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# FREE *CANDIDA RUGOSA* LIPASE-CATALYZED SYNTHESIS OF CITRONELLYL BUTYRATE IN *n*-HEXANE BY DIRECT ESTERIFICATION: EFFECT OF REACTION PARAMETERS

IRVAN DAHLAN[1], AZLINA HARUN @ KAMARUDDIN[2*] & GHASEM D. NAJAFPOUR[3]

**Abstract.** Free and immobilized *Candida rugosa* lipases were investigated for the synthesis of citronellyl butyrate by direct esterification reaction in *n*-hexane as organic solvent. A set of experiments was carried out to verify the influence of various parameters on the formation of citronellyl butyrate by free *Candida rugosa* lipase, such as lipase loading, substrate molar ratio, temperature, three kinds of support for immobilization, and ratio of immobilization. The conversion was increased with increasing lipase loading. The behavior of leveling-off in esterification was observed at higher lipase loading which gave the optimal amount of lipase loading at 3.33 g/l with 92% conversion. This might be due to the excess of lipase active sites, which remained inside the bulk of lipase particles, was not contributing significantly to the reaction. Increasing butyric acid and citronellol concentrations (at fixed citronellol and butyric acid concentrations, respectively) inhibited the lipase activity due to competitive nature of alcohol and acid binding. Optimal acid conversion was obtained at 40°C after 24-h incubation time. Above this temperature, however, the activity of lipase-catalyzed esterification begins to decrease due to denaturation of protein. From the three kinds of supports for immobilized lipase, Amberlite MB-1 showed the highest conversion compared to Amberlite XAD-1180 and Celite 545. The optimal acid conversion was obtained at lipase loading of 10 mg lipase/g support. At this loading, lipase attempts to optimize its contact with the surface of the support whereby optimum active conformation was retained.

*Keywords:* Citronellyl butyrate; *Candida rugosa* lipase; direct esterification; immobilized lipase; organic media

**Abstrak.** Sintesis sitronelil butirat melalui pengesteran langsung telah dikaji di dalam *n*-hexane sebagai pelarut organik yang dimangkinkan oleh lipase bebas dan lipase tersekatgerak daripada *Candida rugosa*. Kajian telah dijalankan untuk mengesahkan pengaruh pelbagai parameter pada pembentukan sitronelil butirat oleh lipase bebas daripada *Candida rugosa*, iaitu kesan kepekatan lipase, nisbah molar substrat, suhu, tiga jenis bahan sokongan untuk lipase tersekatgerak dan nisbah sekatgerak. Peningkatan kepekatan lipase bebas menyebabkan peningkatan kepada penukaran asid. Sifat peningkatan tidak berubah dari tindak balas pengesteran dapat diperhatikan pada kepekatan lipase tinggi yang memberikan kepekatan optimum lipase pada 3.33 g/l dengan penukaran asid sebanyak 92%. Kemungkinan ini disebabkan oleh tapak aktif lipase yang berlebihan yang berada di dalam zarah lipase pukal, yang tidak memberi sumbangan bererti kepada tindak

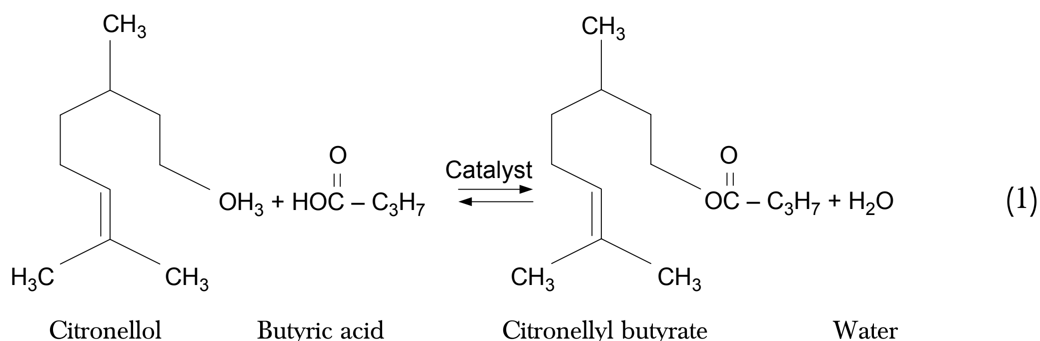
[1,2&3]School of Chemical Engineering, Universiti Sains Malaysia, Engineering Campus, Seri Ampangan, 14300 Nibong Tebal, Pulau Pinang, Malaysia
* Corresponding author: Tel: +60-4-5996417, Fax: +60-4-594 1013. E-mail: chazlina@eng.usm.my

