IMMOBILIZATION OF *CANDIDA RUGOSA* LIPASE ON POLYMER SUPPORT FOR MONOACYLGLYCEROL PRODUCTION AS HALAL EMULSIFIER

BY

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A thesis submitted in fulfilment of the requirement for the degree of Master of Science
(Halal Industry Science)

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JULY 2018

ABSTRACT

Halal emulsifier has a very high demand in the Halal industry market. The production of emulsifier using enzyme-based strategies provides an alternative solution for conventional emulsifier production. Due to the instability of biocatalyst, immobilization technology has been found to be a powerful tool to improve the performance of enzymes. The aim for this research is to optimize the immobilization conditions for *Candida rugosa* lipase attachment on poly (glycidyl methacrylate)-grafted-polyethylene/polypropylene microfibrous sheet (PGMA-g-PE/PP) through covalent bonding. A pretreatment was carried out on the polymer prior to immobilization and the amine group density on the chemically modified PGMA-g-PE/PP microfibrous sheet was found to be 3.33 mmol/g. The chemically modified microfibrous sheet was characterized by Fourier-transform infrared spectroscopy (FTIR-ATR) and field emission scanning electron microscope (FESEM). Response surface methodology (RSM) was applied to model and optimize the immobilization settings represented by three factors including immobilization time (2-6 h), pH (pH 7-9) and enzyme/support ratio (5.0-9.0 mg/cm^2). A well-correlated significant model (*p*-value = 0.0003) was determined for the residual activity of the immobilized lipase (R^2 = 0.9136). The enzymatic activity on *p*-nitrophenyl palmitate (*p*NPP) substrate achieved optimum under the conditions of 4.24 hrs, pH 8 and 8.51 mg/cm^2 ratio of enzyme/support. The optimal reaction temperature and pH value in enzymatic reaction for both free and immobilized lipase were found to be 45 ^{o}C at pH 7 and 55 ^{o}C at pH 6, respectively. The pH endurance, storage and thermal stability of the immobilized lipase were remarkably enhanced. The immobilized lipase can be readily recovered and more than 50% of its activity was retained following 10 cycles. The kinetic parameters study showed that both V_{max} and K_M values were decreased from 0.16 mM/min and 4.69 mM to 0.15 mM/min and 2.86 mM, respectively after immobilization. Lastly, the immobilized lipase was evaluated for monoacylglycerol emulsifier production from Kenaf seed oil. Result showed that monoacylglycerol was detected by GC-TOF/MS. The results of this study suggested that the PGMA-g-PE/PP microfibrous sheet is a promising polymer support for enzyme immobilization with potential for Halal emulsifier production.

