Turkish Journal of Biology
http://journals.tubitak.gov.tr/biology/
Research Article

Turk J Biol
(2013) 37: 538-546
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doi:10.3906/biy-1211-10

# Purification and biochemical characterization of an extracellular lipase from psychrotolerant *Pseudomonas fluorescens* KE38

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**Received:** 06.11.2012 • **Accepted:** 28.01.2013 • **Published Online:** 06.09.2013 • **Printed:** 04.10.2013

**Abstract:** An extracellular lipase producing bacterium was isolated from a soil sample, and identified as a strain of *Pseudomonas fluorescens* by 16S rRNA gene sequencing. It was named *Pseudomonas fluorescens* KE38. KE38 showed psychrotolerant properties with an optimum growth temperature of 25 °C. The lipase enzyme secreted by KE38 was purified 41.13-fold with an overall yield of 54.99%, and a specific activity of 337.3 U/mg. The molecular mass of purified lipase was estimated to be approximately 43 kDa by SDS-PAGE. Although the lipase was active at a temperature range of 15–65 °C, it exhibited maximum activity at 45 °C, at pH 8.0. The enzyme exhibited high stability retaining 100% and 70% of its activity after an incubation period of 45 and 100 min at 45 °C and pH 8.0 respectively. It also showed a broad substrate specificity acting on p-nitrophenyl esters with $C_8$-$C_{18}$ acyl groups as substrates and was activated by $Ca^{2+}$ and $Ni^{2+}$ at 1 mM. While the enzyme retained its activity levels in the presence of a variety of organic solvents, DMSO and dimethylformamide enhanced this. High stability, broad substrate specificity and activity at cold temperatures in the presence of organic solvents, and metal ions make the extracellular lipase of KE38 a candidate for industrial applications.

**Key words**: *Pseudomonas fluorescens*, extracellular lipase, enzyme purification

## 1. Introduction

Enzymes, which may be used in a diverse array of industrial applications, are important biocatalysts. There is always a demand for new enzymes that may offer better properties for specific applications in ever-changing industrial activities (Konarzycka-Bessler and Jaeger, 2006).

Lipases are a particular group of enzymes with many uses such as in textile, food, biomedical, petrochemical, pharmaceutical, detergent, and many other industries (Hasan et al., 2006). Lipases (EC 3.1.1.3, glycerol ester hydrolases) are enzymes that catalyze both the hydrolysis and synthesis of insoluble or poorly soluble long-chain triacylglycerols with an acyl chain length of ≥ 10 carbon atoms based on the presence or absence of water (Jeager et al., 1999; Gupta et al., 2004). In the absence of water, they play a role in esterification, transesterification, acidolysis, and aminolysis reactions (Jeager et al., 1994).

Lipases are produced by both eukaryotes, including animals, plants and fungi, and prokaryotes, including bacteria and archaea (Wang et al., 2008). Microbial lipases have received great attention due to the variety of their biochemical activities and ease of their isolation and production (Schmidt-Dannert, 1999). Microbial lipases are also more stable than plant and animal derivatives and their production is safer for industrial and research applications (Schmidt-Dannert, 1999). Because of their higher activities at neutral or alkaline pH optima, bacterial enzymes are preferred to fungal enzymes. Moreover, genetic and environmental manipulations can be performed more readily on bacterial cells due to their short generation times, their simple nutritional needs and easy screening procedures for desired properties (Hasan et al., 2006). Because of these properties, bacterial lipases have immense applications in food, detergent, pulp and paper, and leather industries; environmental management; and pharmacy (Jeager and Reetz, 1998; Hasan et al., 2006).

Lipases that are capable of catalysing reactions at low or moderate temperatures hold a great potential in certain areas of industry such as detergent, synthesis of heat labile compounds, and biodiesel production. One of the most obvious advantages of using such enzymes is cutting down the cost of energy expenditures for processes that naturally require high temperatures (Joseph et al., 2008).

Here we report the isolation of an extracellular lipase producing *Pseudomonas* sp. from a soil sample collected from Mount Erciyes in Kayseri, Turkey and the purification and characterization of lipase from this bacterium.

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## 2. Materials and methods

### 2.1. Materials

All chemicals were purchased from either Sigma-Aldrich (www.sigmaaldrich.com) or Applichem (www.applichem.com/home/). DNA and protein molecular weight markers were purchased from Fermentas (www.fermentas.com).

