

CITRONELLYL BUTYRATE SYNTHESIS IN NON-CONVENTIONAL MEDIA USING PACKED-BED IMMOBILIZED *CANDIDA RUGOSA* LIPASE REACTOR

I. Dahlan, A. H. Kamaruddin and G. D. Najafpour

School of Chemical Engineering, Universiti Sains Malaysia, Engineering Campus,
Seri Ampangan, 14300 Nibong Tebal, Pulau Pinang, MALAYSIA,

Dahlan_irvan@yahoo.com , chazlina@eng.usm.my , najafpour@yahoo.com

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Abstract The synthesis of citronellyl butyrate by direct esterification reaction catalyzed by immobilized lipase from *Candida rugosa* was studied in a continuous packed bed reactor using *n*-hexane as organic solvent. Parameters such as residence time, temperature, and pH were examined. The optimum conversion was obtained at a flow rate of 1 ml/min (residence time 8 min), temperature of 50 °C, and pH 7.5. At high temperature, biocatalyst was rapidly deactivated with respect to time. The immobilized lipase was stable at pH range of 6 to 9. The deactivation of biocatalyst was increased at pH of immobilized lipase 4 and 10 in comparison with pH range from 5 to 9. The esterification reaction in packed-bed reactor was modeled by ping pong model and ping pong with product inhibition model. The observed kinetics behaviour of esterification reaction was found to follow a ping pong with product inhibition model. In this study, the conversion of butyric acid and stability of immobilized lipase was higher in the system with molecular-sieve than in the system without molecular-sieve.

Keywords Citronellyl Butyrate, Flavor, Esterification, Immobilized Lipase, Non-Conventional Media, Packed-Bed Reactor, Ping Pong Model

چکیده سنتز سیترونیل بوتیرات با واکنش استری شدن به کمک آنزیم لیپاز تثبیت شده ناشی از میکروارگانیزم *Candida rugosa* در راکتور بستر فشرده مورد مطالعه قرار گرفت. پارامترهای مورد مطالعه شامل زمان ماند، دمای واکنش و PH بودند. ضریب تبدیل بهینه یا دبی جریان ۱ میلی لیتر بر دقیقه (زمان ماند ۸ دقیقه) در دمای ۵۰ درجه سانتیگراد و PH برابر ۷/۵ به دست آمد. واکنش در دمای بالا موجب غیر فعال شدن سریع بیوکاتالیست شد. لیپاز ثبت شده در محدوده PH برابر ۶ تا ۹ پایدار بود. این بیوکاتالیست در PH برابر ۴ تا ۱۰ غیرفعال تر از دامنه PH برابر ۵ تا ۹ بود. واکنش استری شدن در راکتور با بستر فشرده به دو روش پینگ پنگ و پینگ پنگ با بازدارنده مورد آنالیز قرار گرفت. نتایج نشان داد که داده های تجربی از مدل بازدارنده پینگ پنگ تبعیت می کند. در این تحقیق تبدیل اسید بوتیریک و پایداری آنزیم لیپاز ثبت شده در سیستم غربال مولکولی به میزان قابل ملاحظه ای بیشتر از بدون غربال مولکولی بود.

1. INTRODUCTION

Citronellyl esters (in particular citronellyl butyrate) are flavor ester of terpene alcohols of short-chain fatty acids. Citronellyl butyrate has a strong "sweet fruity floral like green citrus linalool" flavor, and hence has been used mainly in baked goods, frozen dairy, candy, gelatins and puddings, non-alcoholic

beverages, etc. Formerly, these esters are obtained by various methods such as chemical synthesis, extraction from natural products and by fermentation [1-3]. With the high demand for natural products, flavor industry is more interested in the use of biotechnology to produce natural flavors.

Biotechnology has been an area of major research

activity in the past decade or so for the production of flavor and fragrance compounds by using enzyme-catalyzed reaction and esters produced, however, may be considered natural. Many microbial lipase have been immobilized and a number of studies dealing with advantages of the use of immobilized lipase as biocatalyst in non-conventional media have been reported, for example, the increased in solubility of hydrophobic compounds [4], such as terpene alcohol. However, study of continuous process, especially on lipase-catalyzed synthesis of terpene esters, has received relatively little attention. Although lipase catalyzed reaction has significant benefits, the industrial application of the technology has been slow. Due to their high efficiency, low cost and ease of construction, operation and maintenance, packed-bed reactor have been reported as being the most suitable kind of reactors in industrial scale applications for ester synthesis. The ratio between substrate and enzyme is much lower in a packed bed reactor than in conventional batch reactors, and this result in shorter reaction times [5].

The objectives of this research paper are: (1) to study the reaction parameters of citronellyl butyrate synthesis using an immobilized lipase as catalyst and *n*-hexane as organic (non-conventional) media in a continuous packed-bed reactor, (2) to investigate lipase deactivation mechanism, (3) to determine the kinetic parameters based on ping pong mechanism, and (4) to study the performance of different experimental reactor for the synthesis of citronellyl butyrate, *i.e.* single packed-bed immobilized lipase reactor; packed-bed reactor with bed mixture of immobilized lipase and molecular sieve; and packed-bed immobilized lipase reactor with a separate molecular sieve column.

2. MATERIALS AND METHODS

2.1 Materials

Powdered lipase (EC 3.1.1.3) Type VII from *Candida rugosa* (901 U/mg), DL-Citronellol (95% pure), Amberlite XAD-1180 and molecular sieve were obtained from Sigma-Aldrich (M) Sdn.Bhd. Butyric acid (99% pure) was purchased from Across Organic (NJ, USA). Celite 545

diatomaceous earth, *n*-hexane, phenolphthalein, acetone, and ethanol were supplied by Fisher Scientific (M) Sdn. Bhd. Amberlite MB-1 was obtained from Organo Co. (Japan), and sodium hydroxide was supplied by Merck Co. (Darmstadt, Germany).

