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An extracellular solvent stable alkaline lipase from *Pseudomonas* sp. DMVR46: Partial purification, characterization and application in non-aqueous environment

Vrutika Patel, Shruti Nambiar, Datta Madamwar*

BRD School of Biosciences, Sardar Patel University, Vadtal Road, Satellite Campus, Post Box # 39, Vallabh Vidhyanagar 388 120, Gujarat, India

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ABSTRACT

The biosynthesis of esters is currently of much commercial interest because of the increasing popularity and demand for natural products among consumers. Biotransformation and enzymatic methods of ester synthesis are more effective when performed in non-aqueous media. In present study, an organic solvent stable *Pseudomonas* sp. DMVR46 lipase was partially purified by acetone precipitation and ion exchange chromatography with 28.95-fold purification. The molecular mass of the lipase was found to be ~32 kDa. The partially purified lipase was optimally active at 37 °C and pH 8.5. The enzyme showed greater stability toward organic solvents such as isooctane, cyclohexane and n-hexane retaining more than 70% of its initial activity. The metal ions such as Ca^{2+}, Ba^{2+} and Mg^{2+} had stimulatory effects on lipase activity, whereas Co^{2+} and Zn^{2+} strongly inhibited the activity. Also lipase exhibited variable specificity/hydrolytic activity toward different 4-nitrophenyl esters. DMVR46 lipase was further immobilized into AOT-based organogels used for the synthesis of flavor ester pentyl valerate in presence of organic solvents. The organogels showed repeated use of enzyme with meager loss of activity even upto 10 cycles. The solvent-stable lipase DMVR46 thus proved to be an efficient catalyst showing an attractive potency for application in biocatalysis under non-aqueous environment.

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1. Introduction

Lipases known as triacylglycerol acylhydrolases (E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial prospective. Lipases catalyze the hydrolysis of triacylglycerols to glycerols and free fatty acids. Compared to esterases, lipases are activated only when adsorbed to an oil–water interface [1] and do not hydrolyze dissolved substrates in bulk fluid. Besides, lipases accept a wide range of substrates and are quite stable in non-aqueous solvents, thus they are frequently used for the synthesis of enantiopure compounds for industrial applications [2,3].

Though solvents are extremely lethal to microorganisms, there are numerous advantages of conducting enzymatic conversions in organic solvents such as (i) shifting of thermodynamics equilibrium in favor of synthesis, (ii) regiospecificity and stereoselectivity, (iii) enabling use of hydrophobic substrate, (iv) controlling substrate specificity and (v) thermal stability of enzymes and relative use of product recovery [4]. Solvent stable lipases are obligatory in biotechnological applications, specifically in the production of chiral compounds, high value pharmaceutical substances, modifications of fats and oils, synthesis of flavor esters and food additives, production of biodegradable polymers, biodiesel and in synthesis of fine chemicals [5]. Organic solvents offer advantages on enzyme activity such as synthesis of esters from their constituent acids and alcohol, enzyme stability and possess "pH memory" i.e. catalytic activity of enzyme reflects pH of last solution to which it was exposed [6,7]. So far, the number of solvent tolerant bacterial lipases is inadequate [8], thus it becomes crucial to explore novel lipases with high activity in organic solvents to expand their application in practical catalysis.

Substantial efforts are made to exploit new species of microorganisms that secretes solvent-stable lipase. Baharum et al. [9] reported solvent tolerant lipase produced by *Pseudomonas* sp. strain S5 that was stable in organic solvents such as n-hexane, cyclohexane, toluene and 1-octanol. Rahman et al. [10] described organic solvent tolerant lipase which was not only stable in n-hexane but its activity was stimulated in presence of n-hexane. Cao et al. [8]

* Corresponding author. Tel.: +91 2692 229380; fax: +91 2692 236475; mobile: +91 98256 86025.
E-mail addresses: vrutikaptl.19@yahoo.com (V. Patel), datta_madamwar@yahoo.com (D. Madamwar).

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studied characterization of an organic solvent stable lipase purified from *Pseudomonas stutzeri* LC2-8 and showed its application for efficient resolution of (R, S)-1-phenylethanol. Dandvate et al. [11] purified a solvent tolerant lipase from *Burkholderia multivorans* V2 and showed its application for the synthesis of ethyl butyrate under non-aqueous environment. Conversely, the stability and reusability of the enzyme are of major concern in non-aqueous enzymatic synthesis. The conceivable answer to this problem is recovery and reuse by immobilizing in/on a solid support which makes it cost effective [12,13].

Lipase immobilization is known to allow easier product recovery, flexibility of reactor design and, in some cases, enhanced storage and operational, thermal and conformational stability [14]. Variety of methods has been used for immobilization of biocatalyst such as adsorption, covalent attachment and entrapment in polymer gels, microencapsulation [15] and sol–gel entrapment [16]. One of the well explored methods for immobilization in non-aqueous biocatalysis is encapsulation of enzyme in microemulsion based organogels (MBGs). The arrangement of MBGs consists of solid network of gelatin/water rods stabilized by monolayer of surfactant, in co-existence with a population of conventional water-in-oil microemulsion droplets. Thus, enzyme immobilized in MBGs has considerable advantages in organic media as direct exposure of enzyme to organic solvent is nullified [17,18].

In this paper, we report production, partial purification and characterization of solvent tolerant lipase produced from solvent stable *Pseudomonas* sp. DMVR46 isolated from oil contaminated soil. There are scanty reports for the production of pentyl valerate ester immobilized in MBGs from purified lipase. Thus, we intend to use this lipase for esterification of pentanol and valeric acid to produce pentyl valerate, a compound with fruity aroma used in industries. The main purpose for the study was exploitation of DMVR46 lipase in non-aqueous environment and its reusability.

2. Materials and methods

2.1. Chemicals

DEAE-cellulose and gelatin were procured from Sigma–Aldrich (Germany). Tributyrin oil and bovine serum albumin (BSA) were obtained from HiMedia (India). All p-nitrophenyl esters were purchased from Sigma–Aldrich (Germany). Sodium bis-2-(ethylhexyl) sulfosuccinate (AOT), valeric acid, pentanol and pentyl valerate were obtained from Fluka (Switzerland). All other solvents (methanol, butanol, iso-propanol, ethanol, acetone, cyclohexane, iso-octane, chloroform) used during the experiment were of HPLC/GC grade.

