

UNIVERSITI PUTRA MALAYSIA

EXTRACTION, PURIFICATION, MICROENCAPSULATION AND CHARACTERIZATION OF LIPASE FROM PUMPKIN (CUCURBITA MOSCHATA DUCHESNE EX POIR.) SEED

MUHAINI BINTI MOHD HUSSIN

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

December 2016

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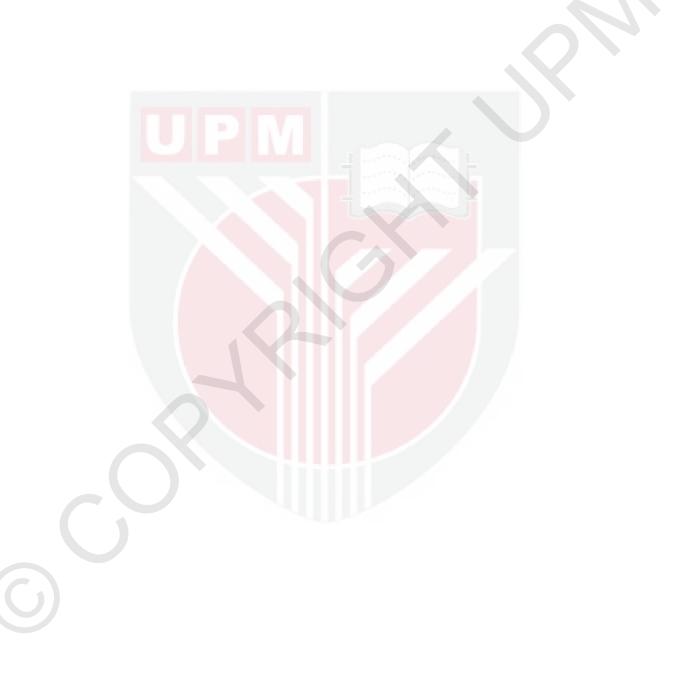
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DEDICATION

I dedicate this thesis to the love of my life... mak, abah and Hanafi..... thank you for being there even in my darkest time...



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

EXTRACTION, PURIFICATION, MICROENCAPSULATION AND CHARACTERIZATION OF LIPASE FROM PUMPKIN (Cucurbita moschata DUCHESNE EX POIR.) SEED

By

MUHAINI BINTI MOHD HUSSIN

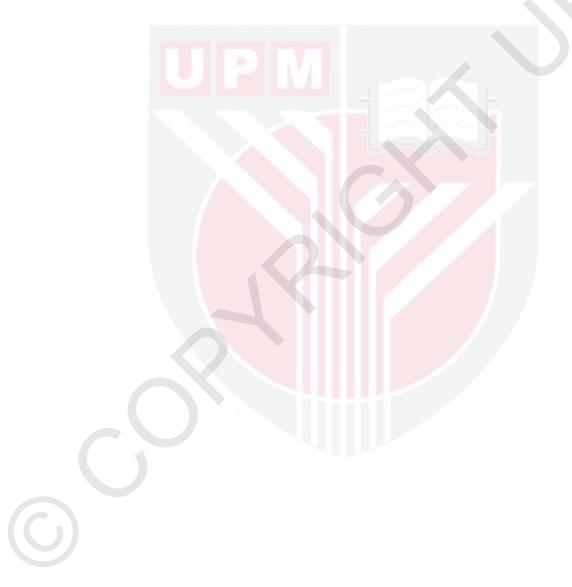
December 2016

Chairman: Associate Professor Dr Mehrnoush Amid,PhDFaculty: Food Science and Technology

Lipase is an enzyme with the presence of hydrolases act on ester bonds of triacylglerols. Most of the enzymes are easily degradable when expose to multistep process and expensive such as conventional purification. Hence it is important to establish and develop simple, low cost and environmental friendly system that could produce lipase to be used in industries such as food, detergent, pharmaceutical, biofuel industries. The pumpkin seed constitutes 30-37% of the whole pumpkin possesses valuable enzyme. Hence, pumpkin seed can be a potential novel source for the valuable and economical natural enzyme such as lipase. In this study, lipase was extracted from pumpkin (Cucurbita moschata) seed and the effects of the main factors affecting enzyme extraction namely, temperature, extraction time, pH of buffer, and buffer to sample (B/S) ratio were investigated for the development of the ultrasound-assisted extraction method. Optimum extraction condition was achieved at 5.5:1 (w/w) B/S ratio, 45 mins extractiong time, temperature 80 °C and pH of buffer 8.0. The yield of the enzyme extracted was 80.1%. Subsequently, the potential pplication of novel aqueous two-phase system (ATPS) composed of Triton X-100 nd xylitol in the purification of lipase from pumpkin seed crude was demonstrated t laboratory scale. In this part of the study, the effect of the main important parameters (such as volume ratio, crude load and pH) on purification of the enzyme was investigated. Optimum condition for purification of lipase from pumpkin seed was obtained After that, optimized extracted sample was purified using aqueous twophase system composed of 22% (w/w) and 25% (w/w) xylitol at 56.2% of tie line length (TLL) and 25% crude at pH 8.0 in order to obtain the purified enzyme. Based on the results it was demonstrated that the temperature TLL, volume ratio, crude load, and pH of buffer influenced the lipase partitioning. In ATPS, it was found that the molecular of lipase was estimated to be 39.2 kDA. Microencapsulation was performed using freeze-drying found that yield of freeze-dried in the trehalose (2%) and Arabic gum (5%) increased to 97.3% \pm 0.3. It was found that during storage encapsulated lipase is stable by 95.2% ±0.1. The immobilized lipase was stable at