2.2 Immobilization Procedure

Immobilization of lipase by adsorption method was carried out by modification according to Omar and Suguna [6]. Two grams of support was washed three times with deionized water and dried in an oven at 80 °C. A 20 mg of lipase was dissolved in 5 ml phosphate buffer solution of pH 7.5 (unless otherwise specified). The dried support was added to the lipase solution. After shaking at 200 rpm for 24 hours at room temperature, then the immobilized lipase preparation was filtered and washed thoroughly with deionized water and rinsed with *n*-hexane. The resultant immobilized lipase was then dried in a vacuum dry desiccator at room temperature and stored in glass vials in refrigerator until further use. The amount of lipase adsorbed to the support was determined by using Pierce BCA (bicinchoninic acid) protein assay with BSA (bovine serum albumin) as standard.

2.3 Reactor Operation

Tubular packed-bed reactor (1.2 cm ID, 24 cm length) was used (Figure 1) for continuous operation which consisted of a jacketed glass tube. The bed was packed with 21 g of immobilized lipase directly into the column. There was a perforated plate at the bottom of the reactor to make uniform flow pattern and to avoid any flow channeling. Density of biocatalyst was experimentally determined given a value of 1.10 g/ml. At zero time, the feed solution (with equimolar concentration of 50 mM) was introduced into the reactor in an up-flow configuration at a flow rate of about 1 ml/min (linear flow rate 3 cm/min) at 37 °C (unless otherwise specified). In immobilized enzyme bioreactor, the external mass transfer is eliminated with high mass flow rate [7]. In the present operating immobilized lipase system, the external mass transfer with the linear flow rate greater than 3×10^{-5} m/s (0.18 cm/min) was omitted. Duplicate

samples of the effluent were periodically taken and acid values were monitored by titration method.

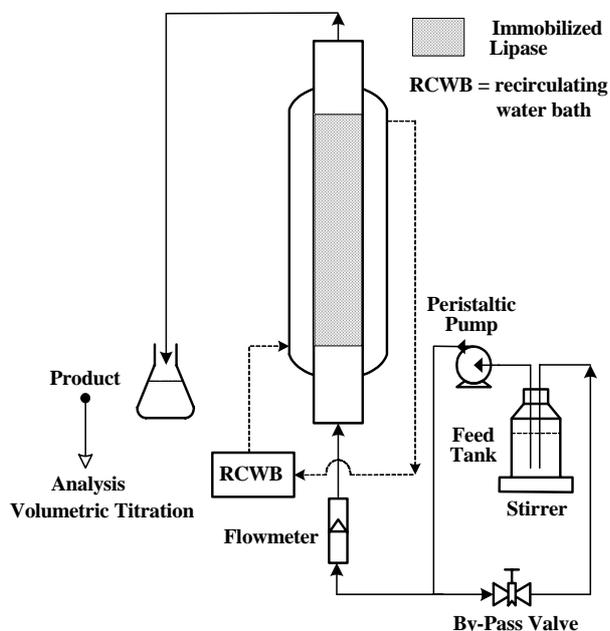


Figure 1. Schematic diagram for experimental set up with single packed bed immobilized lipase reactor.

The water generated through the lipase-catalyzed esterification reactions is considered to be the most important parameter. Adding adsorbents such as molecular sieve to the enzymatic reaction system is the simplest way to remove the produced water. In this case, another two experimental set up were also studied. In the *first case*, two glass reactors with the same size were used. Each reactor was packed with 3.5 g of immobilized lipase directly into the column (4 cm in height), followed by 3.17 g of molecular sieve (4 cm in height) until each reactor is filled with immobilized lipase and molecular sieve. In the *second case*, two glass reactors with the same size were also used. The first reactor was packed with 21 g of immobilized lipase directly into the column. While the second reactor was packed with 19 g of molecular sieve particles (density 1.63 g/ml). The purpose of having two different immobilized lipase reactors set up was to observe and compare the degree of conversion and stability of immobilized lipase.

Duplicate samples of the effluent were also periodically taken to monitor the reactor activities.

2.4 Analytical methods

Samples from the inlet and outlet streams of the reactor were withdrawn periodically for analysis. Acid consumption analysis were conducted and monitored by titration method using Digitrate™ digital burette (Jencons, UK) with NaOH solution, phenolphthalein as the indicator, and ethanol-acetone solution as quenching agent. The difference in the results between duplicates was less than $\pm 5\%$. The average values of replicate data were used in calculation. The mole of acid reacted was calculated from the values obtained for the blank and test samples. The ester produced was determined as being equivalent to the acid consumed. Then the acid conversion was calculated.

3. RESULTS AND DISCUSSION

3.1 Effect of residence time

One of the most crucial parameter that was responsible for the higher yields in the packed bed bioreactor was residence time. Residence time directly relates to the volumetric flow rate when the packed bed used is the same. As a consequence, flow rate is one of the important parameters for the packed bed bioreactor. In this study, the residence time dependence of the immobilized lipase activity in a packed-bed reactor was investigated in the range of 1.3 to 8 min by adjusting volumetric flow rate from 1 to 6 ml/min (with linear flow rate from 3 to 18 cm/min). The effect of residence time on the synthesis of citronellyl butyrate in the packed bed reactor at steady state condition are shown in Figure 2. Experimental results revealed that by increasing residence time (decreasing volumetric flow rate) the conversion of butyric acid to produce citronellyl butyrate was also increased and reached to the highest acid conversion of 95 % at the residence time of 8 min (flow rate of 1 ml/min). This is because, the longer residence time (smaller flow rate) would lengthen the enzyme substrate contact time and create higher esterification activity. High substrate flow rate will reduce mass

transfer of substrate into the supported-enzyme interface, thus less distribution and short contact time of substrate and enzyme active site, yielded lower conversion of substrate.

