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Synthesis of citronellol laurate in organic media catalyzed by immobilized lipases: kinetic studies

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Abstract

Terpene esters of fatty acid are essential oils that find several uses in food, cosmetic and pharmaceutical industries. The current work focuses on the synthesis of citronellol laurate from citronellol and lauric acid, including screening of various immobilized lipases and optimization of reaction conditions such as catalyst loading, speed of agitation, effect of mole ratio and temperature. Among different lipases, Novozym SP 435 was found to be the best catalyst with 53% conversion and 100% selectivity in 2 h at 30 °C. An ordered bi–bi mechanism with dead-end complex of lauric acid was found to represent the kinetic data.

Keywords: Terpenes; β-Citronellol; Lauric acid; Novozym SP 435; Immobilized lipase; Kinetics; Esterification; Non-aqueous enzymology

1. Introduction

Terpene esters of fatty acids find many applications in food, cosmetic and pharmaceutical industries as flavor and fragrance compounds [1–3]. Among the acyclic terpene alcohols, geraniol and citronellol are commercially the most important alcohols [4,5]. Traditionally, these esters are prepared by chemical synthesis and also by fermentation, which require a lot of purification steps and these are often expensive for commercial production [6–10]. Due to the overwhelming interest in natural products, biotechnology should be attractive to produce flavors of natural aroma from natural precursors [11,12]. Enzymes have been used mostly for aqueous phase reactions. However, non-aqueous enzymology has potential applications in fine chemicals, flavor and fragrance, pharmaceuticals and drug industry [13]. It is of particular relevance when reactants are poorly soluble in aqueous media and the hydrolysis reaction is to be suppressed. The use of immobilized enzymes, in particular lipases, in organic media rather than aqueous media has several advantages, such as the shift in thermodynamic equilibria in favor of the product over the hydrolysis reaction, increased solubility of nonpolar substrate, elimination of side reactions, ease of enzyme and product recovery, and increased enzyme thermostability [11,14]. Citronellol and lauric acid are both derived from natural resources and thus it was considered attractive to study the enzymatic synthesis of citronellol laurate in non-aqueous media.

Several mechanisms have been proposed to explain lipase catalyzed reactions; e.g., a sequence of various irreversible consecutive pseudo first orders reactions and ordered bi–bi mechanism. However, the generally accepted mechanism is the so called ping-pong bi–bi mechanism. In the ping-pong bi–bi mechanism, a product is released between additions of two substrates. There are good deal of publications on ordered bi–bi mechanism with a dead-end complex of the substrate in non-aqueous media [15] including fatty acid esterifications [16–18].

The kinetic model of Yadav and Lathi [19] for butyl isobutyrate with *n*-butanol considers ping-pong bi–bi mechanism with inhibition by *n*-butanol whereas that for the synthesis of perlauric acid is an ordered bi–bi mechanism [20]. On the contrary the kinetic model developed by Garcia et al. for the esterification of oleic acid with cetyl alcohol is based on ping-ping bi–bi mechanism with inhibition by both the reactants and both the products [16] whereas the model of Rizzi et al. shows inhibition due to both the substrates [21]. Arcos et al. have not considered any inhibition step in their model [18].

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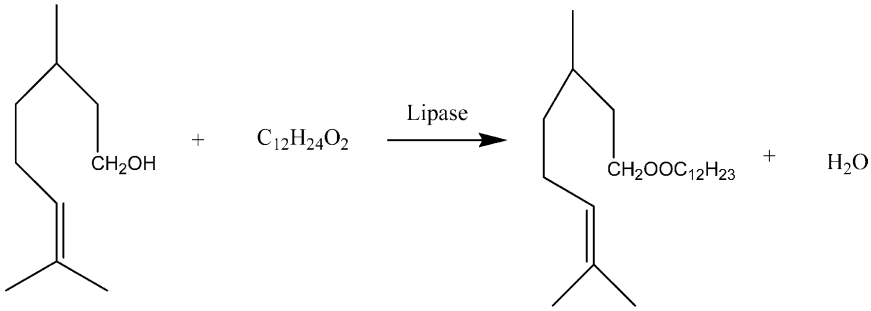
Although several publications have appeared on enzyme-catalyzed reactions in non-aqueous media, there is a dearth of quantitative information on kinetics and modeling of some of the industrially relevant reactions.

