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# Immobilisation of lipase from *Candida rugosa* on layered double hydroxides of Mg/Al and its nanocomposite as biocatalyst for the synthesis of ester

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**Abstract**

Synthesis of layered double hydroxides (LDHs) of Mg/Al-$NO_3^-$ and Mg/Al-sodium dodecyl sulphate (SDS) of molar ratio of Mg/Al (4:1) were done by co-precipitation through continuous agitation. Their structures were determined using X-ray diffractometer (XRD). The spectra showed that basal spacing for synthesized Mg/Al-$NO_3^-$ and Mg/Al-SDS were around 7.8 and 34.3 Å, respectively. The expansion of layered structure was observed to accommodate the surfactant anion between the interlayer for the Mg/Al-SDS. Lipase from *Candida rugosa* was immobilised onto these materials by physical adsorption method. The percentage of protein loaded into Mg/Al-SDS (70.8%) is higher than Mg/Al-$NO_3^-$ (36.9%). The synthesis of butyl oleate using immobilised lipase as biocatalyst was investigated through esterification of oleic acid and 1-butanol as substrate and hexane as reaction medium. The effects of surface area, reaction temperature, thermal stability, leaching study, stability in organic solvent and storage were investigated. The stability was found to be the highest in the Mg/Al-SDS.


*Keywords:* Immobilisation; Lipase; Layered double hydroxides; Esterification

## 1. Introduction

Layered double hydroxides (LDHs) are minerals and synthetic materials with positively charged brucite-type layers of mixed metal hydroxides is generally represented as $[M_{1-x}^{2+}M_x^{3+}(OH)_2]^{x+}[(A^{n-})^{x/n}yH_2O]^{x-}$. Partial substitution of $Mg^{2+}$ cations by other trivalent ones resulted in positive charging of layers. The excess charge of brucite-like sheets is compensated by anions, which together with water molecules are placed in interlayer spaces [1].

In nature, LDH may be important as unstable intermediates during mineral alterations precipitation or corrosion due to their anion exchange capacity and extensive areas of reactive hydroxylated surfaces. Nowadays, LDH find extensive use in applications in areas of pharmaceuticals and catalysis as antacid drugs, gas separations, ionic conductors, precursors for basic catalysts, semiconductors, adsorbents and ion exchangers [2].

Lately, the approach of using LDH has extended in the biotechnology applications such as host materials or support for enzyme. Immobilisation of enzyme on to suitable support has shown to exhibit higher activity and increase stability. Thus, it is one of the method to make enzyme more suitable for the desired purposes of chemical reaction and selectivity of products. Physical and chemical method can be used for immobilising enzymes on to support. Physical method such as adsorption of enzyme on a support is relatively easier and cheaper compared to chemical method such as covalent attachment of the enzyme and support [3].

In this study, Mg/Al-LDH was chosen as support because it is the most common member of the LDH minerals or so-called anionic clays [4]. Roelofs et al. [5] reported that its composition formula is as follows: $Mg_6Al_2(OH)_{16}CO_3{\cdot}4H_2O$. We now reported on the immobilisation of lipase from *Candida rugosa* onto Mg/Al-LDH

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for esterification reaction of oleic acid and butanol. The effects of various parameters on esterification were investigated.

## 2. Experimental procedures

### 2.1. Materials

Lipase from *C. rugosa* (E.C.3.1.1.3 Type VII) was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

### 2.2. Synthesis of Mg/Al-$NO_3^-$ hydrotalcite

The Mg/Al-$NO_3^-$ was synthesized at a molar ratio of Mg:Al (4:1). A solution of 2 M $NaOH/Na_2CO_3$ was added into a 100 mL of solution containing 0.2 M $Mg(NO_3)_2$ and 0.8 M $Al(NO_3)_3$ with continuous stirring using a magnetic stirrer. The dropwise addition was completed until the pH of the solution reached pH 10. The slurry was heated at 65 °C for 18 h in a horizontal water bath shaker (110 rpm). The cooled semi-solid solution was then filtered, washed several times with distilled water and dried in an oven for 24 h at 70 °C.

