ISOLATION, EXPRESSION AND CHARACTERIZATION OF RECOMBINANT α-L-ARABINOFURANOSIDASE FROM *Aspergillus niger* ATCC 120120 IN *Pichia pastoris*

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A thesis submitted in fulfillment of the
requirements for the award of the degree of
Master of Engineering (Bioprocess)

Faculty of Chemical Engineering
Universiti Teknologi Malaysia

2010

Specially dedicated to my beloved family and friends.

- Izawati Che Rashid

ACKNOWLEDGEMENTS

In the name of Allah, The Most Gracious, The Most Merciful. Praise is to Allah S.W.T by whose grace and blessing, I receive guidance in completing this study. Thanks for His greatest love and blessing.

First of all, I would like to extend my deepest appreciation and sincere thanks to my supervisors, Dr. Mohd. Nazlee Faisal bin Md. Ghazali and Prof. Dr. Rosli bin Md. Illias for their invaluable advices, guidance and criticisms throughout this study. Special thanks go to all laboratory staff of the Department of Bioprocess Engineering, UTM and my laboratory colleagues, for their assistances, advices and cooperations. Without their guidance, the lab works might not been successfully completed.

I would like to express my gratitude to Malaysia Genome Institute (MGI) for the financial aid since the research was part of a project under Genomics and Molecular Biology Initiatives Programmes (07-05-16-MGI-GMB12).

Last but not least, my gratitude to my family whose nurturing love, understanding and unstinting support have cheered me up when I am down and kept me going.

ABSTRACT

A recombinant gene encoding α-L-arabinofuranosidase (An*abfA*) amplified from the genomic deoxyribonucleic acid (DNA) of *Aspergillus niger* ATCC 120120 was successfully cloned and expressed in *Pichia pastoris* under the control of alcohol oxidase 1 (*AOX1*) promoter. Molecular analysis of the nucleotide sequence showed that the An*abfA* gene contain eight exons and seven introns. Introns deletion was performed via modified overlap extension-polymerase chain reaction (MOE-PCR). The sequence was found to contain an open reading frame composed of 1887 base pairs (bp) nucleotides and encode 628 amino acid (aa) residues. Amino acids sequence analysis suggested that the mature enzyme was preceded by a 25 aa signal sequences. The effect of cultural conditions on recombinant An*abfA* expression was studied and the enzyme was expressed as soluble protein. The recombinant An*abfA* expressed as an active enzyme at 28°C when cultured in buffered complex methanol medium (BMMY), pH 6 and induced with 2% methanol for every 24 hours. Purified recombinant An*abfA* before and after treatment with PNGase F migrated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a relative molecular mass of 83 kDa and 66 kDa, suggesting that the An*abfA* contains *N*-linked oligosaccharides. The optimum temperature and pH of recombinant An*abfA* were 50°C and pH 4, respectively. The enzyme was stable at pH 2 to pH 6 and retained more than 80% of its activity after pre-incubation at 40°C for 30 minutes. Recombinant An*abfA* activity was stimulated by K^{+}, Mn^{2+}, Na^{2+} and Triton X-100 and significantly inhibited by Cu^{2+} and Fe^{2+}, while the enzyme activity was relatively unaffected by Ca^{2+}, CO^{2+}, Mg^{2+} and ethylenediamine-tetraacetate (EDTA). The Michaelis-Menten constant (K_m) and maximum reaction velocity, (V_{max}) of the recombinant An*abfA* activity towards ρ-nitrophenyl α-L-arabinofuranoside (ρNPA) were 0.93 mM and 17.86 µmol.ml^{-1}.min^{-1}, respectively.

