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Lipase catalyzed transesterification of methyl acetoacetate with *n*-butanol

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Abstract

Keto esters represent an important class of organic building blocks which are used for efficient synthesis of a number of complex natural products. Keto esters are normally produced by chemical catalysis necessitating use of higher temperatures. Alternatively they can be synthesized in non-aqueous media using enzyme catalysis under milder conditions. *n*-Butyl acetoacetate is an important β-keto ester and its synthesis was studied by transesterification of methyl acetoacetate with *n*-butanol using supported lipases such as Novozym 435, Lipozyme RM IM and Lipozyme TL IM. Among these enzymes, Novozym 435 was found to be the most active catalyst in toluene as a solvent. The effects of various parameters on conversion and rates of reaction were studied in the absence of mass transfer limitations. A model based on ping-pong bi-bi mechanism was found to fit the initial rate data very well and the kinetic parameters were evaluated by non-linear regression analysis.

Keywords: Transesterification; β-Keto ester; Methyl acetoacetate; *n*-Butyl acetoacetate; Immobilized lipases; Novozym 435; Kinetic model; Ping-pong bi-bi model

1. Introduction

Non-aqueous enzymology has gained a considerable importance during the past few years. Lipases are the most widely employed enzymes for synthesis of organic chemicals mainly in aqueous media and in some cases non-aqueous media too because they are inexpensive, stable and easy to recycle [1,2]. Lipases possess wide substrate specificity, have an ability to recognize chirality and do not require labile cofactors [3–5]. Of late, lipases have been used to catalyze a number of reactions in non-aqueous media such as esterification, transesterification, amidation, hydrolysis, thioesterification and trans-thioesterification [6–17]. The versatility of lipase catalysis in the synthesis of other group of chemicals needs to be explored. For instance, there is no report on the preparation of keto esters by lipase catalysis including kinetic modeling. Keto esters represent an important class of organic building blocks which are used for efficient synthesis of a number of complex natural products [18–21].

The relevance of enzymatic catalysis vis-à-vis chemical catalysis in ester synthesis should be emphasized since esters are prepared by a variety of techniques [22–24]. Although several new chemical catalysts have been employed to improve the yield of esterification reactions, they share the common disadvantage of high temperature requirements and lack of stereospecificity.

n-Butyl acetoacetate serves as an important synthon since it can be transformed to a chiral building block by chemical and enzymatic transformations. It is used as a tool for chain extension reaction, in the preparation of calcium antagonist, as acetoacetylating agents for polyols and for increasing the storage stability and compatibility with base resins for paints. The current work on synthesis of *n*-butyl acetoacetate by transesterification was therefore undertaken.

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Transesterification is more advantageous over direct esterification using acetoacetic acid due to instability and violent decomposition of the acid in various organic solvents [25]. Also methyl acetoacetate is readily available and thus it serves conveniently as a starting material for transesterification. Hence we thought it was worthwhile to synthesize *n*-butyl acetoacetate via transesterification of methyl acetoacetate and *n*-butanol.

Most of the reported methods for transesterification of β-keto esters are not general and these are equilibrium driven reactions where a large excess of one of the reactants is mandatory to obtain good conversions. Transesterifications catalyzed by DMAP, which is toxic and expensive, require a large amount of catalyst. On the contrary, distannoxanes give excellent yields but the catalysts are very difficult to prepare. Other catalysts such as yttria-zirconia, *n*-bromosuccinimide, lithium perchlorate [26], montmorillonite clay [27], etc. require higher temperatures. Recently Wang et al. reported transesterification of β-keto ester in ionic liquid and sulfamic acid as synergetic catalytic medium [28].

A promising alternative to chemical methods, which obviates the need for high temperature and minimizes waste management problems, is lipase mediated reactions. Enzymatic synthesis of esters can be done by transesterification of an easily available ester or by a direct esterification of the carboxylic acid which can form the initial enzyme–acyl complex [29]. This work focuses on transesterification of methyl acetoacetate, a β-keto ester, with *n*-butanol to prepare *n*-butyl acetoacetate, in the presence of immobilized lipases and the kinetics and mechanism are also established.

