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ORIGINAL ARTICLE

Increasing the catalytic efficiency of *Candida rugosa* lipase for the synthesis of *tert*-alkyl butyrates in low-water media

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Abstract
Reactions involving *tert*-alcohols and their esters are generally not catalyzed by lipases. *Candida rugosa* lipase is one of the few lipases which shows at least limited catalytic activity towards *tert*-alcohols and their esters. Using transesterification of tributyrin with tertiary butyl and amyl alcohols as a model reaction, it is shown that precipitation of lipase by a tertiary alcohol in the presence of a buffer with optimum concentration enhances the catalytic activity 7 fold as compared to rates obtained with lyophilized powders. Optimization of the ratio of triglyceride to tert-alcohols and medium engineering gave an initial rate which was 41 times higher than that obtained with lyophilized powders. Hence, use of a simple enzyme formulation, coupled with optimization of reaction conditions led to *Candida rugosa* lipase becoming a useful catalyst for catalyzing transesterification involving tertiary alcohols.

Keywords: *Lipases, transesterification, tertiary alcohols, enzyme catalysis in low water media, tributyrin, enzyme precipitated and rinsed with propanol*

Introduction

Among the enzymes used in low water media for biotransformation, lipases occupy the most dominant position. Esterification (Dandavate and Madamwar, 2007; Dave and Madamwar, 2008; Dave and Madamwar, 2010), transesterification (Carrea and Riva, 2000), interesterification (Bloomer et al., 1991), kinetic resolution of alcohols and carboxylic acids (Colton et al., 1995), are important types of reactions catalysed by this versatile class of enzymes. One class of compounds which are poor substrates for lipases is *tert*-alcohols and their esters (O'Hagan and Zaidi, 1994; Boseley et al., 1997). There has been considerable interest in tertiary alcohols (and their derivatives) as building blocks (Itoh et al., 1993; Forrat et al., 2005; Forrat et al., 2008). Examples of natural products containing *t*-alcohols as constituents include Cinatrin C, Integerrimine, Herringtonine, Fostriecin, Viridofungin and Zaraqozic acid (Raghavan and Sreekanth, 2008). Few lipases e.g. porcine pancreatic lipase (Zaks and Klibanov, 1994), *Candida rugosa* lipase (CRL, formerly known as *Candida cylindracea* lipase), (O'Hagan and Zaidi, 1994) and *Candida antarctica* lipase A (CAL A) (Krishna et al., 2002) have shown at least low activity towards such substrates. This is due to the presence of the "GGGX" loop and other structural features creating enough space in the binding pocket to accommodate bulky tertiary alcohols (Henke et al., 2002). In most of these cases, either lyophilized powder or "straight from the bottle" (as supplied by the vendor) forms of lipases have been used. It is now well established that lyophilization or drying under vacuum leads to structural changes which are deleterious to the enzyme activity and can not be reversed under low water conditions (Lee and Dordick, 2002; Roy and Gupta, 2004a; Hudson et al., 2005). Cross-linked enzyme aggregates (CLEAs) (Lopez-Serrano et al., 2002; Shah and Gupta, 2007), protein coated microcrystals (PCMCs) (Kreiner et al., 2001; Shah and Gupta, 2007) and cross-linked protein coated microcrystals (CLPCMCs) (Shah et al., 2008) have been shown to be better forms for catalyzing reactions in low-water media. In all such cases, "drying" (removal of excess water) is carried out by precipitation with organic solvents (Shah and Gupta, 2007). An even simpler preparation obtained by precipitation with *n*-propanol called EPRP (enzyme precipitated and rinsed with

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propanol) (Roy and Gupta, 2004b) gave higher rates than lyophilized powders. Organic solvents other than *n*-propanol have been used, so such preparations in general can be called enzyme precipitated and rinsed with organic solvents (EPROS) (Shah and Gupta, 2007). In earlier works, it has been shown that EPROS are also more stereoselective than lyophilized powders in low water media (Shah and Gupta, 2007). In this present work, it is shown that one simple way of catalyzing biotransformations involving *tert*-alcohols (and their derivatives) quite effectively is by using EPROS of CRL. This should pave the way for enzyme based synthetic routes involving *tert*-alcohols and their esters.

