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# Kinetic Behaviour of Free Lipase and Mica-Based Immobilized Lipase Catalyzing the Synthesis of Sugar Esters

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**The utilization of natural mica as a biocatalyst support in kinetic investigations is first described in this study. The formation of lactose caprate from lactose sugar and capric acid, using free lipase (free-CRL) and lipase immobilized on nanoporous mica (NER-CRL) as a biocatalyst, was evaluated through a kinetic study. The apparent kinetic parameters, $K_m$ and $V_{max}$, were determined by means of the Michaelis-Menten kinetic model. The Ping-Pong Bi-Bi mechanism with single substrate inhibition was adopted as it best explains the experimental findings. The kinetic results show lower $K_m$ values with NER-CRL than with free-CRL, indicating the higher affinity of NER-CRL towards both substrates at the maximum reaction velocity ($V_{max,app} > V_{max}$). The kinetic parameters deduced from this model were used to simulate reaction rate data which were in close agreement with the experimental values.**

**Key words:** lipase immobilization; mica; kinetic study; Ping-Pong Bi-Bi model; sugar ester synthesis

Interest in studies of reactions involving various sources of lipase catalysis has grown over the past ten years. Lipases (triacylglycerol ester hydrolase, EC3.1.1.3) have been widely used in biotechnological applications in the dairy, oil processing, pharmaceutical and food industries, and in the production of surfactants.[1)] Immobilizing an enzyme on a solid support allows the use of the enzyme in multiple batches, thereby reducing the production costs. Immobilization also offers several advantages over free enzymes in the possibility of running continuous enzymatic reactions, rapidly terminating reactions, controlled product formation and easy separation of an enzyme from the reaction mixture.[2)] A variety of supports have so far been used for the immobilization of lipase, and comparative studies have shown that a dramatic difference in the activity of a lipase was influenced by the different materials used.[3)] The clay mineral of phyllosilicate, known as mica, was firstly used as a support for enzyme immobilization in our previous work.[4)] We found that mica with almost zero cost exhibited potential for use as a support in enzyme immobilization for bioorganic synthesis.

The lipase-catalyzed production of biosurfactants has been thoroughly demonstrated in the literature.[5–7)] Amongst the biosurfactants, sugar fatty acid esters have attracted the attention of biotechnological researchers due to the vast advantages they offer of their availability under a wide range of hydrophilic-lipophilic balance (HLB) values, biodegradability, non-toxicity, tastelessness and non-irritancy to the skin and eyes.[7)] Sugar esters have an extensive range of applications as emulsifiers, antimicrobial agents in the food industry, personal care and household products. As a promising alternative to chemical methods, the enzymatic synthesis of these compounds can be achieved by coupling a sugar and fatty acid in the presence of lipase as the biocatalyst. The reaction can be carried out under ambient temperature to result in surfactants which can be utilized in medical, food and cosmetic industries without any hazardous effect.[8)]

The most generally accepted method for the lipase-catalyzed reaction of fatty acid esters involves the Ping-Pong Bi-Bi mechanism.[9–14)] However, the kinetic investigation of sugar ester synthesis catalyzed by lipase immobilized on a natural support is still rare. To the best of our knowledge, the use of natural mica as a lipase support in the coupling reaction of lactose sugar with capric acid has not been exploited so far in a kinetic study. The present investigation was therefore focused on the kinetics of lactose ester synthesis in the presence of free lipase and lipase immobilized on the new support of mica clay.

## Materials and Methods

*Materials.* Commercial lipase from *Candida rugosa* (CRL; EC3.1.1.3; 1,150 U/mg of solid, with olive oil hydrolysis at pH 7.2),

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*Abbreviations*: FASEs, fatty acid sugar esters; free-CRL, free lipase; NER-CRL, lipase immobilized on nanoporous mica

glutaraldehyde, capric acid and lactose monohydrate were purchased from Sigma Aldrich. All the chemicals and solvents used were of analytical reagent grade (AR). The immobilization support, mica clay, was collected from the region of Tanah Putih, Gua Musang, Kelantan (Malaysia). Mica with a particle size of about 201 nm and pore size of <50 nm was used for immobilization. The mica was acid-activated by stirring the sample in a solution of HCl for 60 min at 50 °C. An excess acid was removed by repeated water rinsing, and the acid-activated mica was dried at 110 °C.

