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Enzymatic synthesis of methyl adipate ester using lipase from *Candida rugosa* immobilised on Mg, Zn and Ni of layered double hydroxides (LDHs)

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Abstract

The enzymatic synthesis of methyl adipate via green esterification of adipic acid and methanol in hexane has been studied. Lipase from *Candida rugosa* immobilised onto various layered double hydroxides (LDHs) by a reproducible and simple method of physical adsorption was used as biocatalyst with promising result. Mg/Al–NO_3^-, Zn/Al–NO_3^- and Ni/Al–NO_3^- of LDHs with molar ratio of $M^{2+}/M^{3+} = 4{:}1$ were synthesised by co-precipitation method with continuous agitation. The percentages of protein loading on Mg/Al–NO_3^-, Zn/Al–NO_3^- and Ni/Al–NO_3^- were 71%, 67% and 58%, respectively, due to the larger surface area, porosity and basal spacing of the supports. Parameter studies of reaction time, reaction temperature, water activity, thermostability, storage, leaching and reusability were investigated and optimised. Optimum conditions to produce adipate ester upto 80 % were reaction time; 2.5 h, temperature; 50 °C, and water activity; 0.53, respectively. Increased in optimisation conditions and enhanced stability properties were found after immobilisation compared to the native lipase.

Keywords: Adipate; Lipase; Immobilisation; Layered double hydroxides; Biocatalyst

1. Introduction

The excellent properties of adipic esters such as its low toxicity, good thermal stability, low volatility and high biodegradability [1], make it as a very useful compound and significant for industrial application especially in petro-based and lubricant industries. The specially formulated esters such as methyl adipates are widely synthesised due to their relatively low cost and good balance of properties [2] using C6 straight chain dicarboxylic acid, particularly adipic acid. Adipic esters which are produced with alcohol of 1–10 carbon are called adipates and they are most commonly used in manufacturing plasticizers, lubricants, adhesives, paint stripper and coating industry [3]. Methyl adipate esters can be formed by reacting adipic acid with monohydric alcohol namely methanol via esterification reaction under mild condition.

The green synthesis of esters in organic medium catalysed by using immobilised enzyme has greatly expanded the enzyme potential for its use as industrial biocatalyst [4,5]. Compared to biochemical method, the use of homogeneous chemical catalysts may lead to several problems such as separation of products, hazards in handling of the corrosive acids, high energy consumption and degradation of esters, while the enzymatic synthesis offers mild reaction conditions and environmental benign process. Lipase from *Candida rugosa* (E.C. 3.1.1.3 Type VII) has gained considerable importance as versatile biocatalyst in the esterification for the synthesis of a wide range of esters [6]. At present, the main disadvantage for the use of enzyme for the industrial production is the cost of the enzyme. Thus, it is necessary to develop highly stable biocatalyst for the synthesis of high added value of esters. For this reason, the use of immobilised enzyme has become a valid approach due to its special features which allow the reutilisation of the enzyme and separation of products. The previous study on the synthesis of some adipic esters via alcoholysis was reported by Gryglewicz (2001) [2], whereby the immobilised enzymes (Novozym 435 and Lipozyme IM) were

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used as biocatalysts. This work reported that both Novozym 435 and Lipozyme IM showed good reactivity in the alcoholysis of some adipate esters by racemic 2-ethylhexanol as well as neopentyl glycol.

The support used for the immobilisation must provides specific morphological and chemical features, like particle size, pore size and specific surface area [7]. On the other hand, they should also possess mechanical strength, microbial resistance, thermostability, chemical durability and high capacity of enzyme [8]. There are various kinds of material supports, e.g. celite, silica gel, metal oxide, zeolite, and polymer resin that can be used for the immobilisation of enzyme. In this study, layered double hydroxides (LDHs) were chosen as supports for lipase immobilisation based on their extensive used in biotechnology application such as host material or support [9]. Layered double hydroxides (LDHs) are minerals and synthetic materials with positively charged brucite-type layers of mixed metal hydroxides is generally represented as $[M^{2+}_{(1-x)}M^{3+}_{x}(OH)_2]^{x+}[(A^{n-})^{x/n} \cdot yH_2O]^{x-}$. The LDHs can be described as layered compounds of brucite-like structure ($Mg(OH)_2$), with positively charged layers due to a partial substitution of divalent M^{2+} metals by trivalent M^{3+} metals [9–11]. In this reported work, Mg/Al–NO_3^-, Zn/Al–NO_3^- and Ni/Al–NO_3^- were chosen as supports because they were the most common members of the LDH minerals or so-called anionic clays [12].

