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Enhanced Kinetic Performance of CLEA-Lipase Extracted from Skim Latex of *Hevea brasiliensis* upon Immobilization on Magnetic Iron Nanoparticles

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ABSTRACT

In this research, lipase recovered from the skim latex of *Hevea brasiliensis* was immobilized via cross-linked enzyme aggregates (CLEA) technology, while supported by magnetic nanoparticles (MNPs), for properties enhancement. Hybrid immobilization may have affected the kinetic performances of the biocatalysts. The kinetic performance of both MNP supported and unsupported CLEA-lipase, were evaluated based on the Michaelis-Menten model using *p*-nitrophenyl palmitate as the substrate. Three different linearization model equations were used to compute the kinetic properties, v_{max} and K_m, and a hyperbolic regression was conducted with computer software. Based on the best fitted model, v_{max} of MNP-CLEA-lipase, obtained from the Lineweaver-Burk plot (R^2=0.9823), was 0.0023 µmol/min.mL, which is higher than CLEA-lipase (0.0015 µmol/min.mL), indicating it needs much higher substrate concentration to saturate the enzymatic sites to reach its maximum velocity. K_m for MNP-CLEA-lipase was 0.4400 µmol, compared to 0.5188 µmol for CLEA-lipase, inferring that it has a higher affinity towards substrates, whereby its rate will approach v_{max} with lower substrate concentration. Overall, this research demonstrated that wasteful by-products such as skim latex can be converted to useful value-added biocatalyst. A better understanding of the kinetic parameters of this newly produced MNP immobilized biocatalyst is necessary for its further development.

1. Introduction

In Malaysia, natural rubber latex is mostly sourced from *Hevea brasiliensis* tree. Latex is extracted from these trees for the consumption of the various rubber-based industries like examination gloves, condoms, latex thread, tires, gaskets, medical catheters and rubber bands, while the timber can be used in the furniture industries [1]. One of the problems faced by the rubber industries is regarding their waste management that caused mounting environmental problems.

Skim latex is the by-product of the natural rubber processing industry and can be considered as the most polluting waste that comes out of it. This is because, besides the residual 4 to 8% dry rubber content [2], skim latex also contains the pungent-smelled ammonia that was necessarily added to

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stabilize the field latex. As per standard operation procedure, skim latex should be pre-treated in the effluent treatment oxidation pond, before the cleaner water from the pond is periodically released into the main waterways. However, the awful smell from its ammoniated odour [3] affects the community who lived in the surrounding areas, in terms of the air they breathe and their general health, whereby people who are repeatedly exposed to ammonia through inhalation may develop respiratory irritation [4]. Thus, the utilization of skim latex serum, turning it into value-added products would therefore become one of the alternative ways to minimize discharging of effluent. This will make latex processing more environmental-friendly and at the same time can garner additional earnings for the manufacturer themselves.

Skim latex yields 'skim rubber' and 'skim latex serum' upon centrifugation. Skim latex serum contains proteins, carbohydrates, lipids and many other biomolecular compounds. It contains 75% of the total soluble proteins and some of these proteins are enzymes, including hydrolases [5]. According to Yusof *et al*. [6], hydrolases are frequently used in the enzyme-based industries owing to the fact that, these enzymes do not require any co-factors, thus easy to use. Some of the industries that used enzymes are food manufacturing [7, 8], detergents [9–12], pharmaceuticals [13–15] and biodiesel production [16–19].

Lipase, a type of hydrolase, has been purified from the latex serum of *Hevea brasiliensis* [20, 21]. Furthermore, Yusof *et al.* [5] had successfully immobilized this lipase via cross-linked enzyme aggregates (CLEA), a technique that immobilize enzymes without the need of supports [6, 22–36]. CLEA preparation involved precipitants, such as ammonium sulphate $(NH_4)_2SO_4$, that can aggregate the enzymes, and polyfunctional cross-linker, such as glutaraldehyde, that links the enzyme aggregates together. CLEA technique is robust because it can combine purification and immobilization into a single operation to yield highly stable and recyclable catalysts with remarkable catalytic efficiency.

In an attempt to improve the performance, Al Safi and Yusof [37] had adopted a method by Talekar *et al*. [34], by immobilizing the CLEA-lipase onto amino-functionalized magnetic nanoparticles (MNPs). The magnetic property of the nanoparticles helps to ease the separation of CLEA from the reaction mixture by simply using magnetic field instead of the cumbersome centrifugation [38–45]. Besides easy separation of enzymes from the spent media, many researchers have also reported on the higher recovery of the enzymatic activities upon MNPs supported [38–44], including work by Al Safi and Yusof [37].

