Candida rugosa LIPASE SUPPORTED ON BIOMASS-BASED NANOCELLULOSE-SILICA HYBRID POLYETHERSULFONE MEMBRANE FOR SYNTHESIS OF PENTYL VALERATE

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DEDICATION

This thesis is dedicated to my parents *Elias Ismail* and *Zaidah Che Mat*.
I learned from my father, “the way to get started is to quit talking and begin doing”.
I learned from my mother, “where there’s a will, there’s a way”.

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ABSTRACT

The expansion of oil palm plantations to meet global demands has led to new environmental challenges that rose from the production of enormous quantities of biomass. Herein, this study capitalized on extracting nanocellulose (NC) and silica (SiO_2) from oil palm leaves (OPL) using a combination of chemical treatments. The nanoparticles were then used to fabricate the hybrid NC-SiO_2 nanofiller for incorporation into polyethersulfone (PES) to yield the NC-SiO_2-PES support for the immobilization of *Candida rugosa* lipase (NC-SiO_2-PES/CRL). XRD, TGA-DTG and FTIR:ATR characterizations of NC and SiO_2 confirmed the successful isolation of NC and SiO_2 from lignocellulosic resources. A 0.02 cm membrane size with 5% (w/v) of NC-SiO_2 without PVP K30 was optimal for membrane fabrication. Modification of NC-SiO_2-PES was conducted using 3-(aminopropyl)triethoxysilane (APTES) followed by activation with glutaraldehyde that gave the highest conversion of pentyl valerate (91.3%, $p < 0.05$) compared to Glut-NC-SiO_2-PES (73.9%, $p < 0.05$) in 3 h. The optimized Taguchi Design-assisted immobilization of CRL onto NC-SiO_2-PES membrane (5% glutaraldehyde, 4 h of immobilization, 20 mg/mL CRL concentration, 40 °C and pH 5) gave a 90% yield of PeVa in 3 h. Characterization of NC-SiO_2-PES/CRL biocatalyst using FTIR-ATR, XRD, TGA-DTG, FESEM-EDX, TEM, AFM and Raman spectroscopy revealed that the CRL molecules were successfully bound to the surface of the NC-SiO_2-PES membrane via imine bonds formed through a Schiff base mechanism. The results indicated that NC formed intermolecular hydrogen bonds with SiO_2, while OH groups from the resultant NC-SiO_2 forged hydrogen bonds with S=O of PES. The thermal stability of NC-SiO_2-PES/CRL was ~30% higher over the free CRL, with reusability for up to 14 successive esterification cycles. NC-SiO_2-PES/CRL also exhibited extended operational stability, with a robust half-life of ~120 h, excellent storage stability at 4 °C, and the absence of leached protein after 60 min of agitation. Kinetics evaluation showed that the NC-SiO_2-PES/CRL-catalyzed synthesis of PeVa followed the ping-pong bi-bi mechanism (V_{max} of 0.57 mM min^{-1}) with pentanol inhibition ($K_{i,B}$ 78.49 mM). Meanwhile, the Michaelis-Menten constants for substrates, valeric acid ($K_{m,A}$) and pentanol ($K_{m,B}$) were 67.97 mM and 43.53 mM, respectively. The higher values of ΔH°_{d}, ΔG°_{d}, $t_{1/2}$ and activation energy of enzyme denaturation (E_d) conveyed that the NC-SiO_2-PES improved the thermal stability of the CRL and the process followed first-order kinetics ($R^2 > 0.95$). The activation energy (E_a) and activation energy for thermal denaturation (E_d) for the NC-SiO_2-PES/CRL was 6.49 kJ mol^{-1} and 96.8 kJ mol^{-1}, respectively. The NC-SiO_2-PES/CRL's ability to be regenerated chemically and ultrasonically, and reused without significant loss in enzyme activity denotes its potential cost-saving for the production of PeVa. The FTIR, ^{1}H-NMR, gas chromatography-mass spectrometry ($[M]^+$ m/z 130, $C_{10}H_{20}O_2$) verified the enzymatically produced PeVa. The overall findings invariably envisaged the biocompatibility of NC and SiO_2 derived from OPL as a suitable nano-filler to prepare the NC-SiO_2-PES composite for CRL immobilization. Therefore, the NC-SiO_2-PES/CRLs are a potential immobilized biocatalyst to expedite the synthesis of high yields of PeVa.