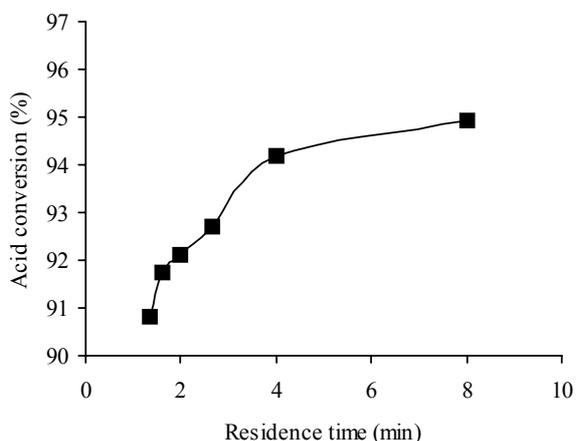


Figure 2. The effect of residence time on citronellyl butyrate synthesis in a continuous packed-bed immobilized lipase reactor at steady-state condition

3.2 Effect of temperature

The temperature dependence of the immobilized lipase activity in a packed-bed reactor was investigated in the range of 20 to 60 °C. The effect of various temperatures on enzymatic synthesis of citronellyl butyrate at steady state condition are shown in Figure 3. Thermal stability of *Candida rugosa* lipase used in a continuous packed-bed reactor was improved and stabilized by immobilization, where the highest conversion of 97 % was obtained at 50 °C. However, as reported that lipase stability was also influenced by temperature, where higher temperature will greatly reduce the lipase stability. As the temperature increase up to 60 °C, lipase activity decreased as reflected in the decrease on acid conversion to 90 %. This results show that any change of temperature causes change in the rate of reaction and degree of conversion.

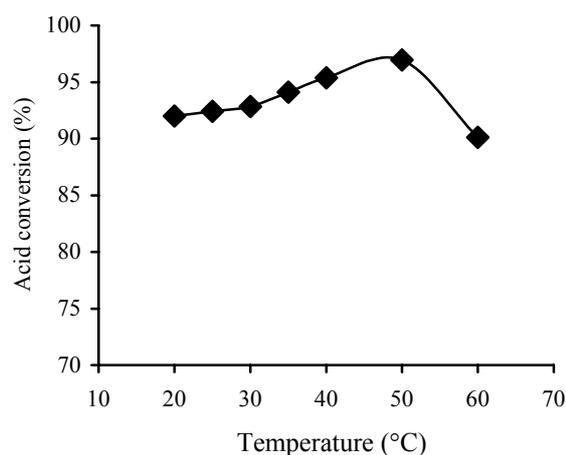


Figure 3. The effect of temperature on citronellyl butyrate synthesis in a continuous packed-bed immobilized lipase reactor at steady-state condition.

It was also found from the experimental data that the enzyme decayed rapidly with time for a period of time. The thermal denaturation has a profound effect on deactivation of the enzyme. It is assumed that thermal denaturation of an enzyme may be modelled by the following serial deactivation scheme [8-9],



the simple first order deactivation expression results

$$a = \exp(-k_d t) \quad (2)$$

where k_d is deactivation rate constant, and can be found from packed-bed equation of first-order reaction and first-order deactivation rate constant given by the following equation [10]:

$$\ln \ln \left(\frac{1}{1 - X_A} \right) = \ln(k\tau) - k_d t \quad (3)$$

From equation (3), the evaluation of the deactivation rate constant with the data at various temperature studied are shown in Figure 4. The deactivation rate constants, k_d , was found to increase (0.61 to 0.92 h⁻¹) with increasing

operating temperature (30 to 60 °C). This means, the conversion of acid decreases more rapidly with increasing temperature. In other words, from the graph of activity of immobilized lipase, *i.e.* according to equation (2), versus time at various

temperatures studied, it was found that deactivation of biocatalyst is more rapid with increasing temperature as reflected in the decrease of biocatalyst activity shown in Figure 5.

