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Isolation, identification, biochemical properties and potential application of an organic solvent-tolerant lipase from *Pseudomonas* sp. strain NEB-1

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Abstract

Using enrichment procedures, 45 organic solvent-tolerant lipase producer bacterial strains were screened from areas contaminated by oil and organic solvents. Among the strains, 15 isolates exhibited extreme stability toward organic solvents and high lipolytic activity. The NEB-1 isolate which was later identified as *Pseudomonas* sp. strain NEB-1 by biochemical tests and 16S rDNA gene sequence analysis was selected based on extremely high tolerance to organic solvents and maximum lipase production. Biochemical studies revealed that the crude lipase was stable at temperatures between 20 ºC and 60 ºC and pH ranges of 4 to 11 for 1 h. Optimum pH and temperature of the enzyme were revealed to be 9.5 and 70 ºC, respectively. The crude lipase showed remarkable tolerance in presence of different organic solvents with a broad range of hydrophobicity characteristics. The solvent stable lipase showed an attractive potency for application in biocatalysis in non-aqueous systems and biodiesel production.

Keywords: Organic solvent-tolerant lipase; *Pseudomonas* sp.; Screening methods; stability; biodiesel

1. Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3), are lipolytic enzymes that catalyze the hydrolysis of ester bonds of triacylglycerols at the oil-water interface (Arpigny & Jaeger, 1999). Various lipases from different eukaryotic and prokaryotic origin have been identified. Lipases from bacterial origin are more attractive due to their versatility, accessibility, high yield possibility, ease of genetic manipulation, regular supply as a result of absence of seasonal fluctuations, and rapid growth of inexpensive culture media (Fang et al., 2006). Some bacterial enzymes are very stable and active in organic solvents. Their remarkable stability in water-restricted environments has made them key enzymes in biotechnological applications (Klibanov, 2001). Using organic solvents in enzymatic reactions has several advantages such as prevention of water dependent undesirable side reactions, reduced microbial contamination, high solubility of substrates, ease recovery of products, shift of thermodynamic equilibrium toward synthetic direction, and enhanced thermostability (Koops, Verheij, Slotboom, & Egmond, 1999; Pencreac'h & Baratti, 2001). More importantly, the substrate, stereo-, regio- and chemoselectivities of the lipases differ markedly in non-aqueous solutions. This strategy is applicable in enantiopure and chiral compounds production which is important in pharmaceuticals and agrochemicals industries (Klibanov, 2001). Besides, biosurfactant and biodiesel production and organic synthesis are other attractive industrial applications of these enzymes.

It has been reported that some wild-type strains can grow in media containing organic solvents (Aono, Ito, Inoue, & Horikoshi, 1992). Based on the hypothesis that the organic solvent-tolerant bacteria can secrete organic solvent-tolerant enzymes, an investigation has been conducted to screen for a lipase producer organic solvent tolerant bacterium, its identification and lipase production. An extracellular lipase produced by *Pseudomonas* sp. strain NEB-1 was shown to be a potentially useful biocatalyst for biodiesel production and organosynthetic reactions. To date, among the high number of lipases described in literatures, only a few studies have been reported on the production of lipases with adequate stability and enzymatic activity in organic solvents especially, ethanol and

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methanol for further consideration in biodiesel production (Fang et al., 2006).

2. Materials and methods

2.1. Materials

Bacterial strains used as control were a gift from Shahid Motahari clinic (Shiraz, Iran). Tributyrin and p-nitrophenyl palmitate (p-NPP) were purchased from Sigma. All other chemicals were obtained from Merck Chemicals (Darmstadt, Germany).

2.2. Sample collection and cultivation

Soil and water samples were collected from different sites contaminated with oil and organic solvents. After removing debris, 1 ml or 1 g of each sample was added to 10 ml sterilized normal saline and shacked. 1% of the suspension as inoculum was transferred to enrichment medium which contained: 0.5% peptone, 0.1% beef extract, 0.2% yeast extract, and 0.5% NaCl, pH 7.0. After autoclaving 70 mg l^{-1} nystatin was added to medium to prevent fungi growth. Cultures were carried out at 37 ºC and 150 rpm for 48 h and transferred to fresh medium three times. Then, samples of the cultures were diluted and spread on nutrient agar plates and restreaked several times to obtain pure bacterial colonies.