ABSTRAK

Perluasan ladang kelapa sawit untuk memenuhi permintaan global telah membawa kepada cabaran persekitaran baru yang timbul dari pengeluaran sejumlah besar biojisim. Di sini, kajian ini memanfaatkan ekstrak nanoselulosa (NC) dan silika (SiO_2) dari daun kelapa sawit (OPL) dengan menggunakan suatu kombinasi rawatan kimia. Nanopartikel ini kemudian digunakan untuk membuat nano-pengisi hibrid NC-SiO_2 untuk dimasukkan ke dalam polyethersulfon (PES) untuk menghasilkan sokongan NC-SiO_2-PES bagi pemegunan lipase *Candida rugosa* (NC-SiO_2-PES/CRL). Pencirian NC dan SiO_2 menggunakan XRD, TGA-DTG dan FTIR:ATR mengesahkan kejayaan pengasingan NC dan SiO_2 daripada sumber lignosellulosa. Ukuran membran 0.02 cm dengan 5% (w/v) NC-SiO_2 tanpa PVP K30 adalah optimum untuk fabrikasi membran. Pengubahsuaian NC-SiO_2-PES dilakukan menggunakan 3-(aminopropil)trietoksisilan (APTES) diikuti dengan pengaktifan dengan glutaraldehid yang memberikan penukaran pentil valerat tertinggi (91.3%, $p < 0.05$) berbanding Glut-NC-SiO_2-PES (73.9%, $p < 0.05$) dalam 3 jam. Pemegunan CRL ke membran NC-SiO_2-PES berbantukan-Rekabentuk Taguchi (5% glutaraldehid, 4 jam pemegunan, 20 mg/mL kepekatan CRL, 40 °C dan pH 5) memberikan hasil 90% PeVa dalam 3 jam. Pencirian biomangkin NC-SiO_2-PES/ RL menggunakan FTIR-ATR, XRD, TGA-DTG, FESEM-EDX, TEM, AFM dan spektroskopi Raman mendedahkan bahawa molekul CRL berjaya diikat pada permukaan membran NC-SiO_2-PES melalui ikatan imina melalui suatu mekanisma bes Schiff. Hasil kajian menunjukkan bahawa NC membentuk ikatan hidrogen intermolekul dengan SiO_2, sementara kumpulan OH dari NC-SiO_2 yang dihasilkan membentuk ikatan hidrogen dengan S=O dari PES. Kestabilan terma NC-SiO_2-PES/CRL adalah ~30% lebih tinggi berbanding CRL bebas, dengan penggunaansemula sehingga 14 kitaran pengesteran berturut-turut. NC-SiO_2-PES/CRL juga memperlihatkan kestabilan operasi yang berpanjangan, dengan jangka hayat yang kuat ~120 jam, kestabilan penyimpanan yang sangat baik pada 4 °C dan ketiadaan larut lesap protein setelah 60 minit pengacauan. Penilaian kinetik menunjukkan bahawa sintesis PeVa yang bermangkinkan NC-SiO_2-PES/CRL mengikuti mekanisme ping-pong bi-bi (V_{max} 0.57 mM min^{-1}) dengan perencatan pentanol ($K_{i,B}$ 78.49 mM). Sementara, pemalar Michaelis-Menten untuk substrat, asid valerik ($K_{m,A}$) dan pentanol ($K_{m,B}$) masing-masing adalah 67.97 mM dan 43.53 mM. Nilai ΔH°_{d}, ΔG°_{d}, $t_{1/2}$ dan tenaga pengaktifan denaturasi enzim (E_d) yang lebih tinggi menyampaikan bahawa NC-SiO_2-PES meningkatkan kestabilan terma CRL dan proses tersebut mengikuti kinetic peringkat pertama ($R^2 > 0.95$). Tenaga pengaktifan (E_a) dan tenaga pengaktifan untuk penyahaktifan termal (E_d) untuk NC-SiO_2-PES/CRL masing-masing adalah 6.49 kJ mol^{-1} dan 96.8 kJ mol^{-1}. Keupayaan NC-SiO_2-PES/CRL untuk dijanasemula secara kimia dan ultrasonik, dan digunakan kembali tanpa kehilangan aktiviti enzim yang signifikan menunjukkan potensi penjimatan kosnya untuk pengeluaran PeVa. FTIR, ^{1}H-NMR, kromatografi gas-spektrometri jisim ($[M]^{+}$ m/z 130, $C_{10}H_{20}O_2$) mengesahkan PeVa yang dihasilkan secara enzimatik. Penemuan keseluruhan secara tak langsung membayangkan kesesuaian bio NC dan SiO_2 yang berasal dari OPL sebagai pengisi nano yang sesuai dalam penyediaan komposit NC-SiO_2-PES untuk pemegunan CRL. Oleh itu, NC-SiO_2-PES/CRLs adalah pemangkin pegun yang berpotensi untuk mempercepat sintesis PeVa berhasil tinggi.

