CHARACTERIZATION OF EXTRACELLULAR PROTEASES FROM *PSEUDOMONAS* SP. 16D4 AND *DERMACOCCUS* SP. 17D6 ISOLATED FROM ARCTIC REGIONS

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A dissertation submitted in partial fulfillment
of the requirements for the award of the
degree of Master of Science (Biotechnology)

Faculty of Biosciences and Medical Engineering
Universiti Teknologi Malaysia

JULY 2015

ACKNOWLEDGEMENT

In preparing this dissertation, I was in contact with many people. They have contributed towards my understanding and thoughts. In particular, I would like to thanks my supervisor, Associate Professor Dr. Zaharah binti Ibrahim, for her encouragement, advices and critics. I was greatly appreciate for receiving all the helpful comments and suggestions. I would also like to thank all my senior colleagues and laboratory assistants, especially Dr. Ang and Dr. Ivy, who gave a lot of precious ideas and guidance whenever I encountered difficulties during the research. Without their continued support, this dissertation might not be the same as presented here. In the end, I am grateful to my family, especially my parents, who had giving me their manual supports and advices.

ABSTRACT

In recent studies, cold-active enzyme has become another subject of interest. It can overcome the limitations of the classical industrial enzymes that normally require higher temperature to function. In this study, 16 bacteria isolated from the Arctic region were screened for their protease activity using Skim Milk agar plate. Two bacteria with lower hydrolysis coefficient (Hc) were selected, they were *Pseudomonas* sp. 16D4 (Hc = 0.339) and *Dermacoccus* sp. 17D6 (Hc = 0.331), and their proteases were produced using "Protease Specific Medium" and were further studied. Both bacteria produced proteases that are associated to growth and the maximal protease activity was reached at 30th hour and 24th hours of incubation, respectively. Protease produced by *Pseudomonas* sp. 16D4 remained stable at temperature ranging from 0^{o}C to 70^{o}C. Its optimum activity was detected at 0^{o}C, pH 9 or 11 with K_m value of 1.37 mg/mL and V_{max} value of 243.90 Unit/L. As for *Dermacoccus* sp. 17D6, the protease activity remained stable at temperature ranging from 0^{o}C to 40^{o}C and its optimum condition for the assay was at 50^{o}C, pH 7 or 9 with K_m value of 0.66 mg/mL and V_{max} value of 344.83 Unit/L. The SDS-PAGE and zymogram analysis further revealed that the molecular mass of the *Pseudomonas* sp. 16D4's protease was 40 kDa. Whereas the size for the *Dermacoccus* sp. 17D6's protease cannot be determined due to the negative result obtained in the zymogram analysis.

ABSTRAK

Enzim sejuk-aktif telah menjadi satu lagi subjek yang menarik pada zaman ini. Ia dapat mengatasi batasan enzim perindustrian klasik yang biasanya memerlukan suhu yang lebih tinggi untuk berfungsi. Dalam kajian ini, aktiviti protease bagi 16 bakteria dari kawasan Artik telah disaring dengan kegunaan Skim susu agar. Dua bakteria dengan pekali hidrolisis (Hc) yang lebih rendah telah dipilih, mereka adalah *Pseudomonas* sp. 16D4 (Hc = 0.339) dan *Dermacoccus* sp. 17D6 (Hc = 0.331). Dengan kegunaan "Protease Specific Medium", protease mereka telah dihasilkan dan digunakan dalam kajian seterusnya. Kedua-dua bakteria menghasil protease yang berkaitan dengan pertumbuhan dan maksimum protease aktiviti boleh dicapai pada jam 30 dan jam 24, masing-masing. Protease yang dihasilkan oleh *Pseudomonas* sp. 16D4 dapat mengekalkan kestabilannya dari suhu 0^oC hingga 70^oC. Aktiviti optimumnya pula dikesan pada 0^oC, pH 9 atau 11 dengan nilai K_m = 1.37 mg/mL dan nilai V_{max} = 243,90 Unit / L. Bagi *Dermacoccus* sp. 17D6 pula, enzimnya dapat mengekalkan kestabilan pada suhu antara 0^oC hingga 40^oC dan keadaan optimum untuk enzim itu adalah 50^oC, pH 7 atau 9 dengan nilai K_m = 0.66 mg mL dan nilai V_{max} = 344,83 Unit / L. Analisis SDS-PAGE dan zymogram telah mendedahkan bahawa molekul saiz bagi enzim *Pseudomonas* sp. 16D4 adalah 40 kDa. Bagi enzim *Dermacoccus* sp. 17D6 pula, saiznya tidak boleh dianggarkan kerana zymogram analisis tidak memberi sebarang data.

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LIST OF ABBREVIATIONS

ABM	-	Antarctic Bacterial Medium
EC	-	Enzyme commission
Hc	-	Hydrolytic coefficient
L	-	Liter
kDa	-	Kilo dalton
M	-	Molarity
min	-	Minute
OD	-	Optical density
pH_{opt}	-	Optimum pH
rpm	-	Rotation per minute
sp.	-	Species
T_{opt}	-	Optimum temperature
U	-	Unit of enzyme
V	-	Volt
w/v	-	Weight per volume
^oC	-	Degree Celsius
μ	-	Micro

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Proteases, also indicated as peptidases or proteinases, are proteolytic enzymes that are responsible in catalyzing the hydrolysis of peptide bonds. These enzymes can break long polypeptide chains into smaller fragments, where this process is essential in the production of proteins by controlling their size, turnover, folding and their composition (Shen and Chou, 2009). From bacteria to human, each of the organisms can produce different kinds of proteases, and each of these proteases has its own physiological function. It is believed that proteases' genes occupy nearly 2% of the human genome, and 1-5% of the bacteria genome (Shen and Chou, 2009). These statistics indicated the importance of proteases in maintaining the life of an organism.