Materials and Methods

Enzyme and chemicals

Candida rugosa lipase (Lipase AYS, Amano) was obtained from Amano Enzymes Inc., Nagoya, Japan. Anhydrous acetone (>99%), methyl tertiary butyl ether (MTBE), cyclopentyl methyl ether (CPME) and *n*-propanol (>99%) were purchased from Sigma-Aldrich, St. Louis, USA. Tertiary butyl alcohol (>98%) and tertiary amyl alcohol (>99%) were purchased from Merck, Mumbai, India and Merck, Honenbrunn, Germany respectively. Tributyrin (>99%) was obtained from Himedia Laboratories Pvt. Ltd, Mumbai, India. Alcohols were distilled and dried over 3Å molecular sieves overnight before use.

Preparation of different formulations of the biocatalyst for low-water media

Lyophilized enzyme preparation. Candida rugosa lipase powder (10 mg) was dissolved in 200 µL of 50 mM phosphate buffer (pH 7.0), frozen at −20°C and was lyophilized for 24 h.

EPROS (Enzyme precipitated and rinsed with organic solvents). Candida rugosa lipase powder (10 mg) was dissolved in 100 µL of phosphate buffer (50–250 mM, pH 7). Precipitation of the enzyme was done by adding the enzyme solution into 1.5 mL ice-cold anhydrous (dried with molecular sieves) organic solvents viz. acetone, propanol, *tert*-alcohols in a 2 mL eppendorf and was allowed to settle at 4°C. After 1 h the precipitate was collected by centrifugation at 9000 × *g* at 4°C for 5 min. The complete precipitation of the enzyme was confirmed by protein assay of the supernatant after precipitation (Bradford, 1976). This precipitation was followed by repeated rinsing (four times) with 1.5 mL of ice-cold precipitating solvent (anhydrous or containing desired amount of water). Each rinsing step was followed by centrifugation at 9000 × g at 4°C for 5 min. Finally, the precipitate was rinsed with *tert*-alcohols (1.5 mL, containing 3% water, w/w enzyme, which were going to be used for alcoholysis) and were directly used for the reaction.

CLEAs (cross-linked enzyme aggregates)

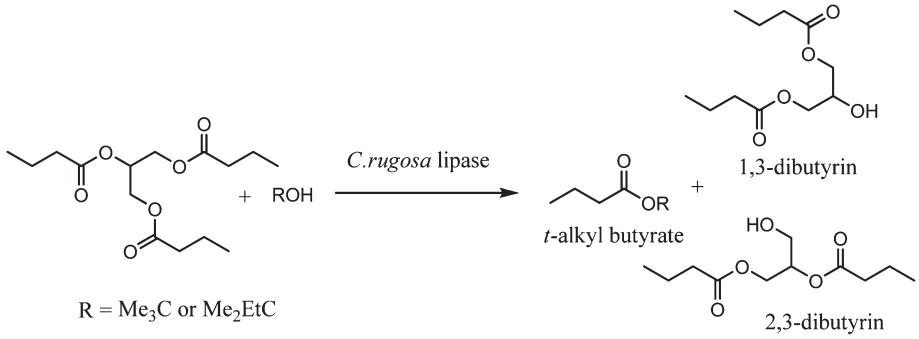
CLEAs were made as mentioned earlier (Lopez-Serrano et al., 2002) using 0.2 M phosphate buffer (pH 7.0). The precipitation followed by cross-linking was done in dimethoxyethane or *tert*-butyl alcohol or in *tert*-amyl alcohol by using 50 mM glutaraldehyde (20 µL of 25% aqueous solution) under constant shaking on an orbital shaker at 300 rpm at 4°C for 17 h.

Enzymatic alcoholysis of tributyrin

In screw-capped vials, tertiary alcohols were taken in excess tributyrin such that, the reaction volume was 1 mL. The concentration of alcohols in the tributyrin (in excess and also playing the role of the solvent) was 1 M. The alcohol was wetted with water which was 3% (w/enzyme weight) i.e. 0.3 mg/10 mg of enzyme (Zaks and Klibanov, 1994). Lipase formulations, prepared from 10 mg commercial powder, were added and the reaction was carried out on an orbital shaker at 250 rpm at 20°C.

