Journal of Molecular Catalysis B: Enzymatic 83 (2012) 16–22

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Microwave assisted lipase catalyzed synthesis of isoamyl myristate in solvent-free system

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ARTICLE INFO

Article history:
Received 14 January 2012
Received in revised form 13 June 2012
Accepted 18 June 2012
Available online 28 June 2012

Keywords:
Enzyme catalysis
Solvent-free synthesis
Novozym 435, Ping-pong bi-bi mechanism
Substrate inhibition
Kinetics
Isoamyl myristate

ABSTRACT

Isoamyl myristate finds many applications in food, cosmetic and pharmaceutical industries as a flavor and fragrance compound. The current work focuses on solvent-free lipase catalyzed microwave assisted synthesis of isoamyl myristate from isoamyl alcohol and myristic acid. The activities of a number of commercially available lipases such as Novozym 435, Lipozyme RM IM and Lipozyme TL IM were evaluated, amongst which Novozym 435 was found to be the most active. Effect of solvent, agitation speed, type of enzyme, enzyme concentration, reactant concentrations and temperature was systematically studied on the esterification of isoamyl alcohol with myristic acid under microwave irradiation. Equimolar quantities of the reactants with 0.38% (w/v) Novozym 435 catalyst loading and agitation speed of 400 rpm leads to 96% conversion at 60 °C in 1 h. Based on the initial rates and concentration profiles, the reaction was found to obey the ping-pong bi-bi mechanism with inhibition by isoamyl alcohol and the kinetic parameters were evaluated by using non-linear regression analysis.

1. Introduction

Solvents are used rampantly in process industries mainly as vehicles for transporting solids, dissolution of immiscible reagents, creation of homogeneous phase to overcome mass transfer effects and enhancing rates of reactions. Solvents also help to dissipate heat of reaction, and are used as diluents to get better selectivities. However, they are a major source of volatile organic compounds (VOCs) and pollution. Solvent-free system offers many advantages. The absence of solvents facilitates downstream processing since fewer components are present in the reaction mass and the elimination of solvents from the production step makes the process cost effective and environmentally friendly [1–3]. Solvent-free energy efficient enzymatic synthesis of wax ester was scaled up by Petersson et al. [4] for industrial purpose. Lipases and supported lipase catalyzed reactions in non-aqueous media have been studied by different groups including ours for industrially significant esterifications [5–10], transesterifications [11,12], oxidations/epoxidations [13–15], optical resolution of pharmaceutical intermediates [16,17], hydrolysis [18], and other transformations [19,20].

Microwave assisted organic synthesis (MAOS) using both chemical and biocatalysts has gained importance in Green Chemistry [21–24]. Microwave irradiation leads to an instantaneous localized superheating due to dipole rotation or ionic conduction and the energy transfer occurs within 10^{-9} s with each cycle of electromagnetic energy and is faster than the rate at which the molecules can relax ($\sim 10^{-5}$ s) which results in non-equilibrium conditions and high instantaneous temperatures. An increase in temperature causes greater movement of molecules leading to a greater number of energetic collisions and hence enhancement in reaction rate and product yield [23,24].

We have reported a number of microwave assisted lipase-catalyzed reactions such as esterification [7,25], transesterification [7,26], epoxidation [14,27], hydrolysis [28] and N-acetylation [29] and found that the reaction rates had increased by 2.5–4.5 times under microwave irradiation. The mechanistic and kinetic aspects have also been discussed in detail in these publications amongst others.

Organic esters are employed as solvents, fragrance, flavors, and precursors in a variety of industries. Particularly, aliphatic esters are greatly used in flavor industry, mainly as fixatives and modifiers, whereas aromatic esters in fragrance compositions [30]. Isoamyl myristate is an important ester which is used as flavor and fragrance agent in a variety of food products. It has also been tested in vitro for antibacterial activity which is found to be comparable with the drug Ciprofloxacin [31].

The synthesis of isoamyl esters has been reported in the literature. However, majority of them have been directed more towards

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http://dx.doi.org/10.1016/j.molcatb.2012.06.011

low molecular weight fatty acid esters, such as acetate, propionate and butyrate. Solvent-free esterification reactions for the synthesis of isoamyl valerate, isoamyl isovalerate and amyl isobutyrate have been attempted earlier [32,33]. Synthesis of isoamyl myristate has only been reported by a reaction of myristic acid with isoamyl alcohol in the presence of sulfuric acid under reflux [31]. Thus, in the current work the esterification of isoamyl alcohol with myristic acid under microwave irradiation was investigated and the effects of various parameters such as solvent, agitation speed, type of enzyme and its concentration, reactant concentration and temperature were systematically studied to deduce mechanism and kinetics.

2. Materials and methods

2.1. Enzymes

Novozym 435 (component B of the lipase from *Candida antarctica* immobilized on macroporous polyacrylate resin), lipozyme RM IM (*Rhizomucor miehei* lipase) immobilized on anionic resin and lipozyme TL IM (*Thermomyces lanuginosus* lipase) were all procured as gift samples from NovoNordisk, Denmark. Olive oil assay and titrimetry methods were used to analyze immobilized enzyme [12]. With this assay method activity was found to be 6900 Units/g for the fresh enzyme which was similar to activity in the validation certificate provided by the manufacturer.