balas. Aktiviti lipase didapati terencat dengan bertambahnya kepekatan asid butirik (pada kepekatan sitronelol tetap) dan sitronelol (pada kepekatan asid butirik tetap). Ini disebabkan adanya persaingan semulajadi pengikatan alkohol dan asid. Penukaran asid optimum diperolehi pada suhu 40°C selepas 24 jam pengeraman. Bagaimanapun, atas dari suhu ini, aktiviti pengesteran yang dimangkinkan oleh lipase mula menurun kerana penyahaslian protein. Daripada tiga jenis sokongan yang digunakan untuk lipase tersekatgerak, Amberlite MB-1 menunjukkan penukaran asid tertinggi berbanding dengan Amberlite XAD-1180 dan Celite 545. Penukaran asid optimum diperolehi pada nisbah sekatgerak 10 mg lipase/g penyokong. Pada nisbah sekatgerak ini, lipase mengoptimumkan sentuhan dengan permukaan penyokong dengan mengekalkan konformasi yang aktif pada tahap optimum.

*Kata kunci:* Sitronelil butirat; lipase *Candida rugosa*; pengesteran langsung; lipase tersekatgerak; pelarut organik

## 1.0 INTRODUCTION

Esters of terpene alcohols of short-chain fatty acids are essential oils that present a great interest as flavor and fragrance compounds, especially citronellyl esters. Among them, citronellyl butyrate, which is the most important and has been used mainly in baked goods, frozen dairy, beverages, candy etc. [1, 2]. Citronellyl butyrate is synthesized by esterification of citronellol with butyric acid in the presence of catalyst as shown below:




Currently, flavor and fragrance compounds are obtained by chemical and extraction methods. But, substance produced by chemical approach cannot be considered natural and do not have the same economical value as chemicals from natural sources. As a result, the majority of flavor and fragrance compounds are still produced by traditional extraction processes from plant and animal sources. However, such sources suffer from a diminishing supply of raw material and expensive product cost [1, 3]. Moreover, these methods exhibit a low yield of the desired product, poor reaction selectivity leading to undesirable side reactions, time-consuming, and high energy requirements [4 – 6]. Because of these disadvantages, enzymatic synthesis with lipase is gaining importance.

Microbial lipases (triacylglycerol acyl-hydrolases, E.C. 3.1.1.3) have been intensively investigated both for the hydrolytic production of fatty acids and for the synthesis of esters [7], especially *Candida rugosa* lipase. The application of free and immobilized lipase in organic media has been the object of numerous studies for more than two decades ago. One advantages of using these organic media is the increased in solubility of hydrophobic compounds [8], such as terpene alcohol.

