UNIVERSITI PUTRA MALAYSIA

MOLECULAR EXPRESSION, CHARACTERIZATION AND POTENTIAL APPLICATIONS OF A THERMOSTABLE ALKALINE PROTEASE FROM Geobacillus thermoglucosidasius SKF4

ALLISON SULEIMAN DAN

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By

ALLISON SULEIMAN DAN

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

January 2022

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DEDICATION

This research work is dedicated with profound gratitude to the memories of my late Father Alhaji Aliu Abe Arekemase and my late wife Odunola Funmilayo Allison Arekemase. To my Mother Oguntuke Seliat Aliu Arekemase (nee Jegede) and also to my four children: Oluwasegun, Oluwaseyi, Oluwassola and Oluwaseye.

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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January 2022

Chairman : Associate Professor Nor'Aini Abdul Rahman, PhD
Faculty : Biotechnology and Biomolecular Science

Protease enzyme catalyses the breakdown of protein molecules into simpler units such as amino acids and peptides. Thermostable proteases are the appropriate enzyme that can be used in industrial processes that require high temperature such as detergent, leather processing etc. Many thermostable proteases have been isolated, but only a few that are both alkaline stable and extremely thermostable have been cloned, completely characterized, and their potential industrial applications fully exploited. The main objective of this research was to clone a thermostable alkaline protease from a thermophilic bacteria and its potential applications as detergent additive, in the production of bioactive peptides and recovery of silver from X-ray film evaluated. In this study a new thermophilic bacterial that produces thermostable protease enzyme was successfully screened and identified using 16S rRNA gene sequence with 99 % identity with other members of *Geobacillus* sp. The organism which was isolated from hot spring in Sungai Klah Perak in Malaysia was identified as *Geobacillus thermoglucosidasius* SKF4 and was characterized using different parameters. The organism was highly thermophilic and produced protease enzyme at optima pH 7 and temperature of 60 ºC. A thermostable alkaline serine protease SpSKF4 gene from the *G. thermoglucosidasius* SKF4 was amplified using polymerase chain reaction (PCR). The gene analysis showed an open frame of 1206 bp coding for a protein of 401 amino acids. The cloned gene was successfully expressed in *Escherichia coli* by T7 promoter using the *pEASY*® Blunt-end E1 prokaryotic expression vector. The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the protein revealed a mature protein of approximately 28 kilo Dalton (kDa) which was also confirmed by the western blot. Optimisation of SpSKF4 protein for soluble expression under different cultural conditions revealed an increase in expression and activity (200 U/mL) at isopropyl β-D thiogalactoside (IPTG) concentration of 0.4 mM and a temperature of 20 ºC after 12 h incubation. The recombinant alkaline serine protease was partially purified and fully characterised. The partially purified enzyme was active between 20-100 ºC with optimum activity (353 U/mL) at optima pH 10 and temperature of 80 ºC. Its activity was

stable at about 40 % capacity at optimum pH 10 and 80 °C after 24 h incubation, with half-life of 15h. Its activity was increased by 60 % with the addition of 10 mM Ca^{2+} and also addition of Mg^{2+} increased the activity by 30 % at concentration of 2.5 mM. However, the activity was reduced by 30 % by copper, a heavy metal. The protease enzyme SpSKF4 was stable in surfactants such as Sodium dodecyl sulphate (SDS). The SDS however, increased the activity of the enzyme by 20 %. The enzyme was inactivated (100 %) by phenylmethylsulfonyl fluoride (PMSF) at concentration of 10mM indicating the enzyme was a serine protease. The kinetic study showed high catalytic efficiency (Kcat/Km) (4.9 mg/ml/min) with casein at 80 ºC, with V_{max} and K_m of 7.1 U/ml and 0.57 mg/ml, respectively. The recombinant enzyme was highly stable in organic solvents and certain oxidising and reducing agents, and also showed high stability (> 90 %) with some commercial detergents. The enzyme showed high capacity as a potential industrial enzyme as a detergent additive, in the recovery of silver from X-ray film and in the production of anti-microbial and anti-oxidant peptides from proteins hydrolysates. The hydrolysates produced from casein and Bovine Serum Albumin (BSA) using SpSKF4 protease showed high 2,2-diphenyl-1-picrylhydrazyl (DPPH) (> 67 %) and 2,2-azino-bis3-ethylbenzothiazoline-6-supfonic acid (ABTS) (> 85 %) radicals scavenging activities. The Fe^{2+} chelating capacity was about 85 %. These have confirmed that the thermostable protease enzyme from *G. thermoglucosidasius* species from hot spring could be used as industrial enzyme in various capacities. In conclusion, a thermostable SpSKF4 protease gene was successfully cloned from a thermophilic organism *G. thermoglucosidasius* SKF4 isolated from hot spring and the enzyme showed a remarkable potential as a prospective enzyme for industrial and biotechnological applications.