The current paper focuses on the synthesis of citronellol laurate from citronellol and lauric acid as the starting materials, using a variety of immobilized lipases and includes kinetics and mechanism.

2. Materials and methods

2.1. Enzymes

Some enzymes were procured as gift samples from reputed firms: Novozym SP 435, Lipozyme IM 20 and Lipozyme TL IM (all Novo Nordisk, Denmark). Novozym SP 435 Novozym 435 is the component B of the lipase from *Candida antarctica*, immobilized on a macroporous polyacrylic resin. Lipozyme IM 20 is *Mucor miehei* immobilized on an anionic resin. Lipozyme TL IM is *Thermomyces lanuginosus* immobilized on silica. *Thermomyces lanuginosus* is produced by genetically modified *Aspergillus oryzae*. Lipase 'Ak' (amano lipase) and Lipase 'PS' (amano lipase) were procured from Amano Pharmaceuticals, Japan.



Citronellol **Lauric acid** **Citronellol Laurate**

2.2. Chemicals

All chemicals were procured from firms of repute: Lauric acid and β-citronellol (98%) (E. Merck, Mumbai, India), heptane, toluene, benzene, 1,4-dioxane, methanol, and other analytical reagents (s.d. Fine Chemicals Pvt. Ltd., Mumbai, India).

2.3. Experimental setup

The experimental setup consisted of a 3 cm i.d. fully baffled mechanically agitated glass reactor of 50 ml capacity, equipped with four baffles and a six-bladed turbine impeller. The entire reactor assembly was immersed in a thermostatic water bath, which was maintained at a desired temperature with an accuracy of ±1 °C.

A typical reaction mixture consisted of 0.005 mol of β-citronellol and 0.005 mol lauric acid diluted to 15 ml with heptane as solvent. The reaction mixture was agitated at 30 °C for 15 min at a speed of 500 rpm and then 53 mg of immobilized enzyme was added to initiate the reaction. Liquid samples were withdrawn periodically from the reaction mixture and filtered to remove traces particles, if any. The filtrate was analyzed by titrimetry with sodium hydroxide using phenolphthalein as indicator. Product was confirmed by GC–MS analysis.

2.4. Analysis

Analysis of reaction mixture was carried out by titrimetry. A 0.2 ml sample was withdrawn from the reaction media at different time intervals, and it was made to 25 ml with methanol and then titrated with 0.025 mol of NaOH with phenolphthalein as indicator. The quantity of free acid was thus known at each time interval from which the fractional conversion was calculated.

3. Results and discussion

The effects of various parameters on conversion and rate of reaction were studied systematically.

3.1. Efficacy of various catalysts

The activities of Novozym SP 435, Lipozyme IM 20, Lipozyme TL, IM Lipase 'Ak' and Lipase 'PS' were evaluated under otherwise similar conditions. The conversion profiles are presented in Fig. 1. The initial activities of the enzymes are given in Table 1.

The conversion varied markedly with the type of lipase because different types of lipases exhibit different substrate specificity. Among these, Lipase 'AK' and Lipase 'PS' did not show any activity. The order of activity is as follows:

Novozym SP 435 > Lipozyme IM 20 > Lipozyme TL IM

Lipozyme TL IM is mainly intended for interesterification of bulk fats in the production of frying fats [22]. Novozym SP 435 and Lipozyme IM are effective. However,