Cloan and Akoh [9] studied the synthesis of geranyl acetate in *n*-hexane as organic solvent with two immobilized *Candida antarctica* lipases (SP382 and SP435). Molar conversion of 95% and 99% were obtained with 2% and 15% (w/w reactants) of SP435 and SP382, respectively. The optimal temperature range of 35 – 40°C was obtained for both lipases. Inhibitory effect was observed when the concentration of acetic acid increased, whereas those of alcohol (geraniol) were not observed. Conversely, Yee *et al.* [10] reported that increasing the citronellol and geraniol concentrations had inhibitory effect on immobilized *Pseudomonas sp.* lipase activity in the synthesis of citronellyl butyrate and geranyl caproate. It was also found that the optimal amount of lipase of 300 units was obtained resulted in yields of 99% and 94% for citronellyl butyrate and geranyl caproate, respectively. Apart from that, Bagi and Simon [11] investigated the operation of differently immobilized forms of porcine pancreas lipase (PPL) in the syntheses of fructose butyrate. From five different immobilized PPL prepared, PPL immobilized on Celite support shows the highest transesterification yield among the other supports, which gave an approximately 10-fold higher yield of fructose ester.

Nevertheless, esterification reaction is still associated with many difficulties to improve the performance of this kind of biocatalyst and several parameters which affect the enzymatic synthesis in an organic solvent have yet to be elucidated. This study focuses on the effect of reaction parameters (lipase loading, substrate molar ratio, temperature, supports for immobilization, and ratio of immobilization) that influence free lipase from *Candida rugosa* in catalyzing citronellyl butyrate by direct esterification using citronellol as alcohol, butyric acid as acyl donor and *n*-hexane as organic solvent.

## 2.0 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), and Amberlite XAD-1180 were obtained from Sigma-Aldrich Sdn. Bhd. (Malaysia). 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 Sdn. Bhd. (Malaysia). 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 [12]. 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.

### 2.3 Esterification Method

Ester synthesis was carried out in 15 ml screw-capped test tubes with a working volume of 10 ml, containing 20 mM butyric acid, 30 mM citronellol, *n*-hexane and an appropriate amount of free lipase (unless otherwise specified). Esterification reaction were incubated at 37°C for 24 hours, under constant shaking at 200 rpm in a Certomat™ orbital shaker (B. Braun, USA) along with blanks as control (samples without enzymes). After the incubation period, duplicate samples were withdrawn from the reaction mixture and the acid values were monitored by titration method.

### 2.4 Analytical Methods

Acid consumption analysis for esterification experiments 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 ± 5%. The averages values of replicate data were used in the 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. The acid conversion was calculated as follows:

$$\text{Conversion (\%)} = \frac{(\textit{initial acid conc.}) - (\textit{acid conc. after reaction})}{(\textit{initial acid conc.})} \times 100 \qquad (2)$$

## 3.0 RESULTS AND DISCUSSION

### 3.1 Effect of Lipase Loading

Study of the effect of free *Candida rugosa* lipase loading were conducted in the range of 0.11 – 7.77 g/l under identical conditions. Figure 1 shows the effect of lipase loading on the synthesis of citronellyl butyrate. The conversion of butyric acid to

produce citronellyl butyrate increased with the amount of lipase. However, at 3.33 to 7.77 g/l of lipase, the observed conversion remained unchanged. This behavior of leveling-off of esterification at higher lipase loading has also been reported by other investigators [9, 10, 13 – 16]. This can be explained by considering that the active sites of the enzyme molecules present in excess would not be exposed to the substrates and remain inside the bulk of enzyme particles without contributing significantly to the reaction [13]. Based on experimental results, the 3.33 g/l of free *Candida rugosa* lipase was defined as the optimal amount of lipase, which resulted in conversion of 92%.

The performance for lipase loading in this study was better than the early results reported in the literature in synthesizing flavor ester. Langrand *et al.* [17] have reported 80% conversion of flavor esters using 50 g/l of free lipase at a substrate concentration of 0.25 M in 24 h incubation time. While Welsh *et al.* [18] have reported 76% conversion in 48 h incubation time using 2% w/v (20 g/l) of free lipase from *Candida cylindracea*. Moreover, the result reported by Razafindralambo *et al.* [19] is still lower than the present study. Razafindralambo *et al.* [19] have reported 80% conversion of flavor acetates using 50 g/l of free lipase from *Mucor miehei* in 24 h incubation time with alcohol/acid ratio of 4.

