Characterization of a lipase from a newly isolated *Pseudomonas* sp.

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ABSTRACT

Background and Objectives: Lipases are valuable biocatalysts which are widely used in the detergent, food, dairy and pharmaceutical industries. The aims of the present study included the isolation of a lipase-producer from industrial zones and the partial characterization of the enzyme.

Materials and Methods: A number of bacteria were isolated from sites related to the oil industries. An isolate forming a halo zone in a selective medium (TW agar) was then selected and grown on a medium suitable for the production of lipase. The isolate was subsequently identified by the 16S rRNA sequencing method, and its enzyme activity was measured by a spectrophotometer using pNPP as a substrate.

Results: The selected isolate was identified by the molecular method as *Pseudomonas* sp. Its extracellular lipase activity was 41.5 ± 1.4 U/ml, and the high affinity of this enzyme for the substrate was indicated by the kinetic parameters of Km and Vm, which were estimated by the the Lineweaver-Burk plot as 0.77 mM and 49.5 U/ml, respectively. Activation energy of lipase calculated from the Arrhenius plot was found to be 20.78 kJ/mol, and a temperature coefficient (Q10) of 4.39 indicated the high catalytic activity of the enzyme and the temperature dependence of the enzymatic reaction.

Conclusion: The results demonstrated that the indigenous isolate could have potential applications in many relevant industries.

Keywords: Lipase, *Pseudomonas*, Kinetic constants, Thermodynamic parameters

INTRODUCTION

Lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) have emerged as key enzymes in biotechnology, owing to their multifaceted properties, which find usage in a wide array of industrial applications, such as food technology, detergent, chemical industry and biomedical sciences (1, 2). These biocatalysts are interesting substitutes for chemical catalysts, because of their faster rates of reaction and better specificity

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under mild conditions (3). In addition to having hydrolytic activity towards triglycerides, lipolytic enzymes are capable of catalyzing many other reactions which include esterification, interesterification, acidolysis, alcoholysis and aminolysis. They are ubiquitous in nature and are produced by various plants, animals and microorganisms. Lipases of microbial origin, mainly bacterial and fungal, represent the most widely used class of enzymes in biotechnological applications and organic chemistry (2). Amongst the important lipase producers, lipases from the *Pseudomonas* genus are widely used for a variety of biotechnological applications (2). In fact, lipases isolated from different sources have a wide range of properties depending on their sources. These include positional specificity consisting of regio- and enantio-specificity, fatty acid specificity, thermal stability, and optimum pH. One could probably find a

lipase from nature that would be suitable for a desired application. It is evident that the enzyme of interest should be characterized to determine its biochemical properties and then employed for catalysis of a specific reaction (3-6).

The main aim of this research was isolation of a lipase-producing bacterium and partial characterization of the enzyme for eventual applications in industry.

MATERIALS AND METHODS

Materials. *para*-Nitrophenyl palmitate (*p*-NPP) was purchased from Sigma Chemicals. All other chemicals were of analytical grade and were purchased from Merck. Olive oil was purchased from Oila Company (Iran).

Isolation. Samples were taken aseptically from various locations, such as the neutralization unit, area beneath the vacuum tower, wastewater from the neutralization process, destaining soil reservoir, transference pool at the Pars Ghoo, Exdaneh and Margarin factories, Tehran, Iran. The collected samples were then maintained at 4°C until further use.

Primary Screening. The samples were transferred to an enriched medium (LB broth) and incubated at 37°C, for 24 h at 200 rpm. Different dilutions (20 ul) of the incubated cultures were then spread onto LB agar plates to isolate single colonies. Each colony were then transferred to TW agar plates (containing (1/L): yeast extract, 5 g; NaCl, 10 g; tryptone, 10 g; Tween 80, 10 ml; tributyrin, 10 ml (pH 7)). The plates were incubated at 30°C for 24 h under aerobic conditions. Lipase production was detected by observation of clear zones around isolated colonies.

