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Purification and Biochemical Characterization of a Novel Magnesium Dependent Lipase from *Trichosporon asahii* MSR 54 and its Application in Biodiesel Production

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ABSTRACT

The present study was aimed to purify and characterize extracellular lipase from *Trichosporon asahii* MSR54. An extracellular 27 kDa lipase from *T. asahii* was purified by anion exchange chromatography with 2.14 purification fold. It was magnesium stimulated. Reversible inhibition of EDTA was found to be by adding 15 mM magnesium chloride confirming its metallo nature. The temperature and pH optima for activity was 40°C and pH 9.0, respectively. It's K_m and V_{max} was found to be 55.5 μM and 6.66 mM min^{-1}, respectively using p-nitrophenyl palmitate as a substrate. The present enzyme hydrolysed a large array of oils and triacylglycerides with better specificity on corn oil and triolein, respectively. It was 1,3-regioselective during hydrolysis of triolein in aqueous as well as in micro-aqueous environment. It was stable towards most of the polar and non-polar solvents including methanol, DMSO, benzene. The present enzyme yielded 87.6% conversion of coconut oil to biodiesel after overnight incubation at 45°C.

Key words: Metallolipase, solvent stable, thiol activation, 1,3 regioselective, biodiesel

INTRODUCTION

Lipases (E.C. 3.1.1.3; triacylglycerol acylhydrolases) are serine hydrolases (Sangeetha *et al*., 2011). They act on the carboxyl ester bonds present in triacylglycerols to liberate fatty acids and glycerol (Islam *et al*., 2008). Under micro-aqueous conditions, lipases possess the unique ability to carry out the reverse reaction leading to esterification, alcoholysis and acidolysis (Padhiar *et al*., 2011). Besides being lipolytic, lipases also possess esterasic activity and have a very diverse substrate range; still they are highly specific, as they are chemo-, regio- and enantio- selective (Gupta *et al*., 2004).

Lipases are widely distributed among plants, animals and microorganisms. Such diversity in the origin reflects variability in the biochemical properties (Saeed *et al*., 2006). However, most of the industrially useful lipases are derived from fungi, yeast and bacterial sources, since they are highly robust and easily produced by fermentation (Dahiya and Purkayastha, 2011). Among these, lipases from yeast such as; *Candida, Geotrichum* and *Yarrowia* find voluminous applications due to their fatty acid specificity and enantioselective nature (Brabcova *et al*., 2010; Maryam *et al*., 2011; Padhiar *et al*., 2011).

Lipases are indispensable catalysts for valuable transformations in the field of oleo-chemistry, organic chemistry and pharmaceutical sectors (Anbu *et al*., 2011). Recently application of lipases

for production of biodiesel has gained momentum due to present day energy crisis. Biodiesel is long chain alky esters which can act as an alternative diesel fuel. It can be produced through esterification of alcohol using cheapest available oils (Nahar and Sunny, 2011; Neelambari *et al.*, 2011). However for the production of biodiesel, lipases which are stable in solvents like methanol, butanol and hexane etc are preferred (Kumari *et al.*, 2009). Hence, due to their versatile uses in the industries the demand for novel lipases with improved properties is ever increasing.

In this respect *Trichosporon asahii* MSR54 was isolated from petroleum sludge which produces two extracellular lipases of varying molecular size viz., 54 and 27 kDa. The 54 kDa lipase has already been reported as a solvent stable enantioselective lipase (Kumar and Gupta, 2008; Kumar *et al.*, 2009). Here, we report the purification and biochemical characterization of another extracellular 27 kDa lipase from *Trichosporon asahii* MSR54 and its application for biodiesel production.

MATERIALS AND METHODS

Chemicals: p-Nitrophenyl esters and triacylglycerides were purchased from Sigma (St. Louis, USA).All other analytical reagents and media components were purchased from Hi-Media or SRL (India). Oils were obtained locally. All the experiments were performed in triplicate.

Yeast strain: The yeast strain was previously isolated from petroleum sludge and deposited to Microbial Type Cultural Collection (MTCC), India with accession number: MTCC 9450.

Inoculum and production medium: The yeast was grown in YPD medium [yeast extract, peptone and dextrose]. 2% (v/v) inoculum was added to the 50 mL of production medium. The medium contained malt extract 1% (w/v); yeast extract 3% (w/v); dextrose 0.2% (w/v); casein 0.2% (w/v); $MgCl_2$ 0.1% (w/v); KH_2PO_4 0.1% (w/v); K_2HPO_4 0.3% (w/v) and corn oil 1% (v/v) (Kumar and Gupta, 2008).