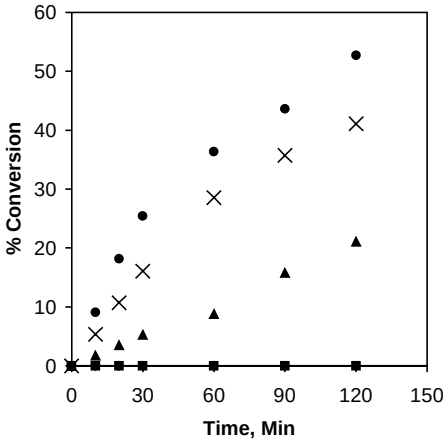


Fig. 1. Screening of different immobilized lipases. Citronellol: 0.005 mol; lauric acid: 0.005 mol; solvent hepatne up to 15 ml; speed of agitation: 500 rpm; catalyst loading: 3%; temperature: 30 °C. (■) Lipase PS; (◆) Lipase Ak; (▲) Lipo TLIM; (●) Novozym SP 435; (×) Lipozyme RM.

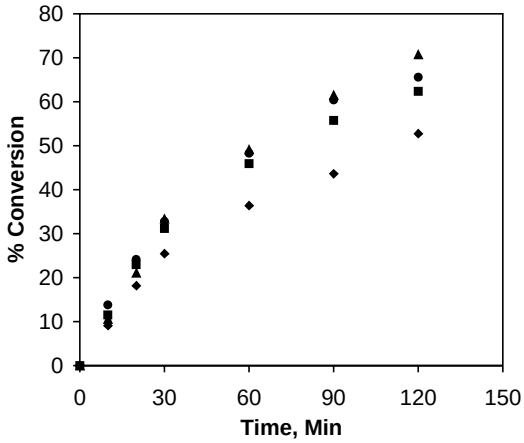


Fig. 2. Effect of speed of agitation on conversion. Citronellol: 0.005 mol; lauric acid: 0.005 mol; solvent hepatne up to 15 ml; catalyst loading: 3%; temperature: 30 °C. (◆) 300 rpm; (■) 500 rpm; (▲) 750 rpm; (●) 1000 rpm.

Novozym SP 435 is a thermostable lipase and mainly useful for the synthesis of esters and amides [23]. Being the most active, Novozym SP 435 was selected for all further experiments.

3.2. Effect of speed of agitation

Effect of speed of agitation was studied in the range of 300–1000 rpm (Fig. 2). The rate of reaction and overall conversion increased with increasing speeds. There was not much difference in the rate and overall final conversion at 500 and 700 rpm but at 1000 rpm, the overall conversion had decreased. It was observed that substantial catalyst particles were thrown outside the liquid phase at higher speed, sticking to the wall of the reactor, which would thereby reduce the effective catalyst loading. Further, it may also be due to the shearing of the enzyme molecule or inactivation of the enzyme due to foam formed at very high speeds.

The observed linear relationship between initial rate and enzyme concentration is consistent with a kinetically controlled enzymatic reaction. However, linearity alone does not rule out the possibility of mass transfer limitations on the rate. A reaction that is controlled by mass transfer would produce a linear dependence of rate upon substrate concentration. It is possible that the reaction is kinetically controlled at high substrate concentrations and mass transfer controlled at low substrate concentrations. To ascertain the role of mass transfer and intra-particle diffusion, it is useful to compare the time constants for reaction (t_r) and diffusion (t_d). These are defined as follows: $t_r = C_0/r(C_0)$ and $t_d = D_S/(k_{SL})^2$, where C_0 is the substrate concentration in bulk organic phase (mol cm^{-3}), $r(C_0)$ the rate of reaction (mol cm^{-3} s^{-1}), D_S the diffusivity of substrate in organic phase (cm^2 s^{-1}) and k_{SL} is the solid–liquid mass transfer coefficient in organic phase (cm s^{-1}). C_0 and $r(C_0)$ are determined experimentally. The mass transfer coefficient could be estimated from the Sherwood number and thus $k_{SL} = 2D_S/d_p$, where d_p is the diameter of the support particle. If $t_r \gg t_d$, it implies that the reaction is not influenced by mass transfer.

