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Optimization parameters for lipase immobilization: effects of time, temperature and cross-linker concentration

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GRAPHICAL ABSTRACT

Immobilized CRL-CS/NC beads

ABSTRACT

In view of the shortcomings associated with the chemical route to synthesize butyl butyrate *viz.* harsh reaction conditions, use of acid catalyst and liberation of unwanted by-products, the development of an alternative biotechnological technique by immobilizing *Candida rugosa* lipase (CRL) onto support has been proposed and may prove beneficial. In this study, nanocellulose (NC) was successfully extracted from oil palm fronds leaves (OPFL) by combination of different chemical treatments such as bleaching, alkaline treatment and acid hydrolysis. The extracted NC was used as nano-filler in the development of stable chitosan (CS)/NC support to covalently immobilize CRL. The immobilization efficacy of CRL onto CS/NC support was evaluated to obtain the optimum conditions for immobilization time, temperature and concentration of cross-linker. The developed CRL-CS/NC may prove advantageous and appears to be a promising substitute to the homogenous acid catalyst as it is more environmental friendly for the synthesis of butyl butyrate.

Keywords: Candida rugosa lipase, nanocellulose, butyl butyrate, immobilization protocol.

1. INTRODUCTION

Butyl butyrate is well-known for its sweet fruity flavor of pineapple used especially for food flavoring, beverage and pharmaceuticals industry [1]. Conventionally, butyl butyrate is prepared using a chemically catalyzed esterification route which is known for various drawbacks [2]. However, such method is still preferred by manufacturers as it allows fast production of large volumes of the ester hence able to meet the high demand for such compound by the flavoring and fragrance industries [3].

In view of undesirable effects of synthesizing butyl butyrate, the biotechnological approach of employing enzymes as the alternative biocatalyst for producing this commercially valuable commodity is therefore, suggested. Among the biotechnologically and commercially relevant enzymes, lipases (triacylglycerol ester hydrolases EC 3.1.1.3) from *Aspegillus sp.*, *Rhizomucor miehei* and *Candida rugosa* (CRL) have been regarded as versatile enzymes to catalyze hydrolytic as well as esterification processes [4]. More importantly, their application for such purposes may prove beneficial in terms of averting energy intensive processes while being more environmentally friendly and safe. Moreover, the ambient conditions for which lipases catalyze can prevents product degradation [4], [5]. However, the high susceptibility of CRL to prematurely deactivate under prolonged harsh industrial conditions of extreme temperature and pH as well as demonstrating low activity in organic solvents has somewhat limited its use [6]. The popular way of averting this problem is by immobilization of the lipase onto an appropriate support matrix; such technique has been proven to impart higher operational stability and consequently improving productivity as well as stability and activity of the enzyme [7], [5]. In this context, CS was selected for its biocompatibility, non-toxicity, biodegradability as well as for having high concentration of amino and hydroxyl groups that facilitates the CRL attachment. Reinforcing nanocellulose (NC) fibers were incorporated into the CS polymer matrix to overcome the inherent low mechanical properties of the pure CS [8].

This research will emphasize on optimization of the immobilization of CRL onto the CS/NC hybrid support to improve efficacy and activity of the CRL to catalyze high yield production of butyl butyrate. Immobilization factors that can affect the efficacy of an immobilization protocol include time, temperature and cross-linker concentration. Maximum intensity of the enzyme-support reaction must be reached to get maximum immobilization efficacy.

2. EXPERIMENTAL

The experimental was divided into three main stages which include extraction of nanocellulose, optimization of the immobilization protocol, and finally the purification and analysis of the enzymatic reaction product i.e. butyl butyrate. In order to extract the NC, OPFL was sorted, cleaned, cut, and dried into finer sizes before undergoes different chemical treatments *viz.* bleaching, alkali treatment and acid hydrolysis. The raw OPFL and extracted NC were then characterized using Fourier-Transform Infrared (FTIR), X-Ray Diffraction (XRD) and Field Emission Electron Scanning Electron Microscopy (FESEM) to study the biochemical and morphological characteristics of the extracted NC. In the second stage, assessment of the

immobilization of CRL was carried out by stirring the CS/NC beads in a 20mL of free CRL (10 mg/mL) at different immobilization time, temperature and glutaraldehyde concentration. The suitability of each step of the immobilization protocol was based on the yield of butyl butyrate catalyzed by the CRL-CS/NC biocatalysts. Later in stage three, the developed CRL-CS/NC was used to synthesize butyl butyrate. The reaction mixtures were pooled and purified by distillation technique. The pure butyl butyrate obtained was monitored by thin layer chromatography and confirmed using FTIR and Nuclear Magnetic Resonance (NMR).

