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Characterization of a novel lipase from *Pseudomonas aeruginosa*

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Abstract

Lipases cleaving oils into fatty acids and glycerol are of great interest for the use in increasing the efficiency of fuels. In this work, a novel lipase from *Pseudomonas aeruginosa*, *P. aeruginosa* A12, was isolated by ion-exchange and hydrophobic chromatography. The purity of lipase was shown by electrophoresis and its molecular weight was estimated to be ~ 31.6 kDa. The whole amino acid sequence was analyzed by an LC-MS/MS method. Temperature- and pH-dependent optimum of the enzyme compiled 30 °C and 7.5, respectively. The obtained enzyme exhibited 79 % similarity of amino acid sequence to a lipase isolated from the same strain of *P. aeruginosa*. Thus, the novel lipase was determined to belong to I.1 subfamily of bacterial true lipases. Three dimensional structure of the isolated lipase isoform was modeled based on obtained sequences. Amino acids forming the catalytic domain were shown in the model. Lid domain is suggested to be in the open conformation. These results provide a potential alternative for enzymatic digestion of fuel oils and serve for the development of fundamental knowledge of lipase activity.

Introduction

Lipases (EC 3.1.1.3) are triglyceride ester hydrolases that cleave ester bonds in glycerides (Balcao *et al.* 1996). Bacteria are considered as their most reliable source that can express them even in coal mines (Ali *et al.* 2015) or geothermal springs (Shahinyan *et al.* 2017). Several isoforms of lipases have been isolated from bacterial cultures (Verma and Sharma 2014) and found applications in food, dairy, and detergent industries (Gupta *et al.* 2004; Sharma *et al.* 2013).

Lipases largely differ by biochemical parameters. The enzymes isolated from *P. aeruginosa* reveal different MW, pH and temperature optimum. A 54 kDa lipase isolated from *P. aeruginosa* was determined to have pH optimum in the range 6 – 9 (Karadzic *et al.* 2006). The 29 kDa lipase with high activity towards olive oil was found to have optimum at moderate temperatures (Gilbert *et al.* 1991). Activity of this lipase, suggested to be a metalloprotein, was inhibited by metal ions such as Hg^{2+}, Zn^{2+}, Cu^{2+}, Ag^{2+} and Fe^{2+}. Its temperature and pH optimum were defined to be 55 ºC and 6.9, respectively (Borkar *et al.* 2009).

The active center of 70 % of lipases isolated from filamentous bacteria includes the amino acid sequence Gly-His-Ser-Leu-Gly-Gly-Ala, and the oxyanion gap consists of the sequence Ile-Val-Val-Tyr-Leu-Val-Ile-Ala-Val-Ser-Phe-Arg-Gly; these two active sites are separated by 72 amino acids (Mancheño *et al.* 2003). The Ser-16, Asp-116

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and His-291 as triad were concluded reveal catalytic activity of lipase isolated from *Aeromonas hydrophila* mainly found in regions of warm climate (Brumlik and Buckley 1996). The three-dimensional structures of lipases show their active site association with α-helices and a central *β*-sheet including active Ser (Arpigny and Jaeger 1999). Lack of disulphide bridges in lipases likely increases their conformational flexibility under cold conditions (Alquati *et al.* 2002).
In this work a new isoform of 31.6 kDa lipase was isolated from *P. aeruginosa* strains. All biochemical parameters were determined and its full amino acid sequence was determined by Mass-spectrometry analysis. Three dimensional structure of the lipase was modeled using SWISS-MODEL software. Lipases with specific properties are promising tools for obtaining biodiesel fuels from fats.

Experimental

Growing bacterial cultures

The cultivation was performed in magnetic rocking chair in 250 mL Erlenmeyer flasks with a circular rotation of 100 rpm, at a temperature of 25 – 26 °C. Then, the resulting cultural liquid was filtered, centrifuged at 6,000 rpm, and lyophilized. Lipolytic activity of three bacterial strains *Pseudomonas aeruginosa*, *Chromobacterium violaceum* and *Bacillus stearothermophilus* were studied at the earliest stage of our studies. Bacteria were grown on a nutrient medium containing 1 % (v/v) cottonseed oil. The growth dynamics and kinetic curves of lipase accumulation in bacterial cultural liquids were observed during 4 d of cultivation in 12 h intervals.