This work focuses on the green synthesis of adipate esters using immobilised lipases (LDHs-lipases). These advanced biocatalysts were optimised and characterised under various reaction parameters via environmental friendly process of esterification.

2. Experimental

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 and solvents used in this study were of analytical grade.

2.2. Synthesis of various LDHs

The Zn/Al–NO_3^-, Mg/Al–NO_3^- and Ni/Al–NO_3^- solutions with molar ratio of 4 were added dropwise by 2 M of NaOH with vigorously stirring using a magnetic stirrer until pH 10. The solutions were aged at 70 °C in a horizontal water bath shaker with continuous shaking at 110 rpm for 18 h. The cooled semi-solid solutions were filtered, washed with distilled water and dried in an oven at 70 °C for 24 h before being characterised using X-ray diffractometer (XRD), accelerated of surface area porosimeter (ASAP) and energy dispersive X-ray (EDX).

2.3. Immobilisation of lipase

Crude lipase from *C. rugosa* (1.5 g) was dispersed into distilled water (15 mL), stirred for 1 h and followed by 15 min centrifuging at 10,000 rpm. The supernatant was used as partially purified lipase. A 15 mL supernatant was added to 2.0 g of LDHs and the mixture was aged in water bath shaker at 100 rpm for 1 h at room temperature. The immobilised lipase was separated from the supernatant by filtering through Whatman No.1 filter paper, freezed at 0 °C and lyophilised in a freeze drier. Then, the immobilisation process occurred was confirmed by energy dispersive X-ray (EDX).

2.4. Protein assay

The amount of protein content before and after immobilisation was determined by Bradford method [13]. Bovine serum albumin was used in this procedure as standard. Define calculation of the total amount of protein adsorbed (immobilised) in percentage as follows;

$$\text{Immobilisation \%} = \frac{(\text{protein amount in supernatant before immobilisation} - \text{protein amount in supernatant after immobilisation})}{\text{protein amount in supernatant before immobilisation} \times 100)}$$

2.5. Esterification assay

The enzymatic reaction system consisted of methanol (4.0 mmol), adipic acid (2.0 mmol) and immobilised enzyme (containing the equivalent protein concentration as in 0.15 g native) was incubated in hexane (10.0 mL) at 30 °C for 2.5 h with continuous shaking at 150 rpm. The reaction was terminated by dilution with 3.50 mL of ethanol:acetone (50:50, v/v) and the remaining free fatty acids in the reaction mixture was determined by titration with 0.15 M NaOH using an Autotitrator (808 Titrando System, Metrohm) to an end point of pH 10. The activities were expressed as specific activity (μmol/(min mg) of protein). All experiments were done in triplicate and control experiments were carried out without LDH support.

2.6. Optimisation study

2.6.1. Time course

The appropriate reaction time catalysed by immobilised enzymes was investigated. The reaction mixtures were incubated in hexane in the presence of lipase. Samples of the reaction were withdrawn at various time (0.5–4 h) and the percentage conversion was measured by determining the remaining unreacted fatty acids in the reaction mixture by titration with NaOH.

2.6.2. Reaction temperature

The reaction mixtures which consist of hexane, methanol, adipic acid and immobilised lipases were incubated at different temperatures ranging from 25 to 70 °C for 2.5 h with continuous shaking at 150 rpm. The percentage conversion was measured by determining the remaining unreacted fatty acids in the reaction mixture by titration with NaOH.