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Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

EKSPRESI MOLEKUL, PENCIRIAN DAN POTENSI APLIKASI PROTEASE ALKALI TERMOSTABIL DARIPADA *Geobacillus thermoglucosidasius* SKF4

Oleh

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Protease enzim memangkinkan pemecahan molekul protein kepada unit yang lebih mudah seperti asid amino dan peptida. Protease termo stabil adalah enzim yang sesuai yang boleh digunakan dalam proses industri yang memerlukan suhu tinggi seperti detergen, pemprosesan kulit dan lain-lain. Banyak protease termo stabil telah dipencilkankan, tetapi hanya sedikit yang bersifat stabil alkali dan sangat stabil termo yang telah diklon dan dicirikan sepenuhnya yang berpotensi dalam aplikasi industri untuk dieksploitasi sepenuhnya. Objektif utama penyelidikan ini adalah untuk mengklon protease stabil alkali termo daripada bakteria termofilik dan menilai aplikasi yang berpotensi sebagai bahan tambahan detergen, pengeluaran peptida bioaktif dan pemulihan perak daripada filem X-ray.

Dalam kajian ini, bakteria termofilik baharu yang menghasilkan enzim protease stabil termo telah berjaya disaring dan dikenal pasti menggunakan jujukan gen 16S rRNA dan menunjukkan identiti 99% dengan ahli-ahli *Geobacillus* sp. yang lain. Organisma tersebut telah dipencilkan dari kolam air panas di Sungai Klah Perak, Malaysia dan dikenal pasti sebagai *Geobacillus thermoglucosidasius* SKF4 dan dicirikan menggunakan parameter yang berbeza. Organisma ini sangat termofilik dan menghasilkan enzim protease pada optima pH 7 dan suhu 60 ºC. Gen protein alkali serine stabil termo SpSKF4 dari *G. thermoglucosidasius* SKF4 telah diperbanyakkan menggunakan tindak balas rantaian polimerase (PCR). Analisis gen menunjukkan bingkai terbuka pengekodan 1206 bp untuk 401 asid amino protein. Gen klon tersebut telah berjaya diekspress dalam *Escherichia coli* oleh promoter T7 menggunakan vektor ekspres prokariotik PEASY® Blunt-end E1. Analisis gel elektrophoresis natrium dodecyl sulfat polyacrylamide (SDS-PAGE) protein menunjukkan protein matang dengan saiz kira-kira 28 kilo Dalton (kDa) yang kemudian disahkan juga oleh blot Western. Pengoptimuman protein SpSKF4 untuk ekspresi terlarut dalam keadaan kultur yang berbeza menunjukkan peningkatan dalam ekspresi dan aktiviti (200 U/mL) pada kepekatan isopropyl β-D thiogalactoside (IPTG) sebanyak 0.4 mM dan suhu 20 ℃

