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Preliminary Studies and Characterization of Oil Palm Frond Leaves Silica-based Bonded Lipase

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ABSTRACT

Despite the benefits of bio-based enzyme biocatalysts, high production-and separation from reaction mixture costs hinders their industrial applications. Whereas enzymes are usually immobilized on solid supports for improving stability, recovery and activity, there are concerns that the synthetic and non-biodegradable nature of the support materials could negatively impact the environment. Thus, there is a need for new enzyme supports to be prepared from sustainable and readily available biodegradable resources. This present study proposed the fabrication of low-cost support comprising co-precipitated magnetic nanoparticles, graphene oxide, and silica extracted from oil palm frond leaves (OPFL) for immobilization of *Candida rugosa* lipase (CRL). The support and immobilized lipase were characterized by Raman spectroscopy**,** atomic force microscopy, FESEM, and FTIR methods. Raman spectral data revealed that the GO was successfully synthesized from graphite. Atomic power and field-emission scanning micrographs confirmed the presence of CRL on the support's surface. The FTIR results showed amide bonds at 1390, 1500, and 1650 cm^{-1}, which corroborated the covalent bonded CRL on the support. The optimum condition to immobilize CRL onto the support was a 16 h immobilization time with pentanoic acid to ethanol molar ratio of 1:1. These conditions favored the highest protein loading of 15.17 ± 0.06 mg/g, and a good immobilization efficiency of 72.34 ± 0.64 %, which gave the highest ethyl pentanoate (EP) conversion of 74.46 ± 0.74 % (specific activity = 56.77 ± 1.42 µmol/min/g) than 48.75 ± 0.70 % for the free CRL. The findings conveyed that the developed support had adequately activated and stabilized the CRL for substantial production of EP and permitted the recovery of the immobilized lipase by induced magnetism.

Keywords: *Silica; Hybrid composite, Support matrix, Immobilized CRL, Esterification*

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INTRODUCTION

Bio-based enzyme catalysis has globally attracted the attention of academics and industry following numerous benefits such enzymes offer, including good selectivity and specificity. These enzymes are less energy demanding than other chemical catalysts, produce fewer side reactions and are more environmentally friendly [1]. However, their solubility in liquids limits their application because of their relatively high production cost and problematic separation from the reaction mixture and recycling. Amongst the enzymes, the *Candida rugosa* lipase is popular for catalyzing an array of biotransformation processes [2]. Soluble CRL is usually immobilized onto solid supports to address the above-said challenges [1-3].

The OPFL-silica is identified as a promising material for replacing the industrially produced, energy-intensive pyrogenic silica for enzyme immobilization [1]. Biosilica is a good support material because of its reactiveness, which stems from its amorphous nature alongside minute pore size and relatively high surface area. The silanol (Si-OH) groups on the silica surface can facilitate functionalization by chemical reactions with enzymes [4]. Likewise, the high surface area of the graphene oxide (GO) imparts high mechanical strength and offers a platform for high enzyme loading [5].

Additionally, incorporating magnetite nanoparticles (MNP) into the composite mixture may improve the support-bonded CRL properties. This is because of the minute size, low toxicity, and paramagnetic feature of the MNP, which could assist the separation of the support-bonded CRL from the media [6]. However, individual nanoparticles (MNP, GO, and SiO_2) tend to aggregate in the reaction medium when used separately as enzyme support. Thus, the study anticipates that a blend of GO, MNP, and SiO_2 could yield a novel ternary composite to sufficiently stabilize and hyperactivate the CRL [3]. Moreover, the lack of studies on the feasibility of ternary composites for activating immobilized lipases deserves further exploration to uncover their effectiveness in catalyzing industrially relevant reactions. It is worth indicating here that the viability of the bio-based MNP@GO/SiO_2 hybrid composite prepared from OPFL to activate and stabilize lipases remains unreported.