2.6.3. Water activity

The enzyme preparations and substrates were pre-equilibrated through vapour phase of saturated salts of known water activities overnight at room temperature. Hydrated salts used were LiCl ($A_w = 0.11$), $MgCl_2 \cdot 6H_2O$ ($A_w = 0.33$), $Mg(NO_3)_2 \cdot 6H_2O$ ($A_w = 0.53$), NaCl ($A_w = 0.75$), KCl ($A_w = 0.86$) and KNO_3 ($A_w = 0.9$). The percentage conversion was measured by determining the remaining unreacted fatty acids in the reaction mixture by titration with NaOH.

2.7. Stability study

2.7.1. Thermal stability

The immobilised enzymes were incubated at various temperatures ranging from 30 to 70 °C in sealed vials for 1 h. The enzymes were left to cool to room temperature before determination of esterification activity and compared to the activity of untreated enzyme. Relative activities were expressed as percentages of the activities at different temperature compared to the maximum activity.

2.7.2. Storage stability

The enzymes were kept for about 60 days at −20, 0, 4 °C and room temperature. The residue activities were determined as percentage of residue activity at 60 days compared to the initial activity at day 1 for each temperature.

2.7.3. Leaching study

The enzymes were carefully washed with hexane at 4.0, 8.0, 12.0, 16.0 and 20.0 mL of hexane with 4.0 mL at each washing. Relative activities were expressed as percentages of the activities at different wash cycle compared to the maximum activity (unwashed enzymes).

2.7.4. Reusability

After each cycle of the reaction, the immobilised enzymes were filtered, washed with hexane and dried at room temperature for 4 h. The immobilised enzymes were reused for further reaction and the activity of the certain cycle was compared to the initial activity (Cycle 1).

3. Results and discussion

3.1. Characterisation of LDHs

The powder X-ray diffraction (XRD) patterns in Fig. 1 showed some intense sharp peaks at the low value of the 2θ angle (~1–30 at position) that corresponded to the basal spacing characteristics of Mg/Al–NO_3^-, Zn/Al–NO_3^- and Ni/Al–NO_3 which were similar to the pattern of natural hydrotalcite [14]. Additional peaks at higher angle (30–60 at position) was associated with the second major phase of magnesium oxide (MgO), zinc oxide (ZnO) and nickel oxide (NiO), respectively. Table 1 showed some similarities of the basal spacing of LDHs before and after calcinations, indicating that the layered structures of the materials were maintained and non-collapsed even after calcinations at 150 °C for 5 h. At the same time, increased basal spacing

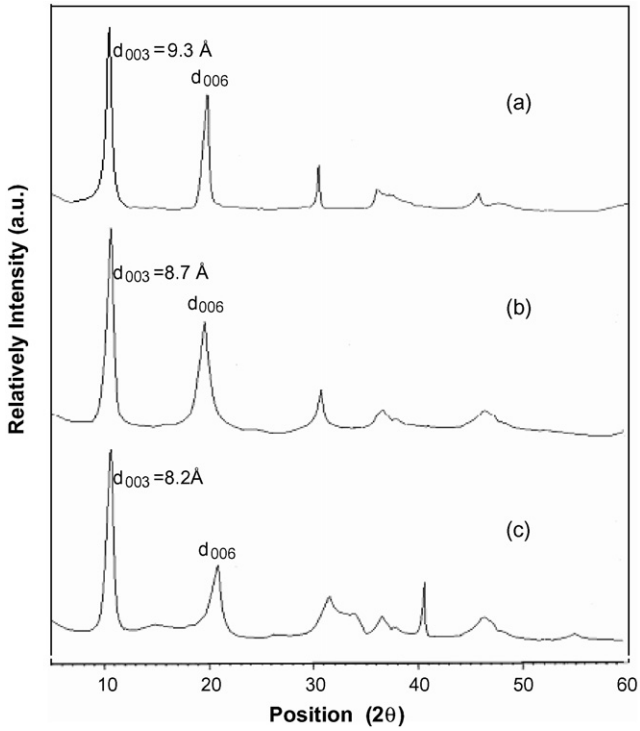

Fig. 1. Powder XRD patterns of: (a) Mg/Al–NO_3^-, (b) Zn/Al–NO_3^- and (c) Ni/Al–NO_3^- of layered double hydroxides. The d_{003} reflections at low angle correspond to the basal spacing.

of Mg/Al–NO_3^- from 8.8 to 9.3 Å showed good improvement of crystallinity, and also shown by Zn/Al–NO_3^- and Ni/Al–NO_3^-. Crystallinity of the precipitated LDHs can be further increased by calcination or hydrothermal treatment [15,16]. As reported by Cardoso et al. (2004), calcination process was able to purify the sample by releasing the carbonate from atmosphere and water in between the layers and thus produced the mixed oxide which can undergo rehydration when in contact with aqueous solution containing anions, regenerating the layered structure of LDH with these anions in the interlayer spaces [17].