The diffusivity of the substrate in organic solvent could be estimated from the Siebel equation [24]. It was observed that when C_0 was 0.33×10^{-3} mol cm^{-3}, $r(C_0)$ was 5.66×10^{-8} mol cm^{-3} s^{-1}. Diffusivity of lauric acid in heptane was calculated as 2.094×10^{-5} cm^2 s^{-1}. The average diameter of the support particle was taken as 0.06 cm since the particle size ranged between 0.03 and 0.09 cm. The value of k_{SL} was calculated from the Sherwood number correlation as 6.98×10^{-4} cm s^{-1}. Thus, t_r and t_d were calculated as 5380 and 42 s, respectively, indicating $t_r \gg t_d$. Further, it is necessary to compare the rate of substrate diffusion per unit interfacial area ($k_{SL}C_0$) with the reaction rate per unit area $\phi r(C_0)/a$. ϕ is the phase volume ratio and a is the interfacial area per volume of organic phase. Since the interfacial area or the phase volume ratio are not known and since the particle is spherical, $\phi/a = R/3$, where R is the radius of the particle, which is 0.03 cm in this case. $k_{SL}C_0$ was found to be 2.303×10^{-7} mol cm^{-2} s^{-1} and $\phi r(C_0)/a$ was found to be 5.66×10^{-11} mol cm^{-2} s^{-1}. Since the rate of substrate diffusion per unit area is greater than the reaction rate per unit area, it is

Table 1
Activities of different enzyme

Sr. no.	Enzyme	Activity (μmol min^{-1} mg^{-1})
1	Novozym SP 435	52.83
2	Lipozyme IM 20	37.73
3	Lipozyme TL IM	9.44
4	Lipase PS	0
5	Lipase Ak	0

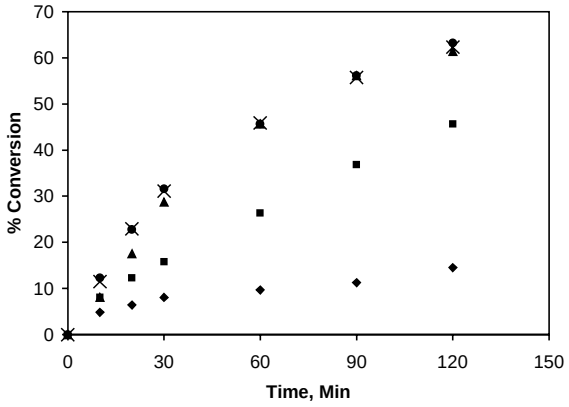


Fig. 3. Effect of type of solvent on conversion. Citronellol: 0.005 mol; lauric acid: 0.005 mol; catalyst: Novozym SP 435; speed of agitation: 500 rpm; catalyst loading: 3%; temperature: 30 °C. (◆) THF; (■) toluene; (▲) hexane; (●) nonane; (×) heptane.

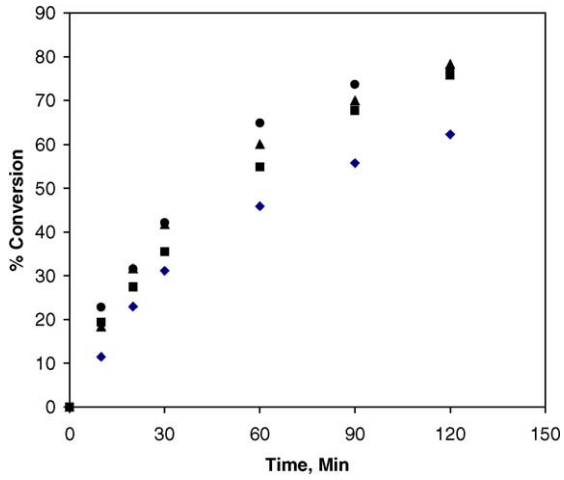


Fig. 4. Effect of temperature on conversion. Citronellol: 0.005 mol; lauric acid: 0.005 mol; catalyst: Novozym SP 435; solvent heptane up to 15 ml; speed of agitation: 500 rpm; catalyst loading: 3%. (◆) 30 °C; (■) 40 °C; (▲) 50 °C; (●) 60 °C.

obvious that mass transfer does not influence the reaction rate and it is solely controlled by intrinsic enzyme kinetics [24].

