Differential induction, purification and characterization of
cold active lipase from *Yarrowia lipolytica* NCIM 3639

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ABSTRACT

The production, purification and characterization of cold active lipases by Yarrowia lipolytica NCIM 3639 is described. The study presents a new finding of production of cell bound and extracellular lipase activities depending upon the substrate used for growth. The strain produced cell bound and extracellular lipase activity when grown on olive oil and Tween 80 respectively. The organism grew profusely at 20 °C and at initial pH of 5.5, producing maximum extracellular lipase. The purified lipase has a molecular mass of 400 kD having 20 subunits forming a multimeric native protein. Further the enzyme displayed an optimum pH of 5.0 and optimum temperature of 25 °C. Peptide mass finger printing revealed that some peptides showed homologues sequence (42 %) to Yarrowia lipolytica LIP8p. The studies on hydrolysis of racemic lavandulyl acetate revealed that extracellular and cell bound lipases show preference over the opposite antipodes of irregular monoterpene, lavandulyl acetate.

Keywords: Cold active lipase; cell bound lipase; extracellular lipase, oligomeric lipase. Lavandulyl acetate.
1. Introduction

Triacylglycerol hydrolases or lipases (EC 3.1.1.3) catalyze ester bond hydrolysis in triacylglycerols with the release of fatty acids, mono- and diglycerides and glycerol. As this substrate presents a very low solubility in water, the catalytic reaction takes place at the lipid–water interface. Although naturally occurring triacylglycerols are the preferred substrates, these enzymes can hydrolyze a wide range of insoluble fatty acid esters. It is well established that the reaction is reversible, and that lipase can catalyze esterification as well as trans-esterification reactions often in nearly anhydrous organic solvents. Many biotechnological processes are expedited by the use of higher temperatures and this generated a lot of research into thermo-stable enzymes. However, more recently there has been a great interest in cold-adapted enzymes for transformations in which substrate and product stabilities require the use of low temperatures and energy savings. Such cold active enzymes are important in many fields including the detergent, textile and food industries, as well as for a variety of bio-catalytic reactions (Alquati et al., 2002).

Due to their broad substrate specificity, lipases are used in versatile industrial applications including their utilization as additives in laundry detergents and catalysts for synthesis of organic compounds. The demand of biocatalysts active at extreme conditions (low or high temperatures, acidic or basic solutions or high salt contents) is increasing. Although, the enzymes from extremophiles are greater attention in recent years (Royter et al., 2009), the cold-active enzymes have also generated considerable interest, since they have potential to improve the efficiency of industrial processes and offer possible
economic benefits through energy saving (Feller and Gerday 2003). Psychrophilic and ectothermic organisms constantly living at low temperature environment possess enzymes adapted to the suitable conditions in the environment, which often show higher catalytic activity at low and moderate temperatures and lower thermostability than their mesophilic and thermophilic counterparts. The high catalytic activity at low temperatures can be favorable in their applications as well (Tutino et al., 2010). So far, very few cold-active lipases from psychrophilic microorganisms have been characterized (Mayordomo et al., 2000; Rashid et al., 2001; Lee et al., 2003; Ryu et al., 2006; Zhang and Zeng 2006; Yang et al., 2008; Park et al., 2009).

An extracellular and two cell-bound activities in Yarrowia lipolytica corresponding to lipase I (39 kDa) and lipase II (44 kDa) were reported (Ota et al., 1982). The cell-bound lipases differed in several properties from the extracellular lipases (Ota et al., 1984). Although much work on lipases from Yarrowia lipolytica has been published (Fickers et al., 2011), there are no reports on cold active lipases from this organism. This effort has been stimulated by the recognition that cold-active enzymes might offer novel opportunities for biotechnological exploitation based on their high catalytic activity at low temperatures, low thermo-stability and unusual specificities. In this study, the production of lipase from Yarrowia lipolytica NCIM 3639 and its purification and characterization are described.

2. Methods

2.1. Chemicals

Peptone, yeast extract, malt extract, glucose, agar were purchased from Hi-Media Laboratories Limited Mumbai, India. Olive oil samples used were obtained from local
market. Tween–80 and Triton X-100 were obtained from Merck India Ltd. P-
Nitrophenylpalmitate (pNPP), Nonidet P-40 (NP-40) and 3-[(3-Cholamidopropyl)
dimethylammonio]-1-propanesulphonate (CHAPS), Sodium dodecyl sulphate (SDS), Q
sepharose, sepharose CL-4B were obtained from Sigma Chemical Co. (USA).

2.2. Microorganism and Culture conditions

Yarrowia lipolytica NCIM 3639 was isolated from refrigerated solution of 5 %
Tween 80 in 50 mM phosphate buffer, pH 6.0. The strain was identified by National
Collection of Yeast cultures (NCYC) England using 26S rDNA D1/D2 sequencing
approach. The strain was maintained on MGYP agar slopes containing malt extract 0.3%,
glucose 1.0%, yeast extract 0.3%, peptone 0.5% and agar 2.0%. Synthetic oil-based
(SOB) medium was used as production medium which contained NaNO₃ 0.05%,
MgSO₄·7H₂O 0.05%, KCl 0.05%, KH₂PO₄ 0.2%, yeast extract 0.1%, bacto-peptone 0.5%
and olive oil 1.0%. The initial pH of the medium was adjusted to 5.5 with 0.1 N NaOH
or HCl prior to sterilization.