2.2. Chemicals

All chemicals were analytical grade reagents procured from firms of repute and used as such without further purification. Myristic acid, isoamyl alcohol, toluene, 1,4-dioxane, *n*-hexane, *n*-heptane, acetonitrile and other analytical reagents were obtained from S.D. Fine Chemicals Pvt. Ltd., Mumbai, India. GC–MS was also done to confirm their purity.

2.3. Experimental setup

2.3.1. Conventional reactor

The studies were carried out in a 3 cm i.d. mechanically agitated glass reactor of 50 mL capacity, equipped with four baffles and six-bladed turbine impeller. The entire reaction assembly was immersed in a thermostatic water bath. The reaction temperature was maintained with an accuracy of ±1 °C.

2.3.2. Microwave reactor

The microwave experiments were conducted in commercially available "Discover" system of CEM Corporation, USA (Model CEM-SP 1245), with proper temperature and pressure feedback systems for complete control of the reaction conditions. It is a mono-mode microwave system. The frequency of microwave generated in the magnetron is 2455 MHz. It automatically regulates the energy consumption to maintain constant temperature at the set temperature with accuracy of ±1 °C. Typically 50 W power input was given and it was continuous with automatic control. The reactor was a 120 mL capacity, 4.5 cm i.d. cylindrical glass vessel with provision for mechanical stirring. A standard six-blade-pitched turbine impeller of 1.5 cm diameter was used for agitation. The actual reactor volume exposed to the microwave irradiation was 45 mL with 5.5 cm height. The temperature in the reactor was digitally controlled with an accuracy of ±1 °C. The quantities of reactants and enzyme for the microwave reaction procedure were identical to those used for conventional heating.

2.3.3. Enzymatic reaction

The range of parameters was determined on the basis of the results obtained in preliminary experiments, considering the experimental set-up limits, and the working conditions limit for each enzyme. Thus, it was possible to fix the upper temperature level at 70 °C, in order to avoid the loss of enzymatic activity caused by temperature effect; there was no deactivation of the enzyme at 60 °C [9]. It has been reported that the external mass transfer resistance was absent at or beyond 400–500 rpm in earlier studies on enzyme catalysis [14–16] and optimum speed of agitation for microwave reactors has been in the range of 300–500 for lab reactors [25–29]; so a speed of 400 rpm was chosen during all experiments. Earlier studies of esterification reaction under microwave reaction have used equimolar quantities of substrates to avoid inhibition by one or both substrates or product(s).

A typical reaction mixture consisted of equimolar quantities of isoamyl alcohol and myristic acid (0.068 mol each) to make the total liquid volume of 25 mL. To make volume of 25 mL in solvent optimization studies, 0.0409 mol of both substrates and 10 mL of solvent were added. Then 0.38% (w/v) of immobilized enzyme was added to initiate the reaction. The reaction mixture was agitated at a speed of 400 rpm at 60 °C under microwave irradiation of 50 W. Samples were withdrawn periodically from the reaction mixture and analyzed by GC. The parameters were optimized by changing one parameter at a time while maintaining others constant. For all the parameter optimization studies, the total volume of the reaction mass was kept 25 mL to maintain uniform volume for all the experiments. This would give the same enzyme loading (mg/cm^3) and mixing conditions (same impeller height) in the microwave reactor.

2.4. Analysis

The concentrations of the reactants and products were determined on Chemito Gas chromatograph (model 8610) equipped with a flame ionization detector. A 2 m × 3.2 mm stainless steel column packed with SE-30 was used for analysis. After an initial hold of 30 s at 55 °C, the column temperature was increased to 235 °C at a ramp rate of 25 °C min^{-1}. Nitrogen was used as the carrier gas at a flow rate of 1 mL min^{-1}. Both injection and detection temperatures were set to 290 °C. Synthetic mixtures were prepared of pure samples, and calibration was done to quantify the collected data for conversions and rates of reactions. Purity of chemicals used and formation of isoamyl myristate was confirmed by GC–MS analysis (Clarus 500 GC/MS, PerkinElmer, USA).

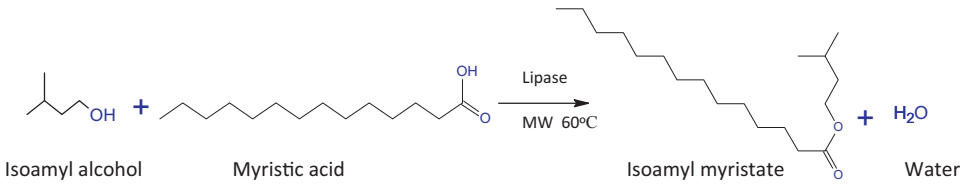
3. Results and discussion

The effects of various parameters on conversion and rates of reaction were studied systematically. The reaction is shown by Scheme 1.