### 2.3. Synthesis of Mg/Al-sodium dodecyl sulphate nanocomposite

The nanocomposite, sodium dodecyl sulphate (SDS) was added into Mg/Al solution before the titration with $NaOH/Na_2CO_3$ to produce LDH with SDS. A 50 mL of solution 0.1 M SDS was added to a 100 mL of solution containing 0.2 M $Mg(NO_3)_2$ and 0.8 M $Al(NO_3)_3$. The dropwise addition was completed until the pH of the solution reached pH 10. The resulting mixture was heated in a horizontal shaker bath (110 rpm) at 65 °C for 18 h, cooled, filtered, and washed several times with distilled water and then dried in an oven at 120 °C.

Both LDHs were characterised using X-ray diffractometer (XRD) and scanning electron micrograph (SEM). Analysis of surface area and porosity of LDHs were done using a Micromeritics, ASAP 2000. This study was carried out to investigate the best support among the LDHs.

### 2.4. Immobilisation of lipase

Crude lipase from *C. rugosa* (1.5 g) was dispersed into distilled water (15 mL), stirred for 1 h and then followed by 15 min centrifuging at 10,000 rpm. The supernatant was used as partially purified lipase. The immobilisation was carried out by continuous stirring at 100 rpm of LDHs (2.0 g) with lipase solution (15 mL) for 1 h at room temperature. The immobilised lipase was filtered and the supernatant was kept for protein assay.

### 2.5. Protein assay

The amount of protein content before and after immobilisation was determined by Bradford method [6]. Define calculation of protein immobilised in percentage as follows:

$$\text{immobilization (\%)} = \frac{\text{total amount of protein adsorbed} \times (\text{before} - \text{after immobilization})}{\text{total amount of protein in supernatant before immobilization}} \times 100$$

### 2.6. Characterisation of the immobilised lipase on the esterification activity

#### 2.6.1. Reaction temperature

This experiment was conducted to investigate the optimum temperature for the esterification reaction. The mixtures were incubated at different temperatures (30, 40, 50, 60, 70 °C) for 5 h with continuous shaking at 150 rpm in water bath shaker. The relative activities are determined as percentage yield of activities at different temperature compared to the activity of reaction at 40 °C as

$$\text{relative activity (\%)} = \frac{\text{\% activity at different temperature}}{\text{maximum \% activity (40 °C)}} \times 100$$

#### 2.6.2. Thermal stability

The immobilised enzymes were incubated at different temperatures (30, 40, 50, 60, 70 °C) in sealed vials for 1 h. After the incubation, the immobilised enzymes were left to cool to room temperature. The relative activities are determined as percentage yield of activities at different treated temperature compared to the activity treated at 40 °C as

$$\text{relative activity (\%)} = \frac{\text{\% activity at different temperature}}{\text{maximum \% activity (40 °C)}} \times 100$$

#### 2.6.3. Leaching study

The immobilised enzyme (0.3 g) was washed consecutively with 4.0, 8.0, 12.0, 16.0 and 20.0 mL of hexane. The relative activities are determined and compared to the activity of the unwashed immobilised lipase as

$$\text{relative activity (\%)} = \frac{\text{\% activity at different mL of hexane}}{\text{maximum \% activity (0 mL)}} \times 100$$

#### 2.6.4. Stability in organic solvent

The immobilised enzymes were incubated in hexane, without shaking for 1–10 days at room temperature. The enzymatic activity was determined at 30 °C. The relative

activities are determined as percentage yield of activities at days 2–10 compared to the activity at day 1 as

$$\text{relative activity (\%)} = \frac{\text{\% activity at number of days}}{\text{maximum \% activity (day 1)}} \times 100$$

