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Decarboxylative aldol reaction catalysed by lipases and a protease in organic co-solvent mixtures and nearly anhydrous organic solvent media

MANALI KAPOOR, ABIR B. MAJUMDER, JOYEETA MUKHERJEE & MUNISHWAR N. GUPTA

Chemistry Department, Indian Institute of Technology Delhi, Hauz Khas, New Delhi, India

Abstract

The effects of the choice of lipase, reaction medium, immobilization, presence of additives and temperature on conversion and stereoselectivity during a lipase catalysed decarboxylative aldol reaction were examined. It was shown that *Candida antarctica* lipase B (CALB) catalysed a decarboxylative aldol reaction between 4-nitrobenzaldehyde and ethyl acetoacetate in a 60% acetonitrile–40% aqueous buffer co-solvent mixture. Interestingly, free and immobilized forms of CALB showed opposite enantioselectivity in this media. The addition of 30 mol% imidazole increased the reaction rate from 8.5 to 55.7 $\mu M\ min^{-1}\ mg^{-1}$. A 98% conversion could be achieved in 14 h (instead of 168 h) by adding imidazole. Other lipases also catalysed this reaction in different reaction media to a varying extent. With *Mucor javanicus* lipase in 30% DMSO, 20% enantiomeric excess (ee) of the (*R*)-product was observed. CALB also catalysed this reaction in nearly anhydrous acetonitrile. In the presence of cross-linked protein coated microcrystals of CALB, 90% conversion was obtained in this media in 24 h. A commercially available protease, alcalase, was also found to catalyse this reaction. While low water media gave poor conversion, the reaction in aqueous–60% acetonitrile co-solvent mixture gave 99% conversion in 72 h, provided imidazole was used as an additive.

Keywords: *decarboxylative aldol reaction, biocatalysis in aqueous-organic co-solvent mixtures, lipases, C–C bond formation*

Introduction

Enzyme promiscuity has attracted considerable attention in recent years (Clinton et al. 2011; Gupta et al. 2011; Hult & Berglund 2007; Khersonsky & Tawfik 2010). Both from the conceptual, as well as practical, point of view, it is important to look at the behaviour of enzymes where no promiscuity has been intentionally engineered. For example, a number of C–C bond formation reactions have been reported with commercially available lipases (De Souza et al. 2009; Li et al. 2009 a,b; Majumder et al. 2009; Torre et al. 2004). Recently, Feng et al. (2009) reported a decarboxylative aldol reaction between 4-nitrobenzaldehyde and ethyl acetoacetate catalysed by immobilized *Candida antarctica* lipase B (CALB) as an example of catalytic promiscuity. Later, Evitt & Bornscheuer (2011) questioned the promiscuity aspect of this reaction and suggested that the results are more likely to be due to normal lipase catalysed hydrolysis of the ester followed by a non-enzymatic aldol condensation step with 4-nitrobenzaldehyde. Evitt and Bornscheuer (2011) suspected that the acetonitrile used by Feng et al. (2009) may not have been dry enough and could have contained sufficient water to promote ester hydrolysis. Later, the Knoevenagal reaction would generate further water to sustain the hydrolysis step (Evitt & Bornscheuer 2011). These conflicting views indicate the need for caution in mechanistic interpretation of results on use of enzymes in organic synthesis. Nevertheless, obtaining an aldol product starting from ethyl acetoacetate (and 4-nitrobenzaldehyde) is a useful synthetic approach (Kourouli et al. 2002). The aim of the present work was to

Correspondence: Munishwar Nath Gupta, Department of Chemistry, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India. Tel: + 91-11-2659-1503. Fax: + 91-11-2658-1703. E-mail: munishwar48@yahoo.co.uk

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search for process conditions in order to achieve better conversions in shorter times. This is important if such approaches are to have any practical utility. We have investigated the same reaction in an excess of water. As an organic solvent was required to dissolve the substrates, a co-solvent mixture of 60% acetonitrile and 40% aqueous buffer was chosen as the reaction medium. Larger numbers of commercially available lipases were also tried.

The same reaction was also studied in some other aqueous–organic co-solvent mixtures. Medium engineering (Laane 1987; Gupta 1992), that is, the composition of the reaction medium was found to have a considerable influence on conversions achieved. Recently, it has been shown that proteases can also catalyse aldol reactions (Li et al. 2010). In the present work, we show that alcalase (a protease available commercially for incorporating in detergents) could also catalyse a decarboxylative aldol reaction. The influence of the reaction medium on the extent of conversion was examined in this case as well.

