Journal of Molecular Catalysis B: Enzymatic 72 (2011) 150–156

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Synthesis of short chain alkyl esters using cutinase from *Burkholderia cepacia* NRRL B2320

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ARTICLE INFO

Article history:
Received 8 April 2011
Received in revised form 30 May 2011
Accepted 30 May 2011
Available online 22 June 2011

Keywords:
Cutinase
Burkholderia cepacia
Short-chain fatty acid
Kinetics
Ping–Pong Bi Bi model

ABSTRACT

Short chain alkyl esters are well appreciated for fruity flavors they provide. These are mainly applied to the fruit-flavored products like jam, jelly, beverages, wine and dairy. Cutinase from *Burkholderia cepacia* NRRL B 2320 was found to be active in catalyzing the synthesis of alkyl esters in organic solvent. The optimal temperature range for the enzyme catalyzed synthesis was found to be from 35 °C to 40 °C. The maximum conversion (%) during synthesis of ester was obtained for butyric acid (C4) and valeric acid (C5) with butanol reflecting the specificity of the enzyme for short-chain length fatty acids. In case of alcohol specificity, butanol was found to be most preferred substrate by the enzyme and conversion (%) decreased with increasing carbon chain length of alcohol used in the esterification reaction. The kinetic analysis for the synthesis of butyl butyrate by varying concentration of one substrate at a time (butanol or butyric acid), showed that Ping–Pong Bi Bi model with acid inhibition and influence of initial water is most suitable model for the prediction of the reaction kinetics.

1. Introduction

Esters of short chain fatty acids and alcohols are important components of natural aromas used in the food industry. They are employed in fruit-flavored products like beverages, candies, jam, jelly, baked food, wines, butter, cream, yoghurt, cheese etc. Among the various esters butyl butyrate, ethyl butyrate significantly contribute the aroma of pineapple, apple, banana while butyl octanoate used for herbal flavor. Hexyl ester (hexyl butyrate) is an ingredient of fruit, beer and citrus aromas. Octyl ester (octyl butyrate) imparts flavor of peach and strawberry [1]. Although these important products are currently synthesized by traditional means of chemical method, which is still the most economical method, but it uses aggressive chemical catalysis and generates by-products during high temperature reaction [2,3]. Natural aromas obtained from plant extract consist of a mixture of different flavor compounds. These natural aromas are very expensive due to their low concentration in the natural product and very low extraction yields [4]. In contrast, the chemical synthesis of these fatty acid esters is economical but these are not classified as natural products as their synthesis requires strong acids or alkali as catalyst and high temperature and pressure. In comparison to the direct synthesis of esters from fatty acids and alcohols, enzymatic means has been suggested as a good alternative due to an energy-saving procedure with high selectivity [4]. The enzymatic approach allows mild reaction conditions and the resulting products are classified as natural by food regulatory agencies, a feature that increased their public acceptance as ingredients for food industry when compared to those synthesized by chemical processes. The synthesis of esters by enzymes can be carried out in a native enzyme suspension in an organic solvent or in a solvent-free system [5].

Until now, lipases and esterases are used for the production of a wide range of ester products in non-conventional media. But most of the lipases have higher affinity for longer-chain length substrates, and low molecular weight substrate may have some inhibitory effect on enzymes [6,7]. In case of esterases, except few, most esterases were lost their reactivity when the chain length of substrate exceeds two [3,8]. Recently, few reports also available where cutinases (EC 3.1.1.74), the hydrolytic enzymes and the smallest member of α/β hydrolase family were employed for synthesis of some alkyl esters [4,9–15]. Cutinases are believed to be a group of enzyme intermediate between lipase and esterase, which are able to hydrolyze cutin polymer, soluble esters and emulsified triacylglycerol [16]. Cutinases mostly belong to the group of serine-hydrolase family, which contains serine group in its active site. As the reaction is reversible, the enzymes can also catalyze the formation of alcohols and fatty acids from esters. In aqueous solutions, the equilibrium is strongly shifted towards the starting reagents and esters cannot be synthesized. To overcome this difficulty, reaction media containing very small amounts of water or comprised of organic solvents can be used to bring about a chemical equilibrium

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doi:10.1016/j.molcatb.2011.05.013

shift towards ester. This reversible reaction was previously shown to follow a Ping–Pong Bi Bi mechanism [14,17].

Cutinase was previously isolated and purified from *Burkholderia cepacia* NRRL B 2320. In this investigation, we have studied the *B. cepacia* cutinase catalyzed synthesis of alkyl esters in isooctane solvents under various conditions. Here, we have also studied the kinetics for the synthesis of butyl butyrate from butyric acid and butanol in isooctane.

2. Materials and methods

Butanol, pentanol, hexanol, octanol, butyric acid, valeric acid, caproic acid and octanoic acid purchased from sigma. Isooctane (Merck) was dried over 4 Å molecular sieve. Tris (hydroxymethyl) amino methane (Tris) was also purchased from Sigma. The cutinase enzyme used for reaction was isolated from *B. cepacia* NRRL B 2320 (previously known as *Pseudomonas cepacia*).

