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Kinetic modulation of *Trichosporon asahii* MSR 54 lipase in presence of organic solvents: Altered fatty acid specificity and reversal of enantio selectivity during hydrolytic reactions

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ABSTRACT

An extracellular *Trichosporon asahii* MSR 54 lipase was purified to homogeneity by ultrafiltration and hydrophobic interaction chromatography. The enzyme is a 54 kDa monomeric protein with PI 5.2. The lipase is optimally active at pH 9.0 and 40 °C and preferred mid to short chain fatty acids during aqueous hydrolysis of triacylglycerides, methyl esters and *p*-nitrophenyl fatty acid esters. It also exhibited *S* enantioselectivity towards hydrolysis of ±phenylethylacetate in aqueous medium. In presence of 50% (v/v) organic solvent of varying log P lipase was stable over a period of 24 h. However in microaqueous environment, the enzyme stability was a direct function of log *P* value. The catalytic efficiency for the hydrolysis of methyl esters of different fatty acid chain length C8:0, C12:0 and C18:0 was altered by addition of any of the organic solvent. There was 3- to 5-fold enhancement for hydrolysis of methyl caprylate in presence of methanol and DMSO and for methyl stearate in presence of ethyl acetate and hexane. On the other hand the efficiency for methyl laurate decreased markedly on the addition of solvent to aqueous system. Interestingly, the fatty acid chain length specificity in an equimolar mixture was inversely related to log *P* of the solvent. The solvent effect on enantioselective hydrolysis of ±phenylethylacetate revealed reversal of enantioselectivity from *S* to *R* enantiomer in the presence of 50% (v/v) 2-propanol and enzyme lost its enantioselective nature in presence of hexane.

1. Introduction

Lipases [EC 3.1.1.3] are enzymes that hydrolyze ester bonds of triacylglycerides at oil–water interfaces and also well known to reverse the reactions in microaqueous conditions. These enzyme are largely exploited for carrying a variety of chemo regio and enantio selective reactions both in aqueous and microaqueous condition [1,2]. Lipases are unique catalyst because of their inherent solvent stability. However the effect of solvent on lipase activity and selectivity varies among lipases from different sources. There are several experimental demonstrations where the substrate specificity has been altered by changing the organic solvent [3]. For their direct application, it is important to study the behavior of lipase in the presence of solvent with respect to kinetic rates and substrate specificity. The results of such investigation will facilitate designing the process for obtaining desired product [4,5]. In this study, we evaluated the effect of solvents on the catalytic efficiency of lipase isolated from *Trichosporon asahii* MSR 54 and also changes in substrate specificity and enantioselectivity upon introduction of solvent during hydrolytic reactions.

2. Materials and methods

2.1. Chemicals

p-Nitrophenyl palmitate for lipase assay, methyl fatty acid ester (C8:0–C18:0), triacylglycerides (C2:0–C18:1), *p*-nitrophenyl ester (C10:0–C16:0), and all the chiral product (*RS*) phenyl ethanol and (*RS*) phenyl ethyl acetate were purchased from Sigma (St. Louis, MO, USA). All other analytical reagents and media components were purchased from Hi-Media, Qualigens or Sisco Research Laboratories (SRL), India.

2.2. Yeast strain and production medium

T. asahii MSR 54 lipase was produced in a medium containing (g/l): yeast extract, 20; malt extract, 6; glucose, 5; KH_2PO_4, 1; K_2HPO_4, 3; Tween 80, 10; casein, 4 and $MgCl_2$, 1 (pH 7.0).The