Materials and methods

Materials

Mucor javanicus lipase (Lipase M) and *Candida rugosa* lipase (Lipase AYS) were kind gifts from Amano Enzyme Inc., Nagoya, Japan. Lipozyme CALB, Novozyme-435 (*Candida antarctica* lipase B immobilized on a macroporous acrylic support), Palatase (*Rhizomucor miehei* lipase), Lipozyme RM IM (*Rhizomucor miehei* lipase immobilized on anion exchange resin) and Alcalase (a crude liquid formulation of proteases produced from *Bacillus licheniformis* containing Subtilisin Carlsberg as the major component) were kind gifts from Novozymes, Bagsvaerd, Denmark. 4-Nitrobenzaldehyde and ethyl acetoacetate (99%, GC) were obtained from Spectrochem, Mumbai, India. Glutaraldehyde (25% v/v) was obtained from Merck, Darmstadt, Germany. Triethylamine was obtained from Merck, Mumbai, India. Imidazole, acetone (99.8%, HPLC grade), acetonitrile (anhydrous, 99.8%), DMF (anhydrous, 99.8%), 1, 4-dioxane (anhydrous, 99.8%) and DMSO (anhydrous, $\geq 99.9\%$) were obtained from Sigma, St. Louis, USA. All other chemicals used were of analytical grade. All the organic solvents were further dried over molecular sieves overnight before use.

Methods

Preparation of denatured enzyme. Twenty microliters of CALB was added to 1 mL 8 M urea and incubated at 100°C for 8 h to denature the enzyme. The denatured enzyme was extensively dialysed against distilled water to remove the urea and then against 10 mM sodium phosphate buffer (pH 7.0). Finally, the enzyme activity was checked by hydrolysis of 4-nitrophenylpalmitate (Jain et al. 2005).

Preparation of enzyme precipitated and rinsed with acetone of CALB. Enzyme precipitated and rinsed with acetone (EPRA) was prepared as described earlier (Shah & Gupta 2007). CALB (20 μL) was diluted with 100 μL of 10 mM sodium phosphate buffer (pH 7.0) and precipitated by drop-wise addition into 6 mL ice-chilled acetone under continuous orbital shaking at 150 rpm. After 20 min, the EPRA was recovered by centrifugation at $8000 \times g$ at 4°C for 5 min and washed twice with ice-chilled acetone (1 mL each time) and then with the anhydrous chilled acetonitrile (1 mL). The final enzyme preparation obtained at this step (EPRA) was kept in anhydrous acetonitrile (1 mL) at 4°C until use.

Preparations of protein coated microcrystals and cross-linked protein coated microcrystals of CALB. Protein coated microcrystals (PCMCs) were prepared by the method of Kreiner et al. (2001). CALB (20 μL) was diluted with 100 μL of 10 mM sodium phosphate buffer (pH 7.0); mixed with 300 μL saturated potassium sulphate solution and precipitated by drop-wise addition into 6 mL ice-chilled acetone under continuous orbital shaking at 150 rpm. After 20 min, the PCMCs were recovered by centrifugation at $8000 \times g$ at 4°C for 5 min and washed twice with ice-chilled acetone (1 mL each time) and then with anhydrous chilled acetonitrile (1 mL). The final enzyme preparations (PCMCs) were kept in anhydrous acetonitrile (1 mL) at 4°C until use. Cross-linked protein coated microcrystals (CLPCMCs) were prepared as described earlier (Shah et al. 2008). The PCMCs of lipase were suspended in acetone (0.5 mL) by mild vortexing and glutaraldehyde (25% v/v, 50 mM) was added. The mixtures were shaken manually during this addition, then kept at 4°C for 1 h with constant stirring at 300 rpm. The CLPCMCs were recovered by centrifugation at $8000 \times g$ at 4°C for 5 min, washed twice with ice-chilled acetone (1 mL each time) to ensure complete removal of unreacted glutaraldehyde and then with the anhydrous chilled acetonitrile (1 mL). The final enzyme preparations obtained (CLPCMCs) were kept in anhydrous acetonitrile (1 mL) at 4°C until use.

Decarboxylative aldol reaction for the synthesis of 4-hydroxy-4-(4-nitro-phenyl)-butan-2-one. 4-Nitrobenzaldehyde (0.125 mmoles) and ethyl acetoacetate (0.75 mmoles) were mixed in 1.25 mL of organic solvent containing the required percentage of 10 mM sodium phosphate buffer, pH 7.0. The reactions were initiated by adding 20 mg lipase or

protease formulation and the mixtures incubated at 30°C with orbital shaking at 200 rpm. Aliquots (20 μL) of reaction mixture were withdrawn at specified time intervals and analysed by HPLC.

HPLC analysis of the reaction for conversion and ee. The 20-μL aliquots were diluted with 800 μL acetonitrile to remove enzymes by precipitation and centrifugation. Supernatants were analysed using a HPLC on Zorbax C-18 reverse phase column with an eluent composed of 25% v/v acetonitrile in water containing trifluoroacetic acid (0.1% v/v) at a flow rate of 1 mL/min, with monitoring by DAD-UV (diode array detector-UV) at 254 nm. Aldol and the 4-nitrobenzaldehyde appeared at 5.9 min and 8.4 min, respectively (Mondal et al. 2006).

The product enantiomers were separated on a Chiralcel OD-RH chiral column with an eluent composed of hexane:isopropanol (5:1, v/v ratio) and detected at 254 nm. The ee values were calculated from the peak areas of these two enantiomers. The absolute configuration was determined by polarimetry using a Rudolph® Autopol V with a 10-mm cell (130 μL). All optical rotations were measured in $CHCl_3$ at 30°C. The optical rotation measurements were confirmed by cross comparison of values obtained with those reported in the literature (Russo et al. 2007).