Lipase assay: Lipase activity was estimated spectrophotometrically by monitoring hydrolysis of p-nitrophenyl-palmitate (p-NPP) to p-nitrophenol at 40°C. The standard assay reaction mixture contained 80 mM p-NPP in 50 mM Glycine-NaOH buffer (pH 9.0) (Kumar and Gupta, 2008). One unit of lipase activity was defined as the amount of enzyme that liberates 1 μmoles/min/mL of p-nitrophenol from p-NPP under the standard assay condition.

Total protein was estimated by Bradford at 595 nm using BSA as the standard protein (Zhang *et al.*, 2008).

Lipase purification: Culture broth was centrifuged at 8000 g for 20 min and filtered through normal blotting sheet. The filtrate was then precipitated by ammonium sulphate (80%) overnight at room temperature followed by centrifugation at 8000 g for 15 min. The recovered supernatant was dialyzed against 10 mM $MgCl_2$ and subjected to anion exchange chromatography on Q-Sephrose column. Further it was eluted by a linear gradient of 100 mM NaCl to 5 M NaCl in 10 mM tris-Cl (pH 8.0). The fractions showing high lipase activity were pooled, dialysed and concentrated.

The relative molecular mass of purified lipase was estimated by SDS-PAGE analysis according to method of Laemmli (1970). The pI of the protein was determined by isoelectric focusing using BioRad Mini IEF Cell Model 111 according to manufacturer's instruction and ampholyte used were

Bio-lyte 3/10. The vacuum concentrated purified protein was sent to protein facility of Iowa state university (USA) for N-terminal sequencing.

Biochemical characterisation of lipase

Effect of pH and temperature on lipase activity and stability: The activity of enzyme was determined in the range 25-60°C in 50 mM glycine-NaOH pH 9.0 buffer having 80 mM p-NPP as a substrate. The activity was determined at different pH ranging from pH 7.0-11.0 using 50 mM of each buffer including sodium phosphate pH 7.0-8.0, glycine-NaOH pH 9.0-10.0 and phosphate hydroxide (pH 11.0) at 40°C.

Effects of different metal ions, EDTA, thiols and solvents on lipase activity: Effect of 10.0- 25.0 mM divalent cations, 2-10 mM EDTA and 2-10 mM thiols (dithiothreitol and β-mercaptoethanol) was studied by incubating the purified enzyme for 1 h at room temperature. Reversible of EDTA inhibition was carried out by incubating 10 mM EDTA treated enzyme in varying concentration of $MgCl_2$ viz., 10.0-25.0 mM for 1 h.

Hundred milligram of lyophilised enzyme was incubated in different solvents (90% v/v) viz., benzene, acetone, acetonitrile, petroleum ether, dimethyl sulphonate, methanol, ethanol, butane, iso-propanol for 1 h at 30°C. Residual activity was determined as per standard assay.

Lipase activity on various substrates: The substrate specificity of yeast lipase was studied using p-nitrophenyl fatty acid esters (p-nitrophenyl esters), triacylglycerides and oils. The activity measurement by p-nitrophenyl fatty acid esters was done spectrophotometrically at pH 9 and 40°C, K_m and V_{max} was calculated as described earlier (Kumar *et al.*, 2009).

Lipase activity on triacylglycerides [trilaurin (C12:0), trimyristin (C14:0), tripalmitin (C16:0), tristearin (C18:0) and triolein (C18:1)] and oils [soybean, mustard, coconut, shikakai, groundnut, rose, olive, corn] was studied using 0.05 M triacylglyceride and 10% oil emulsions, respectively. Emulsion was prepared in pH 9.0 buffer with 2% gum acacia. The reaction was carried out using, 9 mL of substrate and 1 mL of properly diluted enzyme was added in to this and the reaction was incubated at 40°C for 20 min in shaking water bath. The reaction was stopped by adding 1 mL of ethanol: acetone: 1:1. The liberated fatty acids were titrated against 0.1 N NaOH using pH meter. Control reaction was set up using heat inactivated enzyme. The activity was expressed as μmoles of free fatty acid liberated per mL and per minute.

Regio specific hydrolysis of triolein: Hydrolysis of triolein was carried out in both aqueous and micro-aqueous medium. For aqueous medium, 100 mM triolein was emulsified in 100 mL of 50 mM pH 9.0 (glycine-NaOH) buffer using 2% gum acacia. 50 U of purified lyophilized enzyme was added in the 20 mL of reaction mixture and was incubated at 40°C and 100 rpm for 30 min to 3 h. 0.5 mL of aliquot was drawn at regular intervals and reaction was stopped by adding 5 mL of diethyl ether. The extracts were frozen and later was analysed by TLC (silica gel, Merk) using petroleum ether: diethyl ether: acetic acid :: 80:30:1 as solvent system. Spots were visualised by incubating the TLC in a saturated iodine chamber. For micro-aqueous hydrolysis, 100 mM triolein was made in hexane instead of water.

