Journal of Molecular Catalysis B: Enzymatic 99 (2014) 143–149

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Synthesis of isobutyl propionate using immobilized lipase in a solvent free system: Optimization and kinetic studies

Vishakha V. Kuperkar, Vikesh G. Lade, Arushi Prakash, Virendra K. Rathod *

Department of Chemical Engineering, Institute of Chemical Technology, Matunga (E), Mumbai 400019, India

ARTICLE INFO

Article history:
Received 23 July 2013
Received in revised form 30 October 2013
Accepted 30 October 2013
Available online 8 November 2013

Keywords:
Isobutyl propionate
Novozym® 435
Solvent free system (SFS)
Kinetic models
Ping-Pong Bi–Bi mechanism

ABSTRACT

Isobutyl propionate is widely used in food and beverage industries as a rum flavor. This work presents the optimization and kinetic aspects of synthesis of isobutyl propionate by esterification of propionic acid with isobutyl alcohol using immobilized lipase Novozym® 435 in a solvent free system (SFS). Process parameters such as reaction time, temperature, enzyme loading, speed of agitation, water concentration and acid to alcohol molar ratio were optimized to achieve maximum conversion. Higher conversion of 92.52% was obtained with the reaction conditions such as: temperature 40 °C, enzyme loading 5% w/w, acid to alcohol molar ratio 1:3, time 10 h and stirring speed of 300 rpm. The bisubstrate kinetic models of the enzyme catalyzed reactions namely Ordered Bi–Bi, Random Bi–Bi and Ping-Pong Bi–Bi were applied to determine the initial rates and correlated with the experimental findings. Ping-Pong Bi–Bi model with substrate inhibition by both acid and alcohol gives the best fit with parameter values as V_{max} = 0.5 Mol/min/g catalyst, K_A = 0.631 M, K_B = 0.003 M, K_{iA} = 0.0042 M and K_{iB} = 0.1539 M for the concentration ranges of 2.25–10.21 M for propionic acid and 2.55–9.01 M for iso-butanol. The immobilized lipase could be reused for seven times with the % conversion of acid reaching to 83%; signifies that still it can be reused for several more times. SFS is the added benefit to produce such commercially valuable flavor ester.

1. Introduction

The esterification is a reaction wherein alcohol reacts with acid in presence of catalyst to produce ester with elimination of water. Short-chain esters are the class of compounds that are widely distributed in nature and are major components of cosmetics, food flavor, fragrance, pharmaceutical industries due to its natural aroma. Currently commercial market for food flavor is increasing rapidly. Esters obtained by chemical synthesis suffer from drawbacks like high temperature and pressure, harsh reaction conditions involving strong acid catalyst, hazardous chemicals, longer reaction time and low conversion [1]. Other flaws associated are tedious separation processes, toxicity, and unwanted harmful byproducts [2]. Natural flavors extracted from fruits or plants are too expensive and also incapable of fulfilling growing commercial demands [3]. Therefore it is industrially and economically important to synthesize flavors using cheaper and more broadly available material to meet the consumer demand exploring the alternative methods of the production [1,4]. Use of biocatalyst for the production of flavor is green technology based approach and natural, unlike using chemical catalysts. Application of enzymes as a biocatalyst is the most frequently used technique of biosynthesis to produce the flavors with high specificity, mild reaction conditions and greater efficiency [5]. Lipases (triacylglycerol ester hydrolysis EC 3.1.1.3) are enzymes that can catalyze esterification, transesterification, and hydrolysis reactions [6].

Lipases are important class of enzymes because of their properties like regiospecificity, stereospecificity and substrate specificity [7] and their milder reaction conditions that reduce the energy requirements [8]. Recently, lipases immobilized on various supports like macroporous acrylic resin [9] polyacryclic resin, polyurethane foams [5], Amberlite and Celite [10] and cotton cloth [11] have come up for the industrial production of various specialty esters, aroma compounds and active agents. These immobilized techniques have provided more chances to use biocatalysts for various reactions in wider range of operating conditions in terms of pH, temperature and pressure [12,13]. Novozym® 435 is commercial immobilized lipase preparation supplied by Novozymes, immobilized on macroporous polyacrylic resin beads. It has applications ranging from biodiesel production to fine chemistry [14].

The variant shift has occurred in the lipase catalyzed reactions based on the operating media viz. aqueous → biphasic → non-aqueous media (organic). Since, major emphasis was given to organic solvents to produce short-chain fatty acids ester in middle

* Corresponding author. Tel.: +91 22 33612020; fax: +91 22 33611020.
E-mail address: vk.rathod@ictmumbai.edu.in (V.K. Rathod).

1381-1177/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.molcatb.2013.10.024

decades; lot of literature exists with combinations of substrates with experimental and/or statistical determination of optimum reaction conditions for maximum yield in shortest duration [3,14–18]. However the use of toxic organic solvents is being progressively restricted for many applications due to industrial and social implications. Recently the major shift has occurred in the production of the esters where in the reactions are preferred in the solvent free system; as it facilitates the downstream processing thus reduction in cost and environmental hazards [2]. There are few research studies concerning solvent free system for lipase catalyzed production of flavor ester and it is found that the initial rates are found to increase as compared to organic solvent [18,19].

The kinetic studies can provide better insight of the reaction mechanisms of enzyme catalyzed reactions. The kinetics of the specific reaction can follow the specific kinetic model based on its reaction mechanism. The kinetics information (operating conditions and rate parameters) of the esterification reaction is useful for the designing and scale-up of the reactor. However, kinetic studies of lipase-catalyzed esterification in organic solvents or SFS are remarkably rare; most of these are based on the Michaelis–Menten assumptions [5]. By virtue of the importance of kinetic models, the proper assessment of the dynamics of lipase-catalyzed esterification reactions has been done using several models for different combinations of substrates and enzymes over the years. Most lipases are said to follow the Ping-Pong Bi–Bi mechanism [11,20–22] although Ordered reaction mechanism [23] and Random mechanism [24] have also been reported in the literature.

