Isolation, Identification and Enzyme Characterization of Lipase Producing Bacteria from Mucus Layer of *Oncorhynchus mykiss*

Oncorhynchus mykiss'in Yüzey Mukus Tabakasından Lipaz Üretici Bakterilerin İzolasyonu, Tanımlanması ve Enzim Karakterizasyonu

Research Article

Serpil Uğraş[1, 2]

[1]Department of Field Crops, Faculty of Agriculture and Natural Science, Düzce University, Düzce, Turkey.
[2]Department of Biology, Faculty of Arts and Science, Giresun University, Giresun, Turkey.

ABSTRACT

In this study, 13 different bacteria were isolated from the surface mucus layer of *Oncorhynchus mykiss* (rainbow trout). These bacteria were identified as *Exiguobacterium* sp. (*Om1*), *Acinetobacter* sp. (*Om2*, *Om7*), *Microbacterium* sp. (*Om3*), *Arthrobacter* sp. (*Om4*), *Sphingobacterium* sp. (*Om5*), *Stenotrophomonas* sp. (*Om6*, 10 and 11), *Pseudomanas* sp. (*Om8*), *Corynebacterium* sp. (*Om12*) *Aeromonas* sp. (*Om14*) and *Psychrobacter* sp. (*Om15*) based on morphological, physiological, and biochemical characteristics, as well as phylogenetic analysis using 16S rDNA sequences. We report that isolate *Om15* produces a cold-active lipase enzyme. The lipase enzyme was partially purified from the bacterial supernatant and its specific activity was calculated as 64.393 U g^{-1}. Optimal performance of the enzyme occurred at pH 8.0 and 20°C using p-nitrophenyl dodecanoate as a substrate. SDS-PAGE indicated that the lipase enzyme is composed of 2 subunits 58-60 kDa, and that it is possible that the active lipase enzyme is the heterodimer of the subunits, which was confirmed via Native-PAGE. Furthermore, the lipase activity decreased in response to application of Co^{2+} and Cu^{2+} ions; however, no significant difference in the lipase activity was observed via application of other ions.

Key Words
Bacteria, Lipase, *Oncorhynchus mykiss*, SDS-PAGE, Native-PAGE, 16S rDNA.

ÖZET

Bu çalışmada *Oncorhynchus mykiss* (gökkuşağı alabalığı)'in yüzey mukus tabakasından 13 farklı bakteri izole edildi. Bu bakteriler; morfolojik, fizyolojik, biyokimyasal ve 16S rDNA analiz sonuçları dikkate alınarak; *Exiguobacterium* sp. (Om1), *Acinetobacter* sp. (Om2 ve Om7), *Microbacterium* sp. (Om3), *Arthrobacter* sp. (Om4), Sphingobacterium sp. (Om5), Stenotrophomonas sp. (Om6, 10 ve 11), Pseudomanas sp. (Om8), *Corynebacterium* sp. (Om12) *Aeromonas* sp. (Om14) ve *Psychrobacter* sp. (Om15) olarak tanımlandı. Om15 izolatının soğukta aktif lipaz enzimi ürettiği tespit edildi. Lipaz enzimi bakteri süpernatantından kısmi olarak saflaştırıldı ve özgül aktivitesi 64.393 U g^{-1} olarak hesaplandı. Enzimin optimum performansı substrat olarak p-nitrofenil dodekanat kullanıldığında, pH 8.0'de ve 20°C'de gösterdiği tespit edildi. SDS-PAGE analizi ile lipaz enzimin 58-60 kDa büyüklüğünde iki alt birime sahip olduğu tespit edildi. Aktif lipaz enziminin bu alt birimlerin heterodimer yapıları ile oluştuğu Native-PAGE ile tespit edildi. Bunların yanısıra lipazın aktivitesinin Co^{+2} ve Cu^{+2} iyonları uygulaması ile azaldığı diğer iyon uygulamalarının ise aktivitede önemli değişikliklere sebep olmadığı belirlendi.

Anahtar Kelimeler
Bakteri, lipaz, *Oncorhynchus mykiss*, SDS-PAGE, Native-PAGE, 16S rDNA.

Article History: Received: Jan 13, 2016; Revised: Apr 4, 2016; Accepted: Jun 20, 2016; Available Online: Jul 31, 2016.

DOI: 10.15671/HJBC.20164420566

Correspondence to: S. Uğraş, Department of Field Crops, Faculty of Agriculture and Natural Science, Düzce University, Düzce, Turkey.
Tel: +90 380 541 2294 Fax: +90 380 541 2295 E-Mail: serpilkus@gmail.com

INTRODUCTION

Lipolytic enzymes are among the most important enzymes because of their biotechnological potential [1]. Bacteria produce different classes of lipolytic enzymes, including carboxylesterases (EC 3.1.1.1), and lipases (EC 3.1.1.3) [2]. Lipases are the most commonly used group of biocatalysts due to their wide ranging properties that are useful in such biotechnological fields as food technology, detergent production, biomedical sciences, agrochemical activity, and oleo chemical industries [3,4]. Biocatalysts are preferred over chemical catalysts for biotechnological applications [5]. Cold-active lipases have high activity at very low temperatures. Due to these characteristics these enzymes are preferred for the production of especially weak compounds in many fields, including organic chemistry, pharmacology, biophysics, biochemical and process engineering, biotechnology, microbiology, and biochemistry [1]. Nowadays, the number of studies on cold-active lipolytic enzymes in industrial applications is increasing, but still remains limited [1]. Also, the studies on cold active lipases are incomplete and scattered [6]. So, the new studies on cold active lipases are greatly needed.

Furthermore, farming of rainbow trout (*Oncorhynchus mykiss*) is a growing aquaculture industry in Giresun. Unfortunately, the knowledge about symbiotic bacteria living with trout is very limited.

As such, the present study aimed to determine symbiotic bacteria living with trout and to find a new lipase enzyme to use as an alternative to existing enzymes for utilization in various industrial applications. The fourteen different bacteria were isolated from the surface mucus layer of *Oncorhynchus mykiss* (rainbow trout) and these bacteria were identified based on morphological, physiological, and biochemical characteristics, as well as phylogenetic analysis using 16S rDNA sequences. We reported that *Psychrobacter* sp. strain *Om15* (Psp-*Om15*) was produced a cold-active lipase.

