# IMMOBILIZATION OF CLEA-LIPASE OF *Hevea brasiliensis* ONTO MAGNETIC NANOPARTICLES FOR ENHANCED BIOCATALYTIC PERFORMANCE

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## ABSTRACT

Skim latex from *Hevea brasiliensis* consists of many useful proteins and enzymes that can be utilized to produce value-added products for industrial purposes. In this project, to enhance the properties of the recovered lipase from skim latex serum, this enzyme was immobilized via cross-linked enzyme aggregates (CLEA) technology, and supported by magnetic nanoparticles (MNP-CLEA-lipase). MNP-CLEA-lipase was prepared by chemical cross-linking of enzyme aggregates with amino functionalized MNP, which can easily be separated from the spent media after use by magnetic field. The performances of the newly produced MNP-CLEA-lipase were compared with its unsupported counterpart, CLEA-lipase. The optimum conditions for the preparation of CLEA-lipase was carried out by using 45% saturated $(NH_4)_2SO_4$ and 50 mM glutaraldehyde (GA), whereas for MNP-CLEA-lipase, it was carried out by using 70% saturated $(NH_4)_2SO_4$ and 60 mM GA. The optimum pH changes from 6 to 8 when CLEA-lipase is supported, while achieving a maximum of 69.92% residual activity (RA) compared to 20.66% for the unsupported CLEA-lipase. However, the optimum temperature for both are the same (35°C), with MNP-CLEA-lipase achieving 85.89% RA compared to 31.91% for the unsupported ones. Stability studies carried out on both showed that MNP-CLEA-lipase has higher thermal (25–60°C) and pH (5–10) stabilities compared to its unsupported counterparts. As for the reusability of enzymes, MNP-CLEA-lipase retained 56.46% of residual activity after six cycles of reuse. The FESEM results showed that the MNP-CLEA-lipase is a Type 2, which is less structured, and this has bearings towards its activities. FTIR analysis showed the presence of Amide I and Amide II bands in MNP-CLEA-lipase, originating from the newly formed cross-linked between the silanized MNP and CLEA-lipase. Overall, MNP supported CLEA-lipase showed better performance compared to the unsupported biocatalyst.

**Key words:** Skim latex, Skim latex serum, *Hevea brasiliensis*, CLEA-lipase, magnetite, magnetic nanoparticles (MNPs), MNP-CLEA-lipase

## INTRODUCTION

The major source of natural rubber (NR) is from *Hevea brasiliensis* rubber plant. South East Asian countries contribute to 92% of the worldwide NR production, and Malaysia stands as the world's fifth largest producer. Malaysia supplies quality raw rubber of Standard Malaysian Rubber (SMR) grades. This country also produces high quality rubber products such as medical gloves and automotive components (Cataldo, 2000).

Upon tapping, latex exuded from the plant is treated with ammonia to avoid coagulation, however this process leads to the dilution of latex samples. The ammoniated latex is then centrifuged to improve the dry rubber content (DRC) to about 70%, to obtain high concentrated latex for use in the latex-based industries. Centrifugation produces aqueous byproduct, and this is separated from the concentrated rubber portion and discarded into the environment (Mohammadi *et al.*, 2013; Mohamed & Yusof, 2014). This aqueous part, termed skim latex, is deemed to be one of the most polluting wastes in the rubber industry due to its viscosity as well as the strong ammoniated smell. To avoid pollution, skim latex is usually pretreated in the effluent treatment oxidation pond before the cleaner water is released into the main waterways (Mohamed & Yusof, 2014).

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Skim latex still has some amount of rubber particles, at about 4% to 8% DRC (George *et al.*, 2009). By centrifugation, skim latex is separated into skim rubber and skim latex serum. Skim latex serum is the main composition of skim latex, which contains more than 75% of the total soluble protein (Rattanaphan *et al.,* 2012). These hundreds of protein, whereby some of them are enzymes, are waiting to be discovered and identified. Hydrolases are some of the enzymes found in skim latex serum (Yusof *et al.*, 2013; Mardina & Yusof, 2018). According to Yusof *et al.* (2016), hydrolases are easy to use and do not require cofactors, which is one of the reasons why they are frequently used in industries such as food manufacturing (Aravindan *et al.*, 2007; Undurraga *et al.*, 2011), detergents (Rathi *et al.*, 2001; Sarkar *et al.*, 2012; Emtenani *et al.*, 2013; Hasan *et al.*, 2013), pharmaceuticals (Chen & Tsai, 2000; Xin *et al.*, 2001; Gotor-Fernandez *et al.*, 2006), and biodiesel production (Dussan *et al.*, 2007; Raita *et al.*, 2010; Abdelmoez *et al.*, 2013; Stergiou *et al.*, 2013). Lipid acyl hydrolase enzyme (LAH), a subtype of lipase, has been recovered from the serum (Yusof *et al.*, 1998; Mohamed & Yusof, 2014). The recovered lipase was successfully immobilized via cross-linked enzyme aggregates (CLEA) technology (Yusof *et al.*, 2013), a carrier-free immobilization enzyme technique for biocatalysis (Sheldon, 2007a; Sheldon; 2007b; Roessl *et al.*, 2009; Brady & Jordaan, 2009; Mahmod *et al.*, 2015; Khanahmadi *et al.*, 2015; Yusof *et al.*, 2016; Mahmod *et al.*, 2016; Sheldon, 2019). CLEA preparation involves protein precipitation using precipitant, such as ammonium sulphate, $(NH_4)_2SO_4$, followed by cross-linking with polyfunctional reagent, such as glutaraldehyde (GA) (Sheldon, 2007a; Sheldon; 2007b; Yusof *et al.*, 2013; Talekar *et al.*, 2013; Yusof *et al.*, 2016; Sheldon, 2019). CLEA technique combines purification and immobilization into a single operation to provide highly stable and recycleable catalysts with remarkable catalytic efficiency (Sheldon, 2007a; Sheldon, 2007b; Sheldon, 2011; Sheldon & Van Pelt, 2013; Talekar *et al.*, 2013; Gupta *et al.*, 2013a; Barbosa *et al.*, 2014; Sheldon, 2019).