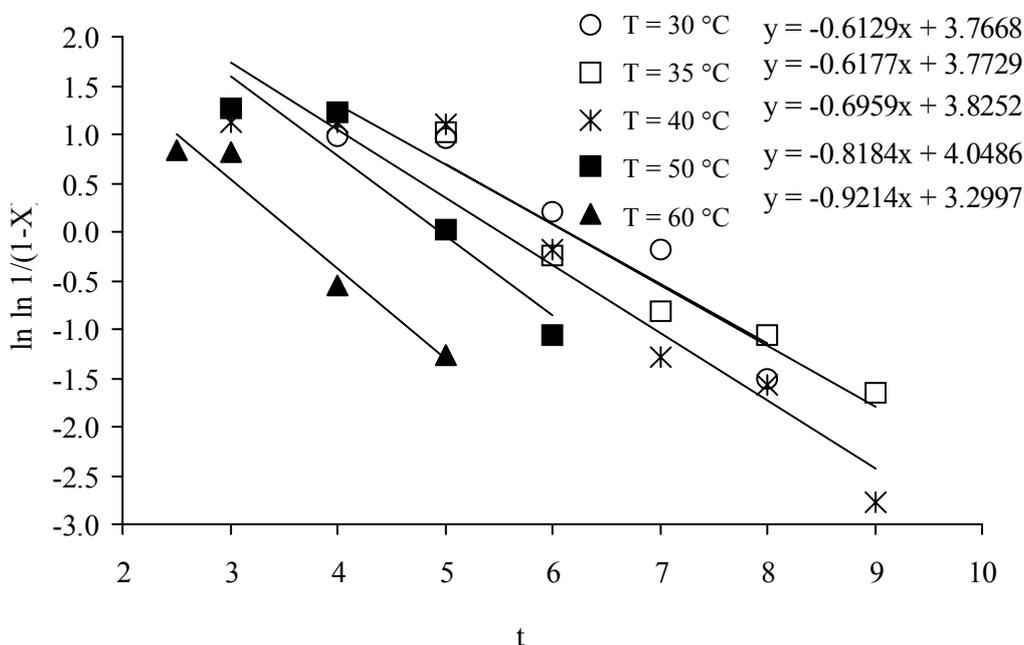


Figure 4. Deactivation kinetics of immobilized lipase in a continuous packed-bed reactor on citronellyl butyrate synthesis at various temperatures studied

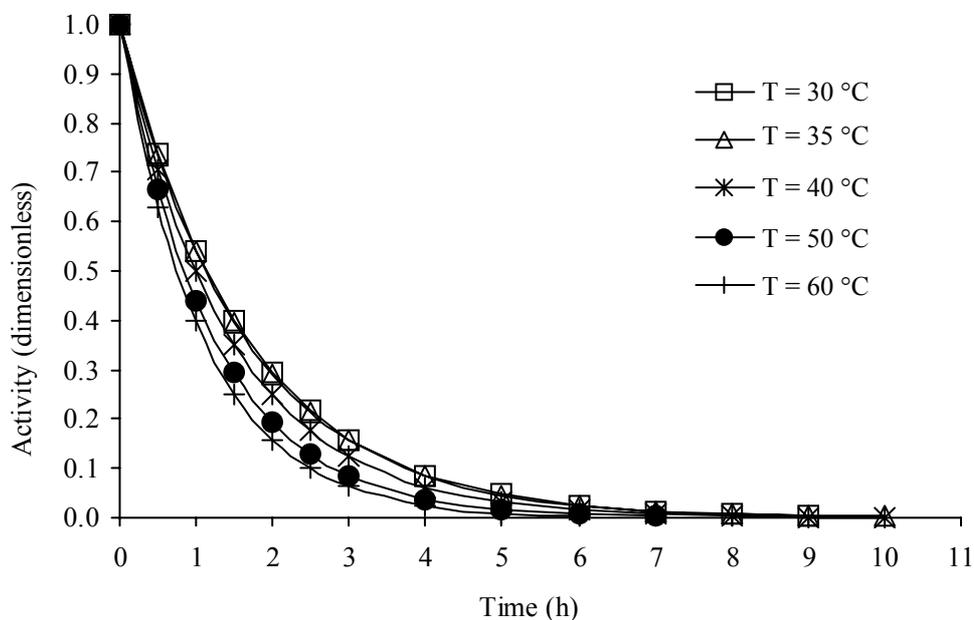


Figure 5. Activity of immobilized lipase in packed-bed reactor in catalyzing citronellyl butyrate as a function of time at various temperatures studied.

3.3 Effect of PH

The effect of pH on the synthesis of citronellyl butyrate was investigated in a continuous packed-bed immobilized lipase reactor in the pH range of 4 to 10. Figure 6 shows the relationship between lipase adsorbed and the acid conversion by immobilized *Candida rugosa* lipase at different pH values in a packed-bed reactor at steady-state condition. About 92 – 95 % of lipases were adsorbed to the support in the range of pH studied. In the pH range from 6 to 10, immobilized lipase were stable, where acid conversion of 88 - 95 % were observed. At pH 4 and 5, the immobilized lipase were more sensitive to pH, where conversion of 66 and 70 %, respectively, were obtained. In this study, the optimal pH was 7.5 with 95 % conversion.

Enzyme deactivation analysis was also carried out for pH dependency using equation (3). Figure 7 shows the evaluation of the deactivation rate constant with the data at various pH studied. The deactivation rate constants (k_d) were found greater at immobilized lipase pH of 4 and 10, *i.e.* 1.5 and 1.07 h^{-1} , respectively. This shows that deactivation of biocatalyst at this lowest and highest pH studied are more rapid in comparison with pH range of 5 to 9. In other words, from the graph of activity of immobilized lipase versus time, it was found that deactivation of biocatalyst was more rapid with extreme pH of immobilized lipase at 4 and 10, respectively, as presented in Figure 8.

