Screening of macromolecular cross-linkers and food-grade additives for enhancement of catalytic performance of MNP-CLEA-lipase of Hevea brasiliensis.

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Abstract. Skim latex from Hevea brasiliensis (rubber tree) consist of many useful proteins and enzymes that can be utilized to produce value added products for industrial purposes. Lipase recovered from skim latex serum was immobilized via cross-linked enzyme aggregates (CLEA) technology, while supported by magnetic nanoparticles for properties enhancement, termed ‘Magnetic Nanoparticles CLEA-lipase’ (MNP-CLEA-lipase). MNP-CLEA-lipase was prepared by chemical cross-linking of enzyme aggregates with amino functionalized magnetic nanoparticles. Instead of using glutaraldehyde as cross-linking agent, green, non-toxic and renewable macromolecular cross-linkers (dextran, chitosan, gum Arabic and pectin) were screened and the best alternative based on highest residual activity was chosen for further analysis. Same goes for additives; the traditional use of expensive BSA was replaced with low cost, food grade additives (egg protein, soy protein and milk protein). The optimum condition parameters such as concentration of cross-linker, concentration of additive and concentration of precipitant were determined using one factor at a time (OFAT) method and structural analysis was done by FTIR. The screening results showed that pectin was the best cross-linker (118 % residual activity) and soy protein was the best additive (129 % residual activity) for MNP-CLEA-lipase with optimum conditions of 180 mg/ml pectin, 0.6 % w/v soy protein and 80 % saturation ammonium sulphate. FTIR of MNP-CLEA-lipase showed an Amide I and Amide V bands which originated from silanized magnetic nanoparticles (MNP) and a new band, Amide II was formed prior to the successful CLEA immobilization of lipase enzyme with MNPs.

1. Introduction

Natural rubber (cis-1,4-polyisoprene) of Hevea brasiliensis is highly used in the latex based industry to produce tires, latex gloves, latex threads and rubber catheters [1]. As a basic raw material in rubber processing, fresh field latex undergoes many procedures during its conversion to dry rubber or high concentrated latex, whereby, the processing usually involves three methods: centrifugation, creaming and evaporation [2–4]. Some of the processes have been carried out by chemical treatment that poses a lot of negative impact on the environment [2]. High concentrated latex is prepared by treating the natural rubber with ammonia and centrifuged to separate the aqueous part from the concentrated rubber. This aqueous part is called skim latex which is one of the most polluting waste in the rubber industry [5]. Before being discharged into the main water system, skim latex is usually pre-treated in the effluent treatment oxidation pond [1].
Manufacturing skim latex into valuable substances would become an alternative to minimize the discharging of effluent into the main waterways, thus making latex processing more environment friendly. Lipid acyl hydrolase (LAH) enzyme is a subtype of lipase and a potential protein that can be recovered from the latex serum [5]. From a study conducted by [5], the highest total activity of LAH in skim latex serum is 61.152 U/ml. Lipase enzyme is immobilized by Cross-Linked Enzyme Aggregates (CLEA) technology which is a carrier-free enzyme immobilization technique for biocatalysis. Traditionally, CLEA preparation involves protein precipitation using ammonium sulphate, followed by cross-linking with glutaraldehyde.

CLEA combines purification and immobilization into a single operation to provide stable and recyclable catalysts with remarkable catalytic efficiency [6]. However, CLEA works when the enzyme has enough lysine (amine residues) which links the enzyme and the cross-linker. Insufficient cross-linking might lead to enzyme leaching into the reaction media and poor stability. CLEA usually involves separation by centrifugation or filtration which would end up with clump formation due to low compression resistance. This might inhibit the internal mass transport of substrate and reduce catalytic activity [9]. Magnetic cross-linked enzyme aggregates (magnetic CLEAs) is a new technique in CLEA technology which has been proven to enhance the catalytic activity of CLEA-immobilized enzymes by increasing its cross-linking ability which leads to improved stability and catalytic activity [7–9]. For the preparation of magnetic CLEAs, amino-functionalized magnetic nanoparticles are added into the enzyme solution for immobilization. The MNPs add more amine residues to generate more cross-linking of the enzyme aggregates which increases the mechanical stability of CLEAs and produces non-leachable CLEAs. Magnetic CLEAs have magnetic properties which helps in separation of MNP-CLEA-lipase from the reaction mixture using a magnet [8,9]. This solves the clump formation problems caused by centrifugation or filtration and makes recycling the immobilized enzyme much easier.

