



UNIVERSITI PUTRA MALAYSIA

***EXPRESSION OF RECOMBINANT THERMOSTABLE W200R
PROTEASE IN *Pichia pastoris****

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IN *Pichia pastoris***

By

MUHAMAD ZARIR BIN MOHAMAD SULTAN

**Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfillment of the Requirement for the Degree of Master of Science**

March 20

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of requirement for the degree of Master of Science

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Chairman: Professor Dato' Abu Bakar Salleh, PhD

Faculty : Institute of Bioscience

Thermostable enzymes are needed in industry because of their stability in harsh industrial processes. As such there is a constant need for new inexpensive thermostable enzymes. Yeast is considered as a good host for large scale protein expression. A thermophilic *Bacillus stearothermophilus* F1 was found to produce an thermostable serine protease. Gene encoding pro-mature thermostable W200R protease was cloned into *Pichia pastoris* expression vector and placed under the control of the methanol inducible alcohol oxidase (AOX) promoter. The recombinant pPICZαB/W200R protease gene was transformed into *E. coli* for plasmid replication before subsequent transformation into *P.*

pastoris strain X-33, GS115 and SMD1168. This expression system efficiently secreted W200R protease into the culture medium driven by *Saccharomyces cerevisiae* α -factor signal sequence. From the initial screening, PpbX1, PpbG2 and PpbS1 recombinants from strain X-33, GS115 and SMD1168, respectively, had the highest expression level of W200R protease. The expression of these recombinants was further optimized under by modifying several parameters such as media composition, concentration of methanol, aeration and induction time. The protease activities detected from these three recombinants were 87.65 U/ml for PpbX1, 98.71 U/ml for PpbG2 and 147.5 U/ml for PpbS1. The W200R protease from PpbS1 recombinant was purified to 11.8 fold with 64% yield using heat-treatment method. The optimum temperature for the activity of this protease was 70 °C and was stable up to 85 °C. The enzyme was stable in the pH range of 7.0 to 10 with optimum pH of 8.0.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Ijazah Master Sains

**PENGEKSPRESAN REKOMBINAN TERMOSTABIL W200R
PROTEASE DI DALAM *PICHIA PASTORIS***

Oleh

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MAC 2012

Pengerusi: Profesor Dato' Abu Bakar Salleh, PhD

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Enzim termostabil diperlukan dalam industri disebabkan oleh kestabilannya dalam keadaan proses industri. Maka enzim termostabil baru yang murah amat diperlukan di dalam industri. Yis merupakan perumah yang baik bagi pengekspresan protein berskala besar. Termofilik *Bacillus stearothermophilus* F1 telah dikenal pasti untuk menghasilkan serine protease yang sangat termostabil. Gen yang mengkodkan W200R protease termostabil pro-matang telah diklonkan dalam vektor pengekspresan *Pichia pastoris* dan diletakkan di bawah kawalan promoter alkohol oksidase (AOX) teraruh metanol. Rekombinan pPICZαB/W200R protease gen ditransformasikan ke dalam *E. coli* untuk proses

replikasi plasmid sebelum ditransformasikan ke dalam *Pichia pastoris* strain X-33, GS115 dan SMD1168. Sistem pengekspresan ini telah berjaya merembeskan W200R protease ke dalam media kultur di bawah kawalan jujukan isyarat α -faktor *Saccharomyces cerevisiae*. Daripada penyaringan awal, rekombinan PpbX1, PpbG2 dan PpbS1, masing-masing dari strain X-33, GS115 dan SMD1168 telah memberikan tahap pengekspresan tertinggi bagi W200R protease. Seterusnya pengekspresaan kesemua rekombinan tersebut telah dioptimumkan dengan mengubah di bawah beberapa parameter seperti media, kepekatan metanol, pengudaraan dan masa aruhan. Aktiviti protease untuk ketiga-tiga rekombinan ini 87.65 U/ml untuk PpbX1, 98.71 U/ml untuk PpbG2 dan 147.5 U/ml untuk PpbS1. W200R protease daripada rekombinan PpbS1 telah ditulenkan sebanyak 11.8 kali ganda, dengan penghasilan sebanyak 64% menggunakan kaedah pemanasan. Suhu optimum untuk aktiviti protease ini adalah 70 °C dan stabil sehingga 85 °C. Enzim ini stabil pada lingkungan pH 7.0 hingga 10.0 dengan pH optimum adalah pada pH 8.0.