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production medium was inoculated with 4% of 24 h old seed culture and incubated at 30 °C; 250 rpm in a New Brunswick Scientific Shaker (Edison, NJ, USA) for 48 h. There after, the cell-free enzyme supernatant was obtained by centrifugation at 14,000×*g* for 20 min at 4 °C (Sorvall® RC 5C Plus). The crude enzyme preparation was subjected to ultrafiltration with 10 kDa membrane (Sartorius, Germany). The retentate solution was adjusted to 2.5 M NaCl and was applied to a 50-ml HiTrap Butyl-Sepharose column (1.2 mg of proteins/ml of column) (Sigma; 1.5 × 12 cm) with a void volume of 8 cm^3 previously equilibrated with step up gradient from 0–2.5 M NaCl in pH 7.0 buffer at a flow rate of 0.5 ml/min. The bound proteins were eluted out from the column by decreasing the NaCl gradient from 2.5 M to zero and finally with 10% isopropanol and fraction of 2 ml each were collected. The protein was estimated by Bradford reagent (Bradford, 1976). The peak of lipase activity was pooled, concentrated in speed vac and then used as source of lipase. The strain has been isolated from petroleum sludge and deposited in Microbial Type Cultural Collection & Gene Bank (MTCC), India with accession No. MTCC 9450.

2.3. Lipase assay

Lipase activity in the culture filtrate was determined by *p*-nitrophenyl palmitate assay [6] and also by titrimetry [7,8] using 1% (v/v) olive oil as substrate. One international unit (U) of lipase was defined as the amount of enzyme required to release 1 μmole of *p*-nitrophenol or fatty acid respectively per minute at 40 °C and pH 9.0.

2.4. Protein assay

Protein concentration was determined by Bradford [9] and Coomasie blue assay procedure using bovine serum albumin (BSA) as standard. The protein concentration was determined spectrophotometrically at 595 nm.

2.5. Solvent stability: aqueous and microaqueous environment

For the aqueous environment, the effect of various polar and non-polar organic solvents on lipase activity was determined by incubating the 10 mg lyophilized enzyme in 1 ml organic solvents (25% and 50% (v/v) in pH 9.0, 0.05 M Tris buffer) for 24 h at room temperature (25–30 °C) where as for the microaqueous environment 10 mg of lyophilized enzyme was taken and incubated in 1 ml of the organic solvent (100%) for 1 h at room temperature. After the incubation time, the volume of the enzyme sample was build up to 2 ml with 1 ml of, pH 9.0, Tris–HCl buffer (0.05 M) to dissolve the lyophilized enzyme completely. For aqueous controls 10 mg lyophilized enzyme was dissolved in 2 ml of pH 9.0 buffer. The enzyme was incubated with the solvents under shaking conditions (100 rpm) to facilitate proper mixing especially in case of water-immiscible solvents. The residual enzyme activity was measured against the aqueous control.

2.6. Hydrolysis of methyl esters, triacylglycerides and p-NP esters

Lipase activity on different methyl esters of fatty acids (C8:0–C18:0), triacylglycerides (C2:0–C18:1), *p*-nitrophenyl ester (C10:0–C16:0), was studied in aqueous system using 0.05 M methyl ester and triacylglyceride emulsions prepared in 0.05 M Tris buffer pH 9.0 using 2% gum acacia. Activity was expressed as % relative activity in comparison to laurate ester, which was considered as 100%.

Effect of solvent on:

(A) Catalytic efficiency of the enzyme for the hydrolysis of methyl esters

Lipase activity on three different methyl esters of fatty acids such as methyl caprylate (C8:0), methyl laurate (C12:0), and methyl stearate (C18:0) was studied in presence of various solvents, 50% (v/v) in 0.05 M Tris buffer pH 9.0. The reaction was initiated with 50 mg (100 U) of purified lipase and terminated after 30 min with 10 ml acetone and ethanol 1:1 and titrated against 0.1N KOH. The K_m and V_{max} of the lipase were determined by using double reciprocal (1/V vs 1/S) plot using varying concentrations (5–400 mM) of substrate. The catalytic efficiency V_{max}/K_m of enzyme for each of the substrate and solvent was calculated and compared with aqueous system.