2.2. Screening of organic solvent tolerant lipolytic microorganism

Soil samples were collected from various oil spilling sites near industrial area of Kadi, Ahmedabad, Gujarat, India. Solvent tolerance was determined by plate overlay method as described by Ogino et al. [19]. Five microliters of overnight grown cultures was transferred to tributyrin agar plates (1% (m/v) tributyrin oil, 0.3% (m/v) yeast extract and 0.5% (m/v) peptone extract, 1.5% (m/v) agar–agar). The plates were kept for 20 min until the drops get dry followed by flooding with 18 mL of different solvents like iso-octane, cyclohexane, toluene, isopropanol, methanol, DMSO. Colonies were examined for solvent tolerance after incubation at 37 °C for 24 h. The ability of the cultures to grow and produce lipase in presence of solvents was observed.

2.3. Identification of solvent stable lipase producing culture using 16S rRNA approach

Bacterial strain designated as DMVR46 was selected on the basis of its solvent tolerance and was identified using 16S rRNA gene sequencing. Genomic DNA was extracted using protocol standardized by Asubel et al. [20] The genomic DNA of DMVR46 was used as template of PCR reaction (30 μL) using universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The amplification of 16S rRNA gene was done in BioRad PCR cycler (Biorad iCycler version 4.006, Biorad, U.S.A.). Each PCR cycle (total 35 cycles) consisted of 1 min denaturation step at 94 °C, followed by 1 min annealing step at 55 °C and 1 min elongation step at 72 °C, with an initial denaturation step at 94 °C for 5 min and a final extension step at 72 °C for 15 min. PCR products were resolved on 1.2% low melting agarose gel in 1× TAE buffer and was visualized with ethidium bromide staining in Gel Documentation (Alpha-Inotech, U.S.A.). The amplified PCR product was subjected to sequencing by automated DNA Analyzer 3730 using ABI PRISM® BigDye™ cycle sequencing kit (Applied Biosystems, USA). The nearly complete sequence (>95%) was submitted to Genbank at NCBI. BLAST (n) program at NCBI server was used to identify and download nearest neighbor sequence from BLAST database [20].

2.4. Optimization of process parameters for maximum lipase production

Optimization of process parameters for lipase production from isolate DMVR46 was aimed to evaluate the effect of a single parameter at a time, and later manifesting it as standardized condition before optimizing the next parameter. The isolate DMVR46 was cultured in 100 mL medium containing 0.5% (m/v) peptone, 0.3% (m/v) yeast extract and 1% (v/v) tributyrin oil with pH 7.0 and incubated at different temperature (30 °C, 37 °C, 40 °C, 45 °C and 50 °C) to determine the effect of temperature. Similarly, to test the effect of initial pH the culture was investigated at varying pH ranging from 6.0 to 11.0 incubated at 37 °C. The standardized temperature 37 °C and pH 8.0 were used in each step for optimizing the effect of additional inducers, nitrogen sources and carbon sources. The effect of different inducers for lipase production was studied by addition of cotton seed oil, soybean oil, sunflower oil, tributyrin oil, maize oil, olive oil and groundnut oil (each at initial concentration of 1% (v/v). Nitrogen sources such as peptone, yeast extract, tryptone, casein, urea, ammonium sulphate and ammonium nitrate were used to observe the influence of nitrogen sources (1%, m/v) on lipase production. To test the influence of additional carbon sources, media were supplemented with dextrose, lactose and sucrose at 1% (m/v). The best carbon, nitrogen and inducer sources obtained were further tested for varied concentration of 0.2%, 0.5%, 1%. 1.5% and 2%. All experiments were performed in triplicates and the results shown are the average of three independent experiments. Data are represented as mean with standard deviation.

2.5. Growth profile for lipase production

A Erlenmeyer flask of 500 mL capacity containing 200 mL of production medium (0.3% (m/v) yeast extract, 0.5% (m/v) peptone as a basal medium and 1.5% (m/v) tryptone, 0.5% (m/v) dextrose and 1% (m/v) cotton seed oil) was inoculated with an overnight grown culture of DMVR46 to obtain an initial culture density (A_{600}) 0.05 and incubated at 37 °C in an orbital shaker (150 rpm). The samples were withdrawn at regular intervals of 24 h and analyzed for cell growth and enzyme activity. The enzyme activity was estimated

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from supernatant (crude lipase) obtained upon centrifugation and cell growth was observed by suspending pellet in distilled water.

2.6. Partial purification of lipase

Solvent tolerant lipase from DMVR46 was purified in two sequential steps. *Step 1*: The culture supernatant was obtained by centrifugation at 10,000 × *g* (Kubota, Model 6500 Japan) for 20 min followed by precipitation using acetone as solvent. Chilled acetone was added slowly to the culture supernatant of strain DMVR46 upto 60% (v/v) concentration with continuous stirring and kept at −20 °C for O/N to allow protein precipitation. The precipitates were harvested by centrifugation at 10,000 × *g* for 20 min at 4 °C and resuspended in 50 mM sodium phosphate buffer (pH 8.0). *Step 2*: The enzyme containing solution was applied to a DEAE-cellulose column previously equilibrated with 50 mM sodium phosphate buffer (pH 8.0). The flow rate was adjusted to 15 mL/h (approx.) having the fraction volume of 1 mL and each fraction was further assayed for enzyme activity and protein content. The lipase preparations (crude and purified fractions) were electrophoresed on SDS-PAGE as described by Laemmli [21]. The protein bands on the gel were visualized upon silver staining.

2.7. Characterization of purified lipase

2.7.1. Effect of pH and temperature on lipase activity and thermal-stability of lipase

Optimum pH of the solvent tolerant lipase was determined by measuring the enzyme activity over a pH value ranging from 6.0 to 10.0 at 37 °C for 30 min. Different buffers used for determination of lipase activity were: pH 6.0–8.5 sodium phosphate buffer, pH 9.0–10.0 glycine–NaOH buffer.

The effect of temperature on the lipase was studied by carrying out the enzymatic reactions at different temperature in the range of 30–50 °C at pH 8.5 (50 mM sodium phosphate buffer). The reactions were carried out for 0–30 min and proceed for enzyme activity. The thermal-stability of the lipase was assayed at various temperatures ranging from 30 to 50 °C for different time interval from 0 to 4 h. At each time interval, 1 mL sample was pipette out and then assayed for residual activity, which was expressed as percentage of initial activity.