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LIST OF ABBREVIATIONS

OPL	-	Oil Palm Leaves
CS	-	Cellulose
NC	-	Nanocellulose
OPLT	-	Oil Palm Leaves Treated
SiO_2	-	Silica
PES	-	Polyethersulfone
CRL	-	*Candida rugosa* lipase
GA	-	Glutaraldehyde
APTES	-	3-aminopropyltriethoxysilane
UTM	-	Universiti Teknologi Malaysia
BSA	-	Bovine Serum Albumin
FTIR	-	Fourier Transform Infrared Spectroscopy
ATR	-	Attenuated Total Reflection
XRD	-	X-ray Diffraction
TGA	-	Thermal Gravimetric Analysis
DTG	-	Derivative Thermogravimetry
DTA	-	Differential Thermal Analysis
TEM	-	Transmission Electron Microscopy
FESEM	-	Field Emission Scanning Electron Microscopy
AFM	-	Atomic Force Microscopy
EDX	-	Energy Dispersive X-ray
NMR	-	Nuclear Magnetic Resonance
GC-MS	-	Gas Chromatography-Mass Spectrometry
IP	-	Immobilized Protein
RA	-	Recovered Activity
OVAT	-	One-Variable-at-Time
ANOVA	-	Analysis of Variances

LIST OF SYMBOLS

°C	-	Degree celcius
g	-	Gram
h	-	Hour
mg	-	Milligram
mL	-	Milliliter
M	-	Molar
nm	-	Nanometer
rpm	-	Rotation per minutes
v/v	-	Volume per volume
w/w	-	Weight per weight
μm	-	Micrometer
%	-	Percentage
U	-	Units
I_c	-	Crystallinity index
K_m	-	Michalis-Menten constant
V_{max}	-	Maximum reaction rate
k_{cat}	-	Turnover number
k_{eff}	-	Catalytic efficacy
k_i	-	Inhibition constant
E_a	-	Activation energy
E_d	-	Activation energy for thermal denaturation
k_d	-	Denaturation constant
$t_{1/2}$	-	Half-life
D-value	-	Decimal reduction time
SF	-	Stabilization factor
ΔH°_d	-	Change in enthalpy
ΔS°_d	-	Change in entropy
ΔG°_d	-	Gibb's free energy

