Candida rugosa LIPASE SUPPORTED ON BIOMASS-BASED NANOCELLULOSE-CHITOSAN COMPOSITE FOR SYNTHESIS OF BUTYL BUTYRATE

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Specially dedicated to *Mak* and *Abah*

Elias Ismail & Zaidah Che Mat

and also my siblings.

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ABSTRACT

The current practices of large-scale oil palm plantations such as passive dumping and open burning of unwanted matured oil palm fronds leaves (OPFL) are aesthetically displeasing and contributes to poor regional air quality along with increased health problems. Improper disposal of such large quantities of agricultural biomass is environmentally challenging and is unsustainable in the long run. In this regard, further research into development of new technological applications for OPFL warrants attention of the scientific community. In this study, OPFL were bleached, alkaline treated and acid-hydrolyzed to obtain the purified nanocellulose (NC). X-Ray diffractogram revealed the extracted NC was crystalline with a crystallinity index of 70.2%. This indicates its suitability as nano-fillers for the preparation of chitosan/nanocellulose (CS-NC) supports to immobilize *Candida rugosa* lipase (CRL) in the production of CRL/CS-NC biocatalysts. Characterization of CRL/CS-NC using FTIR-ATR, TGA, FESEM, XRD, Raman spectroscopy and fluorescence optical microscopy revealed that the CRL molecules were successfully bound to the surface of the CS-NC supports via imine bonds formed through a Schiff base mechanism. The results indicated that CS was highly hydrogen bonded to the NC. The optimum protocol to immobilize CRL onto the CS-NC supports was assessed for factors namely reaction temperature, concentration of glutaraldehyde and pH of buffer, to yield the highest conversion of butyl butyrate in 3 h of incubation. A maximum percent conversion of butyl butyrate at 88% was achieved using an immobilization temperature of 25°C, 0.3% concentration of glutaraldehyde and buffer at pH 7. The efficacy of CRL/CS-NC was compared with the free CRL for conditions viz. incubation time, temperature, molar ratio butanol:butyric acid, stirring rate and enzyme loading. Under optimum conditions (3 h, 50°C, molar ratio of acid/alcohol of 1:2, 200 rpm and 3 mg/mL CRL/CS-NC), the lipase successfully synthesized 90.2% of butyl butyrate as compared to 62.9% by the free CRL (3 h, 40°C, molar ratio of acid/alcohol of 1:2, 200 rpm and 5 mg/mL CRL). Thermal stability of CRL/CS-NC was improved by 1.5-fold over the free CRL, with the biocatalyst reusable for up to 8 successive esterification cycles. FTIR-ATR and NMR analyses on purified butyl butyrate confirmed that the ester was successfully synthesized. Kinetic assessments showed the CRL/CS-NC catalyzed esterification process followed a ping-pong bi-bi mechanism model (V_{max} 4.5 mM min^{-1}) with butanol inhibition ($K_{i,B}$ 69.05 mM) and showed a greater preference for butyric acid ($K_{m,A}$ 155.52 mM) over butanol ($K_{m,B}$ 917.78 mM). In conclusion, NC obtained from OPFL was suitable as raw material for the preparation of a highly functional CS-NC support. Activity of CRL/CS-NC was improved for rapid and high–yield synthesis of butyl butyrate. Hence, the developed CRL/CS-NC was a possible practical substitute to the homogenous acid catalyst in the synthesis of butyl butyrate.