2.7.2. Effects of metal ions, inhibitors and surfactants on the lipase activity

The effects of different metal ions (Ca^{2+}, Mg^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+} and Ba^{2+}) and inhibitors (EDTA and β-mercaptoethanol) were investigated by pre incubating the purified lipase with 10 mM solutions of these ions or inhibitors at pH 8.5 for 30 min at 37 °C. Similarly, the effects of surfactants (SDS, CTAB, Triton X-100, Tween 20 and Tween 80) at the concentration of 0.5% were investigated.

2.7.3. Stability of lipase DMVR46 in different organic solvents

The effects of different organic solvents on the activity and the stability of the purified lipase were investigated following method defined by Ogino et al. [19]. The stability of lipase in organic solvents was investigated by appropriately mixing 3 mL of purified enzyme and 1 mL of solvent in crew cap vials to obtain a final solvent concentration of 25% (v/v). The solution was incubated in shaker (150 rpm) at 37 °C for 0–4 h and lipase activity was assayed in the aqueous phase.

2.7.4. Substrate specificity to various p-nitrophenyl esters

The substrates, p-nitrophenyl fatty acid esters, of varying chain length (C2, C4, C8, C12, C16 and C18) were used at the final concentration of 0.3 mg/mL and the lipase activity was measured according to the pNPP method [22].

2.8. Synthesis of pentyl valerate under water restricted environment using free and immobilized DMVR46 lipase

Pentyl valerate was synthesized by the condensation of pentanol and valeric acid using free as well as immobilized lipase as described by Dandavate et al. [23]. For immobilization, the partially purified lipase was first entrapped in a surfactant solution comprising of 0.1 M sodium bis (2-ethylhexyl) sulfosuccinate (AOT) with Wo = 60 in isooctane by vigorously mixing the two solutions to create a clear reverse micellar system of AOT/50 mM phosphate buffer (pH 8.5) – lipase/isooctane (1 mL). The amount of purified lipase used for immobilization was 108 μL having specific activity of 64.57 U/mg. This system was then polymerized by the addition of 14% gelatin (1.5 mL) maintained at 55 °C followed by vigorous mixing to obtain homogenous AOT based organogel (MBGs). The gel was poured into plastic Petri plates, dried overnight, and cut into small pieces (1 cm × 1 cm with 110 ± 10 μm thickness). The reaction mixture consisted of 20 mL cyclohexane and equimolar concentrations (100 mM) of pentanol and valeric acid. The esterification reaction was initiated by addition of free lipase and the pieces of MBGs to the reaction mixture in glass-stoppered flasks kept on orbital shaker at 37 °C and 150 rpm. At every 24 h interval 100 μL of the reaction mixture was withdrawn and analyzed by gas chromatograph.

2.9. Reusability studies

For reusability studies, upon completion of each cycle for ester production the immobilized enzymes was recovered by washing with neat cyclohexane twice for removal of unwanted substrate or product formed, air dried and reused for next cycle of esterification. This procedure was repeated for several cycles.

2.10. Analytical procedures

2.10.1. Lipase assay

The pH stat method: Lipase activity was measured under controlled temperature using a pH 718 STAT Titrino Titrator (Metrohm, Switzerland). The buffer used for the activity determination comprised of 10% (m/v) Gum Arabic, 0.2% (m/v) bile salt, and 20% (m/v) $CaCl_2$, 5% (v/v) cotton oil as substrate and 0.1 M NaOH as the titrant. The insoluble triglycerides were dispersed by vigorous stirring. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of fatty acid/min at 37 °C [24].

In order to check hydrolytic activity of lipase exclusively for different p-nitrophenyl esters lipase activity was measured with spectrophotometric method using p-nitrophenyl esters as substrate [22]. The substrate with a final concentration of 0.3 mg/mL was dissolved in 1 mL of isopropanol and mixed with 9 mL of 50 mM sodium phosphate buffer (pH 8.5) containing gum Arabic (0.1%) and Triton X-100 (0.6%). The reaction mixture was composed of 240 μL of substrate solution and 10 μL of appropriately diluted enzyme solution, and incubated at 37 °C for 10 min. The p-nitrophenol (p-NP) produced in the reaction mixture was quantified spectrophotometrically at 410 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol p-nitrophenol/min under standard assay conditions.

2.10.2. Protein assay

Soluble protein was estimated by Lowry's method [25] using bovine serum albumin as standard.

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2.10.3. Quantification of ester production by gas chromatography

Quantification of accumulated ester was done by using gas chromatograph using FID as detector described by Raghavendra et al. [26]. After initiation of esterification, 100 μL sample was periodically collected and analyzed by gas chromatograph (Perkin Elmer, Model Clarus 500, USA) equipped with the flame ionization detector and a 30 mRtx-R-20 (cross bond 80% dimethyl-20% diphenyl polysiloxane) capillary column. Nitrogen served as a carrier gas at a split flow rate of 90 mL/min. The injector and detector temperatures were 250 and 280 °C respectively and oven temperature was programmed to increase from 100 to 160 °C at the rate of 20 °C/min, from 160 to 280 °C at the rate of 2 °C/min and from 165 to 175 °C at the rate of 1 °C/min. Ester identification and quantification was done by comparing the retention time and peak area of the sample with a standard. Pure pentyl valerate (>98%) was used as external standard.

3. Results and discussion

3.1. Isolation and identification of solvent tolerant lipase producing bacteria

Lipases are inducible enzymes that are produced in the presence of lipids such as oils, triaclyglycerols, fatty acids and glycerols in addition to carbon sources [27]. Organic solvents are often lethal to microorganisms and thus can denature or inactivate enzymes. Therefore, we assumed that organic solvent tolerant bacteria are ought in producing enzyme that can tolerate the toxic effect of organic solvent to a certain degree and will be very useful in industry. In the present study, we have screened solvent tolerant lipase explored by its ability to grow on tributyrin agar plates flooded with different solvents. Out of all isolates showing clearance zone on tributyrin agar plate, the isolate DMVR46 showed the highest lipase activity (9 U/mL), and hence was used for further studies. Organic solvent tolerant lipase produced by strain DMVR46 was identified as *Pseudomonas* sp. on the basis of 16 S rRNA gene sequencing followed by BLAST analysis which showed sequence homology (100%) with the complete 16S rRNA gene sequence of *Pseudomonas* sp. The sequence of strain DMVR46 was deposited in NCBI with an Accession number KF636492. Phylogenetic affiliation of isolate DMVR46 with related *Pseudomonas* sp. is shown in Fig. 1.