Converting the free enzyme to insoluble form like CLEA and then further supporting it onto solid supports, such as on MNPs, may affect the kinetic performance of an enzyme. A kinetic description of the enzyme activity may lead to better understanding of any enzyme's function [46]. Many researchers have observed changes in the kinetic properties once CLEA was immobilized on MNPs [40, 45, 47, 48]. These kinetic properties include the maximum rate it can achieved and the rate at which the active sites can be saturated by its substrate. In order to study this properties, the enzyme reaction was modelled according to the Michaelis-Menten kinetics, whereby the model equation describes the rate of the enzymatic reactions by relating the reaction rate, v, to the concentration of the substrate, [*S*], as given in equation (1) [49]:

$$v = \frac{v_{max}S}{K_m + S} \tag{1}$$

Equation (1) describes the relationship between the rate of reaction v, to the concentration of substrate concentration *S,* where v_{max} is the maximum rate of reaction and K_m is the Michaelis-Menten constant. v_{max} is proportional to the amount of active enzymes present and K_m is equal to the reaction concentration at which is equal to $v_{max}/2$. Therefore, K_m is considered as a relative

measure of the substrate binding affinity or the stability of the enzyme-substrate complex whereby lower K_m values indicate higher enzyme affinity for the substrate [49].

In this study, the activity of lipase enzyme recovered from the wasteful skim latex was shown to be enhanced upon immobilization by CLEA technology and was further enhanced by immobilizing the CLEA onto MNPs. The changes in the physicochemical properties from one form to the other, may have alter their kinetic properties. For further development of this newly produced biocatalyst, one of the properties that is important to be considered and studied is their kinetic performances. The kinetic parameters of MNP-CLEA were compared to the unsupported counterpart, in order to have a better understanding on the effect of MNPs as support. The expected changes in the kinetic properties, v_{max} and K_m, may have influences on the changes in the final activity of the enzymes. There is no definite changing trend in terms of the kinetic parameters for enzymes upon immobilization [50]; kinetic properties of each newly produced immobilized enzyme need to be determined every time. Different responses in the kinetic parameter upon CLEA immobilization might occur in response to several factors, such as type of enzymes, substrates, reaction systems and procedures and conditions used during the CLEA preparation [51]. Enzyme kinetics can be modelled using the Michaelis-Menten hyperbolic plots, unfortunately, the extrapolation to v_{max} and estimation of K_m using the plot were difficult, but this can be circumvented by using linearizing equations suggested by Lineweaver-Burk, Hanes-Woolf or Eadie-Hofstee equations [49].

2. Methodology

2.1 Materials

Skim latex from *Hevea brasiliensis* was obtained from MARDEC Industrial Sdn. Bhd., Tapah, Perak. All the other chemicals used in this study were obtained from the local suppliers and they were of analytical grade, unless specified otherwise.

2.2 Methods

2.2.1 Preparation of crude enzyme lipase extract from skim latex serum

To reduce the pH of skim latex from 9 to 5, glacial acetic acid was added slowly while stirring. Once the skim latex was acidified, coagulation process started. The sample was centrifuged at 10,000 rpm at 4°C for 30 min to separate the serum from the skim rubber. Solid $(NH_4)_2SO_4$ was added to the serum till it reached 4 M (saturated) to precipitate all protein. For thorough precipitation, the sample was left stirring overnight at 4°C. The precipitated protein was recovered by centrifugation at 5,000 rpm for 15 min, then dissolved in minimal phosphate buffer saline (PBS) at pH 7 to produce a crude free lipase extract [21].

2.2.2 Preparation of magnetic nanoparticles

Iron chloride ($FeCl_3.6H_2O$, 1.351 g) and iron sulphate ($FeSO_4.7H_2O$, 0.6852 g) were added to 3 mL of NH_4OH and 25 mL deionized water. Precipitate was formed and to remove any residual ions, the precipitate was rinsed with deionised water and centrifuged alternately for a few cycles until the rinsing water reached pH 7. Once this is obtained, the precipitate was dried at 100°C for 3 hours to obtain powdered magnetic nanoparticles (MNPs) [38, 40]. For the silanization process, 1 mL of (3-aminopropyl) trimethoxysilane (APTES) was added 2 g of MNPs, 0.25 mL deionized water and 25 mL methanol. The mixture was sonicated for 30 min before adding 15 mL glycerol. The solution was stirred at 200 rpm for 6 hours at 80°C. Precipitate was washed alternately with water and methanol

for three times, and then dried, yielding a fine powder [38, 40]. The functionalized MNPs were suspended in PBS before use.