Lipase activity was compared qualitatively by formation of halo zones on tributyrin agar plates (composition (1/L): tributyrin, 10 ml; Tween 80, 10 ml and agar 1.5 g) after 24 h of incubation at 37°C. Twenty microliters of the supernatant was transferred to this medium by the disk diffusion agar method.

Culture medium for lipase production. The selected isolate was grown in YOP medium (composition (1/L): tryptone, 10 g; NaCl, 10 g; yeast extract, 5 g; olive oil, 10 ml; Tween 80, 10 ml) at 37°C for 16-18 h. The cell suspension was centrifuged at 5000 ×g for 15 min. The resulting supernatant was used for estimation of lipase activity.

Molecular identification. DNA was extracted using the DNA fast protocol (7). Primers used for the PCR amplification of 16S rRNA included   5$^{/}$--AGA GTT TGA TCC TGG CTT AG--3$^{/}$ (forward) and 5$^{/}$ --TAA GGA GGT GAT CCA GC--3$^{/}$ (reverse); provided by CinnaGen Co, Tehran, Iran.

The PCR products were purified using a High Pure PCR product purification kit (Roche Co, Germany). Identification was carried out by sequencing PCR products and blasting the resulting sequence against the NCBI database.

Enzyme assay. Lipolytic activity was determined by the method of Gupta et al. (2002) using *p*NPP as a substrate (8). The substrate solution contained *p*NPP 1.5 mM, acetonitrile 1%, isopropanol 4% and Tris 50 mM (pH 8). The amount of liberated *p*-nitrophenol (*p*PN) was recorded at 410 nm. One unit of lipase activity is defined as the amount of enzyme librating 1 μmol of *p*NP under standard assay conditions.

Determination of optimum pH and temperature. To study the effect of pH on lipase activity, the supernatant containing the enzyme was assayed at various pH values. For this reason, Samples (500 μl) of supernatant were incubated with 10 ml of buffer at various pH values of 5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 10. Then, 100 μl of the resulting solution was added to 900 μl of *p*NPP (as enzyme substrate) and incubated at 37°C for 10 min. The liberated *p*NP was recorded at 410 nm.

To determine the effect of temperature on lipase activity, the enzyme assay was carried out at different temperatures under standard conditions. First, 500 μl of supernatant was incubated with 10 ml of buffer at pH 8, and 100 μl of the resulting solution was added to 900 μl of *p*NPP, and then incubated at different temperatures of 20, 30, 40, 50, 60, 70°C for 10 min.

Temperature stability. Thermal stability of the enzyme was investigated by preincubation of the enzyme at various temperatures (30-90°C) for 10, 20, 30 and 60 min and subsequent measurement of enzyme activity.

All experiments were carried out in triplicate and data were presented as mean values with calculated standard deviations.

Kinetic study. The effect of substrate concentration on the reaction rate was investigated. The Michaelis-Menten constant, K_m, and the maximum velocity for
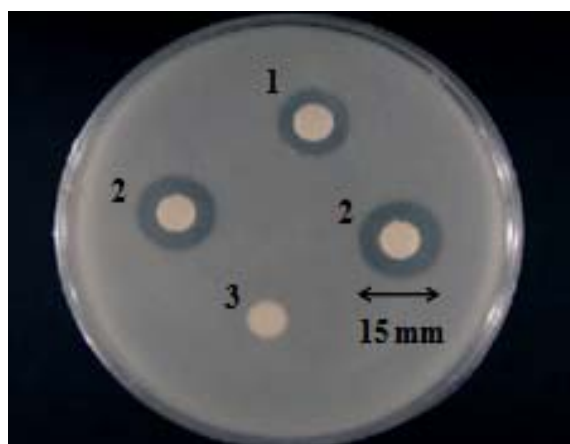
Fig. 1. Halo zone formation on tributyrin agar plate; (1) after 18 h, (2) after 24 h, (3) negative control (*Pichia pastoris* supernatant).