3.1. Screening of different lipases

Immobilized enzymes have various advantages like easy recovery, reusability, and stability and thus can be designed in continuous operations on large scale. Three commercially available lipases, Novozym 435, Lipozyme TL IM, and Lipozyme RM IM, were evaluated to choose the most efficient enzyme for synthesis of isoamyl myristate. Novozym 435 is a versatile enzyme and has been found to be very effective even in solvent-free systems [34]. In the case of Novozym 435, the conversion was 97% compared to 47% with Lipozyme RM IM and 8.5% with Lipozyme TL IM in the first 60 min (data not shown).

Dimensional analysis of *Candida antarctica* lipase B (Novozym 435) based on the crystal structure clearly shows that amino acid



Scheme 1. Synthesis of isoamyl myristate in solvent-free system.

residue A281 is a part of an α-helix (α-10) located at the top of the substrate-binding pocket in a highly hydrophobic environment [35]. This micro-environment around the active site pocket of Novozym 435 favors proper interaction of substrate in non-aqueous media. The flapping lid of *Mucor miehei* (Lipozyme RM IM) and *Thermomyces lanuginosus* (Lipozyme TL IM) projects into the binding pocket of the enzyme [36] thereby creating steric hindrance in binding of myristic acid at the active site. Since Novozym 435 is known for its high activity even in solvent-free media and does not contain a flapping lid, there is less steric hindrance as compared to other lipases and it shows greater activity.

It has been shown that the activity of Novozym 435 was the best amongst all the commercial lipases in a wide range of 30–60 °C for conventional heating [7–10] and also under the influence of microwave irradiation [13]. Lipozyme TL IM is mainly intended for interesterification of bulk fats and production of frying fats [37]. Novozym 435 is a thermostable lipase and mainly useful for the synthesis of esters and amides [38]. The purpose of using these enzymes was to find out if significant activation could be achieved through microwave irradiation, despite their known use. Since Novozym 435 was found to be the best catalyst, it was used in all further studies.

3.2. Synergism of microwave with lipase catalyzed reactions

There is always a trace of residual water remaining in the immobilized enzyme beads, which is heated rapidly due to material–wave interactions [39], leading to thermal effects (connected to dipolar and charge space polarization) and purely non-thermal (accelerating the molecular rotation and electron spin oscillation of the polar parts of enzyme) effects and this leads to improved enzyme activity [40]. The thermal gradients and flow of heat are the reverse of those in materials heated by conventional means and this is mainly responsible for the increased activity and selectivity [41]. Thus, microwave irradiation acts synergistically with lipase catalysis. The reaction at 60 °C with Novozym 435 using conventional heating gave 56% conversion in 60 min while under the influence of microwave (energy input: 50 W) led to a conversion of 96% in 60 min. The initial rate was increased by 2.25 times under the influence of microwave irradiation in the first 15 min of the reaction. All further studies were carried out under microwave irradiation as considerable increase in total conversion was observed using microwave irradiation.

3.3. Effect of solvent

The influence of organic solvents on the enzymatic reactions has been summarized [41]. The enzyme activity is higher in the environment surrounding by non-polar ($\log P > 4$) and mid-polar solvents ($2 < \log P < 4$), whereas the lowest activity is expressed in polar solvents ($\log P < 2$). Solvents having $\log P$ greater than 4 do not distort the essential water coat around the particle, thereby leaving the biocatalyst in an active state. The highest microwave effect is expressed in the polar solvent instead of the non-polar solvents [42], although polar solvent is anticipated as more 'unfavorable' to enzymatic reactions under conventional heating. They deduce that the abnormal solvent effect may be related to the microwave characteristics of selective heating. It is then interesting to investigate how the reactions with and without solvents perform under microwave irradiation. In the present reaction when performed under solvent-free conditions, the micro-environment is surrounded by myristic acid which has a high log *P* value of 5.8 and thus preserves the essential water coat leaving the biocatalyst in an active state. Non-polar solvents (such as toluene, hexane, heptanes and 1,4-dioxane) possess very low dielectric constants, loss tangent ($\tan\delta$) values and dielectric loss values [41]. Non-polar solvents are thus viewed as transparent for microwave radiation, as there is a very weak solvent–microwave interaction leading to direct heating of the substrates [23]. Similar is the fact in the absence of solvents in solvent-free reactions. Microwave irradiation also exerts a stronger selective heating of reactant isoamyl alcohol due to its low log *P* of 1.03 by affecting dipole molecular rotation and ion induction of this polar reactant, thus the energy transfer is direct and fast to the reactant in this solvent-free reaction [42]. The current results showed that combined effects of both substrates in this solvent-free reaction were comparable to results obtained in reactions with non-polar solvents. Under the influence of microwave, the initial rates in this solvent-free reaction were superior to that of reactions in other organic solvents (Fig. 1).