### 2.2. Isolation of extracellular lipase producing bacterium from soil

Soil samples were diluted several times with Luria-Bertani (LB) broth and plated on agar plates of LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1% gellan gum, pH 7.2) supplemented with 1% v/v olive oil as substrate and 1% rhodamine B solution (0.1% w/v) (Kouker and Jaeger, 1987). Lipase producing bacteria were identified on spread plates after several days of incubation at 25 °C by the formation of orange fluorescent halos around the colonies monitored by fluorescence with UV light at 350 nm. Selected bacteria were plated on fresh LB agar and incubated at 25 °C to obtain pure cultures.

### 2.3. Determination of the optimum and highest growth temperature of *Pseudomonas fluorescens* KE38

Growth experiments were performed in 100-mL volumes of LB media, inoculated with 100 µL of the overnight cultures, in 250 mL-Erlenmeyer flasks (1:1000 dilution). Cells were grown aerobically at 6 different temperatures, 5, 10, 15, 20, 25, and 30 °C, for 72 h with shaking at 180 rpm. Bacterial cell growth was monitored with a Multiskan Spectrum microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA) by measuring the optical density at a wavelength of 600 nm ($OD_{600}$) every 4 h for 3 days. Growth rates ($\mu$) at each temperature were calculated by using the formula: $\mu = (\ln I_1 - \ln I_0) / (t_1 - t_0)$, where $I$ stands for optical density at 600 nm ($OD_{600}$) at the beginning and the end of exponential phase of the growth curve, while $t$ is the corresponding time in hours at these $OD_{600}$ values.

### 2.4. Bacterial genotyping by sequencing of the partial 16S rRNA gene and BLAST search

Bacterial genomic DNA was isolated by GeneJE Genomic DNA Purification kit (Fermentas. www.fermentas.com). Then 30 mL of 24 h bacterial culture ($OD_{600}$:1) incubated at 25 °C with shaking (200 rpm) was used for the genomic DNA isolation. The 16S rRNA gene of the isolated strain was amplified from its genomic DNA by PCR using a pair of universal bacterial primers, E334F (5' CCAGACTCCTACGGGAGGCAG3') as forward primer and E1115R (5'CAACGAGCGCAACCCT3') as reverse primer. The amplified DNA fragment was cloned into the pTZ57R/T cloning vector by using the InsTAclon PCR Cloning Kit (Fermentas. www.fermentas.com) according to the manufacturer's instructions. *E. coli* DH5α was used as a host cell. M13F (5'GTAAAACGACGGCCAGT 3') and M13R (5'CAGGAAACAGCTATGACC 3') primers were used to determine the sequence of the cloned DNA fragments. Sequencing was performed at the Biotechnology and Bioengineering Central Research and Application Laboratories, İzmir Institute of Technology, İzmir, Turkey. BLAST analysis was performed on the sequenced 16S rRNA gene via the NCBI search tool Blastn (www.ncbi.nlm.nih.gov/blast) to determine the phylogenetic association of the isolated strain.

### 2.5. Determination of the optimum incubation time for the production of extracellular lipase by *Pseudomonas fluorescens* KE38

*Pseudomonas fluorescens* KE38 was grown at 25 °C with shaking (200 rpm) for 24 h in 10 mL of minimal medium (50 mM $Na_2HPO_4.7H_2O$, 20 mM $KH_2PO_4$, 10 mM NaCl, 20 mM $NH_4Cl$, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$) supplemented with 1% (v/v) olive oil as carbon and energy source. Then 3 mL of this culture was transferred into a 2-L flask containing 300 mL of minimal medium with 1% (v/v) olive oil and grown at 25 °C with shaking (200 rpm) for 1 to 5 days. Aliquots of the culture broth were withdrawn at intervals to determine both the cell growth by spectrophotometer, measuring the OD at 600 nm and the optimum incubation time for maximum extracellular lipase activity in the culture medium. For the measurement of lipase activity, cells were harvested (10,000 × *g*, at 4 °C for 30 min) and the supernatant was passed through a 0.22-µm filter to remove any remaining cells. This filtrate was used for extracellular lipase activity assays.

### 2.6. Purification and determination of the molecular mass of lipase from *Pseudomonas fluorescens* KE38

Lipase was purified from the growth medium after the removal of cells by centrifugation as mentioned above. Purification was performed by ammonium sulfate precipitation, Sephadex G-100 size-exclusion chromatography, and ultrafiltration. Prior to purification studies, optimum ammonium sulfate concentration for the precipitation of lipase was determined by ammonium sulfate fractionation. An appropriate amount of ammonium sulfate to bring the growth medium to 10% saturation was added slowly while stirring until the solution reached equilibrium and then was centrifuged at 10,000 × *g* for 20 min. The supernatant was decanted and subsequently fractioned while the precipitate was resuspended in one-half the original volume with 50 mM Tris-HCl buffer pH 8.0. Ammonium sulfate was added in increments as above in order to obtain the 20%, 30%, 40%, 50%, 60%, 70%, and 80% cut of growth medium. Fractions obtained were assayed for lipase activity, and the 30% ammonium sulfate fraction was found to contain the main lipase activity.