2.3. Screening of lipase producing bacteria

For rapid detection of lipase producing strains, a method using phenol red and olive oil with slight modification was used (R. Singh, Gupta, Goswami, & Gupta, 2006). The medium contained 0.01% phenol red, 2.0% olive oil, 10 mM $CaCl_2$, 2.0% agar, pH 7.3–7.4. After incubation at 37 °C for 15 min, the colonies with obvious color change from pink to yellow were selected and re-spread on tributyrin agar plates with the following composition: 1.0% tributyrin, 0.05% yeast extract, 1.0% $(NH_4)_2SO_4$, 0.35% K_2HPO_4, 0.25% NaCl, 0.05% $MgSO_4$.7H20, and 2.0% agar, pH 7.5. The plates were incubated at 37 °C for 3 days. Lipolytic strains were gathered based on the presence of clear zone around the colonies. Then, one loopful from theses isolates was transferred to basal medium and 20 µl of the overnight cultures were spot inoculated on Rhodamine B agar medium containing 2.0% olive oil (emulsified by 1% polyvinyl alcohol or gum Arabic), 0.8% nutrient broth, 0.05% yeast extract, 0.4% NaCl, and 2.0% agar, pH 7.0. The filter sterilized Rhodamine B solution (0.001%) was added to medium after autoclaving. After incubation at 37 °C for 48 h, the plates were exposed to 350 nm UV irradiation and colonies that exhibited orange fluorescent halo were selected as potential lipase producers.

2.4. Isolation and screening of organic solvent-tolerant strains

1% of preculture basal medium was transferred to Erlenmeyer flasks containing 20 ml of the selective medium (Isken & de Bont, 1998). The medium contained 2.0% olive oil, 0.3% yeast extract, 0.5% $(NH4)_2SO_4$, 0.35% K_2HPO_4, 0.1% KH_2PO_4, 0.25% NaCl, and 0.05% $MgSO_4$.7H20, and 10%-90% organic solvent. The flasks were plugged with aluminum foil-covered stoppers to prevent evaporation of solvent. The cultures were incubated at 37 ºC and 150 rpm for 48 h and strains with high tolerance of toluene were transferred to Nutrient agar (NA) plates for plate overlay assays (Nielsen, Kadavy, Rajagopal, Drijber, & Nickerson, 2005). 20 µl of overnight culture of pure colonies grown in preculture basal medium was transferred onto glass petri dishes containing nutrient agar. The spots were then allowed to dry for 30 min at 37 °C. Solvent was directly poured on top of the agar plate surface to a depth of 5 mm. After 8 h the solvent was pipetted off, and the plates were inverted and incubated at 37 °C for another 24 h. The solvents tested included toluene, and benzene.

2.5. Bacterial strain identification

The isolated strain was identified by combining the analysis of phenotypic and genotypic identification methods. Morphological and physiological characteristics of the isolate were studied either on nutrient agar or in nutrient broth. Gram staining was performed by Burke method and confirmed by KOH test (Barron & Finegold, 1990). Motility was tested using wet mount and staining the flagella (Murray, Doetsch, & Robinow, 1994) and biochemical parameters were checked as recommended by Smibert and Krieg (Smibert RM & NR, 1994). Genotypic identification was done via determining the DNA sequences of partial 16S rDNA gene. Total DNA of strain NEB-1 was extracted by using QIAamp DNA Mini kit (Qiagen). The partial 16S rDNA gene sequence of the selected isolate was amplified by PCR using the primers: HRK1 (5'-ACTCCTACGGGAGGCAGCAG-3') as the forward and HRK2 (5'-TGACGGGCGGTGTGTACAAG-3') as the reverse primer. The amplification was carried out in 50 µl of reaction mixtures as follows: 3 min at 94 ^oC; 35 cycles of 1 min at 93 ^oC, 45 s at 58 ^oC, and 1:30 min at 72 ^oC, and a final extension at 72 ^oC for 10 min. The purified PCR product was sequenced in both directions using an automated sequencer by Bioneer

Company, South Korea. The phylogenic relationship of the isolate was determined by MEGA 4.0 software (Tamura, Dudley, Nei, & Kumar, 2007).