*Enzyme immobilization.* Immobilization of lipase was initially by adsorbing 15.0 mL of the lipase solution (0.1 g/mL) to acid-activated mica (2.0 g) at room temperature under continuous agitation at 100 rpm for 1 h. The lipase-loaded support solution was treated with glutaraldehyde (a 5% aqueous solution) and stirred for 17 h at the end of the reaction. The immobilized lipase was separated by passing through Whatman No. 1 filter paper, before being dried and lyophilized in a freeze drier (Labconco 195, USA). The immobilized lipase is denoted by a nanoscale enzyme reactor (NER-CRL).

*Immobilization efficiency.*

*Protein assay.* Protein was determined according to the Bradford method, using bovine serum albumin as a standard.[15] The amount of immobilized protein was indirectly determined by comparing the difference between the amount of protein introduced into the support and the amount of protein in both the filtrate and washing solutions after immobilization. The degree of protein immobilization (%) was calculated according to Eq. (1):

$$\begin{aligned}&\text{Protein immobilization (\%)}\\&= (\text{Amount of protein introduced before immobilization}\\&\quad - \text{Amount of suspended protein after immobilization}\\&\quad /\text{Amount of protein introduced before immobilization})\\&\quad \times 100 \qquad (1)\end{aligned}$$

*Lipase activity assay.* The lipase-catalyzed esterification reaction was performed at different molar ratios of lactose to capric acid with various time courses. The reaction mixtures consisting of both substrates, the lipase preparation (containing 2.14 mg of protein) and molecular sieves (0.3 g) were incubated in acetone (10.0 mL) at 60 °C with a shaking speed of 250 rpm. The percentage conversion (%) was calculated from the free fatty acid remaining in the reaction mixture as determined by titrating with 0.15 M NaOH. A similar amount of protein (2.14 mg) in the free (Free-CRL) and immobilized (NER-CRL) lipase was used for the esterification reaction. The initial reaction rate was estimated from the slope of the curve for lactose caprate ester formation against time and is reported as mmol/L/min/mg of protein.

*Characterization of the immobilized lipases.* A sample of the mica support itself was characterized by using X-ray diffraction (XRD), nitrogen adsorption-desorption and a particle-size analysis, while the immobilized lipase on the mica support could be clearly viewed and proved by scanning electron microscopy, using the results of an energy dispersive X-ray microanalysis (SEM-EDX) and infra-red (FTIR) spectroscopy results as previously reported.[4]

*Kinetic constants determination.* The values for the kinetic constants were computed using non-linear regression fitting by means of enzyme kinetic (EK-SigmaPlot) software based on data points evaluated from the experimental observations. The proposed kinetic equation was applied to predict the simulated reaction rate for the reaction model assumed.

## Results and Discussion

The main emphasis of this study was to develop a kinetic model focusing on the concentration of each substrate. The effects of a wide range of concentration of both substrates on the rate of reaction were systematically studied. The reaction rate was determined from the results of experiments using Free-CRL or NER-CRL containing the same amount of protein (2.14 mg) with appropriate quantities of capric acid and lactose, the total volume being adjusted to 10.0 mL in acetone. The lactose amount in one set of experiments was varied from 10 to 100 mmol/L with a fixed quantity of capric acid (20 mmol/L), while in another set, the amount of capric acid was varied from 10 to 100 mmol/L with a fixed quantity of lactose (20 mmol/L).

*Initial esterification rate for the free and immobilized lipase systems*

It was found for both Free-CRL and NER-CRL at a fixed capric acid concentration that increasing the lactose concentration resulted in the percentage conversion of the substrate, indicating the formation of a lactose ester, continuously increasing until it reached a maximum level at a 3:1 molar ratio of lactose to capric acid. A further increase in the lactose concentration decreased the percentage conversion of the substrate. A similar effect was also observed for the reaction rate. A drop in the reaction rate at a high concentration of lactose (>50 mmol/L) indicated the lactose inhibitory effect as shown in Fig. 1a and b. This effect can be explained by an accumulation of the polar substrate (lactose) in the aqueous microenvironment surrounding the enzyme, thus causing denaturation of the protein structure.[16] According to Du *et al.*,[17] an alcoholic compound at high concentration is a substrate inhibitor for lipase-catalyzed esterification.