Gas chromatographic analysis

Aliquots from the reaction mixtures were taken at different time intervals and analyzed by GC in an Agilent 6890 N system fitted with a capillary column EQUITY $^{TM-5}$ (30 m × .32 mm × 0.25 µm film thickness) from Supelco (Bellefonte, USA) with flame ionization detection and programmed oven: initial temperature 100°C, ramp 1 at 10°C/min up to 150°C, ramp 2 at 15°C/min up to 250°C. Peaks were assigned after running purified samples of the products (see next section for details of purification of the compounds). In GC analysis, the retention times of the compounds were noted as: tributyrin (11.16 min), dibutyrins (9.26 min and 9.29 min), mono butyrin (6.65 min), tertiary butyl butyrate (6.42 min), glycerol (3.39 min, in traces), butyric acid (3.05 min) when tertiary butyl alcohol was the chosen alcohol. In the absence of available authentic compounds, it was not possible to identify the dibutyrin peaks in terms of 1,3 and 2,3 dibutyrins (these are not commercially available). These dibutyrins are normally obtained by lipase catalysed hydrolysis of triglycerides (Hou, 2005). However acyl migration in lipase catalysed transesterification of tributyrin has been extensively studied (Sjursnes and Anthonsen,



Scheme 1. Lipase catalyzed alcoholysis of tributyrin.

1994; Sjursnes et al., 1995). The focus of the current work was to explore the possibility of making transesterification with *tert*-alcohols more efficient for obtaining corresponding *tert*-alkyl butyrate. However, the product esters, the tributyrin and the mono butyrin could be well separated from each other by column chromatography (details given in the product purification and characterization section) and the GC peak positions of these compounds could be confirmed. Similar pattern was observed in case of *tert*-amyl alcohol with a slight shift in peak position of the ester *tert*-amyl butyrate which was detected at 6.5 min.

Product purification and characterization

The products were purified by column chromatography using a silica gel (200 mesh) column (10 cm length × 3 cm diameter) and eluted with a mixture of hexane, ethyl acetate and acetic acid taken in 80:19:1 v/v ratio. The amount of the crude reaction mixture loaded: 100 mg (~100 micro litre), volume of the eluent: 80 mL, flow rate 1 mL/ min. The fractions were analysed by thin layer chromatogarphy using hexane, ethyl acetate, acetic acid (in 80:20:1 v/v ratio) and developed with iodine. The R_f values were: *tert*-alkyl butyrate (0.83-0.9), tributyrin (0.75), dibutyrins (0.43). The purified fractions were evaporated in rotary evaporator and the identities of the products were confirmed by ^{1}H-NMR analysis. The presence of 1,3- and 2,3- dibutyrins in the mixture of dibutyrins were confirmed by ^{13}C spectra, which showed two carboxyl carbon peaks around $\delta = 173$ ppm in ~1:2 ratio. These dibutyrins appear at 9.26 min and 9.29 min in the GC spectra with more or less in the same ratio (Figure 5). $C_8H_{16}O_2$ (*tert*-butyl butyrate, 24 mg, 17% yield) ^{1}H-NMR δ ($CDCl_3$, 300 MHz, ppm): 0.9 (3 H, triplet, $\mathcal{J} = 7.3$ Hz), 1.3 (9 H, s), 1.7 (2 H, m, $\mathcal{J} = 7.4$, 7.3 Hz), 2.3 (2 H, triplet, $\mathcal{J} = 7.4$ Hz). $C_9H_{18}O_2$ (*tert*-amyl butyrate, 21 mg, 13% yield) ^{1}H-NMR δ($CDCl_3$, 300 MHz, ppm): 0.9-1 (6 H, merged triplet, $\mathcal{J} = 7.3$, 7.2 Hz), 1.3 (6 H, s), 1.6 (2 H, quartet, $\mathcal{J} = 7.2$ Hz), 1.7 (2H, m, $\mathcal{J} = 7.3$, 7.4 Hz), 2.3 (2 H, triplet, $\mathcal{J} = 7.4$ Hz).

Results and Discussion

The earliest attempt at using lipases with *t*-alcohols as substrates was transesterification of tributyrin with *tert*-butyl alcohol (Scheme 1) (Zaks and Klibanov, 1994). Freeze-dried powder of *Candida rugosa* lipase was used as the biocatalyst. In order to investigate whether other enzyme forms could perform better,

Table I. Transesterification of *tert*-butyl alcohol with tributyrin under solvent free conditions.

Entry	*C. rugosa* lipase formulations	Organic solvent used for precipitation	Water added (%, w/w, lipase)	Initial rates* (μM/mg/h)
1	FD	–	0	0
2	FD	–	3	16.6
3	EPROS†	acetone	0	2.2
4	EPROS	acetone	3	68
5	EPROS	*n*-propanol	0	2
6	EPROS	*n*-propanol	3	90
7	EPROS	*tert*-butyl alcohol	0	5
8	EPROS	*tert*-butyl alcohol	3	102

*Synthesis of *t*-butyl butyrate: 1 mL reaction medium containing *tert*-alcohol (1M) in excess tributyrin (Zaks and Klibanov, 1994) was incubated with 10 mg lipase formulations at 20°C under constant shaking at 250 rpm for 72 h. Initial rates were based upon conversions obtained by GC analysis.