Isobutyl propionate is an organic ester having an ethereal, rum-like, fruity odor and therefore it is used as rum flavor to beverages, candies, and baked goods [25]. Thus, this ester flavor has a high commercial demand and it is less reported in literature. Therefore, the objective of the present research work is synthesis of isobutyl propionate in SFS using immobilized lipase as biocatalyst. The optimization of process parameters was carried out based on the investigations relating to the influence of reaction temperature, enzyme load, speed of agitation, water concentration and substrate ratio. Three kinetic mechanisms namely Ordered Bi–Bi mechanism, Random Bi–Bi mechanism and Ping-Pong Bi–Bi mechanism were tested for the validation of the experimental data.

2. Materials and methods

2.1. Materials

Novozym® 435 (lipase B from Candida antarctica; immobilized on macroporous polyacrylic resin beads, bead size 0.3–0.9 mm, bulk density 0.430 g/cm^3) was generously gifted by Zytex Biotech Pvt. Ltd., Mumbai (India). Isobutyl alcohol [*B*] and Propionic acid [*A*] used were A.R. grade (with 99% purity) and were supplied by HiMedia Laboratories Private Limited, Mumbai and Thomas Baker (Chemicals) Pvt. Ltd., Mumbai, respectively.

2.2. Experimental method

The experimental set up consisted of 4.5 cm i.d. three necked baffled glass reactor of 50 ml capacity; provided with six-bladed turbine impeller. The entire assembly was immersed in a thermostatic water bath, which was maintained at the desired temperature with an accuracy of ±2 °C. Electric motor with speed controller was provided for agitation. The experiment was performed as: 0.1 mol of each reactant was added to the reactor and mixture was agitated at 200 rpm for 5 min and then 5% w/w enzyme was added to initiate the reaction. The molar concentration of [*A*] and [*B*] for SFS can be expressed in volume as shown in Figs. 1–4. This is because pure [*A*] having 13.36 M and pure [*B*] having 10.83 M were used directly without any solvent. If equimolar (5.98 Mol) mixture of both reactants [*A*] and [*B*] solution has to be made, the reaction mixture should contain [*A*] 7.5 cm^3 and [*B*] 9.3 cm^3. Accordingly all reaction mixtures were made based on predefined molar ratio. Liquid samples free from catalyst particles were withdrawn periodically and further analyzed to determine the extent of reaction. The procedure was repeated based on criterion for the optimization of reaction parameter (reaction time, temperature, enzyme loading, speed of agitation, concentration of water and molar ratio).

2.3. Analytical method

2.3.1. Identification of reaction product

Identification of synthesized isobutyl propionate in liquid samples was carried out by GC (CHEMITO 8610) equipped with flame ionization detector using 3 m × 0.32 mm I.D. stainless steel column packed with 10% OV-17 stationary phase. Nitrogen was used as carrier gas at pressure 0.8 bar. The temperature program was as follow: 60 °C for 1 min; 5 °C/min up to 100 °C; then steady temperature for 1 min. The injector and detector temperatures were both kept at 150 °C. Injection volume of 2 μl was used. After primary identification on GC, titrimetric analysis (method explained below) was used for routine measurements based on the comparison of both analytical methods which gave about ±3% deviation.

2.3.2. Titrimetric analysis

The isobutyl propionate obtained was expressed in terms of percent (%) conversion i.e. percent of propionic acid converted with respect to the total acid in the reaction mixture by titrating reaction mixture with 0.1N NaOH using phenolphthalein indicator and methanol as a quenching agent.

2.3.3. Determination of initial rates of reaction

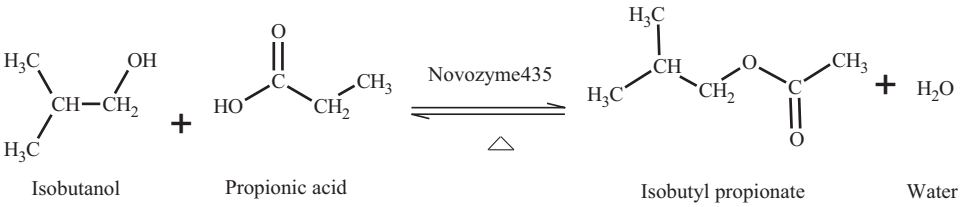
Initial rates of esterification were determined at various reaction conditions depending on the molar ratio. The molar ratio of acid to alcohol was varied from 4:1 to 1:4 in integral successions. The temperature was maintained at 40 °C with 5% (w/w) enzyme, Novozym® 435, loading. Reactions were carried out for 1 h. Aliquots of the reaction mixture were taken every 15 min and analyzed by titrimetric analysis as discussed above. Conversion data for <10% conversion was used to determine initial reaction rates by plotting conversion-time profiles.

2.4. Kinetics and mechanisms of the esterification reaction

Two substrates i.e. propionic acid [*A*] and isobutyl alcohol [*B*] are bound to the immobilized lipase Novozym® 435 [*E*] in either a specific or random order to form an [AEB] complex, which then reacts to form the products viz. isobutyl propionate [*P*] and water [*Q*]. The reaction scheme for the synthesis of isobutyl propionate in a solvent free system (SFS) can be shown as follows:

Reaction Scheme 1. Synthesis of isobutyl propionate by esterification of propionic acid [*A*] with isobutyl alcohol [*B*] using immobilized lipase Novozym® 435 in SFS.

