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DETERMINATION OF THE NUCLEOTIDE SEQUENCE OF RECOMBINANT PLASMID PBX6 CONTAINING A XYLANASE GENE FROM *FIBROBACTER SUCCINOGENES* S 85

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By

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Faculty: Science and Environmental Studies

pBX6 is a recombinant plasmid containing a 3 kb fragment of insert DNA from *Fibrobacter succinogenes* S 85 genomic DNA which encodes xylanase activity. The recombinant plasmid pBX6 has shown xylanase activity when grown on RBB-xylan plates supplemented with ampicillin. Restriction mapping of the plasmid established the presence of single sites for *Eco*RI, *Kpn*I, *Apa*I, *Sac*I, *Eco*RV and *Pst*I. This work was focussed on nucleotide sequence determination of recombinant plasmid pBX6 containing a xylanase gene from *F. succinogenes* S 85. The primer walking strategy was performed to complete manual sequencing, following the protocol cycle sequencing of Sanger dideoxy chain termination. Custom synthesis primer was designed and constructed from 3' end of the preceding sequence on both strands.

The complete nucleotide sequence of plasmid pBX6 has been obtained which overlapped in complementary region. A total of nucleotide sequence of *Pst*I-*Eco*RI DNA fragment has been determined containing bases 3227. Analysis of the whole nucleotide sequence revealed two putative Open Reading Frames (ORFs) i.e; ORF 1 and ORF 2. These ORFs were correspond with other microbial sequences encoding xylanase gene. The ORF 1 starts from bases 129 and ended at 1364, while ORF2 stretchs from 1776 to 3227 bases of the insert DNA. Each of the ORF was preceded by putative *E. coli* -10 and -35 promoters. The ORF 1 was translated into a peptide of 411 amino acid residues, whereas ORF2 translated into 483 amino acid residues, respectively. It was suggested that ORF 2 containing xylanase gene was slightly similar with *xyn*C gene of *F. succinogenes* S 85.

On the basis of amino acid sequence similarity on both ORF 1 and ORF 2, it was shown that the gene involved in the xylan degradation is closely related to the component family 10 catalytic domains of glycosyl hydrolase. The translated product of amino acid residues was expected to have molecular mass about 45.6 kDa for ORF1 and 52.5 kDa for ORF2, with theoretical pI 4.73 and 5.26, respectively. The highest amino acid composition was glycine, whilst the lowest was cystine. Total number of amino acids which showed negatively charged residues (aspartate-glutamate) was 117 residues, whereas total number of positively charged residues (arginine-lysine) was 86 amino acids. The strongest hydrophobic

region was observed at residues 255-256 amino acid of ORF1, and at 17-18 amino acid residues of ORF2. There were found 7 cysteine residues and containing almost an equal amount of α-β helical structure in the sequence, that increase stability of the protein product. The translated product also showed similarity with other prokaryotic xylanases, which have shown putative protein product ranging from 42% to 92%. The ORF 1 residue of xylanase from *F. succinogenes* S 85 has showed homology with other bacterial xylanases such *as Pseudomonas fluorescence* and *Butyrivibrio fibrisolvens,* ranging from 57% to 94% while ORF 2 cluster together and showed homology ranging from 88% to 92% with xylanase from *F. succinogenes* S 85 *(xynC, cmc-xyl)*.

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PENENTUAN JUJUKAN NUKLEOTIDA PLASMID REKOMBINAN PBX6 YANG MENGANDUNGI GEN XILANASE DARI *FIBROBACTER SUCCINOGENES* S 85

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pBX6 ialah plasmid rekombinan yang mengandungi satu DNA selitan dari DNA genomik *F. succinogenes* S 85 yang mengkodkan aktiviti xilanase. Plasmid rekombinan pBX6 telah menunjukkan aktiviti xilanase apabila ditumbuhkan pada petri RBB-xilan yang ditambahkan dengan ampisilin. Pemetaan pembatas bagi plasmid telah mengasaskan kehadiran tapak tunggal untuk *Eco*RI, *Kpn*I, *Apa*I, *Sac*I, *Eco*RV dan *Pst*I. Kajian ini menumpukan perhatian pada penentuan jujukan nukleotida plasmid rekombinan pBX6 yang mengandungi gen xilanase dari *F. succinogenes* S 85. Strategi 'primer walking' telah dijalankan untuk melengkapkan jujukan pBX6 secara manual dengan mengikuti kaedah jujukan rangkaian bagi 'pengakhiran rantai dideosi Sanger'. Primer sintesis telah dirangka dan dibina daripada 3' hujung dari jujukan pendahuluan pada kedua-dua rantai.