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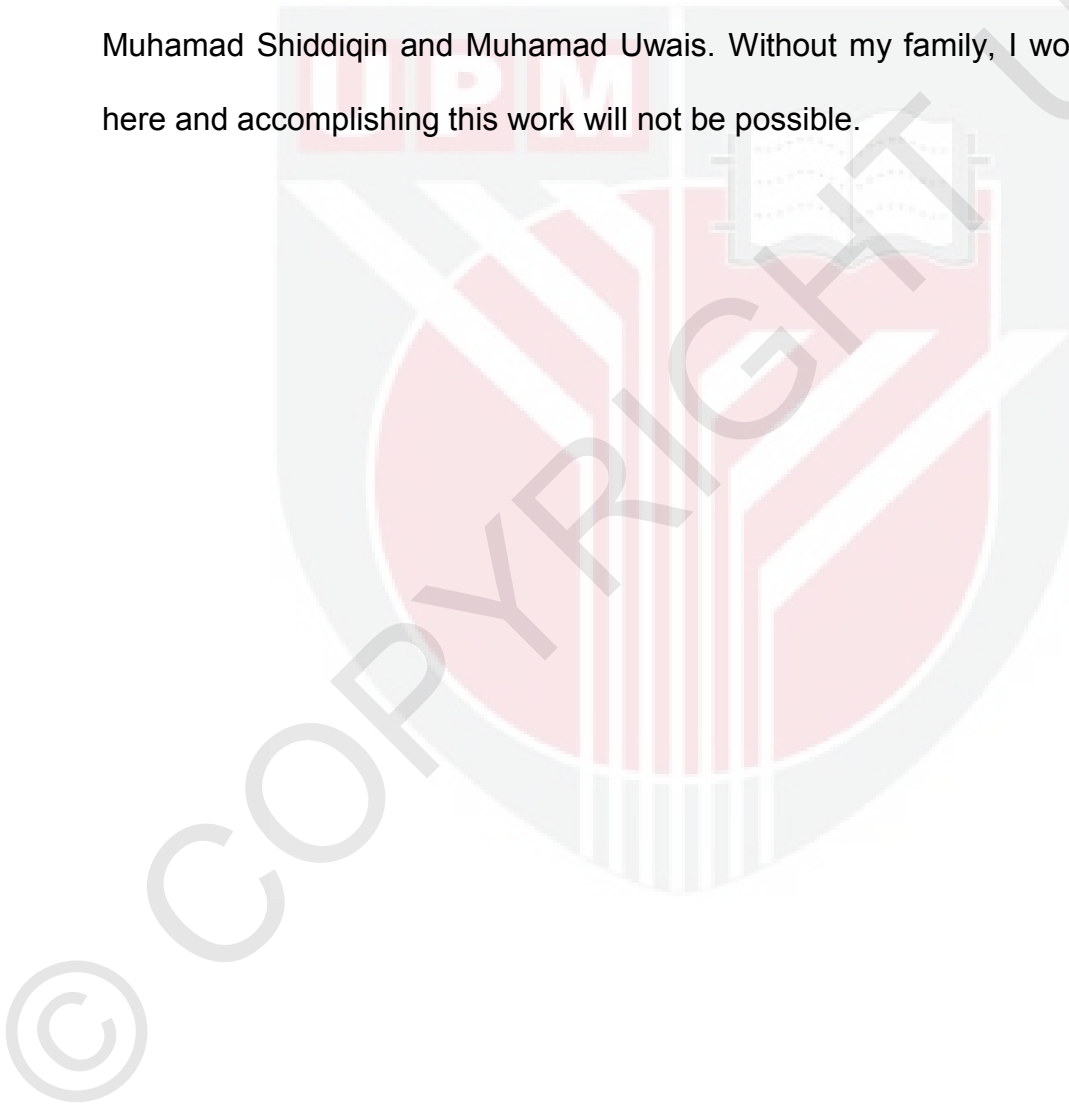
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I certify that an Examination Committee has met on (viva date) to conduct the final examination of Muhamad Zarir Mohamad Sultan on his Master of Science thesis entitled “Expression of Recombinant Thermostable W200R Alkaline Protease In *Pichia pastoris*” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) regulation 1981. The Committee recommends that the student be awarded the Master of Science.