2.2.3 Preparation of CLEA-lipase and MNP-CLEA-lipase

Lipase in the form of CLEA and MNP-CLEA was prepared by methods described in Al Safi and Yusof [37]. To prepare CLEA-lipase, crude lipase extract (1.0 ml, 1.175 mg/mL of protein with lipase activity of 0.008 U/mL) was pipetted into a 15 mL Falcon tube. $(NH_4)_2SO_4$ (saturated, 1.8 mL) and glutaraldehyde (1M, 0.2 mL) were added in and PBS was used to top-up to a final volume of 4 mL. The solution was agitated for 17 hrs at 200 rpm at room temperature. Water (3 mL) was added and centrifuged at 4,000 rpm for 30 mins at 4°C. The supernatant was decanted, and the solid CLEA-lipase was washed three more times with water and then kept in 5 mL of water [5].

To prepare MNP-CLEA-lipase, 20 mg of amino-functionalized MNPs was mixed with 1 mL of crude lipase extract. $(NH_4)_2SO_4$ (saturated, 2.8 mL) and glutaraldehyde (2 M, 0.2 ml) were added and then PBS was used to top-up to a final volume of 4 mL. The solution was agitated at 200 rpm for 17 hours at room temperature. The enzyme preparation was washed with water repeatedly for three times. To recover the magnetic biocatalyst, magnetic field was applied, and the final MNP-CLEA-lipase was kept in 5 mL of water [40].

2.2.4 Lipase Enzyme Activity Assay

In this study, *p*-nitrophenyl palmitate (*p*NPP) was chosen as the substrate for lipase activity assay. The lipase reaction was monitored by observing the production of *p*-nitrophenol (*p*NP). The substrate stock solution was prepared by dissolving 28 mg *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 while heating. Assay mixture contains *p*NPP stock solution (1 mL), Tris-HCl at pH 8.2 (1 mL, 0.1 M) and 1 mL enzyme (or CLEA in 1 mL PBS) and incubated in the water-bath for 30 min at 37°C. 1 mL of 1 M NaOH was added to stop the reaction. The absorbance of the assay was measured at 410 nm wavelength, and a standard calibration curve was used to determine the amount of released *p*NP. One unit (U) of lipase enzyme activity is defined as the amount of enzyme required to release 1.0 µmol of *p*NP per min under the assay conditions. Lipase activity of free enzyme was calculated using equation (2).

$$Lipase\ activity\left(\frac{Units}{ml}\right) = \frac{(Abs\ at\ 410\ nm) \times Total\ vol\ of\ assay(m)}{vol\ of\ used\ enzyme\ (ml) \times Time\ of\ assay\ (min) \times slope} \quad (2)$$

The activity of lipase for CLEA was reported in terms of percentage residual activity (% RA) and was calculated by using Equation (3):

$$\%\ Residual\ Activity\ (RA) = \frac{Activity\ of\ CLEA\ immobilized\ enzyme}{Activity\ of\ free\ lipase} \times 100\% \quad (3)$$

2.2.5 Determination of kinetic parameters

The kinetics experiments of both CLEA-lipase and MNP-CLEA-lipase were modelled using the Michaelis-Menten equation by measuring the initial rates of enzymes with varying amounts of substrate, *p*NPP (0, 0.2, 0.5, 1, 1.5 and 2.0 mM) under the assay conditions [52]. The values of K_m and v_{max} was obtained by linearization plots, namely; Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee [50]. For hyperbolic regression, the Hyperbolic Regression Software (Hyper32 version 1.0.0) was used. The three linearized functions used in this study are:

i. The Lineweaver-Burk plot, where $1/v$ is a linear function of $1/S$:

$$\frac{1}{v} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}} \quad (4)$$

ii. The Hanes-Woolf plot, where $[S]/v$ is a linear function of $[S]$:

$$\frac{[S]}{v} = \frac{[1]}{V_{max}}[S] + \frac{K_m}{V_{max}} \quad (5)$$

iii. The Eadie-Hofstee plot, where v is a linear function of $v/[S]$:

$$v = -K_m\frac{v}{[S]} + v_{max} \quad (6)$$

Lineweaver-Burk plot is a double-reciprocal plot created by plotting $1/v_o$ as a function of 1/[S]. Hanes-Woolf plot is carried out by plotting [S] against [S]/v and Eadie-Hofstee plot is carried out by plotting v_o/S against v_o. The accuracy of K_m and v_{max} for each plot was based on the Coefficient of Determination (R^2). Desirable results are measured by the goodness of fit of the model by determining the value of their R^2 of the lines or curves. R^2 should be maintained within the range of 0 to 1.0. and the model is a better fitted when the value gets closer to 1.0 [6].

3. Results and Discussion

3.1 Lipase activity in crude extract

Lipase was successfully extracted from the *Hevea brasiliensis* skim latex serum using the method prescribed by Mohamed and Yusof [21]. The activity of crude lipase enzyme before immobilization is 0.008 U/mL. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein profile of the extract containing lipase is shown in Fig. 1. Four distinct protein bands can be seen at 30, 37 40 and 45 kDa marks. Lipase from skim latex serum was guesstimated to be any one of the bands or, all of the bands.

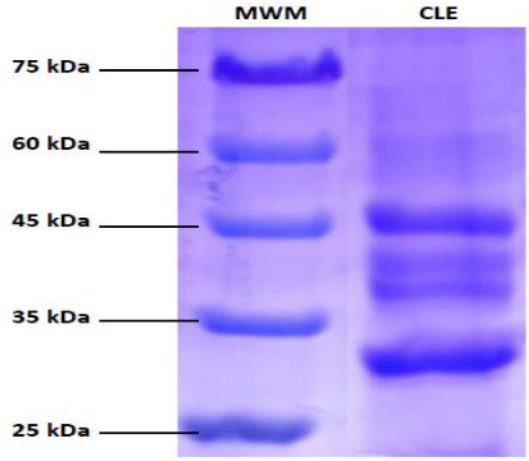

Fig. 1. SDS-PAGE (15% gel) protein profile of crude lipase extract of *Hevea brasiliensis* (MWM-molecular weight markers, CLE-crude lipase extract)

3.2 Lipase activities and comparison of the kinetic parameters of CLEA-lipase and MNP-CLEA-lipase

The conditions to prepare the insoluble biocatalysts, MNP supported and unsupported CLEA-lipase have been optimized previously by Al Safi and Yusof [37]. The results showed that lipase activity was enhanced upon immobilization on MNP, whilst the concentrations of precipitant $(NH_4)_2SO_4$) and cross-linker (glutaraldehyde) used to achieve the highest activities in both systems were different. The optimized conditions to prepare CLEA-lipase that results in the highest residual activity (24.84 % RA) were by using 45% saturated $(NH_4)_2SO_4$ and 50 mM glutaraldehyde. However, the activity of MNP-CLEA-lipase maximised up to 96.38 % RA, when it was prepared using 70% saturated $(NH_4)_2SO_4$ and 60 mM glutaraldehyde [37]. The significant increase in the residual activity of MNP-CLEA-lipase could be due to the presence of MNPs which provide better crosslinking with lipase and glutaraldehyde, leading to better stability and activity [37].

Data plotted in Fig. 2 depicts the kinetic study for both forms of biocatalysts, CLEA-lipase and MNP-CLEA-lipase, respectively, as modelled according to Michaelis-Menten kinetics [49]. The rate of enzyme reactions for both CLEA-lipase and MNP-CLEA-lipase increased rapidly with increased [S], but the rate at which they started plateauing differed. The plots also indicated that both differed significantly in the maximum rates (v_{max}) achieved, whereby, MNPs- CLEA-lipase reached a higher maximum rate compared to the unsupported one. To get the most accurate value of v_{max} and K_m, the data obtained were plotted by four methods, namely Lineweaver-Burk, Hanes-Woolf, Eadie-Hofstee plots and Hyperbolic Regression. The comparison of the kinetic parameters among the plots are shown in Table 1. Results showed that the best model plots were obtained from the Lineweaver-Burk equation observing R^2 values of 0.9823 for MNP-CLEA-lipase. For easy comparison, the value of R^2 for CLEA-lipase was determined to be 0.9610, although it is not the best fitted plot (second best after Hanes-Woolf). Fig. 3 shows the Lineweaver-Burk plots for both enzymes from which the kinetic parameters v_{max} and K_m were determined. The v_{max} for CLEA-lipase is 0.0015 µmol/min.mL, compared to 0.0023 µmol/min.mL for MNP-CLEA-lipase. The increase in v_{max} indicates that MNP-CLEA-lipase required more substrate to convert the reactant to product per unit time. On the other hand, the K_m value for CLEA-lipase is 0.5188 µmol, while it is 0.4400 µmol for MNP-CLEA-lipase, a decrease, indicating that the affinity of the enzyme increased when CLEA-lipase was supported by MNPs. Accordingly, the decrease in K_m value for MNP-CLEA-lipase inferred that the cross-linking of enzyme with MNPs has increased the accessibility of substrate to the enzyme's active sites, which eventually increases the affinity of the substrates to the enzymes.