2.6. Lipase production

One loopful from fresh NA plate of pure isolate was transferred to Nutrient broth (NB). A bacterial inoculum of 4.5% from stationary phase was inoculated into 20 mL of the preliminary optimized lipase production medium in a 125 mL Erlenmeyer flask with the following composition: 2.0% olive oil, 0.03% yeast extract, 0.7% $(NH4)_2SO_4$, 0.25% K_2HPO_4, 0.05% NaCl and 10.0% of mineral stock solution with an initial pH of 7.5. The incubations were performed at 31 °C with shaking at 220 rpm. The culture was harvested after 52 h (based on the relationship between bacterial growth and lipase production) and supernatant was obtained by centrifugation at 8000×g at 4 °C for 20 min. The supernatants were used for lipase assays and biochemical characterization studies.

2.7. Lipase assay

Determination of lipase activity was performed spectrophotometrically by p-nitrophenyl palmitate (p-NPP). The reaction mixture contained 360 µl freshly prepared (2.5% Triton X-100 and 50 mM Tis-HCl buffer, pH 8.0) and 30 µl freshly prepared substrate (10 mM stock in acetonitrile). This mixture was prewarmed and then mixed with 20 µl of enzyme solution. The amount of liberated p-nitrophenol was recorded at A_{410} for 2 min at 30 ^oC. One unit of lipase activity was defined as the amount of enzyme required to liberate 1 µmol of p-nitrophenol released from the substrate per min under standard assay conditions (Moh'd A & Wiegel, 2007).

2.8. Determination of reaction properties

The optimum temperature of lipase was determined at temperature range of 20 °C-80 °C. Thermostability was performed at 20 °C, 40 °C and 60°C during two intervals of 0.5 and 1 h. The optimal pH of the enzyme was determined by using different buffer systems (pH 7.0 to 9.5). For pH stability studies, residual activity in various pH values between 4.0 and 11.0 was measured after 1 h incubation at 30 °C by using 25 mM universal buffer mixture [sodium citrate (pH 3.0-6.2), potassium phosphate (pH 5.8-8.0 and 11.0-13.0), Tris-HCl (pH 7.1-8.9), and glycin-NaOH (pH 8.6-10.6). The substrate emulsion was incubated at desired temperature for 15 min and in each case lipase assay was carried out for 2 min. Each measurement was performed three times.

2.9. Effect of metal ions, inhibitors, and denaturants on lipase activity

The effect of different metal ions at final concentration of 5 mM, and other test components at final concentration of 1 mM and 5 mM was added to p-NPP assay mixture. The residual activity was measured after incubation for 1 h at 30 °C and pH 8.0. All experiments were done in triplicate and standard error was included.

2.10. Enzyme stability in organic solvents

Bacteria was cultured aerobically in the absence of organic solvent and removed by centrifugation at 8000×g at 4°C for 20 min. The supernatant was filtered and residual activity of enzyme was measured after incubation for 6 days at 30°C in various organic solvents at total concentration of 25% using p-NPP as substrate. Remaining activity of lipase was also investigated in presence of 15%, 25%, 35% and 45% of ethanol and methanol after 24 h incubation period at 30 °C.

3. Results and discussion

3.1. Screening of organic solvent-tolerant lipolytic bacteria

The lipolytic activities of all the isolates were qualitatively determined by using plate assays including phenol red agar, tributyrin agar and Rhodamine B agar. Most isolates exhibited lipolytic activity. Tolerance of bacteria to organic solvents was examined in both solid and liquid media (Table 1). The parameter log *P* is defined as logarithm of the partition coefficient of the solvent between n-octanol and water and is used as an index of the solvent polarity. Organic solvents with a log *P* between 2.0 and 3.0 are highly toxic to most bacterial strains even at very low concentrations of 0.1% [8]. Toluene (log *P*=2.5) in total concentration of 10%-90% was added to the medium described earlier. By this procedure, 45 organic solvent-tolerant strains were obtained. Among them, 15 strains showed maximal tolerance (90% toluene). Most of these strains tolerated toluene and benzene (log *P*=2.0) overlays. Out of these, strain NEB-1 showed maximum lipase production (Fig. 1) and was selected for further studies.