†50 mM phophate buffer (pH 7.0) was used during EPROS formation.

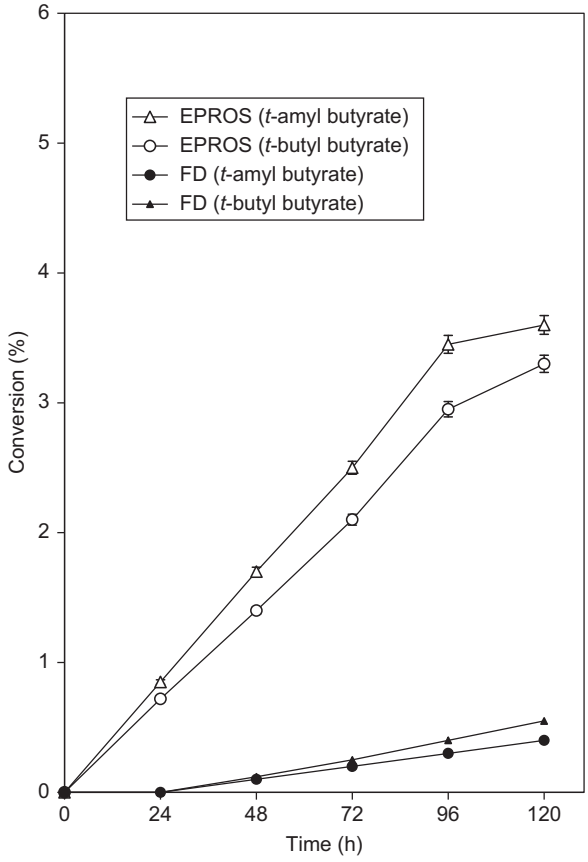


Figure 1. Formation of *tert*-alkylesters by different formulations of *C. rugosa* lipase (prepared in 50 mM phosphate buffer). The conversion (%): The ordinate parameter refers to formation of *t*-alkyl esters only. Reaction conditions were similar to those in Table I and II. Each reaction set was run in duplicate and deviations between pairs of experimental data were within 2%.

the same reaction was investigated in the current work. Table I shows that under the conditions (3% w/w added water) used in Zaks and Klibanov (1994), the CRL freeze-dried powder gave similar initial rates. The purpose here was to compare the initial rates obtained with freeze-dried preparations with EPROS prepared by precipitation with two organic solvents and *tert*-butyl alcohol under identical conditions. Therefore, 3% water originally used by Zaks and Klibanov (1994) was used. Hence, no effort to optimize water concentration was made at this stage. The initial rates were based on the formation of the *tert*-alkyl ester. These were calculated from the linear part of the ester formation vs time plots (Figure 1). All EPROS preparations gave higher initial rates than the freeze-dried powder. Interestingly, *tert*-butyl alcohol (as the precipitating and rinsing solvent) gave the most catalytically efficient EPROS. This may be due to some marginal "imprinting effect" of the kind observed by Stähl et al. (1990) while precipitating an enzyme in the presence of a substrate. To confirm that the observations were valid for *t*-alcohols in general, alcoholysis of tributyrin with *tert*-amyl alcohol was also carried out (Scheme 1, Table II). The results obtained were similar to the first reaction. A dry medium, without any added water was found to produce very poor reaction rates (Zaks and Klibanov, 1994); with EPROS some very poor conversions were obtained while with the freeze-dried powder no conversion occurred even after 72 h. The role of small amounts of water in enhancing protein flexibility and consequently initial rates has been known for a long time (Zaks and Klibanov, 1988). Addition of a small amount of water (3%, w/w enzyme) caused a sharp rise in the reaction rates and, under these low water conditions, EPROS exhibited 5–6 times better rate than the controls (Table I, II; entries 2, 8; Figure 1). Other water concentrations between 0–3% or beyond 3% were not examined. A completely anhydrous condition was tried (0% water) and no reaction took place (Table I). A higher amount of water is likely to cause loss of the "imprinting effect". Earlier results (Roy and Gupta, 2004b) with EPRP, indicated that optimization of the molarity of the buffer (from which the enzyme is precipitated) leads to enhancement of initial rates. Figure 2 shows change in initial rates

Table II. Transesterification of *tert*-amyl alcohol with tributyrin under solvent free conditions.