Jujukan nukleotida penuh bagi potongan DNA *Pst*I-*Eco*RI telah ditentukan mempunyai 3227 bes. Penganalisaan pada seluruh jujukan nukleotida

memberikan 2 anggapan rangka bacaan terbuka (RBT) iaitu RBT 1 dan RBT 2. RBT ini berkenaan rapat dengan jujukan pelbagai mikroorganisma yang mengkodkan gen xilanase. RBT 1 bermula dari bes 129 dan berakhir pada 1364, manakala RBT 2 bermula dari 1776 dan berakhir pada 3227 bes daripada DNA selitan. Setiap RBT didahului jangkaan promoter *E. coli* -10 dan - 35. Masing-masing RBT 1 telah ditranslasi kepada satu peptid yang mengandungi 411 asid amino, manakala RBT 2 telah ditranslasi kepada 483 asid amino. Dicadangkan bahawa ORF 2 mengandungi gen xilanase hampir sama dengan gen *xyn*C dari *F. succinogenes* S 85

Berdasarkan kehampirsamaan jujukan asid amino gen xilanase dari *F. succinogenes* S 85 pada kedua RBT 1 dan RBT 2, ditunjukkan bahawa gen yang terlibat pada penguraian xilan berkenaan rapat dengan kumpulan domain katalitik keluarga 10 glikosil hidrolase. Secara teoritikal, produk translasi dari baki asid amino dijangka mempunyai jisim molekul sebanyak 45.6 kDa untuk RBT 1 dan 52.5 kDa untuk RBT 2, dengan masing-masing mempunyai pI 4.73 dan 5.26. Kandungan asid amino yang paling banyak ialah glisina, manakala yang paling sedikit ialah sistina. Jumlah baki asid amino yang bercas negatif (asid glutamat-asid aspartat) ialah 117, manakala jumlah baki yang bercas positif ialah 86 asid amino. Kawasan hidrofobik yang paling kuat telah dijumpai pada asid amino 255-256 bagi RBT 1, dan pada asid amino 17-18 bagi RBT 2. Terdapat 7 baki sistina dan mengandungi hampir sama jumlah struktur helikal α-β dalam jujukannya, ini meninggikan kestabilan produk protein. Produk protein juga menunjukkan

homologi dengan xilanase dari prokariot lainnya dan dijangka mempunyai kesamaan 42% sampai 92%. Produk RBT 1 menunjukkan homologi dengan xilanase bakteria yang lain seperti *Pseudomonas fluorescence* dan *Butyrivibrio fibrisolvens* (57% sampai 94%), manakala RBT 2 menunjukan kesamaaan dengan xilanase dari *F. succinogenes* S 85 (*xyn*C, *cmc- xyl*) (88% sampai 92%).

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LIST OF ABBREVIATIONS

A	adenine
bp	base pair
C	cytosine
°C	degrees centrigrade
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
G	guanine
g	gravity force
g	gram
h	hour
kb	kilo base pair
kDa	kilo Dalton
M	molar
MCS	multi cloning sites
mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MW	molecular weight
ng	nanogram

ORF	open reading frame
PCR	polymerase chain reaction
pg	pico gram
pI	isoelectric point
RNA	ribonucleic acid
RBB	remazol brilliant blue
RBS	ribosome binding site
rpm	revolutions per minute
SD	Shine-Dalgarno
SDS	sodium dodecyl sulphate
s	second
TAE	tris acetate- EDTA
TEMED N,N,N,N'	tetramethyl-ethylene diamine
T	thymine
Tm	temperature of melting point
U	unit of xylanase activity
µg	micro gram
µl	micro liter
µCi	micro Curie
YT	yeast tryptone

CHAPTER I

INTRODUCTION

Xylans are heterogeneous polysaccharides found in the cell walls of many plants species (Wong *et al.,* 1986). They are important in their structural roles, being applied as polymers in biomass conversion and other uses (Fry, 1986; Mishra *et al.*, 1989; Wong and Saddler, 1992). Complete breakdown of a branched xylan requires the action of endo β-1,4-xylanase that attacks the polysaccharide backbone and exo β -1-4- xylosidase that hydrolyses xylo-oligosaccharides to xylose (Gilkes *et al.,* 1991). They play an important role in physiological plant tissue and plant defense mechanisms (Esteban *et al.,* 1982). In addition, xylanases are also used in the food and feed industry (Wong *et al.,* 1988).

