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ISOLATION OF PROTEASE FROM HALOTOLERANT BACTERIA AND ITS PURIFICATION BY USING AQUEOUS TWO-PHASE SYSTEM

BY

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ABSTRACT

Rising demands on proteases in various applications in industries encouraged the discovery of potent protease sources, especially from microorganisms. Proteases from fermented food have a potential to be used for industrial applications because they may produce halotolerant proteases. Halotolerant proteases can be used in the production of traditional fermented food to shorten the period of the fermentation process compared to the traditional method which took 4-6 months for natural fermentation. Moreover, high risks of protein degradation during purification due to a harsh downstream processing encourage the application of the aqueous two-phase system (ATPS) as a purification method for proteases.This study aimed to isolate and identify the bacteria strain that can produce proteases, and the aqueous two-phase system (ATPS) was applied as the protease purification method. Halotolerant bacteria were isolated from three types of protein-rich fermented food, which are fermented fish sauce (Budu), fermented fish (Pekasam) and fermented soybean (Taucu). The proteolytic activity was observed via a qualitative analysis using a skim milk agar plate. On the other hand, a quantitative analysis was performed on protease assay using casein as a substrate. Protease secreted by the isolated bacteria was concentrated using ammonium precipitation and further purified using PEG/sodium citrate system. Among the samples, only fermented fish sauce (Budu) showed a positive result with the presence of protease-producing halotolerant bacteria. The clear zone observed on skim milk agar indicated the ability of the bacteria to secrete proteolytic enzyme and degrade the casein into small fragments. B7 isolate was selected as the highest protease producer with a specific activity of (3.70±0.06 U/mg) and identified based on morphology, Biolog system and 16S rDNA sequencing. Besides, B7 isolate can also tolerate the presence of sodium chloride (NaCl) up to 10%. Hence, the B7 isolate is classified as moderately halotolerant bacteria. The results indicated that B7 isolate has a 98% similarity with *Bacillus amyloliquefaciens* subs. plantarum FZB42 strain. Thus, the B7 isolate was named *Bacillus amyloliquefaciens* B7 strain. The results of the Biolog analysis also confirm this result. The One factor at time (OFAT) design was employed to determine the central point for each ATPS parameter which resulted in the highest value of responses for enzyme activity, specific activity, and purification factor. Parameters involved in OFAT analysis are molecular weight of polyethelene glycol (PEG), type of salts, concentration of PEG and salt, pH and temperature. Optimization of ATPS conditions based on the face-centered central composite design (FCCCD) in response surface methodology (RSM) with 11 runs showed that the optimal conditions for ATPS are 27% (w/w) PEG 1500, 34% (w/w) sodium citrate, at pH 7 and a temperature of 35 ^{o}C. Analysis of variance (ANOVA) showed the coefficient with determination (R^2) were 0.9546, 0.9465 and 0.9465 for enzyme activity, specific activity and purification factor, respectively. Lastly, the molecular weight of the purified protease was identified as 40 kDA based on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. In conclusion, the isolation of *Bacillus amyloliquefaciens* B7 strain from Budu as a protease producer and the aqueous two-phase system as a suitable protease purification method show the great potential for advancement in the industrial enzyme.

