PROTEOLYTIC ACTIVITY CHARACTERIZATION OF BACTERIA ISOLATED FROM MALAYSIAN TRADITIONAL FERMENTED FOOD

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سُبْحَانَ اللّٰهِ وَ بِحَمْدِهِ ، سُبْحَانَ اللّٰهِ الْعَظِيمِ

"*Maha Suci ALLAH & segala puji bagi-NYA, Maha Suci ALLAH yang Maha Agung"*

To heart of my life Ma, Abah, Abg Zi, Q. Ngah, Q. Chik, Q. Jue, & Pok Pi

To my beloved fiancé Mohd Saiful bin Deraman

To all my awesome friends

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ABSTRACT

Three types of bacteria strains, which are *Bacillus* sp., *Enterococcus gallinarum* and *Bacillus thuringiensis* have been isolated previously from Malaysian traditional fermented food. The proteolytic activities of the three strains were screened on skim milk agar plate. After 24 hours of incubation at 37°C, proteolytic activity was observed based on holozone formation on the skim milk agar plate with a diameter of 0, 1.9, and 3.2 cm respectively for *Bacillus* sp., *Enterococcus gallinarum* and *Bacillus thuringiensis*. The proteolytic activities of all the strains were characterized based on optimum temperature, temperature stability, optimum pH, pH stability, substrate specificity and effect of metal ions towards activity. All three strains showed optimum activity at 50°C. The optimum pH for *Bacillus* sp. and *Bacillus thuringiensis* were pH 8.5, while *Enterococcus gallinarum* showed maximum enzyme activity of 0.068±0.003 U/ml at pH 7.5. The proteolytic activity of the bacteria were stable in the temperature range of 30°C to 50°C and exhibited rapid decrease in activity when incubated at 60°C for 60 minutes. Proteolytic activity of all strains was stable at a broad pH range from pH 4.5 to pH 10.6. The bacteria strains displayed high activity for casein, gelatin and fibrin but showed very low activity for bovine serum albumin. Proteolytic activity of *Bacillus* sp. was enhanced by Cu^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+}, while the proteolytic activity of *Enterococcus gallinarum* was only slightly enhanced by Zn^{2+}.

ABSTRAK

Tiga jenis spesis bakteria daripada makanan terampai tradisional Malaysia telah dipencilkan iaitu *Bacillus* sp., *Enterococcus gallinarum* dan *Bacillus thuringiensis*. Aktiviti proteolitik oleh ketiga-tiga spesis bakteria telah ditentukan menggunakan plat agar susu skim. Selepas inkubasi selama 24 jam pada suhu 37°C, aktiviti proteolitik dapat dilihat melalui pembentukkan kawasan jernih pada plat agar susu skim dengan masing-masing mempunyai diameter (dalam cm) iaitu 0. 1.9, dan 3.2 bagi *Bacillus* sp., *Enterococcus gallinarum,* dan *Bacillusthuringiensis*. Aktiviti proteolitik olehsetiap spesis bakteria dicirikan mengikut suhu optimum, kestabilan suhu, pH optimum, kestabilan pH, pengkhususan substrat dan juga kesan ion logam terhadap aktiviti proteolitik. Setiap spesis bakteria menunjukkan aktiviti optima pada suhu 50°C. pH optima bagi *Bacillus* sp. dan *Bacillus thuringiensis* adalah pH 8.5, manakala *Enterococcus gallinarum* menunjukkan aktiviti enzim yang maksimum iaitu 0.068±0.003 U/ml pada pH 7.5. Aktiviti proteolitik oleh setiap bakteria adalah stabil di dalam linkungan suhu 30°C hingga 50°C dan menurun secara mendadak selepas inkubasi pada suhu 60°C selama 60 minit. Aktiviti proteolitik bagi setiap spesis bakteria adalah stabil dalam lingkungan pH yang luas iaitu daripada pH 4.5 hingga pH 10.6. Ketiga-tiga spesis bakteria menunjukkan aktiviti yang tinggi bagi casein, gelatin dan fibrin tetapi menunjukkan aktiviti yang rendah bagi albumin serum bovin (BSA). Aktiviti proteolitik bagi *Bacillus* sp. dipertingkatkan oleh Cu^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+}dan Zn^{2+}. Manakala aktiviti proteolitik bagi *Enterococcus gallinarum* hanya dipertingkat sedikit oleh Zn^{2+}.

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LIST OF SYMBOLS

%	-	Percentage
°C	-	Degree Celsius
µl	-	Microliter
µmoles	-	Micromoles
cm	-	Centimetre
g	-	Gram
h	-	Hour
M	-	Molarity
mg/ml	-	Milligram per mililiter
Min	-	Minute
ml	-	Mililiter
mM	-	Milimolar
pH	-	Power of hydrogen ion
U	-	Unit of enzyme activity as define.