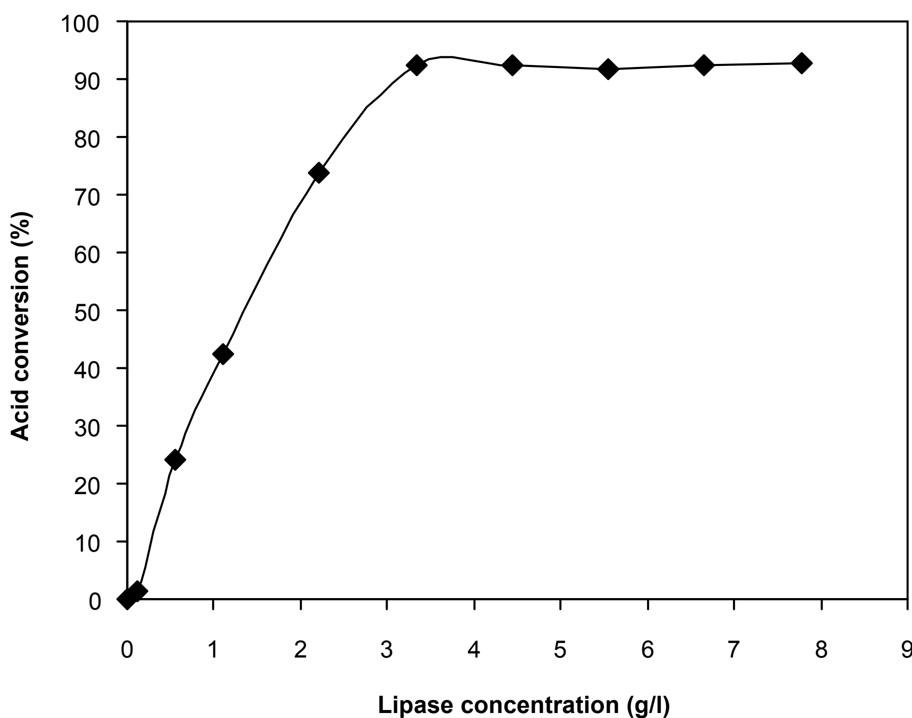



**Figure 1** Effect of free *Candida rugosa* lipase concentration on enzymatic synthesis of citronellyl butyrate

## 3.2 Effect of Substrate Molar Ratio

The effect of substrate molar ratio on esterification activity of free lipase were conducted at a fixed lipase concentration of 3.33 g/l by fixing butyric acid concentration at 20 mM and varying citronellol concentration (20 – 60 mM) and vice versa. As observed in Figure 2, the maximum conversion of 92% was achieved when citronellol was in excess (30 mM) to butyric acid (20 mM) with molar ratio (R) of 1.5. At above 1.5 of alcohol to acid molar ratio, acid conversion decreased slightly from 85% to 81% for alcohol to acid molar ratio of 2 and 3, respectively. Similar result on the effect of alcohol to acid molar ratio (excess nucleophile) was also observed by Krishna *et al.* [16] in the synthesis of isoamyl acetate using immobilized lipase from *Rhizomucor miehei*, where maximum conversion of 91% was achieved when alcohol was used in excess with molar ratio of 2. According to Krishna *et al.* [16] the acyl transfer was affected by the concentration of free alcohol available and, usually, higher concentration of alcohol (acyl acceptor/nucleophile) leads to higher levels of equilibrium conversions due to the availability of excess nucleophile for acyl transfer. However, in this study, alcohol binding probably assumes significance only above a critical level of alcohol. At a ratio of 1.5, maximum acyl transfer appears to take place, giving rise to the maximum extent of esterification. On the other hand, increasing butyric acid concentration at fixed citronellol concentration (acid to alcohol