*2.6.5. Storage stability*

The native and immobilised lipases (0.30 g) were stored in hexane for 60 days at −20, 0, 4 °C and room temperature. The activity of the immobilised enzyme was determined by the esterification activity. The relative activities are determined and compared to the activity at day one for each temperature as

$$\text{relative activity (\%)} = \frac{\text{\% activity at different storage temperature}}{\text{maximum \% activity (storage temperature)}} \times 100$$

*2.7. Esterification assay*

The enzymatic reaction consisted of 1-butanol (4.0 mmol), oleic acid (2.0 mmol), immobilised enzyme (0.3 g) and hexane (2 mL). The reaction mixture was incubated at 30 °C for 5 h, with continuous shaking at 150 rpm in a horizontal water bath shaker. The reaction was terminated by the addition of 3.5 mL of acetone:ethanol (50:50, v/v). The remaining free fatty acid in the reaction was determined by titration with 0.15 M NaOH using an autotitrator to an end point at pH 10. The activities were expressed as specific activity (μmol/min mg protein). All experiments were tested in triplicate. The control experiments were carried out using native lipase of *C. rugosa*.

## 3. Results and discussion

*3.1. Characterisation of LDH*

Both layered double hydroxides, Mg/Al-$NO_3^-$, which anion of the interlayer is $NO_3^-$ and its nanocomposite Mg/Al-SDS formed as white powder have been synthesized with ratio $R = 4$ and pH 10 by direct hydrolysis. They were characterised by XRD, BET and SEM techniques. Analysis of the XRD diffractogram showed that the basal spacing for Mg/Al-$NO_3^-$ is 7.8 Å compared to 34.3 Å for the nanocomposite Mg/Al-SDS. The expansion of layered structure contributes to the maximum of its basal spacing of around 34.3 Å was observed to accommodate the surfactant anion between the interlayer for the Mg/Al-SDS indicating the intercalation process was successfully took place between the interlayer of inorganic LDHs to form layered nanocomposite, of organic–inorganic hybrid type.

The morphologies of Mg/Al-$NO_3^-$ (Fig. 1a) showed some big and rod shaped structures. This will give small surface area and may lead to low protein adsorption and catalytic activity. However, the morphologies of Mg/Al-SDS (Fig. 1b) showed homogenously distributed particles giving a large surface area. This is very important for enzyme work, so that higher protein adsorption could occurred at the surface and will also give high enzymatic activity.