Specific surface area and porosity of LDHs were studied by accelerated of surface area and porosity (ASAP). The BET surface area, pore volume and BJH desorption pore size distribution of different LDHs prepared at ratio 4 were summarised in Table 2. The largest surface area of 52.6 m^2/g of Mg/Al–NO_3^- with pore volume of 22.8×10^{-3} cm^{-3}/g and pore size distribution of 350.5 Å contributed to the higher confinement of enzyme molecules to be scattered on the surface of support. The stability of LDH-lipase could be enhanced by the forces involved between

Table 1
XRD analysis of layered double hydroxides of Mg/Al–NO_3, Zn/Al–NO_3 and Ni/Al–NO_3 prepared at ratio 4 and pH 10.0

Layered double hydroxides	d-Spacing (Å)	
	Before calcination	After calcination
Mg/Al–NO_3^-	8.8	9.3
Zn/Al–NO_3^-	7.9	8.7
Ni/Al–NO_3^-	7.6	8.2

Table 2
BET surface area, pore volume, BJH desorption pore size distribution of LDHs (prepared at ratio 4 and pH 10.0) and activities of LDHs-lipases

Layered double hydroxides	Mg/Al–NO_3^-	Zn/Al–NO_3^-	Ni/Al–NO_3^-
BET surface area (m^2/g)	53	48	24
Pore volume (cm^3/g)	23×10^{-3}	19×10^{-3}	9×10^{-3}
Pore size (nm)	35	22	12
Protein adsorbed (%)	71	67	58
Activity (μmol min^{-1} mg^{-1})	3	2	2

support-enzyme such as electrostatic and hydrophobic interaction, thus can greatly influence its performance as biocatalyst. Porous structure parameters of LDHs were determined on the basis of nitrogen desorption–adsorption isotherm as indicated in Table 2. The result of such parameter has shown that the support is a type of mesoporous materials [7,18].

3.2. Immobilisation of lipase

The percentage of immobilisation indicates the protein loading or adsorption on synthesised supports. The results in Table 2 showed the highest amount of protein adsorbed of 71% on Mg/Al-LDH, followed by 67% on Zn/Al-LDH and the lowest percentage was 58% on Ni/Al-LDH. LDH is more efficient as support for *C. rugosa* lipase as compared to other supports such as Eupergit C, activated carbon and molecular sieve with only 66%, 26% and 19% protein loading, respectively [19]. The increasing in percentage values of immobilisation was due to the larger BET surface area, pore volume and BJH desorption pore size distribution of these supports as reported in Table 2. Based on the BJH average pore diameter of all LDHs, it could be expected that the *C. rugosa* lipase molecule could be entrapped within the pores since typical diameter of a lipase is around 50 Å [7]. Previous study on this support (Mg/Al-LDH) was also reported by Abdul Rahman et al. (2004) [9]. They found that the percentage of protein loading on their support was 36.9% lower than as compared to this work. Thus, they increased the immobilisation percentage by modification of support by added with sodium dodecyl sulphate (SDS).

The choice of a good support material includes its mechanical strength, stable binding ability and high support loading efficiency. The smaller pore size may restrict mass transfer and pore penetration of the protein, which limit the protein interaction with the total surface area of supports [9]. Among the three major forms of immobilisation such as enzymes attached to a support, crosslinked enzyme molecules and enzymes contained within a support, immobilisation of catalyst onto a porous support by adsorption or deposition [20] has been proved to be an easy and useful technique for improving enzymatic activity. It is believed that the immobilised biocatalyst facilitates mass transfer by spreading the enzyme on a larger surface area and by preventing the enzyme particles from aggregation [21] thus help increase dispersion of lipase in the reaction media. In this study, LDHs prepared at ratio 4 was found to exhibit properties which accommodate for adsorption of protein such as better porosity and large surface area.