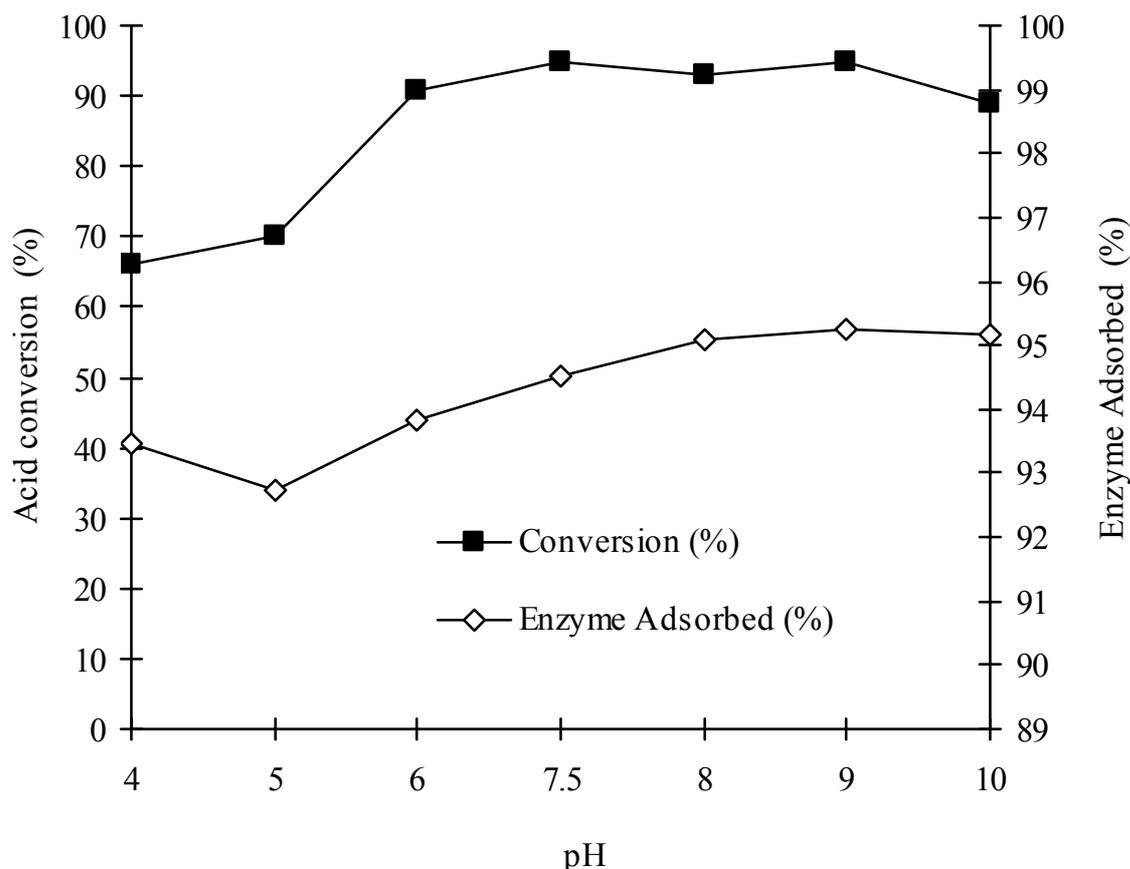


Figure 6. Effect of pH of immobilized lipase during the synthesis of citronellyl butyrate in a continuous packed-bed reactor at steady-state condition.

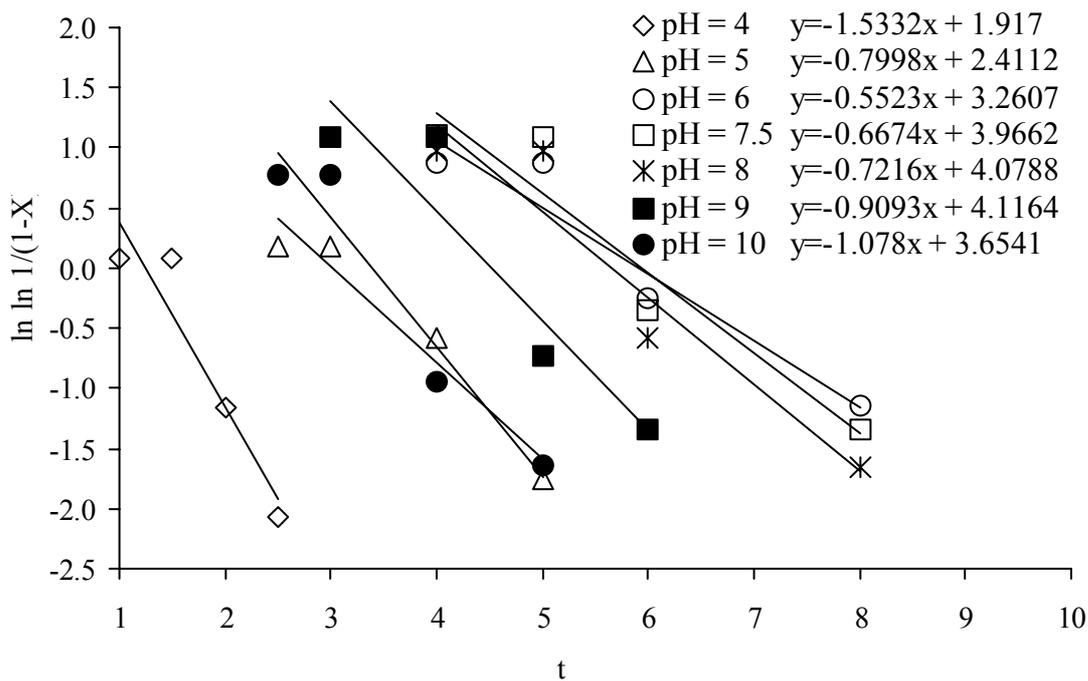


Figure 7. Deactivation kinetics of immobilized lipase in a continuous packed-bed reactor on citronellyl butyrate synthesis at various pH studied.

