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Lipases for use in industrial biocatalysis: Specificity of selected structural groups of lipases

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"Dedicated to Kalle Hult on his 65th birthday".

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ABSTRACT

Lipases for biocatalysis

The substrate specificity of a selected group of lipases was investigated. The enzymes selected were from four structural groups. Group 1: lipases having wide alcohol binding cleft but a narrow acyl binding cleft (*Rhizomucor miehei* lipase, *Thermomyces lanuginosus* lipase, *Fusarium oxysporum* lipase); Group 2: lipases which exhibit strong restriction on the acid part having a narrow tunnel to accommodate the acyl group but wider alcohol binding site (*Candida antarctica* A, *Candida rugosa* lipase); Group 3: lipases having wide acyl binding cleft but narrow alcohol binding cleft (*C. antarctica* lipase B, *Ustilago maydis* lipase), and Group 4: having wider alcohol and wider acyl binding clefts (*Fusarium solani pisi* cutinase, *Humicola insolens* cutinase). Owing to the wide substrate specificity and higher expression levels in recombinant host, these lipases have tremendous importance for hydrolysis and synthesis reactions. Various substrates with substitutions on the alcohol and/or the acid part of the ester molecule were selected. The experimental results support the classification of lipases on the basis of their binding sites. For substrates with heavy alcohol side, *C. Antarctica* lipase A and *R. miehei* lipase type enzymes gave the highest extent of hydrolysis, while for acid heavy substrates the highest conversions were shown by *C. antarctica* lipase B. It is noteworthy that the acid heavy substrates which had aromatic side chains were hydrolyzed only by *C. antarctica* lipase B type of enzymes. Lipases were found to be more active on the alcohol-substituted substrates than acid-substituted substrates.

1. Introduction

Over the last few years, processes have been developed, using hydrolases, oxidoreductases or lyases as biocatalysts in pharmaceutical, agricultural, or synthetic organic chemistry industry. Lipases (triacylglycerol acyl hydrolases) exhibit wide substrate specificity, stereoselectivity and enantioselectivity and are therefore, industrially significant enzymes [1,2]. The use of lipases in non-aqueous environments proves an excellent methodology for the preparation of single-isomer chiral drugs by enzymatic hydrolysis, transesterification or aminolysis reactions. Applications of lipases in asymmetric synthesis include kinetic resolution of racemic alcohols, acids, esters or amines, as well as the desymmetrization of prochiral compounds [3–7]. In the pharmaceutical industry, there has been an ever-increasing trend for chiral drug substances to focus enantiomers instead of racemic mixtures, A major part of the drugs manufactured today contains enantiopure molecules [8]; hence, highly enantioselective reactions for the production of enantiopure building blocks are of great industrial importance. Enzymes are an attractive class of catalysts often used in the synthesis of enantiopure compounds. They usually exhibit high enantioselectivity, operate under mild reaction conditions and have a large substrate scope [9]. However, with substrates that are very different from the natural substrate, enzymes can display low enantioselectivity and/or poor reactivity. A solution to this problem is to genetically modify the enzyme and thereby increase the substrate acceptance or enantioselectivity.

Designing enantioselectivity of enzymes is one of the most attractive but challenging trials in the field of protein engineering for synthesis of enantiometically pure compounds, there is, however, no practical theory for introducing mutations into any enzyme to change its enantioselectivity. One way would be to model substrate lipases and esterases by substrate-imprinted docking, which takes into account the substrate transition states, productive and non-productive hydrogen bonds as well as com-

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Table 1
Classification of lipases on the basis of structural and physico-chemical properties of scissile fatty acid binding sites.