Decarboxylative aldol reaction for the synthesis of 4-hydroxy-4-(4-nitro-phenyl)-butan-2-one in co-solvent mixture of 60% acetonitrile 40% aqueous buffer with urea denatured CALB. A volume of 20 μL of denatured CALB solution in aqueous buffer was added to acetonitrile such that a 60% acetonitrile–40% aqueous buffer co-solvent mixture was obtained. 4-Nitrobenzaldehyde (0.125 mmoles) and ethyl acetoacetate (0.75 mmoles) were added to it and the reaction was monitored as described above.

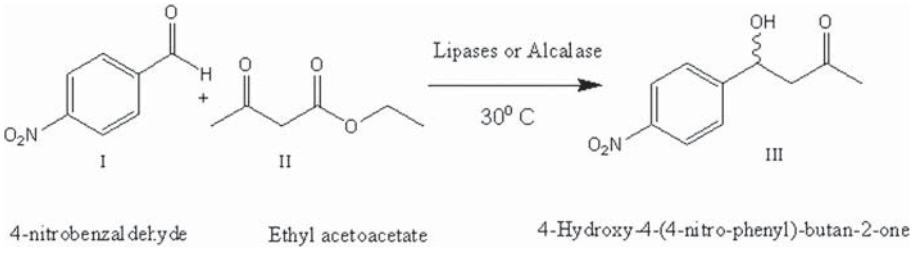
Results and discussion

Catalysis of decarboxylative aldol reaction by different lipases

The decarboxylative aldol reaction is an important C–C bond forming reaction in organic synthesis (Boudoux et al. 2010; Orlandi et al. 2004). The lipase catalysed decarboxylative aldol reaction between 4-nitrobenzaldehyde and ethyl acetoacetate (Scheme 1) was previously carried out in acetonitrile with immobilized CALB (Feng et al. 2009). Retaining acetonitrile as a co-solvent, the identical decarboxylative aldol reaction was studied in the presence of much higher concentrations of water (Table I). A minimum of 60% (v/v) acetonitrile was required to dissolve 0.125 mmoles of 4-nitrobenzaldehyde in 1.25 mL of reaction volume. Nevertheless, all the enzymes screened [*Mucor javanicus* lipase, CALB (free and immobilized), *Mucor meihei* lipase (free and immobilized), *Candida rugosa* lipase] were found to catalyse the decarboxylative aldol reaction in a 60% acetonitrile–40% aqueous buffer co-solvent mixture (Table I). The 4-nitrophenylpalmitate assay showed that denaturation with urea led to almost complete inactivation of the enzyme (data not shown separately). A control with urea denatured CALB preparation showed that only 0.2% aldol product could be obtained if the active enzyme was not present (Figure 1). This shows that some stage of the reaction was enzyme catalysed.

Immobilization of CALB reversed its stereoselectivity

One feature which makes C–C bond formation useful as a synthetic step is that, invariably, new chiral centres are formed. Hence, the possibility of asymmetric synthesis by using enzymatic catalysis is especially attractive (Fessner 2008). No data on the enantioselectivity ('little enantioselectivity') during the decarboxylative aldol reaction catalysed by immobilized CALB was reported (Feng et al. 2009). It was observed that the best conversions were obtained with CALB in free as well as in the immobilized form (Table I). Generally, Novozyme-435 (the immobilized form) performs better than the free form of CALB (Kirk & Christensen 2002; Madeira Lau et al. 2000; Teo et al. 2004). However, in this case, both gave similar conversion in similar time periods. It was decided to look at the enantioselectivity during the aldol decarboxylative reaction with both forms of this enzyme. We observed some limited enantioselectivity with free CALB and its



Scheme 1. Decarboxylative aldol reaction in the presence of enzymes in co-solvent mixtures and nearly anhydrous acetonitrile.

Table I. Use of different lipase formulations for the decarboxylative aldol reaction between 4-nitrobenzaldehyde I and ethyl acetoacetate II in acetonitrile containing 40% v/v aqueous buffer.

Lipase	Time (h)[b]	C (%)[c]	$[\alpha]^{29}_{D}$ ($CHCl_3$)[d]	Absolute configuration[e]	*ee* (%)[f]
Mucor javanicus lipase	144	30	nd	–	nd
Mucor javanicus lipase[a]	144	55	nd	–	nd
CALB (free)	168	98	− 1.9	*S*	6
CALB[a] (free)	48	97	− 1.3	*S*	4
Novozyme-435	168	98	+ 4.4	*R*	14
Novozyme-435[a]	48	97	+ 3.6	*R*	11
Mucor meihei lipase (free)	144	28	nd	–	nd
Mucor meihei lipase (free)[a]	144	44	nd	–	nd
Candida rugosa lipase	144	21	nd	–	nd
Candida rugosa lipase[a]	144	25	nd	–	nd
Lipozyme RM IM	144	6	nd	–	nd
Lipozyme RM IM[a]	144	18	nd	–	nd

