Candida rugosa LIPASE SUPPORTED ON SILICA-COATED MAGNETITE NANOPARTICLES FOR HYDROLYSIS OF OLIVE OIL

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DEDICATION

This dissertation is dedicated to my own sake, my husband, parents and family.

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ABSTRACT

Oil palm leaves (OPL) has high content of silica (SiO_2). SiO_2 has a high surface area and large pore volume which could reduce the aggregation of magnetite (Fe_3O_4). The coating of the superparamagnetic Fe_3O_4 was to enable easy separation from the reaction mixture. SiO_2 extracted from OPL was coated on Fe_3O_4 followed by functionalization of 3-aminopropyltriethoxysilane (APTES) and activation of glutaraldehyde to prepare a nanosupport (G-AP-SiO_2-Fe_3O_4) for immobilisation of *Candida rugosa* lipase (CRL). The feasibility of the biocatalyst (CRL/G-AP-SiO_2-Fe_3O_4) has yet to be tested in aqueous environment. In this research, the CRL/G-AP-SiO_2-Fe_3O_4 was used to determine the optimum condition for hydrolysis of olive oil. The kinetic and thermodynamic properties of the CRL/G-AP-SiO_2-Fe_3O_4 was investigated for the hydrolysis of olive oil. The study first characterised the components and the treated OPL, whereby data of the thermal gravimetric analysis (TGA) indicated that the hemicellulose and lignin components in OPL were successfully reduced by acid treatment and calcination. The morphological and physiochemical facets of the extracted SiO_2 were investigated by fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD) and thermogravimetry analysis-differential scanning calorimetry (TGA-DTG). The results revealed that SiO_2 was successfully extracted from OPL and coated on the Fe_3O_4. Subsequently, it was activated by APTES and glutaraldehyde to yield CRL/G-AP-SiO_2-Fe_3O_4. FTIR, XRD and TGA-DTG data showed that CRL was successfully immobilised on G-AP-SiO_2-Fe_3O_4, as seen with the band arising at 1639 cm^{-1} by C=O and C=N stretching in FTIR. Specifically, immobilisation of CRL onto the G-AP-SiO_2-Fe_3O_4 yielded an enzyme loading and specific activity of 14.7 mg/g and 183 U/g. The CRL/G-AP-SiO_2-Fe_3O_4 was then tested to establish the optimal conditions for catalysing hydrolysis of olive oil. It was found that the optimal conditions for the reaction that yielded the best activity were enzyme loading of 1.00 mg/mL, incubation temperature of 40 °C, pH 8.0, ratio of olive oil: water of 2.5:1, and an agitation speed of 200 rpm. Assessments of thermal stability showed that CRL/G-AP-SiO_2-Fe_3O_4 was more resistant to thermally-induced denaturation, than free CRL. The CRL/G-AP-SiO_2-Fe_3O_4 was kinetically shown to have higher affinity towards the substrate (Michaelis-Menten constant, K_m = 0.583 g/mL) but catalysed at a lower maximum rate of reaction (V_{max} = 833.3 μmol/ml.min) as compared to free CRL (K_m = 6.00 g/mL, V_{max} = 3330 μmol/ml.min), respectively. The thermodynamic parameters based on values of half-life ($t_{1/2}$ = 38.94 min), D-value (129.4 min), thermal deactivation energy (E_d = 112.90 kJ/mol), standard enthalpy of deactivation ($\Delta H_d°$ = 110.10 kJ/mol) and Gibbs free energy ($\Delta G_d°$ = 11.32 kJ/mol) for CRL/G-AP-SiO_2-Fe_3O_4 conclusively showed that the lipase was appreciably more thermostable than free CRL ($t_{1/2}$ = 23.89 min, D-value = 79.67 min, E_d= 93.3 kJ/mol, $\Delta H_d°$ = 87.5 kJ/mol and $\Delta G_d°$ = 9.8111 kJ/mol) at 60°C. The finding shows that SiO_2 extracted from OPL could be coated on Fe_3O_4 to be used as an inorganic support for enzyme immobilisation. The results thus demonstrated that the CRL/G-AP-SiO_2-Fe_3O_4 biocatalyst was a potential candidate for catalysing hydrolytic reactions with good reaction rates, thus envisaging its prospective application as a commercially relevant biocatalyst.