Purification and preparative isolation of lipase

The lipase enzyme was purified from lyophilized bacterial cells as described. Lyophilized culture fluid of *P. aeruginosa* with a specific activity of 129 $U.mg^{-1}$ was used for purification of lipase as a source for enzyme preparation.
At the beginning, an enzyme preparation in the amount of 1 g was dissolved in 20 mM phosphate buffer (pH 8.0, 65 mL) and dialyzed in the same buffer. Further the enzyme solution was concentrated to 10 mL using a 10 kDa membrane (Millipore), to the protein concentration 2.2 $mg.mL^{-1}$.

Chromatographic purification

After dialysis of protein extract, purification was performed on DEAE-TSK 650 column (16 mm – 200 mm) using 20 mM phosphate buffer (pH 8.0). Single-stage gradient of 0 – 0.2 M and 0.2 – 1.0 M NaCl in the same buffer was used. The flow rate was 0.5 $mL.min^{-1}$. The proteins present in the eluate were monitored by measuring the absorption at 280 nm. Further purification of bacterial lipase was carried out on the column (16 mm – 200 mm) filled with Phenyl-TSK 650. Phosphate buffer (20 mM, pH 8.0) was used with a gradient of ethyl alcohol (0 – 60 %; v/v) in the same buffer. The flow rate of eluent was 0.5 $mL.min^{-1}$. The proteins present in the eluate were monitored by measuring the absorption at 280 nm.

Lipase activity

Determination of lipase activity was carried in various buffer systems with a pH from 5 to 11, at room temperature, using the pH-stat method in time-dependent and temperature-dependent manner. Tributyrin (98 %, Sigma Aldrich) was used as a substrate.
Temperature-dependent activity was determined in 0.1 mM sodium phosphate buffer (pH 7.5), and the enzyme solutions were incubated in a water bath at the range of 20 – 60 °C. The enzyme activity was measured after 1 h after incubation. Aliquots were taken every 60 min for 15 h.
pH-dependent lipase activity was studied in various buffer systems in the pH range of 5 – 11 at room temperature. Aliquots were taken every 60 min for 15 h. The quantity of soluble proteins was determined by Lowry method (Lowry *et al.* 1951).

Isolation and purification of proteins from PA gel

Standard SDS-PAGE was performed with 15 μL aliquot of purified proteins and staining was carried out by silver-stain method (Switzer *et al.* 1979).

The spot (fraction) corresponding to the purified lipase was isolated from the gel. SDS residues were removed by treating with 40 % (v/v) methanol, 5 % (v/v) acetic acid, and incubation at room temperature for 15 min. The solution was discarded. The stain was removed by incubating with 50 mM ammonium bicarbonate in 50 % (w/v) acetonitrile for 15 – 20 min at 56 – 60 °C. The procedure was repeated a few times until complete destaining. Then, gel with the sample was washed with deionized water 3-times and were further reduced with 50 – 65 mM DTT in 50 mM ammonium bicarbonate and incubated for 45 min at room temperature. It was subsequently alkylated with 100 µL of 20 mM iodoacetamide in 50 mM ammonium bicarbonate, keeping in dark for 50 – 60 min. Gel samples were dehydrated in acetone incubating until they turned white colour.

Preparation of protein solution for LC-MS/MS analysis

Lipase was digested with trypsin at 37 °C for 15 h. The reaction was terminated by the addition of formic acid. In order to extract enzyme from the gel, gel sample was dried at 45 °C, treated with 0.1 M trifluoroacetic acid, and kept in ultrasonic bath for 10 min and further was centrifuged for 5 min (12,000 rpm). The solution was collected into 1.5 mL tube. The procedure was repeated. Extract was lyophilized (Virtis 2KBTES) for mass analyses.