However, the inhibition effect by lactose was suppressed when immobilized lipase was in used as compared to the free lipase system. The results just described show that the reaction followed Michaelis-Menten kinetics with inhibition due to an excessive amount of lactose, this then being confirmed by a Lineweaver-Burk plot. The graph at a high lactose concentration shows an upward curve, indicating uncompetitive substrate inhibition (Fig. 2a and b). A similar substrate inhibition profile has also been reported by Chulalaksananukul *et al.*[9] and Zaidi *et al.*[10]

On the other hand, when a fixed lactose concentration was used, an increasing capric acid concentration from 1 to 5 molar ratios did not seem to cause any decrease in the percentage conversion or the reaction rate. This determination of rate indicates that the rate initially increased with increasing concentration of capric acid,

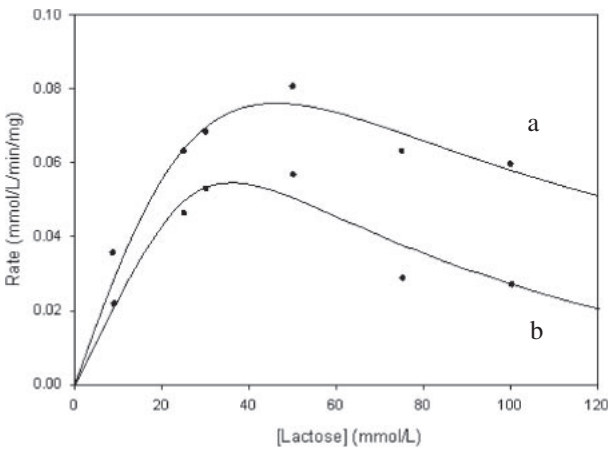



**Fig. 1.** Effect of [Lactose] on the Reaction Rates for (a) NER-CRL and (b) Free-CRL.

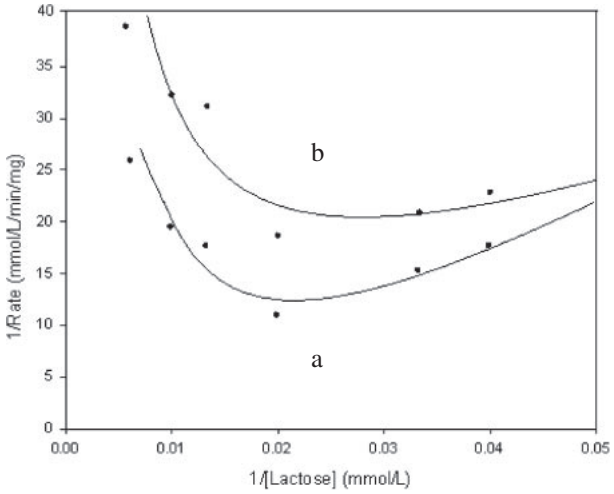



**Fig. 2.** Lineweaver-Burk Plots for Inverse [Lactose] against the Reaction Rates for (a) NER-CRL and (b) Free-CRL.

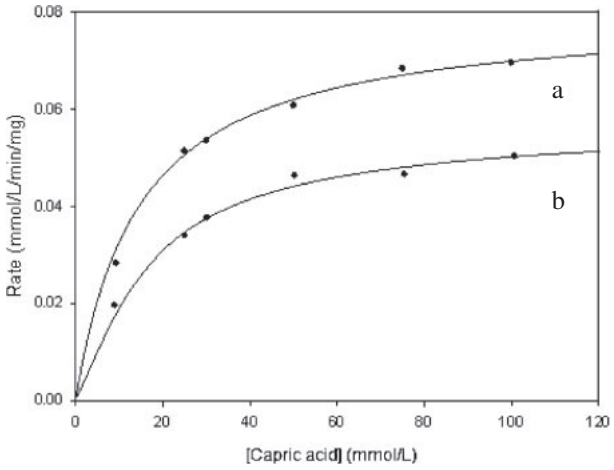



**Fig. 3.** Effect of [Capric Acid] on the Reaction Rates for (a) NER-CRL and (b) Free-CRL.

and there was no evidence of inhibition by the substrate mentioned in the range of values studied, as shown in Fig. 3a and b. As expected, NER-CRL exhibited higher reaction rates than Free-CRL. In this case, the straight line profile shown by the Lineweaver-Burk plot (1/Rate against 1/[Capric acid]) confirmed that the reaction follows Michaelis-Menten kinetics[18] (Fig. 4a and b). The reaction rates were then calculated from the linear portion of the concentration-time profiles.