Cross-linking agents play an important role in locking the enzyme aggregates which forms the solid biocatalyst. Glutaraldehyde is the common type of cross-linker used for CLEA technology due to its low price and availability in commercial quantities [6]. However, glutaraldehyde is not compatible to certain enzymes such as nitrilases which leads to low activity retention. Also, glutaraldehyde’s small sized molecule and high reactivity allows it to leach into the enzyme’s active site which would lead to low activity retention [6,10]. [10] and [22] discovered that macromolecular cross-linkers such as pectin, dextran, chitosan and gum Arabic can replace glutaraldehyde as cross-linkers in CLEA-immobilization while increasing the immobilized enzyme’s activity. They are also non-toxic and would not harm the environment. Therefore, other cross-linking agents namely chitosan, dextran, pectin and gum Arabic were screened to choose the most suitable cross-linking agent that can replace toxic glutaraldehyde. Furthermore, additives are used in CLEA immobilization technology in order to stabilize the enzyme’s activity and to provide additional lysine to react with the cross-linker [11]. Additives can also prevent the leaching of cross-linking agents into the enzyme’s active site [12]. However, common additives such as bovine serum albumin (BSA) are expensive and can increase the final cost of the biocatalyst [13]. [13, 20 and 21] discovered cheaper food grade additives such as soy protein, milk protein and egg white protein that can replace BSA as an additive in CLEA-immobilization. These additives are also rich in lysine residues which can facilitate cross-linking of enzymes. Therefore, alternatives for BSA which are low cost, easily available and non-toxic such as soy protein, milk protein and egg protein were screened. The best additive that can replace BSA is chosen for further analysis. The use of macromolecular cross-linker and food grade additives could be a new discovery in producing eco-friendly and more cost effective MNP-CLEA immobilized enzymes with enhanced stability.

2. Materials and method
2.1 Materials
The skim latex of *Hevea brasiliensis* was obtained from Mardec Industrial Latex Sdn. Bhd. in Tapah, Perak. The chemicals used for this project are of analytical grade. Food grade chemicals used are
pectin from citrus peel, chitosan, dextran, gum Arabic, milk protein powder and soy protein powder. Hen egg white was purchased from a local convenience store.

2.2 Methods

2.3 Preparation of skim latex serum
First, the pH of skim latex was reduced from pH 10 to 5 using glacial acetic acid. Then, the acidified sample was centrifuged at 10000 rpm at 4 °C for 30 minutes to separate the supernatant (skim latex serum) from the coagulated rubber (skim rubber). The coagulated rubber was discarded, and the supernatant was pooled. Next, solid ammonium sulphate, (NH₄)₂SO₄ of 4 M was added to the supernatant and left to precipitate overnight under slow stirring at 4 °C. Then, the samples were centrifuged at 5000 rpm for 15 minutes to collect the precipitate while the supernatant was discarded. The precipitate was further dissolved in minimal PBS at pH 7 [5].

2.4 Protein assay
Protein assay was prepared by using the micro plate procedure by Bradford (1976). The linear range of the assay for BSA is 0.1 to 1.0 mg/ml [14]. 800 µl of each standard and sample solution was pipetted into a clean, dry test tube. Protein solutions were assayed in triplicates. 200 µl of diluted dye reagent was added to each tube and vortexed. The samples were incubated at room temperature for 5 minutes. Then, the absorbance of the assay was measured at 595 nm using Thermo Scientific Multiskan Go Spectrophotometer [15].

2.5 Lipase Enzyme Activity Assay
To make the substrate stock solution, 28 mg of p-nitrophenyl palmitate (pNPP) was dissolved in 100 ml of 1% (v/v) Triton 100-X and 1.7 ml of 1% (w/v) sodium dodecyl sulphate (SDS) with stirring and heating. To start the reaction, 1ml of pNPP stock solution was incubated with 1ml of 0.1M Tris-HCl at pH 8.2 and 1 ml of enzyme in water bath for 30 minutes at 37 °C. Then, 1 ml of 1M NaOH was added to stop the reaction. The absorbance of the assay was measured at 410 nm. One unit 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 [16]. Lipase activity is calculated using equation (1).