Based on the experimental data the initial rates of esterification were determined. These initial reaction velocities were then used for identification of maximum velocity and, Michaelis–Menten, inhibition and dissociation constants using three different bisubstrate kinetic models of the enzyme catalyzed reactions viz. Ordered Bi–Bi, Random Bi–Bi and Ping-Pong Bi–Bi. These three generalized two-substrate two-product i.e. Bi–Bi reactions models were selected as they take into account that the product formation occurs only after the formation of an enzyme–two substrate complex [26].



Scheme 1.

2.4.1. Ordered Bi–Bi mechanism

An Ordered Bi–Bi mechanism is very common with bisubstrate enzymes. This is a ternary-complex mechanism with a strict order of attachment of substrate to enzyme [9,27]. In this mechanism, an enzyme reacts with two substrates in an ordered way affording two products, which are also released in an ordered fashion. If the substrate [*B*] has affinity attaches to the free enzyme and not the enzyme–substrate complex [*E.A*], it forms [*E.B*] (a dead-end complex) by competitively attaching to the active site. This is known as competitive inhibition by substrates. The reaction mechanism is given below

$$E + A \overset{K_{iA}}{\Leftrightarrow} E.A + B \overset{K_B}{\Leftrightarrow} E.A.B \overset{K_{PQ}}{\Leftrightarrow} E.P.Q \overset{K_P}{\Leftrightarrow} E.Q + P \overset{K_Q}{\Leftrightarrow} E + P + Q \quad (1)$$

$$E + B \overset{K_i}{\Leftrightarrow} E.B \quad (2)$$

For an Ordered Bi–Bi system, where the two substrates lead to two products, the initial forward velocity in the absence of products is given by the equation:

$$v_0 = \frac{V_{\max[A][B]}}{[A][B] + K_B[A] + K_A[B](1 + [B]/K_{iA}) + K_{iA}K_B(1 + [B]/K_i)} \quad (3)$$

2.4.2. Random Bi–Bi mechanism

When substrates attach to the enzyme in random order and give products, a random bi–bi mechanism is followed. The attachment of one substrate does alter the affinity of the other substrate towards the enzyme. A rapid-equilibrium random ordered reaction mechanism is outlined below:

$$E + A \overset{K_{iA}}{\Leftrightarrow} E.A + B \overset{K_B}{\Leftrightarrow} E.A.B \overset{k_{cat}}{\Leftrightarrow} E + P + Q \quad (4)$$

$$E + B \overset{K_{iB}}{\Leftrightarrow} E.B + A \overset{K_A}{\Leftrightarrow} E.A.B \overset{k_{cat}}{\Leftrightarrow} E + P + Q \quad (5)$$

The final equation for the initial rate is given by Eq. (6)

$$v_0 = \frac{V_{\max[A][B]}}{[A][B] + K_B[A] + K_A[B]K_{iA}K_B} \quad (6)$$

2.4.3. Ping-Pong Bi–Bi mechanism

The Ping-Pong Bi–Bi model describes a specialized Bi–Bi mechanism in which the binding of substrates and release of products is ordered. In Ping-Pong mechanism, at any point of time only one substrate [*A*] is bound to the enzyme and acyl–enzyme complex [*E.A*] is formed. Then the product [*P*] is detached and formed as it is fragment of the original substrate [*A*]. The rest of the substrate is covalently attached to the enzyme *E*, which is designated as [*F*]. The second substrate [*B*] binds and reacts with the enzyme to form a covalent adduct with the covalent fragment of [*A*] still attached to the enzyme to form product [*Q*] which is released and the enzyme is restored to its initial form [*E*]. A ping-pong mechanism with inhibition by both the substrates is given below

$$E + A \overset{K_A}{\Leftrightarrow} E.A \overset{K_{EA}}{\Leftrightarrow} F.P \overset{K_P}{\Leftrightarrow} F.B \overset{K_B}{\Leftrightarrow} F.B \overset{K_{EB}}{\Leftrightarrow} E.Q \overset{K_Q}{\Leftrightarrow} E + Q \quad (7)$$

According to Ping-Pong Bi–Bi with inhibition by both the substrates mechanism:

$$v_0 = \frac{V_{\max[A][B]}}{[A][B] + K_B[A] + (1 + [A]/K_{iA}) + K_A[B](1 + [B]/K_{iB})} \quad (8)$$

where v_0 is the initial rate of reaction; V_{max} is the maximum velocity; [*A*] and [*B*] are the initial concentrations of the two substrates; K_A is Michaelis–Menten constant for substrate [*A*]; K_B is Michaelis–Menten constant for substrate [*B*], K_{iA} is the inhibition constant for [*A*] and K_{iB} is the inhibition constant for [*B*].

3. Results and discussion

3.1. Optimization of reaction variables

The optimization of the reaction condition such as reaction time, temperature, enzyme loading, alcohol to acid molar ratio, speed of agitation was undertaken. Moreover the reusability of the enzyme was tested for evaluation of economic feasibility of the process. Each of the reaction parameter has been discussed in detail in the following sections.

3.1.1. Effect of temperature

For preliminary analysis, the effect of reaction time on progress of reaction is carried out as it is an important parameter helps to determine optimum time to achieve maximum yield of product. Therefore, the effect of reaction time on lipase catalyzed esterification for isobutyl propionate was carried out in the experimental glass reactor which was placed in a thermostatic water bath with temperature of 40 °C, stirring speed at 200 rpm, enzyme loading 5% w/w and reactant molar ratio 1:1. It was observed that the percentage conversion increased with time and % conversion of 82% was obtained in initial 8 h. However, a marginal change in the conversion was observed after 8 h. This was taken the basis and further experiments were carried out till 10 h.