Nanoparticles have long been used to immobilize enzymes due to its significant surface properties (Ansari & Hussain, 2012), but the application of these nanosupports is limited due to problems with recovery, e.g., via centrifugation or filtration. However, the problem could be solved by using magnetic nanoparticles (MNPs), whereby, enzymes attached to MNPs can be easily separated from the reaction media by using a magnet. MNPs have been commonly used for enzyme immobilization in recent years due to this practical advantage and their low toxicity (Khoshnevisan *et*

*al.*, 2017). Besides single enzymes, CLEAs have also been shown to be successfully supported by MNPs. This 'smart', magnetic CLEA is prepared by adding aminofunctionalized magnetite nanoparticles into the enzyme solution. The enzyme is precipitated to form aggregates and in the presence of a cross-linker, it cross-linked with nanoparticles (Reza *et al.*, 2010; Ansari & Husin, 2012; Talekar 2012; Gupta *et al.*, 2013b; Bhattacharya & Pletschke, 2014; Elkady *et al.*, 2016; Gupta *et al.*, 2017). Besides the ease of separation, many researchers have also reported on the higher recovery of enzymatic activity once supported by MNPs (Reza *et al.*, 2010; Ansari & Husin, 2012; Talekar 2012; Gupta *et al.*, 2013b; Bhattacharya & Pletschke, 2014; Elkady *et al.*, 2016; Gupta *et al.*, 2017).

CLEA-lipase has been produced from the skim latex of *Hevea brasiliensis* by Yusof *et al.* (2013). However it has shown a remarkable loss in activity when compared to its free enzyme system; however, the enhanced stability and recyclability of this cleated biocatalyst compensates for the decreased activity. This project mainly aims at developing a more robust and activity enhanced biocatalyst by producing MNP-CLEA-lipase from skim latex of *Hevea brasiliensis*. The residual activity of MNP-CLEA-lipase was compared to CLEA-lipase. The effects of the concentrations of $(NH_4)_2SO_4$ (as precipitant) and GA (as cross-linker) on the immobilized enzymes, optimum temperature and pH, thermal and pH stabilities, reusability of both immobilized enzymes were compared. The newly produced MNP-CLEA-lipase was submitted for structural characterisation and morphology analysis via FTIR and FESEM respectively.

## MATERIALS AND METHODS

### Materials

The skim latex of *Hevea brasiliensis* was obtained from the MARDEC Industrial Latex Sdn. Bhd. in Tapah, Perak, Malaysia. All chemicals used for this project were of analytical grade.

### Methods

#### Preparation of skim latex serum

To encourage the coagulation of rubber, glacial acetic acid was added to skim latex to reduce the pH from 10 to 5. The sample was then centrifuged at 10,000 rpm at 4°C for 30 min to separate coagulated rubber (skim rubber) from the supernatant, skim latex serum. Skim rubber was discarded and the serum was pooled. Solid $(NH_4)_2SO_4$ was then added to the supernatant until it reached 4 M concentration (or saturation point), and the mixture was left to precipitate overnight

under slow stirring at 4°C. The formation of white precipitate was observed and upon centrifugation at 5,000 rpm for 15 min, the precipitate was collected, while the supernatant was discarded. The precipitate was dissolved in minimal phospate buffer saline (PBS) at pH 7 to yield crude enzyme extract or 'free enzyme'. The protein concentration and lipase activity of the free enzyme were determined (Mohamed & Yusof, 2014).

### Preparation of amino-functionalized magnetic nanoparticles

$NH_4OH$ was added into a mixed solution of 1.351 g of $FeCl_3 \cdot 6H_2O$ and 0.6852 g of $FeSO_4 \cdot 7H_2O$ in 25 mL deionized water at room temperature until a precipitate formed. To remove the residual ions, the precipitate was centrifuged and washed several times with deionized water until it reached pH 7. The precipitate or the magnetic nanoparticles (MNPs) was dried at 100°C for 3 h (Reza *et al.*, 2010; Talekar *et al.*, 2012). The surface of the particles were coated with (3-aminopropyl) trimethoxysilane (APTES) by silanization reaction to obtain aminofunctionalized MNPs. Silanization reaction involved dissolving 1 mL of APTES, 2 g of MNPs and 0.25 mL of deionized water in 25 mL of methanol. The mixture was sonicated for 30 min. Glycerol of 15 mL was then added to the mixture, and the solution was incubated in an incubator shaker at 80°C for 6 h at 200 rpm. The precipitate obtained was washed with water and methanol for three times in each case, and dried, yielding a fine amino functionalized magnetic powder (Reza *et al.*, 2010; Talekar *et al.*, 2012).

### Preparation of CLEA-lipase and MNP-CLEA-lipase

CLEA-lipase was prepared by pipetting 1.0 mL of crude enzyme extract containing 1.175 mg/mL of protein, with a lipase activity of 0.008 U/mL, into a 15 mL Falcon tube. $(NH_4)_2SO_4$ (1.8 mL) and 1M of GA (0.2 mL) were added simultaneously to the enzyme solution, and phosphate buffer saline (PBS) was used to make a final volume of 4 mL. The solution was agitated at 200 rpm for 17 h at room temperature. Then, 3 mL of water was added to the solution and centrifuged at 4,000 rpm for 30 min at 4°C. The supernatant was decanted and the residue was washed three more times with water, centrifuged and decanted. The final CLEA-lipase preparation was kept in 5 mL of water (Yusof *et al.*, 2013).