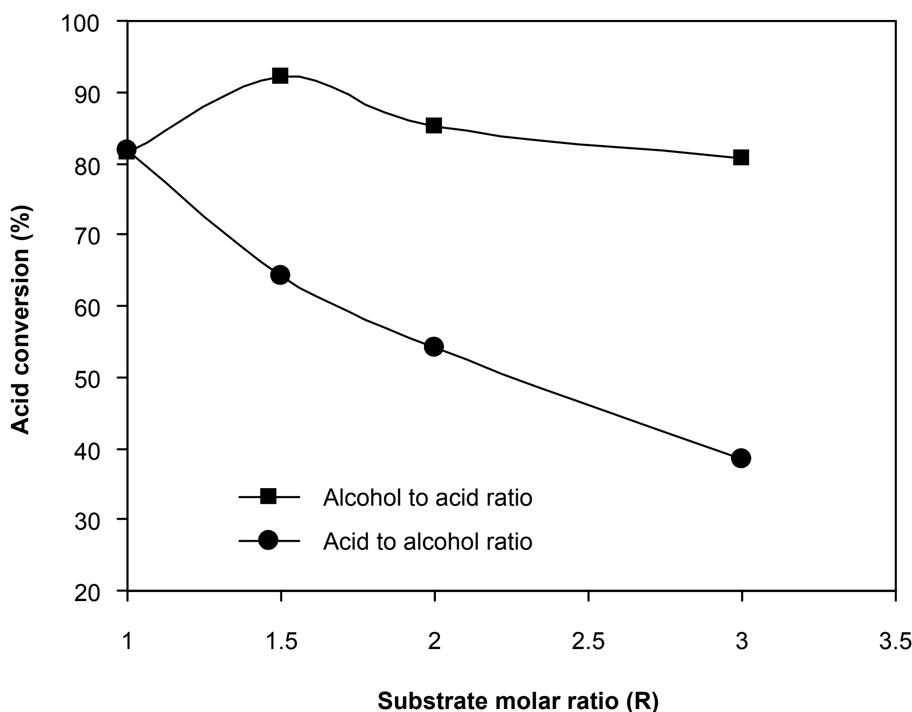



**Figure 2** Effect of substrate molar ratio on enzymatic synthesis of citronellyl

molar ratio) led to a decrease on acid conversion. A maximum conversion of 82% was achieved at equimolar concentrations of citronellol and butyric acid. In esterification, when using butyric acid (being highly polar acid and soluble in water) in excess, some of the acid dissolves in water (produced from reaction). This can alter thermodynamic equilibria, causing hydrolysis of the ester formed and also reducing the activity of free *Candida rugosa* lipase [16].

Based on experimental results, increasing butyric acid and citronellol concentrations (at fixed citronellol and butyric acid concentrations, respectively) inhibited the free *Candida rugosa* lipase activity as reflected in the decrease on conversion. But, study by de Castro *et al.* [20] reported that there was no inhibition when butyric acid was used in excess on lipase-catalyzed esterification of citronellyl butyrate using immobilized *Mucor miehi* lipase (Lipozyme IM$^{20}$). On the other hand, Yee *et al.* [10] reported that only citronellol gave inhibitory effect on enzymatic transesterification reaction of citronellyl butyrate on activity of immobilized *Pseudomonas sp.* lipase. However, Welsh and Williams [21] stated that the concentration of substrate used could affect lipase performance, but the effect was dependent on lipase used to catalyze the reaction.

## 3.3 Effect of Temperature

In this study the effect of temperature on esterification activity of free *Candida rugosa* lipase was monitored in range of 30 to 60°C. A conversion of butyric acid to produce citronellyl butyrate, as well as lipase activity, increased as the temperature was increased from 30°C to 40°C (Figure 3). The optimum conversion of about 94% was achieved at 40°C after 24 hours incubation time. The lipase activity at this condition was 3.9 μmol ester/g lipase/min (0.025 μM ester/unit of lipase/hour). Similar optimum temperature was also reported in the literature for synthesizing terpene esters. Cloan and Akoh [9] found that the optimum temperature range of 35 – 40°C was obtained for *Candida antarctica* lipases. In another study by Yee *et al.* [10], the optimum temperature range of 30 – 50°C was observed for *Pseudomonas sp.* lipase.