2. Experimental

2.1. Enzymes

Supported enzymes Novozym 435, Lipozyme RM IM and Lipozyme TL IM were all procured as gift samples from Novo Nordisk, Denmark. Novozym 435 is the component B of the lipase from *Candida antarctica*, immobilized on macroporous polyacrylate resin (bead size 0.3–0.9 mm, bulk density 430 kg/m^3, activity 7000 propyl laurate units). Lipozyme RM IM is *Mucor miehei* immobilized on an anionic resin and Lipozyme TL IM is *Thermomyces lanuginosus* is immobilized on silica.

2.2. Chemicals

All chemicals were procured from firms of repute and used without any further purification: methyl acetoacetate (Merck, India), toluene, *n*-butanol, benzene, 1,4-dioxane, tetrahyrofuran, and acetonitrile (all A.R. grade from s.d. Fine Chemicals Pvt. Ltd., Mumbai, India).

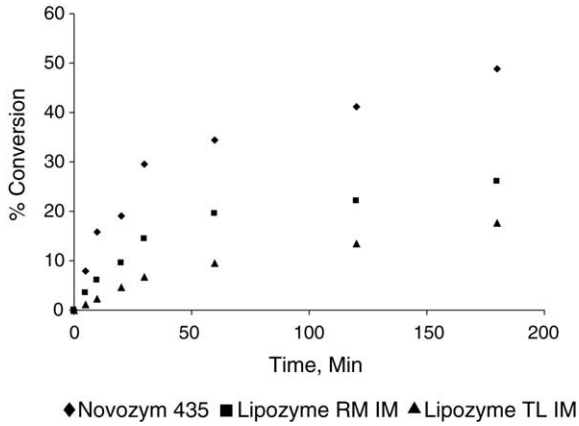


Fig. 1. Screening of catalysts. Reaction conditions: methyl acetoacetate, 0.01 mol; *n*-butanol, 0.01 mol; solvent, toluene up to 10 ml; speed of agitation, 400 rpm; temperature, 30 °C.

2.3. Experimental setup

The experimental set-up consisted of a 4 cm i.d. fully baffled mechanically agitated 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 the desired temperature with an accuracy of ±1 °C.

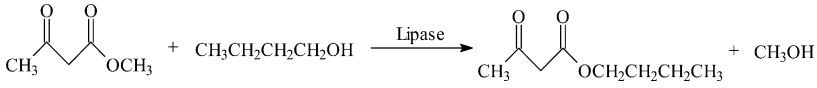
A typical reaction mixture consisted of 0.01 mol *n*-butanol and 0.01 mol methyl acetoacetate diluted to 10 mL with toluene as a solvent, i.e. each reactant with 1.0 mol/L. The reaction mixture was agitated at 30 °C for 15 min at a speed of 400 rpm and 57 mg of immobilized enzyme was added to initiate the reaction. Clear liquid samples were withdrawn periodically and analyzed by using GC.

2.4. Analysis

The concentrations of the reactants and products were determined on a Chemito G.C. equipped with a flame ionization detector. A 3 m × 3.8 mm stainless steel column packed with OV-101 was used for the analysis.

3. Results and discussions

The reaction is represented by the following:



3.1. Effect of various catalysts

Fig. 1 shows the conversion profiles for Novozym 435, Lipozyme RM IM and Lipozyme TL IM under otherwise similar conditions. The conversion varied markedly with the type of lipase. The activities are in the following order:

Novozym 435 > Lipozyme RM IM > Lipozyme TL IM

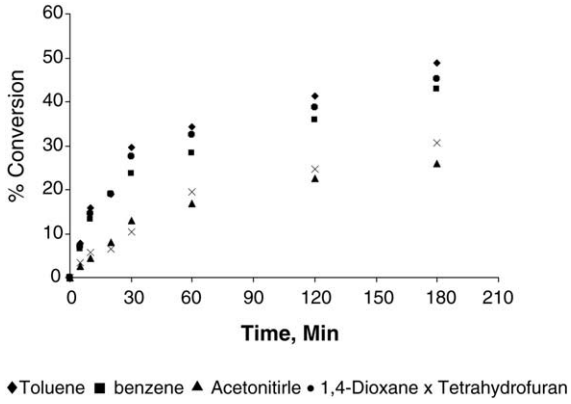


Fig. 2. Effect of different solvents. Reaction conditions: methyl acetoacetate, 0.01 mol; *n*-butanol, 0.01 mol; Novozym 435, 3% (w/w); speed of agitation, 400 rpm; temperature, 30 °C.