2.3. Inoculum preparation and lipase production

Cells of Yarrowia lipolytica were inoculated in 10 ml MGYP liquid medium and
allowed to grow on a rotary shaker with shaking at 150 rpm and at 20 °C for 48 h. This
grown culture (5 ml) was transferred to 250 ml shake flask containing 50 ml MGYP
medium and allowed to grow for 48 h at 20 °C. This was used as an inoculum which
contained approximately 5 ± 1 x 10⁸ cells per ml. For lipase production, conical flasks
(250 ml) containing 70 ml SOB medium were inoculated with 5% inoculum and
incubated on a rotary shaker at 150 rpm at 20 °C for 72 h. The samples were removed
after certain time intervals to determine lipase activity, soluble protein and biomass. The
cells were harvested by centrifugation at 5000g for 10 min at 4 °C and the supernatant was used as extracellular lipase preparation. Cells were washed twice with 50 mM citrate phosphate buffer (pH 5.0) and re-suspended in known amount of buffer solution. This cell suspension was used as a source of intracellular enzyme.

2.4. Enzyme assay, Biomass and protein estimation

The spectrophotometric lipase assay was performed using pNPP as substrate. The substrate solution was prepared by adding solution A (30 mg of pNPP in 10 ml of propane-2-ol) to 9.5 ml of solution B (0.1 g of gum arabic and 0.4 g of Triton X-100 in 90 ml of distilled water) drop wise with intense stirring. The assay mixture consisted of 0.9 ml of substrate solution, 0.1 ml of citrate phosphate buffer (0.5 M, pH 5.0) and 0.1 ml of suitably diluted enzyme. The assay mixture was incubated at 20 °C for 30 min and the p-nitrophenol released was measured at 410 nm in Spectronic-117 spectrophotometer. One unit of activity was expressed as the amount of enzyme that released 1 μmoles of p-nitrophenol per min under the assay conditions. Cell biomass was determined by measurement of the absorbance of cells, after being washed and re-suspended in saline, at 660 nm. Dry weight (DW) was calculated from the absorbance value using a standard curve (18.0 OD corresponds to 10.2 g/l dry weight). Protein concentration was estimated by the method of Lowry with bovine serum albumin as a standard (Lowry et al., 1951).

2.5. Intracellular and cell bound lipase preparations

Wet cells (0.15 g dry equivalent) were washed with 20 ml of citrate phosphate buffer (50 mM, pH-5.0) and suspended in the same buffer at a final volume of 5 ml. The cell suspension was homogenized at 4 °C with a Sonicator (Sonics, Vibra cell) for ten periods of 30 seconds each. The disrupted cells were centrifuged at 5,000g at 4 °C for 10
min. The supernatant was used as intracellular lipase source. The cell debris, after washing with the buffer, was used as cell bound lipase enzyme. For lipase extraction, cell debris was suspended in 10 ml citrate phosphate buffer (50 mM, pH-5.0) with detergents such as 0.5% (v/v) of Triton X-100, Tween 80, NP-40, SDS and CHAPS and the suspension was shaken at 150 rpm, 20 °C for 4h. The debris was removed by centrifugation at 5000g at 4 °C for 10 min and the lipase activity in the filtrate as well as debris was measured.

2.6. Purification of Y. lipolytica NCIM 3639 lipase

All procedure were performed at 4 °C. Cell free culture broth (500 ml) was concentrated to minimum volume by an Amicon Ultrafiltration apparatus equipped with YM-30 membrane (30000 Da cut-off). The Concentrated enzyme solution was applied to a Q Sepharose column (15 x 1.5 cm) pre-equilibrated with 50 mM phosphate buffer (pH 7.0). The column was then washed with 100 mM NaCl in 50 mM citrate phosphate buffer (pH 5.0) till the flow through fractions showed no lipase activity. Enzyme elution was done with 1M NaCl in 50 mM citrate phosphate buffer (pH 5.0) at the flow rate of 12 ml/h. Active fractions were pooled and concentrated by Ultrafiltration. This concentrated fraction was then chromatographed on a Sepharose CL-4B column (150 x 1 cm) pre-equilibrated with 20 mM citrate phosphate buffer (pH 5.0) at a flow rate of 3.6 ml/h. Fractions with high specific activity were pooled, concentrated by Ultrafiltration and the purity of the enzyme was analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE).

2.7. Native molecular weight determination
The native molecular mass of lipase was estimated by gel filtration. The gel filtration column (Sepharose CL-4B, 50 x 1 cm), was equilibrated in 50 mM citrate phosphate buffer (pH 5.0) and calibrated using gel filtration standard molecular weight markers: Bovine serum albumin (Mr 66 kDa); Alcohol dehydrogenase (Mr 150 kDa); β-amylase (Mr 200 kDa), and Apoferetin (Mr 443 kDa). The column void volume was determined with Blue dextran (Mr 2000 kDa). About 2 mg (500 μl) of samples were applied to the column, which was operated at a flow rate of 10 ml/h. In order to achieve optimal calibration, the standards were individually chromatographed and the calibration graph was plotted as elution fractions verses absorbance at 280 nm.