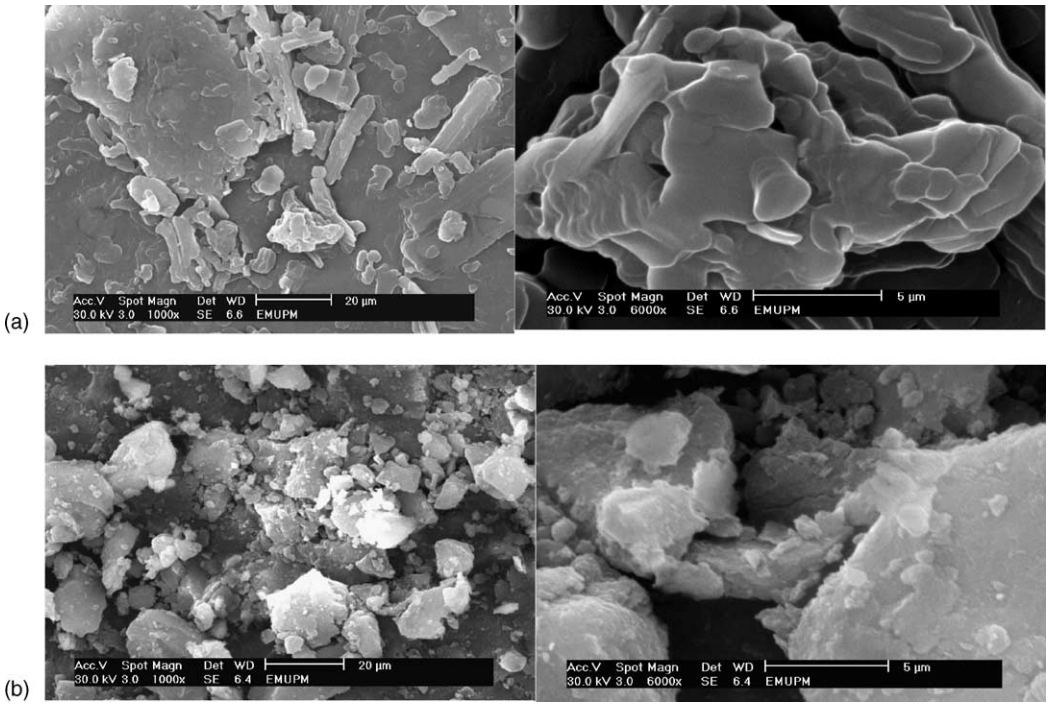


Fig. 1. SEM Micrographs of layered double hydroxide at 1000× magnification and 6000× magnification. (a) Mg/Al-$NO_3^-$, (b) Mg/Al-SDS.

Table 1
BET surface area, micropore volume and BJH desorption pore size distribution of both layered double hydroxides prepared at ratio 4

| Layered double hydroxides | BET surface area ($m^2$/g) | Micropore volume (cc/g) | BJH desorption (Å) |
|---|---|---|---|
| Mg/Al-$NO_3^-$ | 60 | 0.128 | 88.4 |
| Mg/Al-SDS | 71 | 0.275 | 93.5 |

### 3.1.1. Immobilisation of lipase

The BET surface area, micropore volume and BJH desorption pore size distribution of LDHs are summarised in Table 1. The increase of the BET surface area of the support from 60 to 71 $m^2$/g has increased the amount of protein adsorbed on Mg/Al-$NO_3^-$ and Mg/Al-SDS from 24969 and 47861 mg, respectively. Higher amount of protein adsorbed of 70.8% on Mg/Al-SDS was due to the larger surface area and thus increasing the specific surface area of the Mg–Al-SDS support as compared to only 36.9% of protein adsorbed on Mg–Al-$NO_3^-$. During adsorption, the lipase molecules were immobilised within the layer or randomly scattered on the surface of support as they are replacing the water molecules. Furthermore, the expansion of basal spacing between the brucite sheets of the molecules allowed more enzymes to be adsorbed on to it. This situation leads to a better protein adsorption by Mg/Al-SDS. Smaller pore sizes restrict mass transfer and pore penetration of the protein, which limit the protein interaction with the total surface area of the LDHs. In addition for a successful immobilisation by adsorption, an electrostatic interaction is needed between the enzyme and support.

### 3.1.2. Reaction temperature

The effect of the reaction temperature on the enzymatic reaction is shown in Fig. 2. The activity was increased from 30 to 40 °C but decreased when temperature was increased from 50 to 70 °C. The optimum temperature for both immobilised enzymes and native lipase was found at 40 °C. Among these biocatalysts, lipase immobilised onto layered double hydroxide of ratio 4 with SDS showed better results in protecting the enzyme against the excess heat energy from reaction, which may affect enzymatic rate and functional group of substrate involve. The conversion of butyl oleate ester was inhibited in the esterification reaction at higher temperature.

### 3.1.3. Thermal stability

Immobilised lipases showed better thermal stability than native lipase after 1 h incubation at temperatures between 40 and 70 °C as shown in Fig. 3. At temperature above 40 °C, enzymes resistance to adverse heat influences and the ability to maintain their molecular integrity to face denaturants were weakened and destroyed. According to Fagain [7], lipases are easily denatured at high temperature where the peptide bonds and amino acid side chains are reactive and can participate in deleterious reactions at high temperature.