EXPERIMENTAL

Preparation of OPFL Silica and Magnetic Graphene Oxide

Fresh OPFL, after drying (60 ºC, 48 h), was milled and sieved (300 mesh). OPFL (50 g) in HCl (250 mL, 10 mM) was heat-treated for 2 h at 100 ºC and 250 rpm. Afterwards, the sample was rinsed in ultrapure water until a neutral pH was achieved. The treated sample was dried and calcined (600 ºC, 9 h). Then, 20 g of the calcined material was dispersed in 82 mL of NaOH (3.5 M), which produced an aqueous solution of sodium silicate (Na_2SiO_3) [7]. As described by Ranjbari et al. [8], Hummer's method was adopted for GO preparation. A mixture of 5.0 g of graphite and 2.5 g of $NaNO_3$ was added to 115 mL of cold H_2SO_4 (98 %, < 5 °C), then $KMnO_4$ (15.0 g) < 20 °C was added, followed by 10 mL of H_2O_2 (30 %) till the colour of the mixture changed to yellow.

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After 24 h ageing, the sample was rinsed with 100 mL dilute HCl (1.0 M) followed by ultrapure water until the rinsing waters reached a neutral pH. The suspension was air-dried for 72 h to obtain the graphene oxide (GO). MNP@GO was prepared by the chemical co-precipitation technique reported by Jacob et al. [3]. A previously sonicated GO solution (5 g/mL) was added to a 100 mL acetic acid solution that comprised $FeCl_2 \cdot 4H_2O$ (0.006 mol) and $FeCl_3 \cdot 6H_2O$ (0.013 mol) (molar ratio ~1:2). After stirring (500 rpm) in a nitrogen atmosphere, ammonia solution (20 mL, 35% w/v) was added to precipitate the MNP@GO, which was washed thoroughly in ultrapure water and methanol separately before drying (60 ^oC, 10 h). The chemical co-precipitation reaction was performed under the N_2 atmosphere to reduce oxidation.

Preparation and Modification of Hybrid Support

The method of Onoja and Wahab [6] was adopted to prepare the core-shell MNP@GO/SiO_2 hybrid composite. The SiO_2 layers were grown on the surface of the MNP@GO composite. The MNP@GO/SiO_2 hybrid composite was then modified with 3-aminopropyl ethoxy silane (A) and glutaraldehyde (Gl), following the method described by Elias et al.[9] to afford the support matrix (MNP@GO/SiO_2-A-Gl). The steps for preparing the ternary support matrix in this study are depicted in Figure 1.

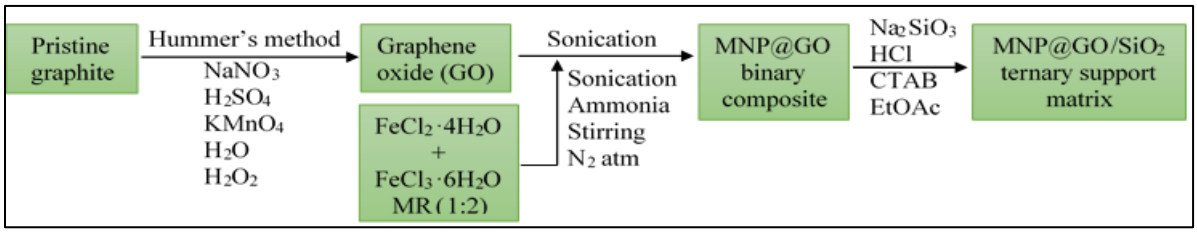

Figure 1: Typical route for the preparation of MNP@GO/SiO_2 ternary support matrix