Entry	*C. rugosa* lipase formulations	Organic solvent used for precipitation	Water added (%, w/w, lipase)	Initial rates* (μM/mg/h)
1	FD	–	0	0
2	FD	–	3	20
3	EPROS†	acetone	0	2
4	EPROS	acetone	3	90
5	EPROS	*n*-propanol	0	3
6	EPROS	*n*-propanol	3	98
7	EPROS	*tert*-amyl alcohol	0	10
8	EPROS	*tert*-amyl alcohol	3	112

*Synthesis of *t*-amyl butyrate: 1 mL reaction medium containing *t*-alcohol (1M) in excess tributyrin (Zaks and Klibanov, 1994) was incubated with 10 mg lipase formulations at 20°C under a constant shaking at 250 rpm for 72 h. Initial rates were based upon conversions obtained by GC analysis.

†50 mM phophate buffer (pH 7.0) was used during EPROS formation.

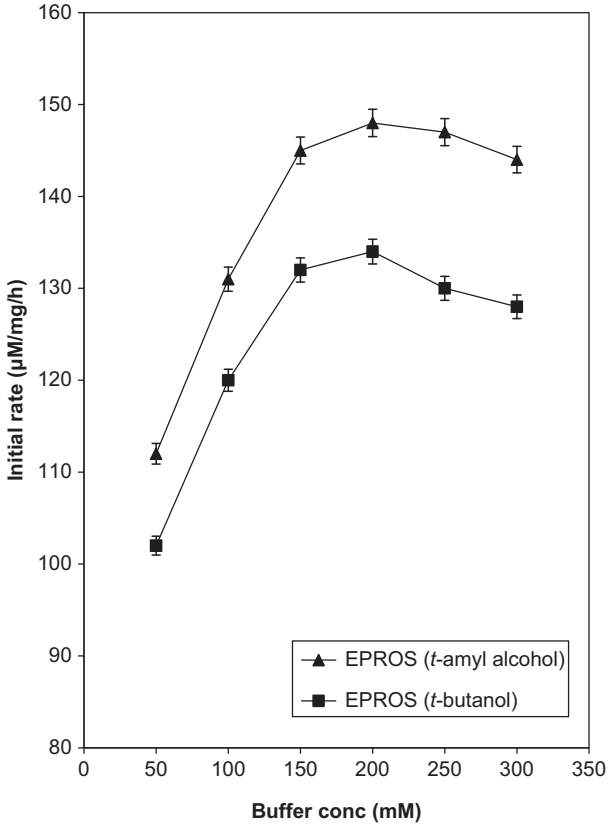


Figure 2. Effect of increase in buffer concentration on the initial rates of EPROS. Reaction conditions were similar to those in Table I and Table II. Each reaction set was run in duplicate and deviations between pairs of experimental data were within 2%.

with a change in the molarity of the buffer. It shows that with an increase in buffer molarity up to 150 mM the initial rate increased sharply, reaching an optimum at around 200 mM followed by a marginal decrease. Use of this optimum buffer molarity led to EPROS which gave initial rates of 134 μM $mg^{-1}h^{-1}$ for *tert*-butyl butyrate synthesis and 148 μM $mg^{-1}h^{-1}$ for *tert*-amyl butyrate synthesis. The increases in rates over those observed without this optimization (Table I and II) were significant, though not dramatic. The conditions of enzyme precipitation with organic solvents have been known to influence the resulting enzyme activity for a long time, even using aqueous solution (Niederauer and Glatz, 1992).

Use of CLEAs for synthesis

Cross-linked enzyme aggregates (CLEAs) of CRL are another biocatalyst formulation used in organic synthesis, which are known to exhibit higher initial rates in low water media compared to the freeze-dried formulations (Lopez-Serrano et al., 2002). CLEAs involve use of organic solvents for precipitating the enzyme as the first step. Table III shows that tertiary butyl alcohol and tertiary amyl alcohol were also the better precipitants in this case. However while the corresponding CLEAs exhibited better lipolytic activity (Decker, 1977) in aqueous medium, when they were used for synthesis they exhibited a lower initial rate by about 30 μM/mg/h (Table III) compared to the EPROs. These results indicate that a more flexible enzyme structure is better for accommodating the bulky tertiary alkyl groups in the active site. Presumably, rigidity introduced as a result of cross-linking prevented accommodation of the tert-alkyl group in the active site. Further experiments were carried out with EPROS only.

