UNIVERSITI PUTRA MALAYSIA

EXTRACTION, PURIFICATION AND CHARACTERIZATION OF POLYGALACTURONASE FROM DURIAN (Durio zibethinus L.) SEEDS

FARHANA AZMIRA BINTI ASMADI

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By

FARHANA AZMIRA BINTI ASMADI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

December 2017

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DEDICATION

This thesis is dedicated to my loving family: For my special person in my life, my father, Asmadi bin Mohamad Sapar and my mother, Suraya binti Mahmud, who have been my inspiration and my strength through all these years, always there for me, never out of reach whenever I needed them. Thank you for your love. To my dear siblings, I am grateful for what you are and have always been to me. To all my friends, my fellow colleagues and to whom I owe more than I can ever repay. Lastly, a very special credit for my dear supervisor, Associate Professor Dr. Mehrnoush Amid for all your care, support and believe in me.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

EXTRACTION, PURIFICATION AND CHARACTERIZATION OF POLYGALACTURONASE FROM DURIAN (*Durio zibethinus* L.) SEED

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FARHANA AZMIRA BINTI ASMADI

December 2017

Chairman : Associate Professor Mehrnoush Amid, PhD
Faculty : Food Science and Technology

Polygalacturonase breaks down pectin chains generally found in the cell wall of plant. Primarily, enzymes have high tendency to be degraded by improper extraction method. Therefore, it is essential to use an economical, simple and efficient extraction method. In this study, polygalacturonase (PG) was extracted from durian seed with ultrasound assisted-extraction. The effects of extraction time, ultrasound temperature, pH of buffer and solvent-to-seed ratio for optimization of the extraction were determined. The optimum combination of extraction was achieved at 30 min extraction time, 50°C temperature and 5:1 ml/g of solvent-to-seed ratio at pH 5.5. Conventional purification processes are multistep, tedious and expensive; thus, it is vital to develop an economical, highly efficient and environmental friendly process for the purification of polygalacturonase with required properties. A novel aqueous two-phase system (ATPS) process composed of surfactant and acetonitrile was employed to purify polygalacturonase from *Durio zibethinus* seed at laboratory scale. In this study, the effect of Tie Line Length (TLL), crude loads and pH on purification of the enzyme were investigated. The results of the ATPS process indicated polygalacturonase was partitioned in the novel method of ATPS composed of 23% (w/w) Triton X-100 and 19% (w/w) acetonitrile, at 55.6% of TLL (tie line length) crude load of 25% (w/w) at pH 6.0. It was determined that the phase components, Tie Line Length (TLL), crude loads and pH effected the polygalacturonase partitioning. This study also showed that ATPS can be used as an economical and effective method for purification of the enzyme from a novel source with potential industrial application and alternative to the conventional ATPS.

Characterization of the purified polygalacturonase was done to determine the polygalacturonase stability in vary conditions. In this study, it indicated that polygalacturonase extracted from durian seed was stable with the presence of some

metal ions, surfactants and oxidizing agents. The metals K^{+}, Mg^{2+}, Na^{+} and Cu^{2+} enhanced the polygalacturonase activity to 135.1%, 108.5%, 94.6% and 86.7% respectively. Meanwhile Zn^{2+}, Ca^{2+} and Fe^{3+} inhibited the enzyme activity to 72.9%, 49.3% and 14.1% respectively. Polygalacturonase showed high stability towards surfactants EDTA (108.1%) and SDS (101.6%). The polygalacturonase was stable in Triton X-100 (97.7%) and Tween 80 (92.5%) meanwhile almost half of the activity was inhibited by oxidizing agent to 66.7%. Based on SDS-PAGE, the estimated molecular weight of this was 34.4 kDa. Hence, as a conclusion, the enzyme with unique characteristics could be extracted and purified from natural source. It has high potential to contribute in some industrial applications such as food and beverages, textile, paper, and other biotechnological applications.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGEKSTRAKAN, PENULENAN DAN PENCIRIAN OF POLYGALAKTURONASE DARIPADA BIJI DURIAN (*Durio zibethinus* L.)

