STATISTICAL APPROACH FOR ENZYME PRODUCTION USING RESPONSE SURFACE METHODOLOGY AND STRUCTURE PREDICTION OF THERMOSTABLE ORGANIC SOLVENT-TOLERANT RAND PROTEASE

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By

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Chairman : Professor Raja Noor Zaliha Raja Abd. Rahman, D.Eng.
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Serine proteases from the *Bacillus* species extensively applied in the biotechnological application. So far, the broad investigation on proteases gave the basic understanding of their catalytic mechanism and their structure-function. Computational structure analysis and homology modelling can be a key process for the 3D structure reconstruction which facilitates the protein-protein interaction research. Protein crystal is the basic necessity to obtain the 3D structure. The crystallisation process requires ample amount of protein. *Bacillus subtilis* strain Rand could only express the low amount of the protein. The Rand protease has unique characteristics and is the first thermostable and organic solvent tolerant protease that has been reported. Therefore, the structural study is needed to understand the enzyme properties. A statistical approach, response surface methodology (RSM), was performed to optimise the production of extracellular Rand protease in bioreactor stirred tank. Consequently, a face-centred, central composite design (CCD) falling under RSM was employed to enhance the protease activity further. The interactive effect of these parameters resulted in a 1.6-fold increase in protease production. An analysis of the variance showed that the adequacy of the model and verification experiments confirmed its validity. Crystallisation of the purified wild-type protein performed under microgravity conditions in space as well as in the ground. There is no crystal form observed in the ground control but, Rand protease protein successfully crystallised under microgravity conditions. A structural prediction for the Rand protease was built using the Yet Another Scientific Artificial Reality Application (YASARA) structure employing a known 3D structure (subtilisin E-propeptide complex; PDB ID: 1SCJ) as a template, which has the highest sequence similarity (96%) to the Rand protease.

The predicted 3D structure of the Rand protease revealed that the topological organization of the α/β-hydrolase fold consisted of 6 α-helices and 13 β-strands. In silico study of docking, the substrate N-succinyl-alanyl-alanyl-prolyl-phenylalanine-4-nitroanilide in Rand protease resulted in 25 clusters whereby 4 clusters observed to involve the catalytic triad of rand protease that is Asp32, His64, and Ser221. This result is in good agreement with the active site prediction and several experimental studies, which shows the same conserved catalytic triad. Molecular dynamics (MD) simulations were performed in two organic solvents with different log*P* values, such as pyridine (log*P* 0.71), benzene (log*P* 2.0) and pure water, for 10 ns to investigate their effect on the Rand protease structure. In conclusion, the production of Rand protease using a bioreactor through RSM could increase the yield of this enzyme compared to using a shake flask. The structure analysis confirmed the unique characteristics of this enzyme and explained the organic solvent stability of the enzyme. The predicted structure clarified the use of HIC as the first step for purification by highlighting the number of hydrophobic residues on the surface of the protein.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
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PENDEKATAN STATISTIK BAGI PENGELUARAN ENZYME MELALUI METODOLOGI PENYELESAIAN RESPONSE DAN JANGKAAN STRUKTUR PROTEASE TERMOSTABIL TAHAN PELARUT ORGANIK

Oleh

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Serine protease dari *Bacillus* sp. telah digunakan secara meluas dalam aplikasi bioteknologi. Justeru itu, protease telah diselidik secara meluas dari sudut pengetahuan asas mekanisma katalisis dan hubungan fungsi struktur. Analisis struktur berkomputer dan permodelan homologi adalah proses utama untuk pembinaan semula struktur 3 dimensi yang memudahkan kajian hubungan protein. Kristal protein adalah keperluan asas untuk mendapatkan struktur 3 dimensi. Proses penghabluran memerlukan jumlah protein yang mencukupi. *Bacillus subtilis* strain Rand telah hanya dapat mengeluarkan jumlah protein yang rendah. Protease Rand mempunyai ciri-ciri yang unik dan merupakan protease termostabil dan toleran kepada pelarut organik yang pertama telah dilaporkan. Oleh itu, kajian struktur diperlukan untuk memahami sifat-sifat enzim. Satu pendekatan statistik, metodologi permukaan tindak balas (RSM), dilakukan untuk mengoptimumkan penghasilan protease di luar sel dalam bioreaktor. Oleh itu, rekabentuk komposit pusat (CCD) yang berpusat di bawah RSM digunakan untuk meningkatkan lagi aktiviti protease. Kesan interaktif parameter ini menghasilkan peningkatan 1.6 kali ganda dalam penghasilan protease. Analisis varian menunjukan ketepatan model tersebut dan pengesahan kajian telah menunjukan ianya berkesan. Enzim yang ditulenkan telah dihablurkan di bawah kondisi mikrograviti di angkasa dan juga di bumi. Tiada hablur terberntuk di bumi, tetapi protein Rand protease berjaya dihablurkan di bawah kondisi mikrograviti. Jangkaan struktur Rand protease telah dibuat menggunakan aplikasi YASARA (*Yet Another Scientific Artificial Reality Application*) berdasarkan struktur 3 dimensi (*Subtilisin E-propeptide complex; PDB ID: 1SCJ*) sebagai rujukan yang mempunyai persamaan jujukan terbanyak (96%) dengan Rand protease. Jangkaan struktur 3 dimensi Rand protease mengandungi organisasi topologi α/β-hydrolase lipatan yang mengandungi 6 helik α dan 13 bebenang β. Dalam kajian silico untuk mengendalikan substrat *N-succinyl-alanyl-alanyl-prolyl-*