Immobilization of CRL on the Support Matrix

Before immobilizing CRL on MNP@GO/SiO_2-A-Gl support, 10 mg/mL of binder-free CRL in sodium phosphate buffer solution (PBS: 100 mM, pH 7.0) was prepared. Then, the MNP@GO/SiO_2-A-Gl (0.5 g) was stirred in the CRL solution (20 mL) for 8–24 h at 4 h intervals. The mixture contained toluene (5 mL), crucial for activating the CRL [3]. After magnetic recovery, MNP@GO/SiO_2/CRL was washed, dried in a desiccator (3 h), and stored at 4 °C for further use. According to the Bradford method, protein content in CRL solution was determined using bovine serum albumin (BSA) as the standard, as described by Hussin et al. [10]. A UV-Vis spectrophotometer (UV-1800, Shimadzu, Toyoko, Japan) was used to record the absorbance at 595 nm in PBS (100 mM), while the Bradford reagent was used as a blank. The amount of the CRL bonded to the MNP@GO/SiO_2-A-Gl support described as protein loading (PL) was calculated by Equation 1.

$$\text{PL (mg/g)} = \frac{(C_i V_i - C_s V_s)}{m} \qquad \text{Equation 1}$$

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Where PL = CRL protein bonded to the MNP@GO/SiO_2-A-Gl (mg/g), C_i = protein content of initial CRL solution (mg/mL), C_s = protein content of supernatant CRL solution (mg/mL), V_i and V_s = initial volume and supernatant volume of CRL solutions (mL), respectively, and m = mass (g) of the support.

MNP@GO/SiO_2/CRL Catalyzed Esterification Production of Ethyl Pentanoate

The activity of the soluble CRL and immobilized CRL (MNP@GO/SiO_2/CRL) was assessed by colourimetric titration, consistent with a method reported in the literature [9], with slight modifications. Briefly, MNP@GO/SiO_2/CRL (1.5 mg/mL) was stirred in a mixture of pentanoic acid and ethanol (molar ratio 1:1) in 3.7 mL of n-heptane at 40 °C. An aliquot was withdrawn at an hour interval (1-3 h) and titrated with NaOH (0.05 M) using phenolphthalein as an indicator. The ester yield and specific activity for MNP@GO/SiO_2/CRL-catalyzed EP production were evaluated as the pentanoic acid percentage converted over the total pentanoic acid in the reaction mixture. The calculations were done using Equations 2 and 3 [9].

$$\text{EP conversion (\%)} = \frac{V_o - V_t}{V_o} \text{ x } 100 \qquad \text{Equation 2}$$

$$\text{Specific activity (}\mu\text{mol/min. g)} = \frac{(V_o - V_t) \text{ x } M_{NaOH} \text{ x } 10^3}{W \text{ x } t} \qquad \text{Equation 3}$$

Where V_o and V_t = initial volumes (mL) of NaOH at time (t = 0) and the volume (mL) of NaOH at each hour (t = t_1, t_2, t_3), M_{NaOH} = molar concentration (mol/L) of NaOH, W = weight (g) of protein in MNP@GO/SiO_2/CRL and t = time of esterification reaction (min).

Characterization of the Support and Immobilized CRL

Raman spectroscopy analysis used the LabRAM HR Evolution Spectrometer (Horiba Scientific, London, UK), equipped with an optical microscope. Briefly, a green laser (12 mW) at 532 nm was focused on the samples using a microprobe equipped with a 100x eyepiece to capture 20 scans. The spectra were acquired at a resolution of 4 cm^{-1}. The samples' morphology was inspected by the NanoWizard 3 Atomic force microscope (AFM) (JPK, France) to record the micrographs of the samples in tapping mode. The micrographs were processed with a JPKSPM Software 4.0 to calculate each sample's root mean square (RMS) roughness. Field-emission scanning electron microscopy (FESEM) micrographs were captured by the JEOL JSM-6700F (Japan) operated at an accelerated voltage and probed current of 5 kV and 10 µA, respectively. Fourier transform infrared: Attenuated total reflectance spectroscopy (FTIR-ATR) analysis was made on a spectrophotometer (Frontier 100, Perkin-Elmer, USA) in one-bounce ATR mode. The spectra were acquired in transmission mode for wavelengths between 400–4000 cm^{-1} at 16 scans and 4 cm^{-1} resolution.