Other than their crucial roles in life cycle, proteases can be considered as one of the most important enzyme for industrial usage; it was estimated that they occupy 40-60% of the industrial enzyme market (Rao *et al.*, 1998; Kuberan *et al.*, 2010; Anuraj *et al.*, 2012; Narendra, 2012). The industries that recruit the usage of proteases including food, pharmaceutical, leather, detergent, and agricultural industries (Pallavi, 2011). Although there were a lot of articles regarding proteases had been reported, this enzyme remains one of the favorite topics in biological research. Scientists aim to find

novel proteases that have higher activity, remain stable at various temperatures, high specificity and can be easily mass produced for industrial usage.

Among different sources of proteases, microbial proteases are the most highlighted as compared with animal and plant proteases. The inability of animal and plant proteases to sustain the demands and their limitations has become reasons for scientists to search more proteases from the microbial source (Rao *et al.*, 1998). Microbial source including fungi, bacteria, and viruses, have broad biochemical diversity, due to this, more proteases with different application can be found. Other advantages including low cost and ease of mass production, the contents of the enzyme products are more controllable, the protease genes are easily subjected for genetic manipulation (Boyer, 1971; Whitaker *et al.*, 1974; Rao *et al.*, 1998; Uhlig and Linsmaier-Bednar, 1998).

Proteases can be classified into acidic, alkaline and neutral proteases based on optimum pH for activity (Pushpam *et al.*, 2011). Based on site of action, they are grouped as exopeptidases and endopeptidases (Rao *et al.*, 1998). And based on their catalytic mechanism, proteases can be categorized as serine, aspartic, cysteine, metallo-, threonine, and glutamic proteases (López-Otín and Bond, 2008).

In the past, thermostable enzymes that can be isolated from thermophiles and mesophiles are always a favorable choice to be used in industries (Turner *et al.*, 2007). But in recent years, some of the scientists had suggested that cold-active enzymes, which can be isolated from psychrophilic, psychrotrophic and psychrotolerant bacteria, can also be a good candidate, where their first benefit is to minimize the energy usage (Margesin, 2002). Cold-active enzymes are mainly sourced from microorganisms from cold habitats such as Arctic regions, polar regions, deep sea, glacier ice, alphine regions and other cold regions on earth (Margesin, 2002; Joshi and Satyanarayana, 2013). Examples of the bacteria strains that found to have the ability to produce cold-active proteases are *Flavobacterium balustinum* P104 (Morita et al.,

1998), *Shewanella strain* Ac10 (Kulakova et al., 1999), *Pseudomonas* strain DY-A (Zeng et al., 2003), *Colwellia* sp. NJ341 (Wang et al., 2005), *Pseudoalteromonas* sp. NJ276 (Wang et al., 2008), *Serratia proteamaculans* (Mikhailova et al., 2014), *Arsukibacterium ikkense* (Gobba et al., 2014) and etc.

1.2 Problem Statement

The usage of classical chemical or petroleum-based reagents in any of the industries, such as detergent and agriculture industry, will eventually create a lot of problems, such as environmental pollution, global warming, eutrophication, and algae bloom. When these types of reagents are release into the water source like river and sea, these toxic compounds will slowly accumulate inside the fishes and in long term consideration, this will also cause a serious human health problem if the fish are being consumed, and food shortage will occur due to poisonous seafood. Due to these reasons, alternative ways or methods, which are more environmental friendly, must be developed to replace the roles of chemical-based reagents, and one of the methods is to replace the chemicals by applying biological enzymes.

Cold-active enzyme is a good candidate for replacing the old-fashioned enzyme, this is because in long term consideration, cold-active enzyme reduce energy consumption. As compared to most of the commercially available enzymes, cold-active enzymes can work efficiently at lower temperature, and are normally produced by psychrophile or psychrotroph. Besides, contamination rate can be decreased during the production as most of the contaminants are inactive at low temperature.

1.3 Research Objectives

I. To screen and select potential extracellular proteases producer from pre-isolated Arctic bacteria.
II. To study the relationship of the extracellular proteases production with respect to its bacterial growth.
III. To characterize the extracellular proteases of the selected bacteria.

1.4 Scope of Study

The scope of this study was to partial purify and characterize the crude proteases extracted from Arctic bacteria. First and foremost, the proteolytic activities of all the pre-isolated bacteria were screened through using qualitative analysis based on skim milk agar plate. Bacteria with high proteolytic activities were then selected and their extracellular proteases were subjected to production for further analysis. From here onwards, quantitative analysis (a standard protease assay) was used to quantify the proteolytic activities of the proteases. The characteristics of the proteases are examined based on their temperature optimum, pH optimum, effect of substrate concentration, thermostability and pH stability. The molecular weight of the proteases was also determined using both SDS-PAGE and zymography methods.

1.5 Significance of Study

The expected outcome of this study was to characterize the extracellular proteases with high proteolytic activity from Arctic bacteria. There are high chances

of isolating cold-active proteases in this research due to cold origin of the bacteria. This type of enzyme might have some potential applications, such as using them in detergent making, and might be able to overcome the limitations of the existing industrial enzymes system. The usage of cold-active enzyme might significantly reduce the energy usage in the industries, and directly affecting the process cost.

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