Novozym 435 was the most active catalyst. It is a thermostable lipase and mainly useful for the synthesis of esters and amides. Lipozyme TL IM is mainly used for transesterification of bulk frying fats and was not as effective as Novozym 435. Hence, Novozym 435 was selected for all further experiments.

3.2. Effect of different solvent

Experiments were carried out with different solvents such as toluene, benzene, 1,4-dioxane, acetonitrile and tetrahydrofuran (Fig. 2). Methyl acetoacetate is immiscible in heptane, hexane, octane and *iso*-octane. A conversion 48% was obtained in toluene, whereas in acetonitrile, it was 25%. The polarity of solvent and its affinity towards the matrix of the lipase support are crucial. The nature of the solvent has been found to have a large influence on both reaction rate and equilibrium. Attempts have been made to rationalize the effects of solvent in term of different solvent parameters, such as log *P*, dipole moment, dielectric constant, etc. [30]. The effect of log *P* value on the rate of reaction is given in Table 1. The rate increases with increasing log *P* values except 1,4-dioxane. The enzyme requires water layer around the support to maintain its activity and the solvents which strip it from the enzyme are the least effective. Toluene does not disturb the water layer to the extent as tetrahydrofuran. Hence toluene was a better solvent and it was used in all further experiments.

Table 1
Effect of solvent on the reaction rate of transesterification of *n*-methyl acetoacetate with *n*-butanol

Solvent	log *P*	Initial rate (mol/L min g of enzyme)
Toluene	2.5	0.21
Benzene	2.0	0.18
1,4-Dioxane	−1.1	0.20
Acetonitrile	−0.33	0.078
Tetrahydrofuran	0.49	0.08

Reaction conditions: methyl acetoacetate, 0.01 mol; *n*-butanol, 0.01 mol; Novozym 435, 3%; speed of agitation, 400 rpm; temperature, 30 °C.

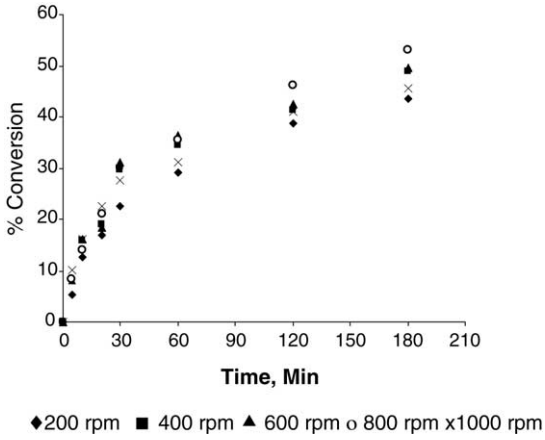


Fig. 3. Effect of speed of agitation. Reaction conditions: methyl acetoacetate, 0.01 mol; *n*-butanol, 0.01 mol; temperature, 30 °C; solvent, toluene up to 10 ml; Novozym 435, 3%.

3.3. Effect of speed of agitation

The effect of external mass transfer resistance for the reactants was studied at five different speeds, namely, 200, 400, 600, 800 and 1000 rpm (Fig. 3). The conversion of methyl acetoacetate increased from 43 to 53%, when the speed was increased from 200 to 800 rpm. However, further increase to 1000 rpm decreased the final conversion to 45%, which was possible due to shearing of enzyme molecules or inactivation of enzyme molecules due to foam formation at this speed. Also some catalyst was thrown out of reaction media which reduced the effective catalyst loading in the liquid phase. There was hardly any change in the overall conversion when the speed of agitation increased from 400 to 800 rpm. The initial rates were practically the same. So further all the experiments were carried out at 400 rpm.

3.4. Effect of catalyst loading

The catalyst loading was varied from 1% (19 mg) to 7.5% (142.5 mg), w/w of the reactants keeping all other parameters constant. It was found that the initial rate of reaction increased from 0.0071 to 0.0151 mol/L-min and the final conversion increased from 31 to 56%, with an increase in catalyst loading from 19 to 142.5 mg. However, an increase in catalyst loading from 57 to 142.5 mg did not have any significant effect on conversion and the trend is non-linear due to availability of more enzyme (Fig. 4), which might be due to the increase in the concentration of catalyst above the substrate concentration which might bring in external mass transfer resistances. Therefore, 57 mg Novozym 435 was used as a catalyst loading in further studies.