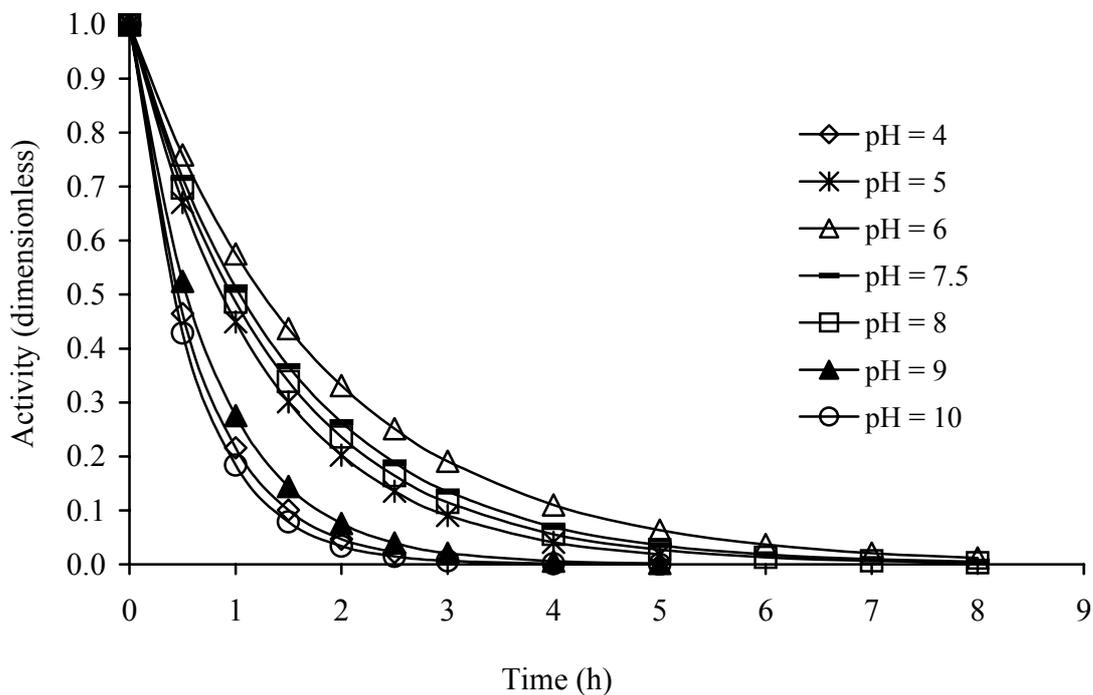


Figure 8. Activity of immobilized lipase in packed-bed reactor in catalyzing citronellyl butyrate as a function of time at various pH studied

3.4 Mathematical models

Various kinetics mechanisms have been proposed for the description of lipase-mediated reactions [11-12]. For two-substrate two-product (bi-bi) reactions, the most generally accepted mechanism for lipase-catalyzed reactions is ping-pong mechanism. This mechanism was first established by Chulalaksananukul *et.al* [13] for the kinetics of esterification of oleic acid with ethanol using immobilized *R. miehei* lipase as biocatalyst and *n*-hexane as organic solvent. After that, many researchers have also proposed kinetic models for lipase-catalyzed esterification and transesterification reactions in organic media based on this mechanism [14-22]. In this study, ping pong and ping pong with product inhibition models, were tested with the experimental data. The experiments were carried out in *n*-hexane with equimolar substrate concentrations of 0.1, 0.05 and 0.02 M (initial butyric acid concentration of 9.35, 4.50 and 1.84 g/l, respectively) at fixed flow rate of 1 ml/min and 37 °C.

Based on a single displacement reaction of ping pong and ping pong with product inhibition mechanisms, the rates of decrease of butyric acid [A] may be given by [16],

$$-\frac{d[A]}{dt} = \frac{V'_m}{1 + \frac{K_3 + K_4}{[A]}} \quad (4)$$

$$\text{and} \\ -\frac{d[A]}{dt} = \frac{V'_m}{1 + \frac{(K_3 + K_4)}{[A]} + \frac{K_3 K_i ([A]_0 - [A])}{[A]}} \quad (5)$$

The decrease of butyric acid concentration as a function of time at various initial concentrations were fitted with these two models by non-linear regression using Polymath software (version 5.1). Figures 9 – 11 shows the comparison of the experimental data and the simulation results for two models tested. The parameters estimated for kinetic constants are given in Table 1. In all the initial butyric acid concentration tested, the ping pong model with product inhibition, *i.e.* equation (5), predicted the observed behaviour more accurately than the ping pong without product inhibition model, *i.e.* equation (4). The estimated value for the second model at various initial butyric acid concentrations has a correlation coefficients (R^2) greater than 0.99 and the sum of squares of errors (SSE) were much less than the first model.

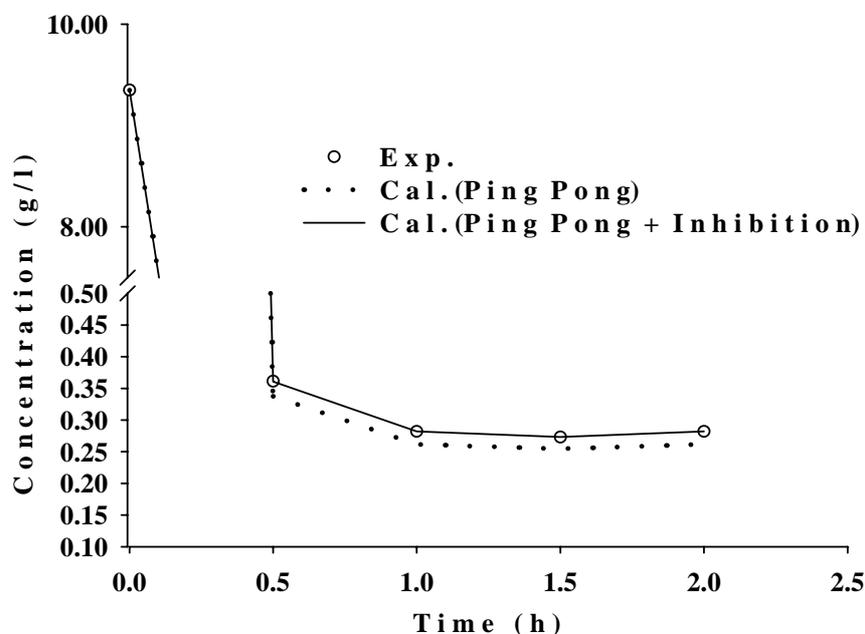


Figure 9. Comparison of experimental data with model predictions of esterification of citronellyl butyrate at initial butyric acid concentration of 0.1 M (9.35 g/l).

