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Biochemical Characterization of Lipases Obtained from *Acinetobacter psychrotolerans* Strains

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Abstract: In this study, extracellular lipases obtained from *Acinetobacter psychrotolerans* strains (Xg1 and Xg2) were characterized. The effects of varying pH values (3.0-10.0) and various temperatures (10-90 °C) on lipase activities were examined. Also the effects of different metal ions, organic solvents and detergents on lipases were studied. The extracellular crude lipases were concentrated using ultrafiltration. Zymogram analysis of these lipases was performed. Lipases exhibited maximum activity at pH 8 and 30 °C. While lipase obtained from the Xg1 strain exhibited the highest stability in the presence of various organic solvents, including hexane, ethyl acetate, chloroform and N,N dietil formamide, lipase obtained from the Xg2 strain was sensitive in the presence of isopropanol, acetonitrile, and butan-1-ol. The lipases of the Xg1 and Xg2 strains were inhibited in the presence of Cu^{2+} and Zn^{2+}. Also, the lipase of the Xg1 strain was inhibited in the presence of Fe^{3+}. In the presence of EDTA, the lipase activities of the Xg1 and Xg2 strains were partially inhibited. In presence of SDS, they were exactly inhibited. According to the zymogram results, the molecular weights of the lipases obtained from the *Acinetobacter psychrotolerans* Xg1 and Xg2 strains have been found approximately 37 and 30 kDa, respectively.

Acinetobacter psychrotolerans Suşlarından Elde Edilen Lipazların Biyokimyasal Karakterizasyonu

Anahtar Kelimeler
Acinetobacter,
Lipaz,
Karakterizasyon

Özet: Bu çalışmada, *Acinetobacter psychrotolerans* suşlarından (Xg1 ve Xg2) elde edilen ekstrasellülar lipazlar karakterize edilmiştir. Farklı pH değerlerinin (3.0-10.0) ve çeşitli sıcaklıkların (10-90 °C) lipaz aktiviteleri üzerindeki etkileri incelenmiştir. Ayrıca farklı metal iyonlarının, organik çözücülerin ve deterjanların lipazlar üzerindeki etkileri de araştırılmıştır. Ekstrasellülar ham lipazlar ultrafiltrasyon ile konsantre edilmiştir. Bu lipazların zymogram analizi yapılmıştır. Lipazlar pH 8 ve 30 °C'de maksimum aktivite gösterdiği bulundu. Xg1 suşundan elde edilen lipaz; hekzan, etil asetat, kloroform ve N,N dietil formamid gibi organik çözücülerinin varlığında yüksek stabilite gösterirken, Xg2 suşundan elde edilen lipaz izopropanol, asetonitril ve butan 1-ol varlığında hassas olduğu bulundu. Xg1 ve Xg2 suşlarından elde edilen lipazlar Cu^{2+} ve Zn^{2+} varlığında inhibe edilmiştir. Xg1 suşundan elde edilen enzim aynı zamanda Fe^{3+} varlığında da inhibe edilmiştir. Xg1 ve Xg2 suşlarının lipaz aktiviteleri EDTA varlığında kısmen inhibe edilmiştir. SDS varlığında, lipazlar tamamen inhibe oldular. Zymogram analiz sonuçlarına göre *Acinetobacter psychrotolerans* Xg1 ve Xg2 suşlarından elde edilen lipazların molekül ağırlıkları yaklaşık olarak sırasıyla 37 ve 30 kDa'dır.

1. Introduction

Lipases are produced by various plants, animals, and microorganisms [1]. These enzymes are included in the EC 3.1.1.3 group. Properties of this group are carboxylesterases. These enzymes catalyze both the hydrolysis and synthesis of long-chain acylglycerols [2]. Among lipases commonly found in nature, microbial lipases are commercially more significant. These lipases are widely applied in industries as they are often more stable, convenient and safe. Microbial lipase production has seen increased use in industrial

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applications in recent years. Industrial applications of the microbial lipases are included such as food, detergent, pharmaceuticals, and cosmetics [3-6]. Bacterial lipases have been reported to be used potentially in industrial applications [7,8]. There are many studies on lipase enzymes obtained from *Acinetobacter* sp. [9-11]. *Acinetobacter* is a genus of aerobic, gram-negative coccobacillus.

In this study, alkaline low temperature lipases obtained from two *A. psychrotolerans* strains isolated from insect [12] were characterized.

2. Material and Method

2.1. Bacterial strains and media

Two *A. psychrotolerans* strains, Xg1 and Xg2, were isolated from insect in a recently study [12]. To detect the lipase activity of these bacteria, three kinds medium were used. The basal medium (0.5% yeast extract, 1% tryptone, 0.5% NaCl, 0.01% $CaCl_2.2H_2O$) was prepared; 1% Tween 80, Tween 20 and tributyrin, respectively, were added [13,14]. The medium for lipase production was prepared rhodamine B agar medium [15]. This medium was observed by fluorescence with UV light at 350 nm.