MATERIALS AND METHODS

Bacterial Strain and Lipase Activity

Fresh *Oncorhynchus mykiss* were obtained from trout farm in Giresun, Turkey. The fishes were packed in iceboxes and transferred to the laboratory within 2 h. Samples were obtained from the surface mucus layer and the gills of the fishes, and were then spread on nutrient agar using a sterile swab. Plates were incubated at 30°C and 10°C for 2-3 d, and then bacterial colonies were chosen according to various morphological characteristics as colony color, and morphology. The lipase activities of the bacteria were determined via two methods [7,8]. The lipase enzyme from the bacterium was obtained as described by Rajan et al. [9].

Identification of the Bacterial Strains

Identification of the bacterial strains was based on their morphological, physiological, and biochemical characteristics, as described in *Bergey's Manual of Systematic Bacteriology* [10]. Gram staining, color and shape of the colonies were determined. NaCl (2%-12%), pH (3.0-11.0), and temperature (4-37°C) tolerance of the bacterium was determined.

For molecular identification of the strain firstly, genomic DNA was extracted according to Sambrook et al. [11]. PCR amplification of 16S rDNA genes using genomic DNA was performed using oligonucleotide primers (UNI16S-L: 5'-ATT CTA GAG TTT GAT CAT GGC TTC A-3' and UNI16S-R: 5'-ATG GTA CCG TGT GAC GGG CGG TGT TGT A-3'), and then sequencing of the amplified DNA fragments was performed by Macrogene, Inc., Europe. Comparison of 16S rDNA gene sequences with entries in the updated GenBank database was performed using the BLAST program.

Lipase Activity Assay Using the Spectrophotometric Method

Lipase activity was determined using a spectrophotometric assay and p-nitrophenyl dodecanoate (Sigma) as a substrate. The enzyme solution (100 μL) was added to the substrate solution (900 μL) that consisted of 1 part 30 mg of p-nitrophenyl dodecanoate in 10 mL of

isopropanol and 9 parts solution of 0.1 g gum Arabic and 2 mL of Triton X-100 in 90 mL of Tris-HCl buffer (pH 8.0). The reaction mixture was incubated for 15 min at 30°C and was immediately cooled for 10 min to 4°C. Lipase activity was read at 410 nm [12], and enzyme activity was calculated as U L^{-1} [12].

The Effect of Carbon Sources and Production Time on the Lipase Production

In order to determine the optimal production time and carbon sources for the production of the lipase enzyme the lipase-producing strain was inoculated into 50 mL of sterile test medium that included various carbon sources, including tween 20, tributyrin, tween 80, and olive oil, and then incubated at 30°C for 72 h. The samples were aseptically removed every 24 h and were stored at 4°C until analysis of enzyme activity [9]. The lipase activity was assayed spectrophotometrically during each condition of optimization [12].

Partial Purification of the Lipase

The culture of the lipase-producing strain was centrifuged (10.000 g for 10 min at 4°C) and the supernatant was used for precipitation. Solid ammonium sulphate was added to achieve 30% saturation at 4°C on the supernatant and was stirred for 24 h. The suspension was re-centrifuged (10.000 g for 10 min at 4°C), the precipitate was re-suspended in 50 mM Tris-HCl buffer (pH 8.0), and then was stored at 4°C. Afterwards, ammonium sulphate was added to reach 50% and 80% saturation of the remaining supernatant. The precipitates were suspended in 50 mM Tris-HCl buffer and were stored at 4°C [13]. The protein concentration was determined calorimetrically using the Bradford assay [14].

Detection of Molecular Weight via SDS-PAGE

SDS-PAGE (12%) was performed according to Laemmli [15]. Samples of the lipase enzyme were run on a SDS-PAGE gel and were compared with the marker containing peptides, ranging from 10 to 225 kDa (Promega, USA). Following electrophoresis, gel was stained with CBB R-250.

Direct Detection of the Lipase Activity via Native-PAGE

Native-PAGE (12%) under non-denaturing conditions was performed as described by Laemmli [15]. Samples of partially purified lipase enzyme and marker were subjected to Native-PAGE. Bovine serum albumin (BSA 1 mg mL^{-1}) was used as a marker. After electrophoresis, the gel was cut vertically. The first part including the samples of partially purified lipase enzyme and BSA were stained with CBB R-250. The other parts of the gel were assayed for direct detection. The direct lipase activity was measured according to Park et al. [16].

The Effect of Various Substrates, pH, and Temperature on the Lipase Activity

The lipase activity was analyzed using various substrates, including p-nitrophenyl acetate, p-nitrophenyl dodecanoate, and p-nitrophenyl butyrate, via the spectrophotometric method [12]. The optimal pH of the lipase enzyme was determined using various buffer solutions (50 mM), including sodium acetate (pH 4.0 and 5.0), potassium phosphate (pH 6.0 and 7.0), tris-HCl (pH 8.0), and glycine-NaOH (pH 9.0 and 10.0) [17]. The lipase activity was analyzed according to Kumar et al. [12]. The optimal temperature of the lipase enzyme was determined at various temperatures ranging from 10°C to 90°C at pH 8.0, and then residual activity was determined. The effect of temperature on the lipase stability was determined via analysis of the residual activity after incubation for 5-60 min at the optimal temperature [17].

The Effect of Metal Ions and Organic Solvents on the Lipase Activity

The enzyme solution obtained from the bacterial supernatant was incubated with 5 mM and 10 mM of such metal ions as $NiCl_2$, $ZnCl_2$, $CuCl_2$, $CoCl_2$, $MgCl_2$, and $CaCl_2$ at 30°C for 1 h [17], and then the residual activity was determined spectrophotometrically [12]. The effect of such organic solvents as hexane, butanol, isopropanol, methanol, ethyl acetate, and ethanol on lipase activity was analyzed according to Lee et al. [17]. Lipase activity was measured spectrophotometrically [12].

Table 1. Characteristics of isolates.