selepas 12 jam inkubasi. Protease alkali serine rekombinan separa tulen ini telah dicirikan sepenuhnya. Enzim separa tulen tersebut didapati aktif antara 20-100 ºC dengan aktiviti optimum (353 U / mL) pada pH optima 10 dan suhu 80 ºC. Aktivitinya adalah stabil pada kapasiti 40 % pada pH optima 10 dan 80 °C selepas 24 jam inkubasi dengan dengan separuh hayat 15 jam. Aktivitinya meningkat sebanyak 60 % dengan penambahan 10 mM Ca^{2+} dan juga penambahan Mg^{2+} meningkatkan aktiviti sebanyak 30 % pada kepekatan 2.5 mM. Walau bagaimanapun, aktiviti itu dikurangkan sebanyak 30 % oleh logam berat tembaga, Enzim protease SpSKF4 stabil dalam surfaktan seperti Sodium dodecyl sulfat (SDS). SDS bagaimanapun meningkatkan aktiviti enzim sebanyak 20%. Enzim ini dinyahaktifkan 100 % oleh fenilmethylsulfonyl fluorida (PMSF) pada kepekatan 10 mM dan ini menunjukkan enzim ini adalah sejenis protease serine. Kajian kinetik menunjukkan kecekapan pemangkin yang tinggi (Kcat/Km) (4.9 mg/ml/min) dengan menggunakan casein sebagai substrat pada 80 °C, dengan Vmax dan Km masing-masing 7.1 U/ml dan 0.57 mg/ml. Enzim rekombinan tersebut sangat stabil dalam pelarut organik dan agen pengoksidaan dan penurunan tertentu, dan juga menunjukkan kestabilan yang tinggi (> 90 %) dengan beberapa detergen komersial. Enzim ini menunjukkan kapasiti tinggi sebagai enzim industri yang berpotensi sebagai bahan tambahan detergen, pemulihan perak dari filem X-ray dan dalam penghasilan peptida anti-mikrob dan anti-oksida daripada hidrolisis protein. Hidrolisis yang dihasilkan daripada casein dan Bovine Serum Albumin (BSA) menggunakan protease SpSKF4 menunjukkan 2,2-diphenyl-1-picrylhydrazyl (DPPH) (> 67 %) yang tinggi dan aktiviti scavenging radikal 2,2-azino-bis3-ethylbenzothiazoline-6-supfonic acid (ABTS) (> 85 %). Kapasiti pengelatan Fe^{2+} pula adalah kira-kira 85%. Kajian ini telah mengesahkan bahawa enzim protease termo stabil dari *G.thermoglucosidasius* dari mata air panas boleh digunakan sebagai enzim industri dalam pelbagai kapasiti.

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This thesis was submitted to the Senate of the 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

Cm	centimeter
dH2O	distilled water
EDTA	ethylene diamine tetraacetic acid
G	gram
g/L	Gram per litre
h	hour
IPTG	isopropyl β-D thiogalactoside
TCA	trichloroacetic acid
kDa	kilo Dalton
L	liter
M	molar
mM	millimolar
Mg	milligram
M	minute
ORF	open reading frame
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
SDS-PGAE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N, N, N, N-Tetramethylenediamine
µg	microgram
µl	microlitre
v/v	volume per volume
w/v	weight per volume
LB	luria bertani
U/mL	unit per milliliter
^{o}C	degree Celsius
Rpm	revolution per minute

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OD	optical density
SMA	skim milk agar
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	(2,2-azino-bis3-ethylbenzothiazoline-6-supfonic acid))
SDS	Sodium dodecyl sulphate
NCBI	National Centre for Biotechnological Information
TAE	Tris-acetate-EDTA-buffer
NaCl	Sodium Chloride
Β	beta
BLAST	Basic Local Alignment Tool
BSA	Bovine Serum Albumin
%	percentage
Α	alpha
Bp	Base pair
APS	Ammonium Persulfate solution
DMSO	Dimethyl sulfoxide

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CHAPTER 1

INTRODUCTION

1.1 Background of the study

Enzymes are highly effective, environmentally benign protein catalysts that are produced by living organisms. Specificity, high catalytic activity, the capacity to function at both moderate and extreme temperatures, and the ability to yield in abundant quantities are just a few of their benefits over chemical catalysts (Gupta et al., 2002). Protease enzyme catalyses the breakdown of protein molecules into simpler units such as amino acids and peptides. Proteases are divided into four categories based on the functional group present in the active site this include; serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley, 1960, Sharma et al., 2019). The pH at which serine proteases are optimally active is at the range of 7 to 11 (Shimogaki et al., 1991). The largest subgroup of serine proteases is serine alkaline proteases, which are active at extremely alkaline pH (Gupta et al., 2002).