MNP-CLEA-lipase was prepared by mixing 20 mg of aminofunctionalized MNPs with 1.0 mL of crude enzyme extract. Then, 2.8 mL of $(NH_4)_2SO_4$ was added to 0.12 mL of 2M of GA and finally PBS was used to make a final volume of 4 mL. The solution was agitated at 200 rpm for 17 h at room temperature. Water (3 mL) was then added to the

mixture and the supernatant was pipetted out and this was repeated twice. Each time, a magnet was used to separate the magnetic biocatalyst from the washings. The final MNP-CLEA-lipase preparation was kept in 5 mL of water (Talekar *et al.*, 2012).

### Protein assay

Protein assay was conducted according to the standard procedure by Bradford (1976). The dye reagent was prepared by diluting one part of dye reagent concentrate with four parts distilled, deionized water. The dye was filtered through the a Whatman #1 filter paper to remove particulates. A series of five dilutions of a protein standard was prepared. The linear range of the assay for bovine serum albumin (BSA) is 0.1 to 1.0 mg/mL. One hundred µL of each standard and sample solution was pipetted into clean, dry Falcon tubes. Protein solutions was assayed in triplicates. Five mL of diluted dye reagent was added to each tube and vortexed. The tubes were incubated at room temperature for 5 min. Then, the absorbance of the assay was measured at 595 nm using Thermo Scientific Multiskan Go Spectrophotometer (BioRad, 2010). The protein content was calculated based on Eq. (1):

$$y = 1.8105x + 0.5484 \qquad \text{Eq. (1)}$$

where, *y* represents the absorbance reading and *x* represents the amount of protein content (mg/mL).

### Lipase enzyme activity assay

The substrate stock solution was prepared by dissolving 28 mg of *p*-nitrophenyl palmitate (*p*NPP) in 100 mL of 1% (v/v) Triton 100-X and 1.7 mL of 1% (w/v) sodium dodecyl sulphate, with stirring and heating. To start the reaction, 1 mL of *p*NPP stock solution was incubated with 1 mL of 0.1 M Tris-HCl at pH 8.2 and 1 mL of enzyme (or if in the CLEA form, CLEA was suspended in 1 mL PBS) in water bath for 30 min at 37°C. NaOH (1 mL of 1M) was added to stop the reaction. The absorbance of the assay was measured at 410 nm using Thermo Scientific Multiskan Go Spectrophotometer and the amount of *p*-nitrophenol released was measured using the standard calibration curve, constructed using *p*-nitrophenol. One unit (U) of lipase enzyme activity is defined as the amount of enzyme required to release 1.0 µmol of *p*-nitrophenol per minute under the assay conditions. Lipase activity of free enzyme was calculated using Eq. (2).

$$\text{Lipase activity} \left( \frac{U}{mL} \right) = \frac{(\text{Absorbance at } 410\,nm) \times \text{Total volume of assay} (mL)}{\text{volume of used enzyme} (mL) \times \text{Time of assay} (min) \times \text{slope of calibration curve}} \qquad \text{Eq. (2)}$$

Residual activity (% RA) was used to report for the lipase activity upon immobilization by CLEA technology and it was calculated by using Eq. (3):

$$Residual\ Activity\ of\ Lipase\ (\%) = \frac{Activity\ of\ CLEA\ immobilized\ enzyme}{Activity\ of\ free\ lipase} \times 100\% \qquad Eq.\ (3)$$

### Effect of preparation parameters on CLEA-lipase and MNP-CLEA-lipase activity

The effect of various process parameters in the preparation of CLEA was studied using the traditional experimental design of one-factor-at-a-time (OFAT) approach. In this study, three important process parameters were selected, namely the amount of MNPs, the concentrations of $(NH_4)_2SO_4$ and glutaraldehyde (GA). For the amount of MNPs, the values tested were varied at 0, 2.0, 4.0, 5.0, 6.0, 8.0, 10.0, 15.0 and 20.0 mg in 4 mL incubation mixture, the concentration of $(NH_4)_2SO_4$ at 0%, 20%, 35%, 45%, 55% and 70% saturation and the concentrations of GA at 0, 15, 25, 35, 50, 65 and 75 mM. Other parameters were kept constant; agitation rate (200 rpm) and incubation time (17 h) (Yusof *et al.*, 2013; Mahmod *et al.*, 2015; Easa & Yusof, 2015). The responses were recorded as lipase residual activity (% RA).

### Optimization of preparation parameters for CLEA-lipase and MNP-CLEA-lipase

The optimization study of the preparation process of CLEA aims at obtaining the best combination of various preparation parameters for maximal lipase activity. In this project, the design of experiments (DOE) was conducted with the aid of Design Expert® (Version 6.0.8) software using face centred central composite design (FCCCD) under the response surface methodology (RSM) approach. The independent variables used were concentrations of $(NH_4)_2SO_4$ and GA, while the lipase residual activity was selected as the response. Other preparation parameters were kept constant, such as, the amount of MNPs (20 mg), agitation rate (200 rpm) and incubation time (17 h). According to the prescribed design, 13 combination runs were conducted, including five replications at the center point. Data obtained from the OFAT study was used to contsruct the DOE. Statistical analysis via ANOVA was used to evaluate the degree of accuracy held by the derived model equations.

### Optimum temperature and pH

Optimum temperature of the CLEA was determined by assessing the lipase residual activity after incubating the mixture in 0.1 M of Tris-HCl at pH 8.2 at different temperatures (25–60°C) for 30 min. Optimum pH was measured by determining the lipase residual activity by incubating CLEA at the optimum temperature at various pH values (5–10) of Tris-HCL buffer.

### Temperature and pH stabilities

Temperature stability of CLEA was determined by measuring lipase residual activity after incubating the enzymes without the addition of substrate at temperatures between 25°C and 60°C at the optimum pH for 30 min. The temperature of the samples was brought back to room temperature, followed by the addition of substrate and the normal lipase activity assay was conducted.

To find the pH stability, lipase residual activity of the samples was determined after incubating the enzymes in substrate-free buffer at various pH values ranging from 5 to 10 at optimum temperature for 30 min. The substrate was then added followed with the normal lipase activity assay.