3. RESULTS AND DISCUSSION

3.1. Extraction and Characterization of NC

The extraction of NC from OPFL was carried out using a combination of different chemical treatments *viz.* bleaching, alkaline treatment and acid hydrolysis. The resultant NC was obtained after centrifugation of the NC suspension in distilled water and were isolated and lyophilized to afford the NC in the form of whitish fibers.

. The characterization of NC were assessed using FTIR, XRD and FESEM. Figure 1 shows the FTIR spectra for raw OPFL, cellulose and NC. A peak at 1721.6 cm^{-1} in the raw OPFL can be assigned to the stretching of C=O. Two peaks that emerged at 1628.74 cm^{-1} and 1533.4 cm^{-1} were contributions from the vibrational stretchings of the C=C bond of aromatic rings in lignin. A band at 1248.7 cm^{-1} was due to the out of plane stretching of C−O−C of aryl-alkyl ether group in lignin. It was apparent the vibrational peaks of both the C=O and C=C functionalities were decreased (**Figure 1b** and **1c**). The peaks that emerged between 2894 cm^{-1} – 2921 cm^{-1} in all spectra were the result of the stretching vibrations of C−H. Notably, the bands at 1628 cm^{-1} – 1635 cm^{-1} (**Figures 1a, 1b** and **1c**) can be allotted to the bending of O−H groups. A broad band in the region of 3500 cm^{-1} – 3300 cm^{-1} in all three spectra can be associated with the vibrational stretching of O−H in the cellulose molecules. The characteristic vibrational stretchings observed in the spectra of the raw OPFL, cellulose and NC within wavenumber regions of 1021 cm^{-1} − 1033 cm^{-1} and 664 cm^{-1} – 665 cm^{-1} can be assigned to the stretching vibrations of the pyranose ring and glycosidic linkages between glucose units in cellulose, respectively.

The XRD pattern (**Figure 2**) for raw OPFL and NC presented a monoclinic sphenodic structural characteristic of Cellulose-I polymorph which strongly suggested the pretreatment of raw OPFL does not significantly influence the natural Cellulose-I structure. From the graph, it can be seen that intensities of peaks at 2θ = 15˚and 2θ = 22˚were slightly increased after the chemical pretreatments. NC showed a good definition that reflected in the greater lattice peak which indicates that NC is more crystalline than the raw OPFL. The crystallinity indices for both raw OPFL and NC was 23.4% and 70.2% respectively. The higher percentage of crystallinity seen in the NC was caused by the breaking of cellulose glycosidic group into two anhydroglucose units. This liberates most of amorphous parts of the cellulose into the acid mixture, leaving only the crystalline parts of cellulose.

The morphology of NC using FESEM is presented in **Figure 3**. From the micrographs, it can be seen that the NC have needle like-shaped structures which affirms the presence of an entangled network of the NC fibers with diameters in the range of 21-25 nm after undergoing several steps of chemical treatment.