3.4. Effect of speed of agitation

This is a solid–liquid system having myristic acid and isoamyl alcohol in the liquid phase and the immobilized enzyme (Novozym 435) as a solid phase. However, as the reaction proceeds, the water

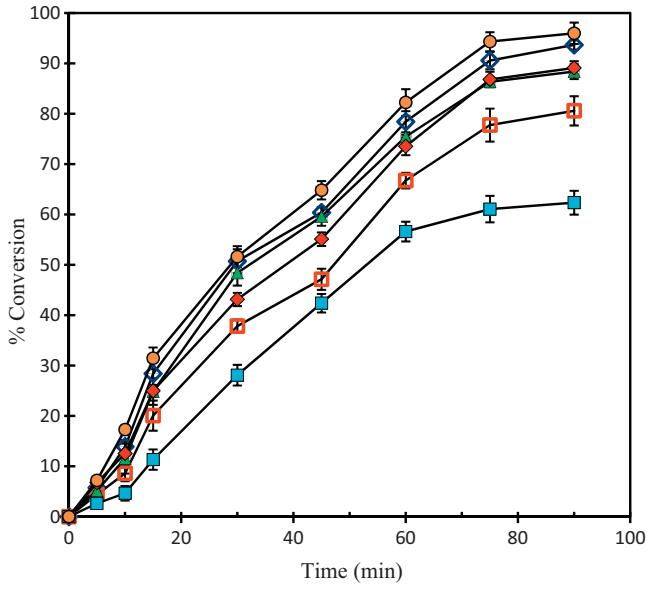


Fig. 1. Screening of different solvents. Reaction conditions – isoamyl alcohol: 0.0409 mol, myristic acid: 0.0409 mol; solvent up to 25 cm^3 (0.068 mol of both reactants for solvent-free reaction); catalyst: 0.38% (w/v); speed of agitation: 400 rpm; temperature: 60 °C; microwave irradiation: 50 W. (●) solvent-free; (◇) *n*-hexane; (▲) *n*-heptane; (◆) toluene; (□) 1,4-dioxane; (■) acetonitrile.

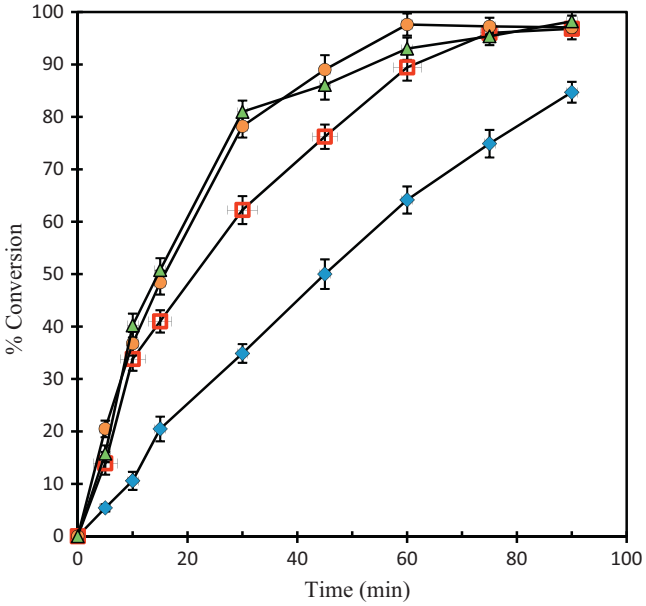


Fig. 2. Effect of catalyst loading. Reaction conditions – isoamyl alcohol: 0.068 mol, myristic acid: 0.068 mol; solvent-free, catalyst: 0.19–0.58% (w/v); speed of agitation: 400 rpm; temperature: 60 °C; microwave irradiation: 50 W. (◆) 0.19% (w/v); (□) 0.29% (w/v); (▲) 0.38% (w/v); (●) 0.58% (w/v).

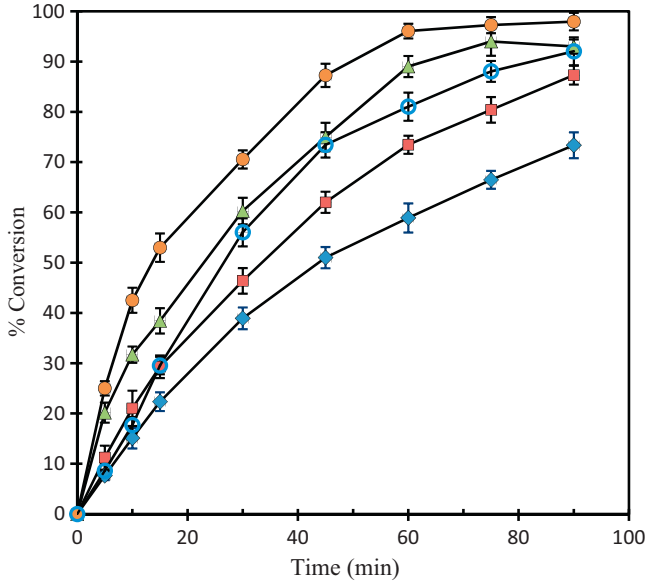


Fig. 3. Effect of temperature. Reaction conditions – isoamyl alcohol: 0.068 mol, myristic acid: 0.068 mol; solvent-free, catalyst: 0.38% (w/v); speed of agitation: 400 rpm; temperature: 30–60 °C; microwave irradiation: 50 W. (◆) 30 °C; (■) 40 °C; (▲) 50 °C; (●) 60 °C; (O) 70 °C.

generated in situ forms immiscible liquid phase and the original two-phase system is converted into a three-phase system. The pores in the enzyme are filled with aqueous phase, and a thin film of aqueous phase which surrounds the matrix should be retained for the enzyme to be active. The effect of external mass transfer resistance to the transfer of the reactants toward the outer surface of the solid particle was studied in the range of speed of agitation of 200–800 rpm. The initial rate of reaction increased with speed of agitation from 200 to 400 rpm and remained constant at higher speeds (data not shown). Indeed, the conversion after 30 min for 400 and 500 rpm remained practically constant at 95%. This indicated that there was no resistance to mass transfer of the reactants to the external surface of the catalyst particle [19]. At speeds beyond 600 rpm, there was a marginal decrease in conversion either due to attrition of enzyme beads or beads sticking to the wall of the reactor [19]. Thus, there was no resistance to external mass transfer. Therefore, further experiments were performed at a speed of 400 rpm.