Lipase class	Group	Active-site properties
RmL type	1	Large alcohol binding cleft but a narrow acyl binding cleft
CaLA type	2	Strong restriction on the acid part having a narrow tunnel to accommodate the acyl group but wider alcohol binding site
CaLB type	3	Lipases having large acyl binding cleft but narrow alcohol binding cleft
Cutinase type	4	Having wider alcohol and wider acyl binding cleft

plete protein flexibility. [10]. Another approach would be to incorporate a desired enantioselectivity to enzymes by directed evolutionary strategy, which comprises iterative cycles of mutation and identification of improved variants by screening or selection [11–14]. In one report, CaLA was made enantioselective for 4-nitrophenyl 2-methylheptanoate, by using the CASTing (Combinatorial active-site saturation test) [15], while another that the enantioselectivity of hydantoinase has been inverted towards D,L-5-(2- methylthioethyl) hydantoin by error-prone PCR and following saturation mutagenesis [16], and enantioselectivity of a lipase from *Pseudomonas aeruginosa* was inverted by the combination of error-prone PCR and DNA shuffling [17].

Lipases have been classified on the basis of structural and physico-chemical properties of scissile fatty acid binding sites to understand the substrate specificity of lipases [18]. In the current study, the substrate specificity of four structural groups of lipases (Table 1): Group 1: lipases having large alcohol binding cleft but a narrow acyl binding cleft (*Rhizomucor miehei* lipase (RmL), *Thermomyces lanuginosus* lipase (TlL), *Fusarium oxysporum* lipase (FoL)); Group 2: exhibit strong restriction on the acid part having a narrow tunnel to accommodate the acyl group but wider alcohol binding site (*Candida antarctica* A (CaLA), *Candida rugosa* lipase (CrL)); Group 3: lipases having large acyl binding cleft but narrow alcohol binding cleft (*C. antarctica* lipase B (CaLB), *Ustilago maydis* lipase (UmL)), and Group 4: having wider alcohol and wider acyl binding cleft (*Fusarium solani pisi* cutinase (FsC), *Humicola insolens* cutinase (HiC)) was investigated.

The work presented here compares the substrate specificity of the various groups of lipases and also looks at the enantioselectivity of CaLB and some of its reported variants, M72L, T103G, and W104H [19].

2. Experimental

2.1. Strains, culture media and growth conditions

Pichia. pastoris CoLS702 [Mut$^-$] strain (this strain is a deletion mutant of *P. pastoris* GS115 strain, wherein the AOX1 gene has been deleted) was used for heterologous expression of CaLA, CaLB, TlL, CrL-1, HiC and CaLB variants T103G, M72L and W104H. The strains were grown and maintained in YPD medium containing yeast extract (10 g/l), peptone (20 g/l), and dextrose (20 g/l). For expression of the heterologous protein, the *Pichia* fermentation medium consisted of yeast extract (10 g/l), peptone (20 g/l), sorbitol (10 g/l), Potassium phosphate buffer (pH 6.0, 50 mM), and yeast nitrogen base without ammonium sulphate and amino acids (3.4 g/l). All fermentations were carried out in baffled Erlenmeyer flasks (1 l) containing 300 ml of fermentation medium at 27.5 °C and 150 rpm. The cells were induced after 24 h of growth with methanol (1%) and the temperature reduced to 22 °C. Methanol induction was followed every 24 h for 6 days after which the cells were removed by centrifugation at 12,000 × *g* for 25 min, the supernatant was filtered through 0.2 μ hollow fibre filter and processed for purification.

2.2. Construction of expression plasmids

A *P. pastoris* vector CoLS789 was used for the expression of CaLA, CaLB, TlL, CrL-1, HiC and CaLB variants T103G, M72L and W104H. This in-house vector has a *HIS4* selection marker, a multiple cloning site, the 3′ and 5′ sequences of a AOX1 gene of *P. pastoris* and a pUC origin of replication. The genes with the native peptide signal were sub-cloned under the control of AOX1 promoter. The resulting constructs were used to transform *Escherichia coli* DH5α. The plasmids were subjected to DNA sequencing for confirming the sequence.

