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Lipase-catalyzed hydrazinolysis of phenyl benzoate: Kinetic modeling approach

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ABSTRACT

Immobilized lipase-catalyzed synthesis of benzoic acid hydrazide from hydrazine and phenyl benzoate is reported in this work. A series of immobilized lipases such as *Candida antarctica* lipase B, *Mucor miehei* lipase and *Thermomyces lanuginosus* lipase were screened to establish that *C. antarctica* lipase B was the best lipase for hydrazinolysis. When phenyl benzoate (0.01 mol) and hydrazine (0.02 mol) in toluene (15 ml) were reacted with *C. antarctica* lipase B (Novozym 435) at 50 °C, 95% of phenyl benzoate was converted to benzoic acid hydrazide after 2 h. The effects of various parameters such as speed of agitation, concentration of the substrates, temperature, enzyme concentration, and reusability of the enzyme were studied to deduce kinetics and mechanism of the reaction. A mechanism based on an ordered bi–bi dead end complex with hydrazine was found to fit the data. Systematic deactivation studies indicated that the enzyme was deactivated due to the hydrazine and phenol, enzyme deactivation obeys first-order series model. The kinetic parameters deduced from these models were used to simulate the lipase activity. There was a very good agreement between the simulated and experimental values.

1. Introduction

Synthesis of esters by enzyme catalysis has several advantages over chemical catalysis since enzymatic reactions need lower energy and provide enhanced selectivity and purity of the product [1]. Although aqueous enzymology has been widely practised for a long time, non-aqueous enzymology has been studied for about two and half decades for synthesis of fine-chemicals, agrochemicals, perfumes, flavors, pharmaceuticals and drugs. Non-aqueous enzymatic catalysis is particularly beneficial when the reactants are poorly soluble in aqueous media and the rates are extremely slow. When hydrolysis is likely to be significant in aqueous media, it can be suppressed by using non-aqueous enzymology [2,3].

Lipases have been employed for the synthesis of organic chemicals, mainly in aqueous media and in some cases non-aqueous media, because they are inexpensive, stable, and easy to recycle [4]. Lipases possess wide substrate specificity, have an ability to recognize chirality, and do not require labile cofactors [5,6]. They have been used to catalyze a number of reactions in non-aqueous media such as esterification, transesterification, amidation, hydrolysis, hydrazinolysis and epoxidation [5,7–16]. The versatility of lipase catalysis in the synthesis of other groups of chemicals needs to be explored. Lipases are known to catalyze the synthesis of amides from non-activated esters. *n*-Octyl alkylamides, for instance, have been synthesized in anhydrous hexane [17]. *Candida antarctica* lipase (CAL) was found to exhibit a very high activity and specificity in the acylation of primary amines with ethyl butyrate [18]. Fatty acid amides have been synthesized by using *Mucor miehei* lipase [19]. The fatty acid analogues of capsaicin have been synthesized by using different lipases and amongst them *Pseudomonas cepacia* lipase was the best [20]. In view of their high reactivity, hydrazides are important starting materials and intermediates in the synthesis of certain amides, aldehydes and heterocyclic compounds that are otherwise difficult to prepare. Hydrazides have also been used in analytical chemistry to identify carboxylic acids and to detect carbonyl compounds that form acyl hydrazones. The reactions between hydrazines and carboxylic acids or their esters are in principle similar to those of carboxylic acids and esters with amines [21].

The current work focuses on enzymatic hydrazinolysis of phenyl benzoate using immobilized lipases and deals with enzyme deactivation and kinetic modeling. There are no literature reports on the hydrazinolysis of phenyl benzoate by enzymes. The product benzoic acid hydrazide is commercially useful and the reaction can be also used as a model for hydrazonolysis of aromatic esters in non-aqueous media. Special emphasis is laid on the analysis of the stability and activity of the lipase under different substrate and product concentrations.

2. Experimental

2.1. Enzymes

All enzymes were procured as gift samples from reputed firms: Novozym 435 (Novo Nordisk, Denmark), Lipozyme IM 20 (Novo Nordisk, Denmark) and Lipozyme TL IM (Novo Nordisk, Denmark). Novozym 435 is *C. antarctica* lipase immobilized on a macroporous polyacrylic resin (activity 10 PLU/g). Lipozyme IM 20 is *M. miehei*

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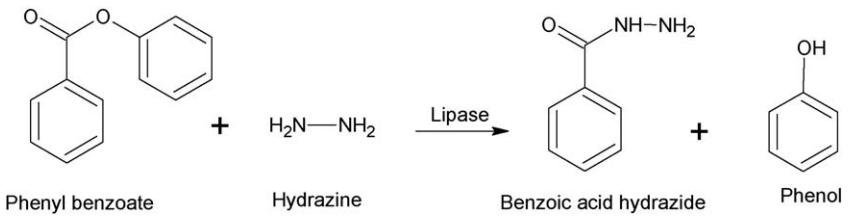

Scheme 1. Hydrazinolysis of phenyl benzoate.

immobilized on an anionic resin (activity 6 BAUN/g). Lipozyme TL IM is *Thermomyces lanuginosus* immobilized on silica (activity 75 IUN/g).