### Reusability test

The enzyme reusability test was carried out by obtaining the lipase residual activity of CLEA for six consecutive cycles. After the first cycle, CLEA was separated and washed three more times with water before they are stored in 5 mL water at 4°C. After 24 h, % RA was measured again and the process was repeated for another five times. For comparison, the first cycle is normalized as 100% and the achievements of the subsequent cycles were calculated based on the activity in the first cycle.

### Morphology analysis by FESEM

The non-functionalized MNPs and MNP-CLEA-lipase samples were sent to Crest Nanosolutions (M) Sdn. Bhd. in Puchong, Selangor, for field electron scanning emission microscopy (FESEM) analysis. The FESEM were operated at 1 kV for the MNPs and at 10 kV for MNP-CLEA-lipase. The morphology of the samples were characterized.

### Structural characterisation by FTIR

Non- and functionalized MNPs, MNP-CLEA-lipase and free lipase samples were subjected to FTIR analysis at the INHART laboratory, IIUM, Gombak. Appearances of new functional groups were deliberated. The analyses were done at 4 cm$^{-1}$ resolution and the scans were done in triplicates.

## RESULTS AND DISCUSSION

### Effect of preparation parameters on CLEA-lipase and MNP-CLEA-lipase activity

The effect of three parameters, namely the amount of MNPs, concentrations of $(NH_4)_2SO_4$ and GA, which are regarded as important parameters for the preparation of MNP-CLEA-lipase was

studied via OFAT. The results, given as lipase residual activity are shown in Figures 1, 2 and 3. Results in Figure 1 showed that the maximum lipase residual activity was achieved by using the highest amount MNPs (20 mg). It is assumed that, if a higher amount of MNP is used, a higher activity can be achieved due to a higher amount of amino group available for cross-linking with CLEA. However, amounts more than 20 mg was not tested due to the limited availability of MNPs. For the concentration of $(NH_4)_2SO_4$, the results in Figure 2 showed a sharp increase of lipase residual activity from up to 35% but it plateaued slowly as it is increased from 35 to 70%, and maximised at 70%. From the results, it can be inferred that at 35% $(NH_4)_2SO_4$ saturation, most of the lipase from the solutions has precipitated.

Although more lipase was seemed to be precipitated as $(NH_4)_2SO_4$ was increased, the results suggest that a higher percentage only increase the activity in minimal amount thus of no real advantage.

Lastly, in Figure 3, the results showed that there was a slight dip in lipase residual activity from 0 to 25 mM GA, but increased sharply as it progressed from 25 to 65 mM. From 65 mM GA, the lipase residual activity increased gently and maximized at the highest GA tested, that is at 75 mM. It is expected that the residual activity can even go higher by using more than 75 mM GA, however, it was not tested in our research. GA acted as the cross-linker in this system by providing the carboxyl groups to react with the amino functionalised MNPs and the amino groups on CLEA. The maximal values in the three parameters tested were considered when preparing the DOE of the following optimization experiments.

**Optimizing the preparation conditions of CLEA**

In this study, the preparation conditions of both CLEA-lipase and MNP-CLEA-lipase were optimized. Due to the limitation of resources, only two parameters were chosen, namely the concentrations of $(NH_4)_2SO_4$ and GA, while keeping the agitation rate at 200 rpm and stirring time for 17 h. As for the supported CLEA, functionalized MNPs were kept constant at 20 mg in 4 mL incubation mixture. As suggested by the software, 13 experimental runs were conducted in triplicates. The ANOVA analysis data are presented in Tables 1 and 2, where A and B are denoted for the concentrations of $(NH_4)_2SO_4$, and GA, respectively. The model regression equations to describe the optimum preparation for both are given as Equations 4 and 5. These regression equations can be applied to investigate the interaction between variables. The optimum level determination of each factor for maximum lipase residual activity can be graphically represented by 3D response surface plots, given by
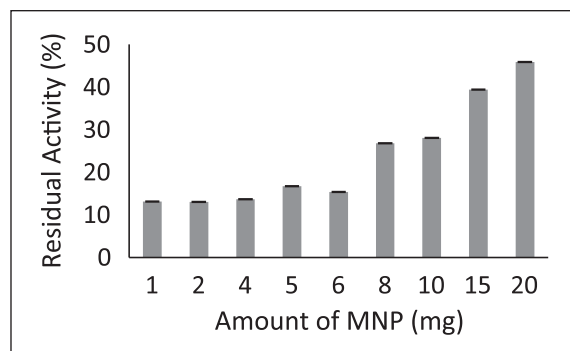


**Fig. 1.** Effect of amount of MNPs on lipase residual activity of MNP-CLEA-lipase.
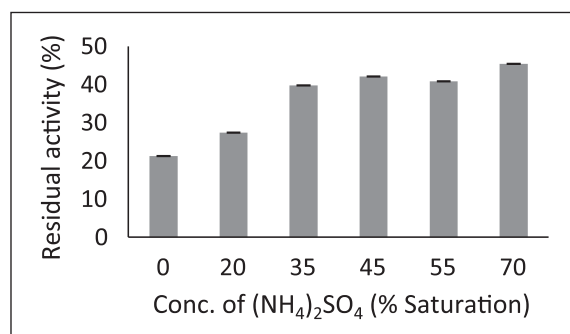


**Fig. 2.** Effect of concentration of $(NH_4)_2SO_4$ (% Saturation) on lipase residual activity of MNP-CLEA-lipase.
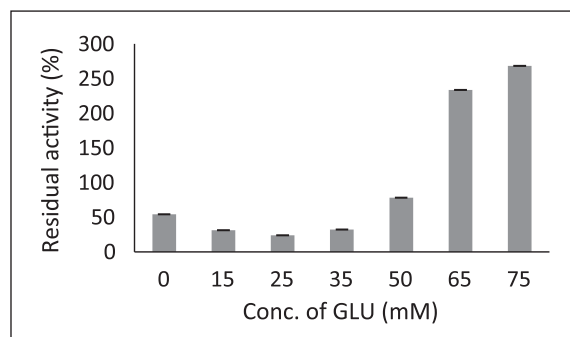


**Fig. 3.** Effect of concentration of GA on lipase residual activity of MNP-CLEA-lipase.

Figure 3 and 4, that showed the level of interaction between the two variables. Based on the value of coefficient determinants, $R^2$, regression equation for CLEA-lipase with $R^2=0.6771$, fitted better than the regression equation for MNP-CLEA-lipase, with $R^2=0.6269$.