Mass spectroscopy analysis

The digested protein samples (2 – 5 µL) were subjected to CHIP (150 µm × 43 mm) Zorbax SB C18 column in Agilent Technologies 1260 Cap Pump with a flow rate of 4 µL/min, elution rate 0.6 $\mu L.min^{-1}$. B solution was managed as following: 5 % (3 min), 80 % (25 – 30 min). The solution was degassed in Agilent Technologies 1260 µ-degasser. Mass detection was performed in CHIP-Q-TOF LC-MS Agilent Technologies 6520B Series mass spectrometer. Mass data was recorded in positive ionization mode (ionization source: CHIP interface, gas flow rate: 4 $L.min^{-1}$, 350 °C, skimmer current power: 65 V, MS/MS: 50-2400 m/z, mobile phase: A – 0.1 % formic acid, B – acetonitrile + 0.1 % formic acid, v/v in all cases), and partial sequences were identified with Spectrum Mill database.

3D structure homology modelling

A 3D model of *P. aeruginosa* A12 was built by SWISS-MODEL modeling program based on crystal structure lipase PAO1. Lid region was also predicted comparing with the same lipase exhibiting 79 amino acid sequence similarity (Nardini *et al.* 2000) with the *P. aeruginosa* A12.

Results and Discussion

Bacterial strains *C. violaceum*, *B. stearothermophilus*, *P. aeruginosa* with high lipase production were cultivated as described. The growth dynamics of cells and also kinetic curves of lipase activity in bacterial cultures were determined during 4 d of cultivation period in 12 h intervals. The data obtained using 1 % (v/v) cottonseed oil as inductor of lipases show that the lipase activity in cultured bacteria *C. violaceum* reached the maximum after 48 h and 84 h were determined as the maximum point for *B. stearothermophilus* and *P. aeruginosa* (Fig. 1). *P. aeruginosa* had the greatest ability to synthesize the enzyme, which is rapidly secreted into the culture medium after 36 h of cultivation, reaching the maximum for 84 h of growth. The lipase secreting abilities of the strains' cultures were as follows: *C. violaceum* < *B. stearothermophilus* < *P. aeruginosa*. (Fig. 1).

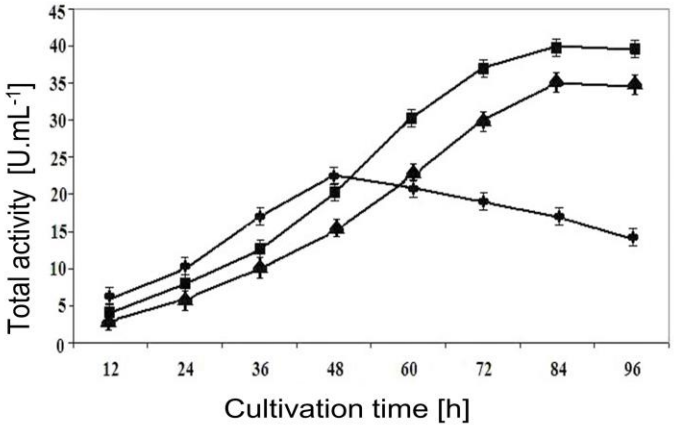

Fig. 1. Total lipase activities of bacterial cultures of strains *C. violacheum* (circles), *P. aeruginosa* (squares) and *B. stearothermophilus* (triangles) (n = 4, RSD ± 5%).