2.1. Production and purification of enzyme

The following medium was used for the production of cutinase ($g\,l^{-1}$): beef extract, 4.0; peptone, 17.8; urea, 5.0; KH_2PO_4, 3.0; KCl, 0.64; $MgSO_4{\cdot}7H_2O$, 5.55 and cutin 10.1. Initial pH of the medium was adjusted at 7.0. A 2% of inoculum from the seed culture was added to 200 ml of the production medium in 1000 ml Erlenmeyer flasks. The flasks were then incubated in a shaking incubator at 28 °C and 180 rpm. After 96 h of fermentation, cells were centrifuged at 8000 rpm for 10 min at 4 ± 1 °C to obtain the cell free broth containing extracellular cutinase. All purification steps were carried out at 0–4 °C unless otherwise indicated. All chromatographic runs were monitored for protein at 280 nm. The enzyme was purified in four steps. The crude enzyme was precipitated with finely powdered ammonium sulphate at 80% saturation. The precipitate was collected by centrifugation at 8000 rpm for 30 min and dissolved in minimal amount of 20 mM Tris–HCl buffer (pH 8.0) and dialyzed against the same buffer for 24 h. Dialyzed ammonium sulphate fraction was applied to a 2 cm × 50 cm CM-650 TOYOPEARL® column (Tosho Corporation, Tokyo, Japan) and eluted using linear gradient of NaCl (0–500 mM). After ion exchange chromatography two steps of gel filtration chromatography was used (Sephadex G100 and Sephacryl S-300 (Sigma)) to get purified homogenous cutinase enzyme. This purified enzyme was used for further study.

2.2. Cutinase assay

The cutinase hydrolytic activity was measured by following the hydrolysis of p-nitrophenyl butyrate (p-NPB) (Sigma) as substrate. An aliquot of (0.02 ml) culture supernatant was added to 0.98 ml of reaction mixture, which was prepared by adding 1 ml 23 mM pNPB in tetrahydrofuran to 40 ml of 50 mM potassium phosphate buffer containing 11.5 mM sodium deoxycholate. The reaction was monitored for 15 min at 37 °C and absorbance of released p-nitrophenol was measured at 410 nm. One enzyme unit is defined as the amount of enzyme required to release 1 μM of p-nitrophenol per min under assay conditions. The cutinase production from *B. cepacia* NRRL B 2320 was confirmed previously (data not shown) using cutinase specific substrate, p-nitrophenyl (16 methyl sulphone ester) hexadecanoate (p-NMSH).

2.3. Enzymatic synthesis of alkyl esters

In the standard protocol, ester synthesis was carried out in screw-capped test tubes as bioreactors. Unless otherwise specified, 0.1 mM of enzyme in lyophilized powder form (calculated on the basis of molecular mass of enzyme as obtained from SDS PAGE 26.5 kDa) was added to 2.5 ml of isooctane containing 0.25 M fatty acid and 0.25 M alcohol. All reagents were previously dried over 4 Å molecular sieves. The tubes were kept in a horizontal shaker at 37 °C. At regular intervals, 300 μl of the mixture (for butyric acid) or 100 μl (for all other acids) were transferred to 2 ml micro centrifuge tube and centrifuged at 10,000 rpm for 1 min to remove the suspended enzyme particles. The progress of reaction was determined by measuring the decreasing profile of acids by titration with 0.01 N NaOH using phenolphthalein as indicator and also with Lowry and Tinsley method [18]. According to Lowry and Tinsley method 700 μl (for butyric acid) or 900 μl (for all other acids) isooctane was added to the samples. Cupric acetate aqueous solution (0.2 ml) containing pyridine (5%, w/v, pH 6.0) was then added into the tube and the solutions were vigorously mixed for 1 min using a vortex mixture. After centrifugation at 2500 rpm for 5 min, the upper organic phase was measured by a UV/visible spectrophotometer (Cary 100, Varian) at 715 nm. The formation of ester was also confirmed by gas chromatography. Water percentage in the reaction mixture was determined by Karl Fisher titration method [2,4]. All reactions and analysis were performed in duplicate.

2.3.1. GC analysis of esters

Synthesis of fatty acid ester was analyzed by gas chromatograph (Varian 390). The diluted aliquots of the reaction mixture were injected into CPSIL 8CB column and compounds were detected by FID. The injector and detector temperature were set at 250 °C. The program (temperature and time) and retention time (t_R) used for different esters are given below.