2.8. Effect of pH and temperature on enzyme activity and stability

The optimal pH of the enzyme was determined by measuring the lipase activity at 25 °C at various pH levels (pH 3-8): citrate phosphate buffer (50 mM, pH 3.0-6.0) and phosphate buffer (50 mM, pH 7.0-8.0). The pH stability was studied by incubating the purified enzyme (20 ug) in various buffers with pH ranging from 3-8 for 30 min at 25 °C. The residual activity was then assayed under standard assay conditions. The optimal temperature of the enzyme was determined by measuring the enzyme activity at various temperatures (5-45 °C) in 50 mM of citrate phosphate buffer, pH 5.0. Thermal stability was determined by incubating the purified enzyme in 50 mM citrate phosphate buffer (pH 5.0) for 24 h at the desired temperatures (5-45 °C) followed by measuring the residual activity.

2.9. Substrate specificity

The substrate specificity was determined using p-NP esters of varying acyl chain lengths from C4 to C18. The following substrates were used: p-NP butyrate (C4:0), p-NP
caprilate (C8:0), p-NP deconate (C10:0), p-NP laurate (C12:0), p-NP myristate (C14:0),
p-NP palmitate (C16:0), and p-NP stearate (C18:0). The substrates were prepared in 2-
propanol at final concentration of 100 μM. Reaction was carried out under standard assay
conditions.

2.10. Effect of metal ions on enzyme activity

For determining the effect of metal ions, EDTA, on enzyme activity, enzyme
assays were performed in presence of various metal ions (5 mM) using pNPP as
substrate.

2.11. Kinetic and temperature dependence

The K\textsubscript{m} and K\textsubscript{cat} values of the enzyme were determined under standard assay
condition using 20-100 μM of pNPP substrate. The constant values were calculated by
fitting data to linear regression using Lineweaver-Burk plot. The effect of temperature on
K\textsubscript{m} and K\textsubscript{cat} of the enzyme was determined by varying the substrate concentration in the
range of 20-100 μM at different temperatures ranging from 5-35 °C. All the assays were
performed by standard assay conditions. Kinetic constants were calculated using
Lineweaver-Burk plots.

2.12. Peptide mass finger printing of lipase

The Peptide mass finger printing of lipase was performed as described earlier
(Mhetras et al., 2009).

2.13. Lipase mediated resolution of (±)-lavandulyl acetate.

Enzyme (extracellular or cell bound, 4U/μmol of lavandulyl acetate) was added to
2 mL in citrate phosphate buffer (50 mM, pH = 5.0) containing 1mg of (±)-lavandulyl
acetate (in 50 μL of methanol). The mixture was incubated at 25 °C on a water bath
shaker (200 rpm). Solid NaCl (0.4 g) was added, and the aqueous phase was extracted three times with 500 µL of tert-butyl methyl ether (TBME). The volume of the extract was reduced to ~50 µL with a stream of dry nitrogen. A 1 µL portion of the extract was analyzed by gas chromatography (Agilent technologies 7890 A GC system, USA) on a column 30 m X 0.32 mm (bore size) X 0.25 µm (film thickness) HP-Chiral (β-cyclodextrin) capillary column (J & W Scientific) with a temperature gradient from 80 °C for 1 min raised to 140 °C at 2 °C per min followed by a temperature gradient of 140 to 230 °C at 25 °C per min with N₂ (flow rate of 1 mL/min) as carrier gas. Samples were also analyzed by GCMS using the same conditions. Control experiments were conducted in parallel with substrate but without lipase. The formation of (R) and (S) lavandulol was monitored by comparing the retention time (Rt : 22.92 and 22.57 min, respectively) and coinjection studies with the (R)-lavandulol obtained by incubating dimethyl allyl diphosphate with chrysanthemyl diphosphate synthase (Thulasiram et al., 2007).

3. Results and discussion

3.1. Cell growth and lipase production

Yarrowia lipolytica NCIM 3639 was isolated from refrigerated Tween 80 samples in our laboratory and we feel that this could be a good source of cold active lipase. Cold active lipases function effectively at cold temperatures with higher catalytic rates in comparison to mesophilic lipases. Although many lipase producing organisms are available, only some bacteria and a few yeasts are known for the production of cold active lipases. Among the psychrophilic fungi and yeasts, Candida lipolytica, Geotrichum candidum Candida antarctica, Penicillium roqueforti (Joseph et al., 2008), and Aspergillus nidulans (Mayordomo et al., 2000) are the potent producers of cold
active lipases. Very recently, solvent stable, cold adapted *Microbacterium* sp. was isolated which produced cold active lipase (Kumar et al., 2011). This cold adapted lipase proved to be efficient in biodiesel production using jatropha oil. Interest in the enzymes produced by cold-adapted microbial strains has recently increased. However, most of the studies have focused on the properties of these enzymes (Feller and Gerday, 1997).