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RESULTS AND DISCUSSION

Characterization of Immobilized CRL

The GO and MNP@GO were analyzed by Raman spectroscopy to determine their chemical compositions and structures, and Figure 1 depicts their spectra. Raman spectrum (Figure 1a) shows two prominent peaks at 1375 cm^{-1} and 1627 cm^{-1}, corresponding to the D and G bands of GO. The presence of these peaks indicates that the GO was successfully synthesized from graphite [3]. Aside from the D and G peaks located at 1341 cm^{-1} and 1590 cm^{-1} in the Raman spectra of MNP@GO (Figure 1b), the three new peaks located at 360, 508, and 668 cm^{-1} are ascribed to magnetite nanoparticles (MNP), as also seen in another similar study [9]. The reduced peak intensity and the higher energy shifts of the D and G bands plus new peaks on spectra of MNP@GO (Figure 1b) imply encapsulation of MNPs in the GO. The above interaction reduced restacking of the graphene layers and consequently prevented oxidation and agglomeration of MNPs. The result was seen to agree with the findings of Li et al. [11].

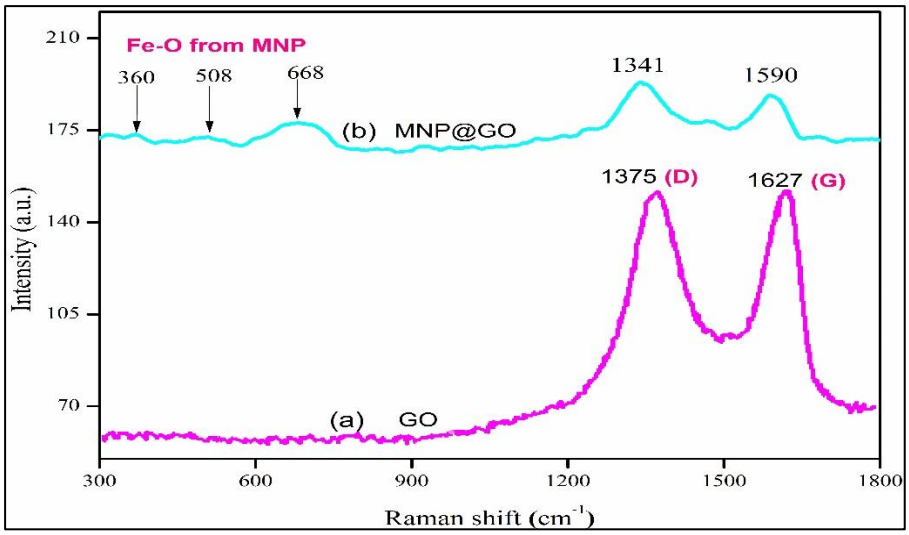

Figure 1: Raman spectra of (a) GO and (b) MNP@GO

Results of the AFM profile on MNP@GO, MNP@GO/SiO_2, and MNP@GO/SiO_2/CRL are presented in Figure 2. As shown, the MNP@GO, MNP@GO/SiO_2, and MNP@GO/SiO_2/CRL revealed their corresponding root mean square (RMS) roughness of 40.21 nm, 30.51 nm, and 29.06 nm for average heights (Hz) of 112.4 nm, 128.9 nm and 137.9 nm (Figure 2). As displayed in Figure 2, the RMS reduced from 30.51 nm to 29.06 nm for the MNP@GO after CRL immobilization. The result above agrees with increased Hz values for all samples (Figure 2) [12]. The collective decline in RMS and increasing values of Hz suggest the formation of molecular interactions between the MNP@GO/SiO_2 support and CRL as reflected in its homogenous surface [10], which suggests that the CRL was successfully immobilized on

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the support. The overall finding of the AFM topographic study proved that the technique was effective in detecting the presence of the enzyme on the support surface after immobilization.