- Butyl butyrate: 150 °C for (0.5 min) −15 °C/min-250 °C (10 min); t_R 2.42 min
- Butyl valerate: 150 °C for (0.5 min) −15 °C/min-250 °C (10 min); t_R 3.14 min
- Butyl hexanoate: 150 °C for (0.5 min) −15 °C/min-250 °C (10 min); t_R 4.38 min
- Butyl octanoate: 150 °C for (0.5 min) −30 °C/min-250 °C (10 min); t_R 6.65 min
- Butyl decanoate: 150 °C for (0.5 min) −30 °C/min-270 °C (10 min); t_R 15.5 min
- Butyl Palmitate: 150 °C for (0.5 min) −30 °C/min-300 °C (10 min); t_R 20.3 min
- Ethyl butyrate: 100 °C for (0.5 min) −15 °C/min-150 °C (10 min); t_R 1.36 min
- Ethyl valerate: 100 °C for (0.5 min) −15 °C/min-150 °C (10 min); t_R 2.55 min
- Ethyl hexanoate: 50 °C for (0.5 min) −30 °C/min-160 °C (10 min); t_R 2.15 min
- Pentyl butyrate: 150 °C for (0.5 min) −30 °C/min-250 °C (10 min); t_R 3.58 min
- Hexyl butyrate: 150 °C for (0.5 min) −30 °C/min-250 °C (10 min); t_R 4.18 min
- Octyl Butyrate: 150 °C for (0.5 min) −30 °C/min-250 °C (10 min); t_R 5.43
- Decyl butyrate: 100 °C for (0.5 min) −30 °C/min-150 °C (10 min); t_R 2.20 min

2.4. Kinetic study for butyl butyrate synthesis

Originally Michaelis–Menten equation was derived for kinetics of single substrate reaction. However, a reaction involving two substrates may also thought to obey the Michaelis–Menten kinetics, if the reaction rate depends on the concentration of both the substrates, so that if one substrate concentration varied while other maintaining constant, the reaction behaves like a single substrate reaction obeying Michaelis–Menten kinetics.

Previously, lipase or cutinase catalyzed esterification reactions have been described by Ping–Pong Bi Bi kinetic model Eq. (1).

$$v = \frac{V_{max}}{1 + (K_{mA}/[A]) + (K_{mB}/[B])} \quad (1)$$

where $[A]$ and $[B]$ are the initial concentration of butyric acid and butyl alcohol, respectively, v is the initial reaction, V_{max} is the maximum reaction rate, K_{mA} and K_{mB} are the Ping–Pong constants for the butyric acid (A) and the butyl alcohol (B).

The competitive inhibition by alcohol [19] or acid [20,21] or both alcohol and acid leads to the use of following modified Michaelis–Menten equations.

$$v = \frac{V_{max}}{1 + K_{mA}/[A](1 + [B]/K_{IB}) + K_{mB}/[B]} \quad (2)$$

Here, K_{IB} is the inhibition constant for alcohol.

$$v = \frac{V_{max}}{1 + K_{mB}/[B](1 + [A]/K_{IA}) + K_{mA}/[A]} \quad (3)$$

Here, K_{IA} is the inhibition constant for butyric acid,

$$v = \frac{V_{max}}{1 + K_{MA}/[A](1 + [B]/K_{IB}) + K_{MB}/[B](1 + [A]/K_{IA})} \quad (4)$$

The presence of water (one of the product) at the start of the reaction modifies the Eqs. (2)–(4) to the following equations (Eqs. (5)–(7)) [19].

$$v = \frac{V_{max}}{1 + (K_A/[A] + K_{AB}/[A]\cdot[B])(1 + [B]/K_{IB}) + K_{BP}/[B]} \quad (5)$$

$$v = \frac{V_{max}}{1 + (K_A/[A] + K_{AB}/[A]\cdot[B]) + K_{BP}/[B](1 + [A]/K_{IA})} \quad (6)$$

$$v = \frac{V_{max}}{1 + (K_A/[A] + K_{AB}/[A]\cdot[B])(1 + [B]/K_{IB}) + K_{BP}/[B](1 + [A]/K_{IA})} \quad (7)$$

where K_{AB} is the constant term contributing forward and reverses reaction of esterification, K_{BP} is the modified K_{MB} due to water inhibition. Microsoft 2003 excel solver used to solve the equations.

3. Results and discussion

The enzyme was purified approximately 89-fold with an overall yield of 17.6% and specific activity of 5360 U mg^{-1} and found to be homogeneous, as evident from SDS-PAGE (data not shown). The molecular mass of cutinase was found to be 26.5 kDa by SDS-PAGE analysis. The amount of enzyme used for esterification reaction was selected on the basis of previous report [15] where kinetic study for the synthesis of ethyl caproate was carried out with 0.1 mM cutinase (*F. solani pisi*).

3.1. Effect of temperature on ester synthesis

The effect of temperature on cutinase activity was evaluated in the range of 25–50 °C, for butyric and valeric acid, with butanol. The conversion (%) was the maximum in the temperature range of 35–40 °C (Fig. 1) and above 40 °C decrease in conversion was observed. The optimum temperature for the esterification reaction was found to be 37 °C. The highest conversion of 94.6% and 87.5% was achieved at 37 °C after 12 h of incubation for butyric acid and valeric acid, respectively. At higher temperatures, the conversion was decreased. For the hydrolysis of synthetic ester, cutinase showed its maximum activity at the range of 35–45 °C (data not shown). Santos and Castro [22] could achieve 75% conversion at 41 °C for the *Candida rugosa* lipase catalyzed reaction of butyric acid and butanol. The maximum of 95% conversion also reported for