3.5. Effect of catalysts loading

The effect of catalyst loading was studied in the range of 0.19–0.58% (w/v) Novozym 435, maintaining all other parameters the same. Both reaction rate and conversion increased with increasing catalyst loading. This indicated that the reaction was kinetically controlled. Increase of catalyst loading from 0.19 to 0.38% (w/v) increased the conversion by around 10%. However, further increase of catalyst loading to 0.58% (w/v) increased the conversion only marginally (Fig. 2). This would suggest that all available active sites of the enzyme are occupied by substrate molecules. Any further increase in enzyme loading will have no effect since no substrate molecules are available and these sites would be vacant [43]. However, beyond 0.38% (w/v) of catalyst loading, there was no substantial increase in final conversion. It appeared that mass transfer limitations were set in at higher loadings beyond 0.38% (w/v). Obviously, the lower the amount of enzyme, the more cost effective the process; thus, 0.38% (w/v) of Novozym 435 was used as optimum catalyst loading in further studies.

3.6. Effect of temperature

The reaction temperature is a crucial parameter in biocatalysis [44]; therefore, we selected four different temperatures in the range of 30–70 °C for the synthesis of isoamyl myristate. It was found that the overall conversion as well as rate of reaction was higher under microwave irradiation vis-à-vis conventional heating at the same temperature. Further, the rate enhancement is due to a combined effect of the microwave absorption properties of reaction mixture, due to their polar and ionic characteristics, as suggested by the "dipolar polarization mechanism" [45]. The enzyme is in the immobilized form, in the proximity of the substrates in solvent-free system and seems to behave slightly differently due to the non-thermal effects of microwave energy. It was clearly observed that initial rates and conversion increased with increase in the reaction temperature. However, a decrease in the conversion was observed with a further rise in the temperature from 60 °C (Fig. 3). This could be attributed to the deactivation of the lipase at high temperatures. Therefore, temperature of 60 °C seemed to be the optimum for the reaction system. Fig. 4 shows the Arrhenius plot. Activation energies calculated respectively as 4.72 and 4.95 kcal/mol for conventional and microwave heating were similar to those reported for most lipase catalyzed reactions [44]. Desnulle [46] has reported a value of 5.3 kcal/mol in an aqueous emulsion system. The lines are almost parallel in the Arrhenius plot (Fig. 4). This would suggest that there is no change in mechanism due to the mode of heating but the rate constant is augmented in microwave heating because of a greater collision frequency of reaction molecules.

3.7. Effect of mole ratio of isoamyl alcohol to myristic acid

The mole ratio of isoamyl alcohol to myristic acid was varied as 1:1, 2:1 and 3:1. Different concentrations of isoamyl alcohol were varied in the range of 2.7, 4.2, 5.1 mol/L, for the known concentration myristic acid of 2.7, 2.1, 1.7 mol/L calculated with respect to a constant total volume of 25 mL of the solvent-free system to achieve the above mentioned mole ratios. Fig. 5 shows that the maximum conversion (96%) and rate of reaction were obtained with a 1:1 mole ratio. The enzyme is immobilized within macroporous

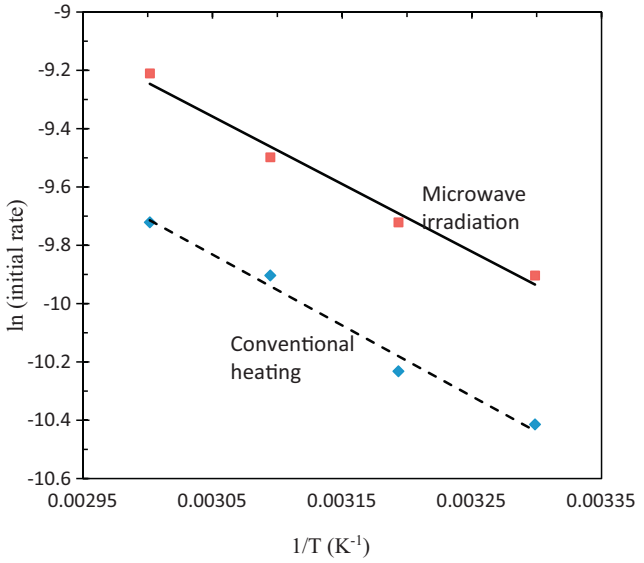


Fig. 4. Arrhenius plot for the synthesis of isoamyl myristate.

