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Research paper

Crude Glycerol for Monoolein Production using Nanotubes Supported Lipase

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Abstract

A growing demand for biofuels as the alternative for fossil fuels' extinction has produced abundance amount of crude glycerol as by-product in biofuel industries. Utilization of crude glycerol into a value-added product such as monoolein has been the focus for this present work. Instead of using free catalyst, the enzyme (lipase) used for this study was immobilized onto the halloysite nanotubes. The immobilized enzyme was characterized using TEM, BET and FTIR analysis. Further, the effect of time, temperature, agitation rate, and substrate molar ratio on monoolein production and fatty acid conversion were studied between free lipase and immobilized lipase using GC analysis. The best working conditions for immobilized lipase to produce monoolein was at 50°C, with 8 to 1 ratio of glycerol to oleic acid, agitated at 250 rpm for 18h. The amount of monoolein produced using immobilized lipase was highly comparable with free lipase, with ability to be reused and thermally stable at higher temperature.

Keywords: *Crude glycerol; Halloysite nanotubes; Immobilized enzyme; Lipase; Monoolein*.

1. Introduction

Glycerol is a hygroscopic, viscous liquid which is non-toxic and well acknowledged as reactive molecule that able to undergo a wide range of reactions to form aldehyde, amine, ester, and ether. Unlike purified and commercial grade glycerol, crude glycerol tends to possess slight impurities such as ash, soaps, fatty acids, glycerides, and solvents, where most of the time do not consumed the major content of glycerol. This crude glycerol can be produced profusely from saponification and hydrolysis reaction in oleochemicals, and transesterification reaction in biodiesel production [1-3]. Rising concerns over depletion of fossil fuel reserves in the near future has urged industries and researchers to opt for alternative energy sources and this biofuel has enticed many for its renewable and environmentally friendly fuel. An upsurge in biofuel production has eventually generated a vast amount of byproduct, which is crude glycerol [3]. The impacts it has on social environment and difficulties face by the industries to dispose this amassed crude glycerol gave impetus to turn it into value-added opportunities. Crude glycerol largely uses as fermentation feedstock for various production, such as butanol, fumaric acid, humectant (1,3-propanediol), and omega-3 polyunsaturated fatty acid (docosahexaenoic acid (DHA)) and also been used to produce monoglycerides (MG) [4-9].

Direct esterification of glycerol with fatty acids and interesterification (glycerolysis) of glycerol with fats or oils are two common routes use to produce MG. Ability of monoglycerides to be manipulated into more lipophilic or more hydrophilic molecules has made it a major ingredient in food, cosmetics, and pharmaceutical industries [10-11]. Interesterification is claimed as more economical for its low-priced substrate (fats) compared to pure fatty acids used in direct esterification. Yet, instead of producing a specific type of MG as in direct esterification, interesterification is known to pro-duce different types of MG while also having the difficulty to ensure complete solubility of glycerol in fats. Since specific and high purity of monoglycerides are required to obtain food or pharmaceutical grade, this present work has opted to produce the monoolein via direct esterification [12-13].

Generally, the downstream stage is dependent to the chosen upstream process, meaning; the good upstream processes will ease the latter downstream stage, and this is what the industries have been aiming for. The upstream phase for monoglyceride production will involve from the selection of reactants or substrates, catalysts, operating systems and the optimum conditions or parameters of reaction to synthesize high yield of monoglyceride. Instead of using chemical catalysts, which are deemed as non-eco-friendly, production of monoolein is being catalyzed by enzyme using non-solvent system. The use of lipase (*Candida rugosa*) to produce monoglycerides comes in varied designs; it can be free lipase or immobilized lipase on a wide range of supports. However, lipase is often not use in its free form because of its price, low stability, and not reusable. Immobilized lipase has been proved by many to attain more ad-vantages over its free form, for instance, improved in stability to-wards heat, solvents, or pH, ease the handling and separation, applicable to be used in continuous fixed-bed reactors and most importantly, this costly enzyme can be recovered and recycle [14-15]. Parallel with industrial purposes, the supports use should exhibit appropriate porosity depending on the size of target enzyme, suitable hardness and density [16]. For this present work, natural clay halloysite nanotubes (HNT) is chosen for its low-priced, larger surface area and pore diameter that appropriate enough to accommodate lipase. This tubular HNT has uniquely different charges on its external and internal surface which able to support molecules on both sides with functionalization accordingly to charged molecules. HNT portrayed as a potential nanocarrier support for its successful immobilization, including for drugs, proteins, and anticorrosion agents [17-19].