ABSTRAK

Daun kelapa sawit (OPL) mempunyai kandungan silika (SiO_2) dan luas permukaan yang tinggi yang boleh mengurangkan pengagregatan magnetit (Fe_3O4). Penyalutan Fe_3O_4 yang superparamagnetik ialah bagi membolehkan pemisahan lebih mudah daripada campuran reaksi. SiO_2 yang diekstrak dari OPL dilapisi pada $F_{e3}O_4$ diikuti dengan fungsionalisasi 3-aminopropiltrietoksisilana (APTES) dan pengaktifan glutaraldehid untuk menyediakan-penyokong nano (G-AP-SiO_2-Fe_3O_4) bagi pemegunan lipase *Candida rugosa* (CRL). Kemampuan pemangkin lipase yang dipegunkan ini (CRL/G-AP-SiO_2-Fe_3O_4) masih belum diuji dalam persekitaran berair. Di dalam kajian ini, CRL/G-AP-SiO_2-Fe_3O_4 digunakan untuk menentukan keadaan optimum untuk hidrolisis minyak zaitun. Ciri-ciri kinetik dan termodinamik CRL/G-AP-SiO_2-Fe_3O_4 disiasat untuk hidrolisis minyak zaitun. Kajian pertama mencirikan komponen dan OPL yang dirawat, di mana data analisis TGA menunjukkan bahawa komponen hemiselulosa dan lignin dalam OPL telah berjaya dikurangkan dengan rawatan asid dan kalsinasi. Sifat morfologi dan fisiologi dari SiO_2 yang diekstrak disiasat oleh FTIR, XRD dan TGA-DTG. Hasilnya menunjukkan bahawa SiO_2 berjaya diekstrak dari OPL dan dilapisi pada Fe_3O_4. Selepas itu, ia diaktifkan oleh APTES dan glutaraldehyde untuk menghasilkan CRL/G-AP-SiO_2-Fe_3O_4. Data FTIR, XRD dan TGA-DTG menunjukkan bahawa CRL berjaya dipegunkan pada G-AP-SiO_2-Fe_3O_4, seperti yang dilihat dengan jalur yang timbul pada 1639 cm^{-1} oleh C=O dan C=N yang ditunjukkan dalam FTIR. Khususnya, imobilisasi CRL ke G-AP-SiO_2-Fe_3O_4 menghasilkan pemuatan enzim dan aktiviti khusus 14.7 mg/g dan 183 U/g. CRL/G-AP-SiO_2-Fe_3O_4 kemudiannya diuji untuk menentukan keadaan optimum untuk menghidrolisis minyak zaitun. Didapati bahawa keadaan optimum untuk tindak balas yang menghasilkan aktiviti terbaik ialah penggunaan enzim 1.00 mg/mL, suhu inkubasi 40 °C, pH 8.0, nisbah minyak zaitun: air 2.5: 1, dan kelajuan agitasi 200 rpm. Penilaian kestabilan terma menunjukkan bahawa CRL/G-AP-SiO_2-Fe_3O_4 lebih tahan terhadap denaturasi yang disebabkan oleh haba, daripada CRL bebas. CRL/G-AP-SiO_2-Fe_3O_4 secara kinetika ditunjukkan mempunyai pertalian yang lebih tinggi terhadap substrat (Michaelis-Menten malar, K_m = 0.583 g/mL) tetapi termangkin pada kadar tindak balas maksimum yang lebih rendah (V_{max} = 833.3 µmol/ml. min) berbanding dengan CRL bebas (K_m = 6.00 g / mL, V_{max} = 3330 µmol/ml.min). Parameter termodinamik berdasarkan nilai-nilai separuh hayat ($t_{1/2}$ = 38.94 min), nilai D (129.4 min), E_d = 112.90 kJ/mol, ΔH_d ° = 110.10 kJ/mol) dan ΔGd ° = 11.32 kJ/mol untuk CRL/G-AP-SiO_2-Fe_3O_4 secara konsisten menunjukkan bahawa lipase lebih tinggi kestabilan termanya daripada CRL bebas ($t_{1/2}$ = 23.89 min, D-value = 79.67 min, E_d = 93.3 kJ / mol, ΔH_d ° = 87.5 kJ/mol dan ΔG_d ° = 9.8111 kJ/mol) pada 60 ° C. Hasil kajian menunjukkan bahawa SiO_2 yang diekstrak dari OPL boleh disalut pada Fe_3O_4 untuk digunakan sebagai sokongan bukan organik untuk enzim terpegun. Hasilnya menunjukkan bahawa pemangkin CRL/G-AP-SiO_2-Fe_3O_4 adalah berpotensi untuk memangkinkan tindak balas hidrolisis dengan kadar reaksi yang baik, dengan itu membayangkan penerapan prospektifnya sebagai pemangkin komersial yang berkaitan.