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DECLARATION

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

MUHAMAD ZARIR MOHAMAD SULTAN

Date: 14 March 2012



TABLE OF CONTENTS

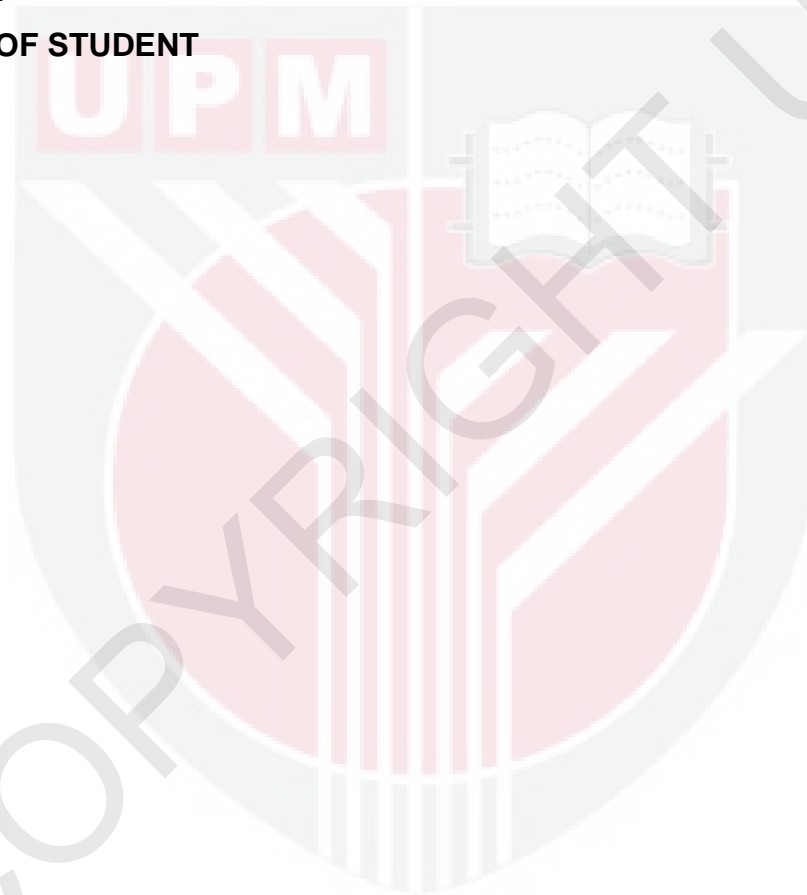
	Page
ABSTRACT	I
ABSTRAK	Iii
ACKNOWLEDGEMENTS	V
APPROVAL	Vii
DECLARATION	Ix
LIST OF TABLES	Xv
LIST OF FIGURES	Xi
1 CHAPTER INTRODUCTION	1
2 CHAPTER LITERATURE REVIEW	4
2.1 Protease	4
2.1.1 Introduction	4
2.1.2 Classification of Proteases	4
2.1.3 Serine proteases	6
2.1.4 Thermostable alkaline protease	9
2.1.5 Application of protease	11
2.1.5.1 Detergent	12
2.1.5.2 Leather industry	13
2.1.5.3 Pharmaceutical industry	13
2.1.6 Recombinant alkaline protease from B. stearothermophilus F1	14
2.1.7 W200R protease	15