2.3. Preparation of P. pastoris competent cells and transformation

YPD 100 mL (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose) medium was inoculated with a single *P. pastoris* colony and grown over night at 30 °C, 200 rpm to an OD_{600} of 0.8–1.0. Cells were collected by centrifugation (10 min, 1500 × *g*, and 20 °C), washed with 50 ml of sterile water and suspended in 2 ml of 100 mM $LiCl_2$. The cells were washed twice with 100 mM $LiCl_2$ and distributed in 100 μl aliquots. To each cell aliquot was added 240 μL of PEG 3350 (50%), 36 μL of $LiCl_2$ (1 M), 25 μL of single stranded DNA and 50 μL of linearized plasmid DNA. The cells were mixed and were incubated at 30 °C for 30 min without shaking and heat shock was given at 42 °C for 25 min. The transformation mix was centrifuged and supernatant was discarded. The transformants were suspended in 100 μL of sterile water spread on SD (Synthetic Dropout) plates (1.34% Yeast Nitrogen Base with ammonium sulphate without amino acids), 4×10^{-5}% (w/v) biotin, 2% (w/v) dextrose) and incubated at 30 °C for 2–4 days. The transformants were selected by their ability to synthesize and utilize histidine.

2.4. Purification

The purification methods for the various proteins are outlined below. Unless otherwise stated all columns used were of 20 ml column volume (CV), and equilibration, washing and elution steps consisted to 10 CV buffer.

2.4.1. CaLA and CaLB

Ammonium sulphate (0.8 M) was added to the fermentation broth and the broth passed through Butyl toyopearl hydrophobic column which was previously equilibrated with Ammonium acetate (0.8 M). The matrix was washed with equilibration buffer and the bound protein was eluted isocratically, first with water and later with ethanol (50%). The eluted protein was dialyzed against HEPES (pH 7.0, 50 mM) and passed through UnoQ (anion exchange). The unbound sample consisted of the purified protein which was concentrated using centrifugal concentrators and used for enzyme assay.

2.4.2. TlL

Sodium chloride (2 M) was added to the fermentation broth and the broth passed through Decyl agarose hydrophobic column which had been previously equilibrated with sodium borate buffer (pH 9.0, 50 mM). The matrix was washed with equilibration buffer and the bound protein was eluted isocratically with equilibration buffer containing Isopropanol (30%). The eluted fraction were pooled, the conductivity was adjusted <7 mS/cm and passed through Q-Sepharose (anion exchange) which had been previously equilibrated with sodium borate buffer (pH 9.0, 50 mM). The matrix was washed with equilibration buffer and the bound protein was eluted using a linear gradient of sodium chloride (1 M) in equilibra-

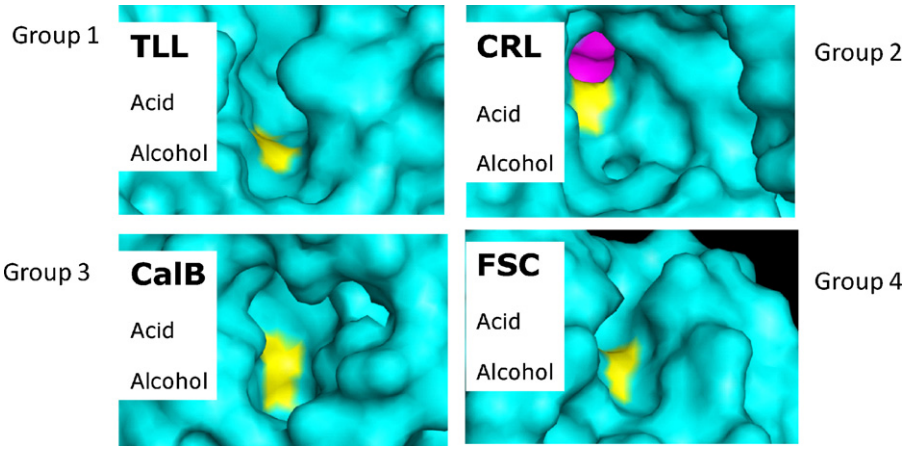

Fig. 1. The four selected structural classes of lipases. Representatives from each group oriented with superimposed active site. Active-site serine is shown in yellow, and with the acid binding part pointing upwards and the alcohol binding part pointing downwards. For the CrL the acid binding part is a deep tunnel going into the structure. Group 1; RmL (TlL: 1GT6), Group 2; CaLA (CrL; 1CrL), Group 3; CaLB (CaLB; 1TCA), and Group 4; FsC (FsC; 1CUS).

tion buffer. The eluted protein was pooled, concentrated and used for enzyme assay.