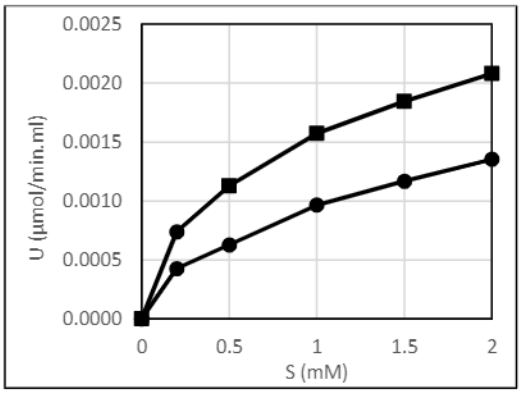

Fig. 2. Comparison of Michaelis-Menten Plots, Unit (U) vs Concentration of Substrate CLEA-lipase and MNP-CLEA-lipase

Table 1
Summary of Michaelis-Menten expression for CLEA-lipase and MNP-CLEA-lipase

Samples	Plots	v_{max} (µmol/min.mL)	K_m µmol	R^2
CLEA-lipase	Hyperbolic Regression	0.0019	0.9592	0.9267
	Lineweaver-Burk	0.0015	0.5188	0.9610
	Hanes-Woolf	0.0019	0.8493	0.9695
	Eadie-Hofstee	0.0016	0.6003	0.8472
MNP-CLEA-lipase	Hyperbolic Regression	0.0027	0.6328	0.8852
	Lineweaver-Burk	0.0023	0.4400	0.9823
	Hanes-Woolf	0.0027	0.6171	0.9695
	Eadie-Hofstee	0.0025	0.4877	0.9267

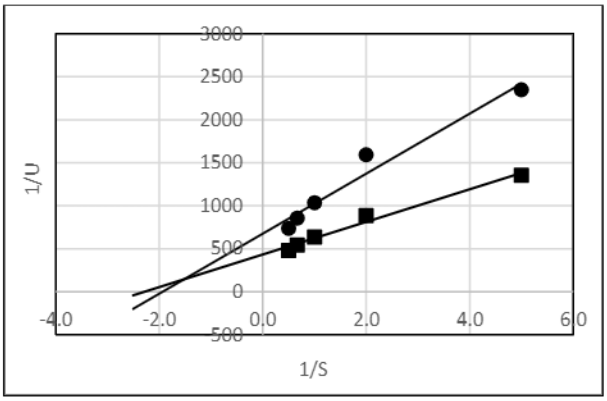

Fig. 3. Comparison of Lineweaver-Burk Plots of CLEA-lipase vs MNP-CLEA-lipase

Accurate estimation of the kinetic parameters (v_{max} and K_m) is very important. Even with the advent of high-end computer and advance computer software that can easily resolve the non-linear regression to find the parameters, linearization of kinetic equation is still popularly used. This is because linearization methods are simple to use; however, researchers should be aware of their drawbacks. Each of these methods has its very own strengths and weaknesses, which are given below:

i. Hyperbolic regression: Although it can be used to accurately predict the kinetic parameters, if powerful computers and regression software is used, such as the Hyperbolic Regression Analysis Software (Hyper32) [28, 50], this method can become unreliable when there are outliers present in the data.
ii. Lineweaver-Burk plot: Errors in this double-reciprocal plot can originate from the *y*-axis when taking the reciprocal of the rate of reaction, which can increase any small errors in measuring the rate. Most points on the plot are found far to the right of the *y*-axis because large [S] (and hence small values for 1/[S] on the plot) are often not possible due to limited substrate solubility, leading to a large extrapolation back to obtain *x*- and *y*-intercepts.
iii. Hanes-Woolf plot: One drawback of this plot is that neither ordinate nor abscissa represent independent variables because both are dependent on substrate concentration.
iv. Eadie-Hofstee plot: Although it gives equal weight to data points in any range of substrate concentration or reaction rate, the problem for this plot is that both ordinate and abscissa are dependent on reaction rate, thus, if there was any measurement error, it will be present in both axes.