(B) Fatty acid specificity by HPLC

An emulsion of 400 mM fatty acid methyl esters with different acyl chain length (50 mM of each (C2:0–C18:2) was prepared in pH 9.0 Tris–HCl buffer/50% (v/v) solvent in buffer containing 2% (w/v) gum acacia as stabilizer. Hydrolysis was carried out at 40 °C by the addition of 50 mg (100 U) lipase (as determined with *p*-nitrophenyl palmitate as substrate). The hydrolytic activity was stopped after 5 min by addition of 100 μl of 85% H_3PO_4. Liberated free fatty acids were mixed with 2 ml of diethylether:*n*-hexane (1:1). The resulting mixture was kept at −20 °C for 15 min and top layer was taken, solvent was heat evaporated. Remaining fatty acids were mixed with 1 ml mobile phase (methanol:0.02 M phosphoric acid; 80:20, v/v). 10 μl of extracted fatty acids was injected into the HPLC (Shimadzu, Japan) having C-18 column (Luna 5u C18 (2) 100A) (250 mm × 4.60 mm) for the estimation of free fatty acids. Concentrations of fatty acids were calculated by comparison of peak areas with those of a reference mixture of fatty acids and subsequently % release of each of the fatty acid was calculated.

(C) Enantioselectivity

Racemic 1-phenyl-ethyl acetate (10 μmoles) was prepared in 15 ml of 50 mM Tris, pH 9.0 buffer at 40 °C and was continuously stirred at 200 rpm. For the solvent effect substrate was prepared in buffer: solvent (1:1) mixture. The reaction was initiated by the addition of 50 mg (100 U) of purified enzyme and reaction time (6 h) was selected after monitoring the progress of reaction by titration till 50% hydrolysis in aqueous system. The same time period was selected for termination of other reactions in presence of 50% (v/v) solvent. The samples for HPLC analysis were prepared by extracting the aliquots (10 ml) saturated with NaCl followed by the addition of an equal volume of ethyl acetate vortexing it and leaving it for 15 min at room temperature. The top layer was extracted, vaccum dried and was re-dissolved in 0.5 ml of HPLC grade hexane: isopropanol mixture 90:10 (v/v), mixture was filtered through 0.2 μm filter and injected into the HPLC having Chiralpak column (AD-H) (0.46 cm × 25 cm) (Diacel Chemical IND., LTD). The retention time of (*S*)-phenyl ethyl acetate, (*R*)-phenyl ethyl acetate, (*R*)-phenyl ethanol, (*S*)-phenyl ethanol are 7.6, 8.3, 10.1 and 10.8 respectively.

(D) Circular dichroism spectroscopy—Circular dichroism (CD)

Spectra were recorded in a spectropolarimeter Jasco J-810, Japan. Protein at a concentration of 0.2 mg/ml in pH 7.0 sodium phosphate buffer (50 mM) was used for spectral analysis. Spectra were recorded, in triplicates, between 190 and 260 nm using a quartz cuvette with 2 mm path-length.

3. Result and discussion

3.1. Substrate specificity

T. asahii MSR 54 produces an extracellular lipase which was purified by hydrophobic interaction chromatography using Butyl-

Table 1
Substrate specificity of *T. asahii* MSR 54 lipase.

Substrate	Relative activity (%)
Triglycerides	
Triacetin C2:0	84
Tributyrin C4:0	68
Tricaprin C6:0	72
Tricaproin C10:0	85
Trilaurin C12:0	100
Trimyrsitin C14:0	64
Tripalmitin C16:0	76
Tristearin C18:0	64
Triolein C18:1	82
p-Nitrophenyl esters	
p-Nitrophenyl caprate	107
p-Nitrophenyl laurate	100
p-Nitrophenyl myrsitate	145
p-Nitrophenyl palmitate	60
p-Nitrophenyl sterate	31
Methyl esters	
Methyl laurate	100
Methyl myrsitate	46.2
Methyl palmitate	15.4
Methyl stearate	15.3