Similar characteristics for the effect of each substrate were observed for both Free-CRL and NER-CRL, whereby lactose acted as an inhibitory substrate, with capric acid serving to advance the reaction. The Ping-Pong Bi-Bi model was selected after careful analysis of the literature to describe the kinetics of this reaction mechanism with dead-end complex inhibition by lactose, as is shown in Scheme 1.

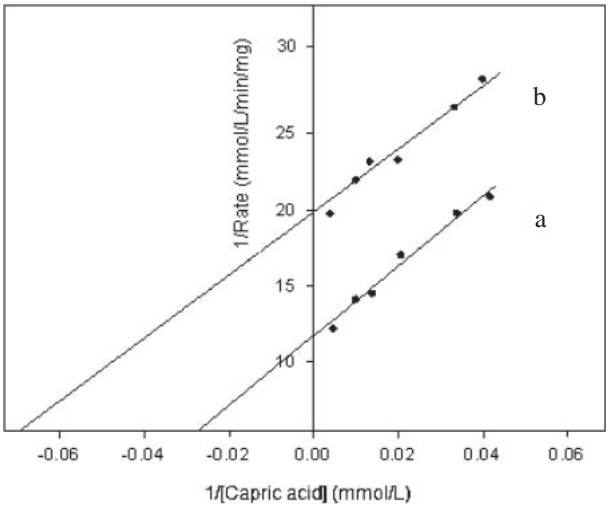
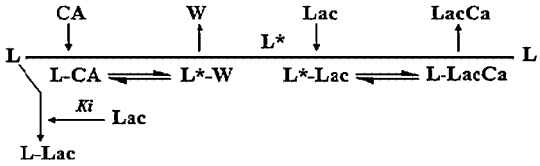



**Fig. 4.** Lineweaver-Burk Plots for Inverse [Capric Acid] against the Reaction Rates for (a) NER-CRL and (b) Free-CRL.




**Scheme 1.** Ping-Pong Bi-Bi Model of Reaction Mechanism.

In this typical reaction mechanism, lipase initially reacts with the capric acid as an acyl donor to form non-covalent lipase-capric acid (L-CA) which is subsequently transformed to an acyl-lipase intermediate by which the first product, water, is released. The modified lipase ($L^*$) then binds to lactose (Lac) and forms a complex which is isomerized by a unimolecular reaction to lipase-lactose caprate (L-LacCa), which then yields an ester product, lactose-caprate (LacCa) and free lipase. Alongside this, lactose also combines with the free lipase and forms a lipase-lactose dead-end complex (L-Lac) that would not interfere with the formation of the product.

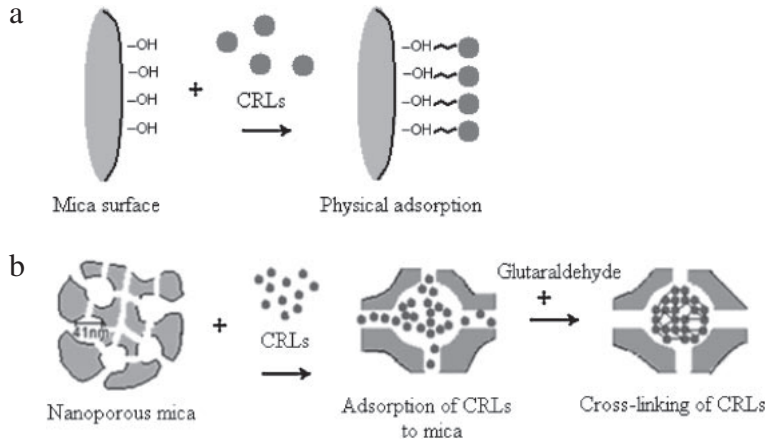
*Kinetic constants*

The rate equation for the Ping-Pong Bi-Bi mechanism with one substrate inhibition, assuming that the experiments were performed under conditions with which the influence of the products can be neglected, is as follows:

$$v = \frac{V_{\max}[\mathrm{CA}][\mathrm{Lac}]}{K_{\mathrm{m(CA)}}[\mathrm{Lac}](1 + [\mathrm{Lac}]/K_{\mathrm{i}}) + K_{\mathrm{m(Lac)}}[\mathrm{CA}] + [\mathrm{CA}][\mathrm{Lac}]} \quad (2)$$

where $v$ is the initial reaction rate, [CA] and [Lac] are the respective concentrations of capric acid and lactose, $V_{\max}$ is the maximum velocity or limiting rate, $K_{\mathrm{m(CA)}}$ and $K_{\mathrm{m(Lac)}}$ are the respective Michaelis constants for capric acid and lactose, and $K_{\mathrm{i}}$ is the inhibition coefficient of the lactose. The kinetic constants in Eq. (2) were estimated by the Lineweaver-Burk plot generated by the nonlinear regression method (EK-Sigma Plot module). The values for the kinetic constants of both Free-CRL and NER-CRL are shown in Table 1.

The kinetic constants in the immobilized system are noted as "apparent" (app), since the interaction between the support and each of the two substrates, which may result in different substrate concentrations near the




**Scheme 2.** Schematic Illustration of Lipase Adsorption to the Mica Support (a), and Cross-Linking of Lipases in the Nanoporous Mica (b).

**Table 1.** Kinetic Constants Obtained for the Esterification Reaction Catalyzed by Free (Free-CRL) and Immobilized Lipase (NER-CRL) from *Candida rugosa*

| Parameters | Free-CRL | NER-CRL |
|---|---|---|
| $V_{max}$ (mmol/L/min/mg)[a,b] | 0.050 | 0.080 |
| $K_{m(CA)}$ (mmol/L/mg)[a] | 14.8 | 14.5 |
| $K_{m(Lac)}$ (mmol/L/mg)[a] | 16410 | 9308 |
| $K_{i(Lac)}$ (mmol/L/mg)[a] | 0.078 | 0.229 |

[a]The kinetic constants are apparent for the immobilized lipase.
[b]$V_{max}$ is expressed per milligram of protein for the free or the immobilized enzyme system.

enzyme, was not accounted for.[19] It was found with both systems that the enzyme affinity for the capric acid substrate was higher than that for the lactose substrate. However, the lower apparent Michaelis constant values ($K_{m,app(CA,Lac)} < K_{m(CA,Lac)}$) showed that NER-CRL had performed with higher affinity for both substrates as compared with the free enzyme at maximum reaction velocity ($V_{max,app} > V_{max}$). The calculated efficiency factor ($\eta$), defined as the ratio of the maximum reaction rate of NER-CRL over that of Free-CRL, was 1.6.

Lipase in its immobilized form would show a decrease in the substrate affinity due to conformational changes to the active site of the enzyme in most cases.[2,20,21] Interestingly, the findings in this work indicate the improved activity of NER-CRL upon immobilization. Lipase molecules with the NER-CRL system (Scheme 2) were initially adsorbed to the surface of the mica support *via* physical adsorption (a). Glutaraldehyde was then added to further cross-link the adsorbed lipase with other lipases. Indeed, the addition of glutaraldehyde enhanced the cross-linking of the lipase molecules to one another inside the nanoporous mica, thus resulting a greater overall size of the cross-linked lipases. The cross-linked lipases were consequently prevented from leaching out of the mica support (b).

The highly porous nature of mica used in this work enabled high protein loading at 8.7 mg of protein/g of support, with high enzyme immobilization at 86.4%. It was assumed that the structural changes in the enzyme induced by this system promoted the enzyme-substrate binding ability on both parts, either on the surface or inside the nanoporous mica support (about 41.0 nm).