2.2	<i>Pichia pastoris</i>	16
2.2.1	Introduction	16
2.2.2	<i>Pichia pastoris</i> as an expression system	17
2.2.3	<i>Pichia</i> strains	18
2.2.4	Expression vector	20
2.2.5	AXO and GAP promoters	22
2.3	Rare codon	23
3	CHAPTER METHODOLOGY	25
3.1	Materials	25
3.2	Culture condition	25
3.3	Extraction and Quantification of recombinant plasmid	26
3.4	Amplification of W200R protease gene	27
3.5	Purification of PCR product	28
3.6	Cloning of W200R gene in <i>P. pastoris</i> expression vector	29
3.6.1	Preparation of expression vector	29
3.6.2	Preparation of <i>E. coli</i> competent cells	30
3.6.3	Digestion of purified PCR product and plasmid	31
3.6.4	Ligation of vector and PCR product	31
3.6.5	Heat-shock transformation of <i>E. coli</i> with ligated DNA	32
3.7	Screening and analysis of positive <i>E. coli</i> transformants	32
3.7.1	Colony PCR	32
3.7.2	Restriction enzyme analysis and sequencing of recombinant W200R protease	33
3.8	Transformation of recombinant W200R protease in <i>Pichia pastoris</i>	34
3.8.1	Linearization of recombinant W200R	34
3.8.2	Preparation of <i>Pichia pastoris</i> competent cells	34
3.8.3	Transformation into <i>Pichia pastoris</i> cells via electroporation	35

3.9	Screening of positive <i>Pichia pastoris</i> transformants via colony PCR analysis	36
3.10	Direct screening of multicopy transformants	36
3.11	Protein expression in <i>Pichia pastoris</i>	37
	3.11.1 Expression of recombinant pPICZ α B/W200R	37
	3.11.2 Assay of recombinant activity	38
3.12	Optimization studies of inducible expression in shake flask	39
	3.12.1 Effect of media on W200R protease expression	39
	3.12.2 Effect of methanol concentration on W200R protease expression	41
	3.12.3 Effect of aeration between baffled and round bottom flasks	41
	3.12.4 Effect of induction times on W200R protease expression.	42
3.13	Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis	42
3.14	Activity staining	43
3.15	Purification of W200R protease by Heat Treatment	44
3.16	Determination of protein concentration	44
3.17	N-terminal sequencing of W200R protease	45
3.18	Characterization of W200R protease	45
	3.18.1 Optimal pH for enzyme activity	45
	3.18.2 pH stability of the enzyme activity	45
	3.18.3 Optimal temperature for enzyme activity	46
	3.18.4 Thermostability of the enzyme	46
4	CHAPTER RESULTS AND DISCUSSION	47
4.1	Growth of <i>E. coli</i> harbouring recombinant W200R F1 protease and plasmid extracted	47

4.2	Cloning of W200R protease gene into <i>Pichia pastoris</i> expression vector	49
4.2.1	Amplification of protease gene	49
4.2.2	Preparation of insert and vector	54
4.2.3	Transformation of <i>E. coli</i>	56
4.3	Screening and analysis of positive <i>E. coli</i> transformants	57
4.3.1	Colony PCR	57
4.3.2	Analysis of recombinant W200R protease	57
4.4	Transformation of expression vector into <i>Pichia pastoris</i>	61
4.4.1	Linearization of recombinant W200R	61
4.4.2	Transformation into <i>Pichia pastoris</i> cells via electroporation	62
4.5	Screening of positive <i>Pichia pastoris</i> transformant via colony PCR analysis	65
4.6	Protein expression in <i>Pichia pastoris</i>	69
4.6.1	Expression of recombinant pPICZ α B/W200R protease	69
4.6.2	Optimization of recombinant W200R protease production in shake flask	70
4.6.2.1	Effect of media on W200R protease	74
4.6.2.2	Effect of methanol concentration on W200R protease	78
4.6.2.3	Effect of aeration between baffled round bottom flasks	83
4.6.2.4	Effect of various induction times on W200R protease	88
4.7	Purification of recombinant W200R protease	94
4.8	Characterization of W200R protease	100
4.8.1	Effect of temperature on activity and thermostability profile of W200R protease	100