Table III. Use of CLEAs for the synthesis of *t*-alkylbutyrates.

Entry no	Precipitant*	**Total Lipolytic activity of the precipitate [U]	CLEA activity (%, free enzyme)	Initial rates† (μM/mg/h)
1	Acetone	302	–	–
2	*n*-propanol	309	–	–
3	DME	326	89	–
4	*tert*-butanol	410	113	112
5	*tert*-amyl alcohol	466	135	116

*Three organic solvents which precipitated 100% or > 100% activity with respect to the specific activity of the control (326 units/ mL/ 10 mg of commercial powder dissolved in phosphate buffer, 200 mM, pH 7) were used for CLEA preparation.
**The total Lipolytic activity in each precipitate was measured by dissolving the precipitate completely in the assay buffer.
†These were initial rates of transesterification of the tributyrin with the t-alcohol (used as the precipitant) under solvent free conditions (and hence can be compared with corresponding transesterification rates given in Table I and II).

Effect of the relative molar ratio of the alcohol to the acyl donor on the synthetic rate

It is expected that concentrations of substrate alcohol should affect the enzyme activity and product yield (as transesterifications are thermodynamically controlled reactions) (Nordblad et al., 2008). Furthermore, alcohols including amyl alcohols are reported to inhibit *C. rugosa* lipase (Bezbradica et al., 2006). The concentration of alcohols will also govern the medium polarity especially under the solvent free conditions used in the present experiments. Figure 3 shows that a two times molar excess of *tert*-butyl alcohol (mol/mol tributyrin) and a four times molar excess of *tert*-amyl alcohol (mol/mol tributyrin) were optimum for *t*-butyl butyrate and *t*-amyl butyrate synthesis respectively. It is interesting to note that, above these optimum alcohol concentrations, the decrease in initial rate was sharper for *tert*-butyl alcohol. Presumably, greater stripping of the essential layer of water by the less polar alcohol was the determining factor (Gupta, 1992; Carrea and Riva, 2000).

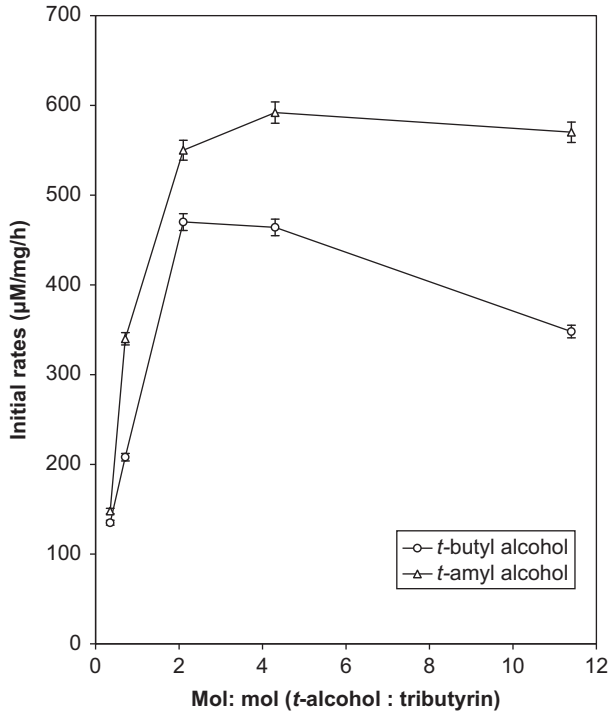


Figure 3. Effect of increase in *tert*-alcohol concentration (x mols tributyrin) on the initial rates of synthesis of *tert*-alkyl butyrates under solvent free conditions. 1mL reaction medium containing tertiary alcohol and tributyrin with molar ratios 0.3 (1M alcohol in 3.2 M tributyrin) to 11.4 (0.7 M tributyrin in 8 M alcohol) was incubated with 10 mg EPROS at 20°C under constant orbital shaking at 250 rpm. Initial rate of *tert*-alkyl butyrate formation was determined by GC. Each reaction set was run in duplicate and deviations between pairs of experimental data were within 2%.