Table 1. Growth and tolerance of strain NEB-1 to different concentration of organic solvents

	10%	30%	50%	70%	90%	100% overlays
Toluene	+++	+ + +	+ + +	+ + +	++	+++
Benzene	+++	+ + +	+ + +	++	++	+++

Note: Growth after 48 h incubation was visually determined: (+) weak, (+ +) acceptable, and (+ + +) good.

Fig. 1. The phenol red plate assay to show extracellular lipase production of the strain NEB-1. The colonies with obvious color change from pink to yellow reveal production of free fatty acid in the olive oil plate after hydrolysis.

3.2. Phenotypic and genotypic identification

Morphological studies were done following the Bergey's manual. Although, cells of strain NEB-1 were found to be gram negative, because of some reports mentioning isolation of organic solvent-tolerant sporulating gram negative bacteria (Roux & Raoult, 2004; Teng et al., 2003), the endospore staining was also carried out (Table 2). The 1037-bp 16S rDNA gene sequence of the present strain has been deposited in the GenBank database under accession number KC951631. According to morphological and physiological characteristics and comparative sequence analysis of the 16S rDNA gene of strain NEB-1 and other 16S rDNA available in the GenBank database, it was tentatively named "*pseudomonas* sp. strain NEB-1". The phylogenetic tree (Fig. 2), constructed by the neighbor-joining method, indicated that isolate NEB-1 was part of the cluster within the genus *pseudomonas*. Among the described sub species, the closest relative of isolate NEB-1 was *pseudomonas stutzeri* RV68 and *p. stutzeri* DSM5190T (AJ288151). There are different genera of bacteria secreting organic solvent-stable lipases. Members of *Pseudomonas* genus are reported to secrete high levels of lipase production and are very attractive biocatalysts due to their catalytic and biochemical properties (Ogino et al., 2000).

Table 2. Morphological and biochemical characterization of the strain NEB-1

Test	Result
Gram staining	Negative
Endospore staining	Negative
Morphology	Rods
Motility	Motile
KOH string	Positive
Pigment	Positive
Growth at 4 °C	Negative
Growth at 41 °C	Positive
Growth on MacConkey	Positive
Growth on EMB	Positive
Catalase	Positive
Oxidase	Positive
Triple sugar iron agar	Lactose negative
OF (dextrose)	Negative
Gelatin hydrolysis	Positive
Starch hydrolysis	Negative
Lysine decarboxylase	Negative

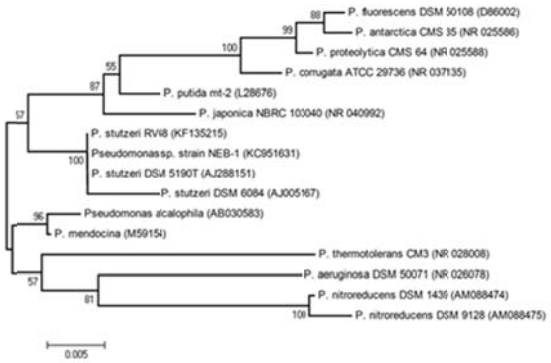

Fig. 2. Neighbor-joining tree based on 16S rDNA gene sequences showing the position of strain NEB-1 relative to some other validly published species from the genus *Pseudomonas*. The bootstrap consensus tree inferred from 500 replicates is shown next to the branches (Bootstrap values > 50 % are indicated). The accession numbers of all 16S rDNA sequence data are shown in parentheses.