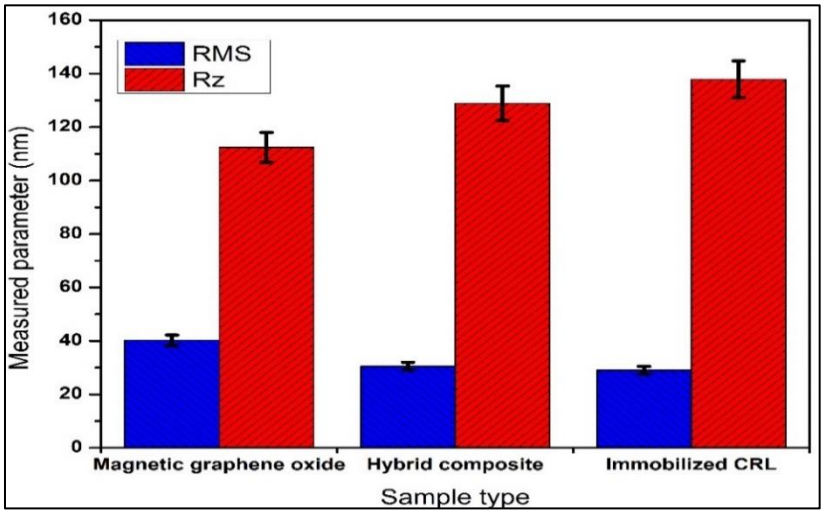

Figure 2: AFM profile of MNP@GO, MNP@GO/SiO_2, and MNP@GO/SiO_2/CRL. Error bars indicate SD (n = 3)

FESEM micrographs of the modified support matrix and immobilized CRL (Figure 3) were acquired to study their morphologies. As seen in Figure 3a, the FESEM micrograph of the modified support matrix (MNP@GO/SiO_2-A-Gl) depicts surface aggregated nanomaterials. This was plausibly due to chemical interactions when modified with 3-aminopropyl ethoxy silane and glutaraldehyde [13]. Figure 3b illustrated a different MNP@GO/SiO_2/CRL morphology when the CRL molecules were covalently attached to the supports' surface. This result was consistent with an earlier report on a similar study by Kai et al. [1].

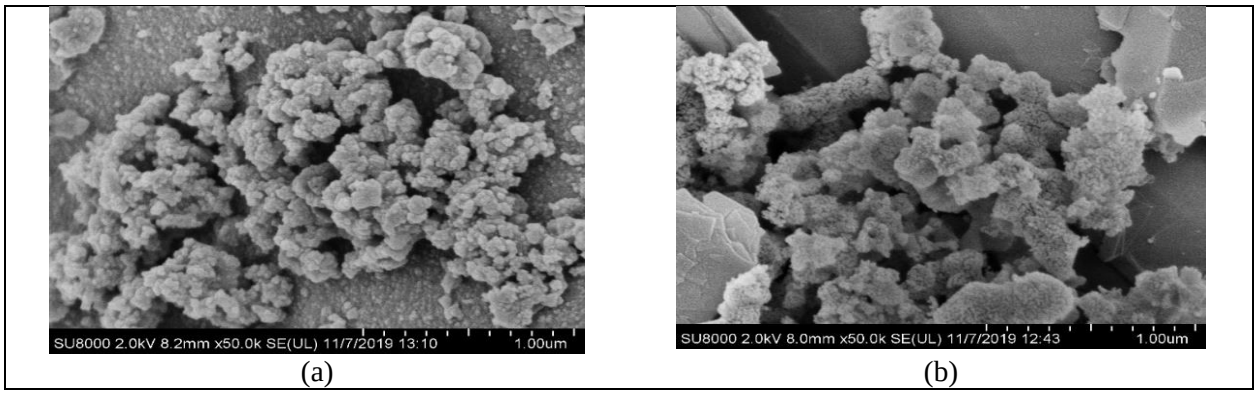

Figure 3: FESEM micrographs (magnification x50 k) of (a) MNP@GO/SiO_2-A-Gl and (b) MNP@GO/SiO_2/CRL