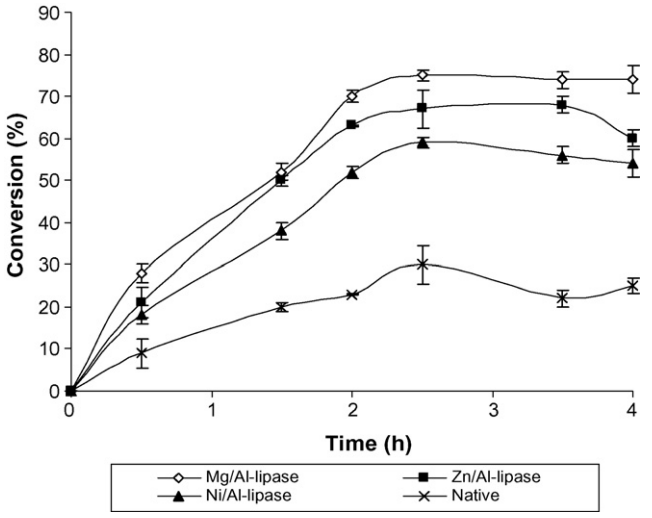

Fig. 2. Effect of reaction time on percentage conversion of ester of immobilised lipases. Reaction condition: agitation speed, 150 rpm; molar ratio of methanol to adipic acid, 2:1; reaction temperature, 30 °C.

3.3. Optimisation study

3.3.1. Effect of reaction time

Time course study is a good indicator of enzyme performance and product yield. A good performance of enzymes should have a short time to obtain good yields. Fig. 2 shows the reaction time profile for the esterification of adipic acid and methanol catalysed by various LDHs-lipases that showed the higher conversion of ester for all various reaction time compared to native enzyme. The rate of reaction and overall conversion increased with increasing reaction time. The result suggested that the reaction proceeded rapidly within 2.5 h (150 min) for all immobilised lipases which giving high yield from 63.0% up to 75.5%. There after, the percentage yields were relatively constant due to production of water molecule, which had increased in an adequate value to accommodate the hydrolysis process, apart from the reaction which had achieved the equilibrium state. As the reaction proceeds, the substrates concentration decreased which led to a fall in the degree of saturation of the enzyme with substrate [22]. In a study by Gryglewicz (2001) [2], it was reported that the reaction proceeded at a very high rate in the presence of Novozym 435 when 30% of 2-ethylhexanol was reacted after 45 min, while a similar percentage was observed for Lipozyme IM only after 17 h.

3.3.2. Effect of reaction temperature

Changing in the reaction temperature can affect the activity and stability of the lipase and thus influenced the rate of reaction [23]. Fig. 3 shows the temperature profile on the influence of temperature on the esterification reaction within temperature range between 25 and 70 °C. The percentage yield was increased with increasing temperature from 25 to 50 °C, as energy received from heat of higher temperature that was used to increase the frequency of collision between the protein and reactants molecules. The percentage yields at optimum temperature (50 °C) were more than 70%, and up to 81% for all immobilised lipases which

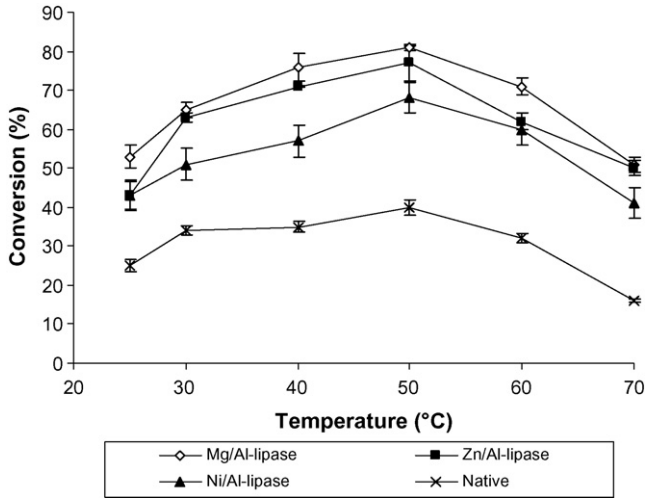

Fig. 3. Effect of reaction temperature on percentage conversion of ester of immobilised lipases. Reaction condition: agitation speed, 150 rpm; molar ratio of methanol to adipic acid, 2:1; reaction time, 2.5 h.