3.5. Effect of concentration of n-butanol

To study the effect of *n*-butanol concentration, the concentration of methyl acetoacetate was kept constant at 1 mol/L and the ratio of *n*-butanol to methyl acetoacetate was var-

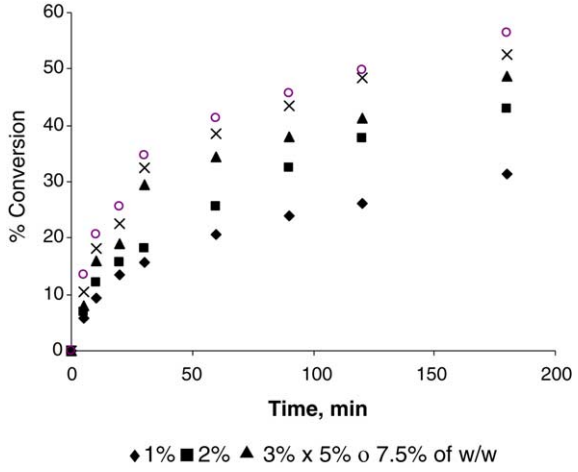


Fig. 4. Effect of catalyst loading. Reaction conditions: methyl acetoacetate, 0.01 mol; *n*-butanol, 0.01 mol; speed of agitation, 400 rpm; temperature, 30 °C; solvent, toluene up to 10 ml.

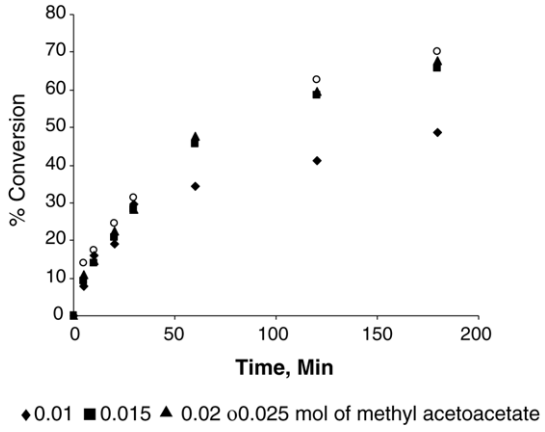


Fig. 6. Effect of concentration of methyl acetoacetate. Reaction conditions: *n*-butanol, 0.01 mol; temperature, 30 °C; speed of agitation, 400 rpm; solvent, toluene up to 10 m; Novozym SP 435, 3%.

ied from 1 to 2.5 mol/L. It was observed that with increasing amount of *n*-butanol 1–2.5 mol/L, the rate of the reaction increased from 0.2140 to 0.3561 mol/L-min-g of enzyme and conversion increased from 48 to 76% (Fig. 5). It was also found that no inhibition occurs by using higher concentrations of *n*-butanol upto 2.5 mol/L and accordingly the conversion of the limiting reactant methyl acetoacetate was found to be maximum of 76% in 3 h.

3.6. Effect of concentration of methyl acetoacetate

In another set of experiments the concentration of *n*-butanol was kept constant (0.01 mol) under otherwise similar conditions and the concentration of methyl acetoacetate was varied from 1 to 2.5 mol/L. The conversion increased from 48 to 70% and rate of reaction increased from 0.2140 to 0.3140 mol/L-min-g of enzyme with increased in the concentration of methyl acetoacetate from 1 to 2.5 mol/L (Fig. 6).

3.7. Effect of temperature

The initial rate was observed to increase from 0.2140 to 0.3508 mol/L-min-g of enzyme and conversion from 48 to 68% with an increase in temperature form 30 to 60 °C (Fig. 7). The Arrhenius plot was made on the basis of ln of initial rates vs. reciprocal of temperature and shown in (Fig. 8). The value of activation energy was obtained as 3.748 kcal/mol, which is a reasonable value for enzymatic reactions in the absence of diffusion limitations [31,32].