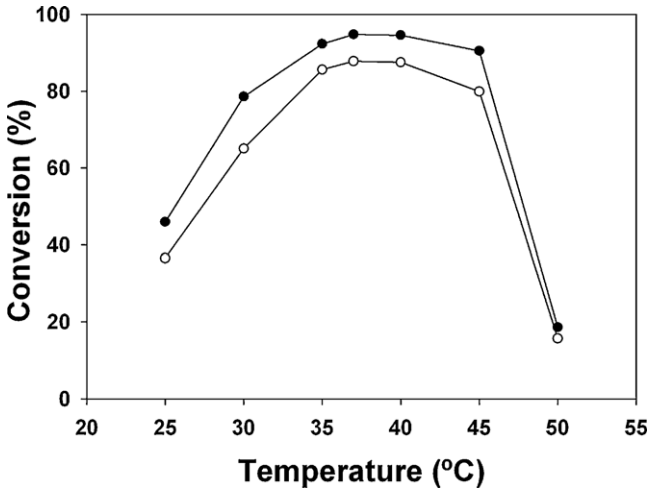

Fig. 1. Effect of temperature on synthesis of butyl esters. Butyl butyrate (●), and butyl valerate (○).

Bacillus licheniformis esterase catalyzed synthesis of ethyl caproate at 37 °C in n-heptane [3].

3.2. Effect of water content

Water content is another important parameter in esterification reactions in non-conventional medium. Water molecules maintain the three-dimensional tertiary conformation of enzymes to retain their activity and it also controls the thermodynamic equilibrium of the enzymatic reactions. However, water is a second product during the reaction of esterification. Generally, low water content favours the synthesis over hydrolysis during esterification reaction. Previously several reports are available on the effect of water content on the esterification reaction by lipase or cutinase [2–4]. To study the effect of water addition on the reaction rate, 0–6% (w/w) was added to the reaction media (Fig. 2). The conversion was increased to 95% upto 2% water and then decreased sharply. This result agrees with the fact that a minimal quantity of water is necessary to maintain the active conformation of the enzyme. At higher water concentrations, the reverse hydrolysis reaction was initiated and therefore decrease in net production of esters was observed. Stimulation of enzyme activity in the presence of a limited concentration of water has also been reported for other lipases [2,3] and cutinases [4] as well.

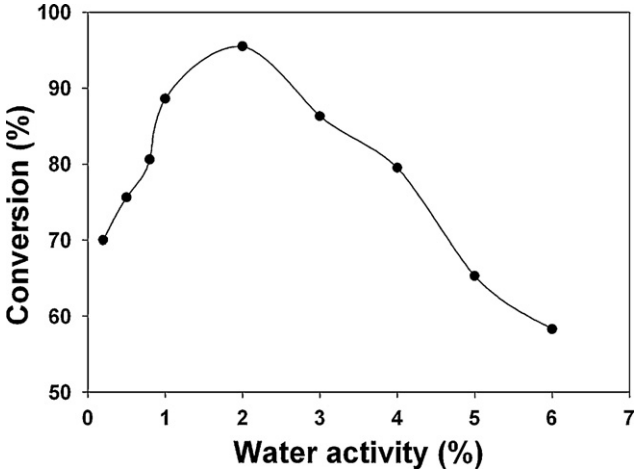

Fig. 2. Effect of water activity (%, w/w) on synthesis of butyl butyrate.

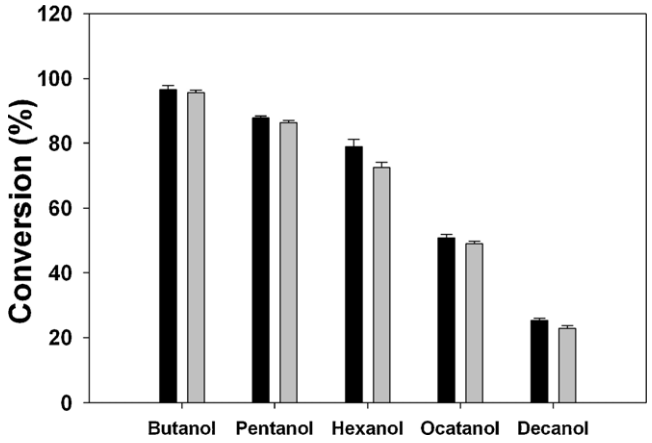

Fig. 3. Effect of alcohol chain length on synthesis of alkyl butyrate. Alcohol/acid molar ratio, R = 1 (alcohol 0.1 M and butyric acid 0.1 M) (■), and R = 2 (alcohol 0.2 M and butyric acid 0.1 M) (▒).

3.3. Effect of alcohol chain length

Fig. 3 shows that the conversion (%) for the synthesis of alkyl butyrate catalyzed by *B. cepacia cutinase* (BCC) at 37 °C decreases with the increasing chain length of alcohol (C4–C10). It was found that BCC showed the highest conversion for butanol among the alcohols tested.