3.3. Optimal condition for lipase production

Various media and culture condition including different pH values (pH 6.5, 7.5, and 8.0), temperatures (25 °C, 31 °C, and 37 °C), and shaking condition (static, 180 rpm, 220 rpm, and 260 rpm) were tested for lipase production each 10 h until 72 h. Lipase activity was investigated spectrophotometrically by using p-NPP as substrate for each time interval. Maximum lipase activity was obtained in a productive medium (2.0% olive oil, 0.03% yeast extract, 0.7% $(NH_4)_2SO_4$, 0.25%

K_2HPO_4, 0.05% NaCl and 10.0% of mineral stock solution) seeded with 4.5% inoculum of cells in stationary-phase from nutrient broth. Optimal physical factors were found to be 31 °C for temperature, pH 7.5 and shaking rate of 220 rpm. Long lag-phase probably occurred because of transferring bacterial cells from a rich medium (inoculum) to a poor one. Like many other hydrolyses, a significant lipase formation was observed during late log-phase of growth when cell density is high. Maximum lipase activity (83.44 U/ml) occurred in 52 h, when the cell growth was also maximum (Fig. 3). This phenomenon is known to be related to quorum sensing activity of bacteria (Rahman, Baharum, Basri, & Salleh, 2005). Maximum lipase activity of *Pseudomonas* strains in most studies have been recorded mainly at the end of log phase (Boran & Ugur, 2010).

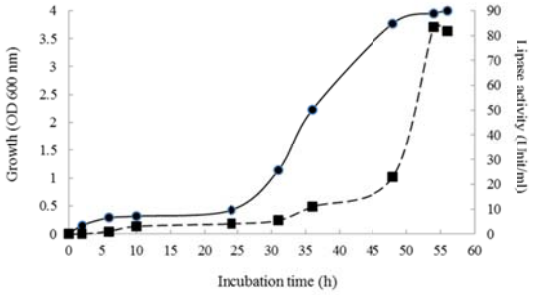

Fig. 3. Kinetics of growth (●) and lipase production (■) in *Pseudomonas* sp. strain NEB-1 in the basal medium. Results represent the means of three experiments. Absence of bars indicates that errors were smaller than symbols.

3.4. Enzyme reaction characteristics

The activity and stability of lipase from *Pseudomonas* sp. strain NEB-1 was measured at different temperatures under standard condition using p-NPP. The optimal temperature was observed to be 70 °C (Fig. 4a). It may be the result of short time interval used for initial velocity measurement of the enzyme. The lipase retained more than 50% of its maximal activity in a broad range of temperatures (40-80 °C). Most lipases from *Pseudomonas* strain have optimum temperatures lower than 70 °C. However, lipase from *Pseudomonas aeruginosa* san-ai has also been reported with optimum temperature of 70 °C (Rikalović, Gojgić-Cvijović, Vrvić, & Karadžić, 2012). To determine thermostability, the lipase activity at the start of incubation was considered 100% and residual activity after 1 h incubation at different temperatures was determined. The lipase from *Pseudomonas* sp. strain NEB-1 was stable at room temperature completely and even retained more than 50% of its initial activity at 40 °C for 30 min (Fig. 4b). Crude lipase with optimal pH of 9.5 showed high activity over alkaline pH values and was stable over a broad range of pH 4.0-11.0 with more than 80% residual activity after 1 h incubation at pH 10.0 (Fig. 5a and b). Most *Pseudomonas* lipases which have been reported so far are alkaline enzymes. Optimal pH for organic solvent-stable lipase from *Pseudomonas stutzeri* LC2-8 and lipase from *Pseudomonas aeruginosa* PseA were pH 8.0 (Cao, Zhuang, Yao, Wu, & He, 2012). An optimal pH 9.0 for lipases from *Pseudomonas* sp. strain S5 and *Pseudomonas fluorescens* JCM5963 was reported (Boran & Ugur, 2010; Rahman et al., 2005). Moreover, optimum pH of lipase from *Pseudomonas aeruginosa* San-ai was reported to be 11.0 (Rikalović et al., 2012).