Oleh

FARHANA AZMIRA BINTI ASMADI

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Pengerusi : Profesor Madya Mehrnoush Amid, PhD
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Polygalacturonase menguraikan rantai pektin yang biasanya terdapat di dinding sel tumbuhan. Enzim mempunyai kecenderungan tinggi untuk direncatkan oleh kaedah pengekstrakan yang tidak sesuai. Oleh itu, adalah penting untuk menggunakan kaedah pengekstrakan yang mudah dan berkesan. Dalam kajian ini, polygalakturonase (PG) diekstraks daripada biji durian dengan bantuan ultrasonik. Efek masa untuk pengekstrakan, suhu ultrasonik, pH dan nisbah pelarut-ke-biji untuk pengoptimuman pengekstrakan telah ditentukan. Gabungan optimum pengekstrakan dicapai pada masa pengekstrakan 30 min, suhu 50°C dan 5: 1 ml/g nisbah pelarut-ke-biji pada pH 5.5. Proses penulenan konvensional adalah berbilang, rumit dan memerlukan kos yang tinggi. Oleh itu, adalah penting untuk inovasikan proses yang lebih ringkas, cekap dan mesra alam bagi penulenan polygalakturonase. Sistem Dua Fasa Berakua (SDFB) yang terdiri daripada surfaktan dan asetonitril digunakan untuk menulenkan polygalakturonase daripada biji durian pada skala makmal. Dalam kajian ini, kesan Panjang Garis Ikatan (PGI), beban mentah dan pH pada pemurnian enzim telah disiasat. Keputusan proses ATPS menunjukkan polygalacturonase dibahagikan kepada kaedah baru ATPS yang terdiri daripada 23% (w / w) Triton X-100 dan 19% (w / w) asetonitril, 55.6% TLL beban 25% (w / w) pada pH 6.0. Telah ditentukan bahawa komponen fasa, Panjang Garis Ikatan (PGI), muatan mentah dan pH mempengaruhi pengasingan polygalakturonase. Kajian ini juga menunjukkan bahawa SDFB boleh digunakan sebagai kaedah yang lebih ekonomik dan berkesan untuk penulenan enzim daripada sumber baru dengan aplikasi perindustrian yang tinggi potensi dan juga sebagai alternatif kepada SDFB konvensional. Pencirian polygalakturonase telah dilakukan untuk menentukan kestabilan polygalakturonase dalam pelbagai keadaan. Dalam kajian ini, ia menunjukkan bahawa polygalakturonase yang diekstrak daripada biji durian sangat stabil dengan kehadiran beberapa ion logam, surfaktan dan agen pengoksida. Logam K +, Mg2 +, Na + dan Cu2 + masing-masing meningkatkan aktiviti polygalakturonase kepada 135.1%, 108.5%, 94.6% dan

86.7%. Sementara itu Zn2 +, Ca2 + dan Fe3 + menghentikan aktiviti enzim sebanyak 72.9%, 49.3% dan 14.1%. Polygalakturonase menunjukkan kestabilan yang tinggi terhadap surfaktan EDTA (108.1%) dan SDS (101.6%). Polygalakturonase stabil bersama kehadiran Triton X-100 (97.7%) dan Tween 80 (92.5%) sementara hampir separuh daripada aktiviti itu dihentikan oleh ejen pengoksidaan kepada 66.7%. Berdasarkan SDS-PAGE, anggaran berat molekul ini ialah 34.4 kDa. Oleh itu, sebagai kesimpulan, enzim yang mempunyai ciri-ciri unik dapat diekstrak dan ditulenkankan daripada sumber semula jadi dah juga bahan buangan. Ia mempunyai potensi yang tinggi untuk menyumbang dalam beberapa aplikasi perindustrian seperti makanan dan minuman, tekstil, kertas, dan aplikasi bioteknologi yang lain.

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I certify that a Thesis Examination Committee has met on 15 December 2017 to conduct the final examination of Farhana Azmira binti Asmadi on her thesis entitled "Extraction, Purification and Characterization of Polygalacturonase from Durian (*Durio zibethinus* L.) Seed" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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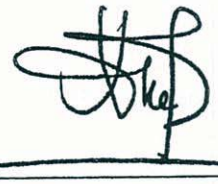

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Name of Member of Supervisory Committee: Nazamid bin Saari

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LIST OF ABBREVIATIONS

ATPS	Aqueous Two-Phase System
BSA	Bovine Serum Albumin
DNS	Dinitrosalicyclic acid
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacitic Acid
kDa	Kilodaltons
LSD	Least Significant Difference
Mt	Metric ton
MW	Molecular weight
PG	Polygalacturonase
RSM	Response Surface Methodology
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis
TCA	Trichloroacetic Acid
TLL	Tie Line Length