For a particular fatty acid, the synthesis of esters will be dependent on its accessibility to the active site, whereas the selection of alcohols for the enzyme-catalyzed synthesis will be more dependent on the region that surrounds the active site. As acyl-enzyme intermediates are not formed by alcohols during the acylation process, the net synthesis of an ester is determined by the diffusion of alcohol molecules into the active site of an enzyme [2].

Small chain alcohols have the advantage over long chain bulky alcohols, as they are capable of diffusing into the active site of the enzyme more readily. So, decrease in reactivity is observed with increase of chain length of the alcohol. Microencapsulated cutinase in AOT reverse micelle also showed preference for alcohols chain length C5 and C6 [10] reflecting both the intrinsic selectivity of the enzyme and the different accessibility of the alcohol substrates to the cutinase active site [23]. Sarazin et al. [9,12] could achieve 80% conversion for the esterification of caprylic acid with butanol with lyophilized cutinase. Previously, similar trend has been found

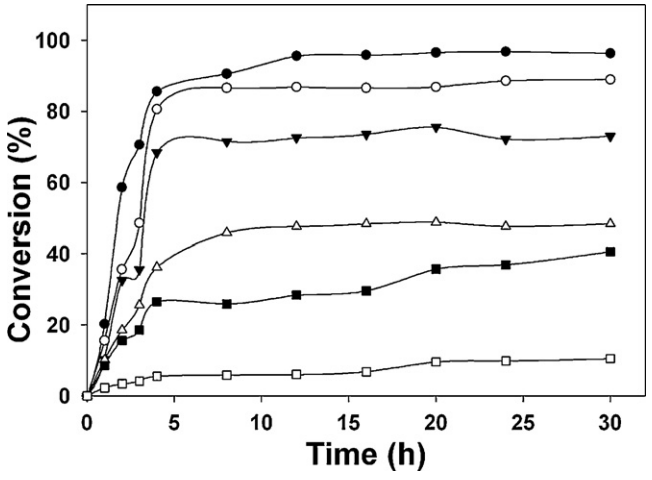

Fig. 4. Effect of acid chain length on synthesis of butyl esters. Alcohol/acid molar ratio, *R* = 1 ([butanol] = 0.1 M). Butyl butyrate (●), butyl valerate, (○), butyl caproate (▼), butyl octanoate (△), butyl decanoate (■), and butyl palmitate (□).

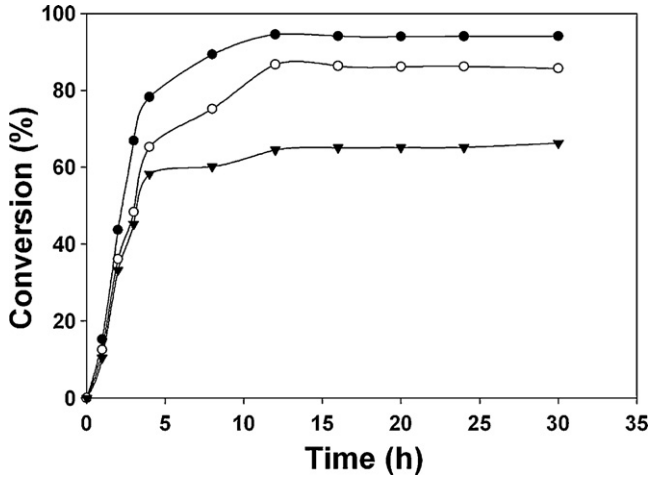

Fig. 5. Effect of acid chain length on synthesis of ethyl esters. Alcohol/acid molar ratio, *R* = 1 ([ethanol] = 0.1 M). Ethyl butyrate (●), ethyl valerate (○) and ethyl caproate (▼).

for lipases from other sources, e.g., *B. licheniformis*, *P. fluorescence*, *M. miehei*, *Aspergillus*, *C. rugosa*, and *Rhizopus arrhizus* [2,24] with maximum activity being seen for C4–C6 alcohols.

3.4. Effect of acid chain length on the synthesis of butyl ester

The selectivity of fatty acid chain-length usually depends on the native properties of the enzymes and mostly found to be similar to that of the hydrolysis reactions. As in case of lipase/esterase-catalyzed synthesis reactions an acyl-enzyme intermediate was formed. So, dependency on the fatty acids chain length will mostly due to the affinity between the fatty acid and enzyme itself.

Fig. 4 shows that the conversion (%) of butyl esters varies with the carbon chain length of the fatty acids. Experiments were conducted for fatty acids of chain length C4–C16. Cutinase showed higher hydrolytic activity in aqueous media with p-nitrophenyl butyrate and valerate than other long chain synthetic esters (data not shown). In case of synthesis reaction in organic media by BCC, almost a similar trend was observed. The decrease in conversion was observed with the increase in fatty acid chain length. The highest conversion was observed for butyric acid and then valeric acid, whereas conversion for octanoic acid and decanoic acid was low. The minimum conversion was observed for the palmitic acid. This may be because the long chain fatty acids react with the active site of cutinase and probably block the access of the alcohol to the intermediate acyl enzyme followed by formation of the product [4]. These results suggest that cutinase from *B. ceapcia* had the highest affinity towards short chain fatty acids (C4–C6), which is in agreement with previous studies performed in organic solvent [4] or reverse micellar systems [10,11,13] using recombinant *Fusarium solani* cutinase. Lipase from *P. fluorescens* also showed similar kind of affinity for short-chain length fatty acid (butyric acid) [25].