3.3. Effect of different solvents

The overall efficiency of the enzyme changes dramatically when the reaction medium is changed from water to an organic solvent. Choosing the appropriate reaction media for the enzymatic reaction is very important because many organic solvents are known to inactivate and denature them. A number of solvents such as tetrahydrofuran (THF), toluene, *n*-hexane, *n*-heptane, *n*-nonane were used (Fig. 3). The activity of Novozym SP 435 increased with $\log P$ value of the solvent. The conversion of citronellol was very low in THF (14%) which has the least $\log P$ of 0.49 among all solvents used in the study. In toluene ($\log P = 2.5$), the conversion was 45%. *n*-Hexane, *n*-heptane and *n*-nonane which have $\log P$ values of 3.5, 4.0 and 5.1, gave conversions of 61, 61.5 and 63%, respectively, which indicate that the chain length of the linear alkane has an insignificant effect. In general, enzymes are more stable when suspended in non-polar solvents that have low solubility for water than in polar solvents [18]. Solvents having $\log P > 4$ do not distort the essential water coat around the particle, thereby leaving the biocatalyst in an active state [25]. Thus, the results are in order. All further experiments were conducted in *n*-heptane.

3.4. Effect of temperature

The effect of temperature on the activity of Novozym SP 435 was monitored in the range of 30–60 °C (Fig. 4) and the final conversions after 2 h were 62, 75, 76 and 77% at 30, 40, 50 and 60 °C. With increasing temperature, the rate of reaction increased. It is well known that Novozym SP 435 is thermally stable at 60 °C and hence there was no deactivation of the enzyme at 60 °C.

3.5. Effect of concentration of lauric acid

The amount of lauric acid was varied in the range of 0.005–0.02 mol, holding constant the amounts of following components of the reaction mixture: citronellol (0.005 mol), Novozym SP 435 (53 mg), *n*-heptane (to make the volume to 15 ml).

Increasing the concentration of lauric acid decreased the rate of reaction and conversion. It could be due to the substrate getting strongly adsorbed on the enzyme active site or inhibition of enzyme due to excess substrate.

3.6. Effect of concentration of citronellol

The effect of moles of citronellol was studied in the range of 0.005–0.02 mol under otherwise similar conditions of the following: lauric acid (0.005 mol), Novozym SP 435 (53 mg), *n*-heptane (to make volume to 15 ml).

It was found that increasing the concentration of citronellol increased the reaction rate. The overall conversion increased with increasing citronellol moles from 0.005 to 0.02 mol, which is discussed with an appropriate model later.

3.7. Effect of catalysts loading

The effect of catalyst loading was studied from 18 to 176 mg under otherwise similar conditions. The rate of reaction increased with increasing catalyst loading and the overall conversion also increased from 43 to 83%. A linear relationship was obtained when the initial rates were plotted against catalyst loading, which indicated that the reaction was kinetically controlled (Fig. 5).

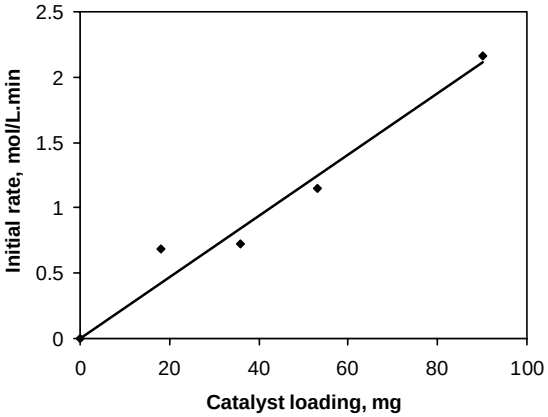


Fig. 5. Effect of catalyst loading on initial rate of reaction.