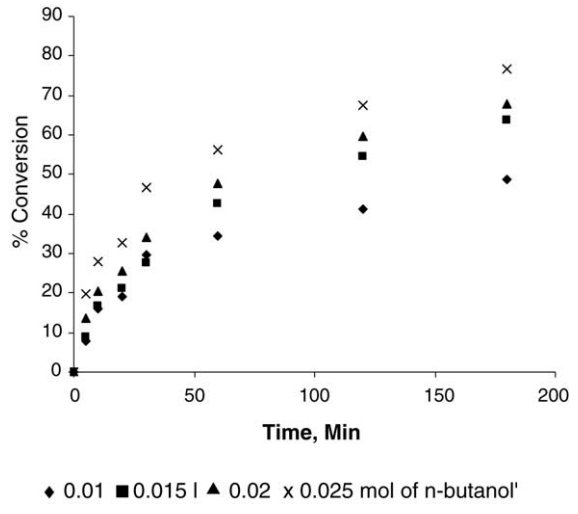


Fig. 5. Effect of concentration of *n*-butanol. Reaction conditions: methyl acetoacetate, 0.01 mol; temperature, 30 °C; speed of agitation, 400 rpm; solvent, toluene up to 10 ml; Novozym 435, 3%.

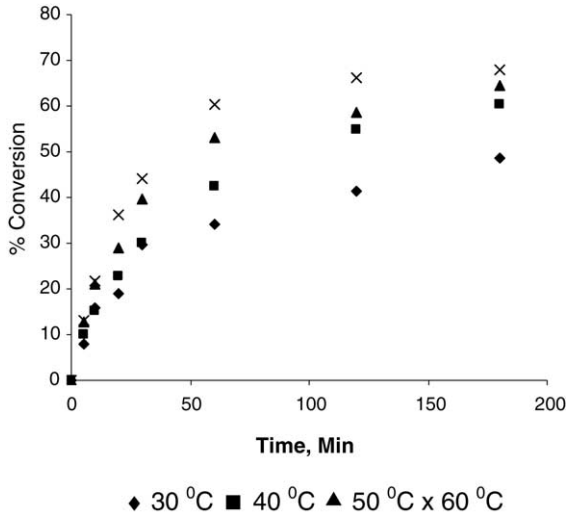


Fig. 7. Effect of temperature. Reaction conditions: methyl acetoacetate, 0.01 mol; *n*-butanol, 0.01 mol; speed of agitation, 400 rpm; solvent, toluene up to 10 ml; Novozym 435, 3%.

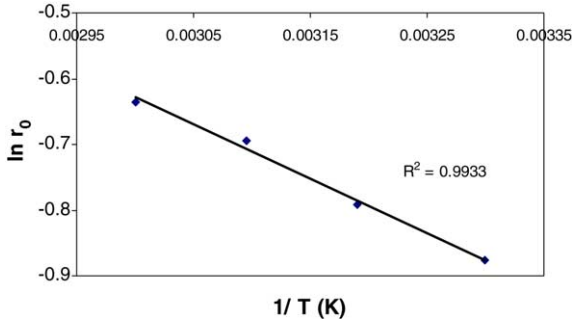


Fig. 8. Arrhenius plot.

3.8. Effect of reusability

The catalyst was filtered, washed with toluene after each use, dried at room temperature and reused. There was a marginal decrease in conversion after three reuses which might be due to the loss of catalyst during handling (Fig. 9). Thus, the enzyme was quite stable.

3.9. Kinetic study

The main emphasis during this study was an optimization of reaction conditions such as speed of agitation, concentration of substrates, enzyme loading, temperature, etc. to maximize the conversion and yield, and also to reduce the cost of production under the most favorable conditions and also to develop a kinetic model. Hence the effects of concentration of both the reactants on the rate of reaction were studied systematically over a wide range. For the determination of initial rates, two set of experiments were conducted by using 57 mg Novozym 435 with appropriate quantities of *n*-butanol and methyl acetoacetate and the total volume was made upto 10 mL with toluene. In one set of experiment, *n*-butanol amount was varied from 1 to 2.5 mol/L at a fixed quantity of methyl acetoacetate (1 mol/L) and in another set, the amount of methyl acetoacetate was varied from 1 to 2.5 mol/L at a fixed quantity of *n*-butanol (1 mol/L). The initial rates were determined from quantified data.