Thermostable enzymes can generate exceptionally high end-product yields and are helpful in various applications such as use of higher processing temperatures, faster reaction speeds, an improvement in the solubility of nongaseous reactants and materials, and reduced incidence of microbial contamination from mesophilic species (Bekler et al., 2015). Due to their strong industrial and research applications such as detergent, and in food, pharmaceuticals, leather, diagnostics, peptide synthesis, waste management, silver recovery, and also being the most molecular exploited and commercialised enzyme group(Banik and Prakash, 2004; Beklet et al., 2015).

Proteases from microbial sources account for around 40 percent of the global enzymes sales (Naveed et al., 2021). It is necessary to select the proper microorganism in order to obtain the desired products. Working under high temperatures the chosen microbes must be able to produce substantial yields, secrete huge amounts of protein, and be free of toxins and other undesirable substances. Thermophilic microorganisms could be of importance in achieving this. Because of their versatility, microbial proteases are commonly utilised in the laundry, food, and textile sectors. Cleaning, textile, leather, and food processing industries are just a few of the sectors that have used alkaline proteases (Gupta et al., 2002). Thermostable alkaline proteases are widely used in a variety of industries, including the detergent and leather industries. However, their use in food and other applications such as recovery of silver from X-ray and photographic films and production of bioactive peptides from food sources have yet to be fully explored (Sharma et al., 2019).

Proteases have been isolated from animal, plant and microbial origins. The latter, however, is the most commercialised because microbial proteases are not susceptible to variations in pH and temperature, and are also tolerant of conditions of detergent and

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organic solvents (Esakkiraj et al., 2016). *Geobacillus* species have recently been identified as one of the major producers of microbial proteases. Examples include *G. stearothermophilus* F1 (Fu et al, 2003), *Geobacillus* sp. PA-5 and PA-9 (Hawumba et al., 2002), *G. toebii* strain LBT 77 (Thebti et al., 2016).

Microbial strain development via traditional mutagenesis (UV or chemical exposure) or recombinant DNA technology (rDNA) is commonly used to accelerate protease synthesis in bacteria. Using appropriate enzyme tools, the recombinant DNA (rDNA) technology as a tool of protein engineering to help in advancing and delivering solutions that address the large needs of customers and markets (Arbige et al., 2019). One of the important recent uses of proteases is the generation of hydrolysates through the process of hydrolysis of the peptide bonds to produce bioactive peptides (Tavano, 2013). Such bioactive peptides includes antimicrobial, antithrombotic, antihypertensive, opioid, immunomodulatory, mineral binding, and anti-oxidative depending on their mode of action (Sanchez and Vazquez, 2017; Jain et al., 2012). On the other hand, proteases can cause very precise and selective protein alterations as opposed to the use of chemicals (Sumantha et al., 2006).

Protease must be stable and active in harsh washing conditions such as high temperature, alkaline pH, metal ions, and high salt concentration as well as stability in surfactants and detergents to be employed in detergent formulations (Gupta et al., 2012, Haddar et al., 2009; Jain et al., 2012). They are used as additives instead of other chemicals which are detrimental to the environment. The demand for highly active and stable proteases in industry is growing, and while site-directed mutagenesis and protein engineering have been used to improve alkaline protease stability, the best strategy appears to be screening microorganisms from extreme environments (Thebti et al., 2016).

The advent of recombinant DNA technology and protein engineering has allowed a microbe to be altered and grown in large amounts in order to meet increased demand (Liu et al., 2013). The isolation and cloning of enzyme-encoding genes from all possible sources, including very difficult-to-manipulate bacteria and other microorganisms, as well as high-yield heterologous protein production, have all been made possible by recombinant DNA techniques (Galante and Formantici, 2003, Rigoldi et al., 2018).Thermophilic and hyperthermophilic organisms have been observed to play a key role in industrial processes, and the enzymes that these microorganisms produce are prized for applications in many fields and produces proteases that are employed in biotechnological processes that operate at elevated temperatures due to their temperature tolerance (Straub et al., 2018) . Because of the above stated criteria, and the advantages that they would have over heat-labile protease producing microorganisms to fulfill industrial application in the future, attempts have been made to screen and isolate heat-stable protease producer from extreme environments such as hot spring and cool environment.