2.2. Chemicals

All chemicals including hydrazine hydrate, phenyl benzoate, dodecane, toluene and other analytical reagents were procured from M/s S.D. Fine Chemicals Pvt. Ltd, Mumbai, India.

2.3. Experimental setup

The experimental setup consisted of a 3 cm internal diameter fully baffled mechanically agitated glass reactor of 50 ml capacity, equipped with four baffles and a six-bladed turbine impeller. The entire reactor assembly was immersed in a thermostatic water bath and temperature was maintained at a desired value with an accuracy of ±1 °C. A typical reaction mixture consisted of 0.01 mol phenyl benzoate and 0.02 mol hydrazine hydrate diluted to 15 ml with toluene as solvent. The reaction mass was agitated at 50 °C for 15 min at a speed of 600 rpm and then 35 mg[1] of immobilized enzyme was added to initiate the reaction. Liquid samples were withdrawn periodically from the reaction mixture, filtered and analyzed by GC.

2.4. Analysis

The concentrations of the reactants and products were determined by GC (Chemito Model 8610) equipped with a flame ionization detector. A 4 m × 3.8 mm stainless steel column packed with SE-30 was used for analysis. Synthetic mixtures were prepared of pure samples and calibration was done to quantify the collected data for conversions and rates of reactions. Benzoic acid hydrazide was confirmed by GC–MS.

2.5. Determination of initial rates

To determine the initial rates of enzymatic reaction, the concentrations of phenyl benzoate were varied from 0.66 to 2.64 M for known concentrations of hydrazine (between 0.66 and 2.64 M) at 50 °C. The total volume was made to 15 ml with toluene, after which 35 mg Novozym 435 was added to initiate the reaction and the reaction was continued until 30% conversion. In brief, concentration versus time plots were made for different substrate concentrations. The derivative of concentration versus time profile at $t = 0$ gives the initial rate.

3. Results and discussions

The reaction is represented by Scheme 1.

3.1. Screening of different lipases

The activity of three commercially available lipases such as Novozym 435, Lipozyme TL IM and Lipozyme RM IM was evaluated in the synthesis of benzoic acid hydrazide from phenyl benzoate using the same conditions (Fig. 1). Novozym 435 was the best enzyme. Lipozyme IM showed very less activity as compared to Novozym 435. *Pseudomonas* lipase has been reported to be a very good catalyst for the hydrazinolysis of aliphatic esters [22], but it was ineffective for aromatic esters. There seems to be a substrate poisoning in the case of aromatic esters. In the case of *C. antarctica* lipase B (Novozym 435) the conversion was about 95% in 2 h as compared to 32% with *M. miehei* lipase (Lipozyme IM) and 25% with *T. lanuginosus* (Lipozyme TL). Therefore, in all further experiments Novozym 435 was used as the catalyst. The micro-environment around the active site pocket of Novozym 435 favors proper interaction of substrate in non-aqueous media. Since hydrazine hydrate was used, an equivalent amount of water was present in the reaction mass. Besides, the supported enzyme contains water in its pore space and thus a thin layer of water is present on the particle external surface. The flapping lid of *M. miehei* and *T. lanuginosus* projects into the binding pocket thereby creating steric hindrance in binding of phenyl benzoate at the active site which is called "interfacial inactivation" [23]. Since Novozym 435 is highly active in anhydrous media and does not contain a flapping lid, there is less steric hindrance as compared to other lipases.

3.2. Mass transfer analysis

The effect of speed of agitation was studied over the range of 200–800 rpm (Fig. 2). Initial reaction rates were nearly independent of speed at beyond 600 rpm. Thus the optimum speed was chosen as 600 rpm for further studies. Above 800 rpm initial rate of reaction was almost constant which indicates that rate of mass transfer is greater than rate of reaction. Although Fig. 2 clearly indicates that there were no external mass transfer limitations beyond 600 rpm, it was necessary to prove it theoretically also. Evaluation of the contributions of external solid–liquid mass transfer and intra-particle diffusion resistances was done by theoretical calculations. The liquid phase diffusivity of hydrazine (B) in toulene ($D_{SL;B}$) at 50 °C was

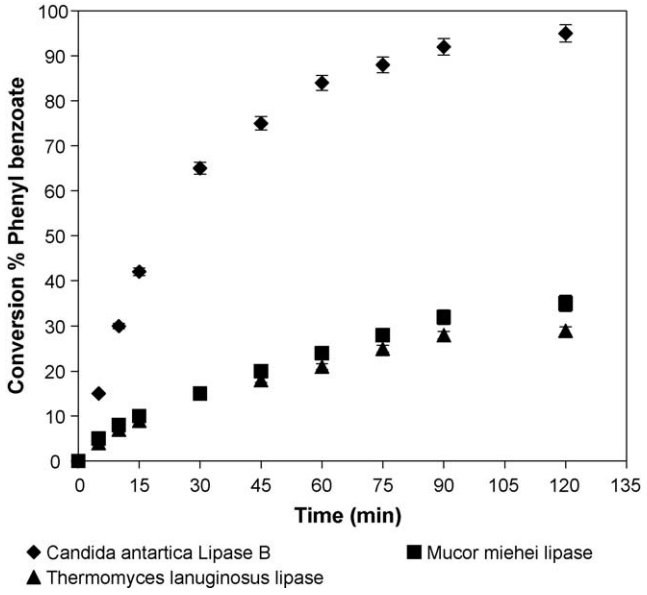

Fig. 1. Screening of different lipases; reaction conditions: phenyl benzoate—0.01 mol, hydrazine—0.02 mol, solvent toluene—up to 15 ml, speed of agitation—600 rpm, lipase—16.22 units/ml, temperature—50 °C.