Isolates	Colony Color	Gram Stain	Temperature Range (°C)	NaCl Tolerance (%)	pH Tolerance	Lipase Activity
Om1	Yellow Orange	G(+)	4-37	2-5	7-9	-
Om2	Pale Yellow	G(-)	4-37	2	5-9	+
Om3	Light Yellow	G(+)	4-40	2-5	7-9	-
Om4	Opaque Cream	G(+)	4-40	2	7-9	-
Om5	Yellow Orange	G(-)	4-37	2-5	7-9	-
Om6	Cream-Yellow	G(-)	4-37	2-5	7-9	-
Om7	Light Cream	G(-)	4-45	2	5-9	+
Om8	Brown Cream	G(-)	4-37	2-5	5-9	-
Om10	Yellow Orange	G(-)	4-37	2-5	7-9	-
Om11	Light Yellow	G(-)	4-37	2	7-9	-
Om12	Opaque Yellow	G(+)	4-37	2-7	5-7	-
Om14	Clear Brown	G(-)	4-37	2-5	7-9	+
Om15	Opaque cream	G(-)	4-37	2-7	7.0-8.0	+++

+; weak activity, +++; high activity

RESULTS AND DISCUSSION

Farming of rainbow trout is a growing aquaculture industry in Giresun. Unfortunately, the knowledge about symbiotic bacteria living with trout is very limited. In this study 13 different bacteria were isolated from the surface mucus layer and the gills of rainbow trout (*Oncorhynchus mykiss*) obtained from a trout farm in Giresun, Turkey. These bacteria were identified as *Exiguobacterium* sp. (*Om1*), *Acinetobacter* sp. (*Om2*, 7) *Microbacterium* sp. (*Om3*), *Arthrobacter* sp. (*Om4*), *Sphingobacterium* sp. (*Om5*), *Stenotrophomonas* sp. (*Om6*, 10 and 11), *Pseudomanas* sp. (*Om8*), *Corynebacterium* sp. (*Om12*), *Aeromonas* sp. (*Om14*) and *Psychrobacter* sp. (*Om15*). Among of them, the four isolates were Gram-positive and the nine isolates were Gram-negative (Table 1). The strains were identified based on molecular characteristics (Table 2).

To qualitatively determine the lipase activity of all isolates were subjected to synthetic esters, including tween 20 and 80, on agar plates (Table 1, Figure 1). In the present study qualitative enzyme activity was indicated by the appearance of white crystals resulting from the deposition of crystals of the calcium salt formed by the fatty acid released in the case of tween 20 and 80 hydrolysis by the lipase enzyme [18]. We showed that *Psychrobacter* sp. strain *Om15* has the highest lipolytic activity. *Psychrobacter* spp., such as *P. immobilis* B10 [19], and *P. cryohalolentis* [20], also exhibited lipase activity.

The present findings show that the optimal production time and carbon source for the production of the lipase from *Psychrobacter* sp. strain *Om15* was 72 h and tween 80, respectively (Table 3). The production parameters, such as production time and suitable carbon sources, of cold-active lipases vary; for example, the lipase enzyme from *Aeromonas* sp. LPB was produced in 8 d using tributyrin as the carbon source [21], the lipase enzyme from *Bacillus sphaericus* MTCC

Table 2. Levels of 16S rRNA sequence similarity between isolates and bacterial species in Databank.

Isolate Code	Accession number	Suggestions of GenBank at species level	Similarity (%)
Om1	EU282459	*Exiguobacterium* sp. TC38-2b	99
	JF505996	*E.sibiricum* strain KNUC9062	
	JX188088	*E. sibiricum* strain TB68	
Om2	JN098518	*Acinetobacter* sp. J1	97
	KF704076	*A. quillouiae* WDL-R5	
	KF561870	*Acinetobacter* sp. E6.2	
Om3	KC768764	*Microbacterium* sp.	99
	JX094161	*M. foliorum* strain N1-12	
	EU714358	*M. foliorum* strain 327	
Om4	DQ365556	*Arthrobacter* sp. GH01	99
	KC153125	*A. nicotianae* strain Dc-06	
	AY635865	*Arthrobacter* sp. BM-3	
Om5	JF327471	*Sphingobacterium* sp. KB46	99
	KC464780	*Sphingobacterium* sp. DS-3PS-9	
	EU375387	*Sphingobacterium* sp. TpI-44	
Om6	JN082748	*Stenotrophomonas* sp. NR17	99
	GU186115	*S.maltophilia* strain IHB B 1365	
	FJ493060	*S.chelatiphaga* strain G-7	
Om7	HF952698	*Acinetobacter* lwoffii	97
	KF228932	*Acinetobacter* sp. JUN-14	
	KC853177	*Acinetobacter* sp. W10	
Om8	JQ317008	*Pseudomanas* azotoformans	99
	JX127246	*P. flourescens* strain 4.9.3	
	KF481916	*P. gessardii* strain KMBMa1	
Om10	AJ551165	*Stenotrophomonas* sp. An27	99
	FM955853	*S. rhizophila*	
	HQ327153	*Stenotrophomonas* sp. TP-Snow-C47	
Om11	JQ977663	*Stenotrophomonas* sp. Awa9	97
	JQ977692	*Stenotrophomonas* sp. Ea21	
	JQ977638	*Stenotrophomonas* sp. Cza24	
Om12	HE979851	*Corynebacterium* sp. KK-5-1	99
	EU798947	*Corynebacterium* sp. JY02	
	KC113136	*C. callunae* strain KSI 1226	

Table 2. Levels of 16S rRNA sequence similarity between isolates and bacterial species in Databank. (continue)

Om13	KC904087	*Acinetobacter* sp. P162	99
	FN395271	*Acinetobacter* sp. FR-W5Bb	98
	AB859741	*A. quillouiae*	98
Om14	KF661547	*Aeromonas* veronii B5	95
	KF358429	*Aeromonas* sobria FGC24	
	KC840855	*Aeromonas*media W52	
Om15	KF186667	*Psychrobacter* sp. SIF3	99
	KF688134	*Psychrobacter* sp. AB2	
	KC884692	*Psychrobacter* sp. L68	