Effect of the solvent on the initial rate

The previous reactions were carried out in solvent free conditions. Here, alcohols predominantly dictated the medium effects. In many earlier studies, non polar media have been reported to give higher reaction rates (Reslow et al., 1987). Thus using a non polar solvent as a medium (instead of solvent free conditions) was also tried. Figure 4 shows that while all other solvents examined decreased the reaction rate, the addition of octane led to a 1.5 fold increase in the initial rate of *t*-butyl butyrate synthesis. Significantly, in the case of *tert*-amyl butyrate synthesis, mixing the *t*-amyl alcohol with other solvents including octane did not improve the reaction rate to a varying degree. This not only reflects the difference in the two alcohols as nucleophiles but also as the reaction medium, in accordance with earlier observations that *tert*-amyl alcohol is an unusually better solvent based purely on the polarity of the solvents as reaction media (Zaks and Klibanov, 1988; Shah et al., 2008). Similar effects have been observed by others. In *Candida rugosa* lipase catalyzed synthesis of amyl butyrate by esterification. Bezbradica et al. (2006) found that the initial rate of esterification was better in amyl alcohol than in isooctane.

What led to such high rates with EPROS while using the *tert*-alcohols in alcoholysis? To start with, unlike most lipases, CRL shows some activity towards *t*-alcohols (and their esters) since the GGGX loop and some other structural features provide enough space in the binding pocket for the quaternary carbon atom (Henke et al., 2002). Colton et al. (1995) reported that "pre-treatment" of crude CRL with 2-propanol gave 2.3 to 25 times higher enantioselectivity (towards seven esters) as compared to the crude enzyme. The pre-treatment involved purification and presumably change in the "closed to open conformation". The initial rates in anhydrous media were not reported, so it is difficult to compare our results with theirs. In the present work, precipitation did not remove any contaminating protein (as all the protein was found to precipitate with the organic solvent), so no purification was involved. While conversion to an open lid structure or a marginal imprinting effect can not be ruled out, the probable cause of obtaining an efficient catalyst was that the enzyme was "dried" (removal of excess water) without lyophilisation, thus preventing structural changes (Lee and Dordick, 2002). Clearly, retaining flexibility would be necessary to accommodate the *tert*-butyl group or *tert*-amyl group in the enzyme binding pocket. Its importance can be appreciated by the fact

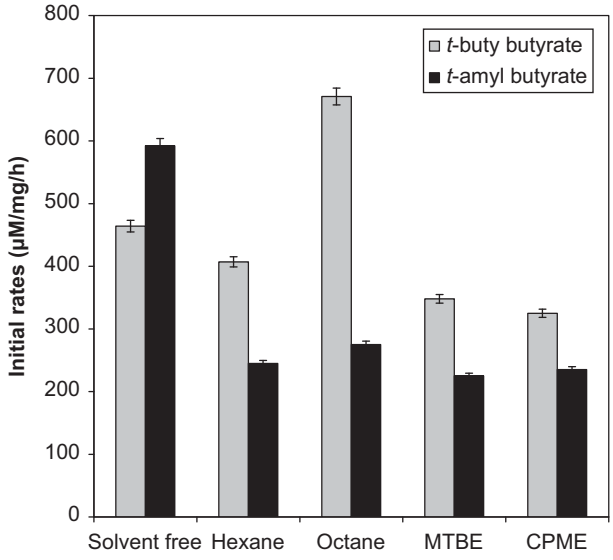


Figure 4. Effect of the presence of solvent on the initial rate of synthesis of *tert*-alkyl butyrates. 2 mL reaction medium containing 1 mL of substrates (*tert*-butyl alcohol and tributyrin in 2:1 molar ratio; *tert*-amyl alcohol and tributyrin in 4:1 molar ratio) and 1 mL of added solvent was incubated with 20 mg lipase formulations at 20°C under constant orbital shaking at 250 rpm. Initial rates of the *tert*-alkyl butyrate formation were determined by GC. Each reaction set was run in duplicate and deviations between pairs of experimental data were within 2%.