LIST OF NOMENCLATURE

A_T	Enzyme Activity in the Top Phase	U/mL
A_B	Enzyme Activity in the Bottom Phase	U/mL
K_e	Partition Coefficient of enzyme	-
K_p	Partition Coefficient of protein	-
P_F	Purification factor of enzyme	-
P_A	Protein concentration of enzyme in top phase	mg/mL
P_B	Protein concentration of enzyme in bottom phase	mg/mL
S	Specific Activity	U/mL
T_A	Total activity of enzyme	U/mL
T_P	Total protein of enzyme	mg
Y	Yield of enzyme	%

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Durio *zibethinus* is known as durian by most consumers in Asia and it is seasonal. Durian tree is currently grown widely all over Southeast Asia (Subhadrabandhu & Ketsa, 2001). A number of durian species are also edible, above all, D. *dulcis*, D. *graveolens*, D. *oxleyanus,* D. *kutejensis*, and D. *testudinarium* are distributed in local markets around Borneo. Durians are produced in the following countries consecutively; Thailand, Malaysia, Indonesia, Vietnam and Philippines.

Durian is usually consumed fresh; but, only one-third of durian can be consumed. The seeds (20–25%) and shell are usually discarded as waste. Durian seeds are highly nutritious and have high fiber content. Amiza *et al.*, (2007) demonstrated that durian seed could be used to produce several food products and used as a thickening agent. Thus, this fruit waste has major potential as a source of raw material useful for the growth of value-added products. Durian seeds can be used as a precious, economical and rich source of media to yield natural enzymes such as polygalacturonase and β-galactosidase

A complex polysaccharide known as pectin, was discovered primarily in the center of lamella and in the main cell walls of higher plants (Kashyap, Vohra, Chopra, & Tewari, 2001). It is part of 35% of main plants walls (Caffall, Pattathil, Phillips, Hahn, & Mohnen, 2009). Pectic substances are degrade by the method of de-polymerization, trans-elimination, or de-esterification with the help of a heterogeneous group of complementary enzymes known as pectolytic enzymes. They are grouped into exo-polygalacturonase and endo-polygalacturonase, pectin lyase and pectin methylesterase enzymes (Fontana & Silveira, 2012). Pectinases are significant enzymes used in the food industry, with 25% share in the world enzyme sales (Jayani, Saxena, & Gupta, 2005). Pectinases can be found in many organisms such as plants, fungi, and bacteria.

Polygalacturonase is a pectin-degrading enzyme complex; one of the richest pectinolytic enzymes. It is functional as a hydrolytic depolymerizing group helps in hydrolyzing polygalacturonic acid chains by addition of water (Schnitzhofer et al., 2007a). Polygalacturonase is the most researched and widely used pectinase in industry. It is used in several industrial and biotechnological processes, for example, fruit and vegetable enzymatic maceration to produce single-cell suspensions and to produce fruit nectars, vegetable purees and baby foods (Rojas *et al.*, 2011). Moreover, polygalacturonases are used to aid in the extraction of essential oils, coffee and pigments. In addition, they are also used to treat indigestion problems in the veterinary field (Palanivelu, 2006). Thus, it is vital to discover new polygalacturonases,

especially in food waste, and optimized their production conditions to meet the growing demand. Moreover, there are some advantages of using agro-industrial residues to produce such as, reduces pollution and produces high-value added products with an economical method (Ruiz, Rodríguez-Jasso, Rodríguez, Contreras-Esquivel, & Aguilar, 2012).

Nowadays, there are several extraction methods was introduced and established for the extraction of plants active components, including ultrasonic-assisted extraction (UAE), enzymatic-assisted extraction (EAE), supercritical fluid extraction (SFE) and dispersive liquid-liquid microextraction (Hardlei, Morkbak and Nexo, 2007; Yang *et al.*, 2009; Campillo *et al.*, 2013;). Conversely, ultrasonic-assisted extraction is the most rapid and effective extraction method. The acoustic cavitation generated in the solvent by the ultrasound wave pathway could enhance ultrasonic extraction (Ghafoor *et al.,* 2009; Zou *et al.,* 2011). A mechanical effect produced by an ultrasound could cause solvent to penetrate highly into the tissue and increase the area of contact surface between the solid and liquid phase. The extraction process can also be further improved by disrupting the cell wall and releasing cellular materials (Vilkhu, Mawson, Simons, & Bates, 2008).