Lipase immobilisation and production of biodiesel: The enzyme was immobilized by physical adsorption on basic alumina. Two hundred milligram of matrics was wet in 2 mL of butanol followed by the addition of 3 mL of glycine-NaOH buffer pH 9.0 (50 mM) and 5 mg lyophilized

enzyme (300 U). The suspension was shaken at 30°C/100 rpm for 12 h after and metrics was dried at room temperature.

Transesterification using *T. asahii* lipase was carried out with coconut oil: methanol ratio of 1:4, 100 U of immobilised enzyme/g of oil using 1,4-dioxane as a solvent with equal oil volume ratio and 0.1% pH 9.0 buffer at 45°C/100 rpm for 24 h. Enzyme was removed by filtration and the methyl esters were recovered by mixing it with hexane. Later on hexane was removed by rota vapour and the methyl ester was recovered from it. The formation of methyl esters was confirmed by TLC using ethyl acetate: hexane: 20: 80 as a solvent as well as by gas chromatography using Stabil wax®-DA column using following conditions: Temp -250°C, Injection mode-split, pressure-126.6 Kpa, total flow -149.4 mL min^{-1}, column flow -2.87 mL min^{-1}, linear flow -50.9 cm sec^{-1}, purge flow -3.0 mL min^{-1}, split ratio -50.0. Total% fatty acid conversion was calculated by comparing the peak areas of standard methyl esters (Kumari *et al*., 2009).

Statistical analysis: All the experiments were repeated three times and are graphically represented as Mean±SD. Microsoft Excel 2007 was used to calculate mean and SD.

RESULTS AND DISCUSSION

Purification of extracellular 27 kDa lipase and N-terminal analysis: Cell-free supernatant was treated with 80% ammonium sulphate resulted in precipitation of three fourth lipase activity along with 63% protein (Kumar *et al*., 2009). However, one fourth lipase activity was recovered from supernatant which was subjected to dialysis using cellulose membrane (SIGMA) against buffer containing 10 mM $MgCl_2$ at neutral pH. $MgCl_2$ was supplemented during dialysis to prevent any significant loss of enzyme activity. The lipase activity was bound to Q sepharose resin and eluted with 0.4 M NaCl in tris-HCl pH 8.0 buffer (Fig. 3). In most of the cases, anion exchange or hydrophobic interaction chromatography has been used to purify lipases from the crude culture broth (Tomizuka *et al*., 1966; Fernandez *et al*., 2006). The lipase was purified to 2.14 fold purity with specific activity of 108.26 U mg^{-1} (Table 1). The purity was checked by HPLC (Fig. 2) using C-18 column and 95% acetonitrile as mobile phase with flow rate of 0.4 mL min^{-1} at 280 nm. On SDS-PAGE (Fig. 1a) a single band having molecular weight of 27 kDa was observed. Literature

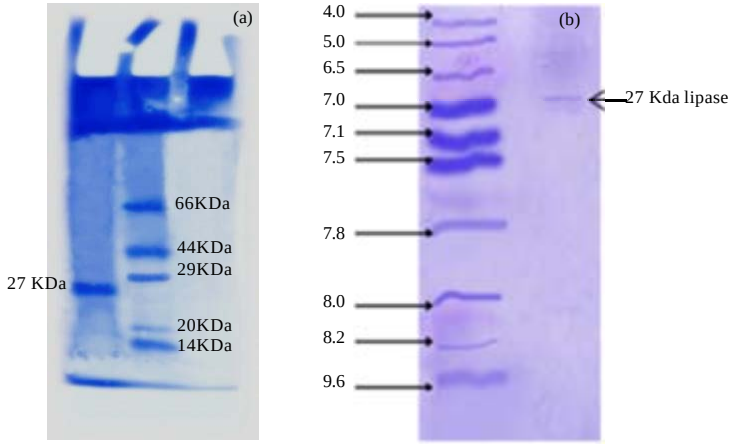

Fig. 1(a-b): (a) SDS-PAGE analysis of lipase and (b) IEF of purified Lipase

Table 1: Purification scheme

Purification method	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Fold purification	Yield (%)
Crude	3464.02	68.5	50.57	1.00	100.00
(a)Ammonium sulphate (80%) (pellet)	2663.72	43.2	61.66	1.22	76.89
(b)Ammonium sulphate (80%)(supernatant)	751.84	10.25	73.3	1.45	29.69
Supernatant was purified by Q-sepharose	541.3	5.46	108.26	2.14	15.64