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This study was designed to establish a set of reaction parameters that affected the monoolein production and fatty acid conversion by using the halloysite nanotubes supported lipase as the biocatalyst compared with the free form of lipase. Characterization studies were done to prove the immobilization of lipase on the HNT. GC-FID was used to determine the monoolein, diolein, triolein and oleic acid qualitatively and quantitatively. Factors that have been identified to influence the monoolein synthesis were studied, such as time, temperature, agitation rate, and substrate molar ratio.

2. Materials and method

2.1. Materials

Crude glycerol was kindly provided by a local biodiesel production plant, Lereno Sdn. Bhd. Lipase from Candida rugosa, halloysite nanotubes, standards for monoolein, diolein, triolein and all chemicals used for immobilization and GC analysis were purchased from Sigma-Aldrich. Oleic acid was purchased from R & M Chemicals, UK.

2.2. Lipase immobilization

HNT was physically incorporated with lipase onto its lumen via simple vacuum entrapment method [20]. C. rugosa was mixed with HNT at 1:1 ratio. After the addition of 10 ml of pH 7 phosphate buffer, the mixture was vortexed until well-dispersed. The sample was placed into a vacuum and the pressure was reduced to 9 in Hg for 30 min at 40°C. After 30 min, the pressure was left to ambient before being agitated and repeated for another two vacuum cycles, to allowing the substitution of nanotubes' inner air with enzyme.

2.3. Characterization studies

The size and morphology of pristine HNT and HNT after immobilization of enzyme were determined by transmission electron microscopy (TEM).
Surface characterization of HNT was carried out at liquid N2 temperature of 77K and specific surface area of samples SBET, was determined using multipoint Brunauer-Emmett-Teller (BET) method, while Barrett-Joyner-Halenda (BJH) method was used to measure the total volume of pores, Vpore. Infrared transmission spectra were obtained using Fourier Transform Infrared Spectroscopy (FTIR) for enzyme, HNT, and enzyme-HNT.

2.4. Esterification

The esterification reaction was carried out in 50 ml screw-capped flask accordingly to the factors investigated. Crude glycerol and oleic acid were added into the flask with studied molar ratio of crude glycerol to oleic acid (4:1 to 10:1) and 0.5 g of immobilized lipase was added into the mixture. The reaction mixture was incubated at varied temperature from 30°C to 50°C at a range of agitation rate (100 to 250 rpm) for period of time (6h to 24h). The obtained reaction mixture was centrifuged at 4000 rpm for 10 min and the upper layer of resulting oil was collected for GC analysis.

2.5. GC analysis

0.1 ml of sample was eluted with 1 ml chloroform and to this, 0.1 ml of internal standard n-tetradecane was added. Samples were analyzed using GC with flame ionization detector. SGE BPX5 (5%-Phenyl Polysilphenylene Siloxane) non-polar capillary column and temperature programmed was set according to the previous study done by Bruschweiler and Dieffenbacber in 1991 with slight modification [21].

Fatty acid conversion was calculated based on the amount of lauric acid converted into products, as given in Equation 1.

$$\text{Fatty acid conversion (\%)} = (A - B) / A \times 100\ \% \quad (1)$$

Where, A and B is the peak area for oleic acid before and after ester-ification, respectively.
Monoolein contents was quantified from standard calibration curves and expressed in % following Equation 2.

$$\text{Monoolein (mg/ml \%)} = \text{Monoolein}/(\text{Monoolein} + \text{Diolein} + \text{Triolein} + \text{Oleic Acid}) \times 100 \quad (2)$$

3. Results and discussion

3.1. Characterization studies

The images of TEM show that HNT have visibly hollow tubular structure with open ends (Fig. 1a and b). The nanotubes had an average length of 0.2–2 μm, an outer diameter in the range of 30–100 nm and lumen diameter were approximately between 10 and 28 nm, while the wall thickness is around 5-8 nm.