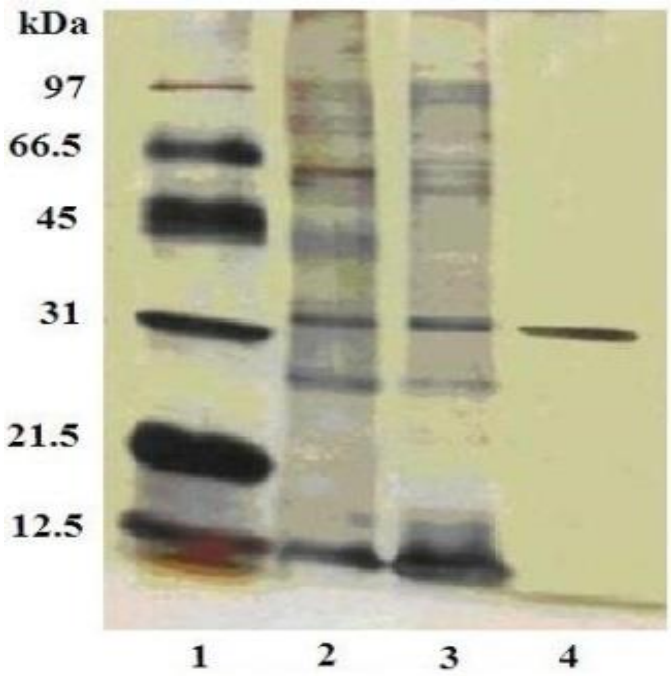

Fig. 2. Electrophoretic analysis of bacterial lysate at individual steps of lipase extraction procedure. Line 1 – marker proteins; 2 – concentrated bacterial extract; 3 – after DEAE-TSK 650 column; 4 – after Phenyl-TSK 650 column.

For further studies, *P. aeruginosa* was cultivated in an optimized nutrient medium for 72 – 96 h; again, 1 % cottonseed oil was used for the induction of the enzyme.The degree of enzyme purification after each stage was determined by electrophoretic analysis. Purification was repeated until a single fraction of 31 kDa lipase was obtained (Fig. 2).

At the first purification process culture liquid, the specific activity of which equaled 129.0 U.mg^{-1}, was dialyzed against water and concentrated. The specific activity of the obtained extract equaled 262.7 U.mg^{-1}. At the next stage of purification ion-exchange chromatography was employed. The quantity of the obtained lipase fractions (12 – 15 fractions in Fig. 3 A) was a total of 22 mg protein in 10 mL, and the specific activity made 723.0 U.mg^{-1}. In the obtained chromatograms five protein peaks were observed. The second peak belonged to a lipase (Fig. 3 A) was used for further purification.

The lipase was completely separated from other proteins by hydrophobic interaction chromatography. The volume of the collected fraction compiled 5.5 ml with a specific activity 1009 U.mg^{-1} and the total protein made 0.825 mg (3 – 6 fractions in Fig. 3 B).

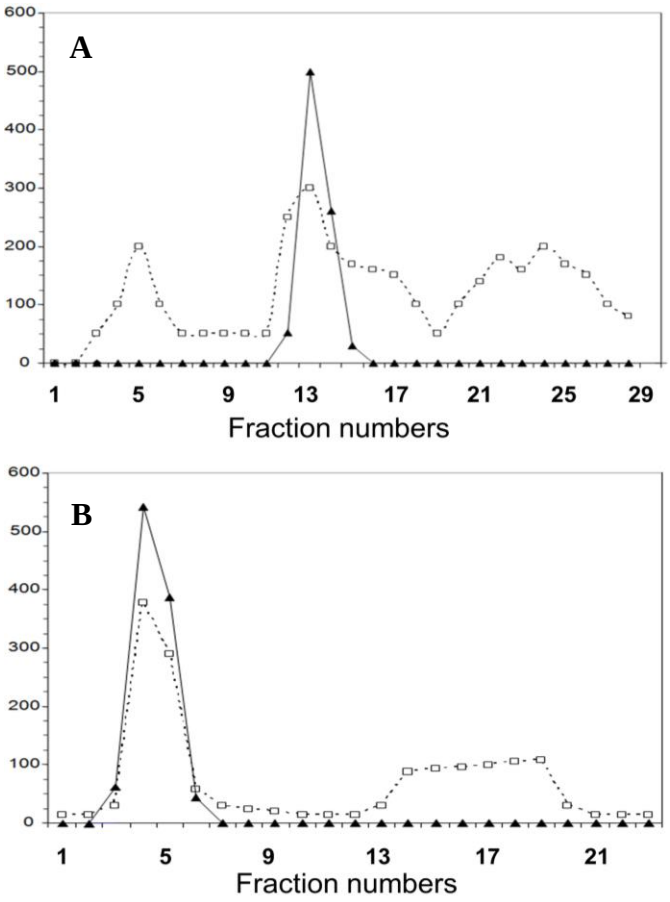

Fig. 3. Column chromatography purification of novel lipase *P. aeruginosa* A12. Chromatograms were obtained by ion-exchange chromatography (**A**) and hydrophobic interaction chromatography (**B**). The optical density of fractions (280 nm) is presented as squares, and corresponding lipase activities (U.mg^{-1}) as filled triangles (n = 4, RSD ± 5 %).