Unfortunately, both model equations were not significant.

$$Y(CL) = 21.12 + 2.35A - 0.13B - 2.70A^2 \qquad \text{Eq. (4)}$$
$$- 3.20B^2 - 1.27AB$$

$$Y(MCL) = 83.23 + 1.96A - 8.68B + 4.80A^2 \qquad \text{Eq. (5)}$$
$$- 9.53B^2 - 0.40AB$$

**Table 1.** ANOVA for CLEA-lipase experimental runs

| Source | Sum of Square | DF | Mean Squares | F value | Prob > F | |
|---|---|---|---|---|---|---|
| Model | 117.72 | 5 | 23.54 | 2.94 | 0.0964 | Not significant |
| A | 33.27 | 1 | 33.27 | 4.15 | 0.0811 | |
| B | 0.094 | 1 | 0.094 | 0.012 | 0.9168 | |
| $A^2$ | 20.08 | 1 | 20.08 | 2.50 | 0.1576 | |
| $B^2$ | 28.37 | 1 | 28.37 | 3.54 | 0.1021 | |
| AB | 6.41 | 1 | 6.41 | 0.80 | 0.4010 | |
| Residual | 56.14 | 7 | 8.02 | | | |
| Lack of Fit | 3.61 | 3 | 1.20 | 0.092 | 0.9608 | Not significant |
| Pure Error | 52.53 | 4 | 13.13 | | | |
| Cor Total | 173.86 | 12 | | | | |

**Table 2**. ANOVA for MNP-CLEA-lipase experimental runs

| Source | Sum of Square | DF | Mean Squares | F value | Prob > F | |
|---|---|---|---|---|---|---|
| Model | 730.65 | 5 | 146.13 | 2.35 | 0.1477 | Not significant |
| A | 23.13 | 1 | 23.13 | 0.37 | 0.5611 | |
| B | 451.71 | 1 | 451.71 | 7.27 | 0.0308 | |
| $A^2$ | 63.69 | 1 | 63.69 | 1.03 | 0.3450 | |
| $B^2$ | 63.69 | 1 | 250.73 | 4.04 | 0.0845 | |
| AB | 0.64 | 1 | 0.64 | 0.010 | 0.9220 | |
| Residual | 434.91 | 7 | 62.13 | | | |
| Lack of Fit | 434.91 | 3 | 144.97 | | | Not significant |
| Pure Error | 0.00 | 4 | 0.00 | | | |
| Cor Total | 1165.55 | 12 | | | | |
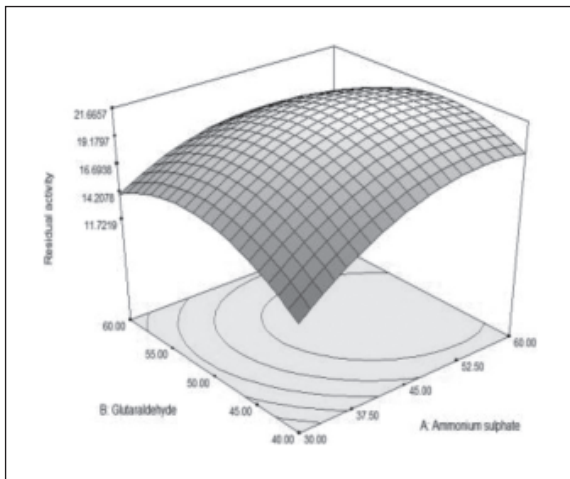


**Fig. 4.** 3-D response surface plots for CLEA-lipase, F-value=2.94, p=0.0964, $R^2$=0.6771.
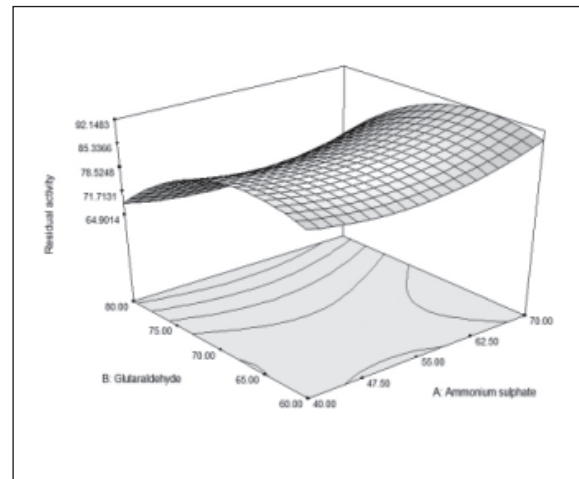


**Fig. 5.** 3-D response surface plot for MNP-CLEA-lipase, F value=2.35, p=0.0181, $R^2$=0.6269.

Referring to the above Equations 4 and 5, *Y(CL)* and *Y(MCL)* represent the equations obtained for CLEA-lipase and MNP-CLEA-lipase, respectively. From the optimization study conducted, the highest response determined was 24.84% RA for CLEA-lipase by using 45% saturated $(NH_4)_2SO_4$ and 50 mM GA. On the other hand, MNP-CLEA-lipase achieved the highest at 96.38% RA by using 70% saturated $(NH_4)_2SO_4$ and 60 mM GA, which means,

overall, MNP-CLEA-lipase showed higher % RA than its unsupported counterpart. The increased in activity can be partly explained by the presence of MNPs. According to Talekar *et al*. (2012), the addition of amino functionalized MNP into the enzyme solution may increase the amine groups concentration and thus, when the precipitated enzyme and amino functionalized MNP were cross-linked via GA, a higher amount of insoluble and

active biocatalysts could be formed due to sufficient amount of amine groups.