3.8. Effect of addition of water

The effect of water addition to the medium was studied from 10 to 100 μl under otherwise similar conditions. With increasing water concentration from 10 to 50 μl, the rate of reaction increased from 0.7169 to 0.7735 mol l^{-1} min^{-1} g^{-1} of enzyme and conversion increased from 62 to 64%. However, further increase in water concentration up to 100 μl resulted in decrease in the rate of reaction to 0.05283 mol l^{-1} min^{-1} g^{-1} of enzyme and conversion decreased to 53% (Fig. 6). Water plays a major role of "molecular lubricant" in enzyme resulting in conformational flexibility of enzyme and the increased hydration leads to enhanced activity in non-aqueous solvents. Water is thought to increase protein flexibility by forming multiple hydrogen bonds with enzyme molecule in organic solvents. There appears to be a critical amount of water necessary for enhancing activity. The addition of more water beyond the critical amount to the system probably increases the thickness of water layer around the catalyst particle to a point

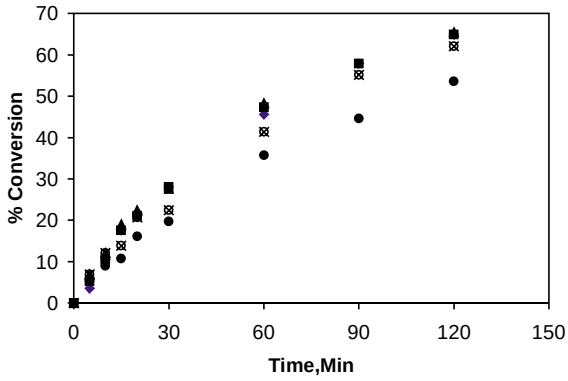


Fig. 6. Effect of addition of water on conversion. Citronellol: 0.005 mol; lauric acid: 0.005 mol; catalyst: Novozym SP 435; solvent heptane up to 15 ml; speed of agitation: 500 rpm; catalyst loading: 3%; temperature: 30 °C. (◆) No water; (■) 10 μl; (▲) 25 μl; (×) 50 μl; (○) 75 μl; (●) 100 μl.

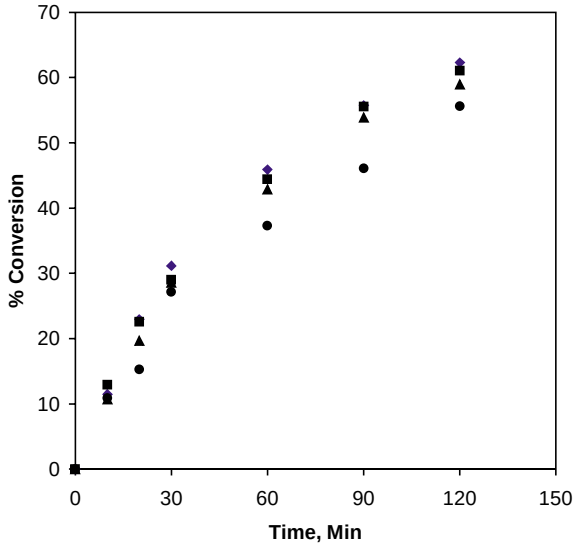


Fig. 7. Effect of catalyst reusability on conversion. Citronellol: 0.005 mol; lauric acid: 0.005 mol; catalyst: Novozym SP 435; solvent heptane up to 15 ml; speed of agitation: 500 rpm; catalyst loading: 3%; temperature: 30 °C. (◆) Fresh; (■) first use; (▲) second use; (●) third use.

where it presents diffusion problems. In thicker water layer, the enzyme becomes more flexible and interacts with the organic solvents, causing denaturation. Organic substrates and product with poor solubility in aqueous medium will diffuse with difficulty through the water layer to the active site of the enzyme.