The mode of lipase induction in *Y. lilolytica* NCIM 3639 is shown in (Table 1). Tween 80 was found to be the best inducer for the production of extracellular lipase. Maximum extracellular lipase activity was obtained in a medium with 2% Tween 80 with a small amount of cell bound lipase activity. Greater or lesser concentrations of Tween 80 resulted in decreased extracellular lipase production. Other substrates such as Tween 20 induced low levels of both cell bound and extracellular lipases, whereas Triton X-100 inhibited both growth and lipase production. The growth of *Y. lipolytica* in olive oil containing media led to the production of high amounts of cell bound lipase with traces of extracellular lipase. Combination of Tween 80 and olive oil produced both extracellular and cell bound lipases. Several lipases have been detected in *Y. lipolytica*, including intracellular, membrane-bound, and extracellular enzymes (Fickers et al., 2005a). In the present study, it is shown that *Y. lipolytica* was found to produce either cell bound or extracellular lipase depending upon the carbon source used for its growth. It produced predominantly cell bound lipase when olive oil is used in the medium. The growth of *Y. lipolytica* on Tween 80 containing medium resulted in the production of significant amount of extracellular lipase. Such differential induction pattern of cell bound and extracellular lipase is not reported so far in the literature. Contradictory results were reported by Dominguez et al., (2003) in which *Y. lipolytica* was found to produce
only extracellular lipase when it was grown in olive oil and sunflower oil. Dominguez et al., (2003) also reported that addition of surfactants such as Tween 80 decreased the extracellular lipase production in *Y. lipolytica*. Time-coursed production of *Y. lipolytica* lipase was examined in minimal media containing 2 % Tween 80 (Fig. 1). Lipase production began to increase after 24 h of incubation, corresponding to the late exponential growth phase, and reached a maximum extracellular lipase activity at 48h. Majority of the lipase activity remained cell bound throughout the period of incubation when the organism was grown in 1 % olive oil based medium for 72 h. The cell bound lipase could not be extracted when the cells were treated with different surfactants such as Triton X-100, NP-40, CHAPS and even Tween 80 (data not shown) indicating the cell bound nature of the lipase. Ota et al., (1982) reported cell bound lipase from *Saccharomycopsis lipolytica* which was extractable using Triton X-100. Fickers et al., (2004) found that the lipase remained cell bound during the growth phase before being released in the media. Lipases YILip7 and YILip8 are two cell-bound lipases which can easily be extracted with phosphate buffer (Fickers et al., 2005b).

3.2. Effect of temperature and pH on lipase production

The optimal temperature and initial pH of the medium for cell growth and lipase production were determined. Maximum activity and biomass were obtained at 20 °C within 48 h of incubation (Fig. 2). The lipase production was delayed when the organism was grown at 15 °C and the maximum activity was obtained after 96 h of incubation. Growth of organism at higher temperatures (25 and 30 °C) resulted in significant decrease in lipase production without affecting the cell growth. Effect of initial pH on biomass and lipase production was evaluated in the pH range from 3 to 8 (Fig. 3). The lipase
production increased up to pH 6.0 and decreased sharply thereafter at pH 7.0. The optimal temperature for lipase production by *Y. lipolytica* is comparable to the Psychrotrophic *Pseudomonas* sp. Strain KB700A (Rashid et al., 2001). *A. nidulans* WG 312 produced extracellular cold active lipase at 30 °C utilizing olive oil as an inducer (Mayordomo et al., 2000). Tween 80 and Tween 20 were the best inducers for cold active lipase production by *Pseudoalteromonas* sp. wp37 at 25 °C (Zeng et al., 2004). The significant lipase activity was obtained in a pH range of 5-6 with maximum activity at pH 5.5. The lipase produced by *A. nidulans* was maximum at pH 6.5 (Mayordomo et al., 2000).

### 3.3. Purification of *Y. lipolytica* extracellular lipase

The *Y. lipolytica* lipase was purified from the culture medium; the supernatant was ultra filtrated with 30 kDa membrane, yielding 25 ml of concentrated lipase solution with 86% yield and a 3.5-fold specific activity. After anion exchange chromatography, a specific activity of 101 IU/mg was obtained with 7.23-fold purification (Table 2). Further purification using gel filtration chromatography (Sepharose CL-4B) resulted in separation of two lipase activity peaks. First peak was eluted with the void volume of the Sepharose CL 4B column which corresponds to very high molecular mass lipase. The second peak was obtained with maximum specific activity of 195 IU/mg and 14 fold purification. SDS-PAGE analysis of second peak showed protein band corresponding to a molecular mass of around 20 kDa (Fig. 4a). The apparent molecular weight of the purified lipase estimated by a Sepharose CL-4B, gel filtration column was 400 kDa (Fig. 4b). These results indicated that the enzyme comprises 20 subunits each with same molecular mass (20 kDa). Several lipases from *Y. lipolytica* have been reported with different forms
LIP1 and LIP3 genes from Y. lipolytica encoding two lipases were identified by Dominguez et al., (2003) that are similar to the lipases of the fungi C. cylindracea and G. candidum. These two lipases were reported to be belonging to the carboxylesterase family. An extracellular and two cell-bound types of activities corresponding to lipase I (39 kDa) and lipase II (44 kDa) were described by Ota and coworkers (Ota et al., 1982). Yu et al., (2007b) expressed lipase Lip2 in Pichia pastoris and the molecular weight of rYlLip2 was found to be 39 kDa which is similar to parent enzyme. Song et al., (2006) expressed YlLip7 and YlLip8 genes encoding two lipases from Yarrowia lipolytica AS 1216 in P. pastotis. These expressed proteins corresponding to two lipases were purified to homogeneity. The purified lipases exhibited molecular weight of 41 kDa each and the optimum temperature of 40 and 45 °C respectively.