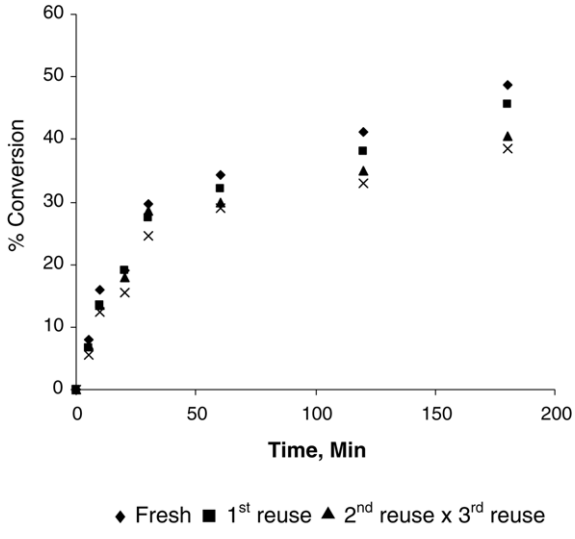


Fig. 9. Effect of reusability. Reaction conditions: methyl acetoacetate, 0.01 mol; *n*-butanol, 0.01 mol; speed of agitation, 400 rpm; solvent, toluene up to 10 ml; Novozym 435, 3%.

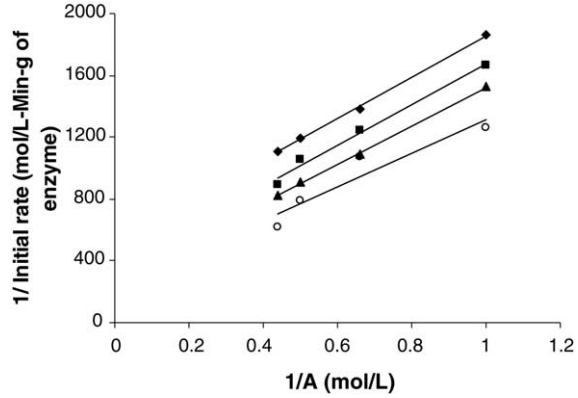


Fig. 10. Lineweaver Burk plot $1/r_0$ vs. $1/[A]$ for transesterification of *n*-methyl acetoacetate with *n*-butanol.

From the initial rate measurements, it was observed that the rate increased with increasing quantities of both the reactants, methyl acetoacetate (A) and *n*-butanol (B). There was no evidence of inhibition by both the substrates. The Lineweaver Burk plot $1/r_0$ versus $1/[A_0]$ for varied initial concentrations of B gives parallel lines (Fig. 10), where r_0 is the initial rate of reaction and $[B_0]$ is the initial concentration of *n*-butanol. The family of lines in Fig. 10 has no common intersection and, therefore a sequential mechanism can be ruled out. In the case of mechanism, one product is released before the next substrate binds to the enzyme [33,34].

In the case of lipase catalyzed reactions, it has been established that the lipase first forms an acyl–enzyme complex with the acyl donor, ruling out the random mechanism. The rate equation for the ternary complex mechanism, for initial conditions is as follows:

$$r_0 = \frac{r_{\max}[A_0][B_0]}{K_{\mathrm{mB}}[A_0] + K_{\mathrm{mA}}[B_0] + [A_0][B_0] + K} \quad (1)$$

The rate equation for the mechanism, for initial conditions is as follows:

$$r_0 = \frac{r_{\max}[A_0][B_0]}{K_{\mathrm{mB}}[A_0] + K_{\mathrm{mA}}[B_0] + [A_0][B_0]} \quad (2)$$

where r_0 is the initial reaction rate, $r_{\max}$ the maximum rate of reaction, $[A_0]$ the initial concentration of methyl acetoacetate, $[B_0]$ the initial concentration of *n*-butanol, K_{mA} the Michaelis constant for methyl acetoacetate, K_{mB} the Michaelis constant for *n*-butanol; $K = K_{\mathrm{mA}}K_{\mathrm{mB}}$.