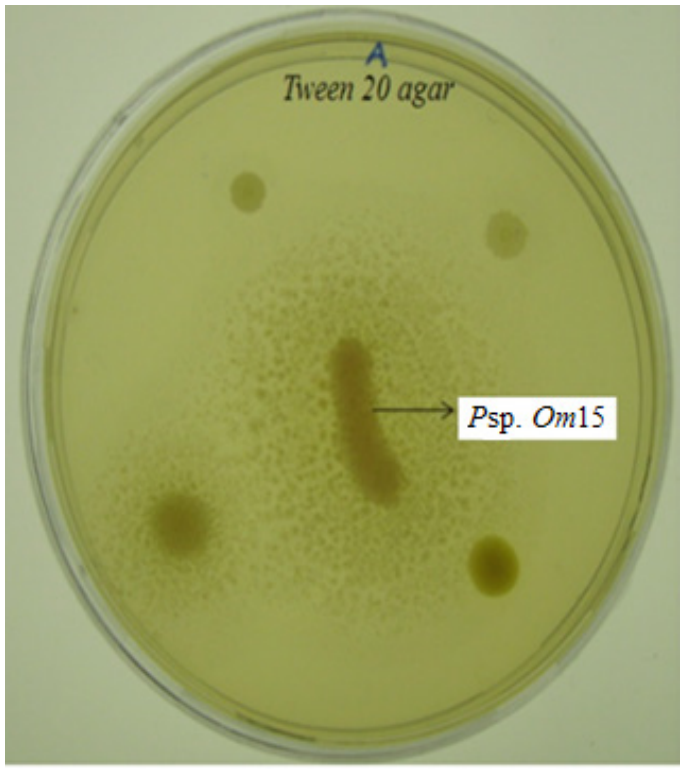

Figure 1. The qualitative activity of lipase enzyme.

7526 was produced in 48 h using lactose/tributyrin as the carbon source [22], the lipase enzyme from *Microbacterium* phyllosphaerae MTCC 7530 was produced in 36 h using tributyrin/lactose as the carbon source [22], and the lipase enzyme from *Psychrobacter* sp. wp37 was produced in 14 d using tween 80 and 20 as the carbon source [23]. Although the production parameters reported in these studies are similar, production time varied in each case.

The lipase enzyme described herein was partially purified from the bacteria supernatant via 80% ammonium sulphate precipitation, and enzyme activity and specific activity were calculated as 12.725 $U.L^{-1}$ and 64.393 $U.g^{-1}$, respectively (Table 4). A clear band was observed via SDS-PAGE. So, different purification stages were not needed in the present study.

Table 3. Effect of carbon sources and incubation times on the lipase production.

Inc. time (h)	Activity (U L^{-1})			
	Tween 20	Tributyrine	Tween 80	Olive oil
24	0.522	0.517	1.593	3.658
48	0.992	0.710	1.885	1.756
72	2.709	0.770	3.831*	2.831

(*); the highest activity

Table 4. Partially purification of the lipase enzyme.

$(NH_4)_2SO_4$ Saturation Rate (%)	Activity (U L^{-1})	Protein (g L^{-1})	Specific Activity (U g^{-1})
0 (control)	3.023	0.0876	34.509
50	12.725	0.4467	28.487
80*	12.724	0.1976	64.393

(*); the highest activity

SDS-PAGE analysis indicated that the lipase enzyme is composed of 2 subunits (58-60 kDa). It is possible that the structure of the active lipase enzyme is heterodimer, because of these subunits (Figure 2). Furthermore, direct detection of the enzyme activity was achieved via Native-PAGE; finally, white clear zones were formed around the active band at approximately 118 kDa (Figure 3). In the present study SDS-PAGE analysis indicated that the lipase enzyme is composed of 2 subunits. Shah and Bhatt reported that the true lipase from *B. subtilis* Pa2 has 2 polypeptide chains that exhibit 2 clear bands near each other that correspond to the molecular weight of 19.4 and 19.8 kDa via SDS-PAGE [24]. Furthermore, *B. subtilis* lipase has 2 chains (LipA and LipB) [25]. These findings are in agreement with those of the present study. In addition, the molecular weight of cold-active lipases was reported to vary from 29 to 85 kDa, including 85 kDa from *Pseudoalteromonas* sp. [1], and *Psychrobacter* sp. [23], 40 kDa from *Bacillus sphaericus* MTCC 7526 [22], 35 kDa from *Psychrobacter* sp. [26], 35.288 kDa from *Psychrobacter immobilis* B10 [19], and 54 kDa from *Psychrobacter cryohalolentis* [20]. The present findings show that the molecular weight of the lipase enzyme obtained from Psp-*Om15* is greater than that of the other cold-active lipases.

In the present study, quantitative enzyme activity was analyzed using various substrates, including p-nitrophenyl acetate (C2), p-nitrophenyl dodecanoate (C12), and p-nitrophenyl butyrate (C4) via the spectrophotometric method, which showed that the lipase enzyme obtained from strain Psp-*Om15* exhibited maximum activity at 20°C using p-nitrophenyl dodecanoate as a substrate in Tris-HCl buffer at pH 8.0 (Figures 4-6). In addition, we think that the enzyme described herein is a true lipase, because it exhibited maximal activity towards water insoluble long-chain triglycerides [2]. Cold-active lipases exhibit maximum activity at varying temperatures and pH levels, and using various substrates. For example, the lipase enzyme from *M. phyllosphaerae* MTCC 7530 exhibited maximum activity at 20°C and pH 8.0, and in the presence of organic solvents, and its activity was compatible with detergents [22], the enzyme from *Acinetobacter* sp. strain no. 6 exhibited maximum activity at 20°C and broad specificity towards the acyl group (C8-C16) of ethyl esters [27], the enzyme from *Psychrobacter* sp. wp37 exhibited maximum activity at 20-30°C and pH 7.0-8.0 [23], the enzyme from *Psychrobacter* sp. 7195 exhibited maximum activity at 30°C and pH 9.0 [26]. These earlier reported temperatures, pHs, and substrates that