The conversion was slightly dropped from 91% to 87% once the temperature increased from 50°C to 60°C. The lipase activity was also decreased from 3.8 to 3.5 μmol ester/g lipase/min at this temperature range. In general, the acid conversion was always greater than 87% over the temperature range studied. It has been reported that most protein denaturation in lipase-catalyzed esterification begins to occur at or above 45°C [9, 22 – 26]. This phenomenon of enzyme denaturation, which is a physical mechanism, can be explained as follows: as the temperature increases, the atoms in the enzyme molecule have greater energies and a greater tendency to move. Because protein structure is stabilized by weak forces, they acquire sufficient energy to overcome such weak interactions holding the globular protein structure together, and the deactivation follows quickly [27].

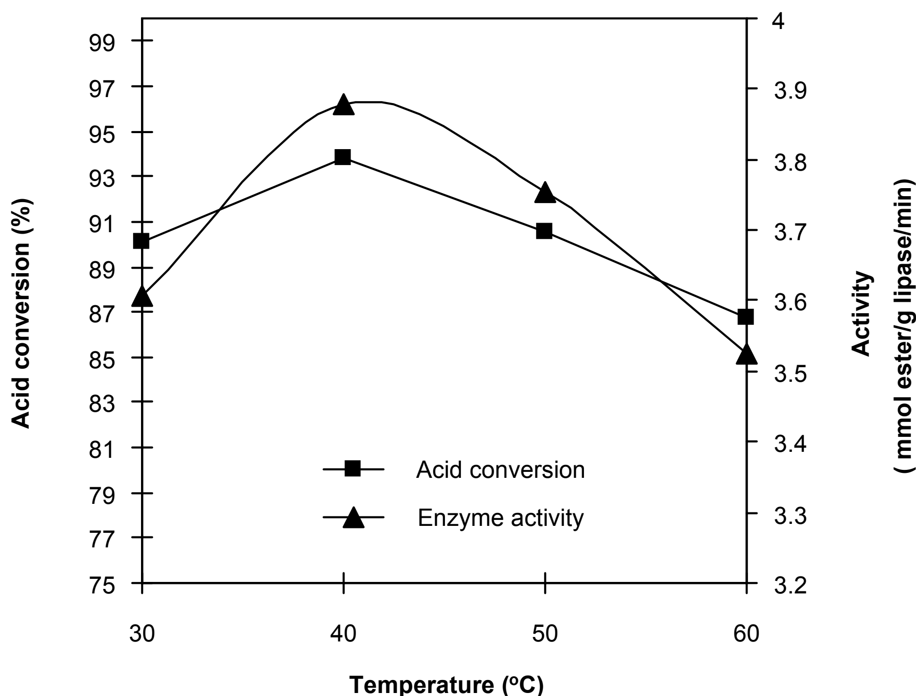



**Figure 3** Effect of temperature on enzymatic synthesis of citronellyl butyrate

## 3.4 Effect of Supports for Lipase Immobilization

The effect of different supports, *i.e.* Amberlite MB-1, Amberlite XAD-1180 and Celite for *Candida rugosa* lipase immobilization on the synthesis of citronellyl butyrate were examined in the range of 0.55 – 38.85 g/l under identical conditions. As shown in Figure 4, the acid conversion was increased with the amount of immobilized lipase for three different supports tested. Amberlite MB-1 showed to be the best overall support matrix, which gave the highest conversion compared with Amberlite XAD-1180 and Celite. Amberlite XAD-1180 and Celite performed poorly, showing the lowest overall conversion over the amount of immobilized lipase studied. The highest conversions of 88%, 29% and 15% were obtained using 38.85 g/l immobilized lipase for Amberlite MB-1, Celite and Amberlite XAD-1180 supports, respectively. Omar and Suguna [12] also reported that Amberlite MB-1 was the best support matrix for immobilization of lipase from *Aspergillus niger* in the synthesis of butyl oleate compared with various supports including Celite. On the contrary, Bagi and Simon [11] showed that porcine pancreas lipase immobilized on Celite gave highest yield of fructose ester compared with various supports.