خلاصة البحث

الطلب على المستحلبات الحلال كبير جدًا وهو في تزايد وتنامي مستمر في السوق من قبل الصناعات الحلال. إن إنتاج المستحلب باستخدام الإنزيمات ليباز يوفر حلاً بديلاً للإنتاج التقليدي للمستحلب. ونظرًا لعدم استقرار المحفز الحيوي في الصناعة، تم العثور على تكنولوجيا التثبيت لتكون أداة قوية لتحسين أداء الإنزيمات. والهدف من هذا البحث هو تحسين ظروف تثبيت إنزيم ليباز المنتج بواسطة الفطر *Candida rugosa* المرتبطة (بعديد غليسيديل ميثاكريلات) المدعمة علي رقائق من عديد الايثيلين وعديد البروبلين (PE/PP-g-PGMA) من خلال الترابط التساهمي كيميائياً. تم إجراء المعالجة المسبقة على البوليمر قبل التثبيت وقدرت كثافة المجموعة الأمينية على رقائق عديد الايثيلين وعديد البروبلين (PE/PP-g-PGMA) المعالجة كيميائياً فوجد أن الكثافة كانت 3.33 مليمول/جم. وقد تم تشخيص الرقائق المجهرية بواسطة جهاز مطياف الأشعة تحت الحمراء (FTIR-ATR) وأيضا بالمجال الميداني لانبعاثات المجهر الإلكتروني (FESEM). تم تطبيق برنامج الحاسوب منهجية سطح الاستجابة (RSM) على النموذج وعلى تحسين إعدادات التثبيت التي تمثلها ثلاثة عوامل، والتي تشمل زمن التثبيت (6-2 ساعة)، ودرجة الحموضة المتوسطة (الرقم الهيدروجيني 7-9)، ونسبة انزيم/دعم (5.0-9.0 ملجم/سم^2). تم تحديد نموذج كبير مرتبط بشكل جيد بقيمة (p-value = 0.0003) للنشاط المتبقي من الليباز المدعم (R^2 = 0.9136). حقق النشاط الأنزيمي على مادة بالميتات البارانيترو فينول (*p*NPP) في ظل الظروف الأمثل التي وجدت من التجربة العملية وكانت 4.24 ساعة، ودرجة الحموضة 8 ونسبة 8.51 ملجم /سم^2. تم العثور على درجة حرارة التفاعل المثلى وقيمة الرقم الهيدروجيني في التفاعل الإنزيمي لكل من إنزيم ليباز الحر والمدعم أي المثبت فكانت 45 درجة مئوية عند درجة الحموضة 7 و55 درجة مئوية عند درجة الحموضة 6، لكل من الإنزيم الحر والمثبت على التوالي. وقد تم تعزيز التحمل للرقم الهيدروجيني، والتخزين والاستقرار الحراري لليباز المثبت بشكل ملحوظ. يمكن استعادة الليباز المثبت لأكثر من 50٪ من نشاطه بسهولة ويسر. وتم الاحتفاظ بإعادة نشاطه بعد 10 دورات. وأظهرت دراسة المعلمات الحركية أن كلا من قيم V_{max} و K_M انخفضت من 0.157 مليمتر/دقيقة و4.686 مليمتر إلى 0.148 مليمتر/دقيقة و2.861 مليمتر، على التوالي بعد التثبيت. وأخيرا، تم تقييم الليباز المثبث لإنتاج مستحلب اسيل الجليسرول الأحادي (MAG) من زيت بذور الكناف. وأظهرت نتيجة التحليل الكشف الموجب للمركب اسيل الجليسرول الأحادي بواسطة GC-TOF/MS. وتشير نتائج هذه الدراسة إلى أن رقائق عديد الايثيلين وعديد البروبلين (PE/PP-g-PGMA) المجهرية تعبر دعامة واعدة لتثبيت إنزيمات الليباز مع إمكانية إنتاج المستحلبات الحلال من الزيوت النباتية.

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 thesis for the degree of Master of Science (Halal Industry Science).

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, 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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For the faith of Islam, my beloved parents, Akbar Tajudin and Hasna Merican, family, lecturers and friends.

ACKNOWLEDGEMENTS

In the name of Allah, The Most Gracious, The Most Merciful.

Alhamdulillah, praise be to Allah, the Almighty and All-Knowing, for giving me strength and guidance to complete this study and Salam to His messenger, Prophet Muhammad S.A.W.

Special appreciation to my supervisor Assoc. Prof Mohamed Elwathig Saeed Mirghani and co-supervisors Assoc. Prof Dr. Ma'an Fahmi Al Khatib and Prof. Hamzah Mohd. Salleh for their motivations, guidance, assistances, thoughtful ideas and great patience throughout this study. Their concerns, encouragement and comments are highly appreciated and will be deeply treasured.

My dedication and gratitude to my beloved parents: Hj Akbar Tajudin and Hjh Hasna Merican and my siblings for all their support, love, patience and motivation that have been given to me throughout my journey as a postgraduate student. Their loving kindness and prayers have been driving me to success. A note of thank to my colleagues and beloved friends for their helps and motivation.