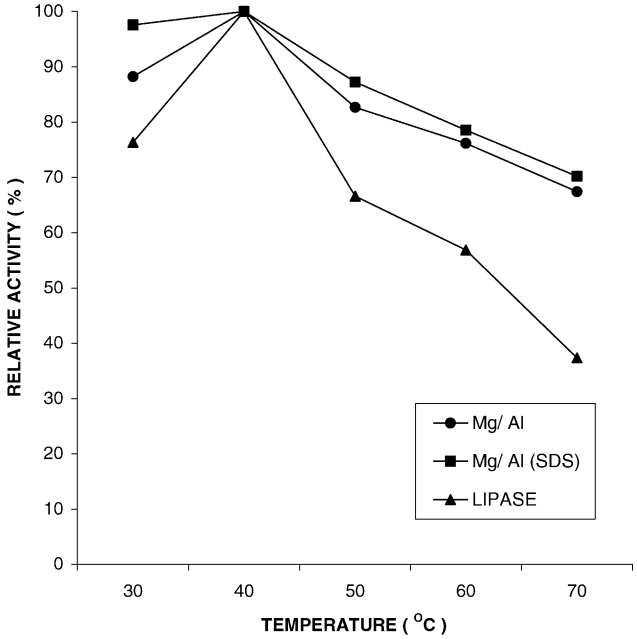


Fig. 2. Effect of reaction temperature on the relative esterification activity of immobilised lipases. Reactions were performed in hexane with 1:1 mol ratio of 1-butanol and oleic acid.

Lipase immobilised onto layered double hydroxide added with SDS showed the highest thermal stability by remaining 60% of its activity at 70 °C. Although heat considerably reduces conformational flexibility of native and immobilised lipase, immobilised lipase is still capable of performing its vibrational and more complex movement required for efficient catalytic activity. Therefore, better thermal sta-

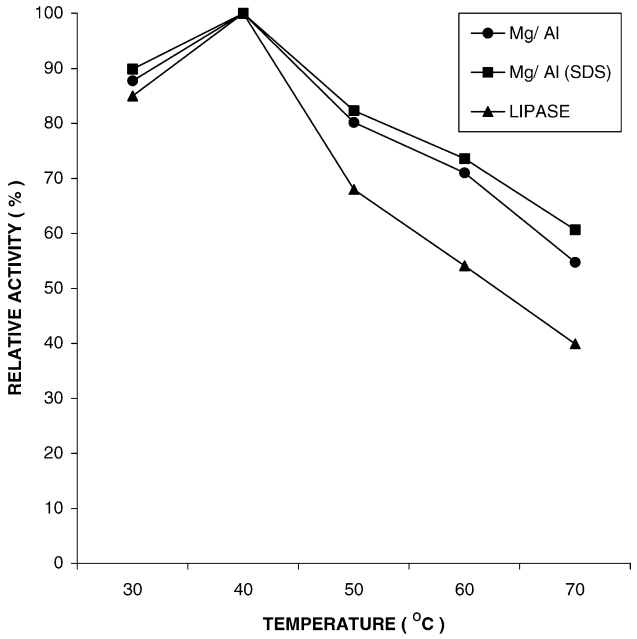


Fig. 3. Percent relative activity of native and immobilised lipases after 1 h incubation at various temperatures. Reactions were performed in hexane with 1:1 mol ratio of 1-butanol and oleic acid at 30 °C.

bility may be achieved by immobilisation on flexible solid support.

### 3.1.4. Leaching study

The effect of leaching of the immobilised enzyme was studied using hexane as washing medium. Both immobilised lipases have retained their catalytic activities at 100% even after careful washing with 20 mL of hexane, indicating that lipase from *C. rugosa* remain immobilised on support. These results have proven that hydrotalcite and its nanocomposite are suitable support for lipase immobilisation and have the ability to prevent the lipase from being leached out from the support. Furthermore, this also proved that the adsorption method used in this study is a suitable method of immobilisation [8].