Fig. 2. Effect of pH on *Pseudomonas* L.M lipase activity.

the enzyme reaction, V_m, catalyzed by lipase were obtained by measurement of enzyme activity at various amounts of the substrate solution (50, 100, 200, 300, 400, 500, 600, 700, 800, 900 µl). Kinetic constants were calculated using the Lineweaver-Burk plot (Eq. 1).

$$1/V = (K_m/V_m)(1/S)+(1/V_m) \qquad [1]$$

Determination of the activation energy and thermodynamic parameters. Activation energy (E_a) was determined from the Arrhenius plot. The values for the activation enthalpy (ΔG^*), activation entropy (ΔS^*) and Q_{10} were calculated according to the following equations:

$$\Delta H^* = E_a - RT \qquad [2]$$
$$\Delta G^* = -RT \ln K_a \quad \text{when } K_a = 1/K_m \qquad [3]$$
$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \qquad [4]$$
$$\log Q_{10} = E_a /(R(1/T_1 - 1/T_2)) \qquad T_1 < T_2 \qquad [5]$$

Where R is the gas constant (8.314 J/mol.K) and T is the temperature (K).

Estimation of molecular weight. Zymography was performed by subjecting lipase samples to electrophoresis on a 13% native polyacrylamide gel (PAGE), without SDS. The gel was then transferred on a tributyrin agar plate to detect lipase activity. After incubation for 12 h at 37°C, lipase activity was visualized as a clear band on the tributyrin agar plate.

RESULTS

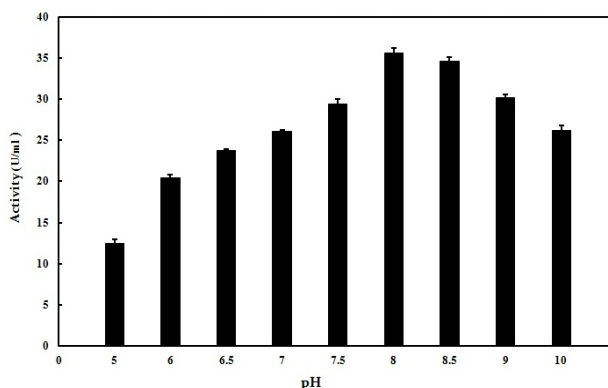Samples from different locations of three oil factories (Ghoo, Exdaneh and Margarin) were first grown on LB broth and then on TW agar plates, to separate the lipase producing-bacteria. Those that were able to produce lipases formed a halo zone on TW agar (data not shown). One of the isolates produced larger halo zones, and was thus selected and used for further studies. Microscopic analysis showed that the isolate was a rod-shaped Gram-negative bacterium, which formed green, shiny colonies that spread extensively on agar plates after 16 h of incubation. Consequently, the selected isolate was identified by the 16S rRNA sequencing method as *Pseudomonas* sp., and was registered as *Pseudomonas* L.M in the EMBL/Genbank under the accession number, FR850044. *Pseudomonas* L.M was subsequently grown on YOP medium in order to induce lipase production. The extracellular lipase activity was qualitatively investigated by transferring the supernatant to tributyrin agar plates as shown in Fig. 1. The enzyme formed a 15 mm halo zone after 24 h of growth, and was found to have an activity of 41.5 ± 1.4 U/ml, when assayed by the *p*NPP method

The lipase activity at different pH values was also assessed. The results showed that maximum activity was obtained at pH 8 (Fig. 2). No significant differences were observed in lipase activity at pH values, 8 and 8.5. The optimum temperature for lipase activity was 60°C, as shown in Fig. 3.

The residual activities of lipase at different pH values and temperatures were measured during 60 min so as to determine the optimum pH and thermo-stability of the enzyme (Fig. 4). The results showed that 68% of lipase activity was present after 30 min of incubation at 40°C and pH 8. The lipase lost 32% and 94% activity when it was incubated at pH 8 for 30 and 60 min, at 30°C, respectively. The enzyme was active only for 10 min at 90°C. The residual activities of 18.1 ± 1.5, 20.9 ± 0.8, 20.9 ± 1.1, 30.9 ± 1.0, 15.2 ± 2.1, 6.5 ± 0.7, 0 U/ml were obtained after 20 min

Table. 1. Thermodynamic parameters for the *Pseudomonas* L.M lipase.