Purification was initiated from a fresh culture. An appropriate amount of ammonium sulfate was added slowly to the growth medium (300 mL) up to 30% saturation (w/v) with stirring. The mixture was allowed to

stand overnight with gentle stirring and then centrifuged at 10,000 × *g* for 30 min. All these steps were performed at 4 °C. The precipitate was dissolved in 6 mL of 50 mM Tris-HCl buffer, pH 8.0, and assayed for lipase activity. Concentrated enzyme was then loaded on to a Sephadex G-100 column (2.5 cm × 50 cm) pre-equilibrated with 50 mM Tris-HCl buffer, pH 8.0, and 1 mL fractions were collected and tested for lipase activity. Active fractions were pooled and then loaded to a microcon YM-100 centrifugal cut off filter unit (membrane pore size, 100 kDa) (Sigma-Aldrich, USA), in order to clear the enzyme solution of impurities. Permeate containing the enzyme solution was stored at –20 °C for further use.

Molecular mass of KE38 lipase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Then 12% polyacrylamide gel (resolving gel) was used together with 4% stacking gel. Purified lipase was loaded on SDS gel. Electrophoresis was performed at 65 V for 30 min and then 105 V for 2 h. Gels were stained with Bromophenol Blue overnight. A Spectra Multicolor Broad Range Protein Ladder was used as a molecular mass marker.

**2.7. Determination of protein concentration**

The protein concentration was determined by Bradford protein assay reagent (Bradford, 1976) (BioRad Protein Assay, BioRad Laboratories GmbH) by using bovine serum albumin as a standard.

**2.8. Assay of lipase activity**

Lipase activity was measured spectrophotometrically using *p*-nitrophenyl laureate (or *p*-nitrophenyl-esters other than *p*-nitrophenyl laurate where needed) as substrate (Gupta et al., 2002). The amount of released *p*-nitrophenyl was measured at 400 nm. The reaction mixture (1 mL) contained 980 µL of 100 mM Tris-HCl buffer including 150 mM sodium chloride and 0.5% triton X-100, pH 8.0, 10 µL of 50 mM *p*-nitrophenyl laurate (or other *p*-nitrophenyl-esters) dissolved in acetonitrile, and finally 10 µL of enzyme solution. The blank contained the same components except for enzyme solution. All experiments were performed in triplicate, the extinction coefficient of p-nitrophenol was determined under each reaction condition, and the effect of nonenzymatic hydrolysis of substrates was subtracted. One unit of lipase activity was defined as 1 µmol *p*-nitrophenol produced per minute under the assay conditions and calculated according to the following formula: Enzyme activity (µmol/min/mg) = dA/dt ($min^{-1}$) / ($\varepsilon_{400nm}$ (mL $\mu mol^{-1}$ $cm^{-1}$) × l (cm) × enzyme (mg $mL^{-1}$)), where ε is the molar extinction coefficient of *p*-nitrophenol expressed in $M^{-1}$ $cm^{-1}$. Molar extinction coefficient is defined as the absorbance of 1 M *p*-nitrophenol measured through a 1-cm cuvette, and was calculated as 14,900 $M^{-1}$ $cm^{-1}$ under standard assay conditions.

**2.9. Determination of the substrate specificity of the lipase enzyme**

Substrate specificity of the lipase was determined by measuring activity towards *p*-nitrophenyl esters of varying chain length (*p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyloctonate (C8), *p*-nitrophenyldecanoate (C10), *p*-nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14), *p*-nitrophenyl palmitate (C16), and *p*-nitrophenyl stearate (C18)) at 50 mM. The lipase activity was assayed at pH 8.0 and 25 °C.

**2.10. Effect of temperature on lipase activity and stability**

The enzyme activity was measured in the range of 4–65 °C with intervals of 10 °C (11 °C for the 4 °C) using the standard assay conditions. In order to study the effect of temperature on enzyme stability, lipase was pre-incubated at different temperatures (35–75 °C) for 45 min in 50 mM Tris-HCl buffer (pH 8.0). It was also incubated at 4 °C for 24 h. Afterwards, the residual lipase activity was tested at pH 8.0 and 45 °C according to the standard assay conditions.

**2.11. Effect of pH on lipase activity and stability**

In order to find the optimum pH for the lipase activity, 4 different buffers (sodium acetate (pH 3.0–5.0), potassium phosphate (pH 5.0–7.0), Tris-HCl (pH 7.0–9.0) and glycine-NaOH (pH 9.0–12.0)) were selected. The enzyme activity was tested at 45 °C at various pH values (pH 4.0–12.0). To determine the effect of pH on lipase stability, lipase was pre-incubated at 45 °C for 100 min in buffers of pH 6.0–10.0. The residual lipase activity was assayed under standard conditions.