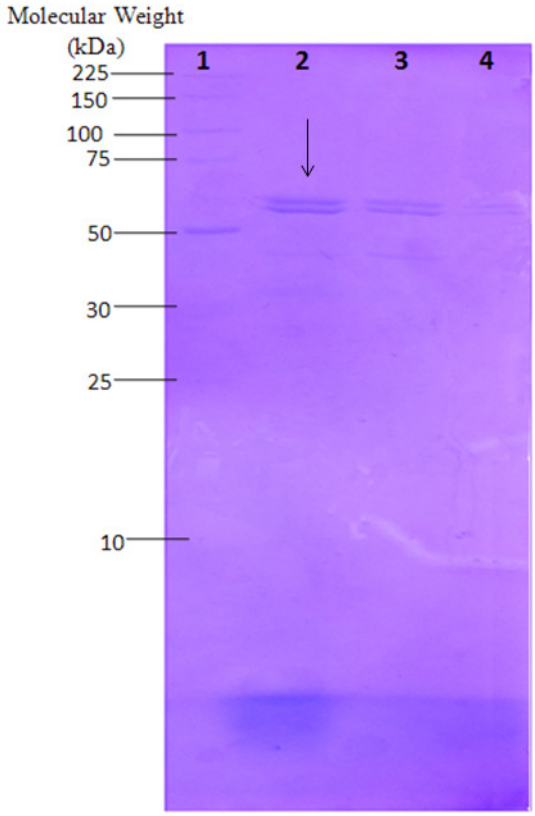

Figure 2. SDS-PAGE analysis of lipase enzyme 1; Marker, 2; 80% ammonium sulphate precipitation, 3; 50% ammonium sulphate precipitation, 4; 0% ammonium sulphate precipitation (control). The arrows correspond to the lipase enzyme.

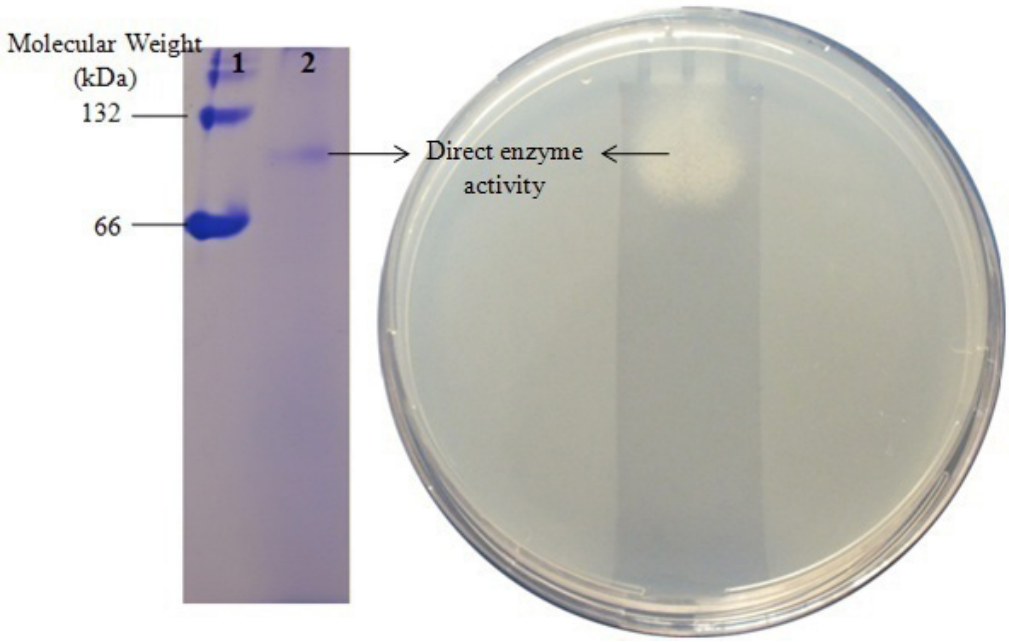

Figure 3. Native-PAGE analysis of lipase enzyme and direct detection of the lipase activity. 1; marker, 2; partially purified lipase enzyme (obtained from 80% ammonium sulphate precipitation).

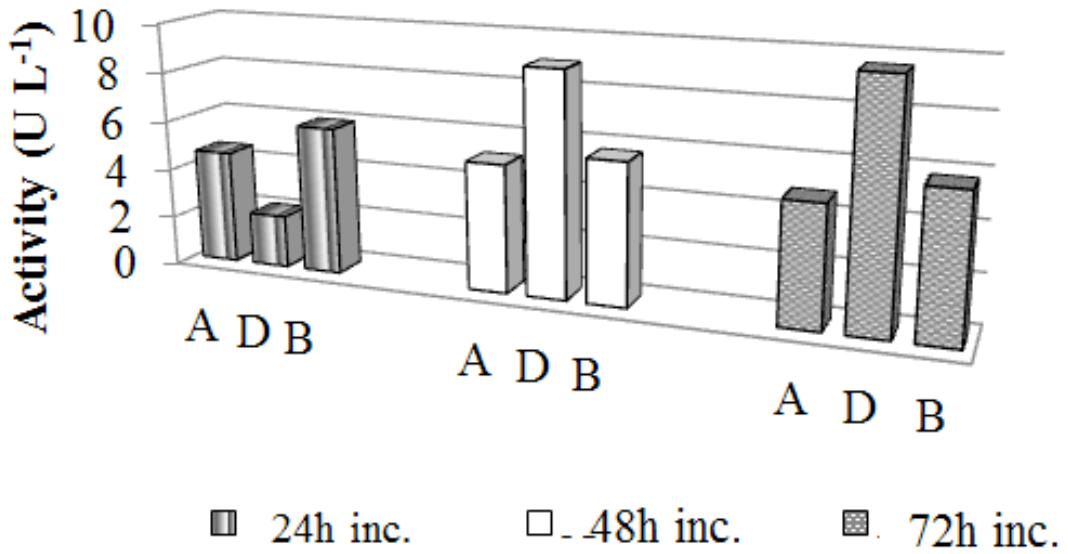

Figure 4. Effect of substrates on the lipase activity. A; p-nitrophenyl acetate, D; p-nitrophenyl dodecanoate, B; p-nitrophenyl butyrate.