Sepharose column. The enzyme was evaluated for the substrate specificity during the hydrolysis of methyl esters (C8:0–C18:0), triacylglycerides (C2:0–C18:1), *p*-nitrophenyl esters (C10:0–C16:0). From the results (Table 1) it was observed that the enzyme showed mid chain selectivity with maximum activity at lauric acid esters. The enzyme was checked for stability towards various organic solvents both in aqueous and microacqueous environment (Figs. 1 and 2). From Fig. 1 it can be observed that *T. asahii* lipase exhibited excellent stability towards large number of organic solvents in aqueous environment at a concentration of 50% (v/v) over a period of 24 h. Among alcohol series there was a trend for decrease in stability with increase in alkyl chain length with least stability in butanol. Similar observation for sensitivity towards alcohol series has been reported for *Yarrowia lipolytica* [10]. Further in 50% (v/v) DMSO, ethyl acetate, diethyl ether, toluene and hexane nearly 1.5-fold increases in its activity was observed. In contrast to this solvent stability of lipase in microaqueous environment was a direct function of log *P* value with better stability in log *P* above 1. Solvents with lower log *P* value are polar solvents and thus quench the

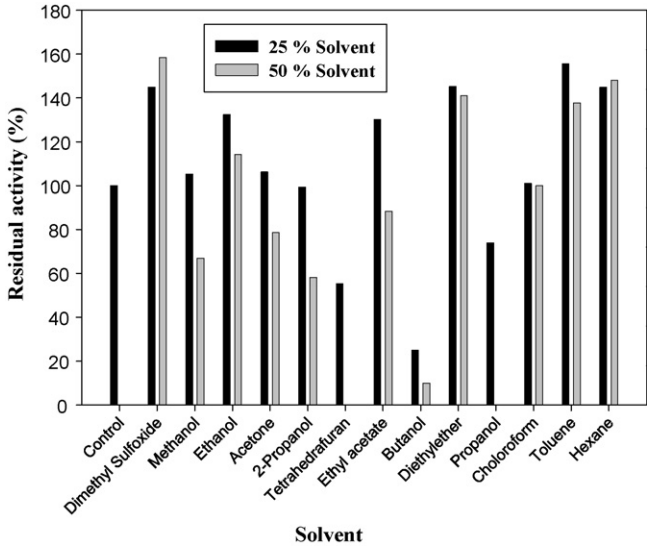

Fig. 1. Effect of organic solvents on the activity of lipase from *T. asahii* MSR 54 after 24 h incubation in aqueous system.

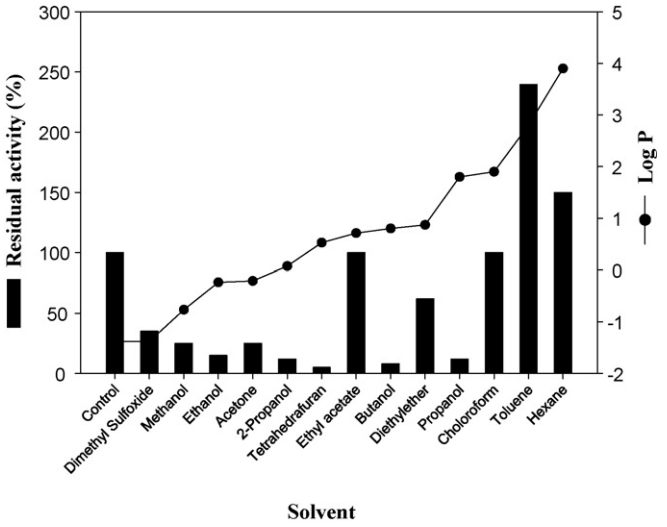

Fig. 2. Effect of organic solvents on the activity of lipase from *T. asahii* MSR 54 after 1 h incubation in microaqueous system.

water pockets of the enzyme readily rendering it inactivated. On the other hand as the log *P* increases the hydrophobicity of the solvent correspondingly increases and the water quenching activity is reduced as in case of toluene and hexane. Similar solvent effects of solvents on lipase stability are well known [11]. It is noteworthy that more than 2-fold enhancement in *T. asahii* lipase was observed after preincubation in toluene.