ملخص البحث

شجّع تزايد الطلب على إنزيمات البروتييز في التطبيقات الصناعية اكتشاف مصادر أخرى لهذه الإنزيمات ، وخاصّة من الكائنات الحية الدقيقة. إنزيمات البروتييز المشتقة من الأغذية المخمرة لها تطبيقات صناعية عدة لقدرتها على إنتاج إنزيمات بروتييز هالوتوليرنت. يمكن استخدام البروتينات هالوتوليرنت في إنتاج المواد الغذائية المخمرة التقليدية لتقصير فترة عملية التخمير بالمقارنة مع الطريقة التقليدية التي تستغرق 4–6 أشهر للتخمير الطبيعي. علاوة على ذلك، فإنّ خطر تحلل البروتين خلال عملية تنقيته بسبب الظروف القاسية، أدّى إلى تشجيع تطبيق نظام التنقية المائي على مرحلتين (ATPS) كطريقة لتنقية البروتييز. لذلك تهدف هذه الدراسة إلى عزل وتحديد السلالة التي يمكن أن تنتج إنزيم البروتييز، ومن ثمّ تنقيته بواسطة النظام المائي على مرحلتين. تمّ عزل بكتيريا halotolerant من ثلاثة أنواع من المواد الغذائية المخمرة الغنية بالبروتين وهي صلصة السمك (Budu) ، الأسماك المخمرة (Pekasam) وفول الصويا المخمّر(Taucu) وقد أُجري التحليل النوعي لنشاط تحلل البروتين باستخدام أجار الحليب الخالي الدسم. من ناحية أخرى، تمّ إجراء التحليل الكمي لفحص البروتييز باستخدام الكازين. وتمّ تركيز إنزيمات البروتييز التي تفرزها البكتيريا باستخدام ترسيب الأمونيوم وتنقيتها باستخدام نظام سترات الصوديوم/PEG. من بين جميع العينات، أظهرت صلصة السمك المخمر (Budu) فقط، نتيجة إيجابية لوجود البكتيريا halotolerant المحللة للبروتين. لوحظت منطقة واضحة في أجار الحليب الخالي من الدسم مما يدل على قدرة البكتيريا على إفراز انزيم البروتييز وتحليل مادة الكازين إلى أجزاء صغيرة. تم اختيار سلالة B7 باعتبارها أعلى منتج للبروتييز مع نشاط محدد عند 0.06±3.70 وحدة/ملجم والتي تم تحديدها على أساس التشكل، اختبار الكيمياء الحيوية باستخدام نظام Biolog وتسلسل 16S rDNA. إلى جانب ذلك، فإنّ سلالة B7 أيضا يمكن أن تتحمل وجود كلوريد الصوديوم لنسبة تصل إلى 10% وبالتالي، تصنف البكتيريا على أنها halotolerantمعتدلة. وتشير النتائج إلى أن سلالة B7 تظهر نسبة 98% من التشابه مع البكتيريا العصوية *Bacillus amyloliquefaciens* متفرعة من سلالة plantarum FZB42. وبذلك سُميت سلالة B7 halotolerant ، *Bacillus amyloliquefaciens* B7 strain كما تأكد ذلك من دراسة Biolog. تمّ تطبيق دراسة عامل واحد في وقت واحد، (OFAT) لتحديد النقاط المركزية لعوامل ATPS الذي ينتج عنه أعلى قيمة من ردود نشاط الإنزيم، النشاط المحدد، وعامل التنقية. تضمنت العوامل المدروسة في OFAT الوزن الجزيئي لبولي إيثيلن الجلايكول (PEG) ، نوع الأملاح، وتركيز PEG والملح، ودرجة الحموضة ودرجة الحرارة. أظهر تحسين ظروف ATPS على أساس FCCCD في RSM مع 11 تجربة أنّ الظروف المثلى للتنقية ATPS عند: PEG 1500 27% (كتلة/كتلة)، سترات الصوديوم 34% (كتلة/كتلة)، درجة الحموضة عند 7.0 والحرارة 35 درجة مئوية. كما أظهر تحليل التباين (ANOVA) قيمة معامل التحديد R^2 كان عند 0.9546، 0.9465 و 0.9465 لنشاط الإنزيم، النشاط المحدد وعامل التنقية ، على التوالي. وأخيراً، فإن الوزن الجزيئي للبروتييز المنقى تحدّد عند 40 كيلو دالتون على أساس تحليل SDS-PAGE . كاستنتاج لمجمل الدراسة فإنّ عزل سلالة *Bacillus amyloliquefaciens* B7 من Budu كمنتج للبروتييز في وسط مائي على مرحلتين كطريقة لتنقية الإنزيم كطريقة ملائمة أظهرت إمكانات كبيرة للتقدم في صناعة الإنزيمات.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Science (Biotechnology Engineering).

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DECLARATION

I hereby declare that this dissertation is the result of my own investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APMSF	Amidinophenylmethane sulfonyl fluoride
ATPS	Aqueous two-phase system
BHI	Brain-heart infusion
BLAST	Basic Local Allignment Search Tool
BSA	Bovine serum albumin
DEAE	Diethyl amino ethyl
DFP	Diisopropyl fluorophosphates
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCCCD	Face centered central composite design
rDNA	Ribosomal deoxyribonucleic acid
DTT	Dithiothreitol
GRAS	Generally regarded as safe
LB	Luria Bertani
MEA	Malt extract agar
MW	Molecular weight
NaCl	Sodium chloride
NA	Nutrient agar
NCBI	National Center for Biotechnology Information
OFAT	One factor at a time
PCR	Polymerase chain reaction
PDA	Potato dextrose agar

PEG	Polyethylene glycol
PF	Purification factor
PMSF	Phenylmethane sulfonyl fluoride
RSM	Response surface methodology
SA	Specific activity
SDA	Sabourad dextrose agar
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TSA	Tryptone soy agar
Vr	Volume ratio