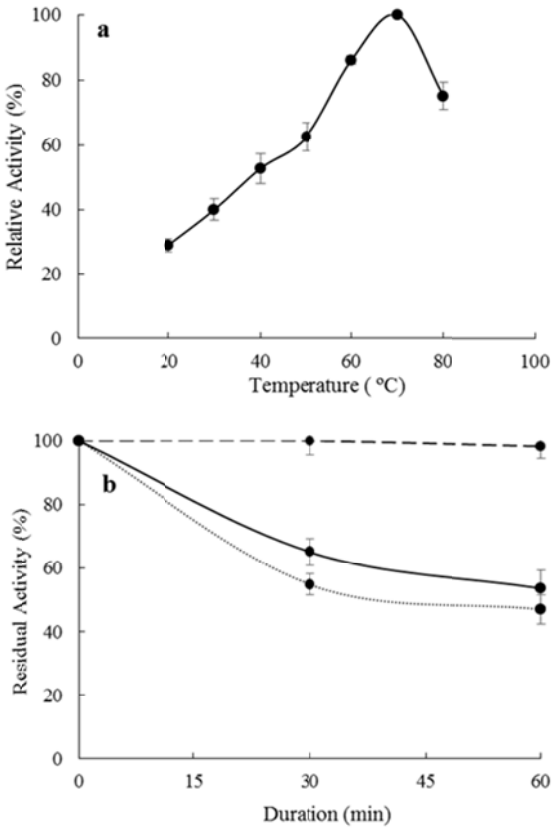

Fig. 4. The effect of temperature on lipase (a) activity and (b) thermostability after 30 min and 1 h incubation at 20°C (---), 40°C (—), and 60°C (…). Results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols. The values are shown as percentages of the maximum activity of enzyme observed at pH 9.5, which is taken as 100%.

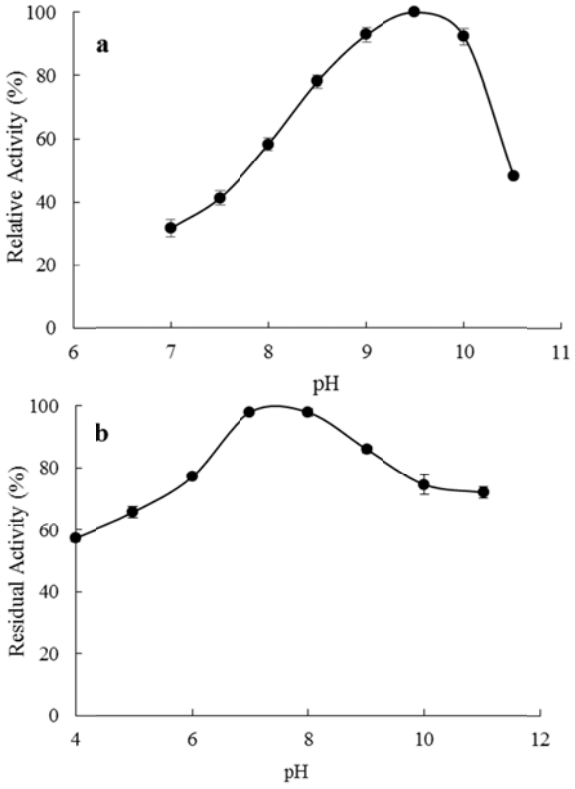

Fig. 5. The effect of pH on lipase (a) activity and (b) stability in strain NEB-1. Results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols. The relative activity was defined as the percentage of activity detected with respect to the maximum lipase activity.