Effect of solvent on lipase activity was further studied through circular dichroism spectral analysis selecting three water miscible solvent among those which affected enzyme activity such as DMSO (activation of enzyme), THF (inactivation of enzyme) and methanol (unaltered activity). CD spectra were superimposed with the aqueous spectrum Fig. 3. Analysis was done in Far-UV at 190–260 nm. From Fig. 2 it can be observed that *T. asahii* lipase protein contains 35.4% α-helix, 53.1% of random coil, 11.6% reverse β-turns and there was no β-pleated sheets and exhibits clear conformational changes in presence of solvent. In the presence of DMSO with native protein, where activation of enzyme was observed, CD analysis revealed a shift from alpha helix to β-pleated sheets (82.2%). However in THF the total secondary structure was lost as was also expected by complete lose of activity. Likewise in methanol the secondary structure was not much altered so was the activity. The activation in DMSO observed in present studies accompanied with increased β-pleated sheets is in contrast to Mahabubur et al. [13] where activation in *Rhizopus oryzae* lipase where attributed to increased alpha helicity in presence of isopropanol. Such varying structural changes on exposure to solvent may be a property of amino acids composition which will vary form lipase to lipase. However overall activation may be a result of differential lid movement due to exposure of hydrophobic residue in presence of solvent favoring better substrate binding as suggested by several researchers [12,14,15]. Catalytic efficiency of the present lipase was compared for the hydrolysis of methyl fatty acid esters of varying chain length C8:0 (methyl caprylate), C12:0 (methyl laurate) and C18:0 (methyl stearate) both in presence and absence of solvents. From Table 2 it can be observed that during aqueous hydrolysis a 9-fold higher catalytic efficiency was observed for the laurate ester 0.92 min in comparison of both caprylate and stearate esters of 0.10 and 0.12 min respectively indicating that the lipase is mid chain fatty acids specific lipase. The introduction of solvent up to 50% showed considerable impact on hydrolysis rates towards different methyl esters. In all the solvents the catalytic efficiency decreased for methyl laurate with least activity in dimethylsulfoxide and methanol. On the other end in the same

Table 2
Effect of organic solvents on the kinetic constants of *T. asahii* lipase for the hydrolysis of methyl fatty acid esters.

Solvent (50%, v/v)	Methyl caprylate			Methyl laurate			Methyl stearate		
	K_m (mM)	V_{max} (μmole/mg/min)	V_{max}/K_m (min)	K_m (mM)	V_{max} (μmole/mg/min)	V_{max}/K_m (min)	K_m (mM)	V_{max} (μmole/mg/min)	V_{max}/K_m (min)
Diethyl ether (0.87)[a]	150	3.2	0.02	33.3	08.7	0.27	05.0	2.85	0.57
Ethyl acetate (0.71)[a]	115	16.3	0.14	30.0	10.0	0.30	05.0	16.6	0.33
THF (0.53)[a]	285	8.33	0.03	33.3	10.0	0.30	16.0	04.0	0.24
Isopropanol (0.07)[a]	200	10.0	0.05	22.2	07.7	0.35	13.3	02.0	0.15
Acetone (−0.21)[a]	100	01.0	0.01	66.6	20.0	0.27	228	1.42	0.03
Methanol (−0.76)[a]	66.0	22.0	0.33	66.6	11.1	0.17	250	4.76	0.02
DMSO (−1.38)[a]	100	55.0	0.56	200	16.7	0.09	83.3	02.5	0.01
Aqueous (−1.38)[a]	100	10.0	0.10	22.2	20.4	0.92	27.0	03.3	0.12

[a] Value in parenthesis indicated log *P*.

Table 3
Effect of solvents on the release and fatty acid specificity during hydrolysis of an equimolar mixture of methyl fatty acid esters by *T. asahii* MSR 54 lipase at pH 9.0, 40 °C after 15 min of incubation.

