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UNIVERSITI PUTRA MALAYSIA

EXPRESSION OF ALKALINE PROTEASE FROM *BACILLUS STEAROTHERMOPHILUS* F1 IN *PICHIA PASTORIS*

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Chairman : Professor Abu Bakar Salleh, PhD.

Faculty : Institute of Bioscience

Studies have revealed that *Pichia pastoris* expression system is able to improve the expression level of heterologous protein. The system has successfully improved the production of foreign proteins from animal, plant and microorganism. This study was designed to produce a thermostable protease in *P. pastoris* expression system in order to obtain higher yields of the enzyme. The thermostable alkaline protease gene from *Bacillus stearothermophilus* F1 was amplified from the recombinant plasmid pTrcHis/F1 protease gene by PCR. Primer pairs were designed to amplify the F1 protease gene with and without its native signal sequence respectively. The former was designed to clone F1 protease native signal sequence by distrupting the Kex2 site of the *Saccharomyces cerevisiae* α-mating factor sequence. The DNA 1.23 kb and 1.16 kb fragments which contain recognition enzyme sites were cloned into expression vectors, pGAPZαB (constitutive) and pPICZαB (methanol inducible). These are shuttle vectors, therefore the

recombinant constructs were transformed into *Escherichia coli* strain TOP 10 and DH5α respectively for propagation purposes prior to transformation into *Pichia* hosts. These recombinant plasmids were later introduced into *P. pastoris* strain SMD1168H (protease deficient strain) and GS115 respectively by electroporation. The selection of positive transformants was done on YPD agar containing 1 M of sorbitol and 100 μg/ml zeocin.

The highest F1 protease yield under the control of *GAP* promoter was obtained after cultivation for 72 h and the maximum yield via *AOX* promoter obtained after 48 h induction with 0.5% methanol. Expression of F1 protease was greater in the recombinant constructs harboring the constitutive system (GE10SM, GE6GS, GX7SM, GX17GS) than in the inducible system (PE56SM, PE16GS, PX57SM, PX20GS). In both systems, the recombinant F1 protease was secreted successfully into the culture medium driven by the α-factor secretion signal and F1 gene native signal sequence. However, the expression and secretion of F1 protease utilizing the former (GE10SM, GE6GS, PE56SM, PE16GS) produced higher results relative to secretion by the native signal sequence of F1 protease (GX7SM, GX17GS, PX57SM, PX20GS).

There were no large differences in yield by both *P. pastoris* strain SMD1168H (protease deficient strain) and GS115 host strains used. However, the best yield was obtained from clone GE6GS, which was strain GS115 harboring the recombinant gene under the control of the constitutive promoter and secretion driven by the α-factor secretion signal sequence. YTPD was found

to be the best medium, producing the highest expression level (4.13 U/ml). The expression level was doubled relative to the expression by *E. coli* system. YTPD was modified from YTPM medium used in the expression of methanol inducible promoter. The F1 protease was constitutively expressed containing C-terminal His_6-tag fusion with 34 kDa molecular weight. These data show that the expression level of F1 protease gene can be increased in *P. pastoris* system without affecting the enzyme function.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memeuhi keperluan untuk ijazah Master Sains

PENGEKSPRESAN PROTEASE ALKALI DARIPADA *BACILLUS STEAROTHERMOPHILUS* F1 DI DALAM *PICHIA PASTORIS*

Oleh

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Kajian terdahulu telah menunjukkan bahawa sistem pengekspresan *Pichia pastoris* boleh digunakan untuk meningkatkan paras pengekspresan protein heterologus. Sistem ini telah berjaya digunakan untuk memperbaiki penghasilan protein asing dari haiwan, tumbuhan dan mikroorganisma. Kajian ini dijalankan untuk meningkatkan penghasilan protease yang termostabil menggunakan sistem pengekspresan *P. pastoris.* Gen termostabil protease alkali daripada *Bacillus stearothermophilus* F1 telah diamplifikasikan daripada plasmid rekombinan pTrcHis/F1 gen protease melalui tindakbalas berantai polimerase. Pasangan primer direka untuk mengamplifikasikan gen protease F1 yang mempunyai jujukan isyarat dan pemotongan pada terminal N gen protease F1, masing-masing. Yang pertama direka untuk pengklonan protease F1 bersama jujukan isyarat asli dengan mengganggu tapak Kex2 pada jujukan faktor α-pengawanan *Saccharomyces cerevisiae*. Fragmen DNA bersaiz 1.2 kb dan 1.16 kb yang mengandungi tapak pengecaman enzim diklonkan ke dalam vektor

pengekspresan pGAPZαB (konstitutif) dan pPICZαB (teraruh metanol). Vektor yang digunakan merupakan vektor ulang alik, maka konstruk rekombinan ditransformasikan terlebih dahulu ke dalam *Escherichia coli* strain TOP 10 dan DH5α sebelum ditransformasikan kedalam hos *Pichia*, bertujuan untuk pembiakan. Plasmid-plasmid rekombinan diperkenalkan ke dalam strain *Pichia pastoris* SMD1168H (strain kurang protease) dan GS115 masing-masing melalui kaedah elektroporasi. Pengecaman transforman positif dilakukan menggunakan agar YPD mengandungi 1 M sorbitol dan 100 μg/ml zeosin.