3.5. Effect of metal ions, reagents, and denaturants on lipase activity

Effect of different bivalent metal ions on lipase activity is presented in Table 3. None of the metal ions showed remarkable inhibitory or stimulatory effect on lipase activity except for Cu^{2+} and Ca^{2+}. There are several reports of inhibitory effect of Cu^{2+} on *Pseudomonas* lipases (Boran & Ugur, 2010; Karadzic, Masui, Zivkovic, & Fujiwara, 2006; Zhang et al., 2009). A possible explanation for stimulatory effect of Ca^{2+} is that the quality of oil-water interference or its stability can be affected by this ion due to fatty acid removal in the form of insoluble calcium salts. The lipase activity was slightly inhibited in presence of Cd^{2+}, Ni^{2+}, Mg^{2+}, and Ba^{2+}. Interestingly, Cd^{2+} and Hg^{2+} did not affect the lipase activity significantly. To our knowledge, until now, tolerances to these toxic metal ions have rarely been reported in literatures. Among the tested reagents, only SDS inhibited NEB-1 lipase drastically. When exposed to concentration of 1 mM and 5 mM of SDS, the enzyme activity reduced to 32.4% and 13.4%, respectively (Table 4). It is possible to assume that the loss of activity could be a result of enzyme conformation destruction. The chelating agent, EDTA did not affect the enzyme negatively which indicates that the lipase is not a metalloenzyme. This is in agreement with the data from metal ions. Similarly, this supports the idea that the Ca^{2+} stimulatory effect on lipase from *Pseudomonas* sp. strain NEB-1 activity is not a result of direct enzyme activation. Based on several reports, EDTA inhibited only a few lipases isolated from *Pseudomonas* genus. Lipases from *Pseudomonas fluorescens* RB02-3 (Boran & Ugur, 2010), *Pseudomonas* sp. strain S5 (Rahman et al., 2005), and *Pseudomonas aeruginosa* (S. Singh & Banerjee, 2007) were not affected by EDTA. The thiol reducing agent, DTT, enhanced the lipase activity. This stimulatory effect suggests that there is no intermolecular disulfide bond in lipase structure which is also consistent with the result of metal ions. Mercury binds to sulfhydryl groups of enzymes (Woods, Kardish, & Fowler, 1981). This observation may explain resistance of lipase to Hg^{2+}. Unlike the disulfide bond, which is not sensitive to treatment with 1 mM and 5 mM concentrations of DTT. It is also possible that putative disulfide bonds are not required for catalytic activity. Pefabloc as a serine residue inhibitor had no negative effect on lipase activity, suggesting that accessible catalytic serine residue is not present in lipase structure. Nonionic detergent Tween 80 in contrast with SDS, strongly stimulated lipase activity presumably due to stabilizing the interfacial area as a surface active agent, facilitating access of the substrate to the enzyme (See Table 4). Lipase from *Pseudomonas aeruginosa* san-ai activity was also stimulated by Tween 80 and inhibited by SDS (Dimitrijević et al., 2011).

Table 3. Effect of different bivalent metal ions on lipase activity

Metal ion	Relative activity (%)*	Metal ion	Relative activity (%)
Cd^{2+}	97.0 ± 0.5	Bo^{2+}	108.0 ± 0.4
Mn^{2+}	110.9 ± 0.7	Zn^{2+}	110.0 ± 0.8
Hg^{2+}	118.0 ± 1.3	Cu^{2+}	74.0 ± 0.9
Ni^{2+}	84.0 ± 1.0	Fe^{2+}	106.0 ± 0.4
Mg^{2+}	94.0 ± 0.5	Co^{2+}	108.0 ± 0.4
Ca^{2+}	122.0 ± 1.0	Ba^{2+}	84.0 ± 1.1

*Lipase activity is shown as values relative to that measured without addition of any metal ions.
All assays were done in triplicate.

Table 4. Effect of different chemicals on lipase activity

Reagent	Relative activity (%)* at a concentration of	
	1 mM	5 mM
Control	100	100
EDTA	129.4 ± 1.1	140.6 ± 1.2
DTT	152.9 ± 9.0	134.4 ± 1.0
Pefabloc	125.1 ± 1.3	135.1 ± 1.3
SDS	32.4 ± 0.7	13.4 ± 0.1
Tween 80	183.0 ± 1.1	134.4 ± 0.6

*Lipase activity is shown as values relative to that measured without addition of any reagents as control. All assays were done in triplicate.