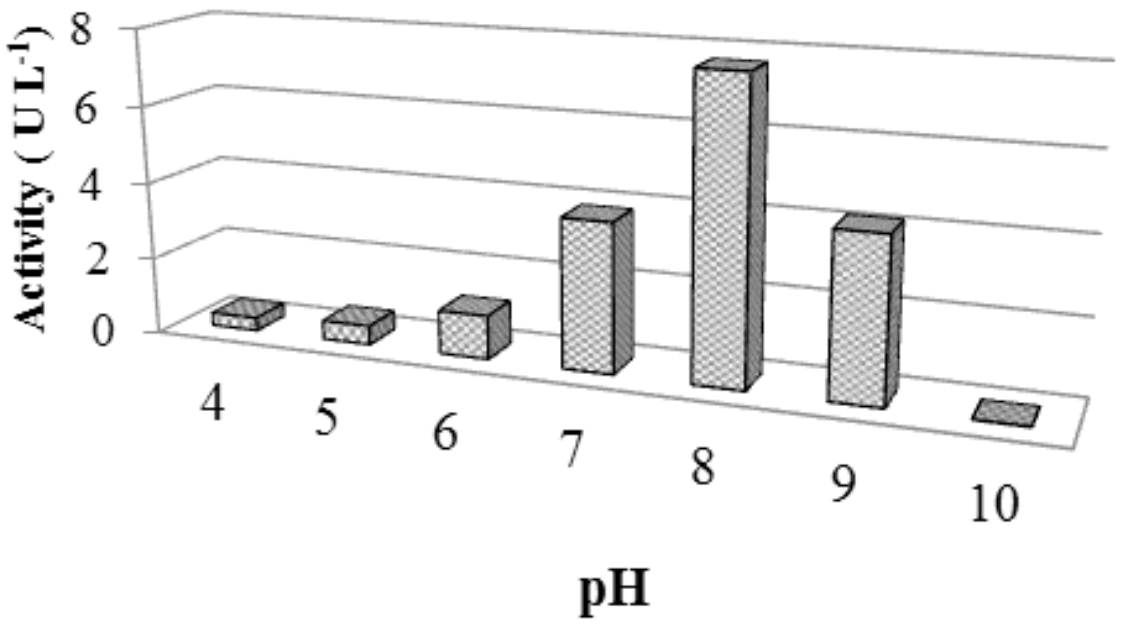

Figure 5. Effect of pH on the enzyme activity.

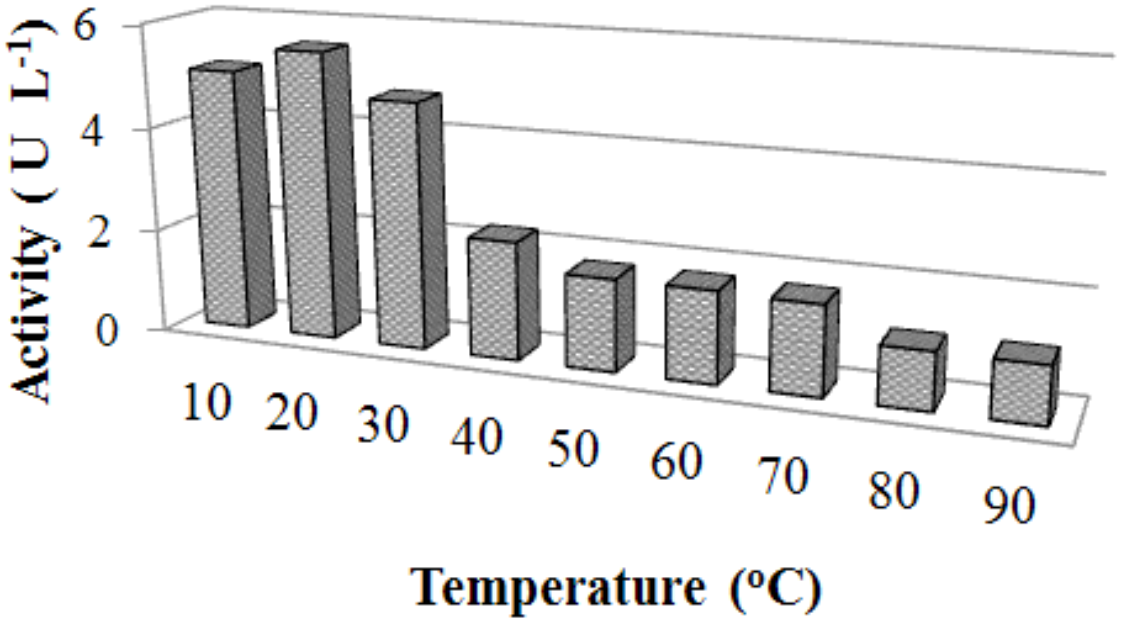

Figure 6. Effect of temperature on the enzyme activity.