ABSTRAK

Amalan semasa ladang kelapa sawit skala besar misalnya pembuangan secara pasif dan pembakaran secara terbuka daun kelapa sawit matang (OPFL) memberi pemandangan yang tidak menyenangkan dan menyumbang kepada penurunan kualiti udara serantau di samping peningkatan masalah kesihatan. Kaedah-kaedah pembuangan sejumlah besar biojisim pertanian demikian adalah mencabar alam sekitar dan tidak lestari dalam jangka masa panjang. Dalam hal ini, penyelidikan lanjut mengenai pembangunan aplikasi teknologi baharu untuk OPFL perlu perhatian komuniti saintifik. Dalam kajian ini, OPFL telah dilunturkan, dirawat alkali dan dihidrolisis berasid untuk mendapatkan nanosellulosa (NC) yang tulen. Difraktogram sinar-X menunjukkan NC yang diekstrak adalah bersifat hablur dengan indeks penghabluran 70.2%. Ini menunjukkan kesesuaiannya sebagai pengisi nano dalam penyediaan sokongan chitosan/nanosellulosa (CS-NC) untuk memegunkan lipasa *Candida rugosa* (CRL) dalam menghasilkan mangkin CRL/CS-NC. Pencirian CRL/CS-NC menggunakan FTIR-ATR, TGA, FESEM, XRD, spektroskopi Raman dan mikroskopi optik pendarfluor mendedahkan bahawa molekul CRL berjaya diikat pada permukaan CS-NC melalui ikatan imina melalui suatu mekanisma bes Schiff. Hasilnya menunjukkan bahawa CS sangat terikat kepada NC melalui ikatan hidrogen. Protokol optimum untuk memegunkan CRL ke atas penyokong CS-NC telah dinilai untuk faktor-faktor iaitu suhu tindak balas, kepekatan glutaraldehid dan pH penimbal, untuk menghasilkan penukaran tertinggi butil butirat dalam pengeraman selama 3 jam. Penukaran maksimum butil butirat sebanyak 88% dicapai menggunakan suhu pemegunan 25°C, kepekatan glutaraldehid 0.3% dan penimbal pemegunan pada pH 7. Keberkesanan CRL/CS-NC dibandingkan dengan CRL bebas untuk keadaan misalnya masa pengeraman, suhu, nisbah molar butanol:asid butirik, kadar pengacauan dan bebanan enzim. Di bawah keadaan optimum (3 jam, 50°C, nisbah molar asid/alkohol 1:2, 200 rpm dan 3 mg/mL CRL/CS-NC), lipasa tersebut berjaya mensintesis 90.2% butilat butirat berbanding kepada 62.9% oleh CRL bebas (3 jam, 40°C, nisbah molar asid/alkohol 1:2, 200 rpm dan 5 mg/mL CRL). Kestabilan terma CRL/CS-NC telah ditambahbaik 1.5 kali ganda berbanding CRL bebas, dengan pemangkin ini boleh diguna semula sehingga 8 kitaran pengesteran berturut-turut. Analisis FTIR-ATR dan NMR terhadap butil butirat tulen mengesahkan bahawa ester ini berjaya disintesis. Kajian kinetik menunjukkan process pengesteran bermangkinkan CRL/CS-NC mengikut model mekanisme ping-pong bi-bi (V_{max} 4.5 mM min^{-1}) dengan perencatan butanol ($K_{i,B}$ 69.05 mM) dan menunjukkan kepilihan lebih tinggi terhadap asid butirik ($K_{m,A}$ 155.52 mM) berbanding butanol ($K_{m,B}$ 917.78 mM). Kesimpulannya, NC yang diperolehi dari OPFL adalah sesuai sebagai bahan mentah untuk penyediaan sokongan CS-NC yang sangat berfungsi. Aktiviti CRL/CS-NC telah dipertingkatkan untuk mensintesis butil butirat dengan cepat dan hasil yang tinggi. Oleh itu, CRL/CS-NC yang dibangunkan boleh menjadi pengganti yang praktikal kepada pemangkin asid homogen dalam sintesis butil butirat.

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LIST OF ABBREVIATIONS

CRL	-	*Candida rugosa* lipase
CS	-	Chitosan
NC	-	Nanocellulose
OPFL	-	Oil palm frond leaves
GA	-	Glutaraldehyde
FTIR	-	Fourier transform infrared spectroscopy
TGA	-	Thermal gravimetric analysis
FESEM	-	Field emission scanning electron microscopy
XRD	-	X-ray diffraction
TLC	-	Thin layer chromatography
NMR	-	Nuclear magnetic resonance
Uv-vis	-	UV-visible spectroscopy
BSA	-	Bovine serum albumin
kDA	-	Kilo dalton

LIST OF EQUATIONS

LIST OF SYMBOLS

°C	-	Degree celcius
g	-	Gram
h	-	Hour
L	-	Liter
mg	-	Milligram
mL	-	Mililiter
M	-	Molar
rpm	-	Rotation per minutes
v/v	-	Volume/volume
w/v	-	Weight/volume
w/w	-	Weight per weight
%	-	Percentage
U	-	Units
I_c	-	Crystallinity index
v	-	Velocity
K_m	-	Michaelis-Menten constant
V_{max}	-	Maximum rate of reaction
K_{cat}	-	Turnover number/catalytic constant
K_{eff}	-	Catalytic efficiency
K_s	-	Specificity constant
$K_{i,B}$	-	Inhibitory constant of alcohol

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

To date, the high demand of food globally has lead the emergent of agricultural land used for cultivation and caused a new environmental challenges related with the disposal of huge quantities of agricultural biomass in the environment (Owolabi *et al.*, 2017). Some irresponsible farmers have resorted to the rapid method but inconvenient method of ‘slash and burn’ to rid-off such biomass and clear their agricultural land. Such drastic measure has contributed to the seasonal hazy season often experienced by the Southeast Asia region (Islam *et al.*, 2016). At present, the current method to rid-off the palm oil biomass from environment is through open burning which leads to the air pollution (Jain *et al.*, 2014; Saliluddin, 2015) as well as causing health complications to the human (Cohen *et al.*, 2005). The problem has been further exacerbated by the fact that Indonesia is the biggest producer of palm oil and hence they are also the annual largest producer of such biomass (Indonesian Palm Oil Advocacy Team-Indonesian Palm Oil Board, 2010; Mahat, 2012). Therefore, this study focused on the utilization of discarded oil palm leaves to find better uses of such biomass may prove beneficial in providing alternative means to dispose as well as to explore the commercial application of this biomass.