C, conversion; CALB, *Candida antarctica* lipase B; ee, enantiomeric excess; nd, not determined.
[a]In presence of triethylamine, NEt_3 (10% mol/mol with respect to substrate, 4-nitrobenzaldehyde) as an additive. Control reactions (without enzyme) in presence of NEt_3 gave less than 8% conversion after 48 h.
[b]This is the time beyond which % conversion did not increase. These were the maximum conversion (%) possible under the reaction conditions.
[c,d,e,f]Conversion and *ee* were determined by HPLC. Enantiomeric excess values were determined for the conversions which were more than 90% (details given in materials and methods section). The reported specific rotation $[\alpha]^{29}_{D}$ of (R)-4-Hydroxy-4-(p-nitrophenyl)-butan-2-one was + 25.6 (Russo et al. 2007).

immobilized form Novozyme-435 (Table I). It is interesting to observe that the free CALB and its immobilized form, Novozyme-435 showed opposite enantiopreference. Dramatic differences in enantiopreferences of the free enzyme and its immobilized form are uncommon but have been reported (Brady & Jordann 2009; Palomo 2008, 2009). The presence of triethylamine, NEt_3 along with the enzyme was found to either enhance percentage conversion or to reach the equilibrium conversion faster (Table I). This is in agreement with earlier observations made when anhydrous acetonitrile was used as the reaction medium (Feng et al. 2009).

Additives improve both conversion as well as enantioselectivity

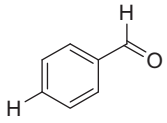
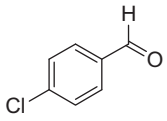
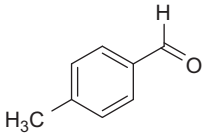
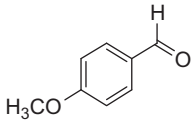
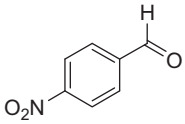
Novozyme-435 is an expensive enzyme; the free form of CALB is much cheaper. As the conversions using CALB (free form) and Novozyme-435 were identical (98% in 168 h) under our conditions, the CALB (free form) catalysed reaction was investigated further. As conversion was observed to be much faster in the presence of NEt_3 (Table I), imidazole was also tried as another base. Imidazole as a part of side chain of histidine is known to act as both acid and base catalyst in the case of many enzyme-catalysed reactions (Berg et al. 2002). In the presence of increasing concentrations of imidazole, the initial rate of the decarboxylative aldol reaction also increased (Table II). At around 20–30% concentration, it reached a plateau; the initial rate was 55.7 μM $min^{-1}mg^{-1}$ at 30 mol% imidazole. The

Table II. Effect of imidazole concentration on the conversion and enantioselectivity of the decarboxylative aldol reaction catalysed by *Candida antarctica* lipase B (CALB) in acetonitrile containing 40% v/v aqueous buffer.

Additive (mol%)[a]	Initial rates (μM min^{-1} mg^{-1})[b]	Time (h)[c]	C (%)[d]	*ee* (%)[e]	$[\alpha]^{29}_{D}$ ($CHCl_3$)
0	8.5	168	98	6	−1.9
5	19.0	38	97	4	−1.3
10	27.1	30	99	12	−3.8
15	42.3	20	99	5	−1.6
20	52.5	16	98	3	−1.0
25	53.9	14	97	0	0
30	55.7	14	98	0	0

C, conversion; ee, enantiomeric excess.
[a]%mol/mol with respect to substrate 4-nitrobenzaldehyde. Control reaction (without enzyme) in presence of imidazole gave less than 5% conversion after 24 h.
[b]Enzyme used to determine initial rate was 9 mg. Initial rates were calculated from aliquots taken within 1–3 h. The percentage conversions during these time periods increased linearly in the range 1–20%.
[c]As given in Table I.
[d,e]Conversion and *ee* were determined by HPLC. The enantiomer present in excess was identified as *S* enantiomer by optical rotation.

Table III. Effect of variation of substrates on reaction catalysed by *Candida antarctica* lipase B (CALB) in acetonitrile containing 40% v/v aqueous buffer.

Entry	Substrate	C (%)[a]
1.		<3
2.		8
3.		<2
4.		<2
5.		98

[a]Conversion (C) (after 168 h) estimated by HPLC.

maximum %conversion reached was between 97 and 99% in all the cases; however, the time required to reach this maximum %conversion decreased as the imidazole concentration increased. It was interesting to observe the effect of imidazole on *ee*% as well. There was a twofold increase in %*ee* in the presence of 10 mol% imidazole. Generally, additives like NEt_3 or imidazole can be assumed to function like chemical catalysts. Hence, the reaction carried out in their presence can be viewed as a sort of chemoenzymatic approach to catalysis. It was also expected that in such cases, the enantioselectivity will be less than in the cases when enzyme alone was used as the catalyst. However, Parker et al. (1998) have also reported that the presence of NEt_3 enhances enantioselectivity of CALB during alcoholysis in low water media.

The decarboxylative aldol reaction with different acceptor aldehydes

When 4-nitrobenzaldehyde was substituted with less active acceptor aldehyde molecules, the %conversions were much less (Table III). The trend in %conversions was in agreement with the known reactivity of these substituted aromatic aldehydes (Morrison & Boyd 1992).