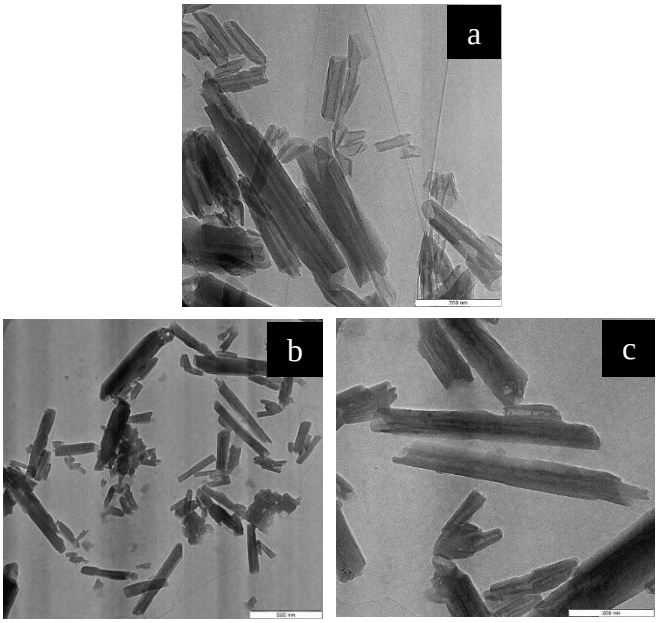

Fig. 1: TEM images of (a) pristine HNT, and (b) – (c) HNT infused with lipase.

TEM scanning images (Fig. 1b and c) revealed the immobilization of lipase on the nanotubes with the dark spots on the surface and inside the lumen attributed to the enzyme that attached on the outer surface and some that infused into the lumen.

Table 1: Porous structure parameters of pristine HNT and HNT after immobilization with lipase.

Samples	[a] S_{BET} (m^2g^{-1})	[b] V_{pore} (cm^3g^{-1})
Pristine HNT	62.85	0.2572
Lipase-HNT	28.49	0.1703

[a] BET (Brunauer, Emmett, and Teller) method
[b] BJH (Barrett, Joynes, and Halenda) method

Textural characterization of HNT as an adsorbent before and after lipase immobilization were studied based on N_2 adsorption-desorption analysis and summarized in Table 1. The pore size of HNT determined by BJH method was in the range of 16 – 24 nm, confirming the mesoporous structure possessed by halloysite nanotubes. The pore size of nanotubes would provide favorable conditions for access and immobilization of lipase since the lipase

size is approximately around 5.1 nm [20], which is smaller than the pore size of HNT. Before immobilization, specific surface area (S_{BET}) of HNT was measured to be 62.85 m^2g^{-1} and after immobilization, it was observed to decrease to more than 50% from the pristine; 28.49 m^2g^{-1}. The total pore volume was also reduced after the immobilization. The reduction in both S_{BET} and V_{pore} has proved that the loading of lipase onto the HNT was successful.

FTIR spectra in **Fig. 2** show comparison of free lipase, pristine HNT, and immobilized lipase on HNT. **Table 2** listed the characteristic bands involved in the spectra with their assigned functional groups. Spectra of free lipase revealed two obvious characteristic bands at 1649-1647 and 1539-1534 cm^{-1} that are both attributed to amide group presents in the polypeptide chain of protein [22-23]. Pristine HNT exhibited spectra of broad bands at between 3693-3691 and 3621-3619 cm^{-1} that are ascribed to the bending and stretching vibrations of OH bonds. Not only the characteristic bands of HNT remained unchanged without any shift, but the appearance of similar bands in the spectra of free lipase proved that the lipase is able to preserve the essential feature of its innate structure on HNT [23-24].

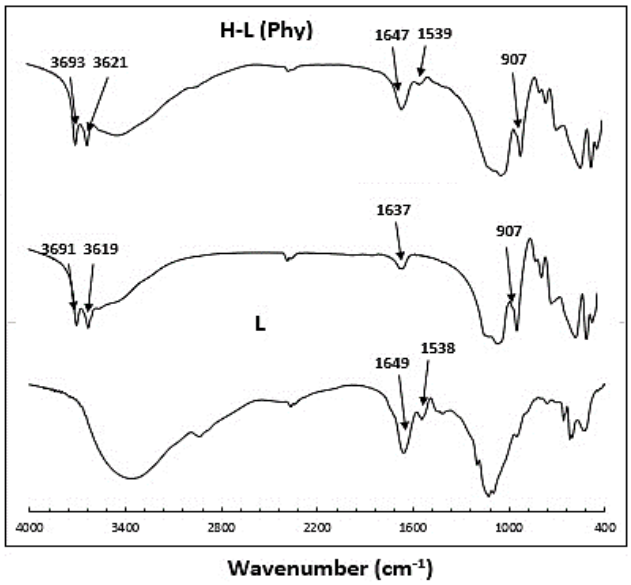

Fig. 2: FTIR spectra of (L) Lipase; (H) HNT; (H-L(Phy) HNT-Lipase physically immobilized.