2.4.3. CrL-1

Ammonium sulphate (0.8 M) was added to the fermentation broth and the broth passed through Butyl toyopearl hydrophobic column which was previously equilibrated with Ammonium acetate (0.8 M). The matrix was washed with equilibration buffer and the bound protein was eluted isocratically first with HEPES (pH 7.0, 50 mM) and later with HEPES (pH 7.0, 50 mM) containing ethanol (50%). The eluted protein was dialyzed against HEPES (pH 7.0, 50 mM) and passed through UnoQ (anion exchange). The matrix was washed with equilibration buffer and the bound protein was eluted using a linear gradient of sodium chloride (1 M) in equilibration buffer. The eluted protein was pooled, concentrated and used for enzyme assay.

2.5. Enzyme assays

The activity of CaLA, CaLB, TlL, CrL-1, HiC and CaLB variants T103G, M72L and W104H were carried out in HEPES buffer (pH 7.0 50 mM) containing $CaCl_2$ (5 mM) and Triton-X-100 (0.4%). The reaction was started by the addition of substrate (40 mM) and enzyme (0.1 mg). All the reactions were performed at 30 °C for 2 h, 6 h, or 24 h to get appropriate conversion. The reactions were stopped with 100 μl HCl (1 M) and the reactants extracted with dichloromethane. The organic phase (20 μl) was diluted with diethylether and analyzed on GC fitted with chiral GC column (Varian CP-Chiralsil-DEX CB 10 m).

Two compounds, one from each substrate categories (A) substrates either branched/large on the alcohol part or (B) substrates either branched/large on the acid part were checked for enantioselective degradation of chiral isomers. The separation procedure for the two isomers on GC is outlined below.

2-ethyl hexyl acetate: Oven $T_1 = 70\,^{\circ}C$, $T_2 = 80\,^{\circ}C$ ($\Delta T = 1\,^{\circ}C$/min), $T_3 = 90\,^{\circ}C$ ($\Delta T = 2\,^{\circ}C$/min), $T_4 = 150\,^{\circ}C$ ($\Delta T = 10\,^{\circ}C$/min); Injector $T = 220\,^{\circ}C$; Detector $T = 250\,^{\circ}C$. Carrier N_2, flow rate = 0.5 ml/min.

Ethyl-2-methyl butyrate: Oven $T_1 = 40\,^{\circ}C$ for 2 min, $T_2 = 52\,^{\circ}C$ ($\Delta T = 2\,^{\circ}C$/min), $T_3 = 150\,^{\circ}C$ ($\Delta T = 20\,^{\circ}C$/min), Injector $T = 220\,^{\circ}C$; Detector $T = 250\,^{\circ}C$. Carrier N_2, flow rate = 1 ml/min.

Enantioselectivity (E) was calculated using below Equation. The activity is given as percentage conversion of the total amount substrate.

$$E = \frac{\ln\{ee_p(1 - ee_s)/(ee_s + ee_p)\}}{\ln\{ee_p(1 + ee_s)/(ee_s + ee_p)\}}$$

3. Results and discussion

Lipases have been of significant interest because of their wide substrate specificity. In this study, the specificity of lipases belonging to four structural groups (Table 1) has been exploited over a range of substrates based on the substrate site binding geometry. Fig. 1 illustrates the four structural classes in this paper. The structural geometry of the lipase is the first indication on the substrate binding possibility in the specific lipase type. Hydrogen bonding, van der Waals contacts and electrostatic interactions are the second important factors for the binding possibility. The X-ray structures indicate the potential main structural appearance of the enzyme under activated and inactivated conditions, respectively. In the present work the X-ray structures in the so-called "open" forms are used. For the practical measurements the structural forms may be different and of course more dynamic, and for the lipases with lids and flaps which need activation (like TlL, RmL, FoL, CrL, etc.) the experimental conditions have special impact on the binding of substrate to the enzyme. The assay condition has thus been focused to work with both lipases that need activation (CaLA) and those that need little or no activation (FsC and CaLB).