**Effect of temperature and pH on CLEA-lipase and MNP-CLEA-lipase activity**

The results in Figure 6 showed that the best working temperature for both CLEA-lipase and MNP-CLEA-lipase occurred at 35°C. However, the achievements of the residual activities at this temperature differs; it is much higher for MNP-CLEA-lipase whereby the RA was 85.89% compared to CLEA-lipase, of 31.91%. This is in agreement with Talekar *et al.* (2012), who suggested that, a higher lipase residual activity can be achieved in MNP-CLEA-lipase, due to the presence of higher amount of covalent cross-linkings between lipase enzyme and amino functionalized MNP. It seems that the formation of higher number of covalent bonds elevate the tolerance of the enzyme against high temperatures.

As for pH, as being shown by Figure 7, the best working pH of CLEA-lipase changes when it is supported by MNP. The highest lipase residual activity for CLEA-lipase, is 20.66% at pH 6, however the best working pH changed to 8, when it is supported by MNP, achieving 69.92% RA. The shift in the best working pH may be due to the changes in the nature of ionization of the acidic and basic amino acid side chains in the microenvironment around the active site of both types of enzymes. Elevated pH of 8 was more preferred by the supported CLEA. The presence of the more interactions between the basic residues of enzyme and GA as a results of cross-linking may have allowed the activity of MNP-CLEA-lipase to supresede its unsupported counterparts (Talekar *et al.*, 2012).

**Thermal and pH stabilities**

As depicted by Figure 8, in terms of thermal stability, MNP-CLEA-lipase showed to be more stable compared to CLEA-lipase at the tested temperature ranging from 25 to 60°C. After both were incubated for 30 min at 60°C, MNP-CLEA-lipase retained 70.09% RA compared to 32.86% for CLEA-lipase. The results indicate that MNP-CLEA-lipase is more thermally stable compared to CLEA-lipase.

The presence of adequate covalent cross-linking between the enzyme molecules and the aminofunctionalized MNP results in better flexibilities in different temperatures and were able to withstand extreme temperature better compared to the unsupported ones (Talekar *et al.*, 2012; Khanahmadi *et al.*, 2015).

For the pH stability study, as shown by Fig. 9, MNP-CLEA-lipase is more stable in the pH range of 5–10 compared to CLEA-lipase. At the highest
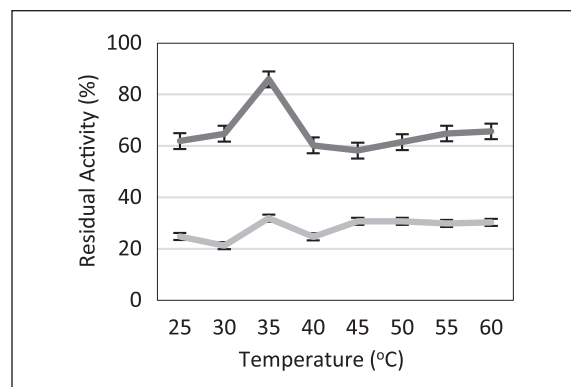


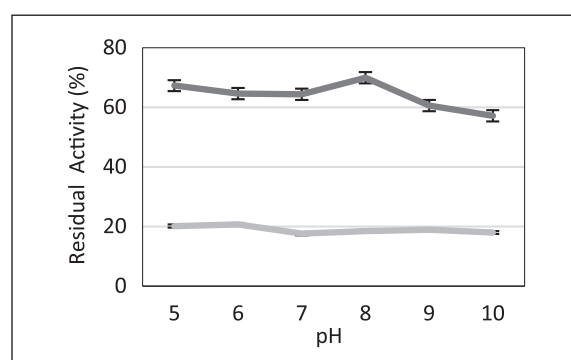**Fig. 6.** Effect of temperature on CLEA-lipase ●— and MNP-CLEA-lipase ●—.



**Fig. 7.** Effect of pH on CLEA-lipase ●— and MNP-CLEA-lipase ●—.
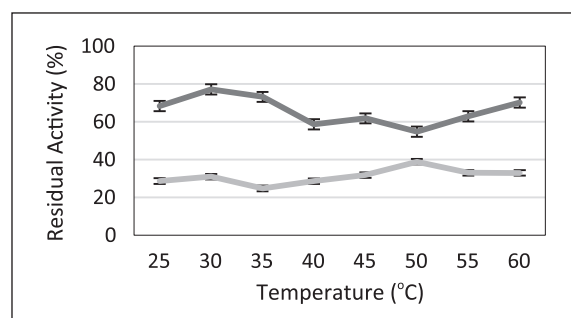


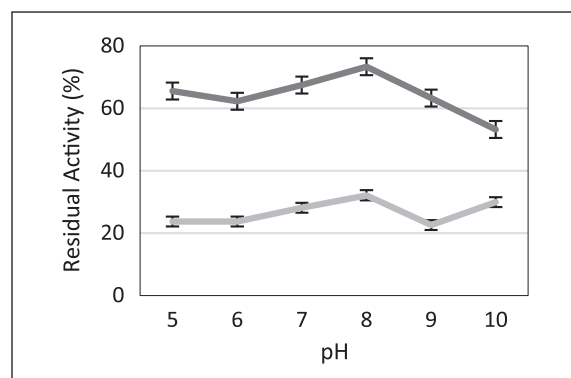**Fig. 8.** Thermal stability for CLEA-lipase ●— and MNP-CLEA-lipase ●—.



**Fig. 9.** pH stability for CLEA-lipase ●— and MNP-CLEA-lipase ●—.

achievement at pH 8, MNP-CLEA-lipase retained 73.32%, whereas CLEA-lipase only retained 32.14% RA.