3.4. Effect of pH and temperature on lipase activity and stability

Maximum lipase activity obtained at pH 5.0 at 25 °C and decreased significantly (50 %) when the pH was increased to 6.5. The enzyme showed stability over a pH range 4 to 6 with complete loss of activity at pH 7.0. The purified enzyme exhibited maximum activity at 25 °C with retention of 40% of activity at 5 °C. The activity decreased sharply at temperatures above 30 °C. The enzyme was found to be stable at 10 and 30 °C. It lost about 40 % of activity after 6 h of incubation at 35 °C, the enzyme was complete inactive at 45 °C after 4 h of incubation. Cold-adapted enzymes are mainly established in manifold applications, which offer potential economic benefits by saving energy (Feller and Gerday, 2003). Furthermore, they also minimize undesirable chemical side-reactions that occur most probably at higher temperatures (Yang et al., 2008). Y. lipolytica lipase displays a temperature optimum of 25 °C. The enzyme is unstable at temperatures above
30 °C. Similar cold-adapted lipases showing temperature optima varying between 25 and 45 °C have been investigated from the other microbial strains such as *Photobacterium* strain (25 °C), two cold active lipase from *Geotrichum* sp. SYBC WU-3 (20 and 15 °C), *Acinetobacter baumannii* BD5 (35 °C), *A. nidulans* (40 °C), *Pseudomonas* sp. Strain KB700A (35 °C) (Mayordomo et al., 2000; Rashid et al., 2001; Ryu et al., 2006; Cai et al., 2009; Park et al., 2009). Moreover, a further psychrophilic lipase Lip 3 obtained from a metagenomic DNA library from deep sea sediment has been described to be highly active at 25 °C (Zhang and Zeng, 2006). A lipase from *Aeromonas* sp. LPB 4 exhibited maximum activity at 10 °C (Lee et al., 2003).

3.5. *Substrate specificity of Y. lipolytica lipase*

The lipase is active against a wide range of *p*-nitrophenyl esters of fatty acids with highest hydrolytic activity with *p*-NP caprylate (C8). The results indicated the clear preference of the enzyme for medium chain length fatty acids (data not shown). Lipases are carboxylesterases displaying maximal activity towards water-insoluble long chain acylglycerols (C8-C18), whereas esterases hydrolyze partially water-soluble ester substrates with short-chain fatty acids (below C8). The lipase produced from a psychrophilic *Photobacterium* strain showed maximum activity towards *p*-NP caprylate (Ryu et al., 2006). The other cold active lipases which showed substrate preference towards medium acyl chain are from *Pseudomonas* sp. strain KB700A (Rashid et al., 2001).


As shown in Table 3, *Y. lipolytica* NCIM 3639 lipase did not show an obligate requirement of metal ions for its activity and EDTA did not affect the activity indicating
that it is neither metal requiring nor a metalloenzyme. However, the enzyme activity was inhibited by Hg\textsuperscript{2+} at 5 mM concentration. Fe\textsuperscript{3+} ions also inhibited significantly the enzyme activity at 5 mM concentration. Other metal ions did not affect the lipase activity to much extent but some of the metal ions did enhance the lipase activity. The presence of DTT, 2-Mercaptoethanol or Triton X-100 had no effect on catalytic activity. Both Fe\textsuperscript{2+} and Fe\textsuperscript{3+} ions were found to inhibit the lipases from Aspergillus niger. (Iwai et al., 1970). Metal ions like Ca\textsuperscript{2+}, Mg\textsuperscript{2+} are well known activators of lipases (Yu et al., 2007a) because Ca\textsuperscript{2+} or Mg\textsuperscript{2+} form complexes with ionized fatty acids which facilitated the removal of free fatty acids formed in the reaction at the water oil interface and changing their stability and behaviors at the interfaces.

3.7. Temperature dependent kinetics

The determination of the kinetic parameters for pNPP hydrolysis revealed an interesting phenomenon of kinetic optimization at environmental temperatures. The lipase displayed highest K\textsubscript{cat} at 25 °C (Table 4), the difference in catalytic efficiency between 5 and 25 °C was 7.7 \times 10^3 \text{min}^{-1}. In contrast, the K\textsubscript{m} values did not show significant variation between 5 and 25 °C (Table 4). At temperatures greater than 25 °C, the k\textsubscript{cat} values decreased and the K\textsubscript{m} values increased. It has been shown that enzymes from thermophilic and psychrophilic microorganisms, show the lowest K\textsubscript{m} values for their substrates at the physiological temperatures of the source organisms (Hochachka and Lewis, 1970). To examine whether this is also the case for lipase from Y. lipolytica, the K\textsubscript{cat} values for pNPP substrate were determined at different temperatures. The highest K\textsubscript{cat} value was observed at around 25 °C which corresponded to the optimal growth and optimal activity of Y. lipolytica lipase with decline of K\textsubscript{cat} value at 30 °C. At
35 °C, the lipase gets inactivated. These results show the cold active nature of Y. lipolytica lipase.