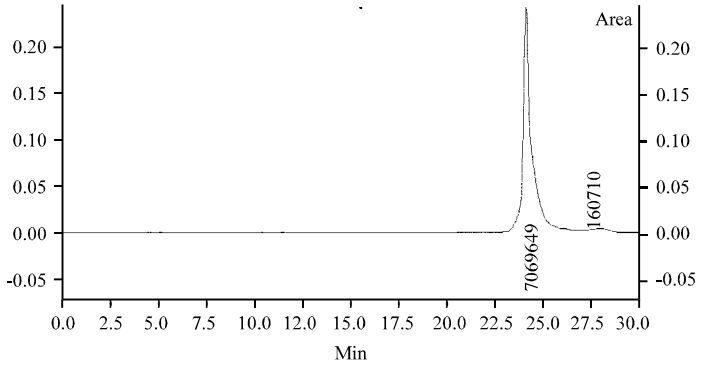

Fig. 2: HPLC chromatogram showing purified lipase

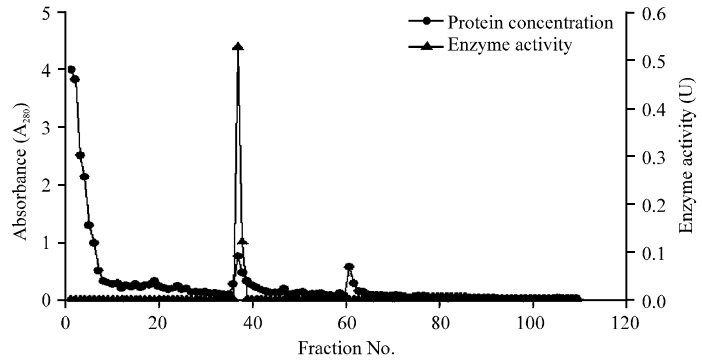

Fig. 3: Elution profile for purification of 27 kDa lipase

suggested that molecular weight of most of the yeast lipases ranges from 45-60 kDa. However, a 22 kDa lipase from *Cryptococcus* sp., *S-2* has also been reported (Kamini *et al.*, 2000).

The pI of the protein (Fig. 1b) was found to be 7 which is in contrast to the earlier reports where most of yeast lipases have pI in the range of 4.4 to 6.4 (Phillips and Pretorius, 1991). The purified protein was subjected to N-terminal analysis for first 10 amino acids. The N-terminal sequence "MQPGNLVILS" shows that it is rich in hydrophobic amino acids and closely related to lipase from *G. geotrichum* species and share 57.1% homology (Fig. 4). Yeast lipases are highly diverse and they at the most share 40-50% homology among themselves.

Effect of temperature and pH: Lipase activity was determined from 20 to 80°C at pH 9.0. Enzyme was active in the range of 40-50°C with optima at 40°C (Fig. 5) where T½ at 40 and 50°C

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CLUSTAL 2.1 multiple sequence alignment

1084959          MQLDPGNAISLLDK 14
CAA57316         MQLDPGNAISLLDK 14
1084958          MQLDPGNAISLLDK 14
GGLIP            MQLDPGNAISLLDK 14
ITHG             MQLDPGNSLTLLDK 14
27KDaMSR54       MQ--PGN-LVILS- 10
                 **   *** : :*.
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Fig. 4: Multiple sequence alignment of N-terminal amino-acid sequence of lipase from *Trichosporon asahii* MSR54. N-terminal sequence alignment of 27 kDa MSR54: 27 kDa lipase from *Trichosporon asahii* MSR54 with GGLIP: *G. geotrichum* BT107 lipase I (DQ414684), 1084959: *G. geotrichum* NRCC 205002, *G. geotrichum* ATCC 34614, CAA57316: *G. geotrichum* CBS 178.71 and ITHG: *G. geotrichum* ATCC 34614