[1] Novozym 435 loading $= \frac{35/1000\,\text{g} \times 7000\,\text{units/g}}{15\,\text{ml}} = 16.33\,\text{units/mL}$.

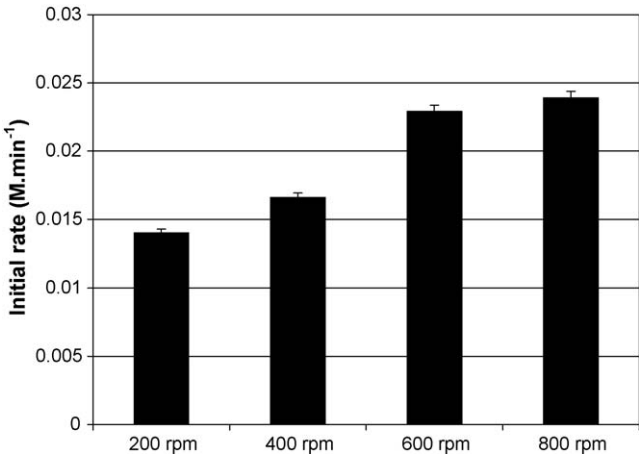

Fig. 2. Effect of speed of agitation; reaction conditions: phenyl benzoate—0.01 mol, hydrazine—0.02 mol, solvent toluene—up to 15 ml, temperature—50 °C. Novozym 435—16.22 units/ml.

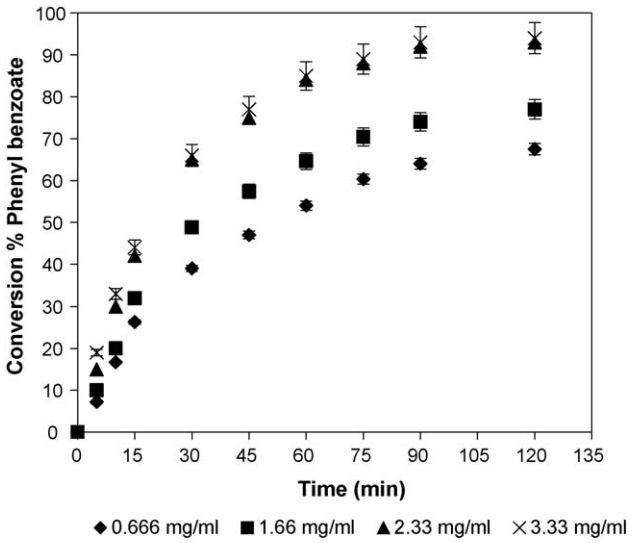

Fig. 3. Effect of catalysis loading; reaction conditions: phenyl benzoate—0.01 mol, hydrazine—0.02 mol, solvent toluene—up to 15 ml, speed of agitation—600 rpm, temperature—50 °C.

calculated by using Shiebel equation [24] as 2.43×10^{-9} m^2 s^{-1}. The value of solid–liquid mass transfer coefficient k_{SL} was calculated by assuming a limiting value of the Sherwood number, $Sh = k_{SL}d_p/D_S = 2$, for non-agitated systems. It should be noted that the actual Sherwood number, which is a function of Reynolds number and Schmidt number, would be much higher in well-agitated systems. However, for the sake of comparison and for orders-of-magnitude calculation, it is safe to take the lowest Sherwood number as 2 which is for quiescent liquids. Thus, $k_{SL;B}$ was calculated as 8.97×10^{-6} m s^{-1} for a particle size (d_p) of Novozym 435 of 0.06 cm. Similarly $D_{SL;A}$ was calculated as 1.54×10^{-9} m^2 s^{-1} for phenyl benzoate (A).

For solid (catalyst)–liquid reactions, the contribution of external mass transfer resistance was calculated in comparison with the reaction within the particle as is given by Yadav and Krishnan [25]. By using these values, external mass resistance (R_D) and internal reaction resistance (R_r) for hydrazine were evaluated as $R_r = 355.5$ and $R_D = 38.67$, indicating $R_r > R_D$. Since $R_r > R_D$ there was no external mass transfer limitation. It was also checked by applying the criteria for external mass transfer, given by Bailey and Ollis [26]. [($\eta_B = 0.68$, $\eta_A = 0.047$, $\phi_B = 1.143$, $\phi_A = 18$, $Bi_{B,} = 392$, $Bi_A = 175$), $(\eta\phi^2/Bi)_B = 0.0022 \ll 1$, $(\eta\phi^2/Bi)_A = 0.087 \ll 1$]. These calculations also indicated that the external mass transfer resistance was absent. Due to uniform particle size of catalyst, we were not able to study the effect of particle of different sizes. It was not advisable to crush the particles to study particle size effects, which would denature the enzyme. The Wiesz–Prater criterion or modulus (C_{WP}) was also used ($C_{WP} = 0.082 \ll 1$) [27]. C_{WP} was less than one suggesting that there was no intra-particle diffusion resistance. These theoretical calculations bolster the experimental observations that the reaction was intrinsically kinetically controlled.