4.8.2	Effect of pH on activity and stability profile of W200R protease	104
5	CHAPTER CONCLUSION AND RECOMMENDATION	109
5.1	Conclusion	109
5.2	Recommendation	111
	REFERENCES	112
	APPENDIX	125
	BIODATA OF STUDENT	130



LIST OF TABLE

Table		Page
1	Classification of proteases	5
2	Commercial bacterial alkaline proteases, sources, applications and their industrial suppliers	10
3	Examples of heterologous proteins expressed in <i>Pichia pastoris</i>	19
4	Expression vectors for <i>Pichia pastoris</i> and other methylotrophic yeasts	21
5	Different media composition	40
6	Composition for SDS-PAGE [12% (w/v) gel separation]	43
7	Purification table of W200R protease	98

LIST OF FIGURES

Figure		Page
1	Serine protease mechanism	7
2	Zone of hydrolysis by protease of <i>E. coli</i> BL21 (DE3) pLySs harboring W200R protease gene on LB- skim milk agar.	48
3	Detection of recombinant Pgex-4T1/ W200R plasmid on agarose gel.	50
4	The PCR product of W200R protease gene visualized on agarose gel.	51
5	Nucleotide and the deduced amino acid sequence of the W200R protease gene.	53
6	The digested pPICZ α B plasmid and W200R protease gene visualized on agarose gel	55
7	The purified PCR product of W200R protease gene using pBF1mFWD for forward and pBF1mREV visualized on agarose gel.	58
8	The double digestion production of plasmid pPICZ α B/W200R protease gene visualize on agarose gel	59
9	Nucleotide of the W200R protease in frame with vector pPICZ α B.	60
10	The single digestion product of plasmid pPICZ α B/W200R protease gene visualized on agarose gel.	63
11	PCR products from the positive colonies of Recombinant <i>Pichia pastoris</i> strain X-33	66
12	PCR products from the positive colonies of Recombinant <i>Pichia pastoris</i> strain GS115	67
13	PCR products from the positive colonies of Recombinant <i>Pichia pastoris</i> strain SMD1168	68

14	Secretion of W200R protease activity by <i>Pichia pastoris</i> strain X-33.	71
15	Secretion of W200R protease activity by <i>Pichia pastoris</i> strain GS115.	72
16	Secretion of W200R protease activity by <i>Pichia pastoris</i> strain SMD1168.	73
17	Effect of media on W200R protease secretion by strain X-33.	75
18	Effect of media on W200R protease secretion by strain GS115.	76
19	Effect of media on W200R protease secretion by strain SMD1168.	77
20	Effect of methanol concentration on W200R protease secretion by strain X-33	80
21	Effect of methanol concentration on W200R protease secretion by strain GS115.	81
22	Effect of methanol concentration on W200R protease secretion by strain SMD1168.	82
23	Effect of flask design on W200R protease secretion by strain X-33.	85
24	Effect of flask design on W200R protease secretion by strain GS115.	86
25	Effect of flask design on W200R protease secretion by strain SMD1168.	87
26	Effect of induction time on secretion of W200R protease by PpbX1	89
27	Effect of induction time on secretion of W200R protease by PpbG2	90
28	Effect of induction time on secretion of W200R protease by PpbS1	91
29	SDS-PAGE analysis of total protein,	93