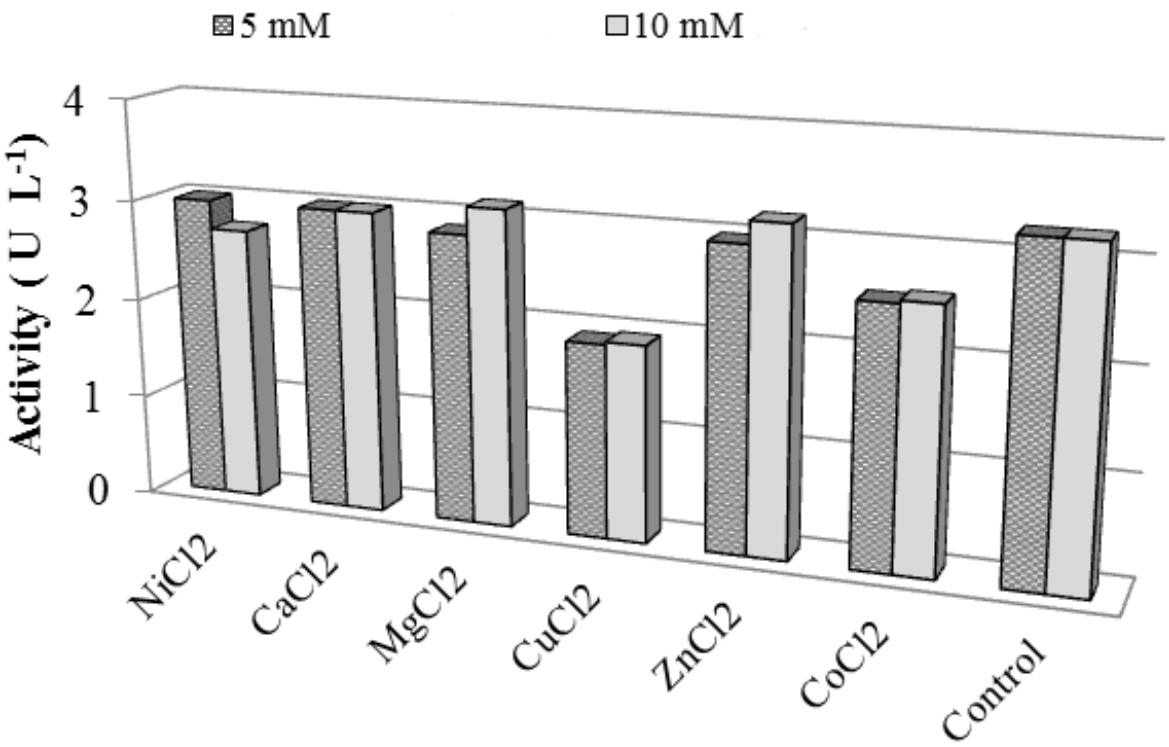

Figure 7. Effect of metal ions on the enzyme activity.

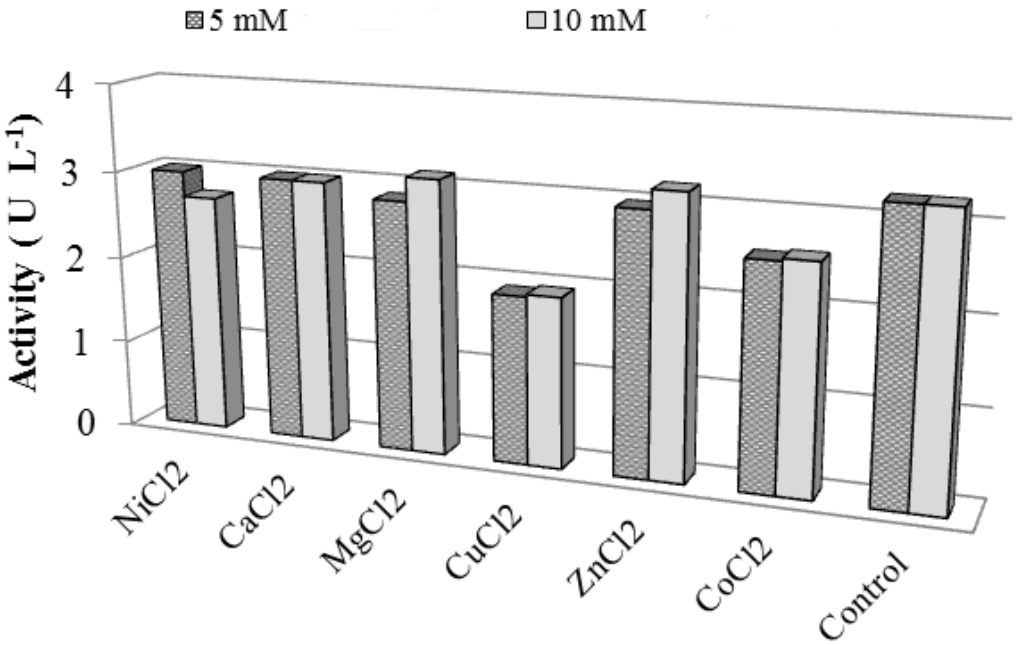

Figure 8. Effect of organic solvents on the enzyme activity.

yielded maximum activity of the described cold-active lipases show that they are very similar to those of the cold-active lipase of the strain Psp-*Om15* presently reported.

The lipase enzyme in the present study was treated with metal ions and organic solvents, and enzyme activity was decreased in the presence of Co^{2+} and Cu^{2+} (Figure 7). No significant differences in the lipase activity were observed with the other ions (Figure 7). Also, organic solvents, except for hexane, decreased the activity of the enzyme when incubated for 2 h, but ethyl acetate, hexane, and butanol increased the activity of the enzyme when incubated for 1 h (Figure 8). The enzymes from *Pseudomonas* sp. strain KB700A (KB-Lip) [28], *Pseudomonas* sp. strain B11-1 (LipP) [29], *Pseudomonas* sp. [30], and *P. fluorescens* HU380 [31] were significantly inhibited by Cu^{2+} [26].

The lipase enzyme in the present study has many advantageous properties, including the fact that production time is very short and it is active at low temperature, thus making it attractive for industrial applications; however, further studies are needed for a better understanding of the molecular characterization and mechanism of activity of the cold-active lipase enzyme obtained from *Psychrobacter* sp. strain *Om15*.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Zihni Demirbag (Blacksea Tecnical University, Turkey) for helping with the bacterial identification analysis and Dr. Hatice Kati (Giresun University, Turkey) for helping with the enzyme analysis.

References

1. F. Kayırhan, S.S. Çelebi, Increase in the enzymatic hydrolysis rate of triacetin using PE particles in a column reactor, Biochem. Eng. J., 1 (1998) 153-158.
2. B. Joseph, P.W. Ramteke and G. Thomas, Cold active microbial lipases: some hot issues and recent developments, Biotechnol. Adv., 26 (2008) 457-470.
3. J.L. Arpigny and K.E. Jaeger, Bacterial lipolytic enzymes: classification and properties, Biochem. J., 343 (1999) 177-183.
4. P. Ranjitha, E.S. Karthy, A. Mohankumar, Purification and characterization of the lipase from marine *Vibrio fischeri*, Int. J. Biol., 1 (2009) 48-56.
5. R. Gupta, N. Gupta, P. Rathi, Bacterial lipases: an overview of production, purification and biochemical properties, Appl. Microbiol. Biotechnol., 64 (2004) 763-81.
6. J.P. Kishore, Z.C. Manojkumar and T.M. Raghunath, Lipase Biodiversity, Ind. J. Sci. Technol., 4 (2011) 971-982.
7. V. Kukreja and M. B. Bera, Lipase from *Pseudomanas aeriginosa* MTCC 2488: partial purification, characterization and calcium dependent thermostability, Ind. J. Biotechnol., 4 (2005) 222-226.
8. E. Haba, O. Bresco, C. Ferrer, A. Marqués, M. Busquets, and A. Manresa, Isolation of lipase-secreting bacteria by deploying used frying oil as selective substrate, Enzym. Microb. Technol., 26 (2000) 40-44.