The resistance to pH changes of CLEA could be due to the covalent cross-linking throughout enzyme aggregates that may have increased the flexibility of the enzyme in different pH conditions (Yu *et al*., 2013; Yusof *et al*., 2013; Khanahmadi *et al*., 2015). However, higher resistance towards pH changes was observed in MNP-CLEA-lipase compared to the unsupported counterparts, inferring the availability of more sufficient covalent cross-linkings.

**Reusability**

Immobilized enzymes should be highly stable to enable its reusability (Mateo *et al*., 2007; Khanahmadi *et al*., 2015). The enzymes can be reusable only if it can retain its activity after several cycles (Garcia-Galan *et al*., 2011). In this research, reusability of CLEA-lipase was studied by carrying out the enzyme activity up to six cycles. Based on the results shown in Figure 10, CLEA-lipase retained 53.13% of the first time activity (considered as 100%) after six cycles while MNP-CLEA-lipase retained 56.46%. By comparison, their loss of enzyme activity during the six cycles followed the same trend, however, there was a slight enhancement for the supported CLEA. This loss may be due to the leaching of enzymes in the reaction medium during the process of washings, which is caused by inefficient cross-linking of the enzymes. On the other hand, adequate cross-linking of lipase and the amino functionalized MNPs enabled it to retain higher activity even after six cycles.

**Morphological analysis by FESEM**

Figure 11 showed the FESEM micrographs of functionalized MNPs before it was used as supports to CLEA-lipase. Due to its magnetic properties, the molecules are seen to form spheres, and with closer inspection the spheres appeared to be loosely aggregated. The estimated sizes for each sphere is about 20 nm.

The FESEM micrographs of MNP-CLEA-lipase is shown by Figure 12. According to Shoevaart *et al*. (2004), the appearance of CLEAs are either a compact spherical shape (Type 1) or less-structured (Type 2). Both have bearings towards the activity of enzymes, which relates to mass transfer limitations. In this research, MNP-CLEA-lipase produced appeared as Type 2, which is loosely structured, as shown by Figure 12.

Loosely structured means the structure were less compact, and this leads to lesser resistance to mass transfer (Yusof *et al*., 2016). This is especially important for larger substrates, for easy accessibility to the active sites of the enzyme. In
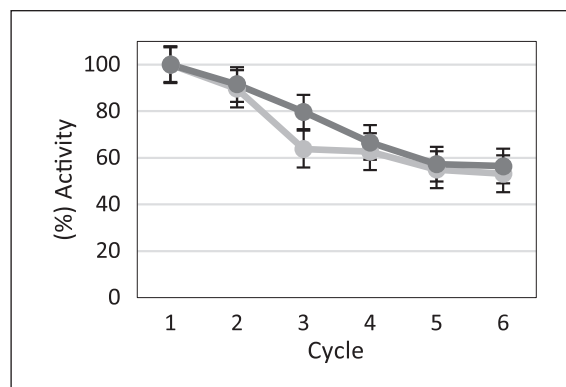


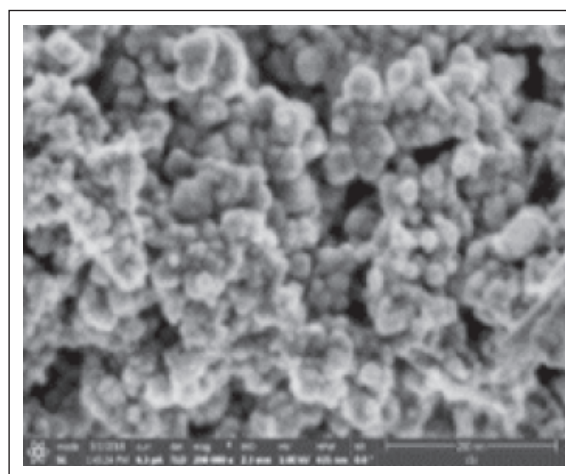**Fig. 10.** Reusability of CLEA-lipase ——— and MNP-CLEA-lipase ———.



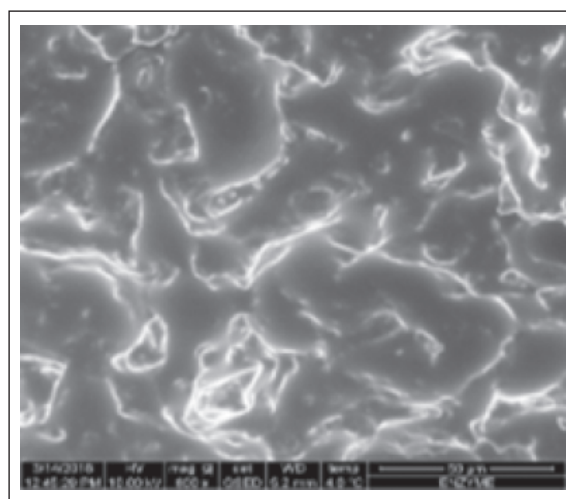**Fig. 11.** FESEM for non-functionalized MNPs.