The enzyme activity and stability are the most important factors to be considered while dealing with the enzymatic reactions. However these factors are the function of the reaction temperature. Generally, an increase in the reaction temperature increases the reaction rate but the stability of the enzyme may decline and even enzyme may be denatured. Therefore, the effect of reaction temperature on the formation of isobutyl propionate was studied at different temperatures ranging from 30 to 60 °C, keeping other parameters constant i.e. stirring speed at 200 rpm, enzyme loading 5% (w/w), substrate molar ratio 1:1. Fig. 1B depicts that the initial reaction rate increases from 0.0028 to 0.0594 Mol/min/g enzyme as temperature increased from 30 to 50 °C. However, % conversion increased from 66 to 82% with an increase in temperature from 30 to 40 °C but at 50 °C final conversion was reduced to 75% as shown in Fig. 1A. This is attributed to thermal denaturation of enzyme at higher temperature and hence decline in conversion with time [6]. An increase in % conversion as well as initial rate with increase in temperature is due to fact that the temperature may reduce mixture viscosity, enhance mutual solubility, improve diffusion process of substrates, and enhance the interactions between catalytic

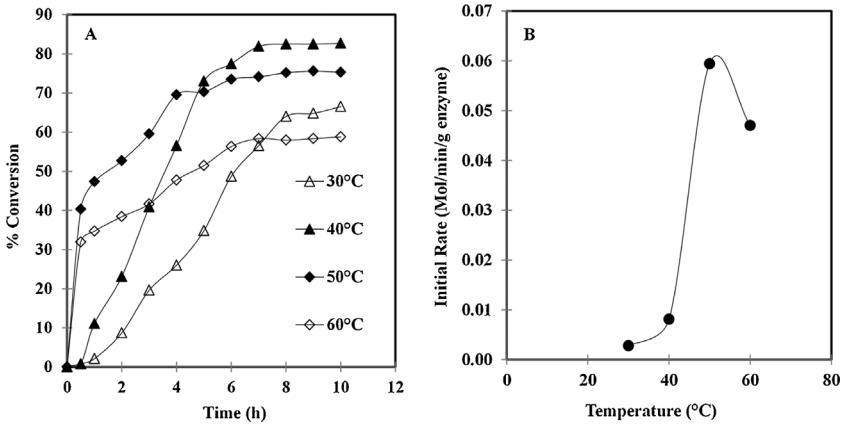


Fig. 1. Effect of temperature for the esterification of propionic acid with isobutyl alcohol using immobilized lipase Novozyme 435 in SFS. (A) % coversion vs. time (B) initial rate vs. temperature.

particles and substrates. However, high temperature may disrupt the active conformation of enzyme which leads to loss of activity and selectivity at higher temperature [7]. Novozym® 435 can work even at 100 °C as it is a heat-tolerant enzyme but, at this high temperature denature enzyme faster with time, so it is recommended to operate at lower temperatures. Moreover, working at lower temperature minimizes thermal energy for economical optimization [8]. Therefore 40 °C was selected as an optimum temperature to carry out further studies.

3.1.2. Effect of enzyme loading

Novozym® 435 is a kind of lipases immobilized on macroporous acrylic resin wherein enzyme molecules could be attached on the surface of the support and/or in the inner part of the support. When the reaction is carried out in a SFS; excessive enzyme particles in the reaction mixture may lead to decrease in mass transfer efficiency due to internal diffusion problem. The optimum enzyme loading has to be found out for any enzymatic reaction as enzymes are main cost component attached to the process. Therefore, keeping high esterification yield and economic feasibility of synthesis of isobutyl propionate using immobilized lipase Novozym® 435 in viewpoint, the effect of enzyme loading was investigated. The range of the enzyme loading was chosen as 2.5–10% (w/w) keeping the other reaction parameters constant. The curves of % conversion with time and initial rate with % enzyme loading are shown in Fig. 2. From Fig. 2A, it is observed that the % conversion was increased up to 82% for 5% w/w enzyme loading, thereafter it was reduced to 75 and 72% for 7.5%, 10% w/w loading respectively. This might be due to high amount lipase making active sites of enzyme unexposed to the substrates [7] or agglomeration of immobilized enzyme in solvent free system [28]. However, the initial rate was found to increase from 0.0042 to 0.0241 Mol/min/g enzyme when % enzyme loading was increased from 2.5 to 7.5 but decreased thereafter (see Fig. 2B). At 5% w/w enzyme loading rapid formation of enzyme–substrate complex explains the higher conversion. Similarly, an excess enzyme also hampers the mixing by increasing viscosity leading to the less effective transfer of the substrates to the active sites of the excess enzyme molecules in turn reducing the overall mass transfer. Considering the above findings, 5% w/w enzyme loading was set to be optimum.

3.1.3. Effect of speed of agitation

In case of immobilized enzyme, the reactants have to diffuse from the bulk liquid to the external surface of the particle and from there into the interior pores of the catalyst where the actual reaction takes place and products are being formed. These, products need to diffuse out from the enzyme particles to the bulk liquid [29]. External mass transfer limitations can be minimized by carrying out the reaction at an optimum speed of agitation and low enzyme loading [30]. Therefore, experiments were carried out at varying speed of agitation from 100 to 400 rpm, so as to study its effect on solvent free esterification reaction. Fig. 3A illustrates that, as speed of agitation increased from 100 to 300 rpm, the % conversion was found to increase from 88 to 92.5% respectively. However, the % conversion