T (K)	ΔG^* (kJ/mol)	ΔH^* (kJ/mol)	ΔS^* (J/mol. K)
293	-0.868	18.34	65.5
303	-0.898	18.26	63.2
313	-0.928	18.18	63.0
323	-0.957	18.10	59.0
333	-0.987	18.01	57.0
343	-1.017	17.93	55.2

of incubation at 30, 40, 50, 60, 70, 80, and 90°C, respectively.

The data indicated that the lipase produced by *Pseudomonas* L.M is an alkaline, thermostable enzyme.

The molecular mass of the lipase was estimated to be approximately 43 kDa by native-PAGE, as shown in Fig. 5.

The kinetic parameters of Km and Vm as obtained by the Lineweaver-Burk plot were 0.77 mM and 49.5 U/ml, respectively, indicating high affinity of the lipase for the substrate (Fig. 6). The activation energy of lipase calculated from the Arrhenius plot (Fig. 7) was found to be 20.78 kJ/mol and the temperature coefficient, $Q_{10,}$ was determined as 4.39, reflecting the high rate and temperature dependence of the enzymatic reaction (Table 1).

DISCUSSION

Oil factories are suitable locations for the isolation of lipase-producing-microrganisms. A *Pseudomonas* sp. was isolated from oil effluent and then identified by molecular methods. Its ability for production of lipase was measured quantitively and qualitively. The pH optimum of the *Pseudomonas* L.M lipase

was found to be 8.0-8.5, which is similar to those reported from other strains of *Pseudomonas* (4, 5, 6, 9, 10). The data regarding the pH optimum prove the alkaliphilic nature of the *Pseudomonas* L.M lipase. Furthremore, the temperature optimum of the *Pseudomonas* L.M lipase was observed to be 60°C, in agreement with lipases from *Pseudomonas* sp. KWI-56 and *Pseudomonas cepacia* DSM 50181 (2). The thermal stability profile of lipase and its alkaline nature indicated that this enzyme has a high potential for use in the detergent industry.

The molecular mass of the lipase from *Pseudomonas* L.M was estimated to be 43 kDa by native-PAGE. There have been different values reported for the molecular mass of *Pseudomonas* sp. Lipases, such as *Pseudomonas aeruginosa* BN-1 with an estimated molecular mass of 60 kD, in agreement with that of *Pseudomonas aeroginosa* PseA (11, 5). Two lipases from *Pseudomonas aeruginosa* have been found to have molecular masses of 59.4 and 54 kDa (12, 13). Lipases from *Pseudomonas fluorescens* MTCC 2421 and *Pseudomonas mendocina* PK-12CS and *Pseudomonas fluorescens* MC50 have been reported
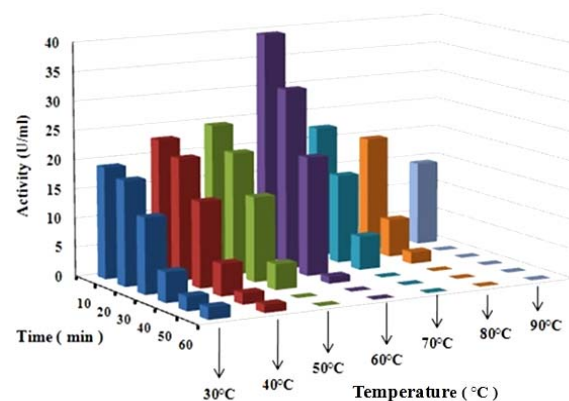

Fig. 3. Effect of temperature on *Pseudomonas* L.M lipase activity.