The overview data of protein quantity and enzyme activity concerning novel *P. aeruginosa* A12 lipase purification steps are presented in Table 1.

We determined the properties of the purified lipase *P. aeruginosa* A12. The results showed that the optimal pH value is 7.5 (Fig. 4), at which the lipase retains 95 % of the initial activity for 15 h (data not shown). High activity of the enzyme was observed in the range of pH 7 – 8 (Fig. 4).

Table 1. Purification of lipase from the culture fluid of *P. aeruginosa* (n = 5, RSD ≤ 4 %).

Purification stage	Volume [mL]	Protein [mg.mL^{-1}]	Total protein [mg]	Specific activity [U.mg^{-1}]
Culture liquid	65.0	0.90	58.5	129.0
Conc. liquid after dialysis	10.0	2.20	22.0	261.7
DEAE-TSK 650	4.0	0.60	2.40	723.0
Phenyl-TSK 650	5.5	0.15	0.825	1,009

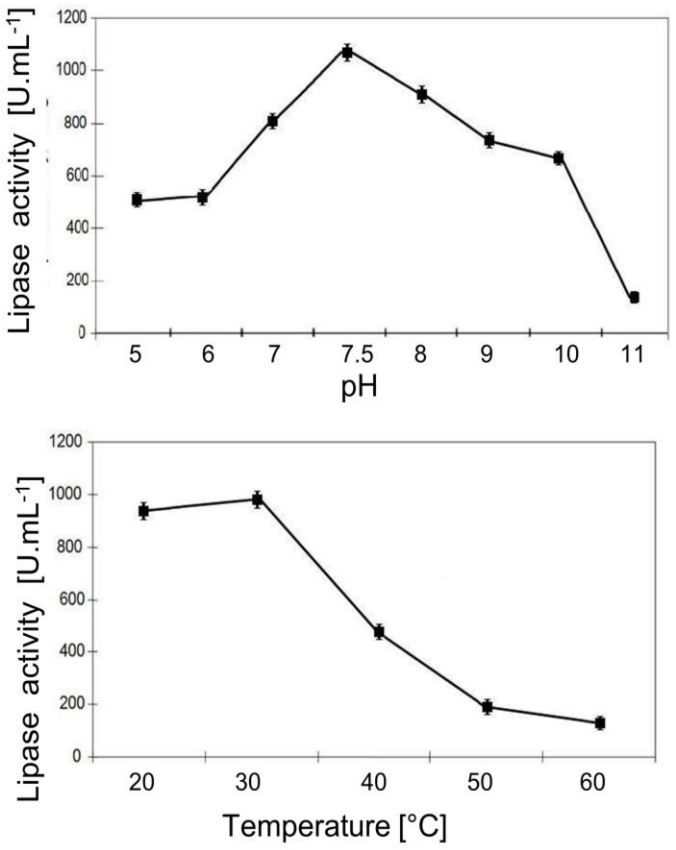

Fig. 4. Optimum conditions of lipase isolated *P. aeruginosa* (n = 4, RSD ± 5 %).

The enzyme incubated for 1 h at a temperature of 30 °C showed a maximum specific activity of 1,009 U.mg^{-1}; after 15 h the activity was reduced to 90 %, equalled to 101 U.mg^{-1} (data not shown). At higher temperatures, the lipase activity rapidly dropped. At 50 °C, for instance, the enzyme activity in one h reduced to 85 % (to 115.2 U.mg^{-1}), and to 99 % in 15 h, respectively (Fig. 4).

In this work, we purified a total of 82.5 mg homogeneous enzyme of 31 kDa with 1,009 U.mg^{-1} specific activity from 10 g of lyophilized bacterial preparation. The obtained parameters for enzymatic activity of this lipase (pH and temperature) correspond to other literature data (Table 2). Lipase isolated from *P. tolaasii* demonstrated highest enzyme activity at pH 7.0 and 35 °C. At 21 °C and pH 7.0, it retained activity for 48 h, and was inhibited by EDTA, *o*-phenanthroline, PMSF (phenylmethylsulfonyl fluoride, a serine protease inhibitor) but reactivated by Ca^{+2} (Baral and Fox 1997).