Enzyme hydrolysis using microbial xylanase is most efficient. Several enzymes, which are capable of degrading xylans, have been isolated from bacteria, yeast and fungi (Gilkes *et al.,* 1991). Although extensive work has been carried out on the purification and characterization of xylanases, more studies at molecular level using recombinant DNA techniques have only been carried out recently (Coughland and Hazlewood, 1993). The rumen bacterium, *Fibrobacter succinogenes* subsp. *succinogenes* S 85 (Montgomerry *et al.*, 1988) (previous name: *Bacteroides succinogenes*), is one of the best xylanase producers. Its xylanolytic complex has been investigated (Sipat *et al.*, 1987; Malburg *et al.,* 1993; Ozcan *et al.,* 1996).

Sipat *et al.,* (1987) cloned a 9.4 kb gene fragment into pBR322 from a genomic library of *F. succinogenes* S 85. This recombinant plasmid pBX1 expressed xylanase activity in *Escherichia coli* HB 101. Matte and Forsberg (1992) purified two endoxylanases from *F. succinogenes,* which differed in substrate specificity from the enzyme coded for xylanase gene cloned by Sipat *et al.,* (1987). Malburg *et al.,* (1995) has demonstrated that multiple xylanase genes were possessed by *F. succinogenes* S 85. Coughland and Hazlewood (1993) have also shown that multiple xylanase genes are produced by a large number of hemicellulolytic fungi.

Studies on xylanase function and structure may be useful in determining the efficiency of microbial xylanase. It comprises various combinations of functional elements; such as catalytic domains, cellulose binding domains, linker regions and repeated sequences of amino acids. It is possible to group xylanases into families according to conserved amino acids sequences in the catalytic domains (Henrissat and Bairoch, 1993). All of the xylanases so far, have been characterized which fall into two classes; xylanases, which have high relative molecular mass-low pI class, and xylanases, which have low molecular mass-high pI class (Wong *et al.,* 1988; Henrissat and Bairoch, 1993; Iyo and Forsberg, 1996).

More detailed study of recombinant plasmid derived from *F. succinogenes* S85 carrying xylanase gene is still necessary to increase its efficiency in the industrial application. Molecular aspects such as cloning and sequencing

are useful tools to obtain the nucleotide sequence involved in xylanase gene product, hence allowing manipulation of the enzyme for various purposes (Malburg *et al.*, 1993).

Recombinant plasmid pBX6 consists of the vector pUC19 with a 3 kb DNA insert from *F. succinogenes* S 85 that was previously cloned by Sipat *et al.*, (1987). This thesis reports the study and characterization of plasmid pBX6 encoding xylanase activity from *F. succinogenes* S 85 by determining the nucleotide sequence and its deduced amino acid sequence in order to understand structure and function of xylanase gene.

CHAPTER II

LITERATURE REVIEW

Xylan and its Chemical Structure

Hemicellulose polysaccharides are the major structure component in the plant cell walls which represent an important energy source for the host ruminant (Dekker and Richards, 1976; Williams and Withers, 1992). Hemicelluloses are generally a mixture of xylans with small amount of various mannans, which can be obtained by alkaline extraction of forages and woody biomass (Hespell *et al.*, 1987).

Xylans are generally represented as complex heteropolysaccharides whose structure varies in nature and degree of branching β-1,4-linked xylopyranosyl main chain. They constitute at least 30% of dry weight in plant cell walls. The general features of the xylans are given in Figure 1. The typical of xylans may derived from various structural type; linear homoxylan (no side chain at all), arabinoxylan (has only one side chain), glucuronoxylan (has α D glucuronic acid or 4-O-methyl ether derivative) and glucuronoarabinoxylan (has α L arabinose, α D glucoronic acid and 4-O- methyl α D glucoronic) (Josselau *et al.*, 1992). Xylans may interact with cellulose, other polysaccharides, and lignin hydroxycinnamic acid through various covalent linkages and non-covalent secondary forces (Ethier *et al.*, 1994). The end product of xylan hydrolysis using alkaline or enzyme, can