30	SDS-PAGE of purified recombinant W200R protease by heat treatment.	95
31	Zymogram analysis of W200R protease	96
32	N-terminal amino acid sequences of W200R protease.	99
33	Optimum temperature for W200R protease	101
34	Thermostability profile of W200R protease	103
35	Optimum pH for W200R protease	105
36	pH stability for W200R protease	107

CHAPTER 1

INTRODUCTION

According to a new technical market research report, Enzymes for Industrial Applications (BIO030E) from BCC Research (www.bccresearch.com), the global market for industrial enzymes has already achieved the USD 2 billion 2007. This is expected to increase to over \$2.7 billion by 2012, a compound average annual growth rate (CAGR) of 4%. A report from McKinsey and Company., showed that the future for sustainable development is clearly a bright one, and enzyme technology will play a major role, along with the use of microorganism, both natural and engineered (Wood and Scott, 2004; Micheelsen *et al.*, 2008). Under the 9th Malaysia Plan, one of the identified areas of growth is the development of biocatalysts such as enzymes for food and feed preparations, cleaning production, textile processing and other industrial processes. In order to make this area of growth successful, production of enzymes locally is necessary because all of the enzymes used in this country are imported and there is a need to reduce the dependency on import (Ibrahim *et al.*, 2006).

Thermostable enzymes are needed in industries because of its stability in harsh industrial processes. These enzymes are able to withstand high temperature, therefore the stability is enhanced and the shelf-life can be prolonged (Haki and Rakshit, 2003). W200R protease is an example for thermostable enzyme. This

enzyme was mutated from F1 protease which was expressed by *B.stearothermophilus F1*. Thermostable proteases are degradative enzymes, which catalyze the total hydrolysis of proteins. Amongst the various proteases, bacterial proteases are the most significant compared with animal, vegetal and fungal proteases (Haddar *et al.*, 2009). Proteases are major industrial enzymes constituting 60 to 65% of the total enzyme sales in various industrial market sectors such as detergent, food, pharmaceutical, leather, diagnostics, waste management, and silver recovery; most of which are alkaline proteases (Genckal and Tari, 2006). Alkaline proteases are enzymes which have the ability to withstand extreme condition such as high pH.

Mesophilic hosts are widely used as expression systems to produce recombinant thermostable proteins (Vieille and Zeikus 2001). They present several advantages compared to thermophiles as host: (i) they are easier to be cultivated, (ii) the recombinant protein can be purified up to 80% using heat-treatment, (iii) the use of strong promoters can lead to good overexpression yields (Sébastien *et al.*, 2003). Moreover, previous work in this field showed that, most of the time, thermostable proteins are properly folded when expressed in mesophilic hosts (Vieille and Zeikus, 1996).

As an eukaryote, *P. pastoris* has many advantages of higher eukaryotic expression systems such as: (i) protein processing,(ii) protein folding, (iii) posttranslational modification and (iv) genetically easy to manipulate as *E. coli*

or *Saccharomyces cerevisiae*. It is faster, easier and less expensive to use than other eukaryotic expression systems such as baculovirus or mammalian tissue culture, and generally gives higher expression levels. As yeast, it shares the advantages of molecular and genetic manipulations with *Saccharomyces*, and it has the added advantage of 10- to 100-fold higher heterologous protein expression levels. Since *P. pastoris* is a mesophilic and eukaryotic host, these features make it very useful as a protein expression system.

Considering the many advantages of using *P. pastoris* as an alternative system to express the thermostable W200R protease, the expression of W200R protease is expected to be higher than *E. coli*. This research was undertaken with the following objectives:-

1. To clone the W200R protease gene from *B. steartotremophilus* F1 in *Pichia pastoris*.
2. To express W200R protease extracellularly in *Pichia pastoris*.
3. To purify and characterize the recombinant protease in *Pichia pastoris*.

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