3.8. Peptide mass finger printing of lipase

Gel bands were manually excised and subjected to automated in-gel chemical modification of cysteine residues with dithiothreitol and iodoacetamide followed by tryptic digestion and the digestion mixture was analyzed directly by QTOF2 hybrid quadrupole mass spectrometer. This mass spectrometric peptide mapping was compared with the theoretical maps of known proteins, twenty three peptides in the spectrum (Table 5) had a mass that was homologous for peptides from YlLip8 of Y. lipolytica, resulting in a sequence coverage of 46.10% (172 aa for a total size of 371 aa for the mature YlLip8 lipase). Six peptides matched with triacylglycerol lipase precursor of Candida deformans, three peptides are homologous to the tryptase inhibitor of chain E - medicinal leech (fragments). Further sixty four of the peptide peaks did not show any match to the known proteins. The molecular weight of Y. lipolytica, YILip8 (Fickers et al., 2005b) is 42 kDa and our purified native lipase from Y. lipolytica NCIM 3639 is of 400 kDa. SDS-PAGE indicated single band with molecular weight of 20 kDa which indicates that the native lipase is comprised of 20 subunits to form oligomeric native protein. This shows that the cold active lipase from Y. lipolytica NCIM 3639 is different from Y. lipolytica YILip8 protein. Such oligomeric lipase with 20 subunits is not yet reported so far. Ota et al., (1982) purified two cell-bound lipases (lipases I and II) with the molecular weights of 39 and 44 kDa, respectively. Both of them had the similar substrate specificity, optimum pH and pH stability. Fickers et al. (2005b) isolated gene LIP7 and LIP8 encoding two cell-bound lipases from Y. lipolytica (YILip7 and YILip8) which have 366 aa and 371 aa,
respectively. Three extracellular lipases (YILip2, YILip7, and YILip8) have been reported in *Y. lipolytica*. Lipase YILip2 was the main extracellular lipase and secreted at the end of the growth phase (Fickers et al., 2004), while YILip7 and YILip8 are mainly associated to the cell wall and easily released by washing the cells with phosphate buffer (Fickers et al., 2005b).

**3.9. Lipase mediated resolution of (±) lavandulyl acetate**

The irregular monoterpene alcohol, lavandulol is a constituent of essential oils and also an important additive in perfumery and cosmetic industry (Seino et al., 2008). Homochiral lavandulol exists naturally in its (R)-form in the essential oil of lavender. The esters of both (R)- and (S)-lavandulol are the segregation pheromones of insects such as *Anthonomus rubi* Herbst (Innocenzi et al., 2001) and *Planococcus ficus*, (Zada et al., 2008) respectively. Cell bound and extracellular lipases isolated from *Yarrowia lipolytica* NCIM 3639 showed the stereospecificity in hydrolysis of the racemic lavandulyl acetate to corresponding (R)- and (S)-lavandulol.

The time course studies with the cell bound and extracellular lipases from *Yarrowia lipolytica* NCIM 3639 for the resolution of (±)-lavandulyl acetate clearly demonstrates that the extracellular lipase hydrolyses the (S)-lavandulyl acetate much faster than the corresponding (R)-enantiomer where as the cell bound lipase hydrolyses the (R)-lavandulyl acetate much faster than the corresponding (S)-enantiomer (Figure A and B). The extent of conversion was determined at various time intervals over 6h of incubation period. The preference for (R)- or (S)-lavandulyl acetate was observed with both lipases till the preferred isomer gets completely hydrolysed to corresponding alcohol. Depending on the incubation time from 15 min to 60 min, the enantiomeric
excess of the (S)-lavandulol was fallen from 80 % to 58% and (R)-lavandulol was fallen from 66% to 42% when racemic lavandulol was incubated with extracellular lipase and cell bound lipase, respectively. In both the cases, the hydrolysis of the preferred lavandulyl acetate will be complete in one hour of reaction time (Fig. 5A and 5B). The results also confirmed that extracellular and cell bound lipases are different.

4. Conclusions

We have shown the differential induction of lipases in Y. lipolytica NCIM 3639 based on substrate used for the growth. Extracellular cold active lipase was purified which exhibited a native molecular mass of 400 kDa. SDS-PAGE showed the molecular mass corresponding to 20 kDa indicative of the oligomeric nature of a lipase comprising of 20 subunits. Lc-MS-MS mass spectrometric analysis showed about 46% homology to LIP8p lipase from Y. lipolytica. Such cold active multimeric lipase with 20 subunits is not reported so far in the literature. The extracellular and cell bound lipases showed the preference over the opposite antipodes of the irregular monoterpene, lavandulyl acetate. The work is progressing on the effect of solvents, reaction conditions, and substrate specificity of both these novel lipases.

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**Figure captions**

**Fig. 1** Time course of lipase production in Tween 80 and Olive oil containing media. Olive oil biomass (□), olive oil cell bound lipase (●), Tween 80 biomass (○), Tween 80 extracellular lipase (■), Tween 80 cell bound lipase (▲).