3.5. Synthesis of ethyl esters

In this study, we have also checked the ability of *B. cepacia* cutinase enzyme to synthesize ethyl esters. Fig. 5, shows the conversion of ethyl esters of butyric acid, valeric acid and caproic acid. Similar kind of profile was observed for ethyl esters as observed before for butyl esters. The maximum (94%) conversion obtained for ethyl butyrate, followed by ethyl valerate (86%) and ethyl caproate (65%). Previously, de Barros et al., [4] also studied the synthesis of ethyl esters for these three fatty acids catalyzed by recombinant *F. solani pisi* cutinase. They have achieved the maximum conversion

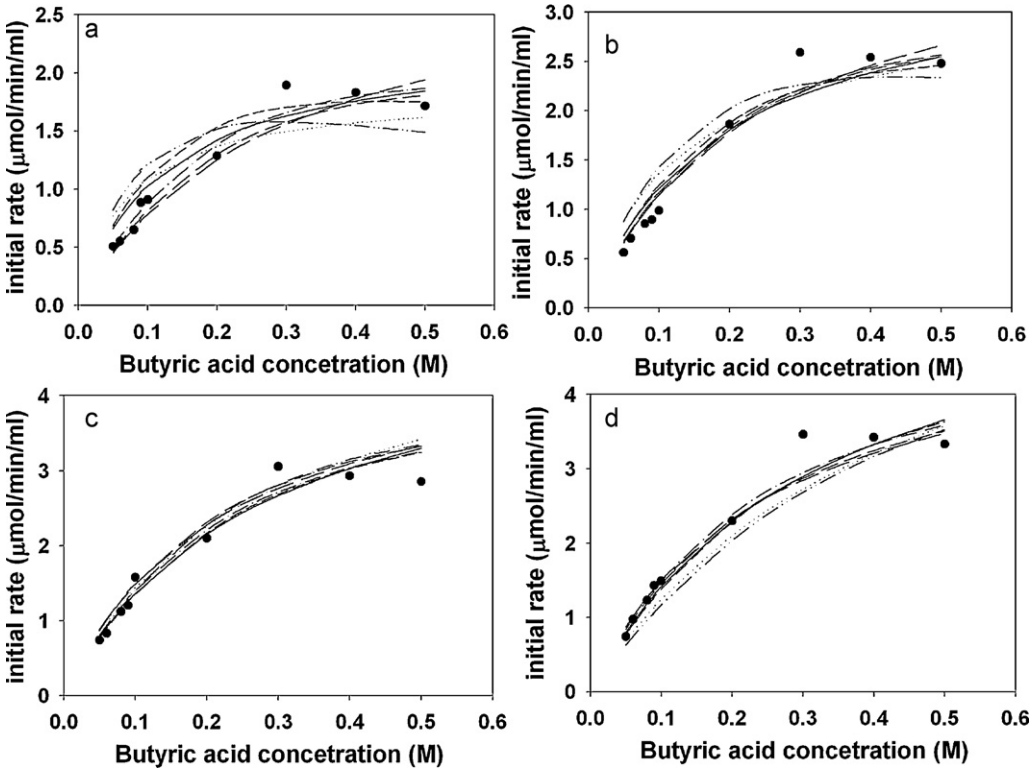

Fig. 6. The initial reaction rates for synthesis of butyl butyrate as a function of the butyric acid concentration at the fixed concentrations of butanol. The experimental data were fitted to Ping–Pong kinetic model equations (Eqs. (1)–(7)) and the lines shown were calculated using the parameters presented in Table 1. (a) 0.05 M butanol, (b) 0.1 M butanol (c) 0.25 M butanol and (d) 0.5 M butanol. Eq. (1) (——), Eq. (2) (·····), Eq. (3) (----), Eq. (4) (–••–••–), Eq. (5) (— — —), Eq. (6) (—•—•—), Eq. (7) (– – – –).