3.6. Enzyme stability in organic solvents

Organic solvent-tolerant lipases are desirable choices in industrial processes, especially in organosynthesis reactions. The effect of organic solvents on lipase activity is related to both the nature of enzyme and the solvent (Hazarika, Goswami, Dutta, & Hazarika, 2002). The stability of crude extracellular lipase in organic solvents with various log *P* after incubation for 6 days is shown in Table 5. All tubes were sealed and covered to prevent solvent loss due to evaporation. The NEB-1 lipase showed good stability toward both hydrophobic and hydrophilic organic solvents in the long period of incubation. Among 12 tested solvents, only acetonitrile reduced the enzyme activity drastically. Lipase from *Pseudomonas aeruginosa* San-ai was also inactivated in 25% acetone after 48 h incubation (Dimitrijević et al., 2011). No considerable enzyme inactivation was demonstrated by n-heptane, xylene, n-hexane, benzene, diethyl ether and acetone. Elevated enzymatic activity in isopropanol and DMSO was observed. Lipase from *Pseudomonas stutzeri* LC2-8 also did not show any notable inactivation in presence of 25% n-heptane and n-hexane and was activated in isopropanol and DMSO (Cao et al., 2012). The most important characteristic of organic solvents is log *P* which gives the best correlation with the enzyme activity. Organic solvents with lower log *P* values are known to be more toxic due to stripping essential water molecules off the enzyme structure. However, lipase from *Pseudomonas* sp. strain NEB-1 did not follow this trend. To explain this behavior and assess the denaturing strength of organic solvents, other solvent related parameters might be helpful (Doukyu & Ogino, 2010). Extreme tolerance of the lipase isolated in our study toward various organic solvents suggests that the lipase from *Pseudomonas* sp. strain NEB-1 can be considered as a potential biocatalyst for organosynthetic reactions.

Table 5. Stability of lipase activity in the presence of various organic solvents

Organic solvent	Log *P*	Relative activity (%)*
Control	-	100
n-Heptan	4.0	97
n-Hexane	3.5	86
Xylene	3.1	90
Toluene	2.5	74
Benzene	2.0	95
Diethyl ether	0.87	85
Isopropanol	0.074	103
Acetone	-0.23	85
Ethanol	-0.24	71
Acetonitrile	-0.34	30
Methanol	-0.76	62
DMSO	-1.37	106

*The lipase was incubated with various solvents (25%) at 30°C and 25 rpm for 6 days.
The activity of the lipase in the absence of organic solvents was taken as control.

3.7. Application of lipase for biodiesel fuel production

Organic solvent-tolerant lipases are frontier enzymes in green chemistry process such as biofuel production. Only organic solvent-tolerant lipases with acceptable stability toward ethanol and methanol can improve biodiesel production (Kaieda, Samukawa, Kondo, & Fukuda, 2001). In order to test the suitability of lipase from *Pseudomonas* sp. strain NEB-1, the enzyme was incubated with different concentrations of methanol and ethanol for 24 h (Table 6). No distinct inactivation happened after 24 h incubation of enzyme even in higher concentration of organic solvents. The residual activity of lipase in presence of ethanol and methanol at concentration of 45% was 70.6% and 152.9%, respectively. High tolerance of the NEB-1 lipase to these solvents, especially methanol proposes the use of this enzyme in biodiesel fuel production by methanolysis from methanol and vegetive oil in crud form such as olive oil.

Table 6. Lipase stability in different concentration of ethanol and methanol

Solvent	Residual activity (%)* at concentration of:				
	-	15%	25%	35%	45%
Ethanol	100	209.8	164.7	90.2	70.6
Methanol	100	243.1	156.9	154.9	152.9

*The lipase was incubated with various concentrations of ethanol and methanol at 30°C and 25 rpm for 24 h.
The activity of the lipase in the absence of organic solvents was taken as control.

4. Conclusion

In our study, an organic solvent-tolerant lipase from *Pseudomonas* sp. strain NEB-1 was obtained. The maximum lipase activity in a production medium that was designed in our laboratory was 83.44 U/ml by using p-NPP as substrate. The enzyme with temperature and pH optimum at 70 °C and pH 9.5 exhibited significant stability in a broad range of temperatures and pH. After incubating the lipase with various metal ions and additives, it was revealed that the enzyme activity can be elevated in the presence of calcium and is inhibited by copper and SDS. The lipase also showed an extreme tolerance to both hydrophilic and hydrophobic organic solvents in long incubation period of 6 days. Stability of lipase even in high concentration to 45% of methanol and ethanol was observed after 24 h incubation. These results suggest the use of enzyme as a potential biocatalyst in organosynthesis and biodiesel fuel production processes.

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