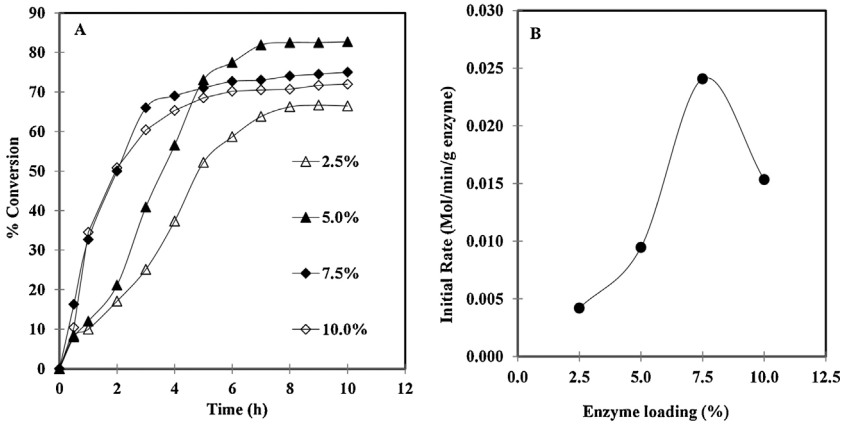


Fig. 2. Effect of enzyme loading for the esterification of propionic acid with isobutyl alcohol using immobilized lipase Novozyme 435 in SFS. (A) % coversion vs. time (B) initial rate vs. % enzyme loading.

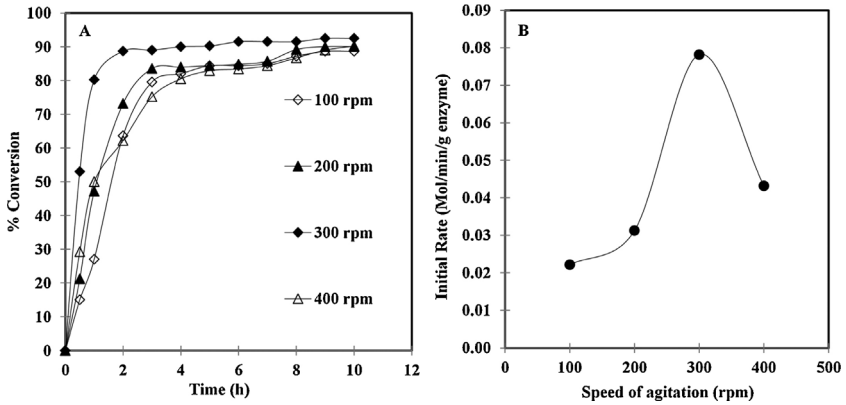


Fig. 3. Effect of speed of agitation for the esterification of propionic acid with isobutyl alcohol using immobilized lipase Novozyme 435 in SFS. (A) % coversion vs. time (B) initial rate vs. speed of agitation (rpm).

decreased at 400 rpm to 90.04%. The similar observations can be made from the initial rate vs. speed of agitation as shown in Fig. 3B. An increase in % conversion and initial rate for increase in the speed of agitation from 100 to 300 rpm can be attributed to the fact that mass transfer resistance has decreased due to the increase in the turbulence. However, the decrease in the % conversion and initial rate at 400 rpm can be reasoned by the shearing effect on enzyme at higher stirring speed. Although highest 92.25% conversion was obtained at 300 rpm in comparison with 200 rpm; enzyme tends to leave its support after 5–6 h thereby losing its recyclability property. Therefore the mild stirring speed of 200 rpm was selected as optimum for the esterification reaction in SFS keeping in view not to hamper the catalytic efficiency (reusability) of the immobilized lipase.

3.1.4. Effect of water concentration

It is well known fact that a large amount of water favors the hydrolysis and inhibits the esterification reaction. However, small amount of water is an important factor to be considered while dealing with lipase catalyzed reactions both for optimal catalytic activity of the enzyme (thermodynamic equilibrium of the reaction) as well as the maintenance of three dimensional structural integrity (reducing probability of contact of inhibitor with enzyme) [3,31]. The effect of initial water on enzymatic activity was examined by adding water ranging from 0.1 M (1.8 cm^3) to 0.3 M (5.4 cm^3) to the reaction mixture moreover it was compared with SFS (i.e. 0 M water concentration). It is clear from Fig. 4A that the highest % conversion of 90.4% is obtained at SFS whereas as we increase the initial concentration of water from 0.1 to 0.3 M the % conversion substantially decreases from 77.3 to 60.5% respectively. Moreover, the initial rate of the esterification reaction is found to be highest (0.063 Mol/min/g enzyme) for 0 M water concentration and it tends to decrease from 0.043 to 0.012 Mol/min/g enzyme when water concentration is increased from 0.1 to 0.3 M. A decrease in % conversion and initial rate for increase in concentration of water can be attributed to the fact that the addition of water shifts the equilibrium towards the backward reaction (hydrolysis) rather than towards forward reaction (esterification) [32].

3.1.5. Effect of molar ratio

The effect of molar ratios of substrates is an important parameter in enzymatic esterification reactions in a SFS. For preliminary analysis, acid to alcohol molar ratios were varied in the range of 1:1–1:4 for concentration of 0.1 M for each substrate. One way of pushing the equilibrium of the reaction in a forward direction is to increase the nucleophile concentration [33] and also negative effect of acid on lipase enzyme was observed in literatures. However, high alcohol concentration may slow down the reaction rates. Therefore, it is necessary to optimize the actual excess nucleophile concentration to be employed in a given reaction. The isobutyl propionate conversion was increased from 82.5 to 90% when molar ratio was increased from 1:1 to 1:3 however only marginal change in the % conversion can be seen at molar ratio 1:4 (Fig. 5). This invariant % conversion at molar ratio of 1:4 indicates the inhibitory effect of alcohol on enzyme which was also shown in case of isoamyl acetate [19], isoamyl myristate [34], geranyl propionate [35]. However, only this evidence does not prove that the inhibitory effect of alcohol on enzyme. This has to be extended to the variation in the acid concentration to analyses the enzymatic kinetic rate data so