A very sincere gratitude I bid to INHART and International Islamic University Malaysia (IIUM) for providing me with all the necessary instruments and facilities for the project. Special acknowledgement to the Ministry of Higher Education for the financial support for research under Fundamental Research Grant Scheme (FRGS). Last but not least, thank you to those who have contributed directly or indirectly to this project. May Allah bless all of your good deeds and *nawaitu*. *Jazakumullahu khairan kathira*.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
DAG	Diacylglycerol
DEA	Diethylamine
EU	European Union
FAME	Fatty Acid Methyl Ester
FCCCD	Face-Centered Central Composite Design
FESEM	Field Emission Scanning Electron Microscope
FTIR-ATR	Fourier-Transform InfraRed Spectroscopy
GC-TOF/MS	Gas Chromatography-Time of Flight-Mass Spectrometry
hrs	Hours
GMA	Glycidyl Methacrylate
GRAS	Genetically Recognized As Safe
K_M	Michaelis Constant
MAG	MonoAcylGlycerol
min	Minute
MUFA	MonoUnsaturated Fatty Acid
PBUH	Peace Be Upon Him
PEG	PolyEthylene Glycol
PE/PP	PolyEthylene/PolyPropylene
PGMA-g-PE/PP	Poly (Glycidyl MethAcrylate)-grafted-PolyEthylene/PolyPropylene
*p*NPP	*para*-NitroPhenyl Palmitate
PUFA	PolyUnsaturated Fatty Acid
RIG	Radiation Induced Grafting
RSM	Response Surface Methodology
SWT	Subhanahu Wa Taa'la
TAG	TriAcylGlycerol
V_{max}	Maximum rate

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

The increase of Muslim population in the world today has urged the production of halal food products. The demand for halal food product is immensely growing as Muslims today are more aware of the importance of halal products in their daily life. Food is said to be closely related to human life and its consumption is essential in providing nutrients to the human body. Food industry consists of a complex, global collective business which contributes to being one of the biggest supplies in the markets around the globe. With the percentage of about 61.3% Muslim in Malaysia, the demand for halal food is accelerating to a bigger number. There are about 16 million Muslim consumers with age ranging from 15-64 years, who have the buying power, are looking for foods which complies with Islamic requirements. However, currently, the local Halal industry is contributing less than 2% of total gross domestic product but it is expected to increase to about 5.8% by 2020. Therefore, Malaysia Halal food sector is now becoming a strong economic force locally and globally (Said et al., 2014). As Muslims are bounded by regulations set by Allah SWT, halal authenticity in the food industry is very crucial in the choice of food produced. Looking at this as an opportunity, Malaysia has developed an aspiration to become a global halal hub and this is clearly outlined in the Third Industrial Master Plan (IMP3) and the Halal Industry Master Plan launched in 2006 and 2008 (retrieved on September 13, 2017:

http://www.miti.gov.my/). To make this a reality, Malaysia is aiming to increase the production of local food as well as developing and promoting its halal food industry to the bigger world (Syed et al., 2012).

The halal food industry is of vital importance to the Muslim worldwide to ensure cleanliness, hygiene and harmless effects to their health and well-being in anything that are purchased, used and consumed. Halal status is able to be the benchmark in term of the quality and safety of a product referring specifically to the permitted products by *shari'ah Islamiyah* (Bohari et al., 2013). In maintaining the halal status of any food product, every aspect of their production needs to be in accordance with halal law. This is based on the Quranic verse**:** "O you People! Eat of what is on earth, Halal and Pure, and do not follow the footsteps of the Satan; Indeed, for he is to you an open enemy (Quran 2:168)".

Food emulsifier is a type of food additive which acts as an interface between the conflicting components of food such as water and oil. Having two elements of the nonpolar hydrophobic region and polar hydrophilic region, its function is to mix both parts of the food together (Hasenhuettl, 2008). With these unique properties, the emulsifier is usually used in the food industry to provide desired texture, mouthfeels and other organoleptic properties in a food product. This food additive is usually added together with any addition of fats into the water which helps in reducing the interfacial tension between the two immiscible liquids, water and oil. The addition of emulsifier is essential in the formation of a more stable and uniform homogenous dispersion. The examples of foods with emulsifier are cakes, biscuits, pastries and margarine. However, the emulsifier which is normally represented by the E- Numbers in food label raises the question of its halal status due to the unknown source of its raw materials (Rahman & Manaf, 2014). The fats and oils

sources which made up the emulsifier can be derived from plants and animal materials. Although plant-based raw materials are preferred, there has always been a possibility for fraudulent practices to include those derived from animal source as raw materials or during processes (Nasyrah, 2012). The doubt on the product appears when animal sources are being used in emulsifier production in which there may be possibility that lard fat is chosen as it provides better choice in term of texture. In fact, even if halal animals were used, it is needed to ensure that they are slaughtered according to *shari'ah*. Therefore, the source of the halal authenticity of emulsifier is ambiguous to Muslim consumers except by referring to its original sources and process of production such as the sources of enzyme used.

Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods which deteriorate the final products. Enzymes are highly specific, efficient and act as 'green' catalysts which accelerate any chemical reactions it undergoes. However, their use is very limited in term of stability and reusability, thus causing an increase in the production cost (Zhang et al., 2015). Immobilization technology is seen to be a new light to overcome the stability, reusability, purification and handling issues of the free enzyme. The enzyme attached to the polymer allows the product to be easily purified in the downstream process and increase the recovery efficiency (Yuce-dursun et al., 2016). This method is known to be very economical especially in a continuous process of production (Alkhatib et al., 2012). The ability of the enzyme to be reused with long reactivity and stability has gained popularity over the use of normal free enzymes. The choice of polymer is crucial in determining the effectiveness of the immobilized enzyme. Lipases from several animal and microbial sources have been immobilized by many different methods and support materials. However, applying functionalized PE/PP

microfibrous matrix, a cheap and abundant polyolefins, is likely to impart various desirable properties to lipase immobilization including high feed diffusion rate and fast kinetics compared to the conventional substrates. This is due to the large surface area and void volumes as well as chemical inertness of PE/PP microfibrous sheets.

1.2 PROBLEM STATEMENT

Enzyme interactions with materials are of important interest in laboratory and industry, especially in food production. However, like other enzymes, the use of soluble lipase causes limitation in terms of low stabilities over wide industrial parameters including pH and temperature, non-reusability, prohibitive cost and the difficulty of separation from products (Kuo et al., 2012; Ma et al., 2016). In order to expand their applications, enzyme needs to be studied to maintain its stability and reusability for continuous usage.

The increasing demand for lipase in the industrial sector has forced this research to be conducted in finding the solution to the limitation problems of the soluble enzyme which is actively being used in industry. Besides, the use of reusable biocatalyst may also give a tremendous decrease in the environmental problems that are mainly generated from industrial chemical wastes. Thus, immobilized enzymes promote a green solution for industrial processes conditions and offer great solutions for other limitations as well.

The production of monoacylglycerol (MAG) used in emulsifiers derived from fats and oils usually involves a continuous process of transesterification in the presence of inorganic alkaline catalysts under a nitrogen atmosphere at a very high temperatures (220-250 °C). The products formed gives low yield, burnt taste and dark-colored products

(Kaewthong & H-Kittikun, 2004). Currently, lipase-catalyzed transesterification is being studied as possible alternatives in producing MAG replacing the conventional method. This is due to the higher yields recovered with much milder reaction conditions giving products with better quality and low energy cost (Rosu et al., 1997). Production of MAG which generally recognized as a form of emulsifier causes doubtful status on its halal authentication due to the raw materials and enzymes used in the process of generating the products. Therefore, this works attempts to solve the problem by providing the clear status of raw materials and process involved in maintaining the status of emulsifier produced by biocatalyst. The choice of polymer matrix for enzyme immobilization is essential in determining the characterization of its stability and reusability and thus poly (glycidyl methacrylate)-grafted-polyethylene/polypropylene microfibrous sheet was used for this research work in producing halal emulsifier.

1.3 RESEARCH OBJECTIVES

The main aim of this research is to generate immobilized lipase on poly (glycidyl methacrylate)-grafted-polyethylene/polypropylene (PGMA-g-PE/PP) microfibrous sheet for the production of halal emulsifier.

1. To optimize the parameters; time, pH and enzyme-to-support ratio for lipase immobilization on PGMA-g-PE/PP
2. To characterize and evaluate the performances of the immobilized lipase in terms of the optimum activities, stability, reusability and kinetic parameters studies.
3. To produce monoacylglycerol using immobilized lipase and Kenaf seed oil.

1.4 RESEARCH SCOPE

The research investigated the production of MAG by enzymatic transesterification approach using immobilized *Candida rugosa* lipase after determining the optimized immobilized conditions and its properties. This research is important considering the benefits it offers in the propensity of immobilized enzymes-catalyzed transesterification to replace chemical transesterification. The use of conventional method causes a reduction in the overall quality of the product formed. Besides, the growing concern of halal authenticity of emulsifier available in the market raises many doubtful questions.