Hasil protease F1 tertinggi di bawah kawalan promoter *GAP* diperolehi selepas pembiakan selama 72 jam dan hasil maksimum melalui promoter *AOX* adalah selepas 48 jam aruhan dengan 0.5 % metanol. Pengekspresan protease F1 adalah lebih besar bagi binaan rekombinan membawa sistem konstitutif (GE10SM, GE6GS, GX7SM, GX17GS) dari yang membawa sistem teraruh (PE56SM, PE16GS, PX57SM, PX20GS). Bagi kedua-dua sistem, rekombinan protease F1 berjaya dirembeskan ke dalam medium kultur dipacu isyarat rembesan faktor-α dan jujukan isyarat asli gen F1. Walau bagaimanapun, pengekspresan dan perembesan protease F1 menggunakan yang pertama (GE10SM, GE6GS, PE56SM, PE16GS) menghasilkan keputusan yang tinggi relatif kepada perembesan melalui jujukan isyarat asli protease F1 (GX7SM, GX17GS, PX57SM, PX20GS).

Tiada perbezaan besar bagi penghasilan protease F1 oleh kedua-dua strain *Pichia* yang digunakan. Walau bagaimanapun, hasil terbaik diperolehi dari

klon GE6GS, iaitu strain GS115 membawa gen rekombinan di bawah kawalan promoter konstitutif dan rembesan dipacu jujukan isyarat rembesan faktor-α. YTPD merupakan medium terbaik iaitu menghasilkan aras pengekspresan tertinggi (4.13 U/ml). Aras pengekspresan telah digandakan relatif kepada pengekspresan melalui sistem *E. coli*. YTPD diubah dari medium YTPM, yang digunakan di dalam pengekspresan oleh promoter teraruh metanol. Pengekspresan protease F1 secara konstitutif mengandungi pertaupan penanda-His_6 pada terminal C dengan berat molekul 34 kDa. Data yang diperolehi menunjukkan bahawa paras pengekspresan gen protease F1 boleh ditingkatkan melalui sistem *P. pastoris* tanpa mengganggu fungsi enzim yang dihasilkan.

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I certify that a Thesis Examination Committee has met on 7 August 2008 to conduct the final examination of Amaliawati Ahmad Latiffi on her thesis entitled “Expression of Alkaline Protease from *Bacillus stearothermophilus* F1 in *Pichia pastoris*” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

AMALIAWATI AHMAD LATIFFI

Date: 4 December 2008

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LIST OF ABBREVIATIONS

$A_{600\ nm}$	absorbance read at wavelength 600 nm
bp	base pair
BMGY	buffered glycerol-complex medium
BMMY	buffered methanol-complex medium
Da	Dalton
dH_2O	distilled water
DNA	deoxyribonucleic acid
x g	x gravity
h	hour
kDa	kiloDalton
kb	kilo base pair
LB	Luria Bertani
M	molar
min	minute
ng	nanogram
nm	nanometre
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
PCMB	ρ-chloromercuricbenzoic acid
Sec	second
SDS	sodium dodecyl sulfate
sp	species
TEMED	N,N,N,N-Tetramethylenediamide

TLCK	L-1-chloro-3-(4-tosylamino)-7-amino-2 heptanone-hydrochloride
TCA	trichloroacetic acid
TSB	tryptone soy broth
U/ml	unit per mililitre
µF	microfarad
µg	microgram
µl	microlitre
V	volt
V/cm	volt per centimetre
v/v	volume per volume
w/v	weight per volume
YNB	yeast nitrogen base
YPD	yeast extract, peptone and dextrose media
YPDS	yeast extract, peptone, dextrose and sorbitol media

CHAPTER 1

INTRODUCTION

The Malaysian government has recognized the potential of biotechnology in creating wealth for the country. The country's strength in manufacturing and processing industries plus the wealth of biodiversity offers a wide range of opportunities for the development of industrial biotechnology. One of the identified areas of growth is the development of biocatalysts such as enzymes for food and feed preparations, cleaning products, textile processing and other industrial processes (9th Malaysia Plan, 2006). Moreover, all enzymes used in this country are imported and thus, it is important for Malaysia to consider enzyme manufacturing within the country as to reduce dependency on import (Ibrahim *et al.*, 2006). The production of enzymes locally will reduce the manufacturing costs and this will give competitiveness to the related industries since enzymes are expensive. It also will generate income for the country because it was estimated that the world demand for enzymes is expected to rise 6.5% annually to nearly USD5.1 billion in 2009 (The Freedonia Group, 2005).