3.3. The effect of carrier concentration

The effect of enzyme carrier concentration was studied from 0.666 to 3.33 mg/ml (Fig. 3). The experiments were conducted at a fixed amount of substrate by varying enzyme concentration. As the concentration of the Novozym 435 increased, the amount of benzoic acid hydrazide formed also increased. The maximum conversion of phenyl benzoate was observed after 2 h. For different concentrations of the enzyme, the initial rate and final conversions were calculated. The initial rate of reaction increased with increasing enzyme concentration and the overall conversion also increased correspondingly from 60% to 95%.

The sensitivity of initial rate of reaction to enzyme concentration is shown by the following power-law model equation:

$$v_i = k_{E1}[E]^{k_{E2}} \quad (1)$$

From the above equation we have

$$\ln(v_i) = k_{E2}\ln([E]) + \ln(k_{E1}) \quad (2)$$

From Eq. (2) kinetic constants were calculated by plotting $\ln(v_i)$ versus [E] (not shown) to get the following.

$$v_i = 0.0045 \times [E]^{0.4038} \quad (3)$$

The above model was valid with the range of 0.66–3.33 mg/ml of enzyme loading. The above power-law model shows an empirical correlation between enzyme activity (the initial rate) and its concentration. It is expected that the rates should be linear in enzyme concentration, if there is no onset of inhibition or the enzyme does not get activated immediately and requires some time for activation if not incubated with the substrates. The proposed mechanism, which is discussed in subsequent section, shows a complex network. Therefore effect of incubation and inhibition was studied independently and is explained systematically.

3.4. Effect of temperature

The hydrazinolysis activity of Novozym 435 was monitored in the range of 30–60 °C (Fig. 4). A maximum conversion of 95% was obtained at 60 °C. A preliminary estimate of the activation energy was done from the observed initial rates at different temperatures under otherwise similar conditions. The initial rates can also be used if all other variables except temperature are maintained constant. It is equivalent to plotting pseudo-rate constants, which are functions of temperature. Fig. 5 shows the Arrhenius plot obtained by plotting the ln(initial rate) values against the reciprocal reaction temperature. The activation energy was found to be 6.07 kcal/mol. Several authors have reported activation energy for enzymatic reaction from 0.9 to 9 kcal/mol. Thus, the activation energy value obtained for enzymatic synthesis of phenyl benzoate is comparable to that reported for enzymatic reaction [26].

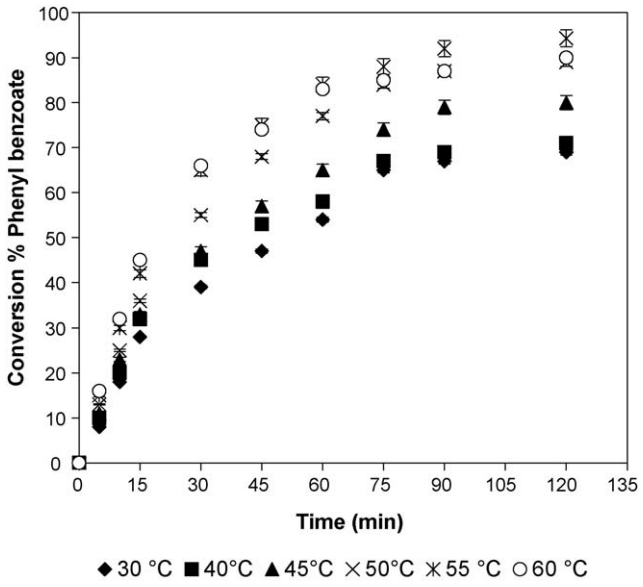

Fig. 4. Effect of temperature; reaction conditions: phenyl benzoate—0.01 mol, hydrazine—0.02 mol, solvent toluene—up to 15 ml, speed of agitation—600 rpm, catalyst—35 mg.

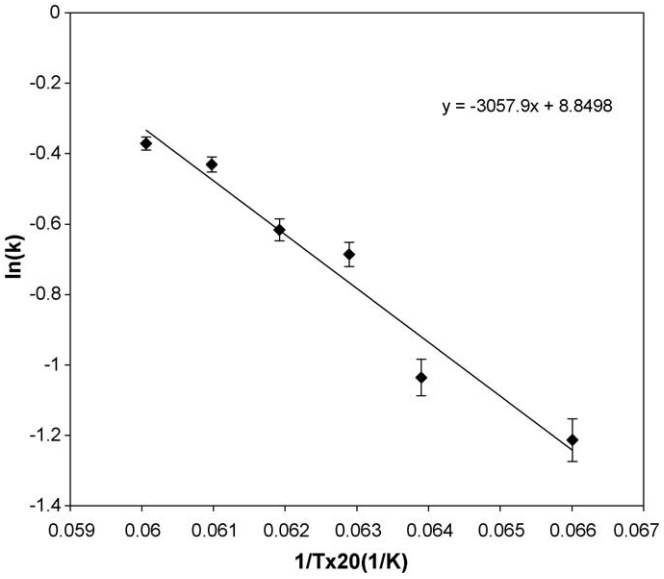

Fig. 5. Arrhenius plot.