9. A. Rajan, D.R.S. Kumar and J. Nair, Isolation of a novel alkaline lipase producing fungus *Aspergillus fumigatus* MTCC 9657 from aged and crude rice bran oil and quantification by HPTLC, Inter. J. Biol. Chem., 5 (2011) 116-126.
10. N.R. Krieg and J.G. Holt, Gram-negative aerobic rods and cocci. In: Palleroni NJ. ed. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, 140-218. 1986.
11. J. Sambrook, E.F. Fritsch, T. Maniati, Molecular Cloning: A Laboratory Manual. 2nd ed. N.Y., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press., 1659 pp. 1989.
12. R. Kumar, A. Sharma, A. Kumar and D. Singh, Lipase from *Bacillus pumilus* RK31: production, purification and some properties, W. Appl. Sci. J., 16 (2012) 940-948.
13. S. Ugras, K. Sezen, H. Kati and Z. Demirbag, Purification and characterization of an antibacterial substance produced by pest-originated *Serratia marcescens* Mm3, Turk. J. Biol., 338 (2014) 177-184.
14. M.M. Bradford, A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem., 72 (1976) 248-254.
15. U.K. Laemmli, Cleavage of structure of proteins during assembly of the head of bacteriophage-T4, Nature, 227 (1970) 680-685.
16. H.J. Park, J.H. Jeong, S.G. Kang, J.H. Lee, S.A. Lee, H.K. Kim, Functional expression and refolding of new alkaline esterase, EM2L8 from deep-sea sediment metagenome, Protein Expr. Purif., 52 (2007) 340-347.
17. D.W. Lee, Y.S. Koh K.J. Kim, B.C. Kim, H.J. Choi, D.S. Kim, M.T. Suhartono, Y.R. Pyun, Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1, FEMS Microbiol. Lett., 179 (1999) 393-400.
18. S.C.B. Gopinath, P. Anbu, A. Hilda, Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments, Mycoscience., 46 (2005) 119-126.
19. J.L. Arpigny, G. Feller, C. Gerday, Cloning, sequence and structural features of a lipase from the antarctic facultative psychrophile *Psychrobacter immobilis* B10, Biochim. Biophys. Acta., 1171 (1993) 331-333.
20. K.A. Novototskaya-Vlasova, L.E. Petrovskaya, E.M. Rivkina, D.A. Dolgikh, and M.P. Kirpichnikov, Characterization of a cold-active lipase from *Psychrobacter cryohalolentis* K5(T) and its deletion mutants, Biochemistry (Mosc)., 78 (2013) 385-94.
21. H.K. Lee, J.A. Min, H.K. Sung, H.S. Won and C.J. Byeong, Purification and characterization of cold active lipase from psychrotrophic *Aeromonas* sp. LPB4, J. Microbiol., 41 (2003) 22-27.
22. B. Joseph, Isolation, purification and characterization of cold adapted extracellular lipases from psychrotrophic bacteria: feasibility as laundry detergent additive. Ph.D thesis, Allahabad Agricultural Institute-Deemed University, Allahabad, India, 2006.
23. X. Zeng, X. Xiao, P. Wang and F. Wang, Screening and characterization of psychrotrophic lipolytic bacteria from deep-sea sediments, J. Microbiol. Biotechnol., 14 (2004) 952-958.
24. K.R. Shah and S.A. Bhatt, Purification and characterization of lipase from *Bacillus subtilis* Pa2, J. Biochem. Tech., 3 (2011) 292-295.
25. M.J. Dröge, Y.L. Boersma, G. Van Pouderoyen, T.E. Vrenken, C.J. Rüggeberg, M.T. Reetz, B. W. Dıjkstra and W.J. Quax, Directed Evolution of *Bacillus subtilis* lipase aby use of enantiomeric phosphonate inhibitors: crystal structures and phage display selection, Chem. Biochem ., 7 (2006) 149-157.
26. J. Zhang, S. Lin and R. Zeng,. Cloning, expression, and characterization of a cold-adapted lipase gene from an antarctic deep-sea psychrotrophic bacterium, *Psychrobacter* sp. 7195, J. Microbiol. Biotechnol., 17 (2007) 604-610.
27. T. Suzuki, T. Nakayama, T. Kurihara, T. Nishino and N. Esaki, Cold-Active lipolytic activity of psychrotrophic *Acinetobacter* sp. strain no. 6, J. Biosci. Bioeng., 92 (2001) 144-148.
28. N. Rashid, S. Yuji, E. Satoshi, A. Haruyuki and I. Tadayuki, Low-Temperature lipase from psychrotrophic *Pseudomonas* sp. strain KB700A., Appl. Environ. Microbiol., 67 (2001) 4064-4069.
29. D.W. Choo, T. Kurihara, T. Suzuki, K. Soda and N. Esaki, A Cold-adapted Lipase on an Alaskan Psychrotroph, *Pseudomonas* sp. strain B11-1: gene cloning and enzyme purification and characterization, Appl. Environ. Microbiol., 64 (1998) 486-491.
30. K. Amada, M. Haruki, T. Imanaka, M. Morikawa, and S. Kanaya, Over production in *Escherichia coli*, purification and characterization of a family I.3 lipase from *Pseudomonas* sp. MIS38, Biochim, Biophys. Acta, 1478 (2000) 201-210.
31. Y. Kojima, M. Kobayashi and S. Shimizu, A novel lipase from *Pseudomonas fluorescens* HU380: gene cloning, overproduction, renaturation-activation, two-step purification, and characterization, J. Biosci. Bioeng., 96 (2003) 242-249.