These results suggested that adsorption capacity [28], water-retaining capacity of the supports [11] and the microenvironment of the *Candida rugosa* lipase molecules

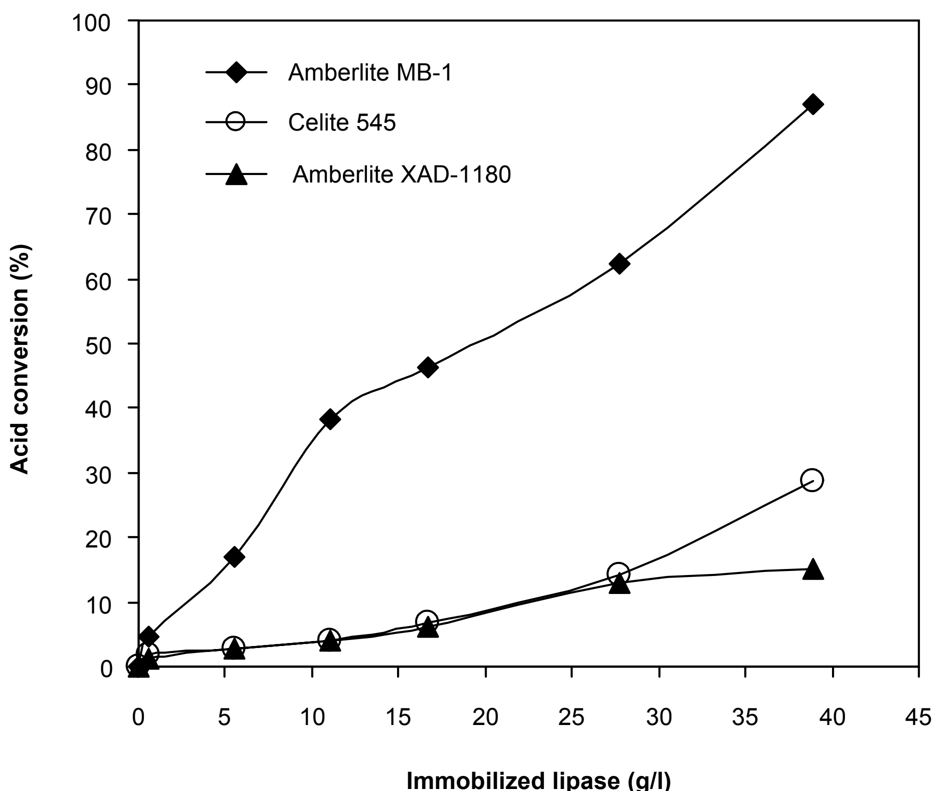



**Figure 4** Effect of support materials for immobilized lipase on enzymatic synthesis of citronellyl butyrate

on the support might cause differences in the equilibrium reached during citronellyl butyrate synthesis. In the present study it is evident that Amberlite XAD-1180 and Celite 545 supports did not work well for *Candida rugosa* lipase. The immobilization method used may also affect the enzymatic synthesis of citronellyl butyrate on these supports. In addition, it was not possible to clearly elucidate the nature of the interaction between the enzyme and the support. This would require a systematic investigation with the aid of protein engineering.