**2.12. Effect of various metal ions on lipase activity**

The effects of various metal ions including $Ca^{2+}$, $Mg^{2+}$, $K^{1+}$, $Na^{1+}$, $Mn^{2+}$, $Ni^{2+}$, $Zn^{2+}$, and EDTA at 1 mM on lipase activity was investigated by incubating the enzyme at 45 °C, pH 8.0, for 60 min. Then the relative activity was calculated by regarding the control (no metal ions in the mixture) as 100%.

**2.13. Effect of various organic solvents on lipase activity**

The effects of organic solvents like ethanol, acetone, methanol, acetonitrile, n-hexane, dimethylsulfoxide (DMSO), dimethylformamide, and 2-propanol at a concentration of 30% (v/v) on the lipase activity were investigated by incubating the enzyme at 45 °C, pH 8.0, for 60 min. Then the relative activity was calculated by regarding the control (no organic solvents in the mixture) as 100%.

## 3. Results and discussion

**3.1. Isolation and identification of lipase producing bacterium from soil**

Soil samples were collected from Mount Erciyes in Kayseri, Turkey, at an altitude of around 3000 m above

sea level. The bacterium that formed the largest halo on rhodamine B-olive oil plates was selected for this study. This bacterium formed yellowish-white, smooth, mucoid, and large colonies on solid media, and further studies on this lipase-producing strain showed that the strain was gram-negative, rod-shaped, aerobic, catalase, and oxidase-positive, indicating that the organism could be a species of *Pseudomonas*. This was confirmed by DNA sequencing and BLAST analysis of its partial 16S rRNA gene sequence, which showed a sequence identity of 99% with *Pseudomonas fluorescens* PfO-1 and a phylogenetic tree constructed using the blast results clustered the organism with *Pseudomonas fluorescens* strains (Figure 1). Thus the organism was referred to as *Pseudomonas fluorescens* KE38. The 16S rRNA sequence was deposited in GenBank with accession number HQ215545. KE38 was capable of growing on minimal medium supplemented with 1% olive oil as the sole carbon and energy source. The optimal and highest temperatures for growth of this strain were 25 °C and 30 °C, respectively. It also exhibited growth at 4 °C. Furthermore, the extracellular lipase activity was detected only when the strain was grown at temperatures between 4 °C and 25 °C. No activity was detected when cells were grown at 30 °C.

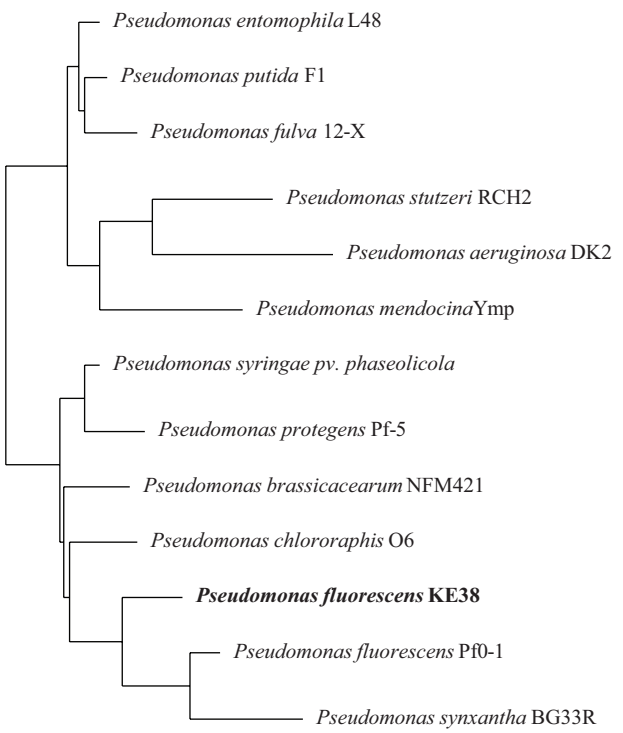


**Figure 1.** Phylogenetic tree of bacteria *Pseudomonas fluorescens* KE38. A neighbor-joining tree showing the phylogenetic position of *Pseudomonas fluorescens* KE38, among bacteria whose whole genome was sequenced, based on 16S rRNA gene sequence.