Table 2: Characteristic bands of the IR for free lipase, HNT and immobilized lipase on HNT.

Characteristic band (cm^{-1})	Functional group
3693-3691	O-H
3621-3619	O-H
1539-1534	C=O
1649-1647	C=O
1637	O-H
909-907	Al-O-OH

3.2. Factors influence monolaurin synthesis

3.2.1. Effect of reaction time

Different esterification reaction times allocated for the samples have shown varied percentage of monoolein produced. Fatty acid conversion for both catalysts (free and immobilized forms) revealed the same trends as the monoolein produced, except for the free lipase where at 12h, the conversion decreased even though the monoolein increased. Free lipase merely managed to increase the monoolein production slightly from 6h to 12h and after 24h, it outweighed the immobilized lipase. Prolonged reaction for free lipase to some extent has allowed the accumulation of product from the beginning. Starting from 6h of reaction time, monoolein produced by immobilized lipase has gradually growing till 18h of reaction time and beginning to plummet from 80% of monoolein to less than 20% as it reached 24h of reaction. At 24h of reaction, the immobilized lipase possibly has fully-fledged into multi-layered adsorption where the substrates could not reach to the catalyst anymore, thus lessen in monoolein produced.

3.2.2. Effect of temperature

Immobilized lipase showed a steady fatty acid conversion after 35°C, while free lipase abruptly decreased after 45°C. A range of temperatures carried out during the esterification has revealed that both immobilized and free lipase steadily increased in monoolein production when the temperature rose. However, at 50°C, the monoolein production catalyzed by free lipase decreased to almost 35% less than the previous production at 45°C and this was due to the protein denaturation. Contrary with immobilized lipase, monoolein showed its higher production when the reaction run at 50°C. This agreed with previous studies where they claimed that immobilization helps the enzyme to retain its functional form and exhibited thermal stability [15].

3.2.2. Effect of agitation rate

Reaction mixtures agitated at different rates showed less significant difference between both free and immobilized lipase. At the beginning more than 40% of monoolein were produced for both and as the agitation rate increased 150 rpm, the production reduced for free lipase and increased for immobilized lipase. Free lipase showed high monoolein production when reaction was agitated at 200 rpm. Immobilized lipase was noticed to slightly improve its monoolein production at 200 rpm and begun to rise tremendously when agitated at 250 rpm. Immobilized lipase favored when the mixture was forcibly agitated at the highest rate (250 rpm) because the contact area between substrates and catalysts would improve, thus allowed more substrate to reach catalyst.

3.2.2. Effect of substrate molar ratio

Substrate molar ratio was set from 4 to 1 till 10 to 1, where it was rude glycerol to oleic acid ratio and not vice versa. Excess glycerol is more attainable and preferable for its ability to force the reaction equilibrium to the right side, which is favoring esterification instead of hydrolysis [25-26]. Both free and immobilized lipase gave higher monoolein production when molar ratio reached 8 to 1. Beyond 8 to 1 ratio, both free lipase and immobilized lipase, showed a fall in monoolein production and this possibly due to surge of mixture viscosity, thus lessen the efficiency of reaction.

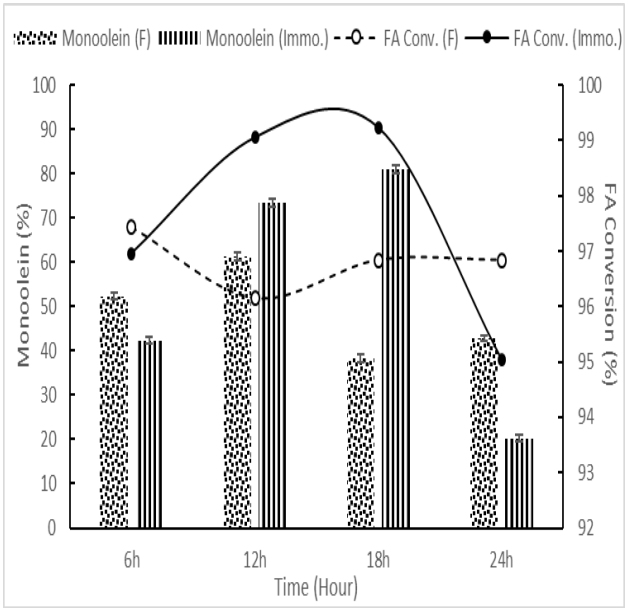

Fig. 3: Effect of different reaction times towards monoolein production and fatty acid conversion