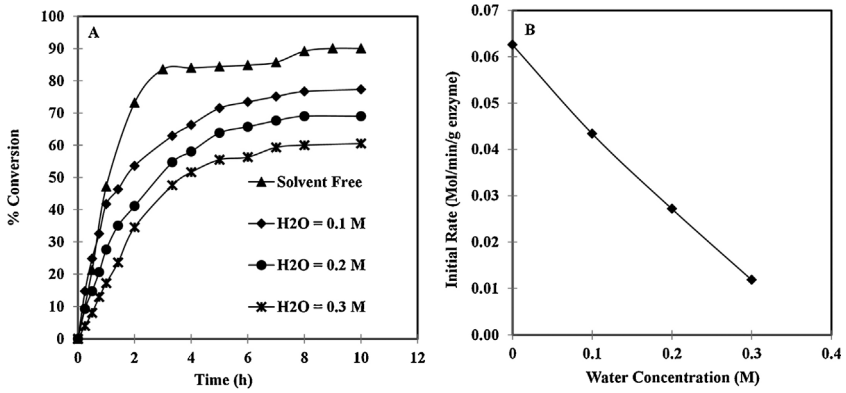


Fig. 4. Effect of addition of water in the esterification of propionic acid with isobutyl alcohol using immobilized lipase Novozyme 435. (A) % coversion vs. time (B) initial rate vs. concentration of water.

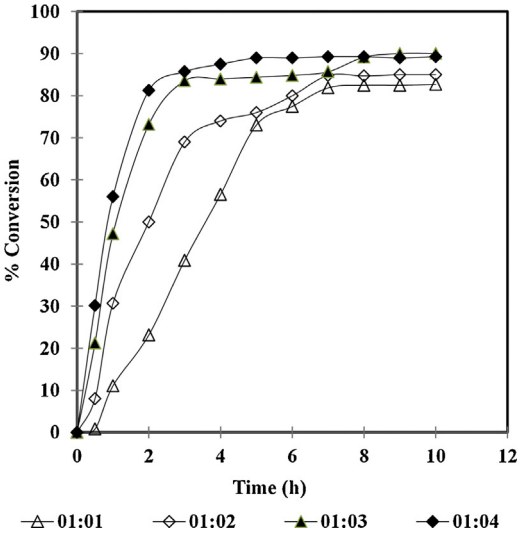


Fig. 5. Effect of acid to alcohol molar ratio for the esterification of propionic acid with isobutyl alcohol using immobilized lipase Novozyme 435 in SFS.

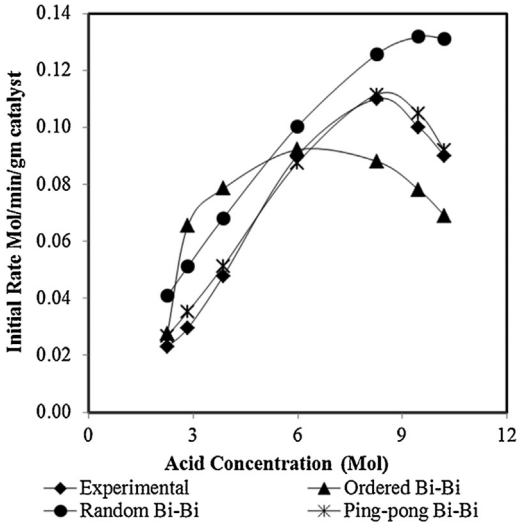


Fig. 7. Comparison of calculated initial rates by the all mechanism with the experimental values.

as to arrive at the actual inhibition effect and mechanism underlying the reaction scheme. This was extended further to study the actual inhibitory effect of both the substrate by kinetic model fitting shown in the Section 3.2.

3.1.6. Enzyme reusability

Novozym® 435 is very efficient enzyme and used for esterification effectively on large extent and hence its reusability is one of the important factor for its use on industrial scale to decide economic viability of the process and to restrict the cost factor. At the end of the reaction, the immobilized lipase was filtered, washed with acetone and kept in desiccator for 24 h and then reused for further batches. With optimized parameters; it was observed that there were marginal changes in percentage conversion up to seven cycles of reuse and further conversion was reduced by difference of 6% only (see Fig. 6). The reusability of immobilized enzyme varies with the reaction systems being used based on the substrates applied in the reaction scheme. This could be a result of the decrease in the amount of effective biocatalyst adsorbed to the solid support or the denaturation of the enzyme by propionic acid after several reaction cycles. Also, decrease in the activity of the enzyme could be because of water loss from the micro-environment of enzyme particles or blocking the active sites by one of the product [34].

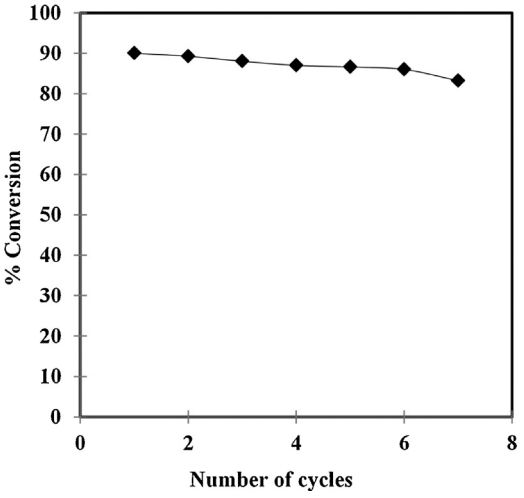


Fig. 6. Reusability of the immobilized lipase Novozyme 435 in SFS for the esterification of propionic acid with isobutyl alcohol.

3.2. Kinetic model based on initial rate measurements

The effect of substrate concentration on the rate of the esterification of propionic acid with isobutyl alcohol using immobilized lipase Novozym® 435 in a SFS was investigated using three different mechanisms. The initial rates of esterification were calculated using data from the reaction times giving less than 10% conversion of the limiting reagent and plotted against acid concentration in Fig. 7. As propionic acid concentration increased from 2.25 M (*B*:*A* = 4:1) to 8.27 M (*B*:*A* = 1:2); the initial velocity reaches to highest value (0.11 M/min/g catalyst) from 0.023 Mol/min/g enzyme and again start decreasing. However the isobutyl alcohol concentration was also variable since the experimentations were done in a solvent free system. The similar trend was observed for the same system when solvent system was used [36].