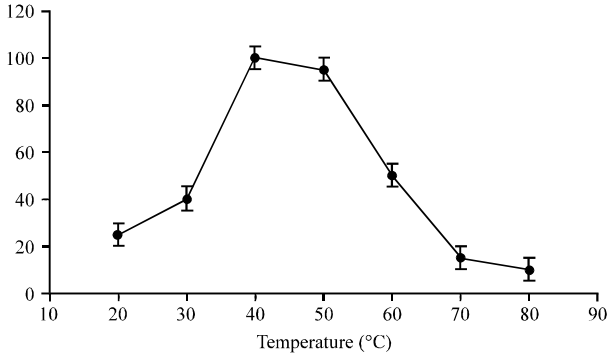

Fig. 5: Effect of temperature on lipase activity at pH 9

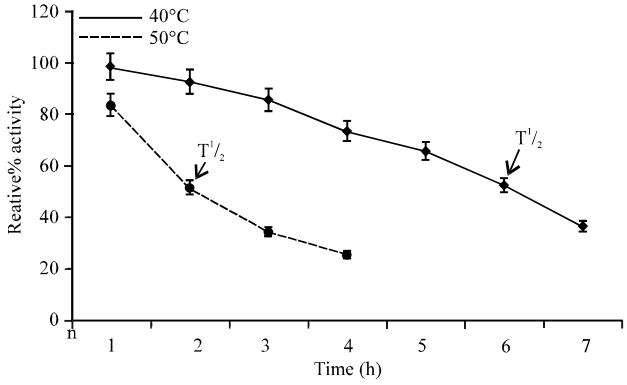

Fig. 6: Thermal stability of lipase

were 6 and 2 h, respectively (Fig. 6). Most of the lipases have temperature optima in the range of 30-40°C (Fujino *et al*., 2006) with T½ 2-3 h. However, few yeast lipases have higher temperature optima and stability like *Kurtzmanomyces* sp., I-11 which was active at 70°C (Kakugawa *et al*., 2002).

The present lipase showed higher activity in the range of pH 7.0-11.0 with optima at pH 9.0 (Fig. 7) with stability of more than 4 h (Fig. 8), whereas it showed 85-78% activity when pH was increased from pH 9.0 to pH 11.0 showing stability in neutral to alkaline pH range. Yeast lipases

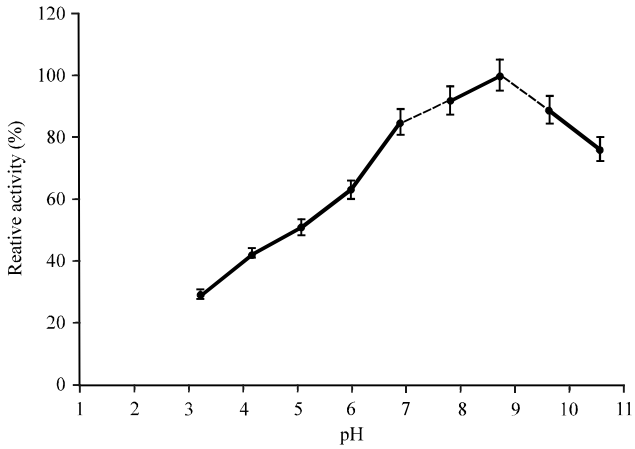

Fig. 7: Lipase activity as function of pH at 40°C. 100% = 107 U mg^{-1} pH (3-7) citrate phosphate buffer; pH (8-9) phosphate buffer and pH (9-10) glycine NaOH buffer

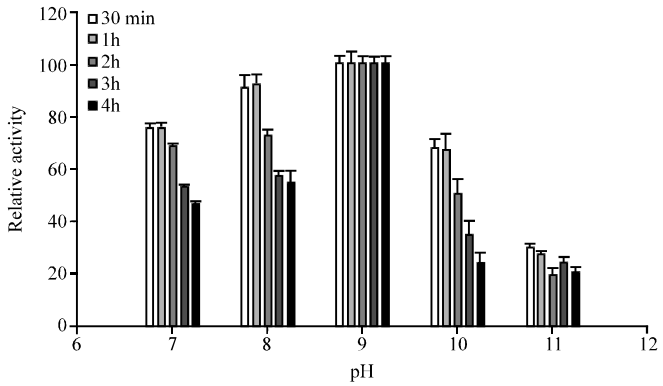

Fig. 8: pH stability of lipase at room temperature

are known to be stable over wide range of pH from 4.0-11.0, but generally lipases have neutral pH. Stability at alkaline and acidic pH for yeast lipases are rare (Gupta *et al*., 2004).