2.2. Lipase production

The Xg1 and Xg2 strains were inoculated in a nutrient broth medium for 24 hours at 30 °C with 150 rpm. For the production of lipases, 1% of the inoculum was inoculated into 100 ml Tween 20 medium, incubated at 30 °C and cultures were collected every 24 h. The crude enzyme was obtained by centrifuging the culture at 10,000 x *rpm* for 10 min at 4 °C. The supernatant was collected and used as a crude enzyme for further studies.

2.3. Lipase assay

Lipase activity of strains was detected spectrophotometrically at 410 nm using p-nitrophenyl palmitate as substrate [16]. One unit of activity was defined as the amount of enzyme needed to release 1 μmol of p-nitrophenol per min.

2.4. Characterization of lipases

The effects of pH on lipase activities, pH range of 3-10 was were determined at 30 °C for 15 min. Sodium acetate buffer at pH 4-5, potassium phosphate buffer at pH 6-7, Tris-HCl buffer at pH 8 and glycine-NaOH buffer at pH 9-10 were used. The stability of the lipases at spesific pH were determined at +4 °C for 24 hours.

Different temperature range (10, 20, 30, 40, 50, 60, 70, 80 and 90 °C) were applied for 15 min at pH 8 to determine the effect of temperature on lipase activities. The thermal stability of the lipase enzymes was observed for 1 h at high activity temperatures. The residual activity of the lipases was measured by the spectrophotometric method.

Different metal ions (Mn^{2+}, Fe^{3+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, NH^{4+}, Ca^{2+}, Mg^{2+}, Hg^{+}) at 5 mM or 10 mM concentrations was added to the enzymes and incubated at 30 °C at pH 8 for 15 min.

The effects of organic solvents on enzyme activities were determined by the spectrophotometric assay after pre-incubation (1 or 2 h) of enzyme (0.3 ml) for 1 and 2 h at 30 °C at pH 8 in 0.1 ml of hexane, ethyl acetate, isopropanol, acetone, ethanol, or butanol-1-ol. Also, under the same conditions, the effects of various compounds (Tween 20, Tween 80, tributyrin, sodium dodecyl sulfate (SDS), β-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), Triton X-100, glycerol, and dithiothreitol (DTT)) on enzyme activities were studied by incubating the enzyme with their different concentrations. All experiments were repeated at least two times.

2.5. Zymogram Analysis

The crude lipases were concentrated using the ultracentrifugal filter with a membrane pore size of 10 kDa.

Native PAGE (10%) was applied to characterize the lipase present in the crude enzyme as described by Laemmli [17]. Electrophoresis was conducted at 50 V for approximately 1 h and then again at 80 V for 4-5 h. After electrophoresis gel was washed in 1% and 0.1 % Triton X-100 (50 mM Tris HCl, pH 8) solutions for 10 min respectively. It was later washed with distilled water for 10 min. The gel was covered with Tween 20 agar medium (1% Tween 20, 20 mM NaCI, 1 mM $CaCI_2$, 0.5% gum Arabic, 1.5% Agar,). After overnight incubation at 30 °C, the lipase activity was visualized as a clear crystal zone. Experiment was repeated at least two times.

3. Results

In this study, two *A. psychrotolerans* strains (Xg1 and Xg2) showed lipolytic zones on Tween 20 and Tween 80 agar after 72 hour incubation at 30 °C (Figure 1). The orange fluorescence produced by these strains on Rhodamine B lipase agar under UV irradiation supported the strains lipase activities (Figure 2).

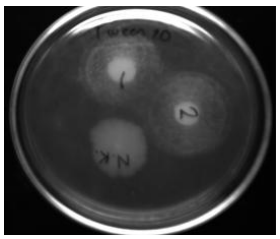

Figure 1. Crystal formation of *A. psychrotolerans* Xg1 and Xg2 strains on Tween 20 agar, 1: Xg1 strain; 2: Xg2 strain; N.C.: Negative control (*E. coli*).

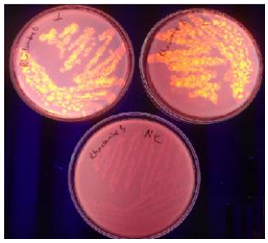

Figure 2. Detection of lipase enzymes of *A. psychrotolerans* Xg1 and Xg2 strains on Rhodamine B plates under UV light, 1: Xg1 strain; 2: Xg2 strain; N.C.: Negative control (*E. coli*).