Oil palm trunk (OPT), empty fruit bunches (EFB) and oil palm frond leaves (OPFL) are the major contributors of oil palm biomass which consist of cellulose, hemicelluloses, lignin (Sauian *et al.*, 2013). From the study done by Mohaiyiddin *et* *al.* (2016), they proposed that the leaves from the oil palm trees are suitable renewable sources for producing functionalized nanocellulose. Hence, acquiring nanocellulose (NC) from such abundant sources meant for enzyme immobilization and subsequently catalyzing synthesis of important esterification reactions, may prove feasible. After extracting and fabricating the nanocellulose from the OPFL, the NC obtained in this study was used as nanofillers for natural polymers chitosan (CS) for developing the CS-NC support and immobilizing lipase onto the nanobioconjugates. NC and CS were chosen in this study for immobilizing lipases as these materials have been found to be biodegradable, biocompatible, displaying excellent mechanical strength, non-hazardous (Abou-Zeid *et al.*, 2015) and did not cause any harm to the environment (Wicklein and Salazar-Alvarez, 2013; Kengkhetkit and Amornsakchai, 2014).

This study chose *Candida rugosa* lipase (CRL) as the lipase of choice as the enzyme has generally been favoured for its ability to catalyze multiple reactions such as oil hydrolysis, transesterification, esterification and interesterification (Houde *et al.*, 2004; Marzuki *et al.*, 2015b). Nonetheless, CRL in its free form have been known to be unstable, show low activity in organic solvents and susceptible to inactivation under conditions of high temperature and extreme pH (Mohamad *et al.*, 2015b). Hence, for a more economical and efficient use of CRL in aqueous as well as in non-aqueous conditions, the activity, selectivity, and operational stability of CRL can be improved by immobilization (Hung *et al.*, 2003; Mateo *et al.*, 2007). Furthermore, it would permit catalyst recovery after the reaction and immediate reuse, especially for multiple catalytic cycles (Zucca and Sanjust, 2014). The study used CRL molecules that were crosslinked to the CS-NC support (CRL/CS-NC) using glutaraldehyde as the cross-linking agent. The resultant interaction is *via* intermolecular covalent bonding between the amino groups of the enzyme and hydroxyl group on the CS-NC composite. The study believes that covalent attachment of the CRL molecules to the surface of the CS-NC supports would result in more favourable interactions due to the inherently strong covalent bond (Romdhane *et al.*, 2011).

The developed CRL/CS-NC nanobiocatalysts were then evaluated for its efficacy for catalyzing a problematic esterification reaction. In this study, the model reaction used was the esterification of butanol and butyric acid to produce butyl butyrate, an important ester that gives the pineapple flavour in food and beverages industries (Martins *et al.*, 2013) as well as used as an additive in the biodiesel production (Ng and Yang, 2016). Butyl butyrate was chosen as the target product mainly due to the current chemical route i.e. Fisher-Speier method is carried out using corrosive acid catalysts that releases harmful by products, uses harsh reaction conditions and require complex separation processes (Ju *et al.*, 2011; Che Marzuki *et al.*, 2015; Mohamad *et al.*, 2015a; Mohamad *et al.*, 2015b). In fact, the commercial Fisher-Speier esterification to produce butyl butyrate has complications in achieving high yield as the elevated temperature (250˚C) used in the reaction tend to counter productively degrade the produced butyl butyrate (Ju *et al.*, 2011). Hence, there is still plenty of room for improving its production process, preferably via biotechnological route. This is because the biotechnological means that uses natural enzymes i.e. CRL to catalyze such reactions would be beneficial to alleviate such drawbacks while being environmentally benign (Charpe and Rathod, 2011). Earlier studies focusing on improving production of butyl butyrate using natural enzymes as the biocatalyst, so far have used *Thermomyces lanuginosus* lipase, requiring 24 h for a 93% conversion of the ester (Salleh *et al.*, 2016). Similarly, Salihu and co-workers achieved a 63% conversion in 12 h of reaction using the commercial *Candida cylindracea* lipase (Salihu *et al.*, 2014). Retrospectively, there is still much to do in developing efficient as well as relatively 'milder and greener' biocatalysts to produce such ester.