phenylalanine-4-nitroanilide dalam protease Rand sebanyak 25 kelompok telah dihasilkan di mana 4 kelompok yang diperhatikan melibatkan triad katalitik protease Rand iaitu Asp32, His64, dan Ser221. Hasilnya adalah seiring dengan berikutan ramalan tapak aktif dan beberapa percubaan kajian, menunjukkan triad pemangkin yang sama dipelihara. Simulasi dinamik molekul telah dilakukan di dalam 3 pelarut organik dengan log *P* berbeza dengan pyridine (log *P* 0.71), benzene (log *P* 2.0) dan air selama 10 ns untuk mengkaji kesannya terhadap struktur Rand protease. Kesimpulannya, penghasilan Rand protease menggunakan bioreaktor melalui RSM mampu meningkatkan penghasilan enzim jika dibandingkan dengan menggunakan kelalang kon. Analisis struktur mengesahkan karakter unik enzim ini dan menjelaskan kestabilan enzim di dalam pelarut organik. Jangkaan struktur telah menyokong penggunaan kromatografi hubungan hidrofobik (HIC) sebagai langkah permulaan untuk penulenan enzim ini dengan memberikan bilangan residu yang mempunyai ciri hidrofobik pada permukaan protein.

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS AND SYMBOL

Å	Angstrom
ANOVA	Analysis of Variance
Bp	Base pair
BSA	Bovine serum albumin
$CaCl_2.2H_2O$	Calcium Chloride
Cm	Centimeter
CCD	Central composite design
Da	Dalton
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DOT	Dissolved oxygen tension
IS	Inoculum size
DTT	Dithiothreitol
EC	Enzyme Commission
EDTA	Ethylene diamine tetra-acetic acid
FID	Free Interface Diffusion
g	Gram
HPLC	High Performance Liquid Chromatography
hr	Hour
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilo Dalton
L	Litre
$MgSO_4.7H_20$	Magnesium Sulphate
µg	Microgram
µl	Microlitre
µmol	Micromole
mg	Milligram
ml	Millilitre

min	Minute
M	Molar
MD	Molecular Dynamics
MEGA	Molecular Evolutionary Genetics Analysis
MW	Molecular Weight
PEG	Polyethylene glycol
pH	Negative logarithm of hydrogen ion concentration
ORF	Open reading frame
PDB	Protein Data Bank
PMSF	Phenyl methyl sulphonyl fluoride
pmol	Picomole
PCR	Polymerase chain reaction
RSM	Response Surface Methodology
RNA	Ribonucleic acid
RMSD	Root Mean Square Deviation
rpm	Rotation per minute
sec	Second
sp.	Species
STDV	Standard deviation
Temp	Temperature
TCA	Trichloroacetic acid
TSB	Tryptone Soya Broth
U	Unit of enzyme activity
U/mg	Unit per milligram
v/v	Volume per volume
w/v	Weight per volume

LIST OF SYMBOLS FOR AMINO ACIDS

A	Ala	Alanine
R	Arg	Arginine
N	Asn	Asparagine
D	Asp	Aspartate
C	Cys	Cysteine
E	Glu	Glutamate
Q	Gln	Glutamine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
L	Leu	Leucine
K	Lys	Lysine
M	Met	Methionine
F	Phe	Phenylalanine
P	Pro	Proline
S	Ser	Serine
T	Thr	Threonine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
V	Val	Valine