polyacrylic resin, which is hydrophilic; it may trap water within the support as well as it may form a film around the resin [37]. External mass transfer limitation due to water was eliminated by increasing the speeds of agitation [47]. The decrease in the conversion and rate of reaction with increasing concentration of isoamyl alcohol could be ascribed to the inhibitory effect of isoamyl alcohol on the Novozym 435. Isoamyl alcohol contains a hydrophobic hydrocarbon tail and a polar head of the alcohol functional group. Since the enzyme is hydrophobic, there may be hydrophobic–hydrophobic interaction between the enzyme and the hydrocarbon chain of isoamyl alcohol, and thus the dissociation of isoamyl alcohol from the enzyme is prolonged. Because of a close contact with the neighboring hydrophobic residues, the enzyme–isoamyl alcohol complex would have to be partially dehydrated, which may destabilize the native conformation of the enzyme; this leads to "molecular lubrication" [27]. Therefore, it may be concluded that

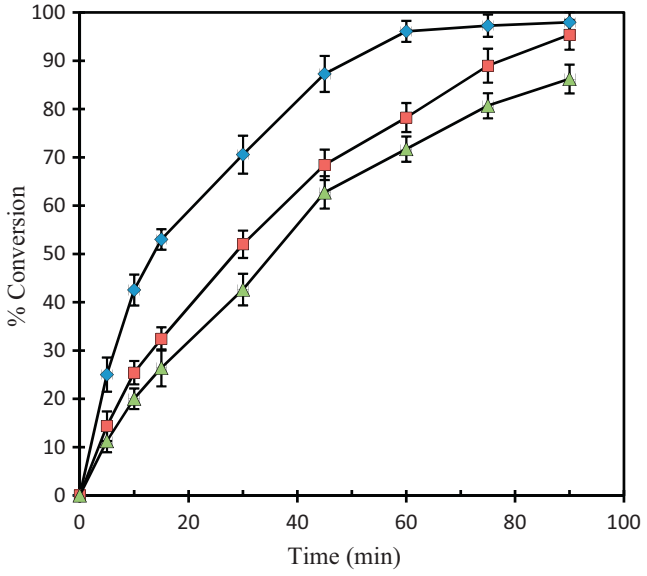


Fig. 5. Effect of isoamyl alcohol concentration. Reaction conditions – isoamyl alcohol: 2.7, 4.2, 5.1 mol/L for myristic acid concentration: 2.7, 2.1, 1.7 mol/L respectively; solvent-free, catalyst: 0.38% (w/v); speed of agitation: 400 rpm; temperature: 60 °C; microwave irradiation: 50 W. (◆) 1:1; (■) 2:1; (O) 3:1.

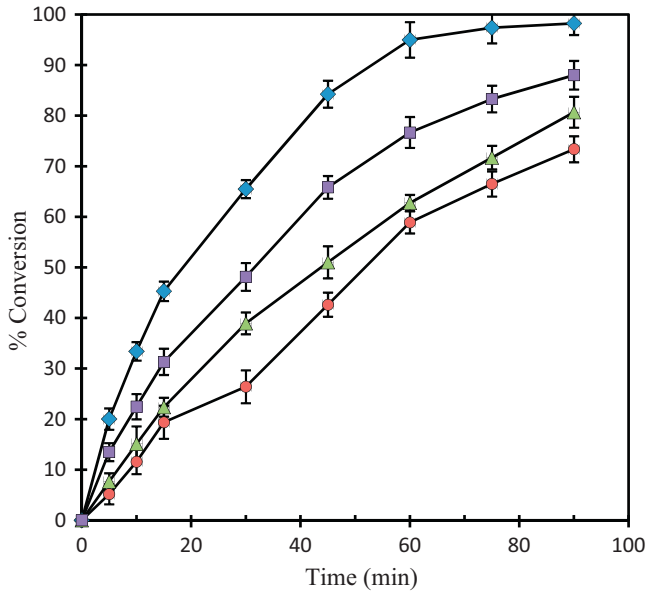


Fig. 6. Reusability of the catalyst. Reaction conditions – isoamyl alcohol: 0.068 mol, myristic acid: 0.068 mol; solvent-free, catalyst: 0.38% (w/v); speed of agitation: 400 rpm; temperature: 60 °C; microwave irradiation: 50 W. (◆) fresh; (O) 1st reuse; (▲) 2nd reuse; (●) 3rd reuse.

isoamyl alcohol at higher concentration interacts with the enzyme to form dead-end inhibitory complex.

3.8. Reusability of the catalyst

Novozym 435 reusability was studied out to ascertain its stability during the reaction. After each run, the enzyme was filtered, washed with *n*-hexane and the solvent was evaporated prior to next run. The dried enzyme was added with the makeup quantity to make the loading 0.38% (w/v) for the further reusability reaction. The activity was reduced after each reuse (Fig. 6).

During reusability study, the enzyme activity was measured after each recycle by olive oil assay [12]. Novozym 435 is *Candida antarctica* lipase immobilized on macroporous polyacrylate resin beads. With this assay method, the activity was found to be 6900 Units/g for the fresh enzyme. After third reuse the activity was reduced to 5400 Units/g, which is equivalent to 21.3% reduction. The probable reason for decrease in activity is a combined effect of different processes simultaneously at play including (i) denaturing of enzyme by the product, (ii) stripping of water from the microenvironment of enzyme particles, (iii) retention of some product blocking the active sites, (iv) onset of solid–liquid mass transfer resistance, and (v) onset of intra-particle diffusion resistance. The detail explanation is as follows.