LIST OF ABBREVIATIONS

BSA	-	Bovine serum albumin
GRAS	-	Generally recognized as safe
NA	-	Nutrient agar
NaCl	-	Sodium Chloride
NaOH	-	Sodium hydroxide
NB	-	Nutrient broth
nm	-	Nanometer
OD	-	Optical density
Rpm	-	Rotation per minute
TCA	-	Trichloroacetic acid
w/v	-	Weight over volume

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Proteolytic enzymes are a group of enzymes in hydrolase class that break down peptide bond of protein into polypeptides or free amino acids through a hydrolysis process (Raja *et al*., 2011; Alnahdi, 2012). Hydrolysis is a process where chemical bond is cleaved by the addition of water molecule (Horton *et al*., 2006). These enzymes are also recognized as proteases, proteinases, and peptidases enzyme (Rani *et al*., 2012).

Proteolytic enzymes possessed several physiological functions and play a vital role in living being where, they involved in food protein digestion, protein turnover, cell division, blood-clotting cascade, signal transduction, and processing of polypeptide hormones. Proteolytic enzymes have the ability to carry out selective modification of proteins by limited cleavage such as activation of zymogenic forms of enzymes, blood clotting and lysis of fibrin clots, and processing and transport of secretory proteins across the membranes (Rani *et al*., 2012).

They are also of great importance in various industries as well as providing lots of economic benefits. Among industrial enzymes, proteases account for 1/3 of the total industrial enzymes used and constitute for about 60% of the total enzyme sale in the world (Bose, 2011). Thus, proteases are commercially being produce for application in detergent, leather, brewing and degumming silk industry. Sources of protease include all forms of life, that is, plants, animals and microorganisms (Khan *et al*., 2011). Some of the well-known proteolytic enzymes from plants are papain, bromelain, keratinases and ficin. Meanwhile, proteases from animal origin are recognized as trypsin, chymotrypsin, pepsin and rennin (Jisha *et al*., 2013).

Due to potential varied applications of proteolytic enzymes in industrial processes and medical therapeutics, microbial protease is much more preferable compared protease originating from plants and animals since they hold the characteristics that are desired in biotechnology applications. Microorganisms are excellent sources of protease, due to their rapid growth, broad biochemical diversity and their susceptibility to genetic manipulation for the generation of new recombinant enzymes with desired properties (Rao *et al*., 1998).

Among the microbial protease, interest have been placed in identifying potential microorganisms producing proteolytic enzyme isolated from traditional fermented food. The fermentation products are good sources for isolation of microorganisms producing useful industrial and food enzymes, e.g. protease and collagenase (Uchida *et al*., 2004). Fermented foods generally preserve pleasant flavour, aroma, texture, enhanced nutritive values and good keeping quality under ambient conditions (Law *et al*., 2011). In Southeast Asian countries, for examples Japan, Korea, China, Indonesia, Thailand and Malaysia are known to possess numerous types of traditional fermented foods from variety of sources. *Natto*, *Douchi* and *Cheongkokjang* are most commonly known soybean types traditional fermented food from Japan, China and Korea respectively (Sumi *et al*., 1987; Peng *et al*., 2003; Jeong *et al*., 2007). Meanwhile, one of the famously soybean fermented food in Malaysia and Indonesia is known as *Tempeh* (Kim *et al*., 2006).

Besides soybean sources, traditional fermented food make from the fish sauce is also known for their protease activity. In Thailand the fermented fish sauce called *Pla-ra*, is known for having potential microorganisms that can produce the protease (Chamroensaksri *et al*., 2008). *Budu* is a famous fermented fish sauce among the state of east- cost Peninsular Malaysia (Ahmad Sanusi and Jamaluddin, 2012). Other than that, traditional fermented food based of shrimp sauce and paste such as *Ka-pi* (Thailand) and *Terasi* (Indonesia) are also shown to govern potential microorganism producing proteolytic enzyme (Tanasupawat *et al*., 2011; Prihanto *et al*., 2013).

1.2 Problem Statement

Numerous types of microorganisms have been isolated from diverse range of traditional fermented foods especially in Southeast Asia region shows potential proteolytic activity. Despite having an essential need to further understand the enzyme in terms of function and structures, a comprehensive understanding of protease will facilitate classification of the enzyme based on the properties they possess for their application especially in industrial processes and medical therapeutics.

1.3 Objectives

The primary aim of this experimental study is to characterize wild type proteolytic enzyme from three types of bacteria which are, *Bacillus* sp., *Enterococcus gallinarum* and *Bacillus thuringiensis* that have been isolated previously from Malaysian traditional fermented food. Therefore the main objectives are:

1) To determine optimum temperature and temperature stability of proteolytic enzyme.
2) To determine optimum pH and pH stability of proteolytic enzyme.
3) To identify substrate specificity
4) To determine the effect of metal ions on proteolytic activity.

1.4 Scope of Study

In this research study, the wild type bacteria that have been isolated from Malaysian traditional fermented food by Krishnan (2010) and Chang (2011) known as *Bacillus* sp., *Enterococcus gallinarum*, and *Bacillus thuriengiensis* were used for the production of proteolytic enzyme. The crude proteolytic enzyme produced then was assayed for its proteolytic activity and characterized according to the optimum temperature, temperature stability, optimum pH, pH stability, substrate specificity and effect of metal ions.

1.5 Significance of Study

All proteolytic enzymes have characteristics properties with regard to temperature, pH, ion requirement, specificity, activity and stability. Studies relating to such properties are imperative and these biochemical parameters will determine the potential application in their respective industries and medical therapeutics fields (Jisha *et al*., 2013).

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