Several mechanisms have been proposed to explain lipase catalyzed reactions. However, in our study we have used three different mechanisms i.e. Ordered Bi–Bi, Random Bi–Bi and Ping-Pong Bi–Bi for evaluation of the initial rates based on their mechanism. For greater accuracy, non-linear regression with minimization of sum of square of errors (*SSE*) was applied using Microsoft Excel 2010 and model parameters were evaluated. The comparison of initial velocity calculated by the rate equation given by mechanism Ordered Bi–Bi, Random Bi–Bi and Ping-Pong Bi–Bi as a function of acid concentration is shown in Table 1 and Fig. 7. It is evident from Table 1 and Fig. 7 that the kinetic parameter values evaluated by Ordered Bi–Bi and Random Bi–Bi are non-significant. Moreover the sum of squares of errors is higher as compared to Ping-Pong Bi–Bi. Although, several mechanisms have been proposed in theory, lipases are generally said to follow the Ping-Pong Bi–Bi Mechanism [21]. Our results indicate the same too.

The esterification of propionic acid with isobutyl alcohol using immobilized lipase Novozym® 435 in SFS includes the reaction steps in which the lipase may react with iso-butanol to yield a dead-end enzyme iso-butanol complex or it may react with propionic acid to yield the effective lipase propionic acid complex. Then the lipase propionic acid complex is transferred to an enzyme–acyl intermediate and water is released. This is followed by the interaction of the enzyme–acyl complex with iso-butanol to form another binary complex, which then yields the ester and

Table 1
Comparison of calculated evaluated kinetic parameters for all mechanisms.

Model/Parameters	Ordered Bi–Bi	Random Bi–Bi	Ping-Pong Bi–Bi	Ping-Pong Bi–Bi (Varma and Madras [36])
V_{max} (M/min/g catalyst)	0.73	67.78	0.500	0.127
K_A (M)	19.65	3159.52	0.631	0.278
K_B (M)	0.50	26.28	0.003	0.0014
K_{iA} (M)	486.66	194.78	0.0042	0.00012
K_{iB} (M)	–	–	0.1539	0.00061
K_i (M)	50.31	–	–	–
SSE	4.3E−03	4.4E−03	9.6E−05	–

free lipase. If the enzyme–acyl complex reacts with acid it forms a dead-end complex leading to acid inhibition. In line with the reaction mechanism discussed above, the best fit model parameters are obtained by non-linear regression for Ping-Pong Bi–Bi mechanism with acid and alcohol inhibition were V_{max} = 0.5 M/min/g enzyme, K_A = 0.631 M, K_B = 0.003 M, K_{iA} = 0.0042 M and K_{iB} = 0.1539 M. The apparent Michaelis–Menten constants obtained from the analysis K_A and K_B, give light to the fact that the enzyme has greater affinity for iso-butanol than propionic acid (specificity (K_s) is given by k_{cat}/K_m where K_m is the Michaelis–Menten constants and $V_{max} = k_{cat}[E_o]$ where $[E_o]$ is the total enzyme concentration). The Michaelis–Menten constant for most of the enzyme catalyzed reactions is in the range of 10^{-1}–10^{-7} M [37] which is consistent with the observed kinetic constants found in this study. The values of the specificity constant suggest that the enzyme specificity is higher in the immobilized lipase system with respect to iso-butanol [38]. The inhibition constants have a direct relationship with inhibitory effect of the substrate.

The above determined model parameters were comparable with those found by Varma and Madras [36] for the same pair of substrates and enzyme but for a solvent system (see Table 1). However, the difference in values is due to the absence of solvent which enables greater interaction between substrates. Our system parameters indicate greater specificity of enzyme towards alcohol and greater inhibition by alcohol when compared to acid. Both systems showed inhibition by both acid and alcohol, with greater affinity of the enzyme towards iso-butanol and greater inhibitory effect of iso-butanol. The values of maximum velocity at substrate saturation in the SFS were much greater (about five times) than that of the solvent system as also observed by Sandoval et al. [39]. This is because SFS has much greater substrate interaction than a solvent system and the maximum velocity can be about six-times that of solvent systems. Since the substrates are always present in large concentrations in the absence of solvents to dilute the reaction mixture there are no diffusion barriers. Moreover the reaction is slow and hence kinetically controlled rather than diffusion controlled.

4. Conclusion

Isobutyl propionate (rum flavor) was successfully synthesized using Novozym® 435 by esterification reaction of iso-butanol and propionic acid in a SFS. Various parameters were optimized in order to achieve better conversion and higher initial rates so as to make process more economical. Maximum conversion of 92.5% was obtained at temperature 40 °C, enzyme loading 5% w/w, acid to alcohol molar ratio of 1:3, stirring speed of 300 rpm. Reusability studies showed that the immobilized enzyme can be reused for seven times without any significant loss of synthetic activity. As the process is solvent free, drawbacks associated with use of organic solvents such as their toxicity, flammability and the most importantly their separation and product purification. Thus overall process becomes more economical and also greener. This work will further help to explore the solvent free processes which generally are not much popular among the literature because of lower yields as compared to those incorporating organic solvents. Three different bi–bi mechanisms are tested for the development of the kinetics of esterification of iso-butanol and propionic acid in SFS. The best fit model parameters obtained by non-linear regression for Ping-Pong Bi–Bi mechanism with acid and alcohol inhibition are V_{max} = 0.5 M/min/g catalyst, K_A = 0.631 M, K_B = 0.003 M, K_{iA} = 0.0042 M and K_{iB} = 0.1539 M.