Generally, purification process depends on polymer/salt system, for instance polymer/polymer, or a polyethylene glycol (PEG)/potassium phosphate system, and PEG/dextran (Goja *et al.*, 2013). However, these traditional aqueous two phase systems have some disadvantages including slow separation, polymers prohibitive cost, difficulties in separating the purified bio-molecules from the polymer as well as phase-forming chemicals are ineffectively to be recycled; causing large chemical/polymers usage and large production costs (Yau et al., 2015). Moreover, these traditional systems require monotonous operations like ultra-filtration, diafiltration and crystallization to remove the phase-forming chemicals/polymers from the recovery of desired proteins. Ideally, an ATPS need to be tremendously cost-effective, eco-friendly and able to maintain the biological activity of enzymes more than the traditional ATPS.

Response surface methodology (RSM) is a group of mathematical and statistical methods that depend on the fit of empirical models (Quiroz-Reyes *et al.*, 2013). In RSM, the effect of independent variables on response variables have to be optimized by thorough experimental design. It also involves an assemblage of techniques for investigating for optimum conditions through experimental methods and has been recognized as an essential technique of statistical design. It is a valuable for design of experiment, procedure of optimization and for data analysis (Morshedi & Akbarian, 2014). RSM is very crucial in the application of development, designing and new scientific formulation such as in industrial, clinical, biological science, food science, social science and physical and engineering sciences (Zhi, Song, Ouyang, & Bi, 2005).

1.2 Problem Statement

Enzymes could be affected to a greater extend by the changes in extraction condition including temperature, time, pH and solvent to sample ratio. Nevertheless, enzyme extraction should be advantageous if it is based on the natural morphology of enzyme which could be destroyed by the undesirable extraction condition. The most popular separation method used in the purification of protein products is chromatography.

Various conventional methods of purification of polygalacturonase have been employed. According to Biazus *et al.*, (2006), prior to chromatography, the crude feedstock of traditional adsorption chromatography or conventional adsorbents need to be clarified. Crucially, solid impurities must be removed from the feedstock as trapping of solid matter could lead to serious operational problems. Even though the possibility of chromatography to provide high selective separation levels in the recovering targeted molecules process, yet due to high cost of purification process, it could demonstrate prohibitive (Clonis, 2006).

Currently, the products of protein including enzymes are high in demand and developing in the market. Hence, it is important to focus on the total cost of the purification process and other succeeding related steps of enzymes. Though, these procedures were multi-step, discontinues, as well as time and labor consuming which could cause significant product loss (Y.-Y. Zhang & Liu, 2010). Nowadays, industry requires fast and cost-effective downstream processes for protein purification, in addition those that provide products with high yield and purity (Guptra *et al*., 2002).

1.3 Significance of Study

Durian growth has been remarkable worldwide, yet, due to its overproduction, the wastage increases. According to Ho and Bhat, (2015), durian has many advantages, values and useful components but it is not currently being utilized commercially. This could lead to pollution and increment in the waste treatment cost (Negro, Tommasi, & Miceli, 2003). Durian seed is normally thrown making it as an agro-industrial residue. The seeds make up to around 5-15 % of the total fruit mass. However, till present, there is no research on the potentiality of durian seed as a source of producing enzymes.

The objective of this study was to establish a successful operational condition for extraction of polygalacturonase from durian seed by reducing the denaturing possibility of the desirable enzyme. There is a need for the development of a rapid and easy process of polygalacturonase purification to improve total yield and purity. Furthermore, scaling up this process should be simple and a continuous steady state. Fundamental findings emphasis on the effects of storage conditions on the activity and stability of the target durian seed-based enzyme and indicate the best method for keeping polygalacturonase active and stable during storage until used in industry. The

novelty of this study is the extraction and purification of polygalacturonase as an important enzyme from waste (durian seed) at elevated level of purification factor and yield by an easy scale-up and rapid processing at low material cost, while benefitting from low interfacial tension and a mild environment.

1.4 Objectives of Study

In this study, the general objective was to study the extraction, purification, and characterization of polygalacturonase from durian (Durio *zibethinus*) seed. The specific objectives of this study were:

1. To establish the optimum condition for extraction of polygalacturonase from durian seed
2. To develop the purification procedure for production of polygalacturonase from durian seed
3. To characterize of polygalacturonase enzyme from durian seed

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