CHAPTER 1

INTRODUCTION

1.1 Background

Currently, enzymes play a significant role, throughout the globe, due to their invaluable applications. Involved in the production of foods, pharmaceuticals and detergents; analysis of clinical samples; alcohol fermentation; and syntheses of organic products; as well as in biotechnology (Malathu *et al.*, 2008; Sanchez and Demain, 2011; Adrio and Demain, 2014). The global market value of enzymes for industrial purposes was approximately $3.3 billion in 2010 (Souza *et al.*, 2015) and from USD 6.4 to 6.9 billion in 2017 (Ferrer *et al.*, 2016). However, the world enzyme demand is forecasted to grow from average 4.6 percent through 2020 to $7.2 billion (Freedonia Group 2016).

Enzymes obtained from microbes serve mainly as resources utilised by industries such as those for food, chemicals and their associates in the production of a variety of biotechnological products (Bhunia *et al.*, 2012). The enzyme subtilisin as one of the proteolytic enzymes, given its vast industrial applications, represents the commercial enzyme available in the most massive quantities. It is mainly produced for the manufacture of detergent additives, and in the processing industries for skin and leather. Subtilisin has, therefore, been extensively researched as a promising target for protein engineering from both the basic and applied perspectives (Hiroshi, 1993).

The genus *Bacillus* is one of the main extracellular protease producers, and industries mostly used the species *Bacillus subtilis* in particular for the production of various enzymes. *Bacillus subtilis* is a rod-shaped Gram-positive bacterium, and its natural habit in soil. It may form a hard or harsh, protective sub-terminal endospore that can withstand high temperatures and other extreme environmental conditions without losing viability when in favourable conditions (Pant *et al.*, 2015).

The protein structures provide sufficient details about its functions, the location of functional sites and clues to find the critical residues (Aloy *et al.*, 2001; Reddy *et al.*, 2001; Yao *et al.*, 2003). Moreover, X-ray crystallography increases the understanding of the functions of a protein and can be used to visualise the protein structure at the atomic level (Rupp, 2009). The Rand protease gene has 96% similarity with the subtilisin-propeptide complex gene (PDB ID: 1SCJ). To understand the structural function of Rand protease enzyme, further studies on structure prediction are necessary. *Bacillus subtilis* strain Rand was reported to have produced a thermo-stable and organic solvent protease. Moreover, the enzyme

showed stability in both polar and non-polar organic solvents, which is a unique property among thermostable proteases (Abusham *et al.*, 2009).

1.2 Problem statement

Rand protease is a thermostable and organic solvent tolerant enzyme with broad applications. However, a study on the structure of such an enzyme is limited. Three-dimensional (3D) structure of a protein is needed to understand their properties. In order to solve the structure, a high concentration of protein is required for protein crystallisation. Production of wild-type Rand protease in shake flask is very low and not enough for protein crystallisation.

1.3 Hypothesis

Production of enough and large quantities of the enzyme for crystallisation purposes is a tall order, and protein crystal is the basic necessity of obtaining the 3D structure. Characterization of Rand protease showed that the enzyme is stable at high temperature as well as in the presence of polar and nonpolar organic solvent, which is unique property among other proteases. Nevertheless, conventional optimisation of production medium in shake flask could not increase the amount of enzyme. A further study on enzyme production in bioreactor stirred tank is necessary to improve the yield of protein, enough for crystallisation, study the structure and to understand the enzyme properties.

1.4 Rational of scope of research

In this work, there were four main steps involved. The first step was the production of Rand protease in 7.5 L bioreactor using RSM technique to produce a high quantity of enzyme. Secondly, purification and crystallisation of the enzyme were carried out. Next, isolation of Rand protease gene was conducted. Finally, a further study on protein prediction was done.

1.5 Objective

This study aims to obtain enough amount of Rand protease for crystallisation and to study the structure-function relationship of the enzyme. To achieve this aim, the following work had to be achieved:

1) To enhance Rand protease production in the bioreactor by applying response surface methodology (RSM).
2) To crystallise Rand protease under gravity and microgravity conditions.
3) To isolate Rand protease gene
4) To predict 3D structure of Rand protease: a silico study
5) To evaluate the effect of organic solvents on overall conformation of Rand protease structure through molecular dynamics (MD) simulations.

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