3.5. Effect of mole ratio of hydrazine

The effect of mole ratio of phenyl benzoate to hydrazine was studied by using Novozym 435. In one set of experiments, different moles of hydrazine were used in the range of 0.01–0.04 mol, whereas the amount of phenyl benzoate was kept constant at 0.01 mol. In all these experiments, the total volume of the liquid phase was the same. The initial rate is plotted against mole ratio in Fig. 6. The initial rate was practically the same for a mole ratio of 1:4 and 1:3. This would suggest that all sites are occupied by hydrazine at 1:3 and thus additional concentration of hydrazine does not increase the rate since no sites are available or hydrazine concentration beyond a certain value has an inhibitory effect. It will be discussed separately.

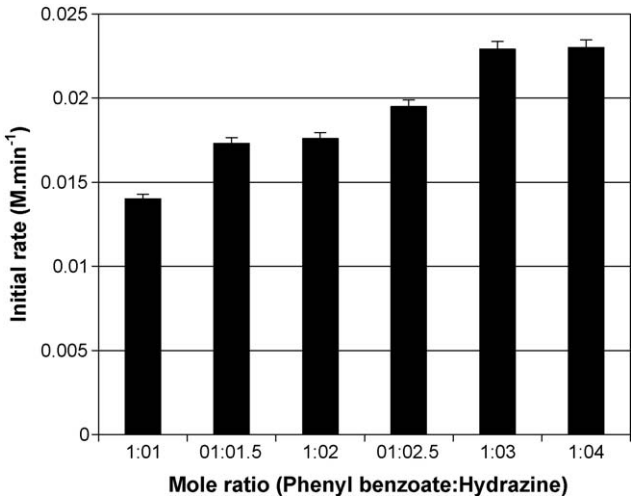

Fig. 6. Effect of mole ratio of hydrazine; reaction conditions: phenyl benzoate—0.01 mol, solvent toluene—up to 15 ml, speed of agitation—600 rpm, temperature—50 °C, catalyst—35 mg.

3.6. Reusability of enzyme

After each reuse, the enzyme was washed with toluene and solvent was evaporated prior to reuse. The activity of lipase was decreased by almost 13% after first reuse and then it was gradually decreased during further reuses. Lipases are known to be deactivated by various physical parameters and chemicals [12]. The reduction in activity after each reuse indicates that the enzyme is strongly inhibited by substrate and product (Fig. 7). However, it is possible that hydrazine, toluene, phenyl benzoate might also have caused denaturation. Extensive study of lipase deactivation in toluene was previously studied by us in whom it was found that there was no deactivation of lipase by toluene [12]. The detailed analysis regarding deactivation due to various factors is discussed in kinetic modeling part.

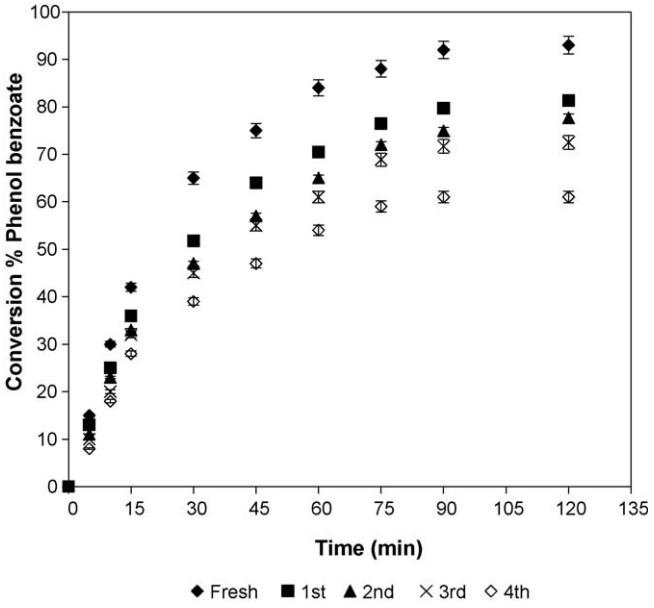

Fig. 7. Reusability of lipase; reaction conditions: phenyl benzoate—0.01 mol, hydrazine—0.02 mol, solvent toluene—up to 15 ml, speed of agitation—600 rpm, temperature—50 °C, catalyst—35 mg.