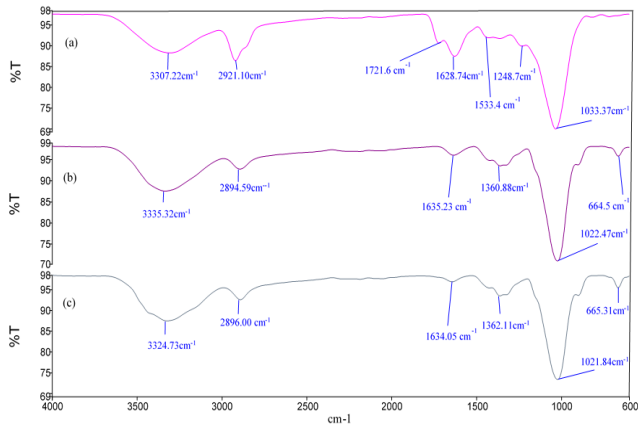

Figure 1 FTIR spectrum of a) raw OPFL, b) cellulose and c) NC

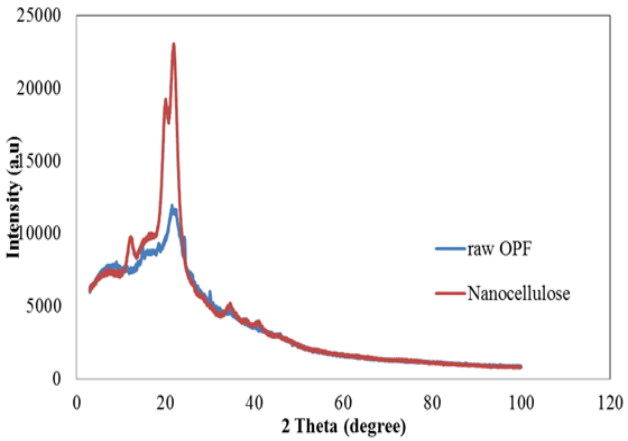

Figure 2 XRD patterns of raw OPFL and NC

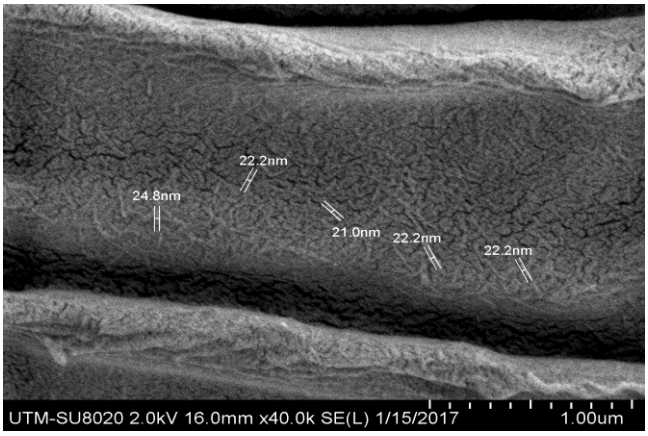

Figure 3 FESEM image of NC at 40,000x magnification

3.2 Optimization of Immobilization Protocol

3.2.1 Effect of Immobilization Duration

The duration for immobilizing CRL were assessed for durations of 4, 8, 12, 16 and 24 h. The effect of various immobilization durations on the efficacy of CRL-CS/NC and the percentage conversion of butyl butyrate within 3 h of reaction is depicted as follows:

Table 1 Effect of various immobilization durations on the efficacy of CRL-CS/NC to synthesize butyl butyrate

Immobilization duration (h)	**4**	**8**	**12**	**16**	**24**
Volume of buffer (mL)	20.00	20.00	20.00	20.00	20.00
Initial CRL concentration (mg/mL)	0.30	0.30	0.30	0.30	0.30
Final CRL concentration (mg/mL)	0.06	0.06	0.12	0.04	0.04
Difference concentration	4.80	4.80	3.60	5.16	5.16
Mass of support (g)	1.00	1.00	1.00	1.00	1.00
Immobilized CRL protein (mg/g)	**4.80**	**4.80**	**3.60**	**5.16**	**5.16**

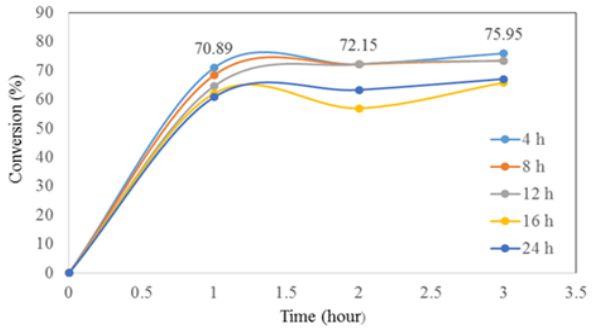

Figure 4 The effect of immobilization time on the percentage conversion of butyl butyrate within 3 h of reaction. [Temp: 25˚C, molar ratio: 2:1, enzyme loading: 3 mg/mL, speed of agitation: 200 rpm]