The maximum activities of the crude lipases(Xg1 and Xg2) were performed from 24 h and 48 h cultures of *A. psychrotolerans* strains in Tween 20 medium with 150 rpm agitation at 30 °C. Lipases exhibited maximum lypolytic activity at pH 8.0 (Figure 3). Activities were absent at pH 3.0, 4.0, and 5.0. Activity at pH 6 was very slightly observed. The stability of the lipases were determined after 24 hours incubation at 4 °C and pH 8. The results indicated that enzyme activities remained stable at pH 8.0.

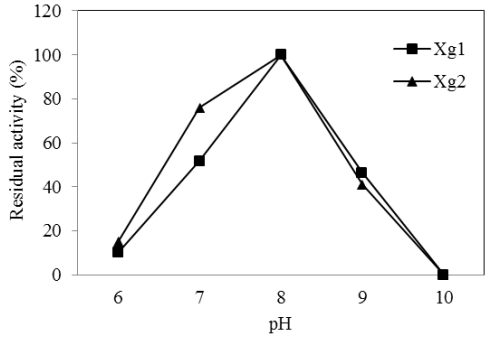

Figure 3. Effect of pH on lipase activity.

In this study, effects of temperature on lipase activities were determined at various temperature. Enzyme activity of the Xg1 strain was detected between 10 and 70°C; for the Xg2 strain, enzyme activity was detected between 10 and 90 °C (Figure 4). The enzymes exhibited the highest activity at 30 °C. Stability of these lipases displayed high activity between 10 and 60 °C for 1 hour (Figure 5).

The effects of metal ions on the lipase enzymes were given in Table 1. As seen in the table, some metal ions did not significantly affect the enzyme activity, except for Cu^{2+}, Zn^{2}+ and Fe^{3+}.

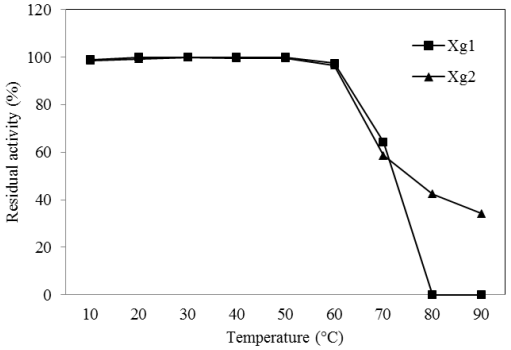

Figure 4. Effect of temperature (for 15 min) on lipase activity.

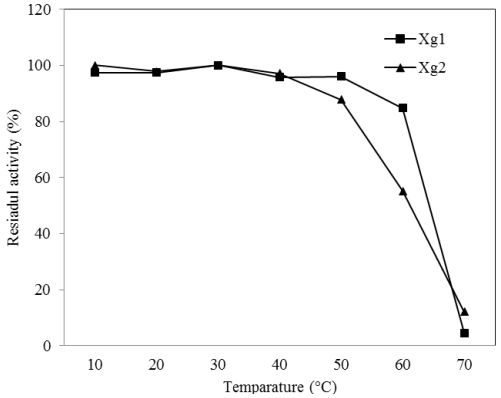

Figure 5. Effect of temperature (for 1 h) on lipase activity.

Table 1. Effect of different metal ions on lipase activity.

Compound	Residual activity (%)			
	Xg1		Xg2	
	5 mM	10 mM	5 mM	10 mM
Control	100	100	100	100
Ca^{2+}	96,84	101,96	99,04	100
Cd^{2+}	99,05	101,96	99,04	101,28
Co^{2+}	95,85	96,09	98,08	99,03
Cu^{2+}	90,53	1,63	61,38	4,1
Fe^{3+}	107,2	24,5	92,01	64,87
Hg^{2+}	94,61	70,75	91,41	58,84
Li^{+}	97,79	100,6	98,84	100
Mg^{2}	95,58	101,9	97,76	100,96
Na^{+}	95,58	103,5	102,5	99,03
NH_4^{+}	99	96,67	109	102,25
Ni^{2+}	100,8	99,01	98,08	99,03
Zn^{2+}	8,28	5,53	13,73	4,5

When organic solvents were tested, Xg1 strain exhibited over 60% activity (Table 2). However Xg2 lipase was sensitive to acetonitrile (1, 2 hours), isopropanol (1 hour) and butan-1-ol (1, 2 hours) (Table 2). At another tested organic solvents, the Xg2 strain had over 80% activity.

Table 2. Effect of different organic solvents on lipase activity.