References

[1] S.M. Radzi, W.A.F. Mustafa, S.S. Othman, H.M. Noor, World Acad. Sci. Eng. Technol. 59 (2011) 677–680.
[2] T.W. Charpe, V.K. Rathod, Waste Manage. 31 (2011) 85–90.
[3] H. Ghamgui, M. Karra-Chaâbouni, S. Bezzine, N. Miled, Y. Gargouri, Enzyme Microb. Technol. 38 (2006) 788–794.
[4] N. Gharat, V.K. Rathod, Ultrason. Sonochem. 20 (2013) 900–905.
[5] P. Pires-Cabral, M.M.R. da Fonseca, S. Ferreira-Dias, Biochem. Eng. J. 43 (2009) 327–332.
[6] R. Ben Salah, H. Ghamghui, N. Miled, H. Mejdoub, Y. Gargouri, J. Biosci. Bioeng. 103 (2007) 368–372.
[7] G.D. Yadav, S. Devendran, Process Biochem. 47 (2012) 496–502.
[8] M.D. Romero, L. Calvo, C. Alba, A. Daneshfar, H.S. Ghaziaskar, Enzyme Microb. Technol. 37 (2005) 42–48.
[9] S. Sun, L. Zhang, X. Meng, Z. Xin, J. Renew. Sust. Energ. 5 (2013) 33127.
[10] N.A. Serri, A.H. Kamaruddin, W.S. Long, Bioprocess. Biosyst. Eng. 29 (2006) 253–260.
[11] C. Shu, J. Cai, L. Huang, X. Zhu, Z. Xu, J. Mol. Catal. B: Enzym. 72 (2011) 139–144.
[12] M.B. Ansorge-Schumacher, O. Thum, Chem. Soc. Rev. 42 (2013) 6475–6490.
[13] K.-E. Jaeger, T. Eggert, Curr. Opin. Biotechnol. 13 (2002) 390–397.
[14] Z. Cabrera, G. Fernandez-Lorente, R. Fernandez-Lafuente, J.M. Palomo, J.M. Guisan, Process Biochem. 44 (2009) 226–231.
[15] M. Liaquat, R.K. Apenten, J. Food Sci. 65 (2000) 295–299.
[16] A. Manjón, J.L. Iborra, A. Arocas, Biotechnol. Lett. 13 (1991) 339–344.
[17] G.D. Yadav, A.H. Trivedi, Enzyme Microb. Technol. 32 (2003) 783–789.
[18] K.P. Dhake, D.D. Thakare, B.M. Bhanage, Flavour Frag. J. 28 (2013) 71–83.
[19] A. Güvenç, N. Kapucu, Ü. Mehmetoğlu, Process Biochem. 38 (2002) 379–386.
[20] M. Martinelle, K. Hult, Biochim. Biophys. Acta 1251 (1995) 191–197.
[21] G.D. Yadav, K.M. Devi, Chem. Eng. Sci. 59 (2004) 373–383.
[22] D. Bezbradica, D. Mijin, S. Siler-Marinkovic, Z. Knezevic, J. Mol. Catal. B: Enzym. 38 (2006) 11–16.
[23] G.D. Yadav, P.S. Lathi, J. Mol. Catal. B: Enzym. 27 (2004) 113–119.
[24] D.K. Daniel, S. Malik, K. Albert, Eng. Life Sci. 11 (2011) 195–200.
[25] I. Alime, L. Hosgun, Turkish J. Chem. 31 (2007) 493–499.
[26] T. Garcia, N. Sanchez, M. Martinez, J. Aracil, Enzyme Microb. Technol. 25 (1999) 584–590.
[27] I. Itabaiana Jr., K.M. Gonçalves, Y.M.L. Cordeiro, M. Zoumpanioti, I.C.R. Leal, L.S.M. Miranda, R.O.M.A. de Souza, A. Xenakis, J. Mol. Catal. B: Enzym. 96 (2013) 34–39.
[28] M.L. Foresti, M.L. Ferreira, Catal. Today 107–108 (2005) 23–30.
[29] J. Sun, B. Yu, P. Curran, S.-Q. Liu, Food Chem. 141 (2013) 2828–2832.
[30] N.R. Sonare, V.K. Rathod, J. Mol. Catal. B: Enzym. 66 (2010) 142–147.
[31] V.K. Garlapati, R. Banerjee, Enzyme Res. (2013), http://dx.doi.org/10.1155/2013/367410.
[32] M. Karra-Chaabouni, S. Pulvin, D. Thomas, D. Touraud, W. Kunz, Biotechnol. Lett. 24 (2002) 1951–1955.
[33] G.V. Chowdary, M.N. Ramesh, S.G. Prapulla, Process Biochem. 36 (2000) 331–339.
[34] G.D. Yadav, P.A. Thorat, J. Mol. Catal. B: Enzym. 83 (2012) 16–22.
[35] N. Paroul, L.P. Grzegozeski, V. Chiaradia, H. Treichel, R.L. Cansian, J.V. Oliveira, D. de Oliveira, J. Chem. Technol. Biotechnol. 85 (2010) 1636–1641.
[36] M.N. Varma, G. Madras, Appl. Biochem. Biotechnol. 160 (2010) 2342–2354.
[37] G.N. Kraai, J.G.M. Winkelman, J.G. de Vries, H.J. Heeres, Biochem. Eng. J. 41 (2008) 87–94.
[38] A.C. Oliveira, M.F. Rosa, M.R. Aires-Barros, J.M.S. Cabral, J. Mol. Catal. B: Enzym. 11 (2001) 999–1005.
[39] G. Sandoval, J.S. Condoret, P. Monsan, A. Marty, Biotechnol. Bioeng. 78 (2002) 313–320.