### 3.2. Growth and lipase production of *Pseudomonas fluorescens KE38*

Maximum extracellular lipase activity for cells grown on minimal medium plus olive oil was observed on day 4 of incubation at 25 °C. After day 4, which corresponds to the end of the stationary phase of growth (Figure 2), lipase activity decreased. The decrease in lipase activity at the later stage could be due to pH change, proteolytic degradation of the enzyme by proteases released into the culture medium at the end of the exponential phase of the growth, or both (Wang and Chen, 1998). Moreover, lipase production was correlated with growth (Figure 2). These results are in accordance with earlier studies, which reported maximum lipase production during the stationary phase of bacterial growth (Chung et al., 1991; Shimizu et al., 2003).

### 3.3. Purification of the extracellular lipase from *Pseudomonas fluorescens KE38*

The purification steps of extracellular lipase secreted by *Pseudomonas fluorescens* KE38 are summarized in Table 1. The molecular weight and purity of lipase were confirmed by SDS-PAGE (Figure 3). The molecular mass of purified lipase was estimated to be approximately 43 kDa after Coomassie Brilliant Blue staining. Most of the lipases from the genus *Pseudomonas* have been reported to have molecular weight in the range of 30–62 kDa (Arpigny and Jaeger, 1999), such as *Pseudomonas monteilii* TKU009 with 44 kDa (Wang et al., 2009), *Pseudomonas pseudoalcaligenes* F-111 with 32 kDa (Lin et al., 1996), *Pseudomonas aureginosa* with 54 kDa (Karadzic et al., 2006), and *Pseudomonas* sp. LP7315 with 59 kDa molecular masses (Sakiyama et al., 2001).

### 3.4. Substrate specificity of the extracellular lipase

In order to determine the effect of fatty acid chain length on the specificity of the purified lipase, various *p*-nitrophenyl

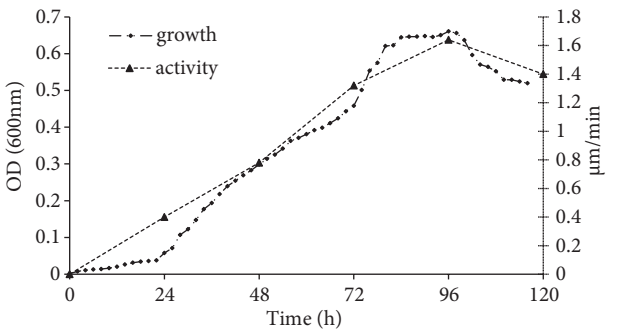


**Figure 2.** Lipase production and growth curve of *Pseudomonas fluorescens* KE38. The bacterium was grown at 25 °C for 5 days with shaking (200 rpm) in the presence of 1% (v/v) olive oil in minimal medium. OD was measured every 2 h. For the assessment of lipase activity, a sample from the culture medium was taken every 24 h and assessed for activity.

**Table 1.** Purification of extracellular lipase from *Pseudomonas fluorescens* KE38.

| Purification method | Total activity (U[a]) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Purification fold |
|---|---|---|---|---|---|
| Crude extract | 334.46 | 40.78 | 8.2 | 100 | 1 |
| Ammonium sulfate recipitation | 229.05 | 1.76 | 130.4 | 68.35 | 15.9 |
| Sephadex G-100 | 196.73 | 0.78 | 252.17 | 58.82 | 30.74 |
| Ultrafiltration | 183.95 | 0.55 | 337.33 | 54.99 | 41.12 |

a: mmole of r-nitrophenol released per min

esters were tested (Figure 4). The lipase preferentially acted on *p*-nitrophenyl esters with $C_8$–$C_{12}$ acyl groups as substrates. The activity was maximal with *p*-nitrophenyloctonate (C8) and 95% with *p*-nitrophenyldecanoate (C10) and 91% with *p*-nitrophenyllaurate (C12). Although the activity of the enzyme decreased on long chain substrates (>14 carbon atoms), it still had 42%, 38%, and 28% activities on *p*-nitrophenylmyristate (C14), *p*-nitrophenylpalmitate (C16), and *p*-nitrophenylstearate (C18), respectively, making the KE38 lipase an enzyme with a broad substrate specificity. The lipase from *Pseudomonas fluorescens* KE38 was found to hydrolyze longer-chain fatty acid esters compared with lipases from other psychrotrophs. For instance, although the lipase from *Pseudomonas* sp. 7323 also had maximal activity towards *p*-nitrophenyl decanoate (C10), it showed less than 20% activity towards *p*-nitrophenyl laurate (C12) and *p*-nitrophenyl myristate (C14), and almost no activity towards *p*-nitrophenyl palmitate (C16) and *p*-nitrophenyl stearin (C18) (Zhang and Zeng, 2008). Cold active lipases from *Pseudomonas* sp. B11-1 (Choo et al., 1998), *Pseudomonas fragi* strain IFO 3458 (Alquati et al., 2002), and *Pseudomonas* sp. strain KB700A (Rashid et al., 2001) were reported to show the highest activity towards short to medium chain (C4–C10) fatty acids with very low or no activity towards longer chain fatty acid esters (C10–C18). Such enzymes are usually are regarded as esterases rather than as true lipases (Jaeger et al., 1999).