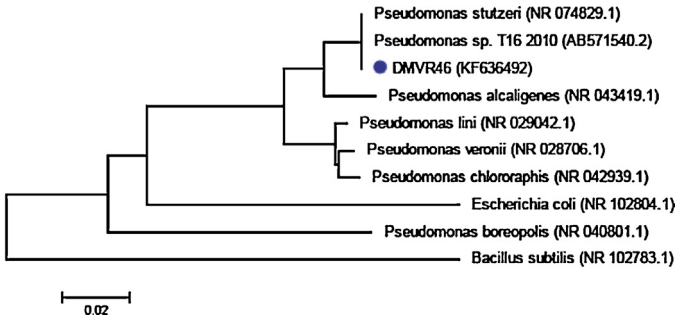

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence of *Pseudomonas* sp. DMVR46. (GenBank accession No. KF636492) and sequence of closest phylogenetic neighbors obtained by NCBI BLAST (n) analysis, numbers in the parenthesis indicate accession numbers of corresponding sequence. The tree was constructed using MEGA 5.0 software. *Bacillus subtilis* strain NR 102783 has been taken as an out-group.

3.2. Growth profile for lipase production

Various physico-chemical parameters were scrutinized for maximum lipase production. The optimum temperature and pH for initial lipase activity was found to be 37 °C and pH 8.0 (Fig. 2(a) and (b)). Cao et al. [8] showed maximum lipase production at pH 8.0 and at 30 °C from *P. stutzeri* LC2-8. Among various inducers tested for lipase production, cotton seed oil (1% (v/v)) with an activity of 48 U/mL (Fig. 2(c)) was the best inducer followed by soya bean and maize oil. The best lipase production (75.54 U/mL lipase activity) was obtained with 1.5% tryptone (Fig. 2(d)) as organic nitrogen supplement. However, all the inorganic nitrogen sources resulted in the reduction of bacterial growth as well as lipase production. Also, as shown in Fig. 2(e) cotton oil used at the concentration of 1% (v/v) and tryptone with concentration of 1.5% (m/v) stimulated lipase activity. Using optimized conditions the maximum lipase activity of 79.54 U/mL was observed on 2nd day with cell mass of 16.571 (including dilution factor) absorbance at 600 nm (assay done using pH STAT method [24]). On 3rd day decline in lipase activity as well as growth was observed (Fig. 3). The decrease of lipase production and growth of the bacterial cells at the later stage could be possibly due to pH inactivation, proteolysis, or both [28].

3.3. Purification of solvent tolerant lipase DMVR46

The extracellular lipase secreted by DMVR46 was purified by acetone precipitation and DEAE-cellulose anion-exchange chromatography. About 28.95 fold purification with 29.74% recovery was achieved (Table 1). The purified lipase showed a single band on SDS-PAGE (Fig. 4). The purified lipase was homogenous and its molecular mass was estimated to be ~32.0 kDa.

Cao et al. [8] reported purification of lipase from *P. stutzeri* by acetone precipitation with 32.8% recovery. Ogino et al. [19] reported purify cation of *Pseudomonas aeruginosa* LST-03 lipase by ion-exchange and hydrophobic interaction chromatography achieving 34.7-fold purification and 12.6% yield. Rahman et al. [10] succeeded in getting higher recovery but they employed affinity chromatography in combination with ion-exchange chromatography. *P. aeruginosa* PseA lipase was purified by ultrafiltration and gel exclusion chromatography with 8.6 fold purification and 51.6% recovery [29]. Meanwhile, *Pseudomonas mendocina* PK-12CS lipase was purified to 240 fold with 14.8% recovery using acetone precipitation and anion exchange chromatography [30].

3.4. Characterization of purified lipase

3.4.1. Effect of pH and temperature on activity and stability of the lipase

The optimum pH for purified lipase was found to be 8.5 (Fig. 5) and it retained 100% of its maximum activity, while remarkable drop was obtained below pH 8.0 and above pH 9.0 indicating alkaline nature of enzyme. Gilbert et al. [31] reported *P. aeruginosa* EF2 lipase with a maximum activity in the range of pH 8.5–9.0.

The optimum temperature for lipase activity was observed to be 37 °C as indicated in Fig. 6(a). Whereas, Cao et al. [8] showed lipase from *P. stutzeri* LC2-8 having optimum activity at 30 °C and organic solvent stable *P. aeruginosa* LST-03 lipase screened by Ogino

Table 1
Purification of lipase produced by *Pseudomonas* sp. DMVR46.

Purification steps	Total activity (Units)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude enzyme	3951	1764.5	2.23	1	100
Acetone precipitation	2780.4	112.5	24.71	11.08	70.37
DEAE-cellulose	1175.3	18.2	64.57	28.95	29.74