It was clear that immobilization duration for up to 4 h was relatively sufficient to immobilize an acceptable amount of the CRL onto the surface of the CS/NC supports with 75.95% conversion of butyl butyrate. This affirmed that a 4 h immobilization was optimum time to produce highly efficient CRL−CS/NCs. The higher activity of the CRL-CS/NC was presumably due to the correct positioning and configuration of CRL molecules. Although, the CRL-CS/NC prepared *via* the 16 h and 24 h immobilization durations showed higher CRL contents (5.16 (mg/g)), the percentages of the produced butyl butyrate was not proportional to the higher deposition percentages of the lipase. Notably, the activity of the CRL-CS/NCs obtained from longer immobilization duration (12, 16 and 24 h) were lower (62−55 % butyl butyrate) than the CRL-CS/NCs obtained from a 4 h immobilization time (75.95%). Such outcome seen can be explained by the fact that after the formation of the first linkages on the surface of CS/NC supports, the CRL itself starts to lose its flexibility resulting in the difficulty for the formation of new bonds. The conformational changes adopted by a lipase upon binding to the surface of the support would cause its lipase structure to become excessively rigid and thus, results in the decline of activity.

3.2.2 Effect of Temperature

The effect of immobilization temperature on percentage conversion of butyl butyrate catalyzed by the CRL-CS/NC was performed at temperatures from 4 ˚C, 25 ˚C, 37 ˚C, 50 ˚C and 70 ˚C. The efficacy of the CRL-CS/NC and the percentage of butyl butyrate conversion is shown in **Table 2**.

Table 2 Effect of temperature on the efficacy of CRL-CS/NC to synthesize butyl butyrate

Temperature (˚C)	**4˚C**	**25˚C**	**37˚C**	**50˚C**	**70˚C**
Volume of buffer (mL)	20.00	20.00	20.00	20.00	20.00
Initial CRL concentration (mg/mL)	0.43	0.37	0.43	0.43	0.43
Final CRL concentration (mg/mL)	0.09	0.06	0.03	0.03	0.03
Difference concentration	6.80	6.20	8.00	8.00	8.00
Mass of support (g)	1.00	1.00	1.00	1.00	1.00
Immobilized CRL protein (mg/g)	**6.80**	**6.20**	**8.00**	**8.00**	**8.00**

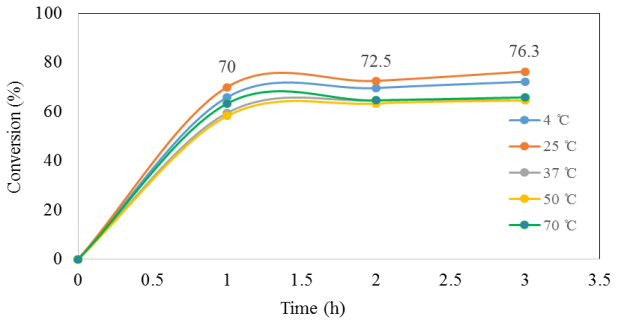

Figure 5 The effect of immobilization temperature on the percentage conversion of butyl butyrate within 3 h of reaction. [Molar ratio: 2:1, enzyme loading: 3 mg/mL, speed of agitation: 200 rpm, immobilization time: 4 h]

Results illustrate the loading of CRL was lower when low immobilization temperatures, 4 ˚C and 25 ˚C, were used, corresponding to 6.8 and 6.2 mg/g compared to the higher immobilization temperature. Maximum conversion of the ester was achieved when the immobilization of CRLs onto the CS/NC supports was carried out at 25 ˚C while the lowest amount of butyl butyrate was obtained at 50 ˚C. However, as the immobilization temperature was further increased, the activities of the CRL-CS/NCs were reduced, though the concentration of CRL on the CS/NC supports was higher at 8.00 mg/g for temperatures, 37 ˚C , 50 ˚C and 70 ˚C. The highest conversion of butyl butyrate achieved in the optimum temperature (25˚C**)** might be due formation of sufficient network of hydrophobic and Van der Waals interactions as well as hydrogen bonds between the CRL and CS/NC support. In contrast, the generally low CRL activity at temperatures, 50˚C and 70˚C was likely due to thermal inactivation of CRL at high temperatures. This subsequently leads to the enzyme losing their catalytically competent form. Thus, the investigation found the optimum immobilization temperature for immobilizing CRL onto the CS/NC supports was 25˚C.

3.2.3 Effect of Glutaraldehyde Concentrations

The effect of various concentrations of cross-linker on the immobilization efficacy of CRL onto the CS/NC beads was carried out in different concentrations of glutaraldehyde from 0.3%, 0.5%, 0.75%, 1.0% and 3.0% (v/v). Results of the efficacy of the CRL-CS/NC and the conversion of butyl butyrate are shown in **Table 3**.