Fig. 4. The vertical axis is enzyme activity, the horizontal axis shows the duration of incubation for 10, 20, 30, 40, 50 and 60 min. The colum from left to right demonstrate the decrease in activity of the enzyme at 30, 40, 50, 60, 70, 80 and 90 OC, during incubation.

Fig. 5. Zymogram of the enzyme after incubating the native-PAGE gel for 12 h at 37oC in the substrate. Column 1- A standard strain with a molecular mass of 43 kDa (*Bacillus thermocatenolatus* lipase in recombinant *pichia pastoris*). Column 2- Lipase activity of *Pseudomonas* L.M was visualized as a clear band on the tributyrin agar plate.

with molecular masses of 65.3 kDa and 80 kDa and 55 kDa (2, 14, 15). Lipases from *Pseudomonas* sp. KWI-56 and *Pseudomonas fluorescens* AK 102 have been shown to have molecular weights of 33 kDa (2, 16), and the lipase from *Pseudomonas aeroginosa* LST-03 was estimated to have a molecular weight of 27.1 kDa (17). Accordingly, the molecular mass of *Pseudomonas aeroginosa* CS-2 was estimated to be 33.9 kDa by SDS-PAGE (9).

The kinetic parameters for *Pseudomonas* L.M were determined by the Lineweaver-Burk plot, as a Km value of 0.77 mM and Vm of 49.5 U/ml, with respect to pNPP as the substrate. However, the K_m and V_m for a lipase from the *Pseudomonas aeruginosa* PseA have been reported as 70.4 mM and 2.24 μmol/(min. mg) with regard to the same substrate, which are also in agreement with those that have been reported for the lipase from another strain of *Pseudomonas*
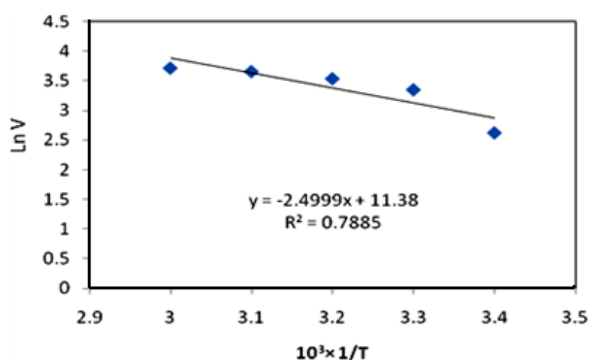

Fig. 7. Arrhenius plot for determination of the activation energy of *Pseudomonas* L.M lipase.
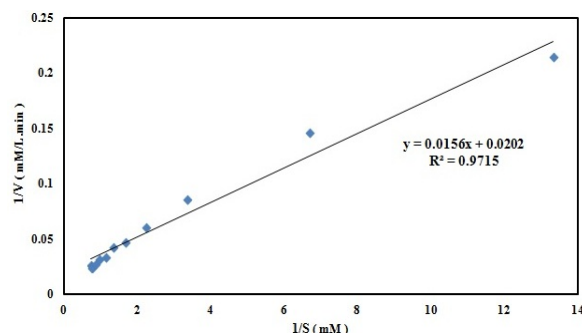

Fig. 6. Lineweaver-Burk plot for *Pseudomonas* L.M lipase.

aeruginosa MB 5001 (5, 18). Therefore, the lipase identified and produced in this study showed a better affinity for the substrate and a better catalytic activity as compared to the previously reported lipases.

According to the Arrhenius plot, the activation energy for the lipase of *Pseudomonas* L.M was estimated as 20.78 kJ/mol, which is in agreement with the lipase from *Bacillus* sp, with an activatiom energy of 20.67 kJ/mol.

To sum up, it can be concluded that the *Pseudomonas* sp. LM has a high potential for use in the production of lipase as a catalyst in transesterification or hydrolysis reactions.