**Fig. 2** Effect of Temperature on growth and extracellular lipase production by *Y. lipolytica* NCIM 3639. The strain was grown in SOB basal medium containing 2% Tween 80, incubated at different temperatures with shaking at 150 rpm. Lipase activity was measured after 48 h. Biomass (□), Extracellular lipase (■).

**Fig. 3** Effect of pH on growth and extracellular lipase production by *Y. lipolytica* NCIM 3639. The yeast strain was grown in SOB basal medium containing 2% Tween 80, incubated at various initial pH with shaking at 150 rpm. Lipase activity was measured after 48 h. Biomass (□), Extracellular lipase (■).

**Fig. 4** Molecular weight of lipase by a) SDS- PAGE, Lane 1-purified enzyme, Lane 2-molecular weight markers. b) Gel permeation chromatography, $V_0$ is void volume.

**Fig. 5** Reaction course of the hydrolysis of (±)-lavandulyl acetate by *Yarrowia lipolytica* NCIM 3639 lipases a) extracellular Lipase b) cell bound lipase

Racemic Lavandulyl acetate (▲), R-Lavandulol (■), S-Lavandulol (●).
Table 1. Effect of Tween 80 and olive oil on lipase production by Yarrowia lipolytica

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Biomass (g/l)</th>
<th>Extracellular activity (IU/ml)</th>
<th>Cell-bound activity (IU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80 (0.5%)</td>
<td>7.5 ± 0.4</td>
<td>3.3 ± 0.2</td>
<td>3.4 ± 0.25</td>
</tr>
<tr>
<td>Tween 80 (0.75%)</td>
<td>7.3 ± 0.5</td>
<td>5.0 ± 0.3</td>
<td>4.2 ± 0.30</td>
</tr>
<tr>
<td>Tween 80 (1.0%)</td>
<td>7.4 ± 0.3</td>
<td>8.5 ± 0.6</td>
<td>6.0 ± 0.50</td>
</tr>
<tr>
<td>Tween 80 (2.0%)</td>
<td>10.8 ± 0.6</td>
<td>15.2 ± 0.8</td>
<td>24.0 ± 1.3</td>
</tr>
<tr>
<td>Tween 20 (2.0%)</td>
<td>8.5 ± 0.6</td>
<td>3.0 ± 0.2</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>Triton X-100 (2.0%)</td>
<td>2.0 ± 0.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Olive oil (1.0%)</td>
<td>8.0 ± 0.4</td>
<td>&lt;0.05</td>
<td>101.3 ± 7.5</td>
</tr>
<tr>
<td>Olive oil (1.0%) +</td>
<td>13.3 ± 0.6</td>
<td>2.8 ± 0.2</td>
<td>69.3 ± 4.5</td>
</tr>
<tr>
<td>Tween 80 (2.0%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The culture was grown in SOB basal medium with lipid substrates and incubated for 48 h at 20 °C with shaking at 150 rpm. The values given in the table are the average of three independent experiments. ND – Not detected

Table 2 Summary of steps of purification of extracellular lipase from Y. lipolytica
<table>
<thead>
<tr>
<th>Purification Steps</th>
<th>Total activity (IU)</th>
<th>Total protein (mg)</th>
<th>Specific activity (IU/mg)</th>
<th>Fold Purification</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture broth</td>
<td>7000</td>
<td>500</td>
<td>14</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>6066</td>
<td>125</td>
<td>48.58</td>
<td>3.47</td>
<td>86.6</td>
</tr>
<tr>
<td>Q sepharose</td>
<td>2026</td>
<td>20</td>
<td>101.3</td>
<td>7.23</td>
<td>28.9</td>
</tr>
<tr>
<td>Sepharose CL-4 B</td>
<td>780</td>
<td>4.0</td>
<td>195</td>
<td>13.9</td>
<td>11.14</td>
</tr>
</tbody>
</table>

**Table 3** Effect of metal ions on *Yarrowia lipolytica* NCIM 3639 lipase
<table>
<thead>
<tr>
<th>Compound (10mM)</th>
<th>Concentration</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>5 mM</td>
<td>97</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10 mM</td>
<td>89</td>
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<tr>
<td>MgCl₂</td>
<td>5 mM</td>
<td>87</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>5 mM</td>
<td>71</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>5 mM</td>
<td>78</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 mM</td>
<td>98</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>5 mM</td>
<td>102</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>5 mM</td>
<td>114</td>
</tr>
<tr>
<td>KCl</td>
<td>5 mM</td>
<td>111</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>5 mM</td>
<td>117</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>5 mM</td>
<td>0</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>5 mM</td>
<td>17</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>5 mM</td>
<td>98</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
<td>105</td>
</tr>
</tbody>
</table>
Table 4  Kinetic parameters of the hydrolysis of pNPP by Y. lipolytica lipase as a function of temperature