In accordance with the above hypothesis, two categories of substrates i.e. fatty acid esters were selected (A) substrates either branched/large on the alcohol part or (B) substrates either branched/large on the acid part. Examples included in first category substrates—Isobutyl propionate, Styrallyl butyrate, 2-ethyl-hexyl acetate and Cyclohexyl acetate. Examples included in the second category substrates—Ethyl-2-methyl butyrate, Ethyl-2-ethyl-hexanoate, Ethyl benzoate and ethyl-2-phenylpropionate. One of these substrates – ethyl-2-phenylpropionate is shown in Fig. 2, which shows how the substrate – a profen core [20] – docks in a substrate binding pocket of CaLB (Fig. 2). Most of the enzymes accept group "A" substrates, however, CaLA type enzymes, which are capable of accepting large alcohol groups, show highest activity. CaLB on the other hand is more stringent with respect to alpha carbon branching on the alcohol side as shown by its activity on styrallyl butyrate (Fig. 3A). 2-ethylhexyl acetate was chosen as a representative substrate for category A substrate and the enantioselectivity of all four groups of enzymes was checked this compound. Most of the enzymes except CaLB could not distinguish between the enantiomers (Fig. 4). Docking studies (not shown here) showed that the different enantiomers bind in a similar fashion, which makes it hard for the

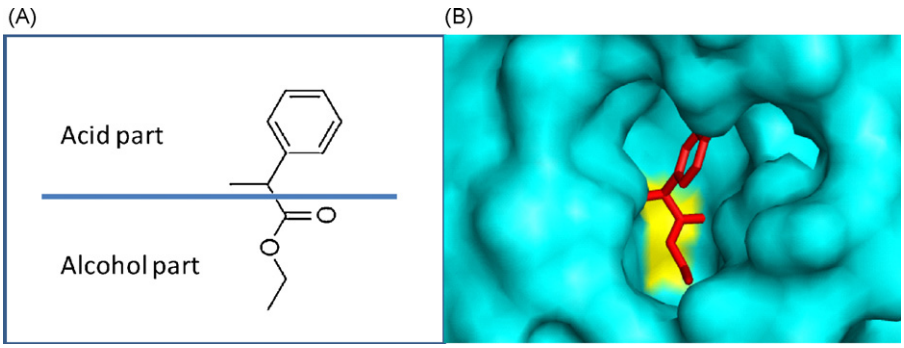

Fig. 2. (A) The acid and alcohol part of the substrate. (B) A profen core compound docked in the active site of CaLB.