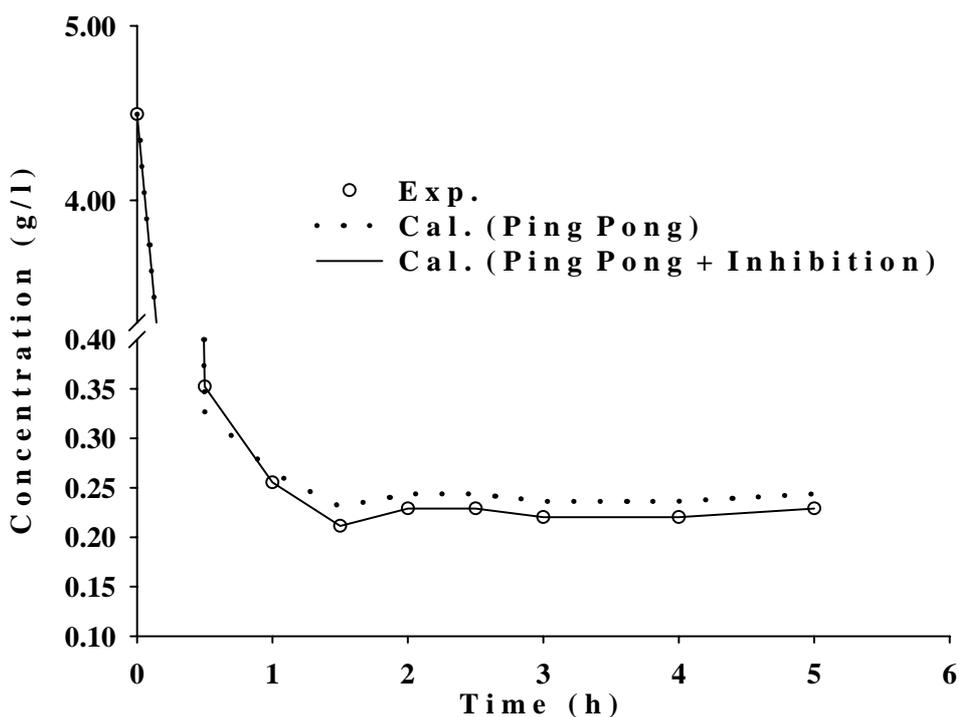


Figure 10. Comparison of experimental data with model predictions of esterification of citronellyl butyrate at initial butyric acid concentration of 0.05 M (4.50 g/l).

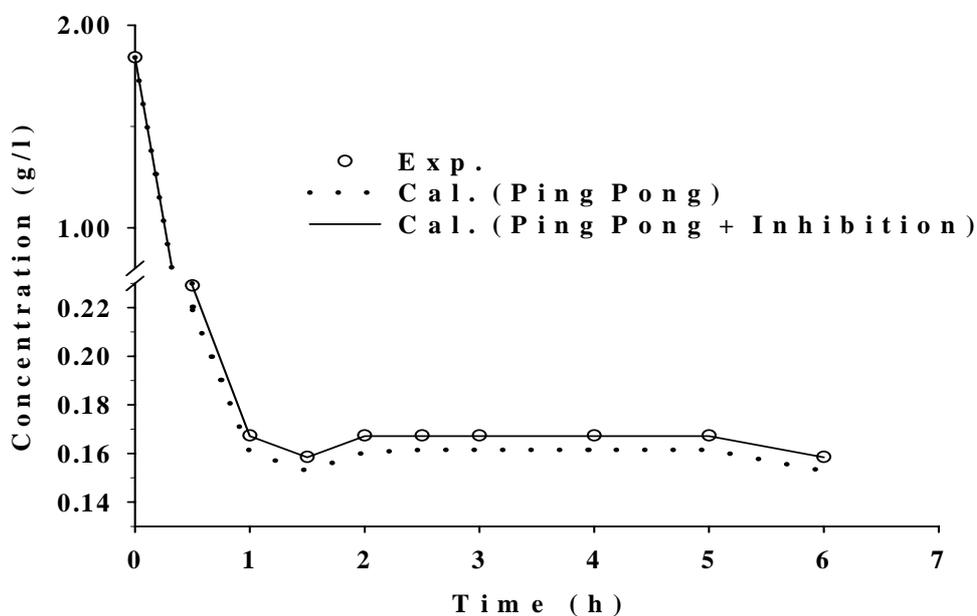


Figure 11. Comparison of experimental data with model predictions of esterification of citronellyl butyrate at initial butyric acid concentration of 0.02 M (1.84 g/l).