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Km</th>
<th>Vmax</th>
<th>Kcat</th>
<th>Kcat/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>28.30</td>
<td>0.033</td>
<td>3.3 $\times 10^3$</td>
<td>132.0</td>
</tr>
<tr>
<td>10</td>
<td>27.57</td>
<td>0.05</td>
<td>5.0 $\times 10^3$</td>
<td>175.0</td>
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<tr>
<td>15</td>
<td>22.22</td>
<td>0.066</td>
<td>6.6 $\times 10^3$</td>
<td>297.0</td>
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<tr>
<td>20</td>
<td>21.10</td>
<td>0.1</td>
<td>1.0 $\times 10^4$</td>
<td>473.9</td>
</tr>
<tr>
<td>25</td>
<td>20.40</td>
<td>0.11</td>
<td>1.1 $\times 10^4$</td>
<td>539.2</td>
</tr>
<tr>
<td>30</td>
<td>18.18</td>
<td>0.09</td>
<td>9.0 $\times 10^3$</td>
<td>495.0</td>
</tr>
</tbody>
</table>

Assays were carried out under standard assay conditions as described in methods.

Kinetic parameters are expressed as $K_m = \mu M$, $V_{max} = \mu$moles min$^{-1}$ mg$^{-1}$, $K_{cat} = $ min$^{-1}$, $K_{cat}/K_m = \mu M^{-1}$ min$^{-1}$. Incubation time was 30’. 
Table 5 Lc-ms-ms analysis of *Y. lipolytica* lipase obtained by chemical modification of cysteine residues with dithiothreitol and iodoacetamide and tryptic digestion.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Protein</th>
<th>Observed</th>
<th>Mr(expt)</th>
<th>Mr(calc)</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Yarrowia lipolytica</em></td>
<td>LIPY8p</td>
<td>402.2057</td>
<td>802.3968</td>
<td>802.3974</td>
<td>IHDGFSK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>414.7268</td>
<td>827.4390</td>
<td>827.4905</td>
<td>FINPPLK</td>
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<tr>
<td></td>
<td></td>
<td>549.3047</td>
<td>1096.5948</td>
<td>1096.591</td>
<td>HFPDIELVK</td>
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<tr>
<td></td>
<td></td>
<td>618.7792</td>
<td>1235.5438</td>
<td>1235.5452</td>
<td>DVISCAGGENSK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>682.3275</td>
<td>1362.6404</td>
<td>1362.7296</td>
<td>VGNKPFaEFink</td>
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<tr>
<td></td>
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<td>455.2310</td>
<td>1362.6712</td>
<td>1362.7296</td>
<td>VGNKPFaEFink</td>
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<tr>
<td></td>
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<td>682.3790</td>
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<td>1362.7296</td>
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<tr>
<td></td>
<td></td>
<td>702.3815</td>
<td>1402.7484</td>
<td>1402.7456</td>
<td>TSITGyLAVDHVK</td>
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<tr>
<td></td>
<td></td>
<td>753.3294</td>
<td>1504.6442</td>
<td>1504.7674</td>
<td>GyDPiLInYGQpr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>753.3857</td>
<td>1504.7568</td>
<td>1504.7674</td>
<td>GyDPiLInYGQpr</td>
</tr>
<tr>
<td><em>Candida deformans.</em></td>
<td>Triacylglycerol lipase precursor</td>
<td>402.2057</td>
<td>802.3968</td>
<td>802.3974</td>
<td>GyDPiLInYGQpr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>753.3294</td>
<td>1504.6442</td>
<td>1504.7674</td>
<td>GyDPiLInYGQpr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>753.3857</td>
<td>1504.7568</td>
<td>1504.7674</td>
<td>GyDPiLInYGQpr</td>
</tr>
<tr>
<td>medicinal leech</td>
<td>tryptase inhibitor</td>
<td>421.7533</td>
<td>841.4920</td>
<td>841.5022</td>
<td>VATVSLPR</td>
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<tr>
<td></td>
<td></td>
<td>530.2791</td>
<td>1058.5436</td>
<td>1058.5720</td>
<td>LSSPATLQSR</td>
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<tr>
<td><em>Burkholderia ambifaria</em></td>
<td>Peptidase M50 precursor</td>
<td>428.7619</td>
<td>855.5092</td>
<td>855.5178</td>
<td>ITAVLSPR</td>
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<tr>
<td><em>Rhodopseudomonas palustris.</em></td>
<td>transcriptional regulator</td>
<td>428.7619</td>
<td>855.5092</td>
<td>855.4926</td>
<td>IDRLSPR</td>
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<tr>
<td><em>Myxococcus xanthus.</em></td>
<td>Hypothetical protein</td>
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<td>855.5092</td>
<td>855.4814</td>
<td>VEGVLSPR</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>regulatory protein trpI</td>
<td>428.7619</td>
<td>855.5092</td>
<td>855.4637</td>
<td>MGPVLSPR</td>
</tr>
<tr>
<td><em>Geobacter uraniumreducens</em></td>
<td>Helix-turn-helix</td>
<td>428.7619</td>
<td>855.5092</td>
<td>855.4749</td>
<td>MPRLSPR</td>
</tr>
<tr>
<td><em>Tetrahymena thermophila</em></td>
<td>Cyclic nucleotide-binding domain containing protein</td>
<td>435.7726</td>
<td>869.5306</td>
<td>869.4971</td>
<td>K.TPISLSPR</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2
Fig. 3
Fig. 4a

Fig. 4b
Fig 5a

Fig 5b

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