### 3.1.5. Stability in organic solvent

The stability of the immobilised lipases in the presence of organic solvent exhibited greater stability than the native lipase for 1–10 days at room temperature as shown in Fig. 4. The Mg/Al-SDS performed the best stability by retaining the conversion of butyl oleate at above 50% of its activities after 10 days of incubation in hexane. The support may prevent the water monolayer from being stripped and maintain the three dimensional structure of its active protein conformation. Therefore, enzymes may be stable in organic solvents than they are in water and this is the reason why they are used as medium reaction in enzymatic reaction [9].

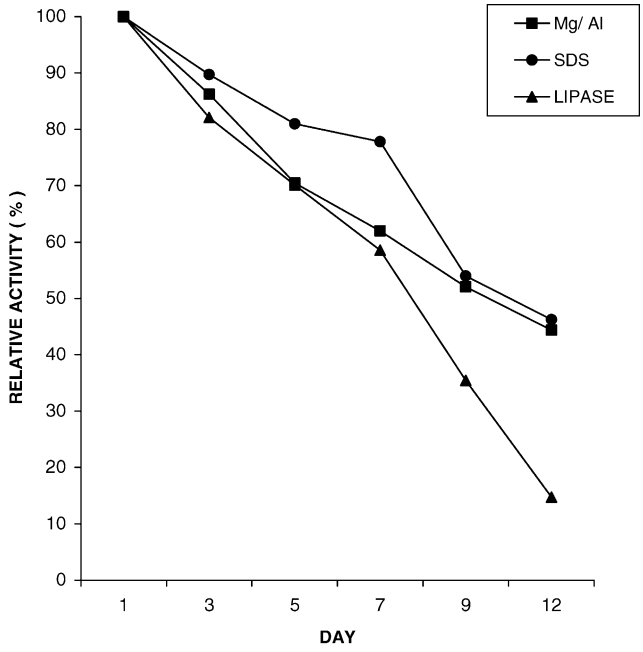


Fig. 4. Percent relative activity of native and immobilised lipase as affected by incubation in hexane at room temperature (25 °C) from days 1 to 10. Reactions were performed in hexane with 1:1 mol ratio of 1-butanol and oleic acid at 30 °C.

Table 2
Percent relative activity of native and immobilised lipase after storing at different temperatures for 60 days

| Lipase | Activity (100%) | | | |
|---|---|---|---|---|
| | RT | 4 °C | 0 °C | −20 °C |
| Native | 55 | 71 | 79 | 100 |
| Mg/Al-$NO_3^-$ | 74 | 82 | 100 | 100 |
| Mg/Al-SDS | 77 | 85 | 100 | 100 |

Reactions were performed in hexane with 1:1 mol ratio of 1-butanol and oleic acid at 30 °C.

### 3.1.6. Storage stability

One of the important criteria that must be considered in developing immobilised enzyme is the stability to store it over a period of time and various temperatures. The enzymatic activity of various lipase preparations determined after storing for 60 days under different temperatures were summarised in Table 2. All lipases showed full catalytic activity after storing them at −20 °C as commonly practised in laboratory. However, only immobilised lipases retained their full catalytic activity when stored at 0 °C as at very low temperatures, lipase is probably locked in its native situation, catalytically active conformation. Immobilised lipases stored at higher temperatures after 60 days still showed good storage stability up to 70% compared to native lipase. The stabilization at this temperature in immobilised enzyme may be due to multipoint attachment of the enzymes to the supports, creating a more rigid enzyme molecule which prevent any intermolecular reaction such as aggregation and proteolysis. Hence, disruption of the active center becomes less likely to occur [10].

## 4. Conclusions

The application of layered double hydroxide of Mg/Al and its nanocomposites as support for enzyme immobilisation have exhibited an increase in stability of biocatalyst and conversion of butyl oleate. In addition, immobilisation can be obtained through simple and inexpensive method of physical adsorption, a promising future of applying biomaterial as a catalyst in any chemical reactions.

## Acknowledgements

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