from 0.7 Kb fragment suggested the presence of distinct catalytic domain in plasmid. As reported previously predicted signal peptide in ORF2 exhibited about 28 residue of amino acid. This fragment was previously estimated under the restriction site of *Sac* I-*Apa* I (0.14 Kb) based on nucleotide sequence study (Suryadi *et al.* 2003).

It was estimated deletion of 0.7 Kb from 3' end of the ORF 2 did not affect xylanase activity; whereas removal about 0.4 Kb from 3' end did. This suggests that the remaining part of the gene was fused out of frame with start codon. It was also pointed out by Yague *et al.* (1990) that deletion of the linker sequence did not affect hydrolysis of soluble xylan sub substrate. It was explained by previous result that the translation initiation may occur downstream from deletion endpoint of ORF1 in region 1.2 Kb (Suryadi *et al.* 2003).

The colony that carried xylanase activity from the replica plate showed clearing zone when grown on 2 YT-RBB-xylan agars. However, yield of the plasmid from the transformant was very low after several times plasmid extraction was carried out by alkaline lysis. This might be due to several reason i.e., the plasmid stability was very poor, or its activity has integrated into genomic, hence explanation to this phenomenon still remain to be resolved.

Xylanases of *F. succinogenes* S 85 have been shown to be extremely complex comprising many different discrete domains within the enzyme (Paradis *et al.* 1993), which are mainly due to either post-translational modification such as proteolysis/glycosylation or multiple genes of the enzyme. Further, they pointed out the xylanase enzymes have molecular weights ranging from 15 kDa to 63 kDa. Berra *et al.* (2000) reported that the *end* B gene which was isolated from *F. succinogenes* S 85 and express xylanase activity had a molecular mass of 62.5 kDa, however the recombinant plasmid pBX6 from *F. succinogenes* S 85 has been shown previously contain a protein with a molecular mass of about 60 kDa that express xylanase activity (Soong 1996).

It was assumed that most of xylanase activity found in extracellular and cytosol fraction of pBX6 (Suryadi *unpublished report*). The present study shows several low molecular protein bands present in SDS-PAGE. It was found that at least two bands of protein, a major thick band and a faint band were

observed from truncated clone at the size approximately of 31 kDa and 42 kDa, respectively.

The result showed that a truncated clone showed less xylanase activity when assayed under the same condition hydrolysis of xylan than the original clone. The lower xylanolytic enzyme activity (41.29 ± 0.025 U/mg) produce from this derivative of pBX6 clone may result from truncation of the enzyme. This reduction in size leads to have lower activity than the original cells (120 ± 0.004 U/mg). The insufficient secretion of the cloned enzyme into the culture fluid could be due to the possible of the signal sequence during the cloning process (Yang *et al.* 1989). It was reported that mutation to Glycine or Serine may have caused changes in its secondary or tertiary structure which in turn may affect the efficiency of the enzyme activity (Hu *et al.* 1991). Suryadi *et al.* (2003) reported that the ORF1 showed bacterial catalytic domain sequence (CDS), whereas ORF2 showed putative promoter sequence of *E. coli* at 26 bp from start codon.

From the present result obtained, it was concluded that deletions up to 2.3 Kb fragments of the insert gene in pBX6 may affect complex loss of transcription; hence to locate region that still express xylanase activity, further work such as mutation study need to be carried out.

CONCLUSION

PBX6 was successfully deleted using PCR sub cloning, in order to determine which part of region is essential for xylanolytic activity. Result of the study

showed a truncated clone showed less xylanase activity when assayed under the same condition hydrolysis of xylan than that of the original clone. The crude xylanase enzyme of truncated recombinant cloned grown in *E. coli* HB 101 showed specific activity 41.29 ± 0.025 U/mg.

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