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Elaeis guineensis, or oil palm, was previously brought over to Malaysia from the central part of West Africa. Since then, the plantations have expanded to many other countries around as global demands for palm oil grew. The suitable soil structure and geographical area are responsible for the largest cultivation area for oil palm in Malaysia and Indonesia, making them the biggest producers of oil palm-derived products and foods. Nonetheless, the rapid expansion of oil palm cultivation has contributed to controversial issues following the implementation of Good Agricultural Practices (GAP), especially among the independent oil palm smallholders (Jelsma et al., 2019). Most of the issues are related to poor practices and low oil palm yields. Also, the constant generation and passive dumping of oil palm biomass from regular plantation harvesting activities further exacerbate environmental issues (Owolabi et al., 2017; Onoja et al., 2018c).

Inter alia, global warming, and the declining petrochemical reserves have led to the need to seek other greener alternatives. The vast oil palm plantations are now viewed from various perspectives, i.e., as a supplementary source of i) biofuel, ii) precursors for the manufacturing process, and iii) renewable polymers. The humble oil palm tree is an excellent reservoir for the commodities above. The oil palm trunk, empty fruit bunch, and shells are rich in biomaterials with potential development into different value-added products. The use of natural fibers, for instance, oil palm leaves (OPL) as renewable materials to replace non-sustainable petrochemical resources, is also on the rise (Ramlee et al., 2019). The cellulose, hemicellulose, lignin- and silica-rich OPLs are keys to its biotechnological feasibility (Onoja et al., 2017; Owolabi et al., 2017). Different uses of OPL-derived cellulose and silica are described in the literature, such as combined organic-inorganic hybrid composites of

magnetized-nanosilica (Onoja et al., 2018b; Onoja et al., 2018a) and the chitosan-nanocellulose composite (Elias et al., 2017; Elias et al., 2018).

Smart materials from cellulose and silica are highly popular for their environmental friendliness, and tunability to nano and micro scales (Qi et al., 2020), following the abundance of polar surface groups (Onoja et al., 2018b). The use of pure cellulose and silica is rather problematic following unsatisfactory mechanical strength and low biocompatibility, respectively (Xiang et al., 2018), thus requiring further modifications. The combination of organic/inorganic materials is an excellent way to resolve each material's shortcoming. The two aforesaid materials' scopes may be improved and expanded by blending with other materials, for instance, membranes constructed from polyethersulfone (PES). A ternary composite may be efficacious to modulate each component's properties, namely the pure PES (Aghababaie et al., 2018), and vice versa for the more hydrophilic nanocellulose-silica (NC-SiO_2) nanoparticles. Relatively hydrophobic support tends to favor lipases' immobilization with increased activity, such as the *Candida rugosa* lipase (CRL). Cellulose and carbon nanotubes' interactions with polymer membrane have improved membrane properties (Costa et al., 2019; Xu et al., 2020). Nonetheless, the versatility of various organic-inorganic hybrids composites is not fully explored and merits further investigations.

It is important to note that lipases (triacylglycerol acylhydrolyases, EC 3.1.1.3) are proponents of greener manufacturing initiatives. They are prized for their broad specificities, high reaction rates, non-toxicity, biodegradability, stability in different reaction media, and reaction reproducibility under ambient conditions. This versatile group of enzymes catalyzes numerous industrial reactions i.e., esterification, transesterification, oil hydrolysis, polymer synthesis, and biodiesel production (Manoel et al., 2015; Aghababaie et al., 2017). The CRL is among the preferred lipases because of its high activity, affordable cost, and broad substrate specificity (Živković et al., 2015; Aghababaie et al., 2017). The activity of free CRL can be modulated by immobilization onto a suitable solid support. This is because the ensuing interaction between the support and the lipase rearranges the enzymes's conformational structure and stabilized it, *inter alia*, triggering the interfacial-

activated opening of the hydrophobic lid that obscures the active site. The active site becomes exposed and facilitates quick entry of the substrates (Aghababaie et al., 2017; Rahman et al., 2018). Therefore, care must is taken when deciding the hydrophobic support material for CRL immobilization, as a too hydrophobic one can inhibit the interaction between the enzyme and substrates (Vitola et al., 2017). Thus, support that combines the hydrophilic NC and the relatively hydrophobic SiO_2 nanoparticles and PES membrane could provide a more compatible contact surface for CRL's covalent attachment. The PES membrane's large surface area, resistance toward mechanical and chemical stresses, and easy preparation in different geometrical configurations make it a suitable carrier for enzyme immobilization (Gupta et al., 2010).