4. Kinetic modeling

4.1. Kinetic modeling for hydrazinolysis

Reactions were carried out under standard conditions in toluene, as described earlier. The double reciprocal plot of reciprocal of rate versus reciprocal of concentration of phenyl benzoate is illustrated in Fig. 8. It was observed that the initial reaction rate had increased as the concentration of phenyl benzoate (A) was increased. When hydrazine (B) concentration was increased, the effect of substrate inhibition by hydrazine was notable which caused the reaction rate to fall. It suggests that both slope and intercepts are affected in a non-competitive inhibition [26]. The plot also shows that as the concentration of hydrazine is increased, the slope increases and intercepts on $1/v$ axis decrease. The inhibition is due to the formation of a dead end complex. A typical reaction sequence is shown below. According to it, the lipase (E) may react with hydrazine [B] to yield a dead end complex (BE) or it may bind to A site to give AE. BE can bind with B to form another dead end complex BEB. Similarly AE can bind with B to form complex EAB which gives rise to either the product P and Q or a complex EB and A. Thus, EB can react with B again to give the dead end complex BEB [28,29]. The reaction sequence may thus be depicted as follows:

$$\begin{array}{ccccccc} BE & \overset{K_i}{\rightleftharpoons} & B & + E + & A & \overset{K_{mA}}{\rightleftharpoons} & EA \\ + & & + & & + & & \\ B & & B & & B & & \\ \downarrow\uparrow \beta K_{mB} & & \downarrow\uparrow K_{mB} & & \uparrow\downarrow \alpha K_{mB} & & \\ BEB & \overset{\beta K_i}{\rightleftharpoons} & B + EB + & A & \overset{\alpha K_i}{\rightleftharpoons} & EAB \xrightarrow{k_p} & E + P + Q \end{array} \quad (4)$$

where, A is phenyl benzoate; B is hydrazine; EA is enzyme phenyl benzoate complex; BE is dead end enzyme hydrazine complex; BEB is dead end enzyme hydrazine complex; EB is effective enzyme hydrazine complex; EA is effective enzyme phenyl benzoate complex; EAB is effective enzyme phenyl benzoate hydrazine complex; P is phenol and Q is benzoic acid hydrazide.

The final equation for the above reaction sequence is [29]:

$$v = \frac{v_m[A]}{\alpha K_{mA}\left(1 + \frac{K_{mB}}{[B]} + \frac{K_{mB}}{K_i} + \frac{[B]}{\beta K_i}\right) + [A]\left(1 + \frac{\alpha K_{mB}}{[B]}\right)} \quad (5)$$

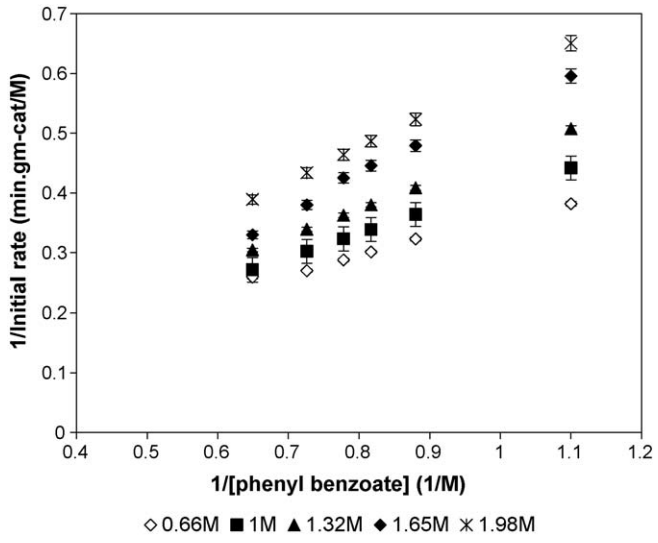

Fig. 8. Linewear–Burk plot for different hydrazine concentrations.

Table 1
Kinetic parameters for hydrazinolysis of phenyl benzoate with hydrazine.

Kinetic constants	Value by Polymath 5.1
v_m (M min^{-1})	2.340
K_{mA} (M)	2.837
K_{mB} (M)	1.263
K_i (M)	4.084
α	0.121
β	0.0623

The values represent an average of duplicates with standard error <10%.

where v is the rate of reaction (M min^{-1}), v_m is the maximum rate of reaction (M min^{-1}), $[A_0]$ is the initial concentration of phenyl benzoate (M), $[B_0]$ is the initial concentration of hydrazine (M), K_{mA} is the Michaelis constant for phenyl benzoate (M), K_{mB} is the Michaelis constant of hydrazine (M), K_i is the inhibition constant of hydrazine (M), α and β are the dimensionless constants.

The data from initial rate measurement were used for the optimization of parameters by least square error estimation using the software Polymath 5.1. The values of the kinetic parameters obtained from non-linear regression analysis are given in Table 1. From this detail kinetic modeling it was observed that affinity of hydrazine is greater as compared to phenyl benzoate. The high values of concentration used are also reflected in the Michaelis constants. These are certainly intrinsic parameters since the mass transfer effects were all eliminated. These values are valid for the concentration range used. A plot of experimental rates versus simulated rates shows that the proposed model fits the experimental data very well (Fig. 9). There was some departure at low values of rates. The reason for the departure at lower value of rate is due to less influence of the inhibition at lower concentrations of the reactants, particularly hydrazine and phenol. Novozym 435 is strongly inhibited by hydrazine. Values of dimensional less parameter indicate that the rate of enzyme–acetate–hydrazine ternary complex formation is greater than that of hydrazine–enzyme–hydrazine ternary complex.