Effect of organic solvents on lipase: Solvent stability of enzyme is important for their usage in synthetic bioconversion in micro aqueous environment. Lipases are known for their ability to work in aqueous and micro-aqueous media. The stability of *T. asahii* lipase in various solvents is depicted in Fig. 9. In water miscible solvents butanol, isopropanol, acetone, ethanol and methanol; lipase retained 81.7, 71.1, 65.3, 83.7 and 93.3% residual activity, respectively when incubated in 90% solvent for 1 h. A slight decrease in the stability with increase in alkyl chain length was also observed with least stability in iso-propanol as compared to ethanol and methanol. Further it retained almost 100% activity in water immiscible solvents like petroleum ether, DMSO and showed 1.8 fold enhancement in presence of benzene. Another 54 kDa lipase from same species showed only 10-30% stability in alcohol series, but was also activated by 50% DMSO, benzene and ether (Kumar *et al*., 2009). This is in contrast to existing reports as yeast lipases are not generally stable in water miscible solvents (Kakugawa *et al*., 2002) and solvent stability is better observed in bacterial lipases (Gupta *et al*., 2004). These results follow the general theory that water miscible

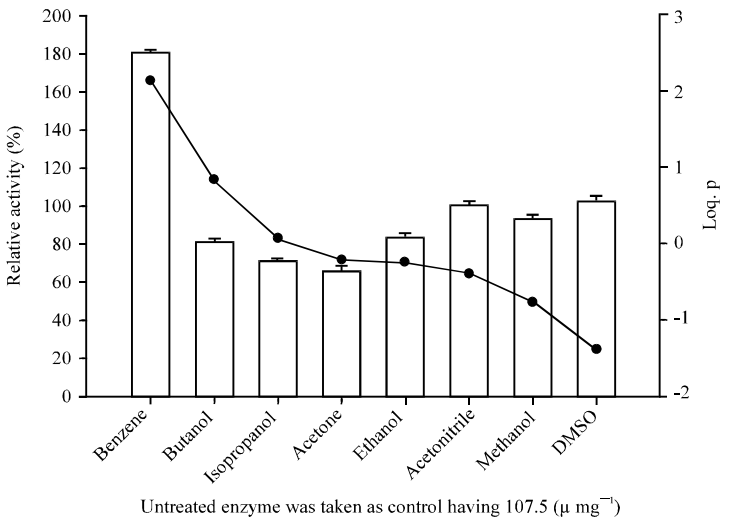

Fig. 9: Solvent stability of lipase for 1 h at room temperature

Table 2: Activation and reactivation of EDTA inhibited lipase in presence of various concentration of magnesium chloride

Compound	Concentration of $MgCl_2$ (mM)	Activation (relative activity %)	Reactivation of 10 mM EDTA treated enzyme (relative activity %)
Control	0	100±3.7	4.17±3.1
$MgCl_2$	10	140±3.7	58.33±2.8
	15	182±0.5	96.86±2.2
	20	249±0.2	140.6±1.80
	25	288±6.1	229.7±0.70

Activity without magnesium chloride was set as 100 % (107 U mg^{-1}), All measurement were repeated three times

solvents are more destabilizing than water immiscible solvents. This is due to the fact that in presence of water immiscible solvents a thin layer of water bound to the enzyme which helps them to retain their native conformation (Klibanov, 1989).

Effect of magnesium ions and EDTA on lipase: The effect of metal ions and EDTA on lipase activity is presented in Table 2. Among the divalent metal ions tested approximately 3 fold enhancement was found in presence of 25 mM magnesium chloride. Similar results have been reported for lipS221 (Zhang *et al*., 2008). It can be explained by the fact that few metal ions have ability to interfere with the bonds between amino acid side chains of the enzyme which results in the denaturation of the active site or alter the activity by stabilising and destabilising the conformation of enzyme (Ebrahimpour *et al*., 2011). The enzyme was completely inhibited in the presence of 10 mM EDTA and it regained its 97% activity when same enzyme was incubated with 15 mM $MgCl_2$ (Table 2), suggesting that it is a metalloenzyme. This character is rarely found in yeast lipases. Lipase from *Arxula adeninivorans* was completely inhibited by EDTA and *Kurtzmanomyces* sp. lipase was unaffected by it while *Candida rugosa* lipase was partially inhibited by EDTA (Kakugawa *et al*., 2002).

Effect of thiols on enzyme: The enzyme showed 2 fold enhancement in presence of 4 mM thiols, **β**-mercaptoethanol and dithiothreitol (Table 3). Thiol activation has also been reported for another

Table 3: Effect of thiols on the activity of lipase

Concentration (mM)	DTT (relative activity %)	β-Mercaptoethanol (relative activity %)
1	96.7±2.30	103.23±2.9
2	139.0±4.40	141.94±3.1
4	213.0±1.40	223.62±2.5
6	165.0±0.85	198.9±0.20
8	51.61±2.6	87.1±2.10
10	39.7±3.80	47.3±1.60

Activity without thiols was set as 100 % (107 U mg^{-1}), All measurement were repeated three times