3.9. Effect of reusability

The catalyst reusability studies were carried out to determine the enzyme stability during the reaction. After each run, the enzyme was filtered, washed with the solvent three to four times, dried at room temperature and reused as such. It was found that there was a marginal decrease in activity from 62 to 55% after three reuses, which might be due to loss of enzyme during filtration and drying since no make-up quantities were added (Fig. 7).

4. Kinetic model

It was observed that the initial reaction rate had increased as the concentration of β-citronellol (A) was increased. A further observation was that when lauric acid (B) concentration was increased, the effect of substrate inhibition by lauric acid was notable which caused the reaction rate to fall.

The Lineweaver–Burk double inversion plot (1/*r* versus 1/[A]) shows that the lines do not cross at the same point (Fig. 8). It suggests that both slope and intercepts are affected in a noncompetitive inhibition [26]. The plot also shows that as the concentration of lauric acid is increased, the slope increases and intercepts on 1/*r* axis de-

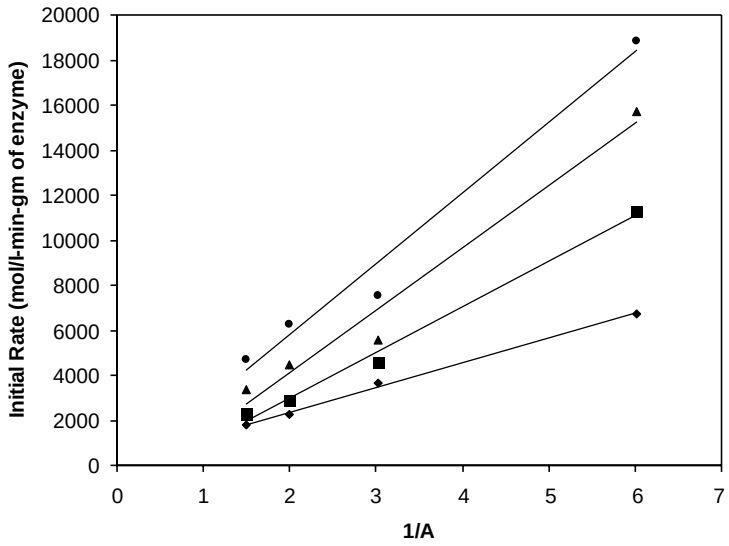
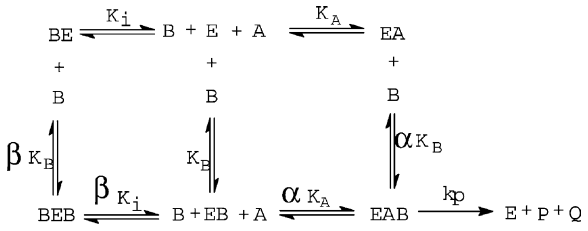


Fig. 8. Double inversion plots: 1/[initial rate] vs. 1/[A] for different concentrations of B. [B]—(◆) 0.166 mol; (■) 0.33 mol; (▲) 0.5 mol; (●) 0.66 mol.

crease. The inhibition is due to the formation of a dead end complex.

A typical reaction sequence is shown below. According to it, the lipase (E) may react with lauric acid [B] to yield a dead end complex (BE) or it may bind to A site to give AE. BE can bind with B to form another dead end complex BEB. Similarly AE can bind with B to form complex EAB which gives rise to either the product P and Q or a complex EB and A. Thus, EB can react with B again to give the dead end complex BEB [27].

The reaction sequence may thus be depicted as follows:



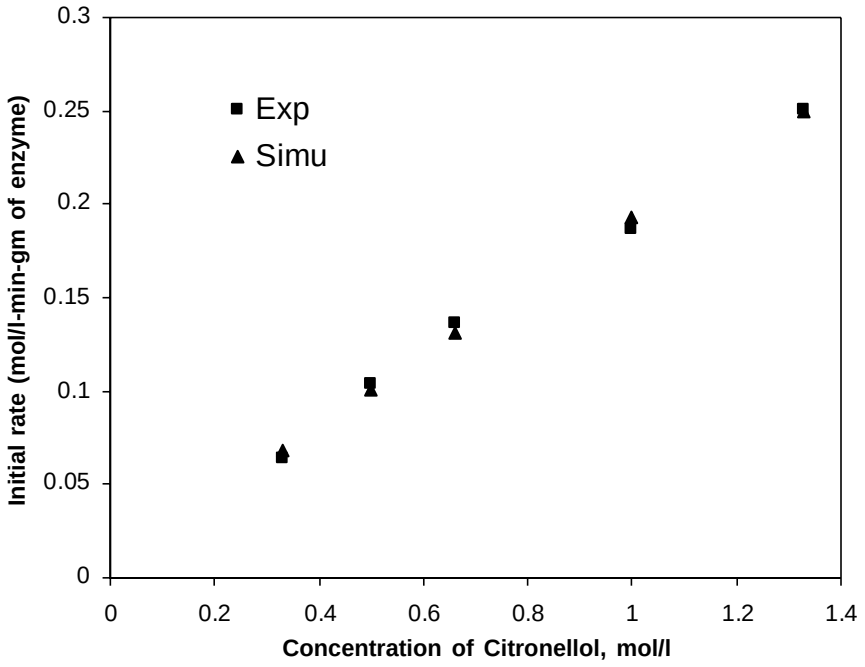


Fig. 9. Comparison of experimental and simulated rates as a function of citronellol concentration.

Table 2
Kinetic parameters

Parameter	Values
r_{max} (mol l^{-1} min^{-1} g^{-1} enzyme)	10.5531
K_i (mol l^{-1} g^{-1} enzyme)	1.6641
K_A (mol l^{-1} g^{-1} enzyme)	38.9845
K_B (mol l^{-1} g^{-1} enzyme)	25.3566

where, A is β-citronellol; B is lauric acid; EA is enzyme citronellol complex; BE is dead end enzyme lauric acid complex; BEB is dead end enzyme lauric acid complex; EB is effective enzyme lauric acid complex; EA is effective enzyme citronellol complex; EAB is effective enzyme citronellol lauric acid complex; P is citronellol laureate; and Q is water.

The final equation for the above reaction sequence is [27]

$$\frac{r}{r_{max}} = \frac{[A]}{\alpha K_A(1 + (K_B/[B]) + (K_B/K_i) + ([B]/\beta K_i)) + [A](1 + (\alpha K_B/[B]))} \quad (1)$$

where r is the initial rate of reaction (mol l^{-1} min^{-1} g^{-1} of enzyme); r_{max} is maximum rate of reaction (mol l^{-1} min^{-1} g^{-1} of enzyme); [A] is initial concentration of citronellol (mol l^{-1} g^{-1} of enzyme); [B] is initial concentration of lauric acid (mol l^{-1} g^{-1} of enzyme); K_B is Michaelis constant for lauric acid (mol l^{-1} g^{-1} of enzyme); K_A is Michaelis constant for citronellol (mol l^{-1} g^{-1} of enzyme); K_i is inhibition constant for lauric acid (mol l^{-1} g^{-1} of enzyme); α and β are 1 (dimensionless) [27].

The data from initial rate measurement were used for the optimization of parameters by least square error estimation using the software Polymath. A plot of simulated and experimental rate versus concentration of citronellol (A) to show that the experimental model fits the data very well (Fig. 9).

The values of the kinetic parameters obtained from non-linear regression analysis are given in Table 2.

5. Conclusions

Synthesis of citronellol laurate was conducted by employing different lipases, among which Novozym SP 435 was found to be the most active catalyst. The effects of various parameters on the conversion and rates of reaction were studied with Novozym SP 435 as catalyst and *n*-heptane as solvent. Initial rate and progress curve data were used to arrive at a suitable model and various parameters were estimated. The apparent fit of the kinetic data to the assumed ordered bi–bi dead end complex with lauric acid provides support for the mechanism. This model was used to simulate the rate data, which were in excellent agreement with experimental values.

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