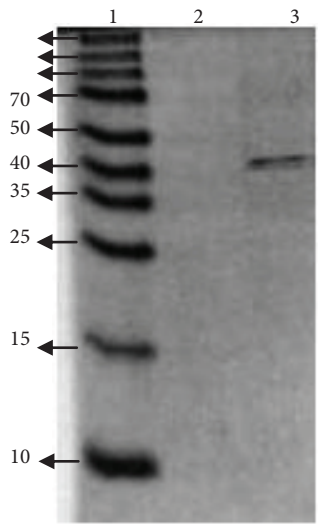


**Figure 3.** SDS-PAGE analysis of purified lipase from *Pseudomonas fluorescens* KE38. The protein was stained with Coomassie brilliant blue. Molecular weight markers are indicated on the left. Lane 1: Protein size marker (260, 140, 100, 70, 50, 40, 35, 25, 15, 10 kDa). Lane 3: purified lipase.

### 3.5. Effect of temperature on the activity and stability of the extracellular lipase

Lipase activity was examined in the temperature range of 4–65 °C, and the maximum activity was observed at 45 °C (Figure 5). The enzyme was also active at cold temperatures, retaining 30% of its activity at 20 °C. It showed no activity at 0 °C. Similar to our isolate, most mesophilic or psychrotrophic gram-negative lipase producers have been shown to have lipases with maximum activity at much higher temperatures than the optimal growth temperatures of their source organisms (Angkawidjaja and Kanaya, 2006). Several cold active *Pseudomonas* lipases were reported to have maximum activity in the temperature ranges of 20–45 °C (Choo et al., 1998; Rashid et al., 2001; Alquati et al., 2002; Zhang and Zeng, 2008). At 55 °C *Pseudomonas fluorescens* KE38 lipase lost 41.8% of its maximum activity, and only 7.4% of its activity remained at 65 °C. Thus, temperatures above 55 °C adversely affected the lipase activity. To examine

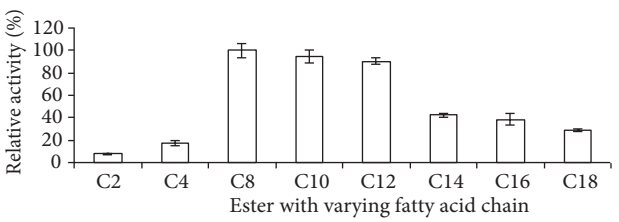


**Figure 4.** Substrate specificity of the lipase from *Pseudomonas fluorescens* KE38. Lipase activity assay was performed at 45 °C, pH 8.0, using *p*-nitrophenyl esters of varying fatty acid chain as substrates. The activity towards *p*-nitrophenyloctonate (C8) was taken as 100%.

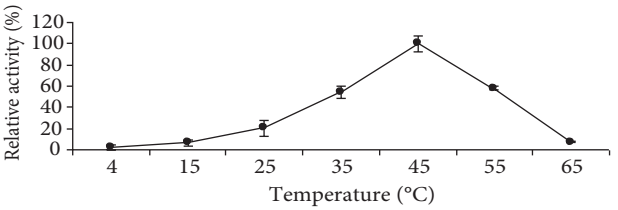


**Figure 5.** Effect of temperature on the activity of the extracellular lipase from *Pseudomonas fluorescens* KE38. Lipase activities were monitored at the indicated reaction temperatures, in 50 mM Tris-HCl buffer, pH 8.0, using p-nitrophenyl laurate as substrate. The maximum activity at 45 °C was taken as 100%.

the thermal stability of the purified lipase, the enzyme was allowed to stand at pH 8.0 for 45 min at different temperatures (35, 45, 55, 65, and 75 °C) and for 19 h at 4 °C. Then the residual activity was measured (Figure 6). The enzyme is highly stable at temperatures below and at 45 °C with almost no loss in activity, and it retained almost half of its activity at temperatures of 55 °C and 65 °C. However, there was no activity at 75 °C. The enzyme exhibited high stability at 4 °C even after 19 h of incubation with 72% residual activity. These results indicate that the stability of the KE38 extracellular lipase is higher than that of other lipases previously reported (Choo et al., 1998; Mayordomo et al., 2000; Lee et al., 2003; Joseph et al., 2006; Zhang and Zeng, 2008; Cai et al., 2009; Yuan et al., 2010; Yadav et al., 2011). This is an important property especially for enzymes intended to be used for long periods in industrial applications.