## 3.5 Effect of Immobilization Ratio

Further experiments on immobilization were carried out to determine an appropriate lipase loading by using a fixed amount of Amberlite MB-1 (2 grams) for different amounts of lipase (5 to 400 mg lipase). Figure 5 shows the different loading of *Candida rugosa* lipases to a fixed Amberlite MB-1 support in catalyzing citronellyl butyrate synthesis. As observed, acid conversion were increased with the amount of immobilized lipase for all immobilization ratios tested. As expected, the acid

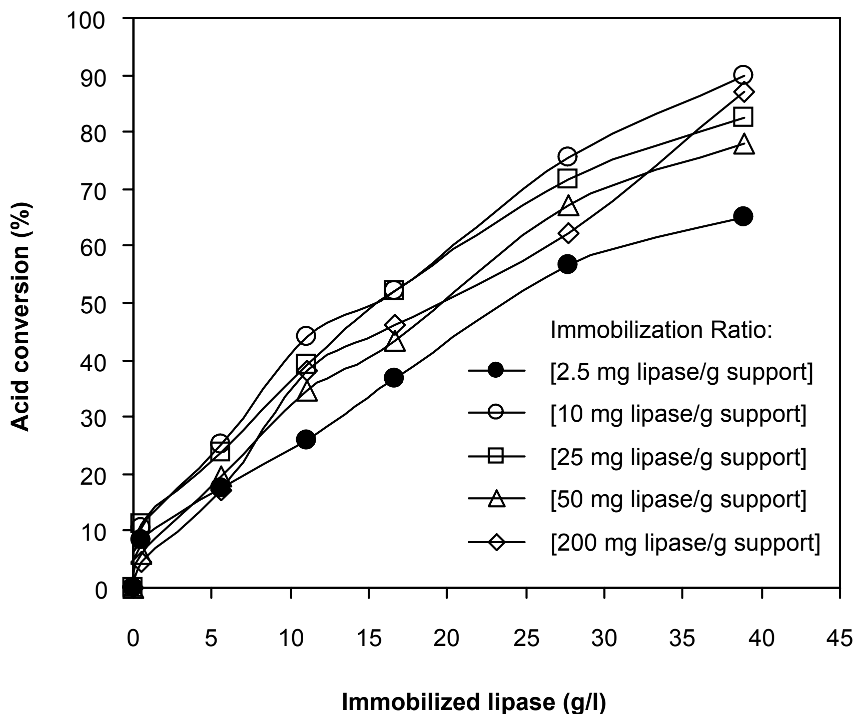



**Figure 5** Effect of immobilization on the amount of lipase and Amberlite MB-1 support ratio on enzymatic synthesis of citronellyl butyrate

conversion of immobilized lipase increased as more lipase was loaded from 2.5 to 10 mg lipase/g support. However, at lipase loading above 10 mg lipase/g support, acid conversion was observed to decrease. The optimal conversion was obtained at lipase loading of 10 mg lipase/g support, where the highest conversion of 90% was achieved using 38.85 g/l of immobilized lipase. These results suggested that at the lowest lipase loading of 2.5 mg lipase/g support, the lipase attempts to maximize its contact with the surface of the support, which results in loss conformation. As the lipase loading increased from 2.5 to 10 mg lipase/g support, less area became available for the lipase to spread itself and more of its active conformation was retained [29]. But, at loadings above 10 mg lipase/g support, a multilayer adsorption might occurred, possibly blocking or inhibiting substrate access to lipase active sites [30].

## 4.0 CONCLUSIONS

In this study, it has been demonstrated that free and immobilized *Candida rugosa* lipases were able to synthesize citronellyl butyrate by direct esterification in *n*-hexane as organic media. By investigating various parameters that affect lipase activity toward citronellyl butyrate synthesis, it has been shown that suitable conditions can be

selected to optimize its biocatalytic activity. Thus, studies with *Candida rugosa* lipase are of great interest since its potential usefulness as biocatalyst in the production of a number of commercially important flavor esters, especially those terpene alcohols of short-chain fatty acid.

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