This is a three phase reaction with organic phase containing reactants (continuous phase) and the product ester, polymer particle containing the enzyme (disperse phase), and water generated in situ as co-product (dispersed phase). The reactants diffuse inside the solid (polymer) matrix containing the enzyme and there is a counter diffusion of the product ester and the co-product water. Water is known to increase protein flexibility by forming multiple hydrogen bonds with the enzyme molecule in organic compounds [47] which has been cited in a number of papers on non-aqueous media reaction [7,27,48]. A critical amount of water is necessary for enzymes to be active in non-aqueous media as well as the outer water layer surrounding the support should not be stripped away by the solvent. Water is originally present in the pores of the support. Novozym 435 contains this critical amount of water. Additional water generated in the system is likely to affect the enzyme

activity. Concentrations of isoamyl alcohol as well as myristic acid are high in the solvent-free media in the current study (2.73 mol each), as compared to other enzymatic reactions in non-aqueous media (concentrations in mmol). Water generated in situ diffuses out of the pore matrix and through the water film surrounding the support (polymer) matrix, and then into the bulk organic phase, which would be saturated with water, and then it forms a separate phase. Similarly the product also diffuses out of the matrix in the same way but is dissolved in the organic phase which is a continuous phase. In this case, intra-particle water and product diffusion is a parallel process and their transfer from external surface through bulk organic phase is a series process. Besides, as the organic ester concentration increases, it is likely to strip off the water from the support. A number of non-polar solvents like hexane or toluene are found to be better than polar solvents for enzymatic reactions in non-aqueous media [9,49]. Generation of water beyond the critical amount in solvent-free system may not affect the ongoing reaction rate in a well agitated system. The denaturing of enzyme sites by high concentration of product is also a possibility. The removal of product during hexane washing would remove all products from active sites.

Once the enzyme is removed from the reaction mixture and is stationary, there is an organic layer surrounding the resin with less water layer. During subsequent runs, the enzyme is denatured due to inhibitory effect of isoamyl alcohol which is used in high concentrations as well as the water layer is also stripped off offering more resistance. Organic substrates with poor solubility in aqueous medium diffuse with difficulty through the intra-particle water layer to the active centers of the enzyme. Once the agitation is stopped and removed from microwave field for filtration and washed, it may not remove the intra-particle product totally thereby reducing the number of active sites.

3.9. Kinetic model

To analyze the reaction mechanism in detail as in other solvent-free systems, reactions were carried out under earlier optimized parameters, and 0.38% (w/v) Novozym 435 was used in each of the experiment. Hence the effects of concentration of both the reactants on the rate of reaction were studied systematically over a wide range. In one set of experiments, the mole ratio of myristic acid to isoamyl alcohol was increased from 1:1 to 2.5:1. In other set of experiments the mole ratio of isoamyl alcohol to myristic acid was increased from 1:1 to 2.5:1. That is – different concentrations of myristic acid were varied in the range of 0.8–2 mol/L, for known concentration of isoamyl alcohol (0.8–2 mol/L). Samples were taken from the reaction mixture periodically and analyzed by GC.

The Lineweaver–Burk plots of reciprocal rate versus reciprocal concentration of myristic acid are illustrated in Fig. 7. The Lineweaver–Burk plots demonstrate that the intercept decreases with increasing concentration of isoamyl alcohol, although the reverse is the case when the concentration of isoamyl alcohol is low, which implies that isoamyl alcohol acts as an inhibitor. Based on the kinetics study including effect of mol ratio and enzyme reusability, it was confirmed that the isoamyl alcohol caused deactivation of the enzyme. For the initial rate analysis, it is assumed that isoamyl alcohol acts as a dead-end inhibitor and the inhibition step is irreversible. The Lineweaver–Burk plot also demonstrates that the isoamyl alcohol inhibition is stronger at low concentrations of myristic acid.

A sequential mechanism can be ruled out as there is no common intersection for the family of lines in Fig. 7. The slope of the lines is not influenced by the concentration of the fixed substrate at low concentrations of isoamyl alcohol and myristic acid [50]. This is indicative of a mechanism that requires the dissociation