### 3.6. Effect of pH on the activity and stability of the extracellular lipase

pH is an important parameter affecting the activity of enzymes due to its effect on the structure of proteins. The lipase activity was examined in the pH range of 4.0–12.0, and the optimum activity was observed at pH 8.0 (Figure 7). The lipase retained 65.11% and 33.14% of its maximum activity at pH 9.0 and 10.0, respectively. In

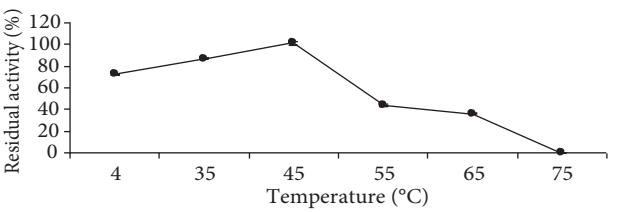


**Figure 6.** Effect of the temperature on the stability of the extracellular lipase from *Pseudomonas fluorescens* KE38. The thermal stability of KE38 lipase was investigated by pre-incubating the lipase at different temperatures (35, 45, 55, 65, and 75 °C for 45 min and 4 °C for 19 h). Residual lipase activity after incubation at each temperature was assayed. Lipase activity at 45 °C, pH 8.0, without any prior incubation was taken as 100%.

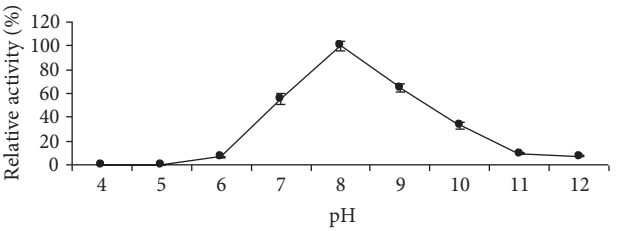


**Figure 7.** Effect of pH on the activity of the extracellular lipase from *Pseudomonas fluorescens* KE38. The lipase activity was assayed in buffers of different pH values at 45 °C, using p-nitrophenyl laurate as substrate. The buffer systems were sodium acetate buffer (pH 3.0–5.0), potassium phosphate buffer (pH 5.0–7.0), Tris-HCl buffer (pH 7.0–9.0), and glycine-NaOH buffer (pH 9.0–12.0). The maximum activity at pH 8.0 was taken as 100%.

the acidic range, significant reduction in enzyme activity was observed. For instance, at pH 6.0 the lipase retained only 6.48% of its maximum activity. The enzyme lost all its activity below pH 6.0. In order to investigate the pH stability, the enzyme was allowed to stand for 100 min at different pH values (pH 6.0–10.0) at 45 °C, and then the residual activity was measured (Figure 8). The purified lipase showed maximum stability at pH 8.0, retaining 65% activity. At pH 7.0, the enzyme retained 50% of its activity and started to lose activity below pH 6.0. Moreover, the enzyme quickly lost most of its activity with remaining activity of 32% and 15% for pH 9.0 and 10.0, respectively. These results showed that the enzyme was an alkaline lipase as with most lipases (Joseph et al., 2008; Rajan and Nair, 2011; Ülker et al., 2011), and highly sensitive to pH changes.

### 3.7. Effect of metal ions on the extracellular lipase activity

Metal ions are responsible for the maintenance of the stability of lipases (Ahmed et al., 2010). Thus, to find out whether the different metal ions stabilize or destabilize the enzyme, the effects of $Ca^{2+}$, $Mg^{2+}$, $K^{1+}$, $Na^{1+}$, $Mn^{2+}$, $Ni^{2+}$,

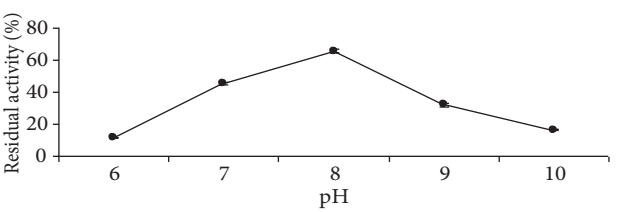


**Figure 8.** Effect of pH on the stability of the extracellular lipase from *Pseudomonas fluorescens* KE38. The pH stability of KE38 lipase was investigated by pre-incubating the lipase at different pH (6.0–10.0 at 1 unit intervals) values for 100 min. Residual lipase activity after incubation at each pH was assayed. Lipase activity at 45 °C, pH 8.0, without any prior incubation was taken as 100%.