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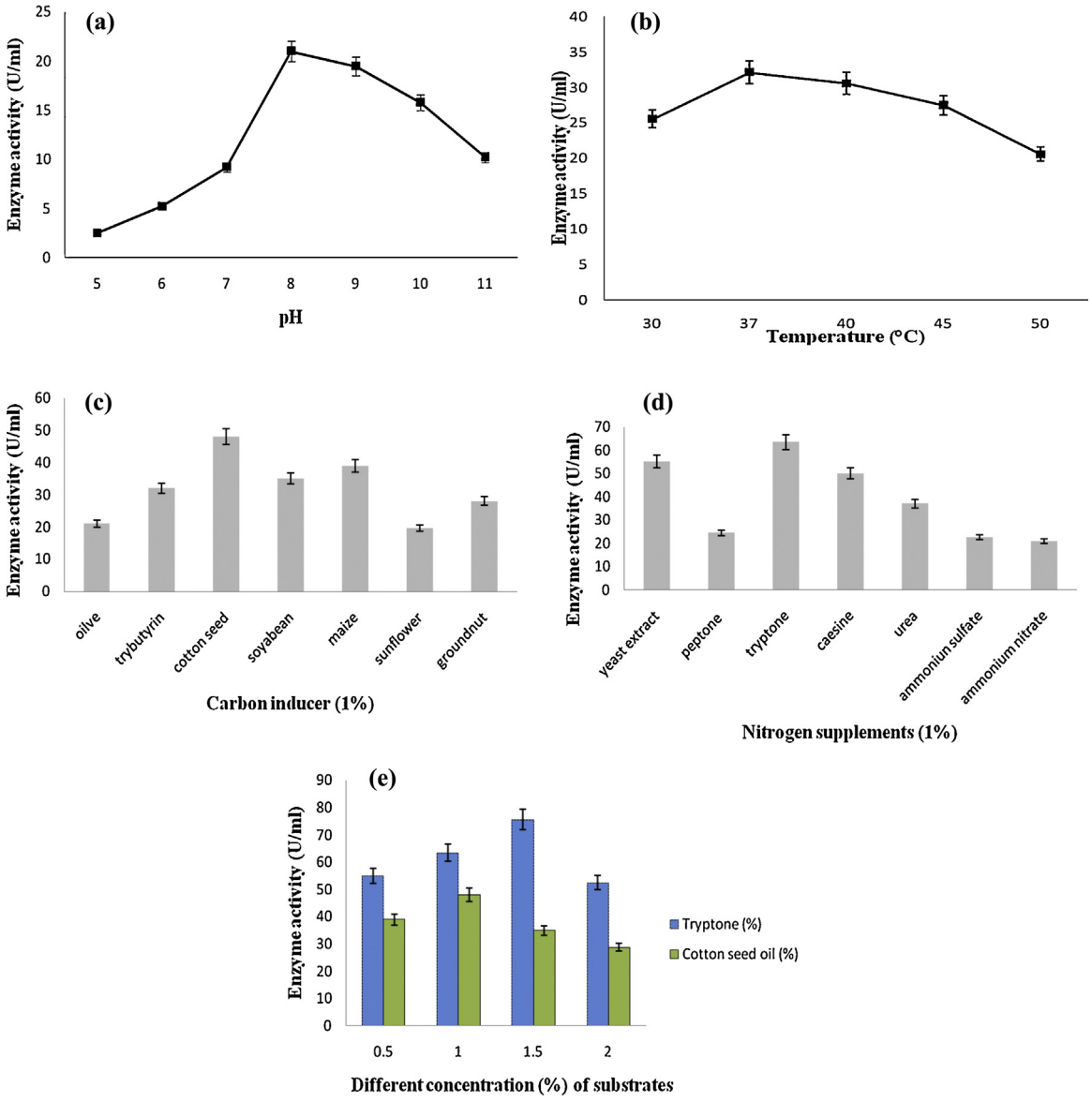

Fig. 2. Effect of (a) pH, (b) temperature, (c) carbon inducer, (d) nitrogen supplements, (e) different substrate i.e. tryptone (%) and cotton seed oil (%) concentration on lipase production by DMVR46.

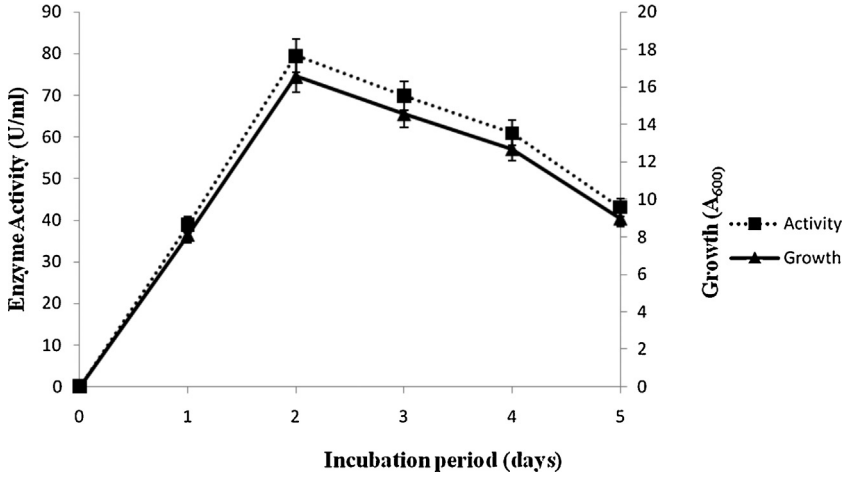

Fig. 3. Growth profile of lipase produced by *Pseudomonas* sp. DMVR46. The production was carried out at 37 °C under shaking condition (150 rpm) in presence of cotton seed oil with pH 8.

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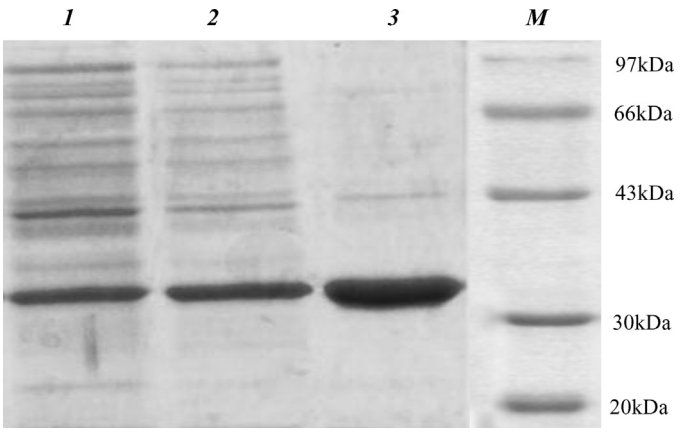

Fig. 4. SDS-PAGE of lipase DMVR46 at different stages of purification. Lane M: protein marker; Lane 1: supernatant; Lane 2: lipase concentrated by acetone precipitation; Lane 3: lipase purified by DEAE-cellulose.