Easa *et al.* [50], who compared the kinetic parameters between free and CLEA immobilized amylase extracted from *Zophobas morio*, suggested that there was no definite changing trend in terms of the kinetic parameters (v_{max}, K_m) for enzymes upon immobilization by CLEA. Xu *et al.* [51] suggested that different responses in the kinetic parameter before and after CLEA immobilization might occur in response to several factors, such as the type of enzymes, substrates, reaction systems and procedures and conditions used during the CLEA preparation.

The results observed in this study concurred with Cui *et al.* [53], with their work involving CLEA-phenylalanine ammonia lyase, extracted from *Rhodotorula glutinis* and using L-phenylalanine as the substrate. They reported that v_{max} and K_m increased and decreased, respectively, upon immobilization on magnetic particles. However, Kumar *et al.* [48] in their work with CLEA-laccase from *Trametes versicolor*, while using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as the substrate, observed that both v_{max} and K_m decreased upon the immobilization of CLEA on the magnetic particles. Talekar *et al.* [40] who worked on CLEA-alpha amylase using starch as the substrate found out that K_m has remained the same, however v_{max} has decreased slightly after immobilization onto magnetic supports. All these reports agree with Easa *et al.* [50] suggestion that there is no definite trend for the changes in the kinetic properties of CLEA upon immobilization. Each enzyme acted differently upon any particular immobilization techniques; the trend of changes in the kinetic parameters for any immobilized enzymes should not be generalized and has to worked out each time.

Table 2 shows the summary of the kinetic performances of MNPs supported versus the unsupported CLEA-lipase and their corresponding catalytic efficiency (v_{max}/K_m). According to Eisenthal *et al*. [54], v_{max}/K_m determines the efficiency of an enzyme converting the substrates into products. The v_{max}/K_m of CLEA-lipase and MNP-CLEA-lipase are 0.0029/min.mL and 0.0052 /min.mL, respectively, inferring that MNP-CLEA-lipase is more efficient than CLEA-lipase as a biocatalyst.

Table 2
Summary of kinetic performance of CLEA and MNP-CLEA lipase according to Lineweaver-Burk Plot

Sample	Plot	v_{max} (µmol/min.mL)	K_m µmol	v_{max}/K_m Catalytic Efficiency ($min^{-1}.mL^{-1}$)
CLEA-lipase	Lineweaver-Burk	0.0015	0.5188	0.0029
MNP-CLEA-lipase		0.0023	0.4400	0.0052

4. Conclusions

The natural rubber manufacturing industry produces skim latex as the major source of pollution and this need to be managed prudently. Part of this problems can be solved by converting it to value-added products. Our research has shown that lipase can easily be recovered from skim latex, however, since lipase in its free form has many disadvantages, this enzyme can be converted to an insoluble CLEA form for stability and it can be further be enhanced for a better stability and efficiency by immobilizing on MNPs. These changes in forms may have affected the kinetic performance of the enzymes which was examined in this study. The kinetic performance of the enzyme reaction was modelled using Michaelis-Menten equation. Various equations were used to linearize the graph to ease the accurate determination of the kinetic parameters. Lineweaver-Burk equation values were finally chosen to best represent the changes based on the observed R^2 values. MNP-CLEA-lipase was determined to have v_{max} higher, but K_m lower than CLEA-lipase, respectively. This indicates that MNP-CLEA-lipase has higher maximum velocity, required more substrate to convert the reactant to product and higher affinity towards substrate compared to its unsupported form. Skim latex is a

serious waste in the natural rubber manufacturing industries, but with the advent of biotechnology, it can be easily transformed to advanced material, such as the versatile MNP-CLEA-lipase, which can be applied in many enzyme-based industries, fulfilling the prophecy of 'waste to wealth'.

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