The genus *Geobacillus* comprises Gram-positive, aerobic and facultative anaerobic bacilli that are thermophiles, growing best at temperatures between 55 and 65 °C (Rao

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et al., 1998). The genus are often isolated from the environment that is hot like desert soil, compost, oil wells and hot springs, though the genus have been found in other areas with temperate climate (Zeigler, 2014). On the other hand, these organisms have long been valued as sources of thermostable proteins that serve as stable catalysts and powerful biomimetic structures (Hussein et al., 2015, Suzuki, 2018).

The separation of enzymes from naturally occurring thermophilic species is another method for extracting enzymes with enhanced thermostability (Sonnleitner and Fiechter, 1983). Therefore, using Recombinant DNA technology to clone and express the thermophilic genes of interest in mesophilic species is the best approach (Maciver et al., 1994). The thermophile *Geobacillus thermoglucosidasius* has a great appeal as a framework for the production of chemicals and fuel (Sheng et al, 2017). The isolation of *Geobacillus thermoglucosidasius* from hot spring in Malaysia and the production of an alkaline serine protease from the organism and its use for anti-microbial and antioxidant bioactive production and as a detergent additive and in X-ray recovery is being reported for the first time. The cloning of a serine alkaline protease gene from *G. thermoglucoidaius*, its expression in mesophilic *E. coli*, purification, and characterization of the recombinant protein and its various applications are described in this research study.

1.2 Problem statement

The current thermophilic bacteria are not yet satisfactory enough in the production of thermostable proteases. Hence there is much focus on genetic engineering of their enzymes to increase their activity and screening of novel enzymes from new thermophilic bacteria sources to obtain the necessary characteristics for industrial and biotechnological applications (Zhu et al, 2020; Aanniz et al., 2015). Due to their extreme growth conditions, it is difficult to cultivate most of the current thermophilic bacteria for the production of protease on a large scale (Liu et al., 2019). Metabolic engineering to improve hydrolysis efficiency and increase thermophile product yield is difficult due to a lack of adequate genetic tools (Liu et al., 2019; Zhu et al., 2020).The loss of functioning capacity and stability of most of the protease enzymes in heat and organic solvents over time still pose a problem (Sharma et al., 2017). The existing thermostable alkaline proteases that find application for industrial purpose face some limitation, such as, lack of enzyme activity, and stability toward modern-bleach based detergent formulation that contain sodium dodecyl sulphate (SDS) and H_2O_2 (Vijayaraghavan et al., 2014; Arya et al., 2021). Antimicrobial resistance of bacteria bioactive agents such as antibiotics have been known to be toxic when used for preservative for animal feeds. Biological agents such as bacterial have been known to develop resistance to antimicrobial agents (Tavano et al., 2013). Most of the industrial processes use chemicals that are not environmental sustainable, leading to environmental pollution and toxicity (Singh and Bajaj, 2017).

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1.3 Hypothesis

The thermostable alkaline serine protease from a thermophilic bacteria may be expressed in *E. coli* and also have the capacities for many industrial and biotechnological applications such as detergent additive, in X-ray silver recovery and in the production of anti-microbial and anti-oxidant bioactive peptides.

1.4 General Objective

To produce a thermostable protease enzyme that will be suitable for industrial applications and have the capacity to generate bioactive peptides that have anti-microbial, anti-oxidant and iron(II) chelating properties from milk proteins.

1.5 Specific objectives

1. To identify thermostable protease enzyme-producing bacteria from different sources.
2. To clone and express the thermostable alkaline serine protease in *E. coli.*
3. To purify and characterise the recombinant enzyme and determine its kinetic properties.
4. To investigate the industrial potentials of the enzyme to generate bioactive peptides e.g. anti-oxidant, anti-microbial and Fe^{2+} chelators from milk proteins, and also as an additive in detergent, and in the recovery of silver from X-ray photographic film.

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