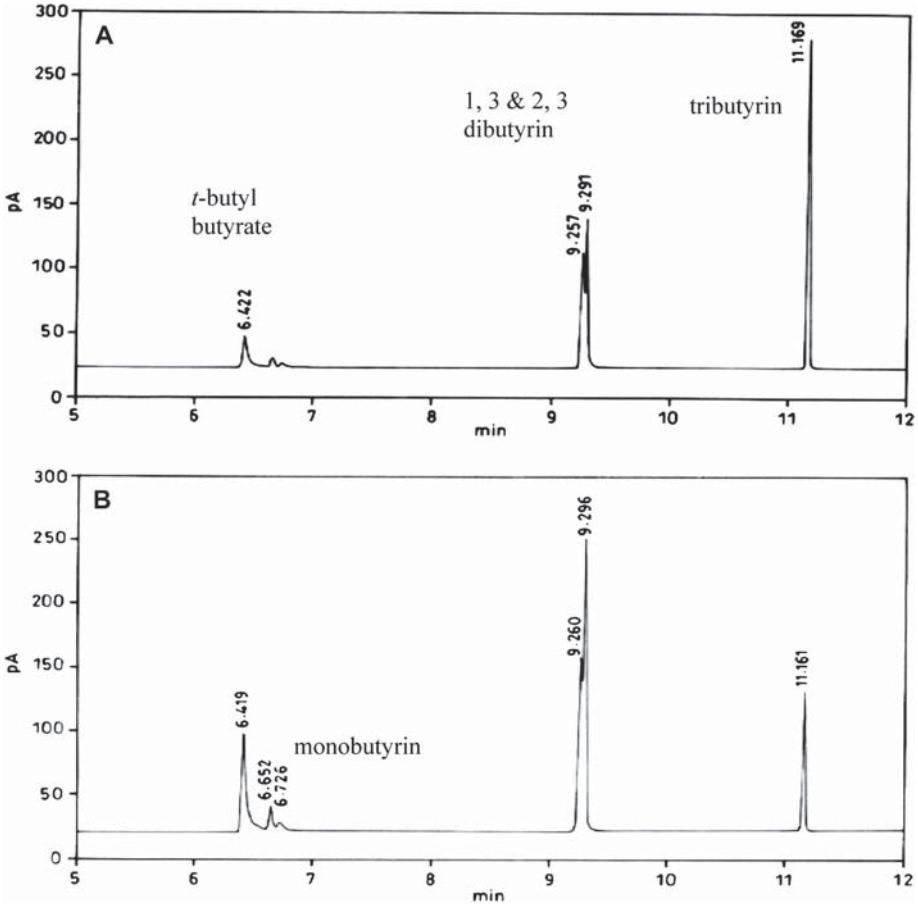


Figure 5. Gas chromatographic analysis: formation of *tert*-butyl butyrate and dibutyrins. A) After 24 h, at 16–17% conversion of tributyrin to the tertiary ester B) after 4 days, at ~20% conversion. The conversion of tributyrin to dibutyrins were 20% after 24 h and > 50% after 4 days. The peak positions were confirmed after running purified NMR grade samples (The results of the NMR of the purified compounds are given in terms of J values in the text).

that when EPROS were cross-linked to form CLEAs, the initial rates in fact decreased. In the case of *tert*-butyl butyrate synthesis, with 1 M alcohol in excess tributyrin, under solvent free conditions, the decrease in initial rate observed was from 134 μM $mg^{-1}h^{-1}$ to 110 μM $mg^{-1}h^{-1}$ and for *tert*-amyl butyrate it was from 148 μM $mg^{-1}h^{-1}$ to 116 μM $mg^{-1}h^{-1}$ when EPROS were cross-linked to form CLEAs (Table III, Figure 2).

Thus while the freeze-dried CRL gave 16 μM $mg^{-1}h^{-1}$ for *tert*-butyl butyrate formation, under the optimized conditions, EPROS of the same enzyme gave 671 μM $mg^{-1}h^{-1}$ as the corresponding rate. This is an increase of 41 fold.

In the present study, the yields were 17% and 13% (both after column purification) for *t*-butyl butyrate and *t*-amyl butyrate respectively. The synthesis of n-butyl butyrate by transesterification of tributyrin with n-butanol using *C. rugosa* lipase is reported to give 92% yield (Chowdary and Prafulla, 2002). This shows that there are still considerable challenges associated with using lipases for reactions involving *t*-alcohols.

Conclusion

By combining improved "drying" the lipase along with optimization of reaction conditions, *C. rugosa* lipase can be a fairly efficient catalyst for alcoholysis with two different commonly used *tert*-alcohols. *C. rugosa* lipase is one of the few lipases which naturally shows at least limited catalytic activity towards tertiary alcohols and their esters. The present work shows that precipitation of the lipase with a tertiary alcohol in the presence of a buffer of optimum concentration enhanced the catalytic activity seven fold compared to the similar rates obtained with lyophilized powders. Optimization of the ratio of triglyceride to tertiary alcohols and medium engineering enhanced the initial rate 41 fold.

The study also demonstrates that for the synthesis of tertiary alcohol esters, a simple preparation like EPROS was better than more complicated formulations like CLEAs which, in many cases, have proved very efficient biocatalysts in low-water media (Lopez-Serrano et al., 2002; Majumder et al., 2008).

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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