1.2 Problem Statement

It is apparent that the surplus of the Malaysian OPL rich in biogenic SiO_2 and NC can be put to good use for numerous purposes (Cohen et al., 2005; Saliluddin, 2015). However, the pure form of the two components lacked the physicochemical versatility and durability to withstand different manufacturing or application conditions. For these reasons, it is impractical to put pristine biogenic SiO_2 and NC derived from the OPL into wide-ranging applications. Therefore, this study proposed a plausible way to profoundly improve the OPL-derived NC and SiO_2 for a wide-ranging purpose. This may be done by combining the PES (NC-SiO_2-PES), a commonplace membrane in many applications. The study believes the resultant NC-SiO_2-PES membrane could have interesting properties, in which the more hydroxyl-rich NC-SiO_2 can modulate the hydrophobicity of the pure PES (Aghababaie et al., 2018) and vice versa.

The study then gauged the feasibility of the newly developed NC-SiO_2-PES membrane by its use as support for CRL immobilization (NC-SiO_2-PES/CRL). The aim was to improve the activity and stability of the immobilized CRL for the esterification production of a model ester, the pentyl valerate (PeVa). PeVa production has a noteworthy technological and commercial importance as a biofuel

to alleviate the global dependence on fossil fuel (Lange et al., 2010; Hu et al., 2017). The reaction used by this study is a part of global efforts to manufacture alternative lean carbon energy sources aside from ethanol production by fermentation of sugars or hydrolysis of vegetable oil (Mishra and Ghosh, 2019; Raj and Krishnan, 2019). Plus, the current process of PeVa synthesis is far from green, being heavily dependent on homogenous catalysts, for instance, sulfuric acid. The process is also energy-intensive with laborious downstream processing (Lange et al., 2010).

The study hypothesized that the hybrid NC-SiO_2-PES membrane could activate CRL activity and stabilizes the lipase for prolonged and efficient esterification production of PeVa. The relatively hydrophobic SiO_2 and PES components could hypothetically induce the CRL lid opening by interfacial activation. CRL immobilization onto the NC-SiO_2-PES membrane could improve the lipase's operational stability and reusability, while insolubilizing the catalyst for easy recovery. Moreover, the NC-SiO_2-PES membrane's ability to activate and stabilize CRL has not been explored, and the feasibility remains to be seen. Finally, PeVa production using immobilized CRL that precludes the use of acids accords well with the 11th Malaysia plan of Sustainable Development Goals towards green growth (Plan, 2015).

1.3 Objectives of Study

This research work was aimed at preparing a functional green biocatalyst using the organic and inorganic components of OPL to give the NC-SiO_2-PES for enhancing CRL activity.

1. To extract and characterize nanocellulose (NC) and silica (SiO_2) from OPL.
2. To fabricate and optimize the NC-SiO_2 reinforced polyethersulfone (PES) membrane (NC-SiO_2-PES) for effective CRL immobilization.

3. To optimize the activity and stability of the NC-SiO_2-PES/CRL biocatalyst and characterize its morphology and operational stability as immobilized biocatalyst in esterification of PeVa.

4. To establish the kinetic and thermodynamic models, as well as the regenerability study of NC-SiO_2-PES/CRL-catalyzed esterification production of PeVa.