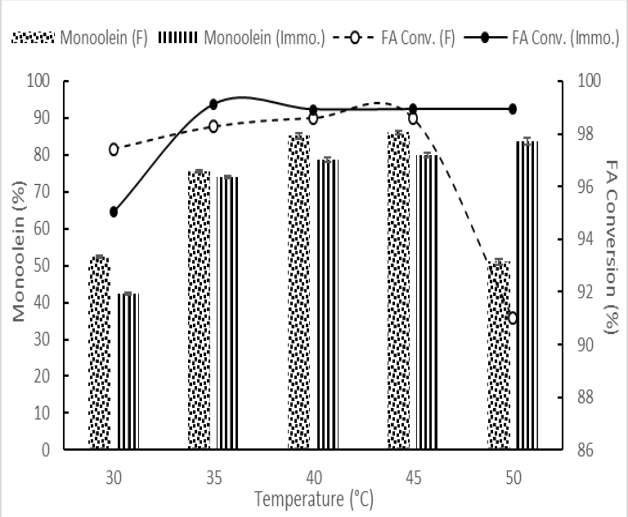

Fig. 4: Effect of different range of temperatures towards monoolein production and fatty acid conversion

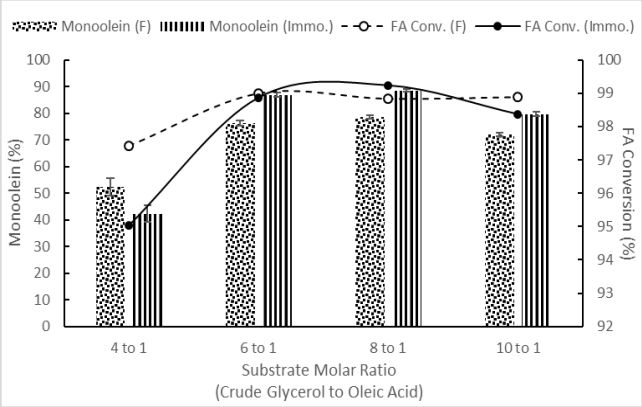

Fig. 5: Effect of different agitation rates during esterification towards monoolein production and fatty acid conversion

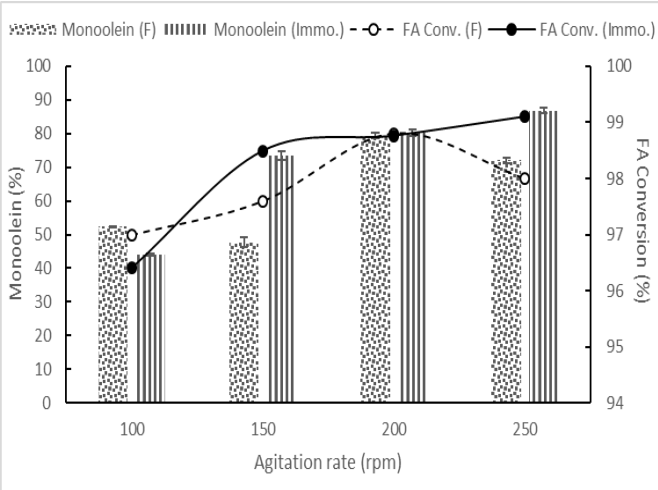

Fig. 6: Effect of four different substrate molar ratios towards monoolein production and fatty acid conversion
(F = Free lipase; Immo. = Immobilized lipase; FA Conv. = Fatty acid conversion)

4. Conclusion

The esterification reaction to produce monoolein from crude glycerol under biocatalyst-driven was found to slant towards the immobilization approach instead of its free form. Ability of immobilized lipase to produce fairly high and comparable amount of monoolein with free lipase approved that the HNT as a promising nanocarrier for lipase without alter or dysfunctional the active form enzyme. Despite of not being enclosed in this study, immobilized lipase still one-steps ahead than free lipase for its ability to be reused. Using immobilized lipase, monoolein production was obtained better at 8 to 1 ratio of crude glycerol to oleic acid, 50°C, agitate at 250 rpm for 18h.

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