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\text{Lipase activity (Units/ml)} = \frac{(\text{Absorbance at 410 nm}) \times \text{Total volume of assay (ml)}}{\text{volume of lipase (ml)} \times \text{Time of assay (min)} \times \text{slope of calibration curve}}
\]

(1)

Specific activity is calculated according to equation (2):

\[
\text{Specific activity (U/mg)} = \frac{\text{Lipase activity (U/ml)}}{\text{Total protein content (mg/ml)}}
\]

(2)

2.6 Preparation of magnetic nanoparticles
Ferric chloride (1.351 g) and of ferrous sulphate (0.6852 g) was added into 25 ml deionized water. Ammonium hydroxide was added into the solution until a precipitate was obtained at room temperature. To remove residual ions, the precipitate was centrifuged and washed several times with deionized water until pH 7 is obtained. The precipitate was dried at 100 °C for 3 hours to form dried magnetite nanoparticles [8]. The surface of the particles was coated with 3-aminopropyl triethoxysilane (APTES) by silanization reaction to obtain aminofunctionalized magnetic nanoparticles. Silanization reaction involves dissolving 1 ml of APTES, 2 g of magnetite nanoparticles and 0.25 ml of deionized water in 25 ml methanol. The mixture was sonicated for 30 minutes. Next, 15 ml of glycerol was added to the mixture, and the solution was incubated at 80 °C for 6 hours at 200 rpm. The precipitate obtained was washed with water and methanol for three times in each case and dried, yielding a fine powder [8,9,17]. For working MNP suspension, 20 mg of MNPs was suspended in PBS.
2.7 Preparation of MNP-CLEA-lipase

MNP-CLEA-lipase was prepared as follows. 20 mg of MNPs were mixed with 1ml of free lipase. Then, \((\text{NH}_3\text{)}_2\text{SO}_4\) cross-linkers and additives were added to the mixture of enzyme solution to make a final volume of 4 ml [18]. The solution was agitated at 200 rpm for 17 hours at room temperature. After immobilization, MNP-CLEA-lipase was separated from the reaction media using a magnet and washed three times. The final MNP-CLEA-lipase preparation was kept in 3 ml of water to measure the residual activity by conducting the lipase assay [9]. The residual activity of MNP-CLEA-lipase was determined using equation (3).

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\text{Residual activity} \, (\%) = \frac{\text{total activity of MNP-CLEA-lipase}}{\text{total activity of free lipase for CLEA preparation}} \times 100\%
\] (3)

2.8 Screening of Different Additives

MNP-CLEA-lipase was prepared following method 2.2.5 by cross linking with glutaraldehyde and addition of different additives namely BSA, soy protein, hen egg white protein and milk protein. The concentration of additives used were 0, 0.2, 0.4, 0.6, 0.8 and 1.0% (w/v) for BSA, soy protein and milk protein while egg protein was prepared using different concentrations due to their different physical properties (0, 0.6, 1.15, 1.72, 2.3, and 2.88%) [13,19–21]. The residual activity of MNP-CLEA-lipase with food grade additives was compared with MNP-CLEA-lipase with BSA to choose the best additive.

2.9 Preparation of Macromolecular Cross-linkers

Pectin, gum Arabic, chitosan, and dextran are not natural cross-linkers. In order to for them to become macromolecular cross-linkers, they need to be derivatized by periodate oxidation. Pectin, dextran, chitosan and gum Arabic were dissolved in volume ratio composition of 80:20 water:ethanol. For oxidation, 3 ml of 0.5 M sodium metaperiodate was added and the pH of the solution was adjusted to 3.5 using dilute hydrochloric acid and sodium bicarbonate solution. Oxidation was carried out under constant stirring for 3 hours in the dark at 60 °C. 3 ml of ethylene glycol was added to stop the oxidation. Then, the macromolecular cross-linkers were precipitated out by adding excess isopropanol and separated by vacuum filtration [10,22–24]. The precipitate was dried at 40 °C overnight [24]. Before being used as a cross-linker, each oxidized dried polysaccharide was dissolved in sodium acetate buffer (0.1 M, pH 6.0) to make 0.42 mol/L aldehyde content which is equivalent to glutaraldehyde [10,22,24].

2.10 Screening of Different Cross-linkers

MNP-CLEA-lipase was prepared following method 2.2.5 without additives. The residual activity of MNP-CLEA-lipase cross-linked with macromolecular cross-linkers was compared to that of MNP-CLEA-lipase cross-linked with glutaraldehyde. The concentration used for macromolecular cross-linkers were (100, 120, 140, 160, 180 and 200 mg/ml) while for glutaraldehyde (30, 40, 50, 60,70, and 80 mM) were used [10]. The best cross-linker was chosen based on the highest residual activity of MNP-CLEA-lipase [10,25].