 80^{0} C compared to the free enzyme, was around 50^{0} C. Characterization of the purified enzyme showed that lipase from pumpkin seed is stable in the presence of metal ions, surfactant and oxidizing agents. The lipase was stable 80^{0} C and pH 8 was found to be its optimum pH. The enzyme showed highest residual lipse activity on calcium chloride (CaCl₂) and EDTA. Whereas, in substrate specificity, 4-nitrophenyl palmitate showed highest enzyme activity compared to corn oil, olive oil, soybean oil, and palm oil. It can be concluded that the valuable enzyme with unique characteristics from a rich, natural and cost-effective source could be made available for use in different types of industries such as food, detergents and also in biotechnological applications.



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

PENGEKSTRATAN, PENULENAN, PEMIKROKAPSULAN DAN PENCIRIAN ENZIM LIPASE DARI BIJI LABU (Cucurbita moschata DUCHESNE EX POIR.) SEED

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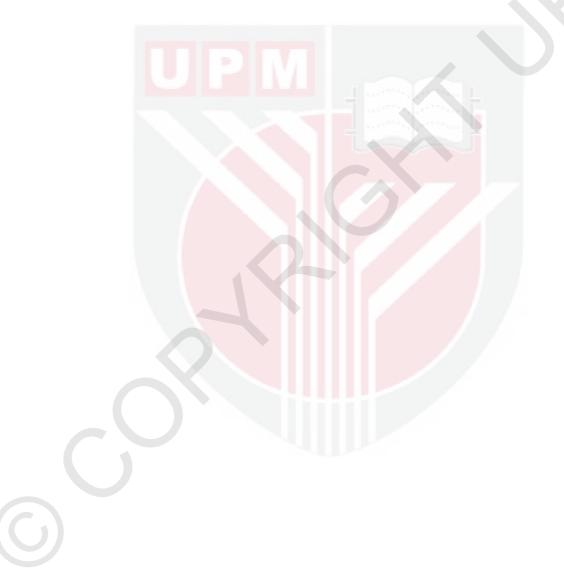
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Pengerusi: Profesor Madya Dr Mehrnoush Amid,PhDFakulti: Sains dan Teknologi Makanan

Lipase merupakan enzim yang berhidrolas bertindak atas bon ester. Kebanyakan enzim terurai apabila terdedah kepada proses berperingkat dan mahal seperti penyucian konvensional. Oleh itu kaedah mudah, kos rendah dan mesra alam boleh menghasilkan lipase yang akan digunakan dalam industri seperti makanan, bahan pencuci, farmaseutikal, industri biofuel. Biji labu merupakan 30-37% daripada keseluruhan labu mempunyai enzim berharga. Oleh itu, biji labu boleh menjadi sumber novel yang berpotensi untuk enzim semula jadi yang berharga dan ekonomi seperti lipase. Lipase telah diekstrak daripada biji labu (Cucurbita moschata) dan kesan faktor utama yang menjejaskan pengeluaran enzim iaitu, suhu, masa pengekstrakan, pH, dan penampan untuk mencuba nisbah telah disiasat untuk pembangunan kaedah pengekstrakan ultrasound bantuan itu. Keadaan pengeluaran yang optimum dicapai pada 5.5: 1 (b/b) B / nisbah S, masa 45 minit pengekstratan, suhu 80 °C dan pH penampan 8.0. Hasil enzim yang diekstrak adalah 80.1%. Selepas itu, permohonan potensi novel akueus sistem dua fasa (ATP) terdiri daripada Triton X-100 dan Xylitol dalam penulenan lipase dari mentah biji labu telah ditunjukkan pada skala makmal. Di bahagian ini, kajian, kesan parameter penting utama (seperti suhu, beban mentah dan pH) kepada pembersihan enzim itu disiasat. keadaan optimum untuk penulenan lipase daripada biji labu telah diperolehi Selepas itu, sampel diekstrak dioptimumkan telah disucikan menggunakan akueus sistem dua fasa terdiri daripada 22% (w/w) dan 25% (w/w) xylitol pada 56.2% daripada tali leher panjang talian (TLL) dan 25% mentah pada pH 8.0 untuk mendapatkan enzim yang tulen. Berdasarkan keputusan itu telah menunjukkan bahawa suhu TLL, nisbah jumlah, beban mentah, dan pH dipengaruhi pembahagian lipase. Dalam novel akueus sistem dua fasa, didapati bahawa molekul lipase dianggarkan 39.2 KDA. Pemikrokapsulan dilakukan dengan menggunakan beku-pengeringan mendapati bahawa hasil beku-kering menggunakan trehalose (2%) dan gum Arabic (5%) meningkat kepada 97.3% \pm 0.3. Ia telah mendapati bahawa semasa penyimpanan