4.2. Kinetic modeling for deactivation of lipase

A number of parameters were studied to understand the deactivation of Novozym 435 [12]. Novozym 435 lipase was

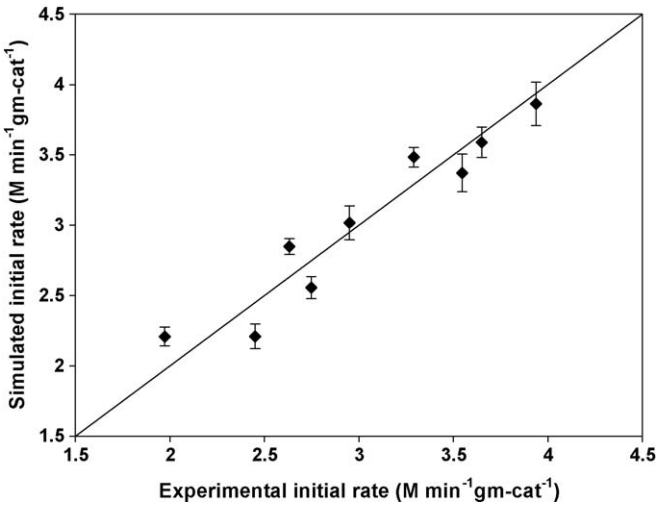

Fig. 9. Parity plot for enzymatic kinetic model.

stirred with the solvent along with hydrazine and phenol individually for 120 min at 30 °C and the reaction was initiated by adding appropriate reactant, and found that the enzyme get deactivated by hydrazine and phenol. Enzyme activity was tested for different time intervals such as 10, 30, 60, 90 and 120 min. The activity of enzyme after 10 min incubation was almost same as fresh enzyme (without incubation). For the above reason we have not integrated kinetic model with deactivation model. The same sets of experiments were carried out to analysis whether the deactivation was due to the other remaining reactants and products. It was found that in the presence of phenyl benzoate and benzoic acid hydrazide, lipase showed no loss in activity.

The multiphasic nature of the protein molecule during the deactivation process strengthens the suggestion that complex internal events take place during its conformational transition. In this way, a series-type deactivation model involving two first-order steps with one active precursor and a final enzyme state with possible non-zero activity was considered for analyzing lipase deactivation. The overall equation for the deactivation of Novozym 435 by hydrazine and phenol is generally represented by the following equations:

$$E + B \xrightarrow{k_{d1}} E'_B \tag{6}$$

$$E'_B + P \xrightarrow{k_{d2}} E'_{BP} \tag{7}$$

$$E_0 \xrightarrow{k_{d1}} E'_B \xrightarrow{k_{d2}} E'_{BP} \tag{8}$$

where E_0, E, E'_B and E'_{BP} are enzyme states at different stages such initial, at time t, deactivation due to hydrazine and phenol, respectively; k_{d1} and k_{d2} are first-order deactivation rate constants (min^{-1}).

From Eq. (6)

$$\frac{dE}{dt} = -k_{d1}E \tag{9}$$

$$\frac{dE'_B}{dt} = k_{d1}E - k_{d2}E'_B \tag{10}$$

$$\frac{dE'_{BP}}{dt} = -k_{d2}E'_B \tag{11}$$

By integrating Eq. (9)

$$\frac{E}{E_0} = e^{-k_{d1}t} \tag{12}$$

Rearranging Eq. (10)

$$\frac{dE'_B}{dt} + k_{d1}E'_B = k_{d2}E \tag{13}$$

which is first-order linear differential equation of the form

$$\frac{dy}{dx} + Py = Q \tag{14}$$

By integrating above Eq. (13) we get

$$\frac{E'_B}{E_0} = \frac{k_{d1}}{k_{d2} - k_{d1}}(e^{-k_{d1}t} - e^{-k_{d2}t}) \tag{15}$$

initial enzyme concentration
= free enzyme concentration
+ deactivated enzyme concentration due to hydrazine
+ deactivated enzyme concentration due to phenolE_0
$= E + E'_{BP} + E'_B$ (16)

Table 2
Different kinetic parameters for deactivation of lipase.

Kinetic constants	Value by Polymath 5.1
k_{d1} (min^{-1})	0.0028
k_{d2} (min^{-1})	1.8079

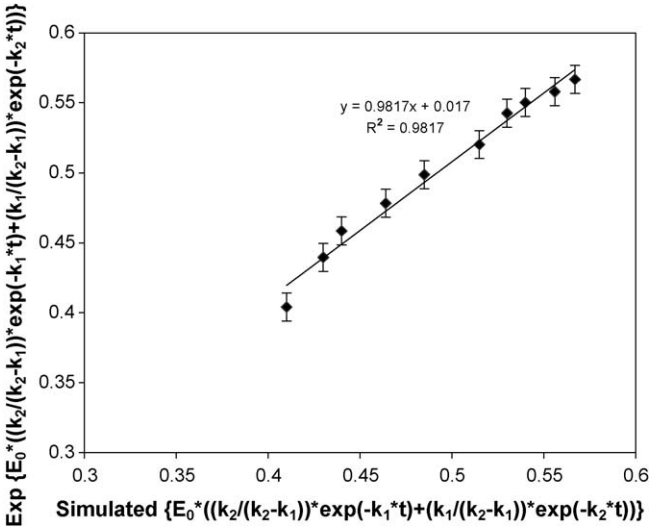

Fig. 10. Validation of deactivation model.