To achieve this proposition, *Candida rugosa* lipase was selected to optimize the immobilized conditions using a face-centered central composite design (FCCCD) of response surface methodology (RSM) by Design Expert software. The research utilized commercially available *Candida rugosa* lipase enzyme which is the common lipase used in industry and one of the most widely studied enzymes (Trbojević Ivić et al., 2016). The lipase produced from microbe provides a halal source and can be considered as generally recognized as safe (GRAS). The method used for immobilization is covalent bonding. Several factors were manipulated during immobilization which are time, pH and enzyme-to-support ratio. The properties of immobilized lipase were determined based on their optimum activities temperature and pH, thermal, pH and storage stability, reusability and kinetic parameters. The immobilized lipase was finally tested on its application to produce MAG by lipase-catalyzed transesterification method for lab scale.

1.5 OUTLINE OF THE THESIS

This thesis consists of five chapters. CHAPTER ONE of the thesis presents the general background of the research work, discussing the objectives devised for the achievement of the research methodology and the scope of the research. CHAPTER TWO presents the review of the findings based on available materials which were found on lipase enzymes, immobilization on different polymers, different methods of immobilization, transesterification using conventional and enzyme-based and various findings considered variables and properties which affect the yields of the products. In CHAPTER THREE the methodology employed in the study was presented and discussed in detail including the materials used and the analytical procedure adopted for the results analysis. CHAPTER FOUR is a presentation of the experimental results and analysis method adopted with discussion explaining and comparing from previous literatures. CHAPTER FIVE, a concise conclusion based on the results and analysis presented were discussed and some recommendations were highlighted.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

Food emulsifier is a type of food additives that are essential in food products. Their importance in the food industry has allowed many researches to be conducted to synthesize it in the most efficient way. Food emulsifier is conventionally being synthesized using a catalyst which requires high temperature giving negative effect to the end product. To overcome these drawbacks, enzymatic catalysis is selected to be the method of choice because of their high competency, specificity, selectivity, mild reaction conditions and environmental-friendly process (Rueda et al., 2015). However, they have several limitations in terms of its stability and reusability which could be further improved through immobilization. Several studies applied enzyme immobilization in order to allow a continuous product formation. However, the best technique to improve enzyme performance is yet to be studied. In this study, an improved methods and conditions to immobilize *Candida rugosa* lipase on a novel PGMA-g-PE/PP microfibrous sheet polymer was employed. With the fact that Muslims were mandated to consume foods that are halal according to *shari'ah*, halal status of food product has become a major concern among Muslims. This research fills the void in producing halal source emulsifier using the most efficient method of immobilized enzyme. The optimization of immobilization conditions

allows further improvement of enzyme loading capability and efficiency in term of its enzyme activity and therefore increasing the yield of the products.

In this chapter, the immobilization technology was discussed briefly, especially on the polymer type and attachments. All the factors which could influence the performance of immobilization were further discussed. The characterization of the immobilized enzyme including their pH and temperature at optimum and stabilities, reusability and kinetic studies were reviewed. The method of emulsifier production, the chosen raw materials and analysis of detection were also discussed.

2.2 HALAL CONCEPT

The definition of halal is that which is permitted, with respect to which no restriction exists and the doing of which the Law-Giver, Allah has allowed. In contrast, haram or the prohibited or unlawful is defined as that which the Law-Giver has absolutely prohibited and anyone who engages in it is liable to the punishment of Allah in the Hereafter as well as a legal punishment in this world (Al-Qaradawi, 1980). Other terms worth mentioning are *masbooh* (doubtful), *makrooh* (dislike), *mustahab* (recommended) and *mubah* (allowed). Many people have the misconception that halal is related only to food while in reality, halal encompasses every aspect of life such as cosmetics, business dealings, clothing and a complete way of life. The status of halal in every aspect of life is very important in Islam as it would affect the Muslim life as a whole. It is mandatory for a true Muslim to only eat, deal and use what is permissible according to what has been mentioned in the Quran and the Hadith (sayings) of Prophet Muhammad (peace and blessings of Allah be upon him, "PBUH"). The concept of *toyyib* is also incorporated with halal which means