2.11 One Factor at a Time (OFAT)

OFAT was carried out by varying one parameter at a time while keeping the others constant. Selected macromolecular cross-linkers and additives were used. The concentrations of selected cross-linker (100, 120, 140, 160, 180 and 200 mg/ml), selected additive (0.2, 0.4, 0.6, 0.8, and 1.0 % w/v) and \((\text{NH}_3\text{)}_2\text{SO}_4\) (50, 60, 70, 80, 90, and 100 % saturation) were varied to determine the optimum conditions for MNP-CLEA-lipase. The parameters that were kept constant were amount of MNPs, agitation rate (200 rpm), amount of lipase enzyme (1ml) and incubation time (17 hours) [18].
2.12 Structural characterization by FTIR

Fourier transform infrared spectroscopy (FTIR) analyzes the structure of the samples by providing information on structural features of peptides and proteins. It also allows us to determine the peptide groups of each sample based on peak values of the wavelengths.\[26\]. The FTIR spectra of free lipase, functionalized MNPs and MNP-CLEA-lipase were recorded using Nicolet iS50 FTIR spectrometer with spectral resolution of 4000-650 cm\(^{-1}\) and spectral resolution 4 cm\(^{-1}\). The samples were placed on a diamond pallet and crushed to obtain an absorption spectrum. The analysis was done in triplicate.

3. Results and discussion

3.1 Screening of Additives

Additives or proteic feeders provide additional lysine which prevents inactivation of enzymes by distorted and insufficient or excessive cross-links. It also prevents enzyme leaching which is due to poor cross-linking with enzymes that contain low lysine groups on their external surface [13,27]. MNP-CLEA-lipase was prepared with addition of BSA and chosen food grade additives namely soy protein, milk protein and egg white protein. The residual activity of MNP-CLEA-lipase with food grade additives were compared with MNP-CLEA-lipase with BSA. The results are illustrated in figure 1 and 2. It is observed from figure 1 and 2 that soy protein showed the highest residual activity which is 130 % at 0.6 % (w/v) compared to the other additives. This might be due to the higher content of lysine residues in soy protein compared to the other additives which provides more sufficient cross-linking [13,28]. According to [13], soy protein possess large molecular sizes which could favor the preparation of CLEAs with lower mass limitations. Soy protein’s low cost and high lysine content (70 mg/g protein) makes it a favorable proteic feeder [28]. At the same concentration, BSA obtained 79 %, milk protein obtained 102 % and egg protein obtained 79 % residual activity. Therefore, from the screening process, soy protein was chosen as the favored additive for further analysis.

![Figure 1](image1.png)

**Figure 1.** Residual activity of MNP-CLEA-lipase with BSA, soy protein and milk protein.

![Figure 2](image2.png)

**Figure 2.** Residual activity of MNP-CLEA-lipase with egg protein.

3.2 Screening of Cross-linkers

In recent years, macromolecular cross-linkers have been recognized as ideal cross-linking agents in CLEA-immobilization which can replace glutaraldehyde. They are generated by periodate oxidation which cleaves the C-2 and C-3 bonds of the monomeric units of the polysaccharide which results in the formation of aldehyde groups. The aldehyde groups formed can cross-link with E-amino groups of lysine of the protein by Shiff’s base formation [10,25]. The effects of macromolecular cross-linkers on MNP-CLEA-lipase were compared to MNP-CLEA-lipase cross-linked with glutaraldehyde. Results show that the highest residual activity for glutaraldehyde was only 94 % at highest concentration (70 mM) as shown in figure 3. Enzymes cross-linked with glutaraldehyde resulted in formation of clumps of enzyme molecules which hinders the active site. This causes mass transfer limitations for macrow-substrates which results to lower residual activity [29]. However, for macromolecular cross-linkers,
pectin showed highest residual activity which is 116 % at 180 mg/ml compared to other cross-linkers. At the same concentration, dextran obtained 55 %, chitosan obtained 67 % while gum Arabic obtained 61 % as shown in figure 4. This makes pectin the most suitable cross-linker that can substitute glutaraldehyde. MNP-CLEA-lipase with dextran, chitosan and gum Arabic showed less cross-linking after immobilization compared to pectin which might be due to mass transfer limitations caused by clump formation. According to [10], each macromolecular cross-linker has different combination of monomers linked to each other by alpha or beta bonding which forms specific structures responsible for the differences in their functional and structural properties.

3.3 **OFAT for concentration of precipitant.**
Precipitation of enzymes is one of the major steps in CLEA-immobilization which creates the aggregated forms of free enzymes. The precipitant used for this study is ammonium sulphate, \((\text{NH}_4)_2\text{SO}_4\). According to [10,30], \((\text{NH}_4)_2\text{SO}_4\) is the best cross-linker for CLEA-immobilization. The concentration of \((\text{NH}_4)_2\text{SO}_4\) was varied at 50–100 % saturation while concentration of pectin and soy protein were kept constant at 180 mg/ml and 0.6 % (w/v) respectively. Residual activity of MNP-CLEA-lipase showed an increase and peaked at 80 % saturation of \((\text{NH}_4)_2\text{SO}_4\) (134 %) as shown in figure 5. Further increase in concentration lowered the residual activity which might be caused by partial deactivation of enzymes due to larger aggregates formed [9,10,31].

