IMMOBILIZATION OF *CANDIDA RUGOSA* LIPASE ON *ZEA MAYS L.* HUSK LEAF ACTIVATED CARBON FOR HYDROLYSIS AND ESTERIFICATION REACTION

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Especially to my beloved parents and family, my supervisor and co-supervisor, fellow friends and lab partners for their encouragement, guidance and assistance
In the name of Allah, the most Gracious and the Most Merciful, Alhamdulillah, all praises to Allah for the strengths and His blessing in completing this thesis. It would not be successful without Allah, who bless me with good health and guides me in every ways in completing the research project.

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Last but not least, million thanks goes to my beloved parents, for their trust, support, love, prayer and encouragement. Thank you very much.
Poor management of the generated waste from the by-products from agricultural land and commercial food industries have contributed to increased ecological burden. The potential of waste biomass in current agriculture practice is not fully utilize the biomass, and left to decompose in field or are burned. Therefore, development of technologies that fully utilize these wastes is, therefore, necessary. In this study, Zea mays L. husk leaves (ZHL) were chemically activate using phosphoric acid (H₃PO₄) under activation temperature of 500°C to obtain ZHL activated carbon (ZHLAC). In order to enhance the immobilization of enzyme by covalent bonding, surface functionalized ZHLAC were prepared using ethylenediamine and glutaraldehyde to increase the functional group on support surface. The biocatalyst study of CRL-FZHLAC using FTIR, TGA and Nitrogen Adsorption revealed CRL were successfully bound to the surface of the FZHLAC support via imine bond formed through a Schiff base mechanism. Thermogravimetric analysis revealed that CRL-FZHLAC was successful prepared with an enzyme loading of 12 % (v/v). The effectiveness of CRL-FZHLAC in enzymatic reaction by hydrolysis of olive oil was performed and optimized under various conditions of temperature, pH of solvent buffer, stirring rate and reusability. Subsequently, enzymatic synthesis of butyl butyrate was also optimized under various conditions of temperature, molar ratio acid/alcohol and stirring rate. Maximum activity of CRL-FZHLAC for hydrolysis (71.24 µmol/min/g) was achieved under an optimized condition of 3 h, 50°C, 200 rpm at pH 8. Under optimum condition [3 h, 40°C, molar ratio of acid/alcohol of 1:2 and 200 rpm], the lipase successfully synthesize 87% of butyl butyrate as compare to 62.9% by the free CRL [3 h, 40°C, molar ratio of acid/alcohol of 1:2 and 200 rpm]. CRL-FZHLAC was reusable for up to 5 cycles the hydrolysis of olive oil and 7 cycles the synthesis of butyl butyrate. In short, it was concluded that AC obtained from waste ZHL was suitable as a raw material to prepare a highly functional FZHLAC. Activity of CRL-FZHLAC was improved to produce high yield of both synthesis of olive oil and butyl butyrate. Thus, the development CRL-FZHLAC was a possible practice in increasing the efficiency of hydrolysis and esterification reaction.
ABSTRAK

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LIST OF ABBREVIATIONS

CRL - *Candida Rugosa* Lipase
ZHL - *Zea May L.*
ZHLAC - *Zea May L.* Activated Carbon
FZHLAC - Functionalize *Zea May L.* Activated Carbon
CRL-FZHLAC - *Candida Rugosa* Lipase- Functionalize *Zea May L.* Activated Carbon
FTIR - Fourier Transform Infrared Spectroscopy
TGA - Thermogravimetric Analysis
XRD - X-ray Diffraction
OVAT - One Variable at-a-Time
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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

The expansion of agricultural land to grow food in meeting the demands of global population has resulted in new environmental challenges. Such drastic change has been mainly attributed to the production of large amounts of agricultural biomass (Owolabi et al., 2017). Biodegradable waste of biomass origin forms the most abundant untapped natural resources on earth. However, when by-products of such waste, albeit from industrial or agricultural activities, are not managed properly, the liberated substances eventually become an ecological burden (Demir et al., 2015). The ecological stress is further heightened when some farmers in certain regions clear up large agricultural lands using the ‘slash and burn’ technique. Although the technique is relatively simple to execute, it can lead to widespread reduction in air quality along with elevated health issues (Islam et al., 2016). In this situation, efforts into developing technologies that fully utilize these unwanted biomass, transforming the wastes into commercially-functional products warrants attention from the scientific community. Studies have shown that aside to improving the way of life, the utilization of unwanted biomass can promote sustainability and alleviate existing pollutions by reducing the rate of waste disposal (Jalil et al., 2012; Demir et al., 2015; Owolabi et al., 2017).
In this perspective, this study was focused on using the lignocellulosic materials from agricultural biomass of *Zea mays* L. (maize) leaf husks (ZHL). Such biomass is available throughout the year, generated in large plantations and as wastes from commercial food industries (Jalil *et al.*, 2012). In Malaysia, cash crops (maize, groundnuts, sugar cane, cassava, yam, sweet potato and yambean) dominate approximately 22.98% of agriculture production, in which 25% are originates from maize (*Zea mays L*). Besides, statistics by Department of Agriculture of Malaysia have shown that maize production have increased for up to 3.8% from 2011 to 2015 (*Department of Agriculture* Putrajaya, Malaysia 2015). However, while ZHL biomass is produced in large quantities, it is typically discarded or left to decompose in fields. Therefore, the full technological potential of this biomass is not fully explored and utilized to its maximum. A matter of fact, the carbon rich ZHL is potentially an excellent source of untapped advanced carbon materials (Gao *et al.*, 2016). Previous studies have shown that plant wastes, such as that from coconut shell, rice husks and the leaves or husks of bamboo are carbon-rich materials that can be fashioned into an array of advanced carbon-based composites suitable for technological applications. Activated carbon derived from *Zea mays* L. is described to possess inherent physicochemical advantages *viz.* a high surface area and porous structure, as well as a
high degree of surface reactivity (Chenenmatchaya et al., 2014; Gao et al., 2016). These properties allow the activated carbon to be manipulated by altering their activation parameter (e.g., type of activation, activating agent, activation/pyrolysis temperature and sequence, and the ratio of impregnation) to produce a plethora of porous structures (Hadi et al., 2015).