Fatty acid & Retention time	C2:0 5.94[a]	C10:0 9.22	C12:0 16.08	C14:0 23.78	C16:0 33.12	C18:0 56.90	C18:1 35.60	C18:2 28.16
Aqueous	0.0	0.0	0.0	**10.0**	0.0	0.0	0.0	0.0
Hexane	0.0	**3.0**	**2.0**	0.0	0.0	0.0	0.0	0.0
Chloroform	0.0	**1.0**	**9.0**	0.0	0.0	**10.0**	0.0	0.0
Diethyl ether	0.0	**1.0**	0.0	0.0	0.0	**17.0**	0.0	0.0
Ethyl acetate	0.0	0.0	0.0	0.0	**8.0**	**9.0**	0.0	0.0
Tetrahedrafuran	0.0	0.0	**3.0**	**8.0**	**3.0**	0.0	0.0	0.0
2-Propanol	0.0	**0.1**	0.0	**8.0**	0.0	0.0	0.0	0.0
Acetone	0.0	0.0	0.0	0.0	**1.0**	**9.0**	0.0	0.0
Methanol	0.0	0.0	0.0	0.0	0.0	**10.0**	0.0	0.0
Ethanol	0.0	0.0	0.0	0.0	0.0	**10.0**	0.0	0.0
Dimethylsulfoxide	0.0	0.0	0.0	**9.0**	**9.0**	0.0	0.0	0.0

[a] Value indicates the retention time of respective fatty acid and number indicates % fatty acid released as quantitated by HPLC.

solvents the catalytic efficiency was increased 3–5 times over aqueous for methyl caprylate and decreased 10 times over aqueous for methyl stearate indicating that introduction of dimethylsulfoxide and methanol makes the lipase selective for caprylate. Likewise ethyl acetate and diethylether introduced long chain selectivity in the lipase where again 3–5-fold improved efficiency for methyl stearate was observed with simultaneous poor catalysis for both C8:0 and C12:0 ester. Overall the effect of solvent on catalytic efficiency of *T. asahii* lipase showed that in aqueous methyl laurate was cleaved, in dimethylsulfoxide and methanol methyl caprylate and in ethyl acetate and hexane methyl stearate was cleaved with highest specificity. The solvent effect on catalytic rate can be attributed to substrate solubility in presence of solvent and also interfacial activation of the enzyme [16]. Further it has also been suggested that organic solvents modulate the catalytic rate by controlling the system interactions between the enzyme and the substrate [17]. The higher rates for stearic fatty acid ester in hydrophobic solvents and that for shorter caprylate fatty acid ester in polar solvent can be explained on the basis of solvation effect of the solvent on the substrate [11]. Thus the catalytic efficiency of the enzyme for individual fatty acid ester varies with the solvent. However it may not exactly translate in a mixture of fatty acid esters and also true fatty acid selectivity can only be known by the release of fatty acids when a mixture of esters is hydrolyzed and such information is important when hydrolysis of fats and oil is taken into account. Therefore it is always suggested that fatty acid selectivity/specificity should always be studied in an equimolar mixture [18]. Hence the fatty acid specificity of the enzyme was also studied in an equimolar mixture of methyl esters both in presence and absence of solvent. Interestingly it was observed that the enzyme released only myristic acid (%) with in 15 min of reaction in an aqueous system showing mid chain selectivity of the lipase (Table 3). The fatty acid specificity not very common among lipases and very few fatty acid specific lipases are known, such as *Geotrichum* sp. lipase showed oleic acid specificity [19,20].

Table 4
Effect of solvents on enantioselectivity of *T. asahii* MSR 54 lipase during hydrolysis of racemic phenyl ethyl acetate.