LIST OF SYMBOLS

g	Gram
h	Hour
l	Liter
kb	Kilo Bases
kDa	Kilo Dalton
kPa	Kilo Pascal
mg	Miligram
ml	Mililiter
min	Minute
nm	Nanometer
rpm	Revolutions per minute
sec	Second
%	Percentage
w/v	Weight over volume
v/v	Volume over volume
V	Voltan
^{o}C	Degree celcius
µl	Microliter
µmole	Micromole
µM	MicroMolar
U/ml	Units/mililiter

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Proteases are known as one of the important hydrolytic enzymes that catalyze peptide bonds of a long chain of proteins into short fragments such as amino acid (Jisha et al., 2013). The ability of proteases to degrade protein can be utilized widely in industrial applications which have attracted researchers to isolate the enzymes from different potential sources such as plants, animals and recently microorganisms (Gupta et al., 2002). Microbial proteases have been studied for the last fifty years whereas in the 1960s the use of popular alkaline proteases, subtilisin, which are mainly secreted from Bacillus genera (Okuda et al., 2013) are well-known in the detergent industry.

Nowadays, there are various applications of microbial enzymes in diverse industries such as the detergent industry, leather industry, food, and animal feed production, peptide synthesis, waste management as well as in therapeutic applications. In previous studies, microbial proteases were found and isolated from bacteria, fungi and yeast (Jones et al., 2007; Kulpreecha et al., 2009; Phetcharat & Duangpaeng, 2012; Sampaio et al., 2008; Sattayasamitsathi et al., 2011). Nowadays, few studies on traditional fermented food from other countries such as Thailand (Choorit & Prasertsan, 1992), India (Singh et al., 2014), Korea (Jeong et al., 2004), Japan (Sumi et al., 1987), and Nigeria (Oke & Onilude, 2014) have revealed that fermented foods are suitable sources in isolating protease-producing microorganisms and are beneficial for humans. For instance, proteases secreted by *Bacillus subtilis* ,known as Nattokinase, are isolated from natto (a traditional fermented food in Japan). This was the first success story of a microbial source of proteases from fermented

food that have specificity acts on fibrin which could help in reducing cardiovascular diseases. Thus, the study to discover isolated bacteria present in fermented food such as *Budu, Pekasam, Cencalok, Belacan* in Malaysia is potent to produce ideal proteases for industrial enzymes is encouraged. In addition, microbial resources from traditional fermented foods consumed all over the world for centuries are generally regarded as safe (GRAS) category and may reduce the potential of isolating bacteria that could produce toxins and are harmful for human consumption such as *Bacillus cereus* (Singh et al., 2014).

On the other hand, the enzyme industry also focused on the upstream and downstream processing of the proteases. In the large scale of enzyme productions, recovering of the enzymes from the fermentation broth is an important part and usually requires many steps resulting in a high tendency of enzymes denaturation. Moreover, downstream processing of enzymes consumes higher costs, especially during the purification stage. Nowadays, aqueous two-phase system (ATPS) has become the modern technique of purification replacing conventional methods such as chromatography (Yang & Ru, 1997), centrifugation, and membrane filtration. Studies conducted by de Silva et al., (2013); Ashipala & He, (2008) found that the production and optimization of protease activity in the aqueous two-phase system (ATPS) using PEG/salt system are indeed practical and cost effective.

1.2 PROBLEM STATEMENT

In recent years, recovery and purification of biomolecules such as enzymes often represents major challenges to the enzyme production process in terms of complexity and high cost (Glyk et al., 2015), which can make up more than 70 % of the total downstream processing cost (Raja et al., 2012). The recovery of the enzymes from

fermentation broth is an important part and usually involves many steps. In each step, a quantity of the target molecule is lost and the many steps involved result in a high tendency of enzymes denaturation. For example, the purification procedures such as chromatography, which needed multiple chromatography steps such as ion exchange, affinity, and gel filtration, are tedious, expensive process and often provide low yields (Arshad et al., 2014). Besides that, the long separation process in ultrafiltration also increases the production cost. The demand on the lower production cost encourages researchers to find efficient and low-cost extraction and purification methods. Hence in this study, the aqueous two-phase system (ATPS) is proposed as an alternative for purification methods. ATPS is a liquid-liquid extraction method formed by polymer/polymer or polymer/salt system. This method is simple, has a rapid separation with little denaturation, easy to scale up and an efficient method to separate various biological products such as enzymes, proteins, nucleic acids, antibiotics and antigens (Ratanapongleka, 2010). Polyethylene glycol (PEG) is used as one of the phase forming polymers in ATPS because it is available at a low cost and forms a two-phase system with other neutral polymers as well as salts (Raja et al., 2012), and it also reduces the potential of enzyme denaturation during the purification process as this method contains a high water content.