Table IV. Effect of water miscible organic co-solvents on the formation of 4-hydroxy-4-(4-nitrophenyl)-butan-2-one by decarboxylative aldol reaction between 4-nitrobenzaldeyhde I and ethyl acetoacetate II catalysed by *Mucor javanicus* lipase in different co-solvents containing 70% v/v aqueous buffer.

Co-solvent[a] added	Initial rate ($\mu M\ min^{-1}\ mg^{-1}$)[b]
DMSO	1.0
DMF	0.6
Dioxane	0.6

[a]Added in minimum amount to make the system homogeneous. [b]Initial rates were calculated from the aliquots taken within 1–3 h. The percentage conversions during these time periods were in the range of 1–20% and were in linear range.

Effect of co-solvent on %conversion and enantioselectivity

Acetonitrile has been described as a 'bad' co-solvent (Khmelnitsky et al. 1991), with a high denaturation capacity. At this stage, it was decided to use some other organic co-solvents. As most of the water miscible organic solvents are 'bad' solvents, the next best option was to use a low co-solvent concentration in the reaction medium. In order to keep the co-solvent concentration low, more polar solvents in which the 4-nitrobenzaldehyde was likely to be more soluble than in acetonitrile were tried. Although DMSO is also a 'bad' solvent, merely 30% v/v DMSO concentration was enough to completely dissolve 0.125 mmoles of 4-nitrobenzaldehyde in 1.25 mL reaction volume (Table IV). The two other (more polar than acetonitrile) solvents, DMF and dioxane were also able to dissolve the 0.125 mmoles substrate at 30% v/v co-solvent concentration. However, DMSO gave higher initial rates than the other two co-solvents (Table IV).

Mucor javanicus lipase showed the next best %conversion among enzymes (in the absence of any additive) (Table I). Hence, it was tested in 30% DMSO along with CALB (free form). Here again 10 mol% of NEt_3 as an additive increased the % conversion (Table V). In 30% DMSO, (a) both *Mucor javanicus* lipase and CALB gave identical conversions in 90 h, (b) even in the presence of 10 mol% NEt_3, both gave somewhat higher but identical conversions in 90 h, (c) the enantioselectivity of *Mucor javanicus* lipase was opposite to that of CALB, (d) *Mucor javanicus* lipase showed higher enantioselectivity (20% *ee*) than even CALB, (e) while conversion was higher in the presence of NEt_3, the enantioselectivity was much lower. In fact, in the presence of NEt_3, CALB showed no enantiopreference (Table V). It is interesting to note that the effect of additives when DMSO was used as a co-solvent was different from what was observed in acetonitrile containing reaction medium (Table II).

Table V. Effect of variation of enzymes and temperature on decarboxylative aldol reaction between 4-nitrobenzaldehyde I and ethyl acetoacetate II in DMSO containing 70% v/v aqueous buffer.

Lipases	Temperature (°C)	Time (h)[c]	C (%)[d]	$[\alpha]^{29}_{D}$ ($CHCl_3$)[e]	Absolute configuration[f]	ee (%)[g]
Mucor javanicus lipase	30	90	54	+6.5	*R*	20
Mucor javanicus lipase[a]	30	90	63	+3.1	*R*	10
Mucor javanicus lipase[b]	30	48	82	+1.3	*R*	4
CALB	30	90	54	−3.8	*S*	12
CALB[a]	30	90	63	0	–	0
CALB[b]	30	48	80	−1.3	*S*	4
Mucor javanicus lipase	40	72	92	−0.7	*R*	2
CALB	40	72	85	−1.1	*S*	4

CALB, *Candida antarctica* lipase B; C, conversion.

[a]In presence of NEt_3 (10 mol%) as an additive.

[b]In presence of imidazole (10 mol%) as an additive. Control reactions (without enzyme) in presence of imidazole or NEt_3 gave less than 8% conversion after 48 h.

[c]This is the time beyond which % Conversion did not increase. These were the maximum conversion (%) possible under the reaction conditions.

[d,e,f,g]Conversion and *ee* were estimated by HPLC (details given in materials and methods section).

Effect of temperature on enantioselectivity

Temperature is an important parameter in enzymatic catalysis. Normally the reaction rate increases with increase in reaction temperature until thermal inactivation of the enzyme starts. In the present study, conversions were higher with both lipases at higher temperature (40°C) (TableV). However, the enzymes showed lower enantioselectivity at higher temperature. Lower enantioselectivity at higher temperature is common and considered to be the result of a more flexible enzyme conformation at higher temperature (Sakai et al. 1997).