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LIST OF ABBREVIATIONS

APTES	-	3-aminopropyltriethoxysilane
BSA	-	Bovine serum albumin
CRL	-	*Candida rugosa* lipase
E_d	-	Deactivation energy
$FeCl_2.4H_2O$	-	Iron(II) chloride tetrahydrate
$FeCl_3.6H_2O$	-	Iron(III) chloride hexahydrate
$FeSO_4.7H_2O$	-	Iron(II) sulphate heptahydrate
FTIR	-	Fourier transform infrared spectroscopy
Fe_3O_4	-	Magnetic Nanoparticles
G	-	Glutaraldehyde
H_2SO_4	-	Sulphuric acid
HCl	-	Hydrochloric acid
K_2HPO_4	-	Dipotassium hydrogen phosphate
KH_2PO_4	-	Potassium dihydrogen phosphate
K_d	-	Deactivation constant
K_m	-	Michaelis-Menten constant
NaOH	-	Sodium hydroxide
$-NH_2$	-	Amine group
NH_4OH	-	Ammonium hydroxide
OPF	-	Oil palm fronds
OPFL	-	Oil palm frond leaves
OPL	-	Oil palm leaves
OPLA	-	Oil palm leaves ash
OPT	-	Oil palm trunks
OVAT	-	One-variable-at-a-time
R^2	-	Coefficient of determination
-SH	-	Thio group
SiO_2	-	Silica
TGA	-	Thermogravimetric analysis
V_{max}	-	Maximum rate of reaction

LIST OF SYMBOLS

°C	-	degree Celsius
E_d	-	denaturation activation energy
g	-	gram
h	-	hour
K	-	kelvin
kDa	-	kilo Dalton
k_d	-	deactivation rate constant
kJ/mol	-	kilojoules per mole
l	-	litre
mg	-	miligrams
min	-	minute
ml	-	mililitre
mM	-	milimolar
mg/g	-	miligram per gram
rpm	-	rotation per minutes
s	-	second
SF	-	stabilisation factor
$t_{1/2}$	-	half life
U	-	units
µmol	-	micro mole
v/v	-	volume per volume
w/v	-	weight per volume
w/w	-	weight per weight
%	-	percentage
$\Delta H_d°$	-	standard energy of deactivation
$\Delta S_d°$	-	standard entropy of deactivation
$\Delta G_d°$	-	standard free energy of deactivation

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Oil palm or known as *Elaeis guineensis* is known as the most important plant species found in the Elaeis genus that belongs to the Palmae family. It is planted in large plantations in many tropical countries, for instance, Malaysia, Thailand and Indonesia (Nordin, Sulaiman, Hashim, & Mohamad Kassim, 2016). Palm oil extracted from pulp and kernel of the fruit are useful as edible oil to manufacture soap, food products, flavours and etc. Nonetheless, the high production rate of palm oil has taken its toll on the environment, as these large plantations leave behind large quantities of biomass, approximately 50-70 tonnes per hectare of the plantation (Shuit, Tan, Lee, & Kamaruddin, 2009). Oil palm fronds (OPF) including the oil palm leaves (OPL) and petioles constitute which is approximately 47% of the total oil palm waste (Nordin, Sulaiman, Hashim, & Mohamad Kassim, 2017). So far, these wastes are not fully utilised or recycled effectively, and are often eliminated by land-filling and open burning. Open burning in particularly, pollutes the environment and damages the ecosystem (Sidik, Jalil, Triwahyono, Adam, Satar, & Hameed, 2012). Review of the literature conveyed that agricultural biomass i.e. OPL are potential raw materials for the manufacturing a myriad of value-added products such as animal food, fertiliser and absorbent. This is because of the abundance, readily available, and low-cost material of OPL. OPL in most part is used as a pelleting feed stock pulp and for the manufacturing of paper (Onoja, Attan, Chandren, Abdul Razak, Abdul Keyon, Mahat, & Wahab, 2017; Onoja, Chandren, Abdul Razak, Mahat, & Wahab, 2018a; Onoja, Chandren, Razak, & Wahab, 2018b).