**Fig. 12.** FESEM for MNP-CLEA-lipase.

this research, pNPP was used as the substrate for the lipase activity. pNPP is a big molecule, having 18 carbons as its hydrophobic tail. Loosely structured biocatalyst will ease the manuevaring of this substrate molecules towards the enzyme's active-sites. According to Yu *et al*. (2006) and
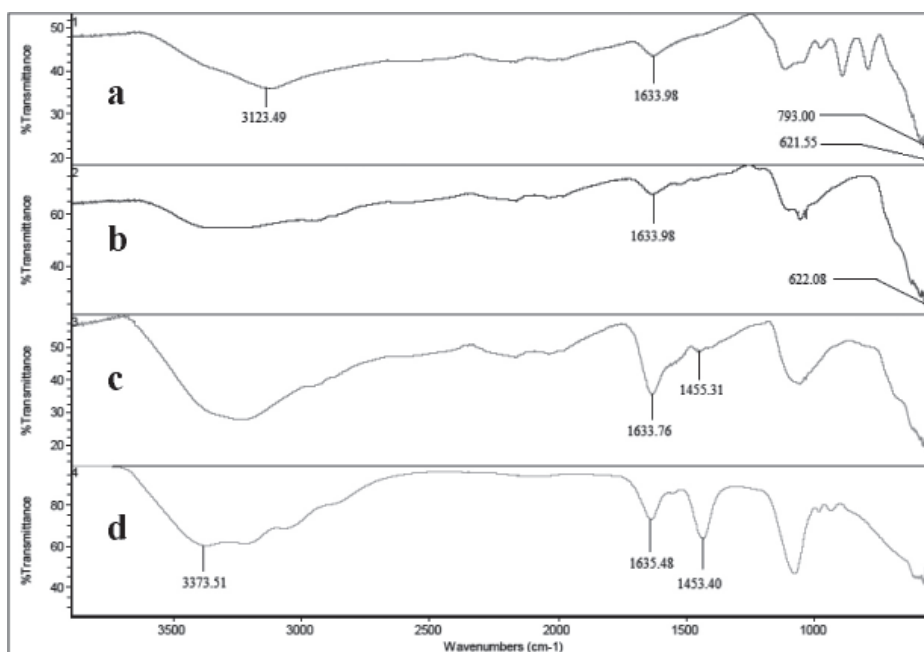
**Fig. 13.** FTIR analysis (a) non-functionalized MNPs, (b) functionalized MNPs, (c) MNP-CLEA-lipase, (d) free-lipase.

Sheldon (2019), the morphological appearances and the particle sizes of CLEA are also influenced by other parameters such as the concentrations of enzymes, the types of precipitants, cross-linkers and additives. On the other hand, particle sizes of CLEA also affect filterability, especially in the non-magnetic system. Sheldon (2019) emphasized that the molar ratio of cross-linker to enzyme influences the activity, stability and the particle size of the resulting CLEA. Other important factors that influence the recovery activity as well as the particle size and morphology of CLEA are the rate of addition of the cross-linker and the cross-linking time. Pchelintsev *et al*. (2009) reported that the particle size of a CLEA can also increase on ageing, which suggest that the cross-linking process may not have been completed.

**Structural characterisation by FTIR**

FTIR spectroscopy analyzes the samples by providing information on the structural features of molecules. It also allows us to determine the presence of functional groups and bonds of each sample based on peak values at the designated wavelengths (Fabian & Mantele, 2002).

Figure 13 shows the FTIR comparison spectra for MNPs, functionalized MNPs, MNP-CLEA-lipase and free lipase. Figure 13 (a) shows a typical FTIR spectrum of $Fe_3O_4$ containing magnetite. The spectrum contains characteristic bands of the Fe–O at around in 621 cm$^{-1}$. When functionalized by APTES, the FTIR spectrum of the magnetite

shifted to higher wavenumbers, to account for the presence of Fe–O–Si bonds as shown in Figure 13 (b). This is because, once functionalized, the Fe–O–H groups on the surface of magnetite were replaced by Fe–O–Si(O–)$_2$–R. The higher electro-negativity of –Si(O–) compared to H leads to an enhancement of the bond forces for the Fe–O bonds, shifting the absorption bands to high wave numbers (Ma *et al*., 2003). Results also showed that, once CLEA-lipase was immobilized on the MNP, the FTIR spectrum of the immobilized biocatalyst appeared quite similar to its corresponding enzymes [compare Figure 13 (c) for MNP-CLEA-lipase with Figure 13 (d) for free lipase]. The presence of a more pronounced broad peak around 3373 cm$^{-1}$ corresponding to the N–H asymmetric stretching is contributed by amine H–bonds. The presence of new peaks in the immobilized biocatalyst, at wavelength 1633 cm$^{-1}$ are ascribed to Amide I (C=O stretching) and, at 1455 cm$^{-1}$, to Amide II (CN stretching and NH bending). In general, the spectra in Figure 13 showed that MNP has been successfully functionalized by APTES and finally cross-linked to CLEA-lipase via GA.

**CONCLUSION**

In conclusion, the management of waste from the rubber manufacturing industry problem can be partly solved by utilizing the skim latex to produce important biocatalysts. Skim latex, a byproduct of

the high concentrated latex industry, instead of being thrown away, can be value-added by recovering an important enzyme such as lipase. Lipase was chosen because of its versatility and wide use in industrial applications. However, the use of free lipase suffers from many disadvantages, in terms of stability. It has been shown that the activity of lipase can be enhanced by immobilization by CLEA technology, and in the present research, it is shown that it can be further enhanced by use of MNPs support. The optimum process conditions to prepare MNP-CLEA-lipase and CLEA-lipase for maximal enzyme activity differs in terms of the concentrations of precipitant and cross-linker used. Overall, MNP-CLEA-lipase was shown to perform better in many aspects compared to the unsupported one; it is more stable at high temperature and at more extreme pHs. This is especially important if these biocatalyst are intended to be used in the industrial settings, whereby the temperature and pH of reactions can shift to the extremes during the processes. Upon testing, MNP-CLEA-lipase was able to be recycled as much as six times, retaining higher residual activity than its unsupported counterpart. Separation of the magnetic biocatalyst from the reaction media was made easy, by using magnetic field, instead of the cumbersome centrifugation and filtration processes for the unsupported CLEA. The FESEM results showed that the MNP-CLEA-lipase is a Type 2, which is less structured, and this may be an advantage because it leads to less resistance towards mass transfer during the enzymatic reaction. FTIR showed that MNP-CLEA-lipase was successfully produced, proven by the increased presence of Amide I and Amide II bands, that represents the increase of amide bonds in the new biocatalyst. Overall, the production of value-added products from skim latex such as MNP-CLEA-lipase with enhanced properties, demonstrated that the wasteful byproducts could easily be converted to innovative biomaterial, which has potential to be used in many biotechnological applications.

## ACKNOWLEDGMENT

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