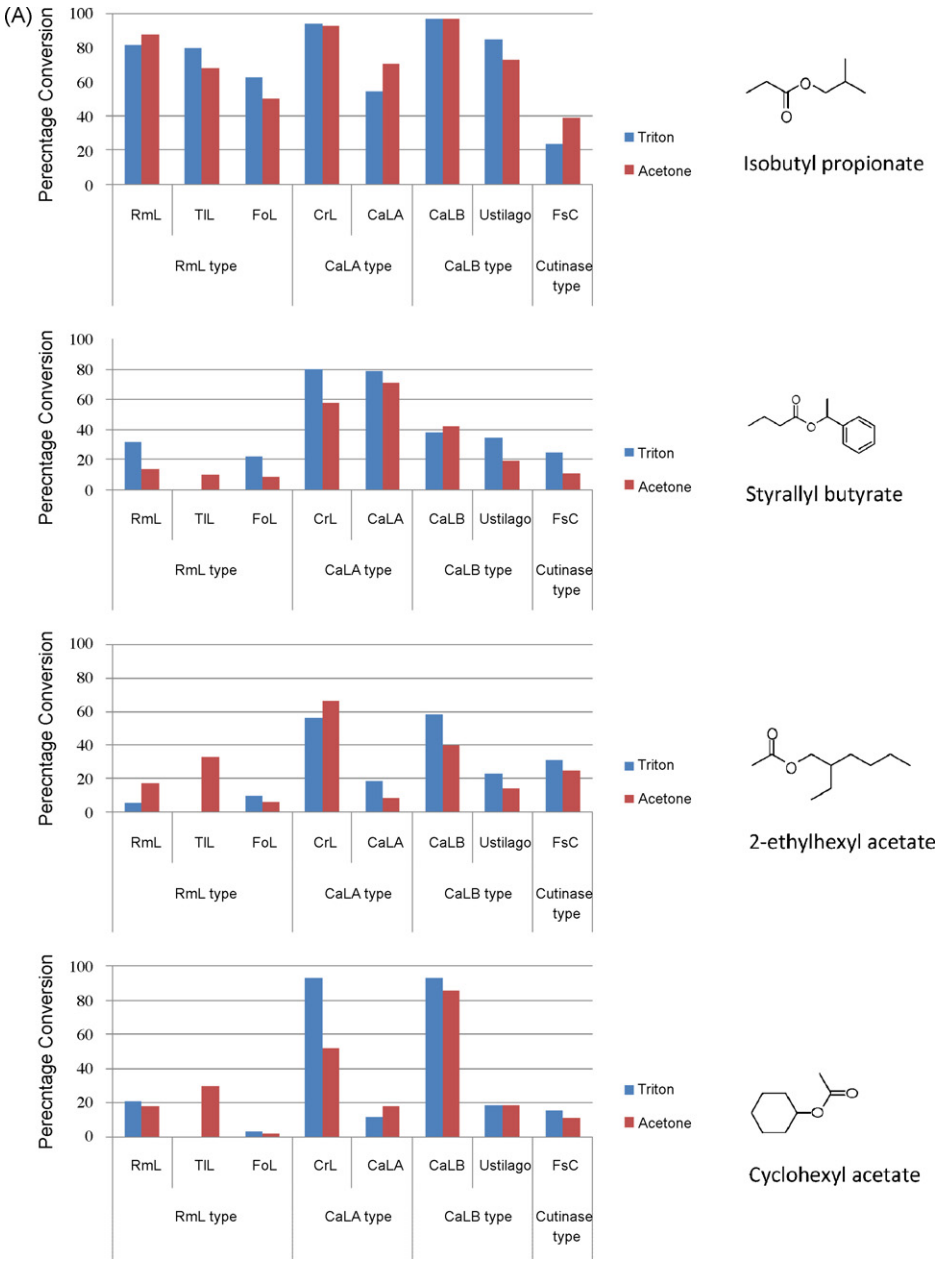

Fig. 3. Enzyme activity on "branched"/"large" substrates on alcohol part (A) and acid part (B). The activity is given in conversion percentage of total amount substrate. Two conditions were chosen: Triton 0,1% or Acetone 5%, Buffer: 50 mM Tris-buffer, pH 7, Amount of enzyme: 0,5 mg, Substrate volume: 50 μl, All the reactions were performed in 30 °C and 24 h. The reactions were stopped with 100 μl 1 M HCl and the reactants are extracted with dichloromethane. 20 μl of the organic phase was diluted with diethylether and put on the GC.