$Zn^{2+}$, and metal chelator EDTA at 1 mM on lipase activity were investigated and the results are summarized in Table 2. Based on the results, $Ca^{2+}$ and $Ni^{2+}$ stimulated the enzyme activity by 3.5% and 9.5%, respectively. However, not much decrease in the lipase activity was observed in the presence of $Mn^{2+}$, $K^{1+}$, $Mg^{2+}$, or $Na^{1+}$ as the enzyme retained more than 83% of its activity. $Zn^{2+}$ inhibited the activity by 21.4% as for the other *Pseudomonas* lipases (Choo et al., 1998; Rashid et al., 2001; Alquati et al., 2002; Zhang and Zeng, 2008) but to a lesser extent. In the presence of EDTA, the enzyme lost about half of its maximum activity, suggesting that the lipase was a metalloenzyme. These results indicate that the extracellular lipase from *Pseudomonas fluorescens* KE38 is highly stable in the presence of various metal ions. Moreover, the activity of KE38 lipase in the presence of $Ni^{2+}$ was a distinguishing characteristic compared to other *Pseudomonas* lipases, since they were generally activated only by $Ca^{2+}$, but not by $Ni^{2+}$ (Joseph et al., 2008).

### 3.8. Effect of various organic solvents on the extracellular lipase activity

Organic solvent stability of enzymes is a desirable feature especially in synthesis reactions such as esterification and transesterification (Gupta et al., 2004). In order to investigate the effect of organic solvents on lipase activity, ethanol, acetone, methanol, acetonitrile, n-hexane, DMSO, dimethylformamide, and 2-propanol were used at a concentration of 30% (v/v). DMSO and dimethylformamide activated the enzyme by 191% and 113%, respectively (Table 3). The enzyme retained about 25%, 75%, and 59% of its activity in the presence of ethanol, acetone, and methanol, respectively. Acetonitrile and 2-propanol strongly inhibited lipase activity, whereas n-hexane completely inhibited enzyme activity. There are some possible explanations for the stimulatory effects of organic solvents on enzyme activity. The solvent may enhance enzyme activity without causing any denaturation by modifying the oil–water interface. Secondly, solvents may make changes in the 3-dimensional structures of the enzyme or may prevent the disaggregation of lipase (Ahmed et al., 2010). The solvents showing negative effects on lipase activity may distort the active site of the enzyme, thus preventing the substrate from accessing the enzyme active site efficiently (Peng et al., 2010). DMSO and dimethylformamide preference of the enzyme was a distinguishing feature compared to the lipases from other psychrotolerant *Pseudomonas* species such as *Pseudomonas* sp. B11-1 (Joseph et al., 2008). The lipase from *Pseudomonas fluorescens* KE38 may have potential use in organic synthesis and chiral resolution reactions that involve the use of DMSO and dimethylformamide as solvents.

**Table 2.** Effect of various metal ions on extracellular lipase activity from *Pseudomonas fluorescens* KE38. Lipase activity assay was performed at 45 °C, pH 8.0, in the presence of 1 mM various metal ions. Activity of control (no metal ions in the mixture) was taken as 100%.

| Metal ions | Relative activity (%) |
|---|---|
| Control | 100 |
| $CaCl_2$ | 103.49 |
| $MgCl_2$ | 86.94 |
| KCl | 87.02 |
| NaCl | 83.14 |
| $MnCl_2$ | 94.78 |
| $NiCl_2$ | 109.5 |
| $ZnCl_2$ | 78.6 |
| EDTA | 52.22 |

**Table 3.** Effect of various organic solvents on extracellular lipase activity from *Pseudomonas fluorescens* KE38. Lipase activity assay was performed at 45 °C, pH 8.0, in the presence of 30% (v/v) various organic solvents. Activity of control (no organic solvents in the mixture) was taken as 100%.

| Organic solvents | Residual activity (%) |
|---|---|
| Control | 100 |
| Ethanol | 24.76 |
| Methanol | 58.74 |
| Acetone | 74.71 |
| Acetonitrile | 1.56 |
| n-hexane | 0 |
| DMSO | 291 |
| Dimethylformamide | 213.37 |
| 2-propanol | 1.61 |

In conclusion, an extracellular bacterial lipase with high stability, broad substrate specificity, and activity in the presence of organic solvents and metal ions was successfully purified and its preliminary biochemical characterization was performed. Studies with the psychrotrophic *Pseudomonas* lipases have been very limited (Joseph et al., 2008). Therefore, this study indicates the production of an organic solvent tolerant alkaline lipase with a broad substrate specificity and high stability from a new *Pseudomonas* species. These results suggest that the extracellular lipase enzyme produced by KE38 has the potential to be used as a biocatalyst in synthesis reactions or in industrial applications.

**Acknowledgment**

This work was funded by the State Planning Agency of Turkey.

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