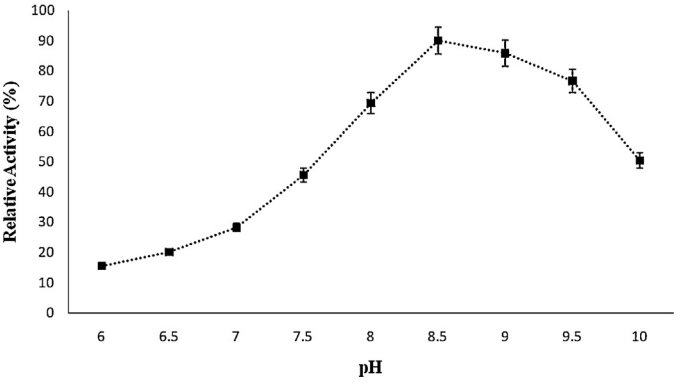

Fig. 5. Effect of pH on activity of lipase obtained from purified *Pseudomonas* sp. DMVR46. The enzyme was added to various buffer systems (pH 6.0–10.0) of 50 mM at 37 °C for 30 min. The buffer systems used were pH 6.0–8.5 sodium phosphate buffer and pH 9.0–10.0 glycine–NaOH buffer.

et al. [19] showed maximum activity at 37 °C. The trend for thermal stability of purified lipase on different temperature is shown in Fig. 6(b). Lipase DMVR46 retained approximately 68% of its initial activity at 37 °C when incubated for 4 h. With increase in temperature, at 40 °C enzyme retained only 28% of initial activity, while at 50 °C enzyme was drastically inactivated as shown in Fig. 6(b). Similar trends of reports were seen in organic solvent-stable LST-03 lipase having maximal activity at 37 °C as reported by Ogino et al. [19]. Thermal stability profile of PseA purified by Gaur et al. [29] was found to be stable at 40 °C upto 4 h. Sharma et al. [32] reported that *Pseudomonas* sp. AG-8 lipase has optimal activity at 45 °C. In contrast, organic solvent stable LST-03 lipase showed a lower stability (below 40 °C for 10 min) then DMVR46 lipase.

3.4.2. Substrate specificity

Substrate specificity of lipases may be attributed to alterations in the geometry and dimensions of their active sites [33]. The substrate specificity of purified lipase was determined by testing the lipolytic activities against p-nitrophenyl fatty acid esters according to the spectrophotometric method [22]. The trend for hydrolytic activity of enzyme toward substrate is evident from Fig. 7. The result indicates higher hydrolytic activity for long chain substrate (p-nitrophenyl palmitate) p-NPP; this can be explained in the terms of lipase structure. Lipases exist in two forms, closed (inactive) and open (active) forms. *Pseudomonas* sp. lipase has been characterized of lacking interfacial activation and 'lid' in contrast to most of other lipases [34]. The CMC (critical micelle concentration) values for

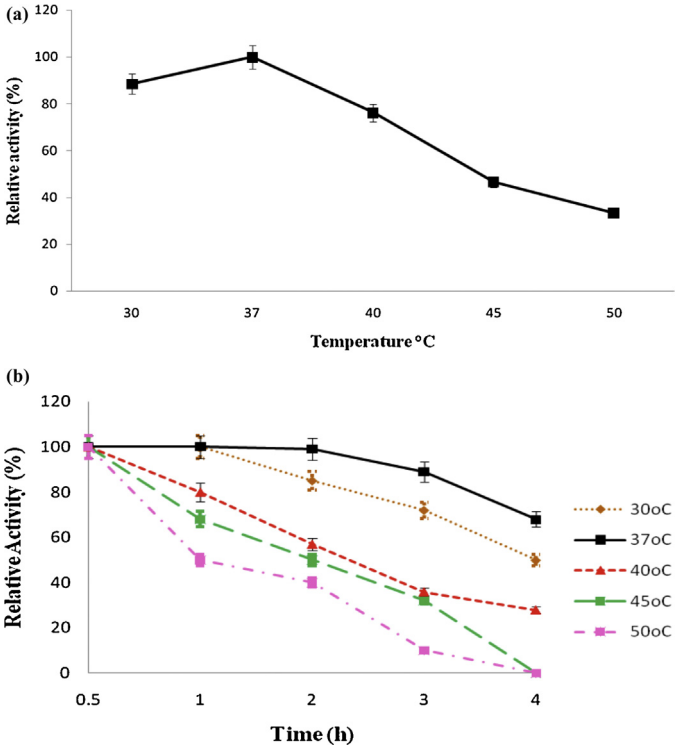

Fig. 6. (a) Temperature profile of purified DMVR46 lipase. The effect of temperature on lipase activity was studied by carrying out the enzyme reaction at different temperature in the range of 30–50 °C at pH 8.5 using sodium phosphate buffer (50 mM). The reaction was carried out for 4 h. (b) Thermal stability of purified lipase was measured by incubating the lipase in 50 mM sodium phosphate buffer (pH 8.5) at 30 °C, 37 °C, 40 °C, 45 °C and 50 °C for 4 h. Residual lipase activity (%) was calculated relative to the initial activity.

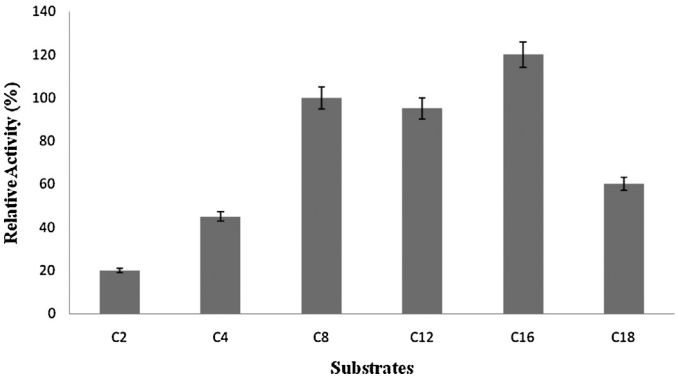

Fig. 7. Substrate specificity of purified DMVR46 lipase against various fatty acid esters. The activity of lipase toward different p-nitrophenyl esters were determined and calculated relative to the maximum activity measured toward p-nitrophenyl caprylate (taken as 100%). The composition of the reaction mixture was 0.1 mg/mL of lipase and 0.3 mg/mL of synthetic pNP esters (where C2 = p-nitrophenyl acetate, C4 = p-nitrophenyl butyrate, C8 = p-nitrophenyl caprylate, C12 = p-nitrophenyl laurate, C16 = p-nitrophenyl palmitate, C18 = p-nitrophenyl sterate).

different substrates were found to be (p-nitrophenyl butyrate) p-NPB 200 μM, (p-nitrophenyl caprylate) p-NPC 5 μM, (p-nitrophenyl laurate) p-NPL 100 μM, p-NPP 50 μM and (p-nitrophenyl stearate) p-NPS 20 μM. Thus, from the results it could be concluded that i) no interfacial activation was noted with short and moderate chain substrates, while activation was observed at point using substrate of long chain (p-NPP) and ii) the lack of interfacial activation of lipases could be caused not only by the structural features of the enzyme but also by very low CMC values of the substrate [34–36]. Generally, lipases from *Pseudomonas* sp. prefer

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Table 2
Effect of various metal ions, inhibitors and surfactants on DMVR46[a] lipase activity at pH 8.5 and 37 °C.