1.2 Problem Statement

The current strategies for disposing such biomass in large plantations are aesthetically displeasing and have significantly contributed to elevated occurrence of serious regional air pollution in the Southeast Asia as well as around the world (Mike, 2015; Islam *et al.*, 2016). Pollution of this magnitude is hazardous to both the environment and well-being of human (Cohen *et al.*, 2005). The drastic method adopted by unscrupulous farmers or plantations to dispose of the biomass do not fully utilize the leaves of the palm oil tree to its full potential. It is evident that there is invariably still much work to be done on exploring the possible uses of oil palm leaves. Hence, concerted efforts in researching for possible alternative applications of leaves from the oil palm tree constitute an interesting scientific study while being a proponent of the 'Zero Waste' initiative outlined by the Malaysian Palm Oil Board (Tan, 2006; Ng *et al.*, 2012). This study is interested in exploring the feasibility of extracting nanocellulosic materials acquired from renewable raw sources. Such possibility has been reported for other types of agricultural biomass (Lani *et al.*, 2014; Marino *et al.*, 2015) but not for the oil palm leaves. In this context, the study hereby proposed the use of NC extracted from the abundant oil palm leaves to be incorporated as fillers in the CS-NC matrix for immobilizing the CRL lipase. Such technique proposed here would simultaneously afford better uses to the discarded oil palm leaves.

Studies utilizing the CS-NC matrix for enzyme immobilization have never been reported and the efficacy of such support for activating CRL reactions remains to be seen. It is hypothesized that the employment of NC extracted from OPFL as a component in the CS-NC composite may result in upgraded esterification production of butyl butyrate owing to the formation of stable interactions between the enzyme and the support. The developed CRL/CS-NC nanobiocatalysts were evaluated for the esterification of butanol and butyric acid to produce butyl butyrate. The study believes that such reaction is suitable for evaluating efficacy of the CRL/CS-NC as the current Fisher-Speier esterification method to produce butyl butyrate has been nothing but problematic, producing low yields of the ester (Ju *et al.*, 2011).

1.3 Objectives of Study

This research work is aimed to develop novel biocatalysts from natural biowaste for characterization and application in non-aqueous biocatalysis. The objectives of this study are as follow:

1. To prepare and characterize nanocellulose extracted from the OPFL.
2. To characterize the morphology and optimize the immobilization protocols for immobilizing CRL onto the OPFL derived CS-NC support.
3. To characterize the developed CRL/CS-NC nanobiocatalyst.
4. To optimize the esterification condition and develop kinetic model of CRL/CS-NC biocatalysts for the production of butyl butyrate.

1.4 Scopes of Study

The scopes of this project include the pretreatment of the OPFL such as cleaning, cutting, drying and grinding before extraction of NC using bleaching, alkali treatment and acid hydrolysis method. The extracted NC was characterized using fourier transform infrared spectroscopy (FTIR) and x-ray diffaction (XRD). The fabrication of CS-NC support was done to develop stable biodegradable support. Next, the study characterize the physicochemical properties and optimize the immobilization protocol catalyzed by CRL/CS-NCs for the production of butyl butyrate using the method of one-variable-at-a-time (OVAT) for parameters immobilization time, temperature, concentration of crosslinker and pH. The determination of protein content and efficacy of immobilization were examined prior to the immobilization.

The study subsequently assessed the optimization of the CRL/CS-NCs aided synthesis of butyl butyrate using the method of OVAT based on incubation time, temperature, substrate molar ratio (acid:alcohol), stirring rate, enzyme loading, reusability and thermal stability. The kinetic study for the CRL/CS-NCs catalyzed esterification of butanol and butyric acid based on different concentrations of the substrates were carried out to establish the mechanism of the developed biocatalysts and to find out the kinetic parameters of the lipase viz. V_{max}, K_m, K_{cat} and K_{eff}.

The following part of the study the morphological characteristics of the CS/NC and CRL/CS-NCs beads by:

a. FTIR spectroscopy: attenuated total reflection (ATR)
b. Thermal gravimetric analysis (TGA)
c. Field emission scanning electron microscopy (FESEM)
d. X-ray diffraction (XRD)
e. Optical Fluorescence microscopy
f. Raman spectroscopy

Lastly, the purification and characterization of the esterification products were done using TLC, FTIR:ATR and NMR to confirm the formation of desired ester. The statistical analysis was conducted by using IBM SPSS version 20.0 software.

1.5 Significance of Study

Converting the OPFL into multifunctional solid supports may prove useful and allow the maximum utilization of oil palm tree which is abundant throughout Malaysia. Significantly, this study also proposing an alternative usage of OPFL instead of being dump into the environment as well as disposing through open burning which related to the seasonal environmental problem i.e. haze. Moreover, the employment of current chemical method in production of butyl butyrate can be replaced by an alternative method using CRL/CS-NC biocatalyst *viz*. the use of lower reaction temperature that indirectly increase the production yield of butyl esters.

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