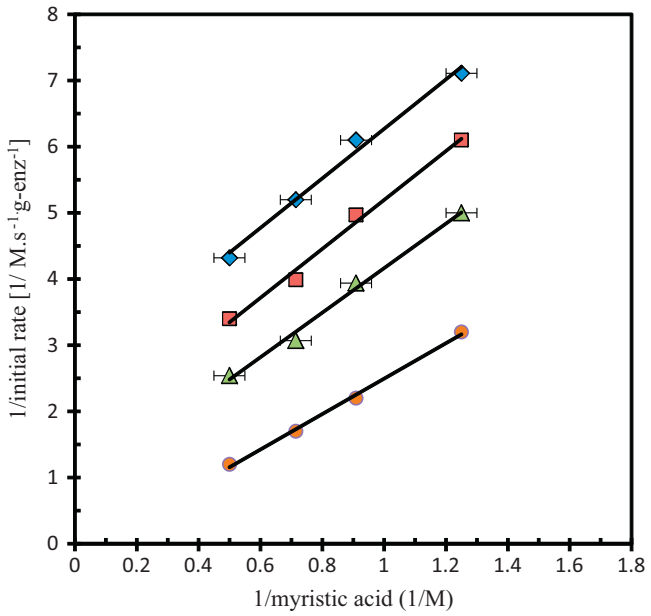


Fig. 7. Lineweaver–Burk double inversion plot for different concentrations of isoamyl alcohol at 60 °C. The initial rate of reaction is (M s^{-1} g-enz^{-1}) for a fixed catalyst loading of 0.38% (w/v). Concentrations of isoamyl alcohol: (◆) 0.8 mol/L; (■) 1.1 mol/L; (▲) 1.4 mol/L; (●) 2 mol/L.

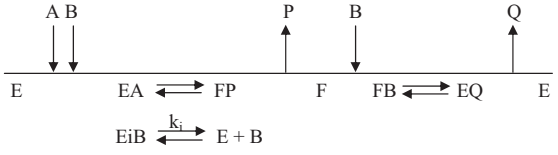
of one product before the second substrate can associate to the enzyme–substrate complex. The shape of the curve indicates substrate inhibition at high concentrations of isoamyl alcohol.

A mechanism in which a product is released between additions of two substrates is common in group transfer or "substituted enzyme" reactions. The mechanism is called "ping-pong bi-bi" mechanism as each substrate addition is followed by a product release. From these observations, a ping-pong bi-bi mechanism with alcohol inhibition is postulated. These assumptions are used to design a reaction mechanism that is depicted in Cleland's notation (Scheme 2).

By analogy to the classical mechanism of esterification by lipases, it is assumed that myristic acid (A) binds first to the free enzyme (E) and forms a noncovalent enzyme-acid complex (EA), which releases the first product, water (P) and F modified enzyme. The second substrate, isoamyl alcohol (B), reacts with F to give the complex FB and gives the product isoamyl myristate (Q) and free enzyme (E). Along with this, B also forms the dead-end complex [EiB] by binding to free enzyme [E]. The rate equation for the ping-pong bi-bi mechanism is as follows [50]:

$$\nu = \frac{\nu_m[A][B]}{K_{mB}[A] + K_{mA}[B](1 + [B]/K_i) + [A][B]} \quad (1)$$

where ν is the initial rate of reaction, ν_m is maximum rate of reaction, [A] is initial concentration of myristic acid, [B] is initial concentration of isoamyl alcohol, K_{mA} is the Michaelis constant for myristic acid, K_{mB} is the Michaelis constant for isoamyl alcohol and K_i is the inhibition constant due to isoamyl alcohol.



(1)

Scheme 2. Cleland's notation for a ping-pong bi-bi mechanism with single substrate inhibition.

Table 1
Values of the kinetic parameter for ping-pong bi-bi mechanism with isoamyl alcohol inhibition.

Kinetic parameter	Value
Apparent v_m (mol L^{-1} min^{-1})	7.05
Apparent K_{mA} (mol/L)	1.45
Apparent K_{mB} (mol/L)	1.07
Apparent K_i (mol/L)	1.23

The initial rates were calculated from the linear portion of the concentration–time profiles. In Fig. 7, the Lineweaver–Burk double inversion was examined. One intersection at *X*-axis (inverse of rate) for isoamyl alcohol of 2 mol/L is marginally negative. It has been reported [51–53] that Lineweaver–Burk double inversion plot has not been very accurate in the estimation of kinetic parameters and in some cases negative values are obtained, which is clearly meaningless [52,53]. To verify the application of ping-pong bi-bi mechanism the data was analyzed by non-linear regression using the Software Package Polymath 5.1 [9,25,53]. The apparent kinetic parameters determined by Polymath 5.1 are given in Table 1. These parameters were utilized to simulate the initial rates. A plot of simulated versus experimental data is made at various concentrations of A to show that the experimental model fits the data very well (data not shown). This demonstrates that the proposed model is valid.

4. Conclusions

Isoamyl myristate was successfully synthesized by solvent-free esterification reaction of isoamyl alcohol and myristic acid, catalyzed by an immobilized lipase, under microwave irradiation. Novozym 435 was found to be the best catalyst and microwave irradiation reduced the reaction time. There was a synergism between enzyme catalysis and microwave irradiation. Isoamyl alcohol at high concentrations acted as an inhibitor. A kinetic model was proposed by collecting both initial rate data as well as concentration–time profiles for the reaction. A ping-pong bi-bi mechanism was found to fit the data well for enzyme catalysis under microwave irradiation in this solvent-free system.

Acknowledgments

G.D.Y. acknowledges support for the personal chair from R.T. Mody distinguished Professor Endowment and J.C. Bose National fellowship from DST-GOI. P.A.T. acknowledges University Grants Commission for awarding junior Research fellowship under its SAP program. The authors thank Novo Nordisk, Denmark, for the gifts of enzymes.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2012.06.011.

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