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In this study, structural analysis of samples was done using FTIR spectroscopy in attenuated total reflection mode. This was to detect the presence of distinct functional groups and to correlate the changes in the support's and biocatalyst's compositions following immobilization. The results are presented in Figure 4a–c. In Figure 4a, the main functional groups of Fe_3O_4, SiO_2, and GO were found, where a peak at 560 cm^{-1} corresponds to the Fe–O stretching mode of Fe_3O_4. Conversely, peaks centred at 800 cm^{-1} (Si–O–Si), 1054 cm^{-1} (Si–O–Si), and 970 cm^{-1} are correlated to symmetric, asymmetric stretching vibrations and bending vibrations of the siloxane and silanol groups of SiO_2 particles [2].

The carbonyl group (C=O) of GO is at a peak at 1640 cm^{-1}, while the broad peak at 3450 cm^{-1} represents the H–O–H bending vibration of loosely bonded water molecules. Similar peaks were observed by Jacob et al. [3] in their study. New peaks were detected on the FTIR spectrum of the MNP@GO/SiO_2-A-Gl (Figure 4b). Due to the methylene group from A and Gl, the C-H stretching mode produced a peak at 2940 cm^{-1}, while a peak at 1635 cm^{-1} corresponded to stretching vibrations of C=N bonds. Peaks that describe C–H and C=N bonds in the MNP@GO/SiO_2-A-Gl spectrum indicate the successful covalent bonding of 3-aminotriethoxysilane at the surface of MNP@GO/SiO_2.

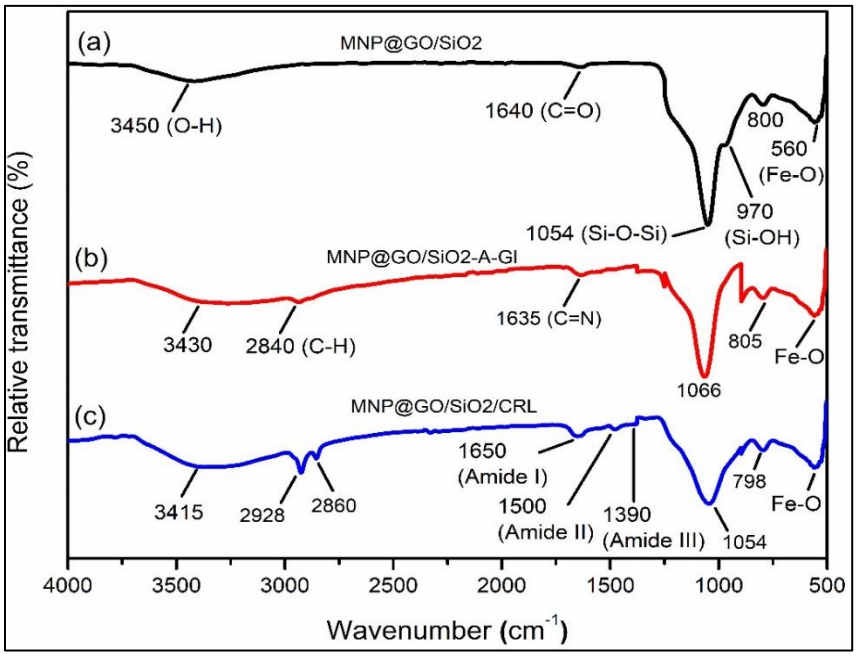

Figure 4: FTIR spectra of (a) MNP@GO/SiO_2, (b) MNP@GO/SiO_2-A-Gl, and (c) MNP@GO/SiO_2/CRL

This produces the necessary aminated surface and glutaraldehyde moieties for the subsequent CRL covalent immobilization. Figure 4c illustrates the MNP@GO/SiO_2/CRL spectrum in which the peaks at 2928 and 2860 cm^{-1} are ascribed to the stretching vibration of C–H bonds [14]. Other vibrations also include the bending mode of the N-H (Amide II) bond and the stretching vibrations of C–C, C–N, or the bending vibration of N–H (Amide III) bonds, respectively [6]. Generally, Amides I, II, and III are helpful to verify

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the presence and ultimately the successful immobilization of enzymes. Accordingly, the combined FTIR spectral results corroborate the successful fabrication of the support and the covalent immobilization of CRL.