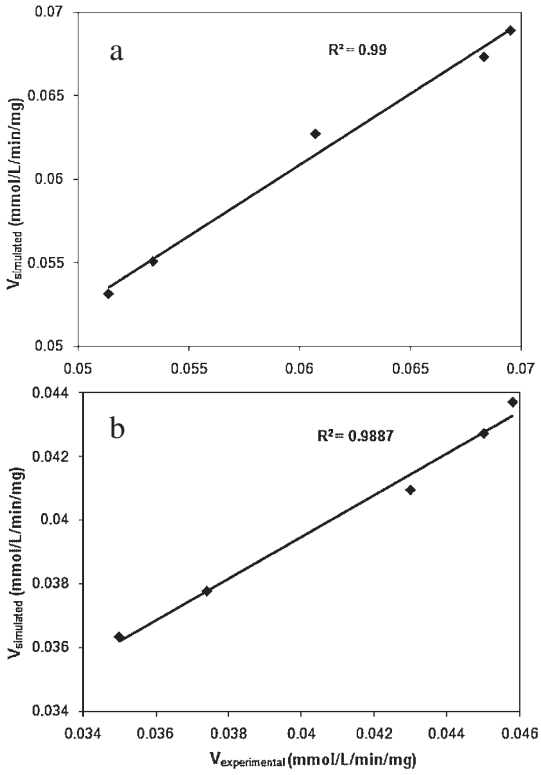



**Fig. 5.** Parity Plots of the Experimental and Simulated Rates for the Ping-Pong Bi-Bi Mechanism for (a) NER-CRL and (b) Free-CRL.

This phenomenon could be explained by the change in protein conformation upon immobilization which may have opened or provided a more favourable position of the active sites to be accessed by the substrates.[22]

The validation of the Ping-Pong Bi-Bi model for this reaction has also been evaluated. The kinetic constants obtained (Table 1) were applied to the proposed model (Eq. (2)) for both enzymatic systems. The coefficient of determination and $R^2$ values between the simulated and experimental rates were respectively determined from Fig. 5 as 0.9900 and 0.9887 for NER-CRL (a) and Free-CRL (b). The plots for the experimental and simulated rates *versus* the concentration of capric acid are respectively shown in Fig. 6 for NER-CRL (a) and Free-CRL (b). These results indicate that the predicted model precisely fitted the estimated data. The kinetics of

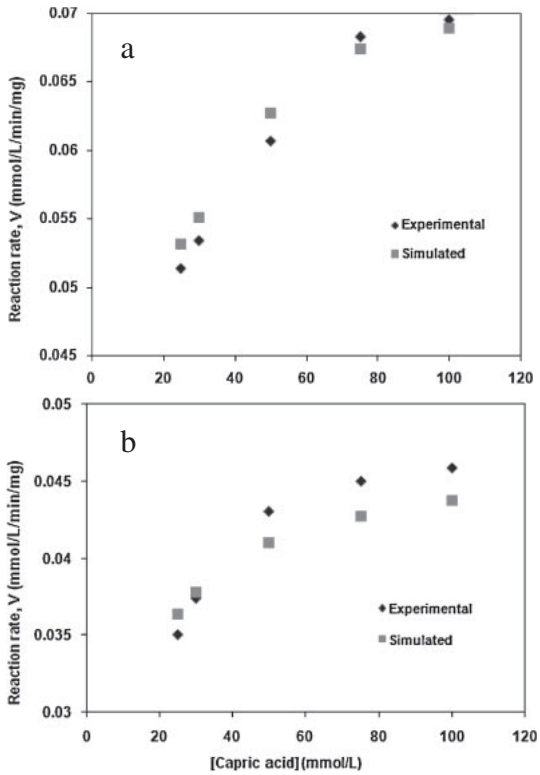



**Fig. 6.** Plots of the Experimental and Simulated Rates against [Capric Acid] for (a) NER-CRL and (b) Free-CRL.

glucose ester synthesis have been performed in recent studies by Flores and Halling[23] and Arcos *et al.*[24] in 2-methyl 2-butanol and in acetone, respectively, who had proposed the similar Ping-Pong Bi-Bi model.