3.4 **OFAT for concentration of additive.**
From the screening results, soy protein was chosen as a suitable additive which can replace BSA. For OFAT, concentration of soy protein was varied at 0–1.0 % (w/v) (figure 6) while concentration of
pectin (180 mg/ml) and (NH₄)₂SO₄ (80 % saturation) were kept constant. MNP-CLEA-lipase achieved highest residual activity at 0.6 % (w/v) of soy protein (120 %). The residual activity decreased at higher concentration which might be due to competition between the free amino groups of lipase with free amino groups of soy protein [13].

3.5 **OFAT for concentration of cross-linker.**

The cross-linker chosen to replace glutaraldehyde was pectin based on the screening results. For OFAT, the concentration of pectin was varied at 100–180 mg/ml (figure 7) while concentration of soy protein and concentration of (NH₄)₂SO₄ was kept constant at 0.6 % (w/v) and 80 % saturation respectively. The variation of pectin concentration alters the extent of cross-linking by controlling the reaction between ε-amino groups of surface lysine side groups of the enzyme molecule [25]. The residual activity of MNP-CLEA-lipase increased as the concentration of pectin increased until it reached its peak at 180 mg/ml (110 %). Higher pectin concentration reduced the residual activity due to excessive cross-linking which might result to loss of enzyme’s minimum flexibility needed for the activity of MNP-CLEA-lipase [25].

![Figure 6. Residual activity of MNP-CLEA-lipase with different concentrations of soy protein.](image)

![Figure 7. Residual activity of MNP-CLEA-lipase with different concentrations of pectin.](image)

3.6 **Structural characterization by FTIR**

Fourier transform infrared spectroscopy (FTIR) analyzes the structure of the samples by providing information on structural features of peptides and proteins. It also allows us to determine the peptide groups of each sample based on peak values of the wavelengths [26]. Changes in secondary structure of MNP-CLEA-lipase with pectin and soy protein was analyzed by FTIR. Figure 8(a) illustrates the peaks for functionalized MNPs while figure 8(b) illustrates the peaks for MNP-CLEA-lipase. MNPs and MNP-CLEA-lipase showed similar bands at 1633.62 cm⁻¹ and 1645.23 cm⁻¹ respectively which contributes to Amide I bands (N-H stretching). Also, they have similar bands at 689.75 cm⁻¹ and 702.29 cm⁻¹ which belongs to Amide V bands (N-H bending). However, MNP-CLEA-lipase showed an increase in wavelength which might be caused by the enhancement of the bond forces [32]. MNP-CLEA-lipase also showed a new bond formation at peak 1549 cm⁻¹ which contributes to Amide II band (N-H bending and C-N stretching) [33]. According to figure 9, free lipase also showed presence of Amide I band at 1635.48 cm⁻¹. The wavelengths for MNP-CLEA-lipase and free lipase appeared to be quite similar which might be due to the same attributes. Although, the strength of the peaks for MNP-CLEA-lipase were more prominent compared to free lipase. Generally, MNP-CLEA-lipase showed characteristics originating from silanized MNPs and free lipase based on the similar Amide groups present. This shows that free lipase enzyme was successfully immobilized by CLEA with addition of magnetic nanoparticles and using pectin and soy protein.
Figure 8. FTIR analysis. (a) Functionalized MNPs. (b) MNP-CLEA-lipase crossed linked with pectin and added soy protein.

Figure 9. FTIR analysis of free lipase

4. Conclusion
The study on MNP-CLEA immobilized enzymes have been rising throughout the years and is still expanding as it is a very efficient immobilization method due to its ease in separation process by using a magnet and can be easily recycled for reuse. However, to be able to be utilized in food-based industries and for environmental purposes, green, low-cost and non-toxic materials should be used throughout the immobilization process. Fortunately, low-cost food grade cross-linkers and additives are proven to be better alternatives that can counter the problems faced by using traditional cross-linker such as glutaraldehyde and additive such as BSA while enhancing the enzyme’s biocatalytic activity. Pectin and soy protein appeared to be a suitable combination for MNP-CLEA-lipase as results showed high residual activity with optimum conditions of 180 mg/ml pectin, 0.6 % w/v soy protein and 80 % saturation of ammonium sulphate. MNP-CLEA immobilization with macromolecular cross-linkers and food grade additives can become a new discovery which not only can produce more stable biocatalysts but also eco-friendly and cost-effective biocatalysts.
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