Biochemical analysis and RNA sequencing revealed a new *P. aeruginosa* strain, T1, revealing a lipase activity in the presence of mineral salts, which can degrade different types of edible oils at 30 °C. Noteworthy, it was active in the broad range of 15 – 50 °C (Hasanuzzaman *et al.* 2004). Further, the pH and temperature optima of partially purified lipase from *P. aeruginosa* AAU2 was reported as 7.5 and 40 °C, with preference for longer carbon chain fatty acid esters (Bose and Keharia 2013).

Depending on growing conditions of bacterial strains, lipases differ by their pH and temperature optimum (Table 2). The novel lipase, studied in this work, was found to be strongly inhibited by methanol; efficiency of transesterification was dramatically reduced by concentrations of 1 – 4 M (data not shown). On contrary higher concentration of ethanol enhanced formation of mono-, di-, and triglycerides (data not shown). *P. aeruginosa* A12 revealed maximum activity at 30 °C.

Table 2. Biochemical parameters of lipases isolated from *Pseudomonas* strains.

Bacterial strain	MW [kDa]	pH [optimum]	Temperature [optimum]	Reference
P. aeruginosa A12	31.6	7.5	30 °C	This work
Pseudomonas aeruginosa	54	11.0	70 °C	Karadzic *et al.* 2006
Pseudomonas tolaasii		7.0	35 °C	Baral and Fox 1997
Pseudomonas aeruginosa KM110		8.0	45 °C	Mobarak-Qamsari *et al.* 2011
Pseudomonas aeruginosa EF2	29			Gilbert *et al.* 1991
Pseudomonas aeruginosa SRT 9	29	6.9	55 °C	Borkar *et al.* 2009
Pseudomonas aeruginosa AAU2		7.5	40 °C	Bose and Keharia 2013
Pseudomonas aeruginosa		7.0	30 °C	Zouaoui *et al.* 2012
*Pseudomonas sp.*Lp1		8.0	40 °C	Kanimozhi *et al.* 2010
Pseudomonas gessardii		7.0	37 °C	Veerrapagu *et al.* 2013
Pseudomonas fluorescens KE38	43	8.0	45 °C	Gokbulut *et al.* 2013
Pseudomonas aeruginosa sp.	58	9.0	55 °C	Gaonkar *et al.* 2018
Pseudomonas aeruginosa Ps-1,2	50	7.0	37 °C	Saadatullah *et al.* 2018