1.4 Scopes of Study

The project first begins with the collection and pretreatment of the OPL before extracting the NC and SiO_2. For these activities, methods using bleaching, alkali treatment, acid hydrolysis method acid, and thermal treatment were used. The extracted NC and silica were then characterized by several techniques, *viz*. FTIR Spectroscopy: Attenuated Total Reflection (ATR), X-ray diffraction (XRD), and Thermal gravimetric analysis-derivative thermogravimetry (TGA-DTG).

The NC-SiO_2 nanoparticles (2:1) reinforced the polyethersulfone (PES) membrane to give rise to more stable and yet biodegradable support for CRL activation. Fabrication of the membrane was optimized for dope solution preparation and fabrication of NC-SiO_2-PES membrane, for parameters pore former, size of the membrane, and the amount of NC-SiO_2 nanoparticles. Modification and activation of the NC-SiO_2-PES used the 3-(aminopropyl)triethoxysilane (APTES) and glutaraldehyde to increase the hybrid membrane's biocompatibility (NC-SiO_2-PES/CRLs). The study then screened the parameters that could influence the immobilization of CRL onto NC-SiO_2-PES membrane. The investigated parameters were the concentration of crosslinker (1-7%), immobilization time (4-24 h), immobilization temperature (10-70 °C), concentration of CRL solution (1-10 mg/mL) and the pH (5-10). The best-identified parameters were then employed in the subsequent investigation. The statistically -assisted Taguchi design optimization experiment to immobilize CRL onto NC-SiO_2-PES membrane employed the best parameters identified from the screening study.

The determination of protein content and efficacy of immobilization were examined prior to the immobilization. After this, the morphology of NC-SiO_2-PES/CRL is characterized as the following:

a) FTIR spectroscopy: attenuated total reflection (ATR)
b) Thermal gravimetric analysis-derivative thermogravimetry (TGA-DTG)
c) Field emission scanning electron microscopy (FESEM)
d) X-ray diffraction (XRD)
e) Transmission electron spectroscopy (TEM)
f) Atomic force microscopy (AFM)
g) Raman spectroscopy

The operational stability such as reusability, thermal stability, half-life, and leaching study was investigated in this study. Plus, storage stability at room and cold temperature were also studied here. Part of the study assessed the kinetic parameters for the NC-SiO_2-PES/CRL-catalyzed esterification production of PeVa. This investigation was pertinent to determine the mechanism of esterification by the NC-SiO_2-PES/CRL and identify the values of the maximum velocity on an enzymatic reaction (V_{max}), Michaelis-Menten constant (K_m), the number of substrate molecules turnover per enzyme molecules per minute (K_{cat}) and catalytic efficiency (K_{eff}). Next, the thermodynamic parameters, involving activation energy of thermal denaturation (E_d), half-life ($t_{1/2}$), decimal reduction time (D–value), standard enthalpy of denaturation (ΔH^o_d), the standard free energy of denaturation (ΔG^o_d), as well as standard entropy of denaturation (ΔS^o_d), were assessed in the study. The next part of the study assessed the regenerability study of the reused NC-SiO_2-PES/CRL after inactivation following repeated usages from the reusability study. The regenerability study was performed by two different methods; using acid treatment and ultrasonic method. The enzymatically produced PeVa was purified and characterized using FTIR:ATR, NMR, and GC-MS to prove its production. For this part of the study, the IBM SPSS version 20.0 software was employed to evaluate the significance of each treatment method to regenerate the reused NC-SiO_2-PES/CRL.

1.5 Significance of Study

This study successfully established the protocol for producing a novel and highly functional NC-SiO_2-PES support to activate an enzyme, wherein the support was fabricated from silica and cellulose sourced from OPL. The study's findings also found another use for the discarded OPL by converting the biomass into a multifunctional support material. This will create a new portfolio for sustainable commodity manufacturing, which would contribute to the nation's economic development. Most importantly, the NC-SiO_2-PES/CRL developed by this study may prove to be an eco-friendly alternative to manufacture PeVa while promoting sustainable biofuel production and reducing its carbon footprint.

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