According to a few reports, approximately 72.6% silica (SiO_2) exists in palm oil fuel ash and, as much as 46.0 % is found in oil palm ash (Adam, Sulaiman, Baharuddin, Mokhtar, Busu, & Tengku Zainal Mulok, 2017; Faizul, Abdullah, &

Fazlul, 2012). In addition, another study reported that 95.2 % of SiO_2 found in acid treated OPL (Onoja *et al.*, 2017). These works highlighted the potential of OPL as a renewable SiO_2 source, aside to SiO_2 sources that are mined from the earth's crust (Faizul *et al.*, 2012). SiO_2 is prized for its multiple applications, largely because of its natural abundance of silanol groups (-SiOH), which contributes to compatibility for interaction with different types of proteins. SiO_2 is generally used for improving the stability, biocompatibility, hydrophilicity and surface functionality of the nanoparticles, i.e. magnetite (Fe_3O_4) (Abbas, Torati, Soo Lee, Rinaldi, & Kim, 2014). Onoja *et al.* (2018a) also reported the use of SiO_2 from OPL ash (OPLA) as a nanocoating material over nanoparticles of Fe_3O_4 to covalently bind *Candida rugosa* lipase (CRL). The resultant biocatalyst was appreciably activated and stabilised by the SiO_2-Fe_3O_4 hybrid nanosupport (Onoja *et al.*, 2017; Onoja *et al.*, 2018a; Onoja *et al.*, 2018b).

Studies have shown that enzyme immobilisation onto solid supports can effectively distribute the enzyme molecules and prevent formation of inactive aggregates, in conjunction to stabilising their structures (Prlainovic, Bezbradica, Rogan, Uskokovic, Mijin, & Marinkovic, 2016). For such a purpose, inert polymers and inorganic materials are the preferred choice of carrier matrices. This has to do with their stability, inherent physical strength, regenerability, ability to increase catalytic activity, microbial resistance and reduce inhibition by the product during reactions (Datta, Christena, & Rajaram, 2012). In this milieu, the immobilisation of enzymes onto nanostructures such as nanoparticles, nanofibers, nanotubes and nanocomposites have been a topic of active research in enzyme technology. The high specific surface area of nanosupports is useful in enhancing the binding capacity of the enzymes, lowering transfer resistance with minimum diffusion limitation and lower operational cost (Singh & Mukhopadhyay, 2014). In fact, Fe_3O_4 are quite popular supports for immobilising enzymes, as the nanoparticles facilitate easy separation of the biocatalyst from the reaction mixture using an external magnetic field. This approach permits the reuse of enzymes in continuous operations (Singh *et al.*, 2014). It was previously demonstrated that the coating of SiO_2 over Fe_3O_4 (G-AP-SiO_2-Fe_3O_4) before the covalent binding of CRL onto the surface of the support (Onoja *et al.*, 2018a) yielded a more activated and stabilised biocatalyst (CRL/G-AP-

SiO_2-Fe_3O_4) for catalysing an esterification production of butyl butyrate. This work proves that CRL/G-AP-SiO_2-Fe_3O_4 was highly active in a water-free system. However, many questions remain unanswered in terms of stability and activity of the lipase when catalysing in a water-based system, i.e. a hydrolytic reaction (Onoja *et al.*, 2017; Onoja *et al.*, 2018a; Onoja *et al.*, 2018b).

1.2 Problem Statement

Although the G-AP-SiO_2-Fe_3O_4 nanosupport was shown to be applicable for activation and stabilisation of CRL for an esterification reaction, the same cannot be assumed for its capability to improve lipase activity for a hydrolysis reaction. This is because conditions in an enzyme-catalysed esterification reaction are highly different to that in an aqueous reaction system. CRL/G-AP-SiO_2-Fe_3O_4 is adversely affected by water molecules in an esterification reaction in which the produced ester is counterproductively hydrolysed in the presence of excess water (Abd Rahman, Abd Manan, Marzuki, Mahat, Attan, Abdul Keyon, Jamalis, Aboul-Enein, & Wahab, 2017; Elias, Chandren, Razak, Jamalis, Widodo, & Wahab, 2018; Manan, Attan, Zakaria, Keyon, & Wahab, 2018). Likewise, the high quantities of alcohol and acids of the starting materials can affect the conformation of CRL, causing the lipase to alter its activity and stability. In an aqueous system, all the above are absent and, the CRL/G-AP-SiO_2-Fe_3O_4 was predicted to act differently, thereby influencing its stability, as well as the reaction kinetic and thermodynamic properties.