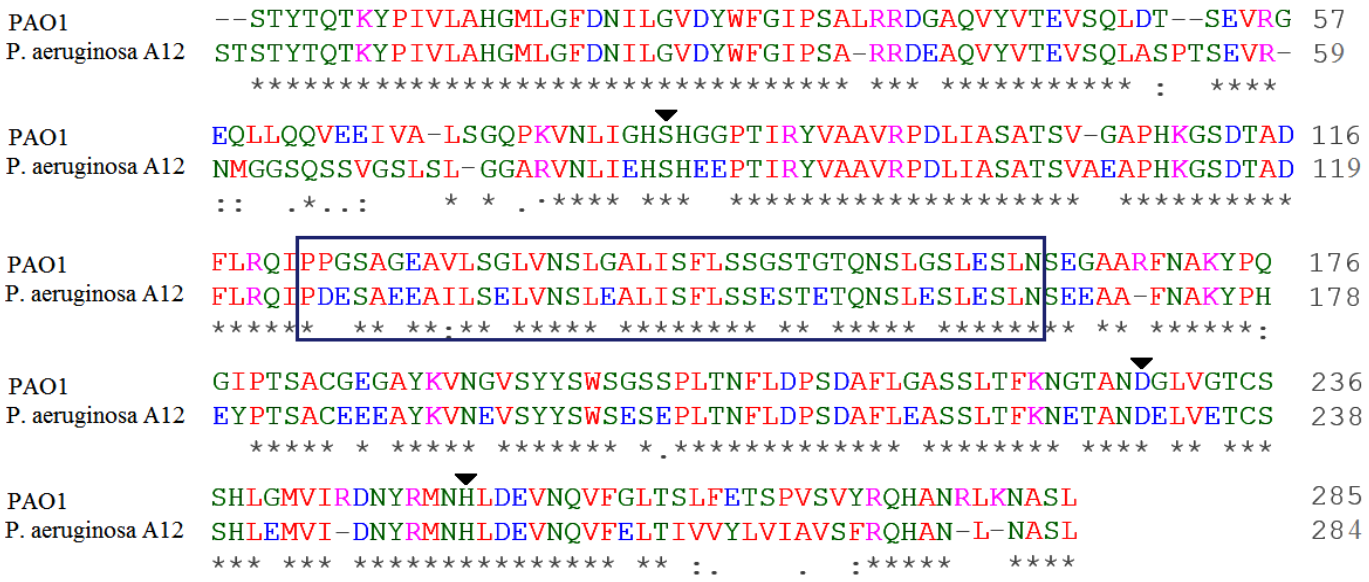

Fig. 5. Comparison of amino acid sequences of lipases isolated in this work *P. aeruginosa* A12 and PAO1 (by Nardini *et al.* 2000; Protein Data Bank deposit number 1EX9). *Amino acids determining catalytic triad such as Ser82, Asp229, and His251 are shown with a black triangle. Lid region was indicated as shown by Khan *et al.* 2017.

For comparison, enzyme activity of the lipase from *P. aeruginosa* reached maximum at 45 °C and pH range 6 – 9, and was inhibited by Zn^{+2} and Cu^{+2} to 32 and 17 %, respectively (Mobarak-Qamsari 2011).

The other 54 kDa lipase from *P. aeruginosa* had highest activity at pH 11 and 70 °C, while was inhibited strongly by Zn^{2+}, Hg^{2+}, Cu^{2+} and slightly also by Ca^{2+} and Mg^{2+} (Karadzic *et al.* 2006).

Methyl *p*-nitrophenyl *n*-hexylphosphonate was also reported to inhibit lipase activity (Rotticci *et al.* 2000). pH and temperature tolerance of *P. aeruginosa* A12 correspond to other lipases isolated from other strains of *P. aeruginosa*, found in pH interval 6 –10 (Mobarak-Qamsari *et al.* 2011; Zouaoui *et al.* 2012; Gokbulut *et al.* 2013).

Structure of the lipase from P. aeruginosa

LC MS/MS analysis revealed 14 fragments covering the whole sequence of the peptide, proved by comparison with amino acid sequence of other lipase from *P. aeruginosa*. The amino acid sequence of *P. aeruginosa* A12, identified in this work, was compared with the 32 kDa lipase from *P. aeruginosa* PAO 1 consisted of 285 amino acids (Nardini *et al.* 2000), the structure of which was earlier established in crystal. Results showed 79 % similarity between these two proteins with 60 amino acid difference (Fig. 5).

In BLAST analysis, the isolated isoform of lipases showed similarity to I.1 subfamily of EC 3.1.1.3 bacterial true lipases (Messaoudi *et al.* 2010). Besides, it exerts high amino sequence similarity to the *P. aeruginosa* lipase consisted of 311 amino acids (identity ~ 77.5 %, *E*-value $\leq e^{-140}$).

Three dimensional structure of the isolated lipase was modeled using SWISSMODEL software analysis (Fig 6 A). The catalytic triad involving Ser-Asp-His is indicated in dot and line mode (Fig 6 B). Spatial proximity of these three amino acids explains its structure-activity relationship of the novel lipase. Protein modeling enables to suggest the conservation of catalytic triad – Ser82, Asp229, and His251 (Fig. 5 and Fig.6). Lid domain in open conformation that make the active site cavity to solvent and substrate (Nardini *et al.* 2000) is suggested to be similar in *P. aeruginosa* A12. Homology modeling revealed all α-helices and β-sheets, available in PAO1 lipase, are conserved in *P. aeruginosa* A12 which is 79 % identical. Similar trends were suggested in lipases from *Rhizopus chinensis* (Yu *et al.* 2014). Lid regions in lipases have been reviewed well and by their lid domains they were divided into three groups: ones without lids, seconds lipases with one loop or one helix and lipases with two or more helixes lids (Khan *et al.* 2017). Homology modeling enables us to conclude that the novel lipase *P. aeruginosa* A12