## Conclusions

Nanoporous mica clay was prepared and employed for immobilizing lipase to synthesize lactose caprate. Interestingly, the use of the newly immobilized lipase (NER-CRL) as a biocatalyst showed a better result than the use of free lipase. The reaction was found to follow Michaelis-Menten kinetics with inhibition by the lactose substrate and was further described by the Ping-Pong Bi-Bi mechanism. The following values of $V_{max}$, $K_{m(CA)}$, $K_{m(Lac)}$ and $K_{i(Lac)}$ were respectively obtained for Free-CRL: 0.050 mmol/L/min/mg of protein, and 14.8, 16410 and 0.078 mmol/L/mg of protein. The apparent kinetic constants were also estimated for NER-CRL. A good correlation between the experimental data and the model was obtained for both enzyme systems before achieving equilibrium in the esterification reaction. Lipase immobilized on the nanoporous mica support possessed efficient biocatalytic performance as a catalyst for the production of lactose caprate, a high added value product.

## Acknowledgments

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## References

1) Schmid RD and Verger R, *Angew. Chem. Int. Ed.*, **37**, 1609–1633 (1998).
2) Ghiaci M, Aghaei H, Soleimanian S, and Sedaghat ME, *Appl. Clay Sci.*, **43**, 289–295 (2009).
3) Sanjay G and Sugunan S, *Catal. Commun.*, **6**, 81–86 (2005).
4) Zaidan UH, Abdul Rahman MB, Basri M, Othman SS, Abdul Rahman RNZA, and Salleh AB, *Appl. Clay Sci.*, **47**, 276–282 (2010).
5) Dandekar PP and Patravale VB, *Indian J. Chem. Technol.*, **16**, 317–321 (2009).
6) Piao J, Kishi S, and Adachi S, *Colloids Surf. A Physicochem. Eng. Aspects*, **277**, 15–19 (2006).
7) Rahman RA and Herawan T, *J. Oil Palm Res.*, **12**, 117–122 (2000).
8) Sabeder S, Habulin M, and Knez Z, *J. Food Eng.*, **77**, 880–886 (2006).
9) Chulalaksananukul W, Condoret JS, and Combes D, *Enzyme Microb. Technol.*, **14**, 293–298 (1992).
10) Zaidi A, Gainer JL, Carta G, Mrani A, Kadiri T, Belarbi Y, and Mir A, *J. Biotechnol.*, **93**, 209–216 (2002).
11) Yadav GD and Devi KM, *Chem. Eng. Sci.*, **59**, 373–383 (2004).
12) El Rassy H, Perrard A, and Pierre AC, *J. Mol. Cat. B: Enzymatic*, **30**, 137–150 (2004).
13) Yadav GD and Lathi PS, *J. Mol. Cat. B: Enzymatic*, **32**, 107–113 (2005).
14) Al-Zuhair S, Ling FW, and Jun LS, *Process Biochem.*, **42**, 951–960 (2007).
15) Bradford M, *Anal. Biochem.*, **72**, 248–254 (1976).
16) Ghamgui H, Karra-Chaabouni M, and Gargouri Y, *Enzyme Microb. Technol.*, **35**, 355–363 (2004).
17) Du W, Xu Y, Liu D, and Zeng J, *J. Mol. Cat. B: Enzymatic*, **30**, 125–129 (2004).
18) Seong BL, Han MH, Park JM, and Choi CY, *Biotechnol. Lett.*, **3**, 607–612 (1981).
19) Oliveira AC, Rosa MF, Aires-Barros MR, and Cabral JMS, *J. Mol. Cat. B: Enzymatic*, **11**, 999–1005 (2001).
20) Hung TC, Giridhar R, Chiou SH, and Wu WT, *J. Mol. Cat. B: Enzymatic*, **26**, 69–78 (2003).
21) Tukel SS and Alptekin O, *Process Biochem.*, **39**, 2149–2155 (2004).
22) David AE, Wang NS, Yang VC, and Yang AJ, *J. Biotechnol.*, **125**, 395–407 (2006).
23) Flores MV and Halling PJ, *Biotechnol. Bioeng.*, **78**, 795–801 (2002).
24) Arcos JA, Hill Jr CG, and Otero C, *Biotechnol. Bioeng.*, **73**, 104–110 (2001).