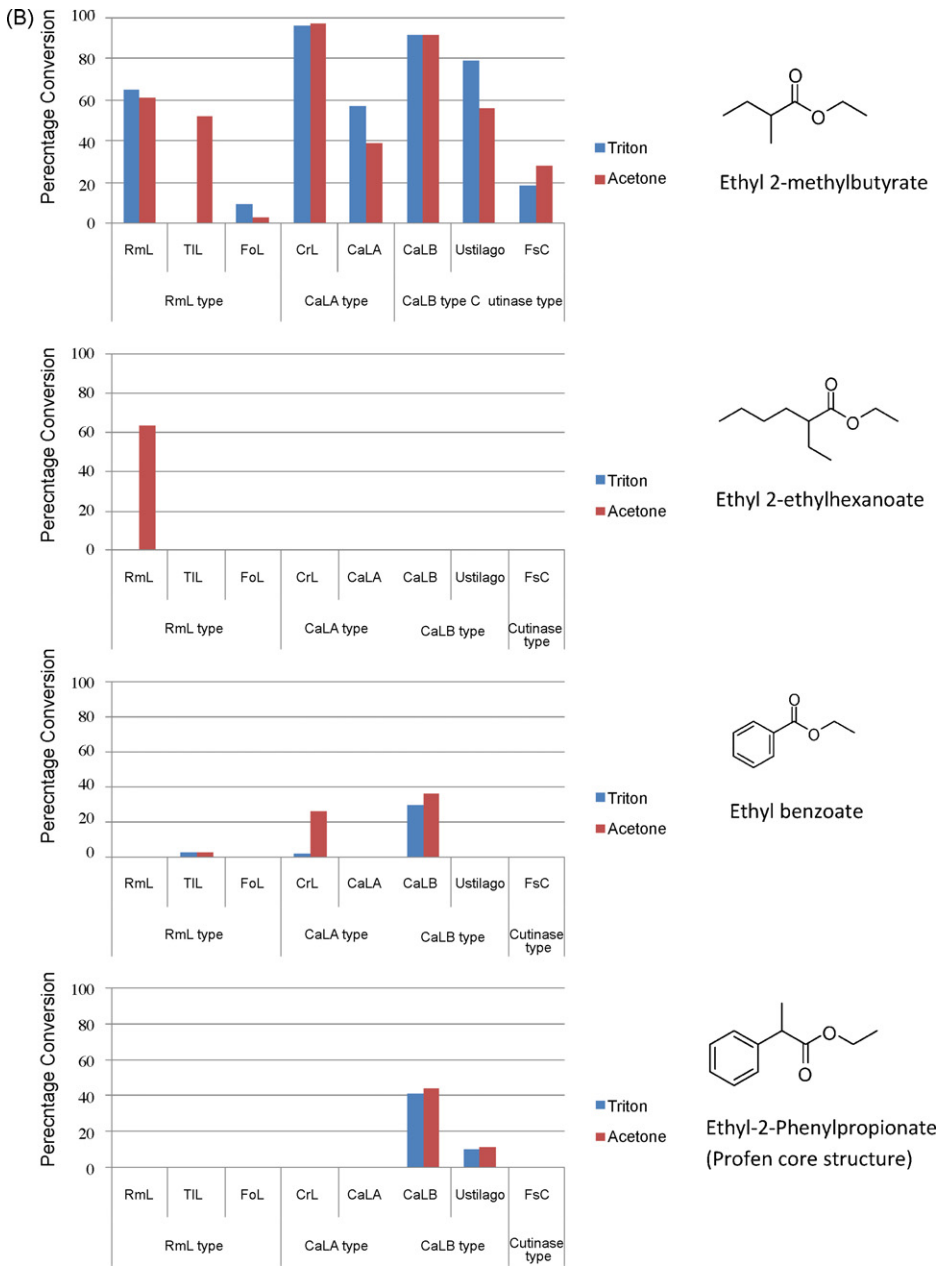

Fig. 3. (*Continued*).

enzyme to experience any difference. This is probably due to the negligible size differences of the substituents in the branching. CaLB showed very high enantioselectivity on 2-ethyl hexyl acetate. Therefore, it was decided to check the enantioselectivity of CaLB variants T103G, M72L and W104H [19] on the same substrate. The CaLB variant T103G which introduced the consensus mutation G-X-S-X-G in CaLB, thereby increasing thermostability, has shown reduced enantioselectivity towards 2-ethyl hexyl acetate (Fig. 4). The CaLB variant W104H in which more space is introduced into the active site has shown 6.7-fold increase in enantioselectivity (Fig. 4). The CaLB variant M72L having higher oxidation stability has also shown decrease in the enantioselectivity (Fig. 4).