ACKNOWLEDGMENTS

REFERENCES

1. Jaeger KE, Ransac S, Dijkstra BW, Colson C, Heuvel M van, Misset O. Bacterial Lipases. *FEMS Microbiol Rev* 1994; 15: 29-63.
2. Gupta R, Gupta N, Rathi P. Bacterial lipases: an overview of production, purification and biochemical properties. *Appl Microbiol Biotechnol* 2004; 64: 763-781.
3. Hasan F, Shah AA, Hameed A. Industrial applications of microbial lipases. *Enzyme Microb Technol* 2006; 39: 235-251.
4. Dharmsthiti S, Kuhasuntisuk B. Lipase from *Pseudomonas aeroginosa* LP602: biochemical properties and application for wastewater treatment. *J Ind Microbiol Biotechnol* 1998; 21: 75-80.
5. Gaur R, Gupta A, Khare SK. Purification and characterization of lipase from solvent tolerant *Pseudomonas aeruginosa* PseA. *Process Biochem* 2008; 43: 1040-1046.
6. Kojima Y, Shimizu S. Purification and characterization

of the lipase from *Pseudomonas fluorescens* HU380. *J Biosci Bioeng* 2003; 96: 219-226.

7. Sambrook J, Fritsch E.J, Maniatis. (2002). Molecular cloning, A laboratory manual. Cold Spring, Harbor Laboratory Press.

8. Gupta N, Rathi P, Gupta R. Simplified para-nitrophenyl palmitate assay for lipases and esterases. *Anal Biochem* 2002; 311: 98-99.

9. Peng R, Lin J, Wei D. Purification and characterization of an oraganic solvent-tolerant lipase from *Pseudomonas aeruginosa* CS-2. *Appl Biochem Biotechnol* 2010; 162: 733-743.

10. Rashid N, Shimada Y, Ezaki S, Atomi H, Imanaka T. Low temprature lipase from psychrotrophic *Pseudomonas* sp. strain KB700A. *Appl Environ Microbiol* 2001; 67: 4064-4069.

11. Syed MN, Iqbal S, Bano S, Khan AB, Qader SA, Azhar A. Purification and characterization of 60 D lipase linked with chaperonin from *Pseudomonas aeruginosa* BN-1. *Afr J Biotechnol* 2010; 9: 7724-7732.

12. Karadzic I, Masui A, Zivkovic LI, Fujiwara N. Purifi-cation and characterization of an alkaline lipase from *Pseudomonas aeroginosa* isolated from putrid mineral cutting oil as component of metalworking fluid. *J Biosci Bioeng* 2006; 102: 82-9.

13. Singh M, Banerjee UC. Enantioselective transesteri-fication of (RS)-1-chloro-3-(3,4-difluorophenoxy)-2-propanol using *Pseudomonas aeroginosa* lipases. *Tetrahedron: Asymmetry* 2007; 18: 2079-2085.

14. Chakraborty K, Paulraj R. Purification and biochemical characterization of an extracellular lipase from *Pseudo-monas fluorescens* MTCC 2421. *J Agric Food Chem* 2009; 57: 3859-3866.

15. Jinwal UK, Roy U, Chowdhury AR, Bhaduri AP, Roy PK. Purification and characterization of an alkaline lipase from a newly isolated *Pseudomonas mendocina* PK-12CS and chemoselective hydrolysis of fatty acid ester. *Bioorg Med Chem* 2003; 11: 1041-1046.

16. Kojima Y, Yokoe M, Mase T. Purification and characterization of an alkaline lipase from *Pseudomonas flourescens* AK 102. *Biosci Biotechnol Biochem* 1994; 58: 1564-1568.

17. Ogino H, Nakagawa S, Shinya K, Muto T, Fujimura N, et al. Purification and characterization of organic solvent-stable lipase from organic solvent-tolerant *Pseudomonas aeruginosa* LST-03. *J Biosci Bioeng* 2000; 89: 451-457.

18. Chartrain M, Katz L, Marcin C, Thien M, Smith S, et al. Purification of a novel bioconverting lipase from *Pseudomonas aeruginosa* MB 5001. *Enzyme Microb Technol* 1993; 15: 575-580.