ABSTRAK

Gen rekombinan yang mengkodkan α-L-arabinofuranosidase (An*abfA*) diamplifikasikan daripada asid deoksiribonukleik (DNA) genomik *Aspergillus niger* ATCC 120120 berjaya diklonkan dan diekspreskan di dalam *Pichia pastoris* di bawah kawalan pengaruh alkohol oksidase 1 (*AOX1*). Analisis molekular terhadap jujukan nukleotida menunjukkan gen An*abfA* mengandungi lapan ekson dan tujuh intron. Pemadaman intron dilakukan melalui pengubahsuaian pemanjangan pertindihan-tindakbalas rantaian polimerase (MOE-PCR). Jujukan didapati mengandungi rangka bacaan terbuka 1887 pasangan bes (bp) nukleotida dan mengkodkan 628 asid amino (aa). Analisis jujukan asid amino mencadangkan bahawa enzim matang didahului oleh 25 aa isyarat jujukan. Kesan keadaan pengkulturan terhadap pengekspresan An*abfA* rekombinan dikaji dan enzim diekspreskan sebagai protein larut. An*abfA* rekombinan diekspreskan sebagai enzim aktif pada 28°C apabila dikulturkan di dalam media metanol kompleks berpenimbal (BMMY), pH 6 dan diaruhkan dengan 2% metanol pada setiap 24 jam. An*abfA* rekombinan tulen sebelum dan selepas rawatan dengan PNGase F bergerak melalui natrium dodesil sulfat-poliakrilamida elektroforesis gel (SDS-PAGE) dengan berat molekular relatif 83 kDa dan 66 kDa, mencadangkan bahawa An*abfA* mengandungi rantaian *N*-oligosakarida. Suhu dan pH optimum bagi An*abfA* rekombinan ialah pada 50°C dan pH 4. Enzim stabil pada pH 2 hingga pH 6 dan mengekalkan lebih daripada 80% aktivitinya selepas pra-pengeraman pada 40°C selama 30 minit. Aktiviti An*abfA* rekombinan ditingkatkan oleh K^{+}, Mn^{2+}, Na^{2+} dan Triton X-100, direncatkan oleh Cu^{2+} dan Fe^{2+}, sementara aktiviti enzim tidak berubah dengan penambahan Ca^{2+}, CO^{2+}, Mg^{2+} dan etilenediamina-tetraacetat (EDTA). Nilai pemalar Michaelis-Menten (K_m) dan halaju tindakbalas maksimum (V_{max}) bagi An*abfA* rekombinan tulen terhadap ρ-nitrofenil α-L-arabinofuranosida (ρNPA) ialah 0.93 mM dan 17.86 $\mu mol.ml^{-1}.min^{-1}$.

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LIST OF SYMBOLS/ABBREVIATIONS

A. niger	-	*Aspergillus niger*
An*abfA*	-	*A. niger* ATCC 120120 α-L-arabinofuranosidase
ATCC	-	American Type Culture Collection
A_{600}	-	Absorbance at 600 nm
bp	-	Base pairs
BLAST	-	Basic Local Alignment Search Tool
BMG	-	Buffered minimal glycerol medium
BMGY	-	Buffered complex glycerol medium
BMM	-	Buffered minimal methanol medium
BMMY	-	Buffered complex methanol medium
BSA	-	Bovine serum albumin
E. coli	-	*Escherichia coli*
EDTA	-	Ethylene diamine tetraacetic acid
F	-	Fragment
g	-	Gram
g/l	-	Gram per litre
HCl	-	Hydrochloric acid
IPTG	-	Isopropyl-β-D-thiogalactoside
kb	-	Kilo base
kDa	-	Kilo Dalton
L	-	Litre
LB	-	Luria-Bertani

mg	-	Miligram
min	-	Minutes
ml	-	Mililitre
mM	-	Milimolar
M	-	Molar
MD	-	Minimal dextrose plate
MM	-	Minimal methanol plate
MOE-PCR	-	Modified overlap extension-PCR
MW	-	Molecular weight
nm	-	Nanometer
NCBI	-	National Center for Biotechnology Information
PCR	-	Polymerase chain reaction
rpm	-	Revolution per minute
U	-	Unit (enzyme activity)
V	-	Volt
YPD	-	Yeast potato dextrose medium
ρNPA	-	ρ-nitrophenyl α-L-arabinofuranoside
µg	-	Microgram
µl	-	Microlitre
µm	-	Micrometer
µmol	-	Micromole
°C	-	Degree celsius
%	-	Percentage

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The composition of plant cell wall is complex and variable. Plant cell wall polysaccharides are the most abundant organic compounds found in nature which make up 90% of the plant cell wall. Lignocelluloses of plant cell walls are mainly found in the form of long chains celluloses (the main structural component of the plant cell wall), hemicelluloses (comprising various β-xylan chains), pectin substances (consisting of galacturonans and rhamnogalacturonans, arabinans, galactans and arabinogalactans) and lignin. Compounds also acting as protective barriers against phytopathogenic organisms (Carlos *et al*., 2004).