Enzyme supports fabricated from biomaterials have attractive and potential applications largely due to their biodegradability, renewability, low cost and low carbon dioxide release (Elias et al., 2017). Herein, this study proposes the preparation of a support consisting of chemically-functionalized activated carbon derived from Zea mays L. leaf husks (FZHLAC). The process of chemical-assist surface functionalization on ZHLAC was crucial to improve its biocompatibility as the support to immobilize Candida rugosa lipase (CRL). Chemical activation is the preferred technique in this study to activate ZHLAC as it has been proven promising and gave rise to new types of supports exhibiting exceptionally high specific surface area (Ros et al., 2006; Hadi et al., 2015). Surface activation of ZHLAC have been widely describe to enhance the capacity of the support to accept higher loadings of protein materials (Ehrhardt et al., 1989; Ros et al., 2006). Moreover, the highly porous nature of activated carbon increases activity and the stability of enzymes to function under extreme conditions of pH, temperature and pressure (Furegon et al., 1997; Marzuki et al., 2015). In our case, it can favorably lead to a more stable and rigid structure of the immobilized CRL, and potentially increase the operational stability of the lipase for extended usages (Marzuki et al., 2015b). Other benefits also include facile recoverability and reusability of the biocatalyst (Mohamad et al., 2015a; Marzuki et al., 2015; Manan et al., 2016; Isah et al., 2017) and potential cost savings when used in large-scale manufacturing processes (Rani et al., 2000).

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1.2 Problem Statement

Considering that ZHL is constantly produced as an agricultural waste and the biotechnological potential of this biomass is not fully explored, its utilization for producing a value-added product i.e. support for CRL immobilization appears feasible and commercially attractive. Moreover, the cost for large-scale production of commercial activated carbons is very expensive (Safa *et al*., 2007; Cronje *et al*., 2011). Activated carbons developed from low cost raw biomaterials (Dias *et al*., 2007) i.e. ZHL may prove attractive as a cheaper alternative. Moreover, the existing chemical activation technique used to produce activated carbon is far from being eco-friendly as well as require a complicated and a costly synthetic route (Kumar *et al*., 2016; Yorgun & Yildiz., 2015). In this regard, the protocol to prepare activated carbon using ZHL biomass in this study is potentially more sustainable to overcome the abovementioned drawbacks.
Assessment on the feasibility of CRL-FZHLAC as biocatalyst was focused on synthesizing butyl butyrate as current attempts to produce high yield of the ester has been problematic. Furthermore, activated carbon prepared from ZHL as support for CRL immobilization and subsequently used for such reaction, remains unreported. ZHL was chemically activated and converted to activated carbon before undergoing surface functionalization to introduce active sites to covalently attach the CRL via ethylenediamine and glutaraldehyde. The use of crosslinkers, ethylenediamine and glutaraldehyde on FZHLAC support can increase the number of functional groups on the surface as well as favorably altering its stability and mechanical properties (Ramani et al., 2012). The method developed here is more eco-friendly and would complement existing technologies for preparing commercial activated carbons. It is hypothesized that the covalent attachment of CRL onto FZHLAC may improve biocompatibility of FZHLAC to receive CRL and increase structural integrity of the CRL, potentially improving rate of hydrolysis of olive and yield of butyl butyrate.

1.3 Objectives

The objectives of this study are:

i. To immobilize CRL onto FZHLAC supports.

ii. To characterize the morphological properties of CRL-FZHLAC.

iii. To optimize CRL-FZHLAC for the hydrolysis of olive oil and esterification synthesis of butyl butyrate and assess the stability of CRL-FZHLAC.
1.4 Scope of Study

The scope of this project involved preparation of activated carbon from ZHL husk leaf using phosphoric acid as the activating agent to afford ZHLAC. This is followed by the covalent immobilization of CRL onto the surface of FZHLAC using glutaraldehyde as the crosslinker.

The study subsequently assessed the morphological characteristics of the ZHLAC, FZHLAC and CRL-FZHLAC by Fourier Transform Infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), Nitrogen adsorption, and X-ray diffraction (XRD). In order to check the surface area and crystallinity of the sample of sample, nitrogen adsorption and XRD was used.

The following part of the study is the optimization of the CRL-FZHLAC for the hydrolytic reaction of olive oil emulsion and the esterification synthesis of butyl butyrate using the OVAT method. The parameters evaluated were temperature, stirring rate, pH and molar ratio of the substrates. The reusability and thermal stability of the CRL-FZHLAC were also established.

1.5 Significances of Study

The protocol for the development of FZHLC support from ZHL for immobilization of CRL may prove useful for future utilization of the support for immobilization of other types of enzymes, and not just lipases like CRL. Immobilization of CRL onto functionalized FZHLAC can improve the physico-chemical and catalytic properties of the enzyme. Most importantly, the study offers information on how the highly porous and rich surface groups (Zhang et al., 2012; Kennedy et al., 2007) of FZLAC can improve stability and activity of CRL for two
important industrial reactions catalyzed by lipases (Kahveci et al., 2012; Salihu et al., 2013).
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