Solvent 50%	Time (h)	Conversion[a] (%)	Enantiomer	e.e.[b] (%)
Aqueous	6	45	*S*	76.0
Diethyl ether	6	25	*R*	67.0
Ethyl acetate	6	20	*S*	98.2
Hexane	6	25	*R*	17.5
2-Propanol	6	15	*R*	82.0

Purified lipase (50 mg) was added to ±phenyl ethyl acetate emulsion in aqueous/50% (v/v) solvent at 40 °C; pH 9.0.

[a] Percent hydrolysis was calculated by quantification of ±phenyl ethyl acetate and ±phenyl ethanol by HPLC.

[b] Enantiomeric excess (e.e.%) was determined by HPLC (CHIRALPAK AD-H, Daicel).

The fatty acid specificity of *T. asahii* as exhibited in aqueous system was unaltered in presence of 50% (v/v) 2-propanol. However it changed to longer chain fatty acid with initial release of stearic acid from the mixture in presence of methanol, ethanol, diethylether,

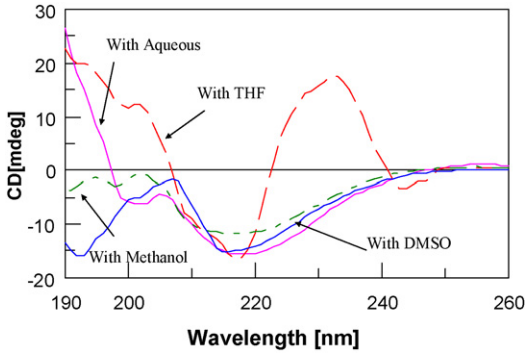

Fig. 3. Comparative circular dichroism spectral analysis of *T. asahii* MSR 54 lipase in aqueous system versus 50% (v/v) solvent.