In group B substrates, ethyl-2-methyl butyrate was easily hydrolyzed by most of the enzymes (Fig. 3B). The analysis based on molecular docking of CrL and TlL with this substrate showed that the R-enantiomer docked in an active fashion while the S docked with the oxygen orientated in the wrong direction. This has also been shown by the results in which CaLA is showing better enantioselectivity than all the other groups (Fig. 4).

The hydrolysis of all other substrates was enzyme specific. RmL was the only enzyme which was capable of hydrolyzing Ethyl 2-ethylhexanoate (larger branch on acid part) (Fig. 3B). This is probably due to the large hydrophobic area of the active site of the RmL. Ethyl benzoate which had benzene ring on acid part was hydrolyzed only by CrL, TlL and CaLB (Fig. 3B) whereas phenyl propionic acid ethyl ester having profen core structure was hydrolyzed only by enzymes in the CaLB group (Fig. 3B).

In conclusion, lipases are found to be more active on the alcohol branched substrates than acid-branched substrates. As depicted in the figures, for alcohol side large substrates, CaLA and RmL type enzymes give the highest extent of hydrolysis, while for large acid substrates the highest conversion was shown by CaLB. It is note-

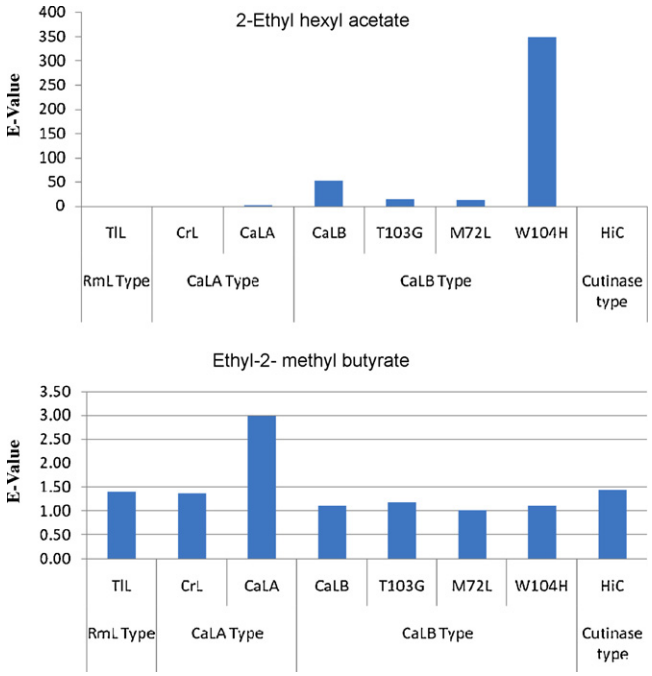

Fig. 4. Enantioselective ratio of CaLA, CaLB, CaLB (T103G), CaLB (M72L) and CaLB (W104H), HiC, TIL and CrL-1 on 2-ethyl hexyl acetate and Ethyl-2-methyl butyrate.

worthy that the large acid substrates which had aromatic side chains were hydrolyzed only by CaLB type of enzymes.

4. Conclusions

Structural space for acceptance of substrates of different shapes in lipases from the groups of RmL, CaLA, CaLB and Cutinase has been explored. The RmL group allows large alcohol part substrate, but more limited/narrow acyl binding cleft on the acid part. CaLA group exhibits strong restriction on the acid part having a narrow tunnel to accommodate the acyl group from the acid part of the substrate and a much wider space on the alcohol part allowing highly branched and large groups on the alcohol part. The CaLB group shows more space on the acid part and less on the alcohol part and with restriction on the branching at CA carbon. Cutinase group has a reasonable open space on both the alcohol and the acid part of the substrate. This structural understanding was tested with the 8 substrates giving an overall result pointing in the same direction as the structural understanding.

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