Organic solvent	Residual activity (%)			
	Xg1		Xg2	
	1 h	2 h	1 h	2 h
Control	100	100	100	100
N,N-diethylformamide	103,8	98,59	100	100,31
Ethanol (96%)	102,23	89,25	97,12	97,77
Acetonitrile	100,95	96,41	30,35	32,58
Isopropanol	100,63	95,48	35,46	91,42
Hexane	99,68	100	102,5	101,27
Ethylacetate	97,54	98,77	101,25	100
Chloroform	97,54	95,7	102,5	102,5
Butan-1-ol	97,52	60,48	25,87	35,14
Acetone	61,38	81,27	97,12	80,83

The presence of lipase enzymes at various detergents were measured after 1 h at 30 °C. The lipase enzymes exhibited over 93% activity against some detergents used in this study (Table 3). However, the enzymes were found to be sensitive to EDTA and SDS, which yielded approximately 55-95% loss in activity (Table 3).

Table 3. Effect of different detergents on lipase activity.

Detergent	Residual activity (%)	
	Xg1	Xg2
Control	100	100
Triton X-100 (1%)	99,5	100
Mercaptoethanol (1%)	98,68	94,8
Tributyrin (1%)	97,37	94,8
Glycerol (1%)	97,86	95,87
Tween 20 (1%)	97,37	98,47
Tween 80 (1%)	95,9	94,8
DTT (10 mM)	93,11	96,94
EDTA (10 mM)	18,03	44,49
SDS (1%)	0,65	6,42

In order to detected the molecular weight of the lipase, we analyzed the protein using native PAGE. The molecular weights of lipases from *A. psychrotolerans* Xg1 and Xg2 strains were estimated approximately 37 and 30 kDa by comparison of marker size and zymogram analysis results, respectively (Figure 6).

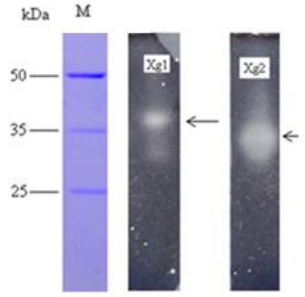

Figure 6. Zymogram analysis of lipases. M:Marker

4. Discussion and Conclusion

In this study, extracellular lipases obtained from *A. psychrotolerans* strains (Xg1 and Xg2) were characterized.

Optimum pH for lipases of Xg1 ve Xg2 strains were pH 8. Lipase activities of the Xg1 and Xg2 strains were 51% and 76% at pH 7.0, respectively. Activities were 46% and 41% at pH 9.0, respectively. The optimum pH for lipases were slightly different such as pH 8.5 for *A. lwoffi* 016 [9] and pH 7.8-8.8 for *A. calcoaceticus* BD413 [10].

The enzymes exhibited the highest activity at 30 °C. The optimum temperature of the lipases characterized in this study were lower than the others studied in the literature, such as 40 °C for *Acinetobacter* sp. CR9 [11] and *Acinetobacter* sp. ES-1 strains [19], 37 °C for another *Acinetobacter* sp. strain [20] and 60 °C for *Acinetobacter baylyi* [21]. Temperature stability of the lipases of both Xg1 and Xg2 strains retained 97% and 100% at 10 °C, respectively. However, the lipases of the Xg1 and Xg2 strains retained 85 and 55% at 60 °C, respectively. Results reported by Wang et al. [18] have great similarity with our own. Lipases activity appearing in low temperature and thermostability is limited.

The structures of enzymes are usually deteriorated in the presence of organic solvents [22]. In this study, activity of Xg2 lipase was found to reduce in the presence of acetonitrile, isopropanol and butan-1-ol. However, in presence another organic solvents, the Xg1 and Xg2 strains had over 60% activity. For enzyme reactions, the stability of organic solvents are important.

Wang et al. [23] reported that 1% Tween 20, Tween 80 detergents displayed approximately 50-60% activity. Our study showed close results with Cherif et al. [24] in several detergents.

The molecular weights of lipases from *A. psychrotolerans* Xg1 and Xg2 strains were found approximately 37 and 30 kDa, respectively. In previous studies, the molecular weight of lipase from *Acinetobacter* sp. was variable from 23 to 62 kDa [18]. The molecular weights of lipases at other strains was reported as: 40 kDa at *A. junii* SY-01 [25], 60 kDa at *Acinetobacter* sp. B2 [26], 23 kDa at *A. calcoaceticus* LP009 [27], 45 kDa at *A. radioresistens* CMC-1 [28], 62 kDa at *Acinetobacter* sp. KM109 [29], 38 kDa at *A. radioresistens* CMC-2 [30] and 53 kDa at *Acinetobacter johnsonii* LP28 [18].

The results obtained in this study showed that lipases of two *A. psychrotolerans* strains exhibited high activity at 30 °C at pH8. The lipases were also highly stable in the presence of various minerals, some detergents and different organic solvents. All these results showed that lipases of Xg1 and Xg2 strains have the potential for the application in the detergent and various industries.

Acknowledgment

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