Catalysis of decarboxylative aldol reaction by alcalase

Both lipases and serine proteases involve catalytic triads in their mechanisms. The active site of lipases is chemically similar to that of serine proteases**.** Many proteases have been known to show esterase and esterification activity (typical reactions catalysed by lipases) (Gupta 1992, 2000; Khmelnitsky & Rich 1999). In view of this, it was decided to try a protease as a catalyst for decarboxylative aldol reaction. Alcalase is an inexpensive detergent protease which is commercially available (Chen et al. 1991). It was also found to catalyse the decarboxylative aldol reaction between 4-nitrobenzaldehyde and ethyl acetoacetate (Scheme 1). Initially, the reaction was attempted in solvent free condition, with an excess of ethyl acetoacetate acting as the reaction medium. The reaction was found to be slow and 89% conversion was obtained after 15 days (this was in the presence of an optimum ~5% v/v water (data not shown). As Feng et al. (2009) originally studied the lipase -catalysed reaction in acetonitrile, the protease catalyst was examined in that medium. However, as seen in Figure 1, low water conditions showed poor conversions. Co-solvent mixture of 40% aqueous buffer–60% acetonitrile gave the best conversion. As the Figure 2 (inlay) shows, 47% conversion could be obtained after 6 days. It was not possible to use greater than 40% water as 4-nitrobenzaldehyde started precipitating beyond this concentration of water.

Imidazole as an additive improved the %conversion. A 20 mol% of imidazole gave 99% conversion after 72 h (data not shown). A control run with just 20 mol% imidazole without enzyme gave less than 7% conversion during the same time. DMSO as a co-solvent was better than acetonitrile (Figure 3). With 35% aqueous–DMSO co-solvent mixture as a reaction medium, 99% conversion could be obtained in about 62 h. DMF was found to be equally good as a co-solvent and gave similar results (Figure 4). Addition of imidazole again enhanced the conversion with both DMSO and DMF as co-solvents (Table VI).

Catalysis of decarboxylative aldol reaction by enzymes in nearly anhydrous acetonitrile

Feng et al. (2009) used acetonitrile as a reaction medium for carrying out the reaction. Evitt and Bornscheuer (2011) suspected that [as the grade of the acetonitrile was not mentioned by Feng et al. (2009)] their acetonitrile may have contained enough water to hydrolyse the ester. We decided to reinvestigate this by using 'dried' or nearly anhydrous acetonitrile as reaction medium. While the presence of traces of water can be neither ruled out nor is desirable (Gupta 1992, 2000), the results obtained by us may be of interest to other workers in the context of

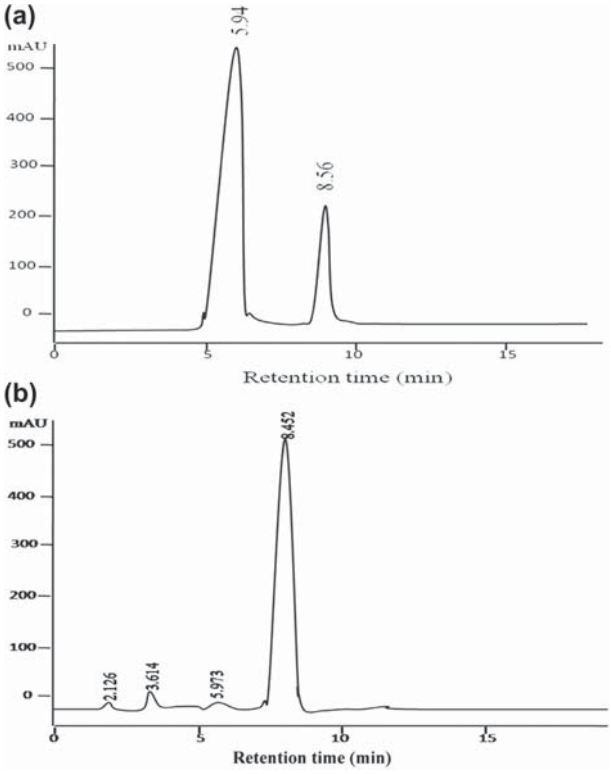


Figure 1. HPLC chromatogram for decarboxylative aldol reaction in aqueous organic co-solvent after 24 h using (a) free CALB in aqueous organic co-solvent after 24 h (b) denatured CALB.

further investigations on the mechanism of the reaction. For maintaining nearly anhydrous medium, CALB precipitated with acetone (Shah & Gupta 2007), protein coated microcrystals (PCMCs) (Kreiner et al. 2001) and cross-linked protein coated

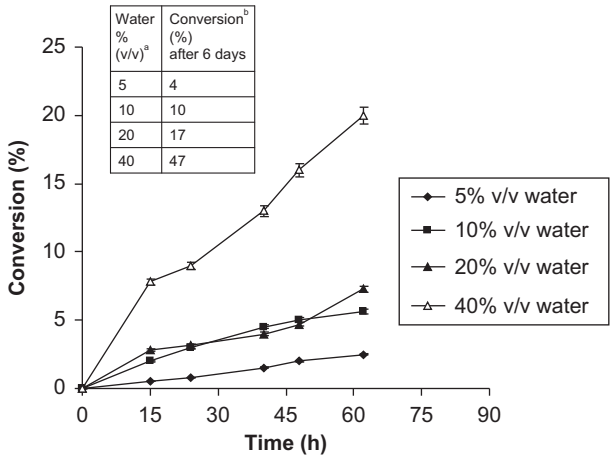


Figure 2. Effect of increasing percentage of water in reaction medium containing acetonitrile as a solvent and using alcalase as an enzyme. [a]beyond 40% water (v/v) in the reaction medium, 4-nitrobenzaldehyde becomes insoluble. Experiments were carried out in triplicates and percentage error in set of readings was within 5%.