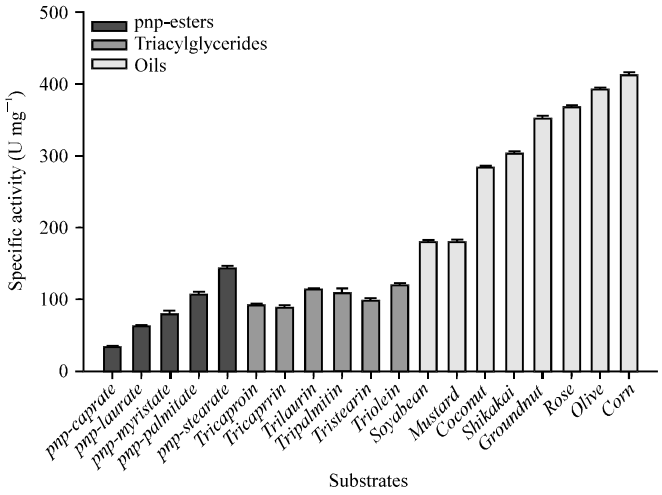

Fig. 10: Substrate specificity of lipase at pH 9 and 40°C

lipase from the same organism (Kumar *et al.*, 2009). However generally lipase are not known to be stimulated by thiols.

Substrate specificity of lipase: Lipases are known for their varying substrate specificity which is very important regarding their biotechnological applications. In this regard present lipase was evaluated towards various oils, p-nitrophenyl esters and triacylglycerides. It showed higher activity towards substrates having longer carbon chains. It effectively hydrolysed corn oil>olive oil>rose oil>groundnut oil>shikakai oil>coconut oil>mustard oil (Fig. 10). With respect to p-nitrophenol esters best hydrolysis was observed on stearate followed by palmitate> myristate>laurate>caprate whereas on triacylglycerides the maximum hydrolysis was observed on triolein followed by trilaurin>tripalmitin>tricaproin>tristearin>tricaprin (Fig. 11). Hence, the wide substrate specificity indicated that the enzyme preferred long chain fatty acids. This is in contrast to 54 kDa lipase from the same organism which showed preference for the short to mid chain fatty acids (Kumar *et al.*, 2009). Most of the yeast lipases prefer mid chain length esters as reported from *Geotrichum candidum* 4013 sp., (Brabcova *et al.*, 2010), *Candida* sp. and *Yarrowia* sp., (Fickers *et al.*, 2005). However, lipases from *Geotrichum candidum* as well as *Pichia burtonii* showed preference for longer chain p-NP esters (Brabcova *et al.*, 2010).

The kinetic parameters K_m and V_{max} were calculated for the hydrolysis of p-nitrophenyl palmitate. The Lineweaver-Burk plot was linear and depicted that it follows the Michaelis-Menten kinetics on p-NPP (Fig. 11). The K_m and V_{max} were 5.55 μM and 6.66 mM mg^{-1} which is similar to most of the lipases from *G. candidum* IMI387428 (Loo *et al.*, 2007).