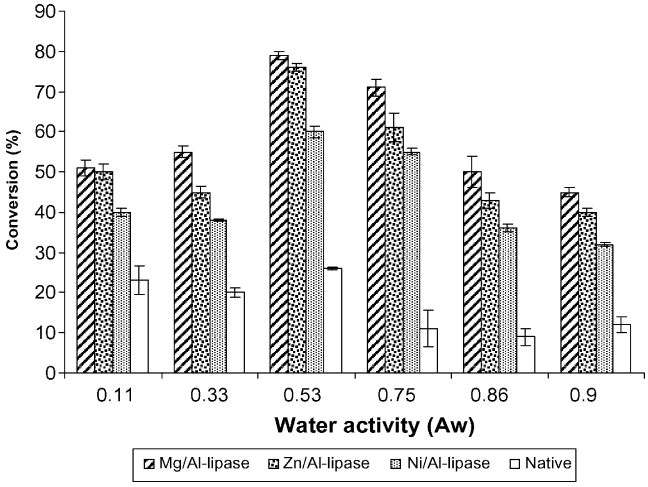

Fig. 4. Effect of water activity on percentage conversion of ester of immobilised lipases. Reaction condition: agitation speed, 150 rpm; molar ratio of methanol to adipic acid, 2:1; reaction time, 2.5 h. $A_w = 0.11$ (LiCl), $A_w = 0.33$ ($MgCl_2 \cdot 6H_2O$), $A_w = 0.53$ ($Mg(NO_3)_2 \cdot 6H_2O$), $A_w = 0.75$ (NaCl), $A_w = 0.86$ (KCl) and $A_w = 0.9$ (KNO_3).

has the advantage of conferring stability to the lipase [24], while the native lipase gave the lowest percentage at 50 °C which due to the low activation energy of the enzymatic reactions [25]. The percentage yield of ester was decreased at temperature between 60 to 70 °C and this might be due to the thermal deactivation of the enzyme [26]. However, the immobilised lipases showed the higher conversion of esters compared to the native lipase.

3.3.3. Effect of water activity

The water activity (A_w) describes the mass action of water on equilibrium and necessary for the optimal tertiary confirmation of the enzyme. The amount of water in the system is one of the most important parameters to achieve an efficient synthesis reaction [27]. Water activity is strongly influenced by the hydration level of the enzyme and affected the activity. The effect of initial water activity (A_w) on dimethyl adipate ester production via esterification using immobilised lipases is shown in Fig. 4. In this study, a similar trend was observed for this reaction whereby all immobilised lipases and native lipase showed maximum activity achieved when the substrates, enzyme and solvent were pre-equilibrated in the hydrated salt with water activity (A_w) of 0.53, followed by other four hydrated salts with higher water activity. These salts exhibited almost similar profile in the system, giving broad and bell-shaped curves for the esterification study. At higher water activities ($>A_w = 0.53$), percentage conversions of product decreased which may be due to the negative effects of water, which strongly hydrate matrices, as well as the microenvironment of the immobilised lipases.