Rearranging

$$E'_{BP} = E_0 - E - E'_B \tag{17}$$

From Eqs. (9) and (14) we get

$$E'_{BP} = E_0\left(1 + \frac{k_{d2}}{k_{d1} - k_{d2}}e^{-k_{d1}t} + \frac{k_{d1}}{k_{d2} - k_{d1}}e^{-k_{d2}t}\right) \tag{18}$$

total concentration enzyme
= concentration of deactivated enzyme
+ concentration of active enzyme
$E_0 = E'_{BP} + E_{BP}$ (19)

Therefore

$$E_{BP} = E_0 - E'_{BP} \tag{20}$$

From Eqs. (18) and (20)

$$E_{BP} = E_0\left(\frac{k_{d2}}{k_{d2} - k_{d1}}e^{-k_{d1}t} + \frac{k_{d1}}{k_{d1} - k_{d2}}e^{-k_{d2}t}\right) \tag{21}$$

This is the final equation active enzyme concentration. It should be noted that E_{BP} is the concentration of active enzyme. Quantification of active enzyme is based on initial rate measurement. Activity based on initial rate of fresh enzyme without any deactivation is considered as (E_0). Thus, E_{BP} is the concentration of active enzyme after deactivation ($E_0 - E'_{BP}$). Here E'_{BP} is the total enzyme deactivated by hydrazine and phenol.

Kinetic parameters were refined by using Polymath 5.1. Enzyme was incubated in hydrazine and phenol for different time intervals. For each incubation time the activity was evaluated. Knowing the experimental initial rates with respect to incubation time, all the rate constants were calculated by applying non-linear regression to rate equation using Polymath 5.1. The best-fit values obtained are tabulated in Table 2.

These values were then used to generate simulated initial rate values. Simulated values of lipase activity so obtained were compared with experimental values (Fig. 10). From these rate

constants it is clearly seen that the rate of deactivation with respect to phenol is greater than that of hydrazine hydrate.

5. Conclusions

Synthesis of benzoic acid hydrazide was conducted by employing different lipases, amongst which Novozym 435 was found to be the most active catalyst. The effects of various parameters on the conversion and rates of reaction were studied with Novozym 435 as the catalyst and toluene as the solvent. Initial rate and progress curve data were used to arrive at a suitable model and various parameters were estimated. The apparent fit of the kinetic data to the assumed ordered bi–bi dead end complex with hydrazine provides support for the mechanism. This model was used to simulate the rate data, which were in excellent agreement with experimental values. The deactivation study demonstrated that the enzyme was deactivated by hydrazine and phenol. The rate constants clearly indicate that the rate of deactivation of enzyme with respect to phenol is greater than that of hydrazine hydrate. The kinetic parameters deduced from deactivation model were used to simulate the initial rate, which are in good agreement with the experimental values.

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Appendix A. Nomenclature

A	phenyl benzoate
ABE	ternary complex of the enzyme, hydrazine and phenyl benzoate
AE	enzyme–phenyl benzoate complex
B	hydrazine
Bi	Biot number
$D_{SL;A}$	liquid phase diffusivity of phenyl benzoate ($cm^2\ s^{-1}$)
$D_{SL;B}$	liquid phase diffusivity of hydrazine ($cm^2\ s^{-1}$)
d_p	diameter of enzyme particle (cm)
E	free enzyme
E_0	initial concentration of enzyme,
EQ	enzyme–benzoic acid hydrazide complex
E_{BP}	concentration of active enzyme
E'_{BP}	total enzyme deactivated by hydrazine and phenol
K_i	inhibition constant
k_{d1}	deactivation constant due to hydrazine (min^{-1})
k_{d2}	deactivation constant due to phenol (min^{-1})
k_{E1},k_{E2}	constants in Eq. (1)
K_i	inhibition constant
K_{mA}	Michaelis constant for phenyl benzoate (M)
K_{mB}	Michaelis constant for hydrazine (M)
$k_{SL;A}$	liquid side mass transfer coefficient for phenyl benzoate ($cm\ s^{-1}$)
$k_{SL;B}$	liquid side mass transfer coefficient for hydrazine ($cm\ s^{-1}$)
P	phenol
Q	benzoic acid hydrazide
R	gas constant ($KJ\ mol^{-1}\ K^{-1}$)
R_D	external mass transfer resistance
R_r	internal reaction resistance
v	rate of reaction ($M\ min^{-1}$)
v_i	initial rate of reaction ($M\ min^{-1}$)
v_{max}	maximum velocity in enzymatic step ($M\ min^{-1}$)
X	fractional conversion

Greek letters

η	effectiveness factor
ϕ	Thiele modulus
α	dimensionless constant
β	dimensionless constant

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