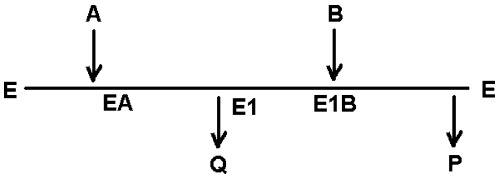
The initial rates were calculated from the linear portion of the concentration–time profiles and the kinetic constants were obtained by non-linear regression analysis for the above models (Table 2). It was observed that the sum of the squared residuals was minimum in the case of the ping-pong bi-bi model. According to the ping-pong bi-bi mechanism,

Table 2
Kinetic parameters for transesterification of *n*-methyl acetoacetate with *n*-butanol

Kinetic constants	Ping-pong bi-bi	Ternary complex
r_{max} (mol/L min g of enzyme)	0.9068	8115.77
K_{mA} (mol/L g of enzyme)	754.73	732416
K_{mB} (mol/L g of enzyme)	5.087	2034
K (mol/L g of enzyme)	NA	−5696
SSE	3.43×10^{-6}	3.48×10^{-6}

methyl acetoacetate (A) first binds with the lipase (E) and gives the methyl acetoacetate–enzyme complex (EA), which then intermediately transfers to carboxylic-lipase (E1) and methanol (Q) is released. This is followed by the interaction of carboxylic-lipase with *n*-butanol (B) to form another binary complex (E1B), which then yields the butyl acetoacetate (P) and free lipase (E).

The reaction mechanism may be illustrated as follows:



At low concentrations of A, the reaction E + A ⇔ EA will be rate limiting. Increasing the concentration of B will convert more E1 to E1B and thereby decrease the concentration of enzyme to form E1. The reduction of E1 would pull the E1B ⇔ E1 + B reaction to the right reducing the concentration of E1B which, in turn, would pull EA ⇔ E1B to the right which would have the same effect on the rate limiting step. The reduction in E1 would seem to pull this to the right because the loss of E1 would decrease the rate of the reverse reaction. It has no direct effect on the rate of the forward reaction. The rate of this reverse reaction is already zero under the normal conditions of the assay so it cannot be slowed down any more. Effectively then the irreversibility of the reaction isolates the rate limiting step from the influence of B and a change in the concentration in B has no effect on the rate at low A concentrations. The slope of the line is unchanged, which is a typical characteristic of the ping-pong bi-bi mechanism. Hence, in this system effect of concentration was studied the range of 1–2.5 mol/L.

In our earlier work [18] on using *n*-butanol for esterification of isobutyric acid, it was found that at a concentration 0.03 mol, *n*-butanol acted as the inhibitor and hence it was advisable to have concentrations lower than this value in the current system. Hence we thought it desirable to study the kinetic mechanism for that particular range of concentration.

Recent publications of Yadav and Sivakumar [16], Lee et al. [35] and Palomo et al. [36] have reported that only at high concentrations, methanol acts as an inhibitor. However, the amount of methanol generated during the current reaction was very less as compared to amount required for inhibition. In the case of kinetic model, it was based on initial rate and hence methanol inhibition was not considered.

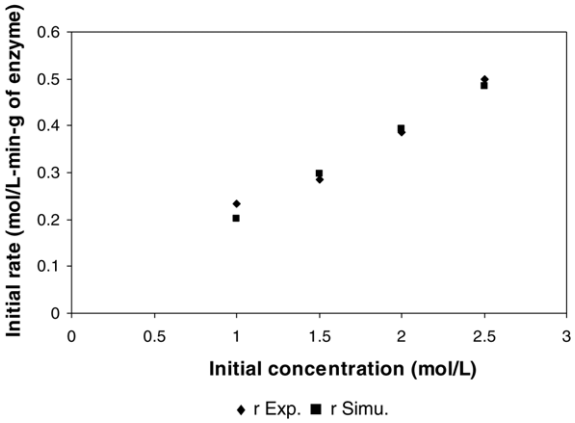


Fig. 11. Plot of initial concentration of B vs. initial rate.

A plot of initial concentration of B against initial observed rate and simulated rate shows an excellent fit thereby proving the validity of the model (Fig. 11).

4. Conclusion

β-Keto esters are important compounds which serve as synthons in a number of industries. These are typically produced by using chemical catalysis at higher temperatures. Synthesis of *n*-butyl acetoacetate, a β-keto ester, was accomplished by transesterification of methyl acetoacetate with *n*-butanol using different lipases, among which Novozym 435 was found to be the most active catalyst. The effects of various parameters on conversion and rates of reaction were studied systematically with Novozym 435 as catalyst and toluene as solvent. Initial rate and progress curve data were used to fit the ping-pong bi-bi mechanism and various parameters were estimated. This model was used to simulate the rate data, which were in excellent agreement with experimental values.

Acknowledgments

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