Thermostable enzymes are great interest to the industry because its can tolerate high temperature, therefore use of these enzymes at elevate temperature is possible. These enzymes able to withstand high temperature, therefore the enzymes stability are enhanced and the shelf-life can be prolonged. Reactions at high temperature often resulted in higher reaction rates and subsequently allow the use of higher substrates concentrations.

These properties have made thermostable enzymes an attractive component example as an additive in detergents.

Proteases namely the alkaline proteases are utilized predominantly as cleansing additive in detergents because the ability to withstand high pH conditions that occurs in washing environment. Of all the microorganisms that have been screened for the use in various industrial applications, members of the genus *Bacillus* were found to be predominant and an abundant source of alkaline protease. The commercially available alkaline proteases are mostly derived from *Bacillus* strains, although several fungal sources are being increasingly employed. The former are also producers of extracellular proteases (Singh *et al*., 2001)

A locally isolated thermophilic *Bacillus stearothermophilus* F1 strain was found to secrete thermostable alkaline protease (Rahman, 1993). This alkaline protease is a good candidate for future commercialization because the enzyme has high catalytic efficiency and stable at pH 7 and 10 for 24 h at 70^{o}C, hence this property indicates the possibilities for the use of F1 protease in the detergent industry.

In the industrial processes, it is impractical to obtain thermostable enzymes by isolation and purification from naturally occurring thermophilic microorganism. The maintenance of thermophilic microorganisms in bioprocessing fermenters is tedious and costly, as it requires special equipment suitable for high temperature fermentation. The advances of

molecular biology techniques have made it possible to produce thermostable enzymes in mesophilic microorganisms. Genetic engineering greatly improves productivity and cost-effectiveness in existing processes.

Fu (2001) has succeeded in cloning and expressing *B. stearothermophilus* F1 alkaline protease gene in *Escherichia coli* expression system. The nucleotide sequence analysis and the deduced amino acid sequence showed that F1 protease produced as a prepropeptide precursor consisting of a signal peptide of 25 amino acid residues, 97 amino acid residues of N-terminal propeptide and 279 amino acid residues of the F1 protease mature domain. The sequence of F1 protease gene was submitted to Genbank and the assigned accession number is AY028615.

The F1 protease gene expression by *E. coli* system utilized the bacteriocin release-protein system to release the recombinant F1 protease into the culture medium. The advantage of producing extracellular protease is larger enzyme volume can be obtained as the culture medium provides larger space for accumulation of protein. Without having to rupture the cells, the enzymes can be obtained with simple purification steps and the cells can be utilized continuously. However, productivity of *E. coli* clone is insufficient to produce this protease within the context of an economically viable process therefore the *Pichia pastoris* expression system was suggested to enhance the production of thermostable F1 alkaline protease.

P. pastoris is a methylotrophic yeast and was reported able to produce 10-100 fold higher levels of expression for foreign gene. Various recombinant proteins have been successfully and functionally expressed by *P. pastoris* expression system. Comparable to *E. coli* system, *P. pastoris* is genetically easy to manipulate and does not require a complex growth medium. *Pichia* cells can be grown to very high cell densities; hence through large scale fermentation, higher yield of heterologous proteins can be obtained. The integrated vectors help genetic stability of the recombinant elements, even in continuous and large scale fermentation processes, unlike the instability of plasmid in *E. coli* system. Furthermore, in *Pichia* expression system, the proteins can be expressed intra- or extracellularly, therefore the production of extracellular protein is possible. *Pichia* hosts secrete relatively low levels of native proteins, thus the purification of the recombinant proteins is easier and cheaper as compared to *E. coli* expression system (Cereghino and Cregg, 2000).

Due to the advantages reported, the primary objective of this study is to utilize molecular approach to improve expression level of F1 protease through the *P. pastoris* expression system relative to *E. coli* system. Such aims entail several stages; these are:-

1. To clone and extracellularly express the F1 protease gene.
2. To identify suitable plasmids and *P. pastoris* strains for expression.
3. To identify the best conditions for higher-level production of F1 protease production by *P. pastoris.*