Table 3 Effect of concentration of glutaraldehyde on the efficacy of CRL-CS/NC to synthesize butyl butyrate

Concentration of glutaryaldehyde (v/v%)	**0.30**	**0.50**	**0.75**	**1.00**	**3.00**
Volume of buffer (mL)	40.00	40.00	40.00	40.00	40.00
Initial CRL concentration (mg/mL)	0.43	0.43	0.43	0.43	0.43
Final CRL concentration (mg/mL)	0.08	0.07	0.07	0.04	0.08
Difference concentration	14.00	14.40	14.40	15.60	14.00
Mass of support (g)	2.00	2.00	2.00	2.00	2.00
Immobilized CRL protein (mg/g)	**7.00**	**7.20**	**7.20**	**7.80**	**7.00**

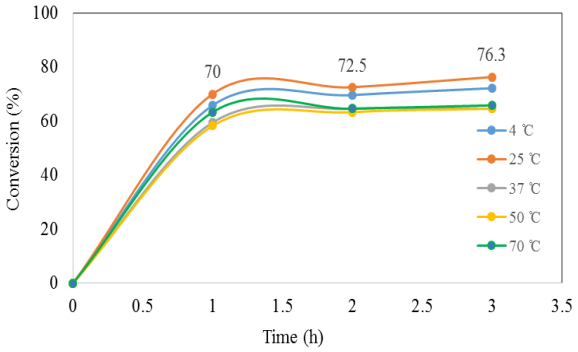

Figure 6 Effect of various concentrations of glutaraladehyde on the percentage conversion of butyl butyrate within 3 h of reaction. [Temp: 25˚ C, molar ratio: 2:1, enzyme loading: 3 mg/mL, speed of agitation: 200 rpm, immobilization time: 4 h]

It was apparent that a 1 % (v/v) concentration of glutaraldehyde was favorable resulted in the highest loading of CRL at 7.8 mg/g. Utilization of low and high concentrations of glutaraldehyde gave the lowest CRL loading (7.00 mg/g). But the study was not interested in this outcome as a high enzyme loading does not always produce good enzyme activity. Assessments on the esterification reactions showed that as the concentration of glutaraldehyde increase, the activity of CRL-CS/NC exhibited an ascending tendency throughout the 3 h incubation time. Using a 0.30% concentration of glutaraldehyde gave the best immobilization effect that resulted in the CRL-CS/NCs showing the highest activity at 75.0% ($p < 0.05$) conversion of butyl butyrate. This indicates a 0.30% v/v of glutaraldehyde was optimum for immobilizing sufficient amounts of the CRL on to the CS/NC supports, without reducing activity of the CRL. In contrast, the percentages of the synthesized butyl butyrate were among the lowest when high concentrations of glutaraldehyde were used. The study believes the use of high concentrations of glutaraldehyde would result in the excess binding of CRL molecules on the surface of the support that increases the steric hindrance and consequently interfere with the access of substrates into the active sites of the CRL-CS/NCs.

3.3 Purification and analysis of butyl butyrate

In this study, to confirm butyl butyrate was the main product of the esterification reactions catalyzed by the CRL-CS/NC, the reaction mixtures were pooled and purified by distillation technique and was identified using Thin Layer Chromatography (TLC), Fourier-Transform Infrared (FTIR) and Nuclear Magnetic Resonance (NMR).

3.3.1 Thin Layer Chromatography (TLC)

The purified butyl butyrate was monitored over a TLC plate after undergoing neutralization, liquid-liquid extraction and distillation. The two spots observed on the TLC profile (**Figure 7**) indicates the treatments produced the purified butyl butyrate (Rf = 0.78).

3.3.2 Fourier-Transform Infrared (FTIR) Analysis

The FTIR spectrum of the purified butyl butyrate produced by CRL-CS/NC is represented in **Figure 8**. The absorption band at 2956.38 cm^{-1} refers to asymmetric and symmetric stretching vibrations of C-H sp^3 and the characteristic absorption band at 1729.79 cm^{-1} could be assigned to C=O of ester. The results affirm that butyl butyrate was successfully synthesized by the CRL-CS/NC. The peak assigned at 1179.96 cm^{-1} – 1085.73 cm^{-1} can be allotted to the stretching vibration of C-O ester. A peak that appeared at 1457.51 cm^{-1} is the contribution of the stretching vibrations for the sp^3 of –C−H (alkane) bonding. A slightly broad peak in the region of 3200 cm^{-1} was likely due minute amounts of butanol which indicates the purification protocol *via* the use of distillation was not capable to remove all traces of butanol.