terkandung lipase adalah stabil dengan $95.2\% \pm 0.1$. Lipase stabil pada 80° C berbanding enzim percuma, adalah sekitar 50° C. Pencirian enzim yang tulen menunjukkan bahawa lipase daripada biji labu adalah stabil di hadapan kakisan, surfaktan dan agen pengoksidaan. Lipase itu 80° C stabil dan pH 8 didapati pH optimum. Enzim ini menunjukkan aktiviti lipse sisa tertinggi kalsium klorida (CaCl₂) dan EDTA. Manakala, dalam substrat kekhususan, substrat 4-nitrophenyl palmitate menunjukkan aktiviti enzim tertinggi berbanding dengan minyak jagung, minyak zaitun, minyak kacang soya dan minyak sawit. Dapat disimpulkan bahawa enzim berharga dengan ciri-ciri unik dari sumber yang kaya, semula jadi dan kos efektif boleh disediakan untuk digunakan dalam pelbagai industri seperti makanan, bahan pencuci dan juga dalam aplikasi bioteknologi.



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I certify that a Thesis Examination Committee has met on 23 December 2016 to conduct the final examination of Muhaini binti Mohd Hussin on her thesis entitled "Extraction, Purification, Microencapsulation and Characterization of Lipase from Pumpkin (*Cucurbita moschata* Duchesne ex Poir.) 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.

Members of the Thesis Examination Committee were as follows:

Russly bin Abdul Rahman, PhD Professor Faculty of Food Science and Technology Universiti Putra Malaysia (Chairman)

Anis Shobirin binti Meor Hussin, PhD Associate Professor Faculty of Food Science and Technology Universiti Putra Malaysia (Internal Examiner)

Alfi Khatib, PhD Associate Professor International Islamic University Malaysia (External Examiner)

NOR AINI AB. SHUKOR, PhD Professor and Deputy Dean School of Graduate Studies Universiti Putra Malaysia

Date: 28 February 2017

The thesis submitted to the Senate of University Putra Malaysia has been accepted as fulfillment of the requirement for the degree Master of Science. The members of the Supervisory Committee were as follows:

Mehrnoush Amid, PhD

Associate Professor Faculty of Food Science and Technology Universiti Putra Malaysia (Chairman)

Mohd Yazid Manap, PhD

Profesor Institut Penyelidikan Produk Halal Universiti Putra Malaysia (Member)

Badlishah Sham Bahrin, PhD

Associate Professor Faculty of Food Science and Technology Universiti Putra Malaysia (Member)

ROBIAH BINTI YUNUS, PhD Professor and Dean

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Signature:	
Name of Chairman	
of Supervisory	
Committee:	Associate Professor Dr. Mehrnoush Amid
	PM
Signature:	
Name of Member	
of Supervisory	
Committee:	Professor Dr. Mohd Yazid Manap
Signature:	
Name of Member	
of Supervisory	
Committee:	Associate Professor Dr. Badlishah Sham Bahrin

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LIST OF ABBREVATIONS

ATPS	Aqueous Two Phase System
BSA	Bovine Serum Albumin
p-NPP	4-nitrophenyl palmitate
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacitic Acid
Mt	Metric ton
MW	Molecular weight
RSM	Response Surface Methodology
SD	Standard Devation
SDS- PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis
ТСА	Trichloroacetic Acid
TLL	Tie Line Length

	centimeter
:	gram
I	molarity
1	milligram
I	milliliter
1	millimeter
I	millimole
	micromillimeter
UPN	nanometer
	kilovolt
	Unit
	volume
	weight