microcrystals (CLPCMCs) (Shah et al. 2008) of CALB were used as catalysts. These formulations have been reported to be more efficient biocatalysts in low water as compared to lyophilized powders (Kreiner et al. 2001; Shah & Gupta 2007; Shah et al. 2008). All CALB formulations showed significant levels of aldol reaction. Both PCMC and CLPCMC of CALB gave ~90% conversion in 24 h (Figure 5). Interestingly, similar formulations of *Mucor javanicus* lipase gave less than 5% conversion even after 16 h. The 'dry' preparations of α-chymotrypsin, papain and alcalase obtained by precipitation with dried

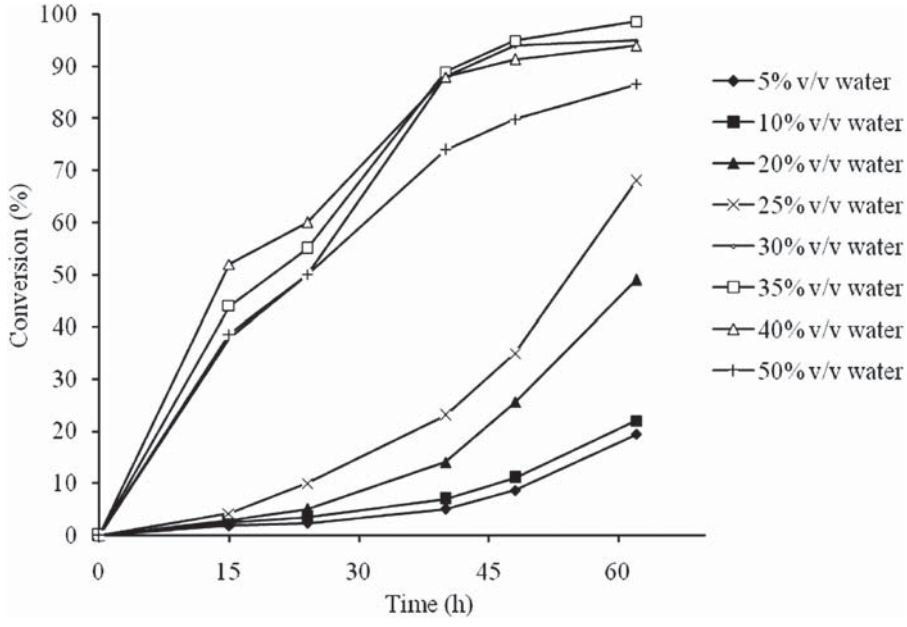


Figure 3. Effect of increasing concentration of water in reaction medium containing DMSO as a solvent and using alcalase as an enzyme. Reaction in which no aqueous buffer was added (i.e. contains 1.6% v/v water due to alcalase) shows less than 5% conversion after 62 h. Experiments were carried out in triplicates and percentage error in set of readings was within 5%.

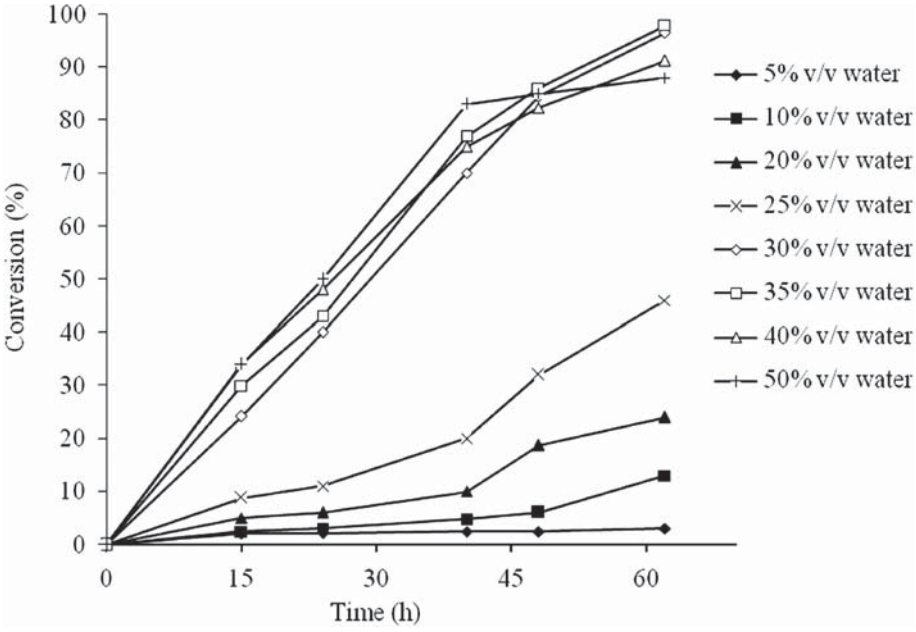


Figure 4. Effect of increasing concentration of water in reaction medium containing DMF as a solvent and using alcalase as an enzyme. Reaction in which no aqueous buffer was added (i.e. contains 1.6% v/v water due to alcalase) shows less than 5% conversion after 62 h. Experiments were carried out in triplicates and percentage error in set of readings was within 5%.