3.4. Stability study

3.4.1. Effect of thermostability

Utilisation of enzymes in processes often encounters the problem of thermal inactivation of enzyme. At high temperature, enzyme undergoes partial unfolding by heat-induced destruction of non-covalent interactions [21]. The effect of temperatures on the stability of immobilised lipases was carried out after 1 h incubation at various temperatures. As shown in Fig. 5, immobilised lipases retained their catalytic activities of 60% to 75% of its original activities even after incubation at highest temperature of 70 °C, while the native retarded to 11% at the same temperature. According to Dave and Madamwar (2006), lipases were easily denatured at high temperature where the peptide bonds and amino acid side chains were reactive and can participate in deleteriouse reactions at high temperature. The immobilised lipases may be more rigid in terms of conformation and therefore were able to remain stable even at higher temperature. In addition, stability of enzyme was proportional to the number of physical adsorption between enzyme and immobilisation matrix that locks the enzyme into the active conformation and thus prevent irreversible unfolding of the protein [28]. Among these, lipase immobilised onto Mg/Al-LDHs exhibited highest thermal stability and still capable of performing its vibrational and more complex movement required for efficient catalytic activity.

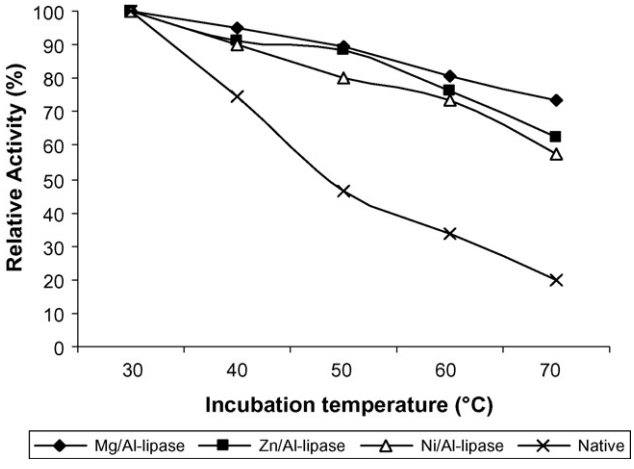

Fig. 5. Percent relative activity of native and immobilised lipases after 1 h incubation at various temperatures. Reactions were performed in hexane with 2:1 molar ratio of methanol and adipic acid at 30 °C.

Compared to native enzymes, thermal stability of many lipases can be significantly enhanced by immobilisation [29,30].

3.4.2. Effect of storage stability

The storage stability of immobilised enzymes without appreciable loss of enzyme activity are important for the economic viability of a biosynthetic process [31]. The storage stability of native and immobilised lipases were determined after showed for 60 days under various temperature conditions (Fig. 6). The immobilised lipases showed higher storage stability compared to native lipase. All lipases exhibited full catalytic activity after storing them at −20 °C in laboratory refrigerator, but a decrease in stability of them was found at higher storage temperature. Upon storage at 0 °C, Mg/Al-lipase and Zn/Al-lipase seemed to retain their activity by more than 80% of its initial activity. One of the most important aims of the enzyme engineering is to enhance the conformational stability of enzymes. It is generally known that protein which physically adsorbed is more stable than protein which has highly cross-linked structure that is more resistant to denaturation [31]. Lipases immobilised on LDHs were found to retain their activity more than 50% even stored at 4 °C and this indicated that the enzyme is easily handled after immobilisation. Immobilised lipases however, retained their higher catalytic activity of 38% and above, while the native only retained about 6% after 60 days of storage at room temperature.

3.4.3. Effect of leaching study

Leaching study shows that all immobilised lipases preparations retained their catalytic activity by more than 90% even after 6 washing cycles (24.0 mL of hexane). Interestingly, all of them retained full catalytic activity (100%) up to third washing cycles, except for Ni/Al-lipase that showed slightly leached effect. However, their activities only showed a gradually 10% decreased from 4th to 6th washing cycles (16.0–24.0 mL hexane). Since the enzyme is immobilised, the support protects the enzyme from any damaging conditions, so that it is not easily affected by the polarity of solvent. Some lost of enzymes during washing can be inherent consequence of its immobilisation on each support surface. It may also be due to the inactivation of enzyme caused by the leakage of protein from the support, not only because of washing by hexane. This result indicated that lipase remained immobilised to support even after leaching test up to 24.0 mL of hexane. It has been proven that layered double hydroxides was suitable supports for immobilisation of enzyme as it protected lipase protein from being leached away by washing with hexane.