Effect of Immobilization Time

Enzyme immobilization improves the stability of immobilized enzymes, enabling their separation from the reaction mixture to facilitate product purification [14, 15]. This approach is key to lowering the total cost of production of the biocatalyst for synthetic reactions. An extended immobilization time is often vital for properly orienting interacting groups in enzymes and support [16]. The effects of immobilization time on protein loading, immobilization efficiency (IE) and EP yield were tested between 8–24 h (Figure 5). The MNP@GO/SiO_2/CRL biocatalyst offered protein loading (PL) ranging from 5.2 ± 0.52–15.2 ± 0.82 mg/g (Figure 5a), gave immobilization efficiency between 70.4 ± 1.45–79.5 ± 2.13 % (Figure 5b). The highest protein loaded on the MNP@GO/SiO_2-A-Gl support was 15.2 mg/g (Figure 5a), and a 16 h immobilization time gave the highest IE (79.5 ± 2.13 %) (Figure 5b).

The effect of immobilization time (8–24 h) on EP yield for the esterification time (1–3 h) was also assessed. As seen in Figure 5c, the ester yields of the MNP@GO/SiO_2/CRL-catalyzed esterification of ethanol and pentanoic acid tested at 1 h, 2 h, and 3 h intervals were found to be 29.07 ± 0.61–55.82 ± 1.22%, 38.18 ± 0.73–65.55 ± 1.53 %, and 49.45 ± 1.41–74.46 ± 2.74 %, respectively (Figure 5c). A 16 h of immobilization yielded the highest EP (72.2 %) in 3h. Consequently, the study found that 16 h is sufficient for immobilizing substantial quantities of the CRL molecules on the support, in which the lipase was adequately stabilized and activated. The specific activity of MNP@GO/SiO_2/CRL was higher than soluble CRL by ∼30 %, as depicted in Table 1. The general outcome of the study implies that MNP@GO/SiO_2/CRL exhibited good catalytic activity to produce ethyl pentanoate.