Futhermore, another way proposed in this study to reduce the risk of enzyme denaturation during enzyme production includes isolating the bacteria that can produce proteases from fermented food. The presence of a high concentration of NaCl (5-10%) in fermented food such as Budu, Pekasam and Taucu could help in isolating bacteria that can withstand the harsh environment especially in high salinity during enzyme processing.The protein rich fermented food in Malaysia such as fermented fish sauce (Budu), fermented fish (Pekasam) and fermented soybean (Taucu) have the

potential to possess microorganisms that could produce proteolytic enzymes because the main ingredient of the food is protein itself.

Interestingly, Zakaria et al., (2015), reported that fermented food products are a good source for the isolation of bacteria with serine protease enzymes including the strains of *Bacillus, Lactococcus, Lactobacilli, Bifidobacteria, Enterococcus, Pseudomonas, Streptococcus,* and *Pediococci* (Montville & Matthews, 2007). Peng et al., (2005) reported that serine proteases possessed a significant potential in reducing cardiovascular diseases, thus these microorganisms are a suitable thrombolytic agent. Moreover, contamination by other mesophilic bacteria (bacteria that could not live in the presence of NaCl) during enzyme production could be reduced. Thus, the isolation and identification of ideal bacteria that could produce active and stable proteases that can withstand harsh conditions of enzyme production from the protein rich fermented food source are explored in this study.

1.3 RESEARCH OBJECTIVES

This research consists of three specific objectives which are as follows:

i. To isolate and identify halotolerant bacteria producing protease from fermented food.
ii. To screen the parameters of ATPS for purification of protease secreted by isolated halotolerant bacteria.
iii. To optimize the ATPS conditions for protease purification.

1.4 RESEARCH METHODOLOGY

This research was started by isolating and screening the potential bacteria that can produce protease from fermented food. The protease assay was conducted by using qualitative and quantitative assays. For the qualitative assay, the sample of fermented food was spread on nutrient agar and the grown bacteria were further screened by streaking the bacteria on the skim milk agar. After that, the protease activity of the bacteria was screened using casein as a substrate in protease assay. Only one selected isolate was further identified by using a morphological test which involves gram staining and microscope observation. Other tests were employed to identify he phenotype and the name of the strain using 16S rDNA sequencing and phenotypic fingerprint, Biolog analysis.

After that, the selected bacteria was cultured in nutrient broth and the protease produced by the bacteria was concentrated using ammonium precipitation. Next, purification of the protease was carried out by aqueous two phase system (ATPS) using PEG/salt system. Six parameters of ATPS conditions were involved to identify the suitable conditions that could obtain a high protease activity. After that, the optimization of the ATPS conditions was evaluated by using Design Expert 6.0.11 software. Then, the purified protease was characterized by using SDS-PAGE analysis to identify the molecular weight of the protease.

1.5 SCOPE OF RESEARCH

The scope of the research is described below:

i. Isolation and screening of halotolerant bacteria producing proteases from protein- rich fermented food (budu, pekasam and taucu) using spreading and streaking techniques on nutrient and skim milk agar plates.

ii. Identification of the halotolerant bacteria strain produced the highest protease activity using morphological, biochemical and molecular analysis.

iii. Protease from B7 isolate is concentrated by ammonium precipitation followed with the purification by aqueous two-phase system (ATPS).

iv. Investigation on the effects of physical parameters is carried out by One Factor at Time (OFAT) method and the selected parameters are; molecular weight (MW) of PEG, type of salt, concentration of salt (w/w%), concentration of PEG (w/w%), temperature and pH on the enzyme activity of the purified protease enzyme.

v. Optimization of ATPS conditions based on two factors; pH and temperature, using the face centered central composite design (FCCCD) in response surface methodology (RSM) where the responses are the enzyme activity, specific activity and purification factor of protease.

1.6 THESIS ORGANIZATION

The thesis is divided and organized into five main chapters. Chapter 1 is an introduction of the research work including the problem statement, research objectives and a short description of the research methodology, and also the scope of the research. Next, chapter 2 provides a literature review on previous researches related to the protease enzyme and their applications in the industry. In addition, it also includes the methods involved to purify proteases in recent years. Then, chapter 3 briefly described the methodology involved in this research including the materials utilized and experimental procedures while chapter 4 reported all the results and discussion based on the findings obtained in this research. The final chapter provides a conclusion of the research and some recommendations for future works to improve the research.