organic solvent were also tried in nearly anhydrous acetonitrile and gave less than 1% conversion in 24 h (data not shown). This indicates that the results with CALB formulations are not artifacts. Evitt and Bornscheuer (2011) believe the hydrolysis step to be the first only step which is enzyme catalysed. If the enzyme-catalysed reaction does involve hydrolysis as the first step, it would need at least stoichiometric amount of water in the reaction medium. A trace would not do. Based upon 0.75 mmoles of ethylacetoacetate as the starting material, at least 1.04% (w/w) water would be needed for complete hydrolysis. Considering that our conversion was ~90% even in dry anhydrous medium, that level of residual water is considered unlikely.

Table VI. Effect of variation of imidazole concentration on decarboxylative aldol reaction in DMSO and DMF containing 35% v/v aqueous buffer and using alcalase as an enzyme.

Solvent	Imidazole (mol%)[a]	Time (h)[b]	Conversion (%)[c]
DMSO	5	48	99
	10	30	98
	20	24	98
DMF	5	48	98
	10	48	99
	20	40	99

[a]Additive added was % mol/mol with respect to substrate 4-nitrobenzaldehyde. Control reaction (without enzyme) in presence of imidazole (20 mol%) in either of the solvents gave less than 8% conversion after 48 h.
[b]This is the time beyond which % conversion did not increase.
[c]These were the maximum conversion (%) possible under the reaction conditions.

Conclusion

There is growing evidence emerging from various groups that enzymes catalyse promiscuous reactions (Chen et al. 1991; De Souza et al. 2009; Feng et al. 2009; Fessner 2008; Li et al. 2009b, 2010; Madeira Lau et al. 2000; Majumder et al. 2009; Teo et al.

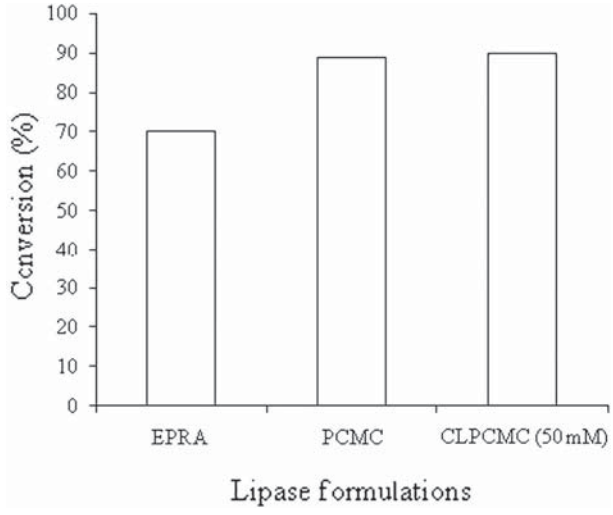


Figure 5. Use of different lipase formulations of CALB to catalyse decarboxylative aldol reaction in nearly anhydrous acetonitrile. Conversions (%) were determined after 24 h using HPLC.

2004). In particular, that lipases catalyse C–C bond formation (Kapoor & Gupta 2012).

In a recent review entitled 'What makes an enzyme promiscuous?' Babtie et al. (2010) observed 'Kinetic analyses of promiscuous enzymes reveal rate accelerations, $(k_{cat}/K_m)/k_2$, of up to 10^{18} for their secondary activities. Such large values suggest that binding and catalysis can be highly efficient for more than one reaction, challenging the notion that proficient catalysis requires specificity.' Admittedly, enantioselectivity so far reported in the literature has been rather poor. Given that enzyme structure represents a trade-off between stability, catalytic efficiency and enantioselectivity (Majumder & Gupta 2011), this should in fact reassure us that catalytic promiscuity is a true facet of enzyme behaviour. The k_{cat}/K_m and stability of enzymes from thermophilic sources reflects this trade-off.

The purpose of this current work was not to prove that catalytic promiscuity occurs, but to outline a more efficient way for a conversion. Whether the route is promiscuous or not is debatable. Evitt and Bornscheuer (2011) feel that it is a normal catalysis, admitting 'The mechanism detailing why acetoacetate will undergo aldol reaction, while ethyl acetoacetate does not, has not been investigated by us.' We have reinvestigated the reaction in as dry a condition as possible and feel extensive study of that detailed mechanism may only settle the issue.

In order to prove that active enzyme was necessary [either for catalysing a normal reaction – a mechanism outlined by Bornscheuer, or promiscuous reaction – a mechanism favoured by Feng et al. (2009)], we carried out a control with urea denatured lipase and found that the decarboxylative aldol reaction no longer took place at a significant rate. Whether lipase/protease catalyses all the steps of the conversion or their role stops at the hydrolysis level is a separate issue. It is important enough that a high level of conversion to a product resulting from decarboxylative aldol reaction is possible. Both lipases and a protease alcalase catalyse this in both co-solvent mixtures and nearly anhydrous medium.

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