Herein, this study investigates the ability of CRL/G-AP-SiO_2-Fe_3O_4 to catalyse a hydrolytic reaction, i.e. hydrolysis of olive oil. It is worth noting here that data on the ability of CRL/G-AP-SiO_2-Fe_3O_4 to catalyse hydrolytic reactions remain unavailable. Thus, the findings of this study would greatly contribute to the body of knowledge in terms of the versatility of CRL/G-AP-SiO_2-Fe_3O_4 as a biocatalyst. This study hypothesised that the catalytic properties CRL/G-AP-SiO_2-Fe_3O_4 in a water-based system would be differ to that of the aqueous-free i.e. esterification. The presence of water would greatly change the stability of CRL/G-AP-SiO_2-Fe_3O_4 in catalysing the reaction, alongside changes in its kinetic and thermodynamic properties.

1.3 Research Objectives

The objectives of this study are:

(i) To prepare and characterise the morphology of SiO_2 and Fe_3O_4.

(ii) To characterise the morphology and physiochemical properties of CRL immobilised onto oil-palm leaves ash (OPLA)-based magnetite-silica matrix (CRL/G-AP-SiO_2-Fe_3O_4).

(iii) To optimise and compare the free CRL and CRL/G-AP-SiO_2-Fe_3O_4-catalysed hydrolysis of olive oil.

(iv) To assess the stability, as well as the kinetic and thermodynamic properties of the CRL/G-AP-SiO_2-Fe_3O_4-catalysed hydrolysis of olive oil.

1.4 Scope of Study

The study begins with the collection of OPL from Universiti Teknologi Malaysia (UTM). The OPL collected were washed and ground into powder for acid treatment. It was then calcined to obtain treated oil palm leaves ash (OPLA). Untreated OPL and treated OPLA were characterised using thermo gravimetric analysis (TGA) and fourier transform infrared (FTIR) spectroscopy. The SiO_2 extracted from OPLA was coated onto Fe_3O_4 to produce SiO_2-Fe_3O_4 nanosupport. It was then activated with 3-aminopropyltriethoxysilane (APTES) and functionalised by glutaraldehyde to give G-AP-SiO_2-Fe_3O_4. Subsequently, CRL was immobilised onto G-AP-SiO_2-Fe_3O_4 to produce the biocatalyst (CRL/G-AP-SiO_2-Fe_3O_4). The morphology and physiochemical properties SiO_2-Fe_3O_4, G-AP-SiO_2-Fe_3O_4 and CRL/G-AP-SiO_2-Fe_3O_4 were characterised by using FTIR, TGA and X-ray diffraction (XRD).

The next step involved the optimisation of CRL/G-AP-SiO_2-Fe_3O_4 catalysed hydrolysis of olive oil for parameters temperature, pH and stirring rate. This part of the work attempts to establish and compare the best conditions to yield the highest percentage of the liberated free fatty acids. It was, in actual, to gauge which lipase was more efficient and activated to carry out the hydrolysis reaction.

Finally, the study assessed the stability of CRL/G-AP-SiO_2-Fe_3O_4 by carrying out the reactions under different temperatures and leaching study. Subsequently, kinetic and thermodynamic parameters for the CRL/G-AP-SiO_2-Fe_3O_4-catalysed hydrolysis of olive oil emulsion were assessed. The kinetic study assessed the values of Michaelis-Menten constant (K_m) and maximum rate of reaction (V_{max}) whereas the thermodynamic investigation were to estimate the values of thermal deactivation energy (E_d), half-life ($t_{1/2}$), standard enthalpy of denaturation ($\Delta H_d°$), standard entropy of denaturation ($\Delta S_d°$) and standard free energy of denaturation ($\Delta G_d°$).

1.5 Significance of Study

In this research work, the kinetic and thermodynamic parameters, as well as the ability of CRL/G-AP-SiO_2-Fe_3O_4 to catalyse a hydrolytic reaction was established. The findings can further add to the body of knowledge with regards to the efficacy and versatility of CRL/G-AP-SiO_2-Fe_3O_4 to catalyse reactions from two different systems, *viz* the water-based or water-free (organic-solvent based) system.

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