Metal ions	Relative lipase activity (%)
Control[b]	100
Ca^{2+}	135.1
Mg^{2+}	119.4
Fe^{2+}	98.3
Co^{2+}	7.7
Zn^{2+}	3.6
Ba^{2+}	110.3
EDTA	40.44
2-Mercaptoethanol	98.89
PMSF	95.7
Iodoacetate	36.12
SDS*	80.65
Triton X-100*	137.5
Tween 20*	92.49
Tween 80*	96.39
CTAB*	12.5

[a] Lipase DMVR46 was incubated with various metals, inhibitors (10 mM) and detergents (0.5%) with the superscript * in 50 mM sodium phosphate buffer (pH 8.5) at 37 °C for 30 min.
[b] Lipase activity is shown as value relative to the initial activity without addition of effectors (control).

small or medium chain fatty acids triglycerides or their methyl esters [19,37,38]. However, lipase from *P. aeruginosa* AAU2 [39], *Pseudomonas alcaligenes* EF2 [31], *Pseudomonas* sp. S5 [10] and *Pseudomonas cepacia* [33] shows specificity for longer chain fatty acids substrates.

3.4.3. Effects of metal ions, inhibitors and surfactants on lipase activity

Among the tested metal ions Ca^{2+}, Mg^{2+} and Ba^{2+} significantly stimulate activity while Zn^{2+} and Co^{2+} strongly inhibit lipase activity (Table 2). Many lipases have been found to display enhanced activity in the presence of Ca^{2+} [10,11,29,31]. The presence of Ca^{2+}, Ba^{2+} and Mg^{2+} increase lipase activity this may be due to binding of metal complex to the active site of the enzyme which leads to the conformational changes in protein [10]. Alternatively, Ca^{2+} might complex with fatty acids produced during catalysis eliminating the possibility of product inhibition [40]. The chelating agent EDTA significantly reduce the lipase activity suggesting that purified lipase may be a metalloenzyme, whereas in presence of disulphide reducing agent β-mercaptoethanol slight reduction in activity was observed. PMSF, a serine inhibitor has a marginal effect on DMVR46 lipase at 10 mM concentration exhibiting 95% of relative lipase activity. This may be attributed to the fact that the catalytic serine residue is inaccessible owing to the presence of lid covering the active site which is a characteristic feature for most of the lipases [8,29,39].

Surfactants facilitate access of substrate to the enzyme by reducing the interfacial tension between oil and water increasing the lipid–water interfacial area where catalytic reactions takes place [41]. Non-ionic detergent Triton X-100 stimulated lipase activity (Table 2). Whereas, Tween 80 has modest influence on lipase activity by reducing it, while anionic surfactant sodium dodecyl sulphate (SDS) showed 20% decrease in activity. Cationic surfactant (CTAB) completely inactivated the enzyme, as CTAB has been thought to destroy the conformation of lipase [42].

3.4.4. Stability of lipase in organic solvents

High activity and stability of lipases in organic solvents is considered as novel attributes. Effects of different organic solvents (25%) on the stability of *Pseudomonas* sp. DMVR46 lipase are shown in Table 3. The enzyme was found to be quite stable and active in most of the organic solvents. The highest stability was achieved in isooctane, cyclohexane and n-hexane with the relative lipase activity of 120%, 80% and 78% respectively after 4 h. The activation of lipase could be explained by the interaction of organic solvents with hydrophobic amino residues present in the lid that covers the catalytic site of the enzyme, thereby maintaining the lipase in its open conformation [43]. When organic solvents such as toluene, butanol and chloroform was added to purified lipase solution and incubated for 1 h, the enzyme got drastically inactivated. The possible reason for this may be the incubation of enzyme in polar solvents (Log *P* values <3.0), remove water molecules necessary for its catalytic function which leads to decrease in activity of enzyme. The results suggested DMVR46 lipase exhibited fairly good stability, retaining more than 70% of its activity in presence of organic solvents with Log *P* value of 3.00 or higher. The reasonably high stability of DMVR46 lipase in organic solvents makes it potentially useful for practical application in many synthetic reactions in non-conventional media.

Table 3
Organic solvent stability of *Pseudomonas* sp. DMVR46 lipase at pH 8.5 and 37 °C.

Organic solvents (25%)	Log *P*	Relative lipase activity (%)
Control	–	100
Methanol	−0.76	8.90
Ethanol	−0.24	30.5
Butanol	0.89	0
Iso-propanol	0.28	40.8
Toluene	2.64	0
Acetone	−0.23	45.23
Iso-octane	4.7	120.41
Cyclohexane	3.2	80.52
n-Hexane	3.5	78.98
Chloroform	2.0	0

Note: Data are means of triplicate determinations. The purified enzyme and organic solvents were mixed in a 3:1 ratio, and the mixture was incubated at 37 °C with shaking at 150 rpm for 4 h and assayed for lipase activity. Activities of DMVR46 in presence of various organic solvents are shown as a value relative to those in the absence of organic solvent (control).

3.5. Kinetic study

The maximum specific activities (V_{max}) and Michaelis constants (K_m) for free and immobilized lipase were estimated from double reciprocal plots of thee initial rates of pNPP hydrolysis. The K_m of immobilized lipase (0.45 mM) was ~3-folds lower than that of free lipase (1.437 mM), while the V_{max} of immobilized lipase (257 μmol/mg/min) was ~9-folds higher than that of free lipase (25.18 μmol/mg/min). The lower K_m value indicated the higher affinity of enzyme for substrate and higher V_{max} value indicates higher activity of enzyme. The immobilized enzyme was found to be 35 times more efficient catalyst for pNPP hydrolysis in comparison to that of free lipase. Immobilization of lipase in organogels induces some structural changes, which probably is responsible for enhancing its esterification activity. The changes in parameters suggest that immobilization of lipase resulted in an increased affinity for the substrate by improving accessibility of the active site [23].