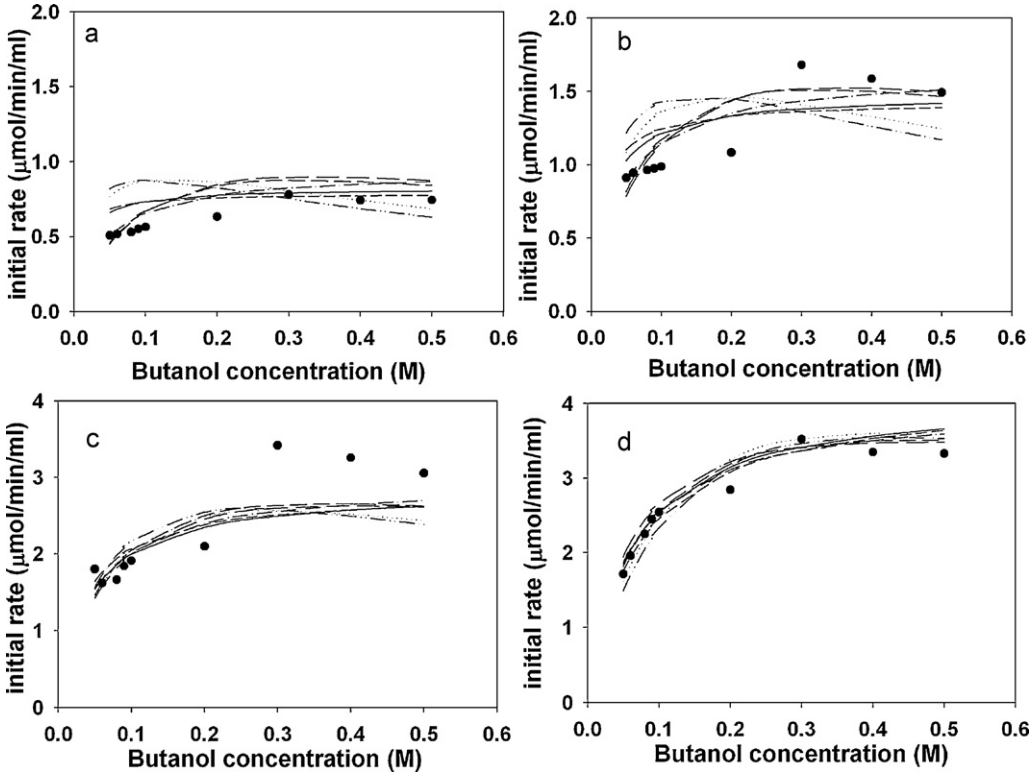

Fig. 7. The initial reaction rates for synthesis of butyl butyrate as a function of the butanol concentration at the fixed concentrations of butyric acid. The experimental data were fitted to Ping–Pong kinetic model equations (Eqs. (1)–(7)). The lines shown were calculated using the parameters presented in Table 1. (a) 0.05 M butyric acid, (b) 0.1 M butyric acid (c) 0.25 M butyric acid (d) 0.5 M butyric acid. Eq. (1) (——), Eq. (2) (·····), Eq. (3) (----), Eq. (4) (–••–••–), Eq. (5) (— — —), Eq. (6) (—•—•—), Eq. (7) (– – – –).

Table 1
Estimated kinetic constants for synthesis of butyl butyrate using different kinetic models.

	V_{max} (μmol/min/ml)	K_{mA} (M)	K_{mB} or K_{BP} (M)	K_{IA} (M)	K_{IB} (M)	K_{AB} (M^2)	R^2 (%)
Eq. (1)	7.40	0.40	0.11	–	–	–	94.7
Eq. (2)	9.60	0.34	0.21	–	0.57	–	90.4
Eq. (3)	7.86	0.45	0.07	0.58	–	–	94.6
Eq. (4)	11.7	0.45	0.16	0.58	0.55	–	88.5
Eq. (5)	5.64	0.11	0.04	–	0.58	0.02	95.0
Eq. (6)	6.27	0.28	0.03	0.35	–	0.01	96.6
Eq. (7)	6.13	0.13	0.03	0.35	0.58	0.02	95.5

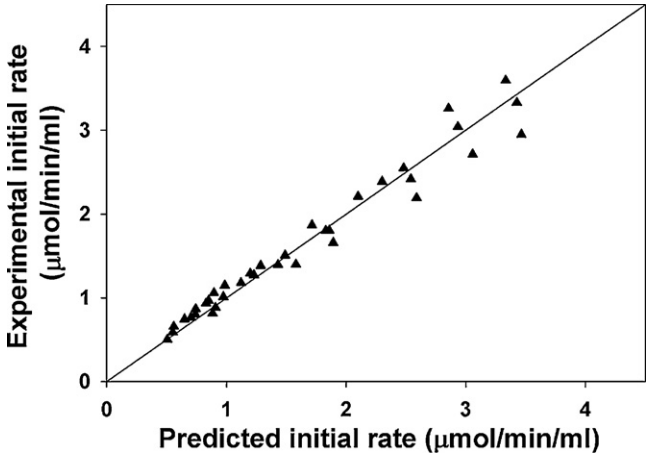

Fig. 8. Parity plot for predictions of initial reaction rate for best fitted model (model Eq. (6)).

for valeric acid (95%) followed by butyric acid (84%) and caproic acid (62%), though they found the rate of ester synthesis is higher for butyric acid than that of valeric acid. The specificity for cutinase from two different sources may slightly vary with the fatty acid chain length, but both enzymes prefer short chain length fatty acid (C4–C6) for esterification reaction. In the present study, it was observed that the maximum conversion obtained (94.6%) for butyric acid, followed by valeric acid (87.5%) and caproic acid (72.5%) for the synthesis of butyl ester. The efficiency of *B. cepacia* cutinase is similar for the synthesis of both butyl and ethyl esters.