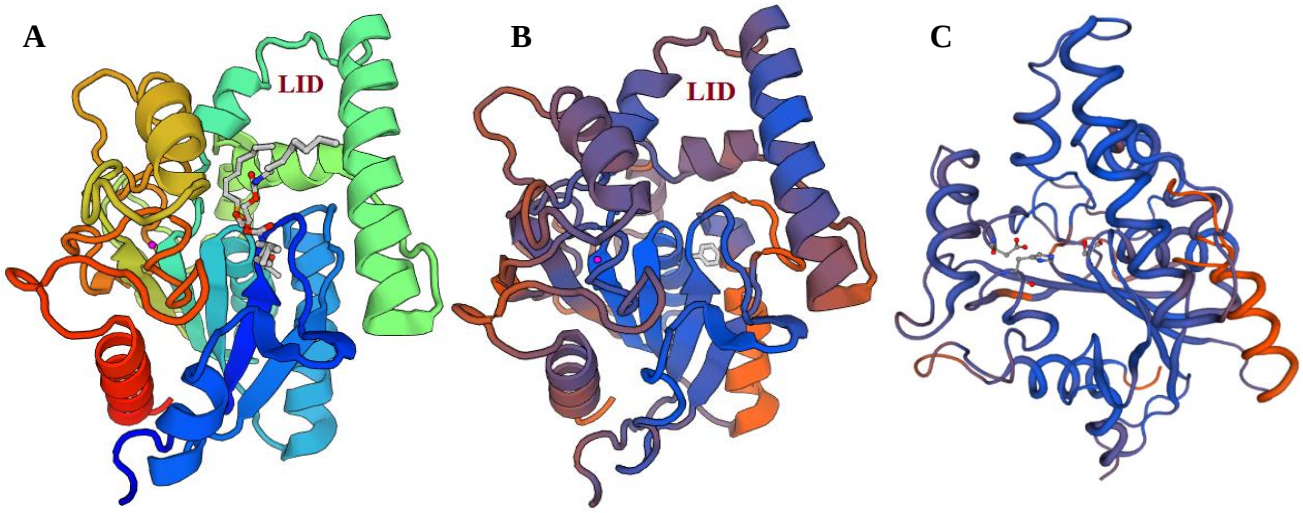

Fig. 6. 3D structure homology modelling of the lipase. (**A**) Crystal structure of PAO 1 lipase (Nardini *et al.* 2000). (**B**) 3D cartoon model of *P. aeruginosa* A12. (**C**) 3D tube model of *P. aeruginosa* A12 with structural positions of catalytic triads, indicated in dot and line mode. LID region among three α-helices was demonstrated in PAO and *P. aeruginosa* A12.

belongs to the third group that at least three α-helixes are located over the active site (Fig. 6). Based on observations lipases containing larger lipases with two or more α-helixes are expected thermostable (Khan *et al.* 2017). *P. aeruginosa* partially agrees with these observations that at 40 °C still twice lower activity was detected. However, at 50 °C lipase activity of *P. aeruginosa* A12 strongly dropped, 5-fold lower enzyme activity than that at 30 °C was determined.

Conclusions

In conclusion, a novel 31.6 kDa lipase isoform was isolated from *P. aeruginosa* strain. The pH and temperature optima were determined as 7.5 and 30 °C (respectively). Enzyme activity was found to be induced by ethyl alcohol and inhibited by methyl alcohol. Modeling three dimensional homology structure proved the spatial proximity of Ser-Asp-His catalytic triad revealed open conformation of lid region. The enzyme can be potentially interesting for biotechnological means because of its pH tolerance in the interval 6 –10 and temperature tolerance with a 2-fold difference in activity from 20 till 40 °C.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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