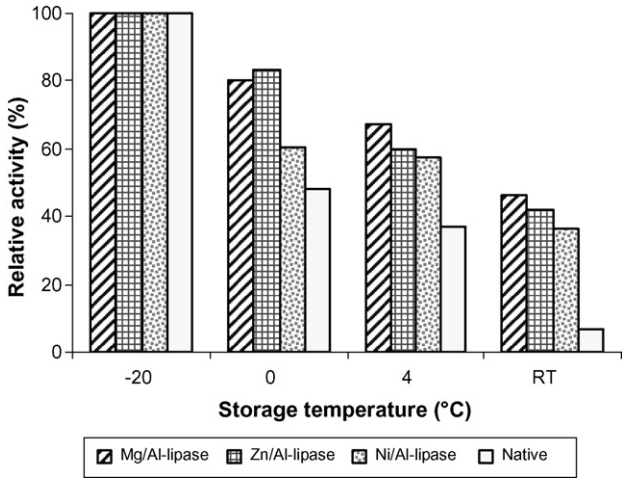

Fig. 6. Percent relative activity of native and immobilised lipases after storing at different storage temperatures for 60 days. Reactions were performed in hexane with 2:1 molar ratio of methanol to adipic acid at 30 °C.

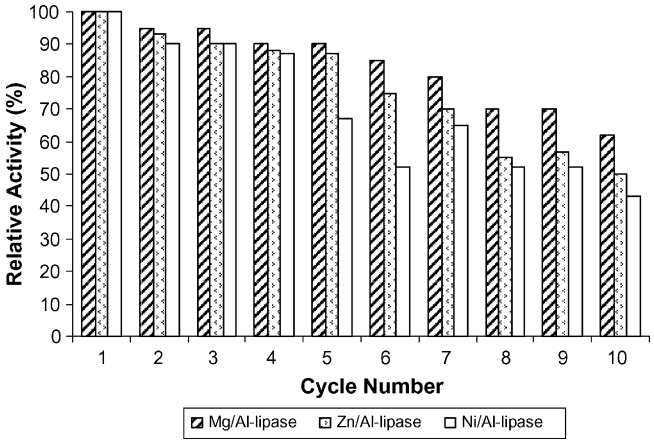

Fig. 7. Percent relative activity of immobilised lipases on reusability. Reactions were performed in hexane with 2:1 molar ratio of methanol to adipic acid at 30 °C.

3.4.4. Effect of reusability

The use of immobilised enzyme (for repeated use) might help to drive down the product cost and make the enzymatic process economically viable [32]. Fig. 7 shows the profile of enzyme reusability for methyl adipate syntheses within reaction time 2.5 h in each cycle. Operational stability or reusability is of high importance in determining immobilised enzyme efficiency. High percentages relative activity of methyl adipate were maintained at more than 85% from cycle 1 to 5 for all LDH-lipases. However, the activity started to decrease gradually thereafter from cycle 8 to 10, which might be due to loss of enzyme during filtration and drying since no make-up quantities were added. Among them, Mg/Al-lipase was found to be the most stable biocatalyst which might able to retain more than 70% activity even at 9th cycle, while the high remaining activities of Zn/Al-lipase and Ni/Al-lipase, respectively were 57% and 52%. The decrease in the conversion after several runs were due to the turnover of large quantities of substrates that resulted in the production of substantial quantities of water (as co-product) [21]. From this study, immobilisation of lipase onto porous support through physical adsorption has been proven to be a useful technique for improving enzyme activity through direct interaction with the lipase beside protecting it from direct inactivation by solvent [33].

4. Conclusion

An effective means for achieving an organic production of methyl adipate esters using immobilised LDHs-lipases as bio-

catalysts have been achieved. Modified advanced material of layered double hydroxides of Mg/Al, Zn/Al and Ni/Al seemed to be a well-suited support for immobilisation of lipase for esterification in hexane. Approach of physical adsorption technique for immobilisation is relatively more easier and inexpensive. The properties of supports and stability of immobilised lipases exhibited interesting characteristics that may be suitable for industrial biotransformations. Optimised reaction conditions have been successfully carried out by performing an excellent conversion of methyl adipate ester via the environmental benign process. This work suggested with the greener and safer materials design for more practical use of sustainable resources.

Acknowledgements

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