3.6. Effect of substrate concentration and kinetics for synthesis of butyl butyrate

The rate-determining step for an enzyme-catalyzed hydrolysis reaction in an aqueous emulsion system is acylation. But in the presence of excess water, the rate of deacylation will be faster than the rate of acylation. So, the relative activity of the enzyme against different substrates will be dependent on the interaction between the substrate and the active site of the native enzyme [2]. Where as, in case of enzyme-catalyzed esterification reaction, where both substrates are soluble in a single phase having equal opportunity to reach the enzyme active site, and thus the kinetics of the reaction will be more complex than those of the hydrolysis reaction.

The effect of different concentrations of butyric acid (Fig. 6) or butanol (Fig. 7) on the initial rates of butyl butyrate synthesis was studied in the range of 0–0.5 M for both the substrates. Fig. 6 showed the effect of increasing butyric acid concentration on initial reaction rate at four different concentrations of butanol (0.05 M, 0.1 M, 0.25 M and 0.5 M). Fig. 7 shows the effect of increasing butanol concentration on initial rate of synthesis of butyl butyrate at four different concentrations of butyric acid (0.05 M, 0.1 M, 0.25 M and 0.5 M). From Figs. 6 and 7 it is observed that the rate of reaction increases with the increase in substrate concentration up to 0.3 M. When the substrate concentrations increases further (>0.3 M), the initial reaction rate started to decrease. The loss of activity at high alcohol concentrations might be due to its dehydrating effect on the surface of the enzyme in organic media, which inhibits the cutinase activity. On the other hand decrease in reaction rate at high acid concentration may be due to the change in catalytic environment at higher concentration of acids. It was previously found that cutinase showed very little hydrolysis activity at pH less than 5.5, as increase in acid concentration reduce the pH of the organic media, and hence cause the loss of esterification activity too. The esterification using other lipase or cutinase (recombinant *F. solani* cutinase) were also showed similar trend at different substrate concentrations [4].

For kinetic study of butyl butyrate synthesis, the effect of concentrations of both substrates on the rate of reaction was investigated. To estimate the kinetic parameters, the experimental data were fitted to the model Eqs. (1)–(7). The kinetic parameters obtained by fitting the data to these equations are given in Table 1. The variation of experimental initial reaction rate as a function of initial substrate concentration and the fitted curves are presented in Figs. 6 and 7 for butyric acid and butanol, respectively. The model Eq. (1) gives the simple Michaelis–Menten equation for two substrate Ping–Pong Bi Bi kinetic model. But this model may not predict the kinetics of the reaction appropriately after certain concentration of substrates, where increasing concentration of one or both substrates has inhibitory effect on reaction rate. So, the introduction of one or more extra terms may be helpful for proper prediction of the reaction kinetics with inhibitory effect of one or more substrates. K_{IB} and K_{IA} are inhibition constant for butanol and butyric acid, respectively. In Eqs. (2) and (3) are obtained when inhibition due to butanol or butyric acid are considered, where as Eq. (4) has both K_{IA} and K_{IB} considering inhibition by both the substrates. From the results obtained by fitting the experimental data to the above mentioned four equations it was observed that kinetic parameters does not vary much for the other three equations than the classical Michaelis–Menten equation. But the decrease in R^2 value is due to incorporation of one or more extra terms. The Eq. (3) (with acid inhibition term) is found to be more suitable with R^2 value of 94.6% for our data than other two equations. In the esterification reaction, water is one of the products that is also present at the start of the reaction, because minimum water is required for the enzyme activity. The incorporation of effect of water, converts Eqs. (3)–(5), which contains an extra term K_{AB} and modified K_{MB} to K_{BP}. We have studied the influence of water for all three above mentioned cases of alcohol, acid and both alcohol and acid inhibition. The modified model equations are given by Eqs. (5)–(7). It has been clearly observed from the kinetic parameters and the regression coefficient that incorporation of the effect of water improves the fit for all three conditions. Parity plot (Fig. 8) showing estimated initial reaction rate by different models that fit to the entire data versus experimental initial reaction rate. The best fit obtained for the reaction with Eq. (6) with R^2 of 96.6%. This depicts that the inhibition by butyric acid in the experimental range is more prominent than other substrate. Pinto-Sousa et al., [11] had also studied the kinetics of esterification reaction of butyric acid and 2-butanol catalyzed by

microencapsulated cutinase in nonionic surfactant, phosphotidylcholine. They have also observed that the substrate inhibition by 2-butanol and butyric acid above the concentration of 0.5 M and 0.2 M, respectively.

4. Conclusion

Cutinase from *B. cepacia* was found to be efficiently catalyzing reaction for synthesis of short-chain length fatty acid esters. The optimum range of temperature was found to be from 35 to 40 °C for the esterification with the maximum conversion at 37 °C. The better conversion (%) could be obtained for short-chain length fatty acid (butyric acid) and alcohol (butanol) than that of long-chain acid or alcohol. The similar fatty acid chain length specificity was observed for both butyl esters and ethyl esters. The kinetic parameters for the synthesis of butyl butyrate were also estimated using simple and modified Ping–Pong Bi Bi kinetic models. Among the tested models, Ping–Pong model with acid inhibition and water influence was found to be the best fitted model for the experimental data.

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