NOMENCLATURE

A_{T}	Enzyme activity in top phase	U/mL
A_B	Enzyme activity in bottom phase	U/mL
K _e	Partition Coefficient of enzyme	-
K _p	Partition Coefficient of protein	-
\mathbf{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

Pumpkin is derived from the genus *Cucurbita* of the family *Cucurbitaceae* and is grown in the tropical and sub tropical regions. Pumpkin is processed into byproducts such as fried, frozen, candied, dried or pickled (Mayor *et al.*, 2007) which have gained attention and popularity not only from the consumer but also from the manufacturer. In Malaysia, *C.moschata* is the most common pumpkin and it is known as *Labu manis* in Malay language. *C.moschata* was selected for extraction and purification of lipase in this study.

Lipase acts as a catalyst during hydrolysis of triacylglycerols which release fatty acids and glycerols. Lipase is important as it involves in a number of reactions such as esterification, interesterification, acidolysis and aminolysis and making it the most versatile biocatalyst (Pandey *et al.*, 2010). Besides that, lipase is characterized with its ability to operate in mild conditions. It also possesses unique specificities that direct the reaction course towards a desired product (Villeneuve, 2003). The lipase market has been growing rapidly and up to this date, its is increasing 8% annually and in the future it is expected to reach 30 billion Euros (Villeneuve, 2003). Therefore, it is very important to establish an alternative especially from different natural source such as plant seed.

The conventional classical extraction methods such as soxhlet extraction and solvent extraction have many disadvantages, one of them is the requirement of several hours of contact times (Albu *et al.*, 2004). Application of ultrasound in extraction is an alternative to overcome this drawback as it is proven to give greater impact in extraction process. The efficiency of extraction is increased by ultrasound due to cavitation (Vilkhu *et al.*, 2008).

Generally, the ultrasound assisted extraction (UAE) of enzymes is followed by conventional purification steps. This two-step process makes the downstream processing consumed up to 50% to 80% of the final cost of the industrial products (de Brito Cardoso *et al.*, 2014). Therefore, an alternative system is used to simplify downstream processes and remove clarification and desalting steps. Based on this description, aqueous two-phase system (ATPS) was used to purify lipase from pumpkin (*C.moschata*) seed.

ATPS has been an attractive technique for purification and recovery of biomolucles namely proteins (Asenjo and Andrews, 2012), enzymes (Barbosa *et al.*, 2011), nucleic acids (Luechau *et al.*, 2009), and other compounds such as alkaloids (Passos

et al., 2013) and antioxidants (Reis *et al.*, 2012). In the past decades, ATPS was formed by combination of two polymers (dextran/polyethylene glycol) (Antov, 2004) or by combination of polymer-salt (PEG/phosphates, sulphates, or chlorides) (Zhao *et al.*, 2011). However, this conventional ATPS requires additional steps such as ultrafiltration, diafiltration and crystallization to eliminate phase-forming elements from the targeted biomolecules (Amid et al., 2015). To improve this conventional ATPS, an economical and environmental friendly method of ATPS with ability to retain enzyme biological and chemical activity is introduced.

Microencapsulation is a technique used for protection, isolation and controlled release (Anjani *et al.*, 2007). Freeze drying is a technique to encapsulate enzyme as it dehydrate all heat-sensitive material (Ezhilarasi *et al.*, 2013). It is important to use and choose stabilizers to coat the enzyme to minimize the risk of deactivation and destabilization of enzymes (Ezhilarasi *et al.*, 2013).

1.2. Statement of Problem

Lipase is easily degraded under extreme pH, temperature and exposure to industrial chemicals which leads to changes in its natural morphology. Another challenge in utilizing enzyme in industries is during its purification and recovery stages where the protocols involved increase the costs of final product by 60-90% and decrease the yield of desired sample (Barbosa *et al.*, 2011) At least four chromatographic steps are required to determine the purity of lipase (Palekar *et al.*, 2000). The current conventional purification processes are basically multistep, with discontinuous stages and above all are time and labor consuming which lead to higher cost and decrease the storage of the enzyme. Enzymes are very sensitive and for that there are many factors responsible for its instability and inactivation such as exposure to pH, temperature, binding of metal ions and oxidative stress. These factors could lead to decrease in the lipase activity and stability (Simpson, 2010).

1.3. Significance of Present Study

Brian (2008) reported that approximately 5,500 metric tonnes of pumpkin are generated in food processing industry. The increased demand of pumpkin and its co-products will shoot up the crop's utility and versatility (Aziah and Komathi, 2009). Hence, the pumpkin crop versatility and profitability could be expanded by diversifying its use and utilizing its agricultural by-product waste, seed (Hameed and El Khaiary, 2008).