The effect of temperature on affinity of free and immobilized enzyme can be seen in Arrhenius plot (Fig. 8) The free and immobilized lipase exhibited a linear relationship in the temperature range of 30–40 °C and the corresponding activation energies were calculated to be 9.2 kJ/mol for free lipase and 2.48 kJ/mol for immobilized lipase. The activation energy of the immobilized lipase is lower in comparison to that of free lipase which suggests the change in conformation of enzyme during immobilization.

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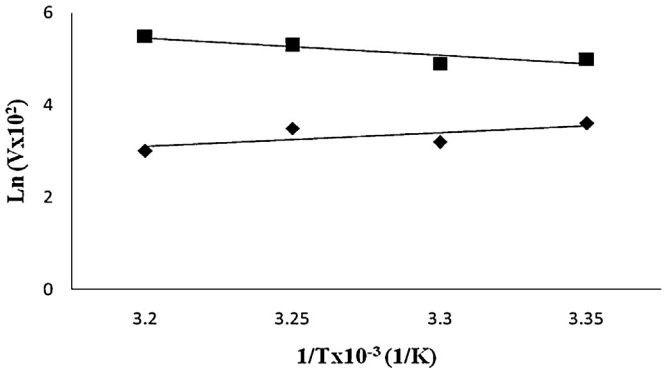

Fig. 8. Arrhenius plot for initial hydrolysis rate of pNPP catalyzed by free and immobilized lipase. Activation energy for free (♦) and immobilized lipase (■) was calculated to be 9.2 and 2.48 kJ/mol, respectively.

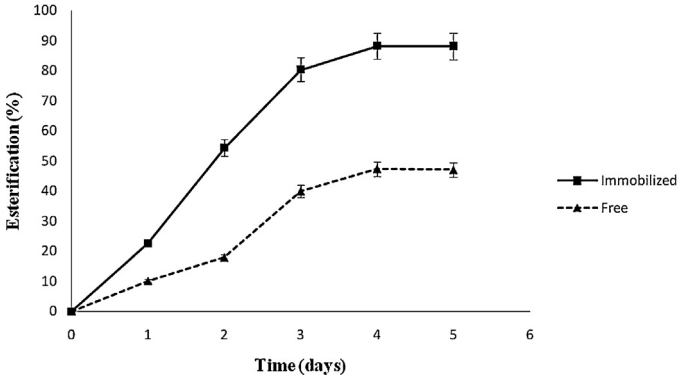

Fig. 9. Time course of pentyl valerate production catalyzed by free and immobilized DMVR46 lipase using pentanol (0.1 M) and valeric acid (0.1 M). The reaction was carried out in presence of cyclohexane as solvent at 37 °C with shaking (150 rpm).

3.6. Application of purified lipase for ester synthesis under water restricted environment using free and immobilized DMVR46 lipase

The biosynthesis of esters is currently of much commercial interest as the fatty acid esters synthesized using enzymes offer better odor and flavor characteristics when compared to those produced chemically [44]. Lipase from DMVR46 was used for the synthesis of industrially important ester "pentyl valerate". Fig. 9 shows the trend of synthesizing ester using free and immobilized lipase. The partially purified lipase from DMVR46 exhibited significant esterification efficiency with 88% ester production after 4 days of reaction when immobilized into AOT-organogels. While the free lipase showed only 47% of ester synthesis after 4 days of reaction. The immobilized enzyme has been more active than crude extract. This phenomenon can be attributed to the fact that the catalytic activity of lipase extract is lower than immobilized form due to the better dispersion of enzyme molecules on the support surface which improves its activity in organic medium [45]. Moreover, the immobilization of lipase in MBGs has been reported to improve the catalytic efficiency of enzyme by providing protection against inhibitory effect of organic solvents as well as aid in reusability of lipase facilitating easy recovery by simple filtration [23]. Hence, DMVR46 lipase may prove to be promising in esterification and standardization of reaction parameters may increase its efficiency as a biocatalyst.

3.7. Reusability of DMVR46 lipase

To prove the potential of an immobilized enzyme for application in industry, the most important criterion is to demonstrate its high reusability. The MBGs were subjected to reusability examination for determining the efficiency of immobilization. The enzyme preparation was given solvent washes after every cycle where the reaction time for each cycle was 4 days and reused in fresh media supplemented with substrates. The reusability studies showed that immobilized enzyme was stable up to 6 cycles of esterification, during which no significant decrease in esterification efficiency was noticed. However, activity started declining slowly after 6th cycle (Fig. 10). This may be due to accumulation of water formed as a by-product of reaction. Lipase DMVR46 exhibited 70% of its residual activity even after 10th run indicating meager loss of activity.

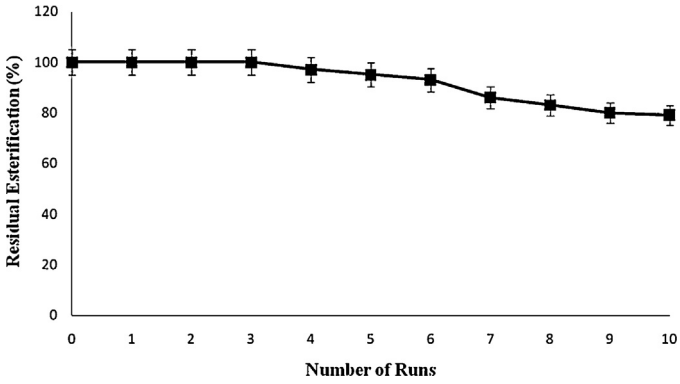

Fig. 10. Reusability of DMVR46 lipase immobilized into AOT-based organogels. The reaction was carried out at 37 °C and 150 rpm.

4. Conclusion

In this study, an extracellular lipase from solvent tolerant *Pseudomonas* sp. DMVR46 was purified following simple purification procedure with 29.74% recovery. The molecular mass of the lipase was found to be ~32.0 kDa by SDS-PAGE. It exhibited optimum activity at pH 8.5 and 37 °C. Among various p-nitrophenyl esters with different chain lengths, the lipase showed maximum activity on p-nitrophenyl palmitate (C16). The enzyme exhibited significant stability in presence of iso-octane and cyclohexane and was activated by Ca^{2+}, Ba^{2+} and Mg^{2+} but SDS and EDTA has negative influence on its activity. The partially purified lipase showed significant esterification activity for synthesis of pentyl valerate and revealed improved catalytic efficiency upon immobilization in microemulsion based organogels. These results make solvent-tolerant lipase DMVR46 more potentially valuable for biotechnological applications in non-aqueous catalysis.

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