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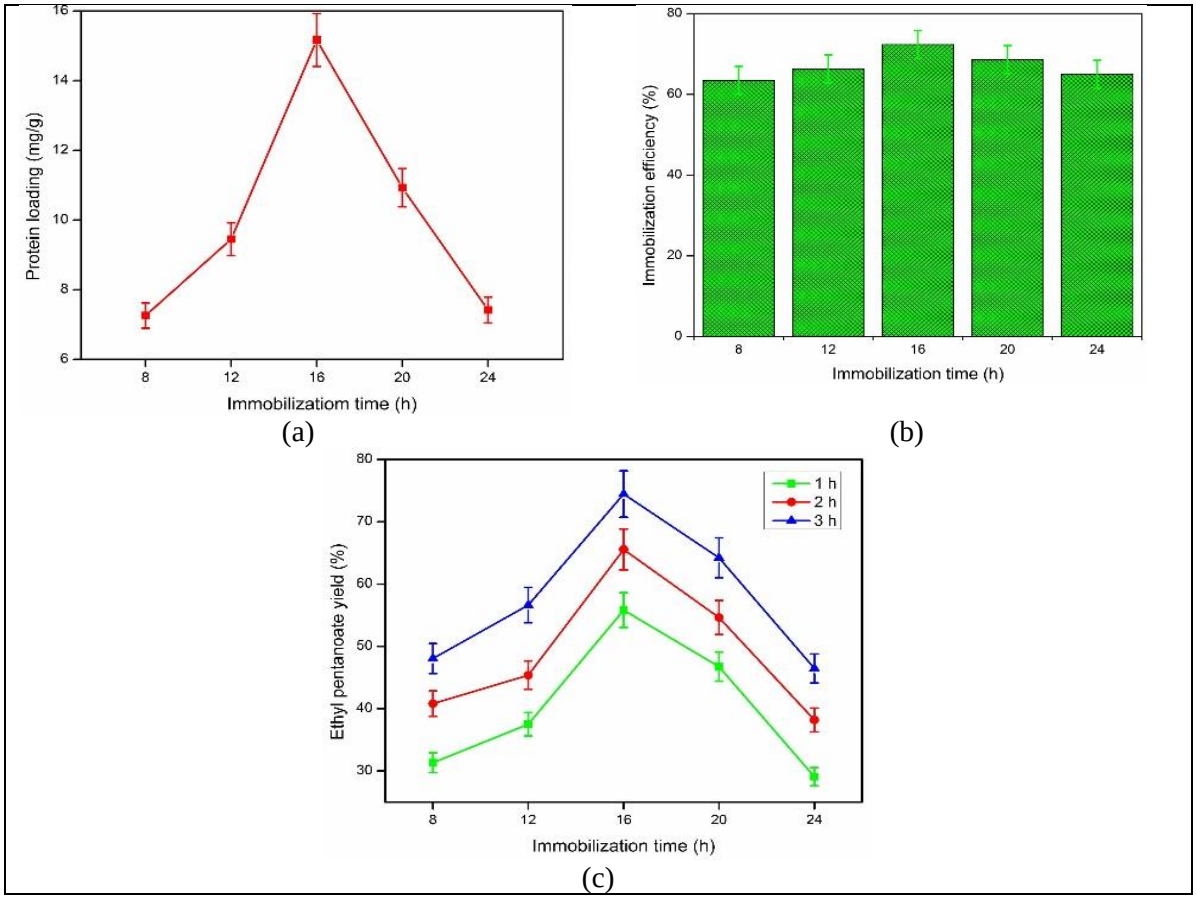

Figure 5: Effect of immobilization time on (a) protein loading, (b) immobilization efficiency, and (c) ethyl pentanoate yield. Error bars indicates SD (n = 3)

Table 1: Lipase activity at pH 7.0 and 40 °C

Type of lipase	**CRL concentration (mg/mL)**	**Specific activity (µmol/min.g)**	**Residual activity (%)**
Soluble CRL	10	39.74±0.65	100
Immobilized CRL	10	56.77±1.42	143

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CONCLUSION

In this study, the MNP@GO/SiO_2-A-Gl support was successfully fabricated and applied to catalyze the esterification production of ethyl pentanoate in n-heptane with a relatively high yield. The MNP@GO/SiO_2/CRL biocatalyst was more active than the soluble CRL to synthesize EP because the tendency for soluble CRL to aggregate in reaction media circumvented their immobilization onto the solid support (MNP@GO/SiO_2). Furthermore, MNPs in the MNP@GO/SiO_2/CRL eased the biocatalyst recovery from the reaction medium without further centrifugation or filtration, thus reducing the ester's overall production cost. The outcome indicates the MNP@GO/SiO_2/CRLs' potential as biocatalysts to produce EP in high yields. This is possible by stabilizing the CRL molecules in their microenvironment on the solid support. Further studies on the effect of other immobilization parameters were needed to fully comprehend changes brought about by the immobilization process.

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AUTHOR'S CONTRIBUTION

Jacob designed the research, carried out the laboratory tests, wrote the original draft and revised the article. Jacob also anchored the virtualization and formal analysis. Wahab conceptualized the central research idea and provided the funding. Wahab also supervised and reviewed the research progress and approved the article submission.

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CONFLICT OF INTEREST STATEMENT

The authors agree that this research was conducted without any self-benefits, commercial or financial conflicts and declare the absence of conflicting interests with the funders.

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