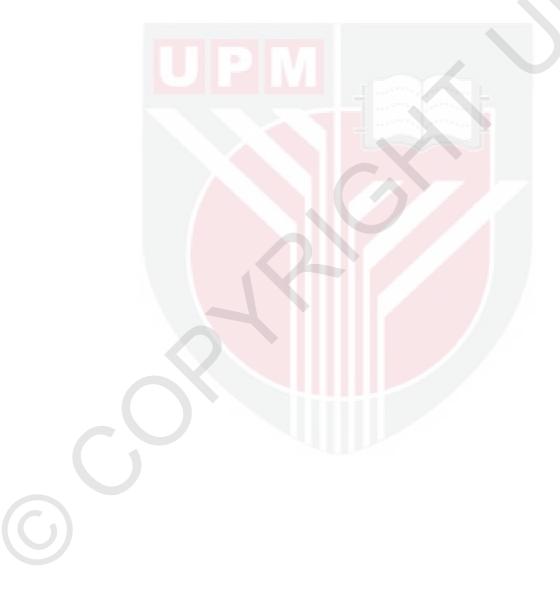
There is an urgency to develop a relatively fast and cheap process for purification and recovery of the lipase with a high yield and purity to meet the industrial requirement. The important findings that will be investigated is the effects of storage conditions on activity and stability of the pumpkin seed- based enzyme and obtain the best technique to maintain the lipase activity and stability during storage until further use in industry.



1.4 Objective of Study

The aim of this research are:

- 1. To optimize extraction condition of lipase from pumpkin (*Cucurbita moschata*) seed using Ultrasound Assisted Extraction (UAE)
- 2. To develop and optimize the purification procedure of lipase from pumpkin (*Cucurbita moschata*) seed using Aqueous Two Phase System (ATPS)
- 3. To microencapsulate lipase pumpkin (*Cucurbita moschata*) seed using Freeze-Drying Method
- 4. To characterize enzymatic properties of Lipase from pumpkin (*Cucurbita moschata*) seed.



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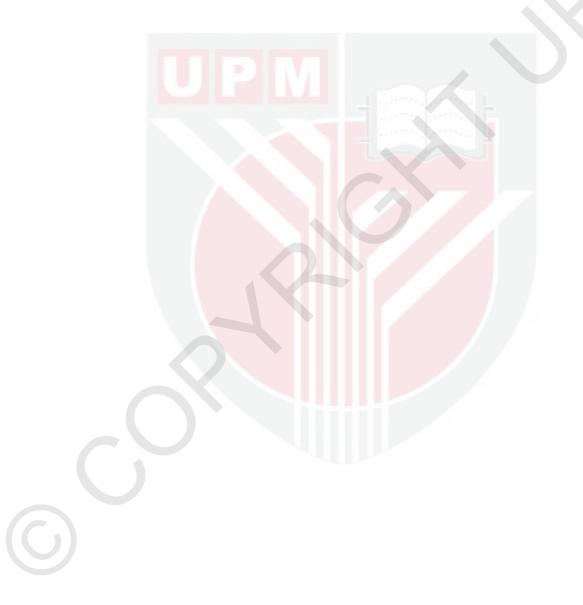
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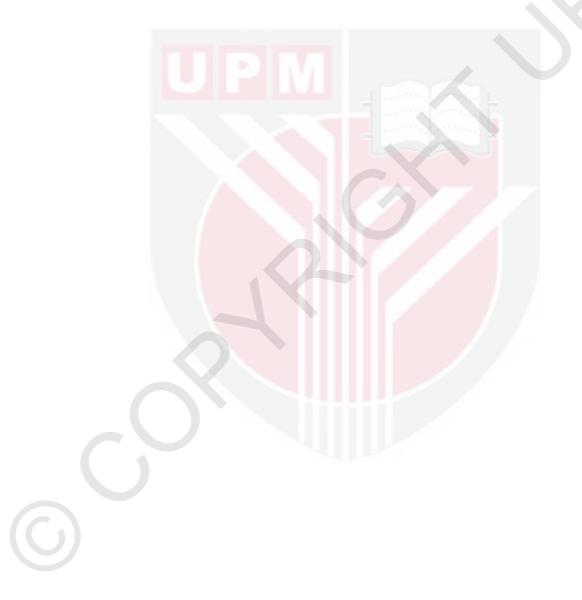
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PUBLICATION

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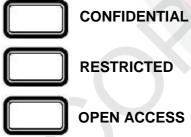
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