Table 1. Estimated kinetic constants for esterification of citronellyl butyrate

Butyric acid concentration	Models	Parameters				
		V_m' (g/l.min)	$K_3 + K_4$ (g/l)	K_3K_i (g/l)	R^2	SSE ($\times 10^9$)
0.1 M	Ping pong	0.1758	1.1010	0.9428	4320.0
	Ping pong with product inhibition	1.1010	1.3679	0.7894	0.9997	5.8
0.05 M	Ping pong	0.1580	1.0101	0.9409	13200.0
	Ping pong with product inhibition	0.6091	0.3395	1.0101	0.9999	2.0
0.02 M	Ping pong	0.1481	1.0101	0.9690	1820.0
	Ping pong with product inhibition	0.2366	0.0577	0.9973	0.9999	4.4

3.5. Performance of packed-bed immobilized lipase reactor with molecular-sieve

In this study, citronellyl butyrate synthesis was conducted in the packed-bed immobilized lipase reactor with mixed and separated molecular-sieve particle. The comparison in the synthesis of citronellyl butyrate by using different experimental reactor set up are shown in Figure 12. As observed, the reactions were quite fast and reached a steady state within 1 to 1.5 hours for all experimental reactor set up studied. A conversion of about 92 - 93 % was maintained over a period of 15 hours at steady state condition for the system with molecular-sieve. For the system without molecular-sieve, only 90 % of conversion is maintained over a period of 10 hours. Based on this experiment on the performance of different reactor set up, the conversion of butyric acid to produce ester and the stability of immobilized *Candida rugosa* lipase increased more in the system with molecular-sieve than in the system without molecular-sieve. Although higher conversion of 93 % was obtained by using packed-bed reactor with a bed mixture of immobilized lipase and molecular-sieve, however, this

experimental set up was not practical and extensive effort was required for regeneration. Hence, packed-bed immobilized lipase reactor with a separate molecular sieve column was preferred in carrying out this study. The main advantage of this system was that regeneration of the molecular-sieve column could be independently carried out upon saturation without disrupting the immobilized enzyme reactor.

4. CONCLUSIONS

Continuous esterification of citronellyl butyrate by immobilized *Candida rugosa* lipase in a packed-bed reactor with and without molecular-sieve particle has been carried out in non-conventional media (*n*-hexane). The esterification reaction was simulated by two models namely, ping pong model and ping pong with product inhibition model. The second model was found to predict the observed behaviour accurately. In order to improve the efficiency, packed-bed immobilized lipase reactor with a separate molecular sieve column was preferred than packed-bed reactor with a bed mixture of immobilized lipase and molecular sieve.

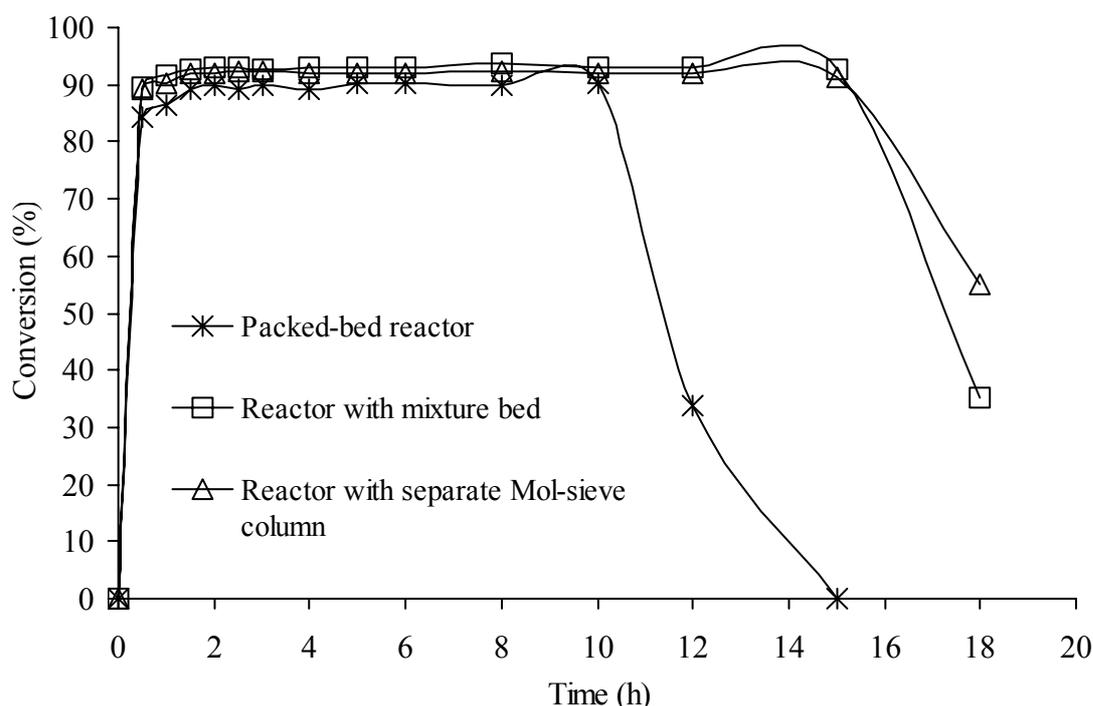


Figure 12. Comparison of experimental reactor set up in the synthesis of citronellyl butyrate.

5. ACKNOWLEDGEMENTS

The authors acknowledge the research grant provided by Universiti Sains Malaysia that has resulted the present paper.

NOTATION	
a	enzyme activity
[A]	concentration of butyric acid (mole/l)
[A] ₀	initial concentration of butyric acid (mole/l)
E, E ₁ , E _d	active, intermediates and deactivated enzyme (g/l)
k	rate constants (mole/l.h)
K ₃ , K ₄	constants related to reaction rate constants
K _i	inhibition constant (mole/l)
k _d , k _{d1} , k _{d2}	first-order deactivation rate constants (h ⁻¹)
t	time (h)
V _m	constant related to maximum velocity (mole/l.h)
X _A	conversion of butyric acid

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