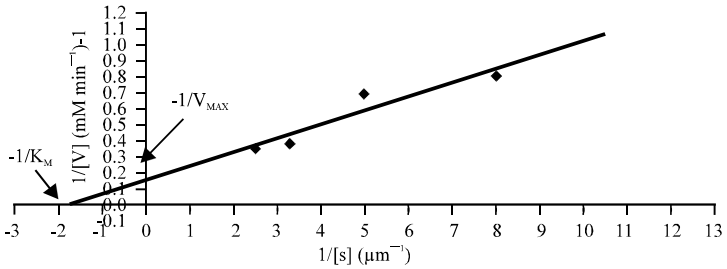

Fig. 11: Lineweaver-Burk plot of lipase

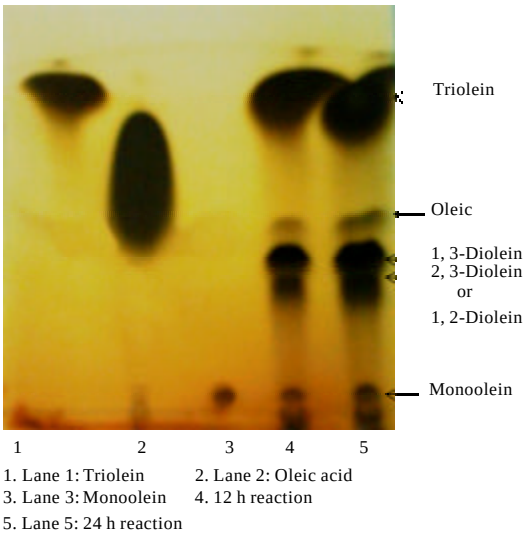

Fig. 12: Regioselectivity of the lipase using triolein in micro-aqueous medium

Regioselective hydrolysis of triolein: Regioselectivity is an important characteristic of lipases for food industries to design nutraceutical or functional lipids. Regioselective nature directly depends on the molecular property of the enzyme. On the basis of regioisomeric excess (RE% = % (1,3-diglyceride)-%(1,2-diglyceride)) enzymes have been characterised into three groups viz., non-specific (RE<70), 1,3-selective (90>RE>70) and 1,3-specific (RE>90). Non-specific enzymes are known for random cleavage of acyl group during triglyceride hydrolysis for example; lipase from *Candida antarctica* (Watanabe *et al*., 2009) whereas regioselective enzymes are generally known from fungi viz., *Rhizopus arrhizus* and *Fusarium heterosporum* (Meghwanshi *et al*., 2006).

The regioselectivity of lipases obtained from studies in aqueous or bi-phasic media are inherently unreliable due to the notorious problem of acyl group migrations associated with these systems where as in aprotic or organic solvents mono- and diglycerides are stable towards acyl group migration. Hence, the present lipase was analysed in both aqueous (data not shown) and hexane system for triolein hydrolysis. Products of triolein hydrolysis released monoolein, diolein and oleic acid after 12 and 24 h. It indicated that enzyme is likely to be a 1,3-regioselective enzyme (Fig. 12).

Lipase immobilization and biodiesel production from lipase: Lipases are efficient biocatalyst for the synthesis of alkyl esters by alcoholysis of lipids. For the ease of convenience enzyme was

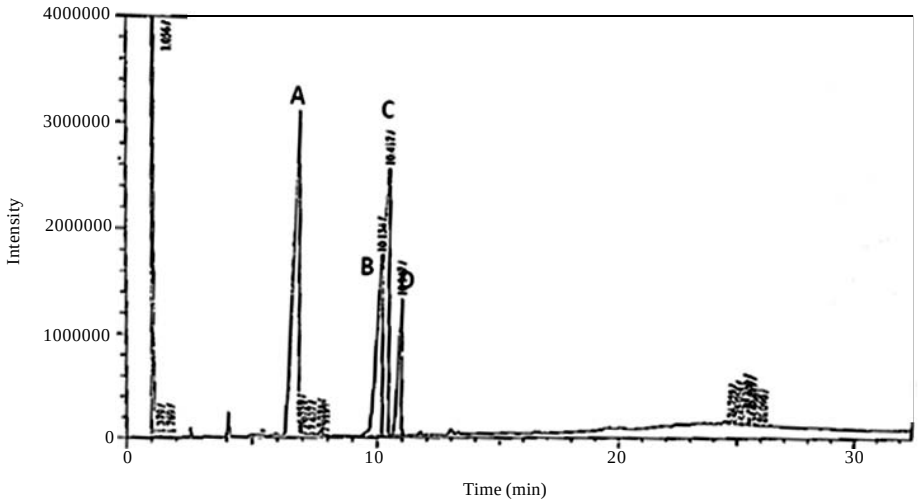

Fig. 13: GC chromatogram showing the formation of different methyl esters by lipase using coconut oil and methanol. Peak A, B, C and D are methyl palmitate, methyl stearate, methyl oleate and methyl linoleate, respectively

immobilized on basic alumina through physical adsorption and the immobilisation yield was found to be 72% where 1 mg of immobilised metrics had 75 U enzyme activity (data not shown).

Transesterification of coconut oil in methanol confirmed the formation of four different methyl esters viz., methyl palmitate, methyl oleate, methyl stearate and methyl linoleate in decreasing order (Fig. 13). The conversion rate was found to be 87.6%. Biodiesel production from bacterial lipases are well reported but with lower conversion rate (Kumari *et al*., 2009). However lipase from *C. antarctica* has been reported for the production of biodiesel from tallow, soybean and rapeseed oil but with the conversion rate of 61.2-83.8% (Fukuda *et al*., 2001). This is due to the reason that most of the lipases are inhibited by methanol and glycerol; a by-product during biodiesel production (Fukuda *et al*., 2001).

CONCLUSION

A minor extracellular 27 kDa lipase from *Trichosporon asahii* MSR54 was purified from fermentation broth. It is magnesium dependent metallo enzyme. The optimum temperature and pH was 40°C and pH 9.0, respectively. The lipase showed preference for long chain triglycerides i.e., C:12 to C:18. It was a thiol activated and 1,3 regioselective lipase. It efficiently produced biodiesel from coconut oil with conversion rate of 87.6%.

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