3.3.3 Nuclear Magnetic Resonance (NMR) Analysis

The ^1H NMR spectra of butyl butyrate with peak assignment is represented in **Figure 9**. The important peak that corresponded to the structure of butyl butyrate was observed at $\delta = 3.97$ ppm assigned to $-CH_2OC=O-$.An additional peak with a chemical shift, $\delta=3.54$ ppm was consistent with the $–CH_2OH$ that shows the presence of a trace amount of butanol, hence agrees well with the weak, slightly broad peak at 3200 cm^{-1} in the FTIR spectrum.

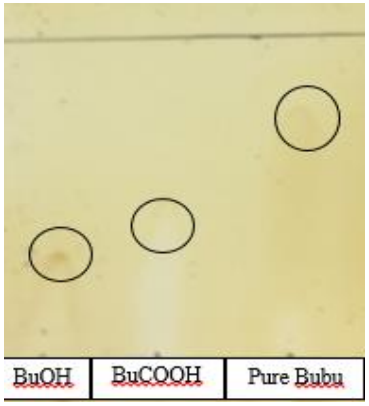

Figure 7 TLC plate for pure butyl butyrate

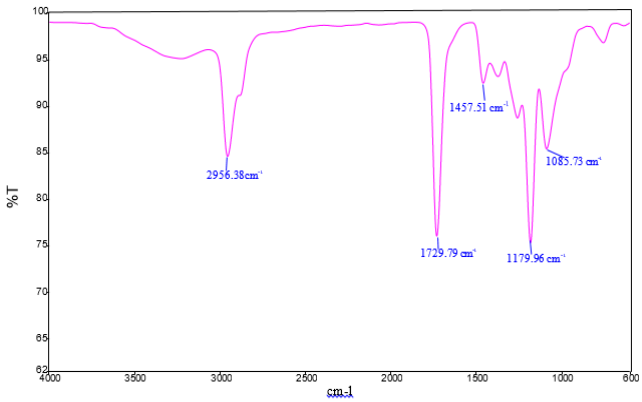

Figure 8 FTIR spectra of purified butyl butyrate

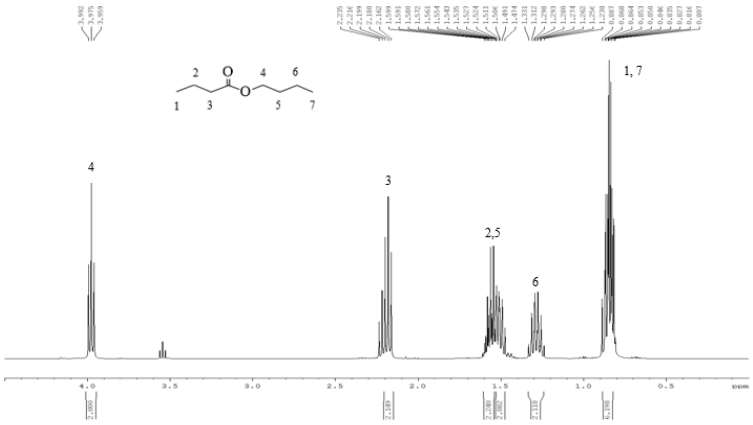

Figure 9 Spectrum of ^{1}H NMR for pure butyl butyrate

4. CONCLUSION

This study has attempted to describe the optimal conditions of CRL-CS/NC for synthesizing butyl butyrate. NC was successfully extracted from raw OPF leaves and the morphological and physicochemical characterizations of the extracted NC successfully carried out using FTIR, FESEM and XRD. The results confirmed that the NC from the pretreated OPF leaves was a good source of NC, which crystallinity index as high as 70%. The study found the optimum condition that gave the highest percent conversion of butyl butyrate as high as 76.3% ($p < 0.05$) within 3 h of reaction using an immobilization time of 4 h at 25˚C ($p < 0.05$) using 0.30% (v/v) concentration of the crosslinker ($p < 0.05$).The product of the enzymatic synthesis of butyl butyrate catalyzed by the CRL-CS/NC were successfully purified and characterized. Results of analyses by TLC, FTIR and NMR strong indicated butyl butyrate was the product of the enzymatic esterification reaction.

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