Hemicelluloses are one of the most abundant renewable polymers on earth. Moreover, celluloses, hemicelluloses, pectins and lignin are the key components in the degradation of lignocelluloses. Many enzymes are involved in the degradation of these polymeric substrates (Ward and Moo-Young, 1989).

Fungal plant pathogens are able to produce a variety of enzymes hydrolyzing these compounds, facilitating the penetration and colonization of their hosts. Xylan is a predominant hemicelluloses polysaccharide composed of a back bone of β-1,4-xylopyranosyl residues, some of which are substituted with arabinosyl, acetyl and glucuronosyl residues. L-arabinosyl residues are widely distributed in these polymers as side chains. L-arabinosyl residues are widely distributed in some hemicelluloses, such as arabinan, arabinoxylan, arabic gum and arabinogalactan (Carlos *et al*., 2004). The presence of these side chains restricts the enzymatic hydrolysis of hemicelluloses and pectins (Rahman *et al*., 2003; Saha, 2000; Saha and Bothast, 1998). The complete breakdown of these compounds requires the cooperative action of several microbial enzymes, particularly xylanases, β-xylosidases and arabinofuranosidases.

Further, it also represents a formidable technological barrier that retards the development of various industrial processes (Saha, 2000). The use of a single accessory enzyme for partial or specific modification of lignocelluloses might offer new interesting options for the utilization of these low cost raw materials (Leathers, 2003; Sknchez-Torres *et al*., 1996).

The α-L-arabinofuranosidases are accessory enzymes that cleave α-L-arabinofuranosidic linkages and act synergistically with other hemicellulases and pectic enzymes for the complete hydrolysis of hemicelluloses and pectins (Spagna *et al*., 1998; Margolles-Clark *et al*., 1996). The actions of α-L-arabinofuranosidase alone or in combination with other lignocelluloses degrading enzymes represent a promising biotechnological tool as alternatives to some of the existing chemical technologies such as chlorination in pulp and paper industry (Gomes *et al*., 2000; Mai *et al*., 2000; Gobbetti *et al*., 1999), synthesis of oligosaccharides (Rémond *et al*., 2004; Rémond *et al*., 2002) and pretreatment of lignocelluloses for bioethanol production (Saha, 2003; Saha and Bothast, 1998).

Nowadays, cellulases and hemicellulases have numerous applications and biotechnological potential for various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agricultural. A great deal interest in the enzymology of hemicelluloses degradation has reinitiated for a number of application, most notably biofuel production (Foreman *et al*., 2003) and because of the biotechnological interest in the hydrolysis of hemicelluloses for the pulp and paper or the feedstock industry (Herrmann *et al*., 1997).

From the standpoint of the food industry, the pectic substances, arabinans in particular, have become one of the most important constituents of plant cell walls. Arabinans consists of a main chain of α-L-arabinose subunits linked α-(1→5) to one another. Side chains are linked by α-(1→3) or sometimes α-(1→2) to the main α-(1→5)-L-arabinan backbone. In apple, for example one third of the total arabinose is present in the side chains. The molecular weight of arabinan is normally about 15 kDa.

Generally, heterologous expression for a recombinant protein expression is often a convenient way to produce large amounts of proteins (Kallas, 2006). Prokaryotic hosts (*Escherichia coli*, *Bacillus* etc.) are generally easy to work with and generate large amounts of proteins. Besides, commonly used eukaryotic expression systems are mammalian cells, plant cells, insect cells, filamentous fungi and yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris*. Thus, due to their utility in industrial applications, cloning and expression of α-L-arabinofuranosidase gene in *P. pastoris* expression system should been studied in details.

1.2 Objectives of Research

The objectives of this research are to clone and express α-L-arabinofuranosidase (An*abfA*) gene from *Aspergillus niger* ATCC 120120 in *P. pastoris* and to characterize the purified recombinant enzyme.

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