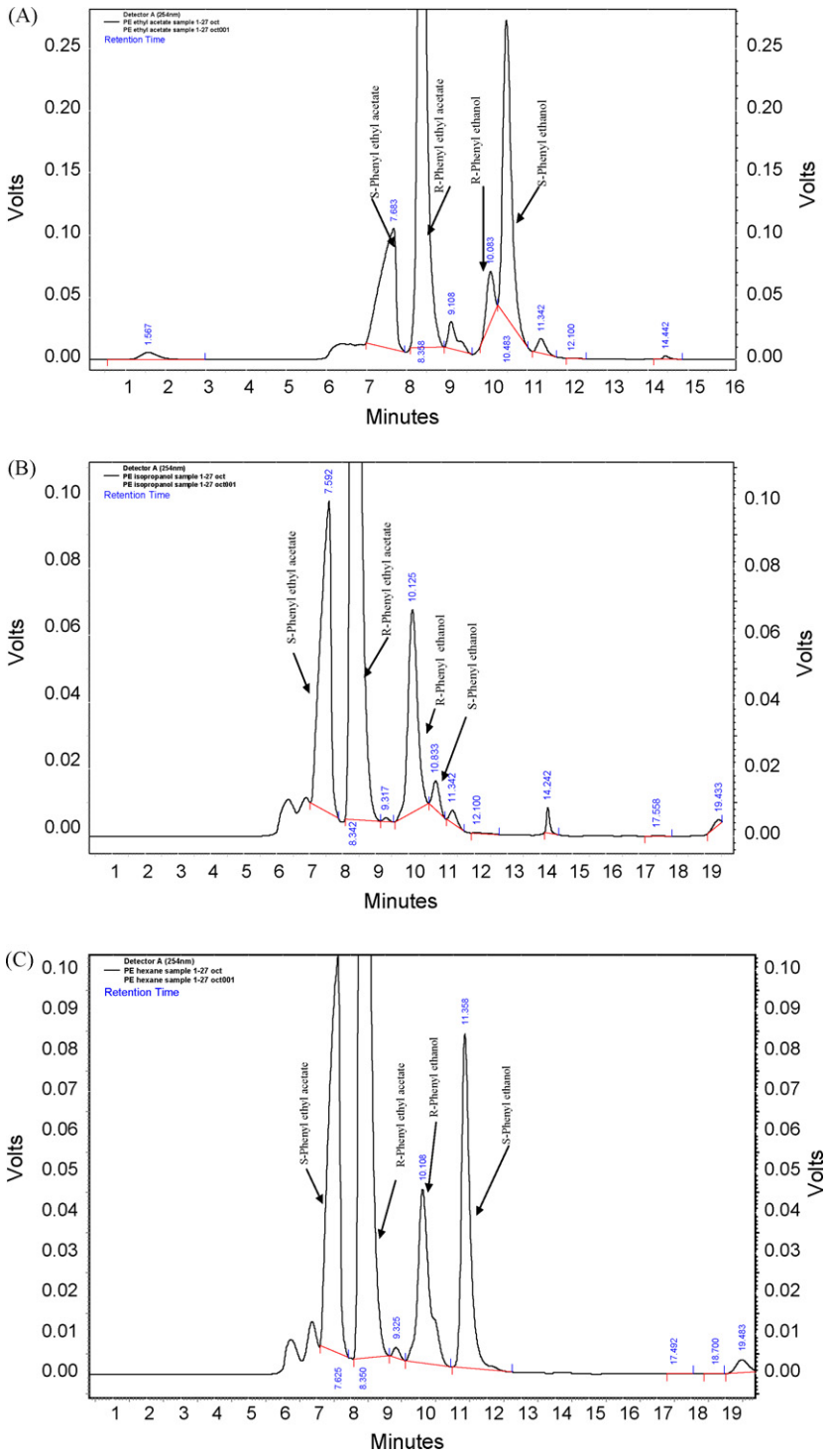

Fig. 4. HPLC chromatogram of chiral product (*RS*)-phenyl ethanol obtained after hydrolysis of (*RS*)-phenyl ethyl acetate in (A) aqueous system, (B) 50% of 2-propanol and (C) hexane using *T. asahii* MSR 54 lipase at 40 °C and pH 9.0.

and acetone. Likewise in presence of hexane the release of only mid-chain fatty acid such as caprylate and laurate was observed. Thus in presence of solvents the enzyme exhibited specificity for mid to long chain fatty acids. Unsaturated fatty acid was not cleaved in any of the solvent. In presence of solvent DMSO, THF, ethyl acetate, diethyl ether and chloroform quantitative release of fatty acid was higher than that in the aqueous system showing that solvent enhances the catalytic rate. Thus the behavior of enzyme during hydrolysis of a mixture of esters was entirely different and cannot be extrapolated from its efficiency on individual ester. This is in confirmation with Secundo et al. [21] and Torres and Otero [22] who attributed such differences to limitation of diffusion and conformational changes of *Candida antartica* CALB and *Pseudomonas cepacia* lipases and also to differential displacement of lid altering the substrate accessibility to the active site [23].

T. asahii MSR 54 lipase exhibited *S*-enantioselectivity with 76 e.e. during aqueous hydrolysis of racemic mixture of phenyl ethyl acetate. The presence of organic solvent 50% (v/v) markedly influenced the enantiomeric preference with improved *S*-enantiomeric access to 98.2 in presence of ethyl acetate. In presence of 2-

propanol the enantio preference was completely reversed to the *R*-enantiomer with 82 e.e. while in hexane the enantioselective property was lost (Table 4) (Fig. 4). This suggests that the presence of solvent affects the enzyme conformation rendering the changes in enantio preference [24–28]. A similar complete reversal of enantioselectivity has also been observed by a transition from water to organic solvents in lipase by Tawaki et al. [28,29].

4. Conclusion

Thus it can be said *T. asahii* MSR 54 lipase is a mid chain fatty acid specific lipase and its fatty acid specificity and enantioselectivity can be altered by choice of solvent during aqueous hydrolysis. The property of the solvent effect on lipase substrate specificity is of great biotechnological importance and can be exploited for developing desired hydrolytic reactions.

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