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Characterization of Lipase Producing Thermophilic Bacilli Isolated from Armenian Geothermal Springs

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Keywords: *Lipase production, lipase activity, lipolytic activity, thermophilic bacilli, hot springs.*

Introduction. Lipases (E.C. 3.1.1.3) catalyze the hydrolysis of acyl-glycerol long chains. They are widely present in nature and are found in plants, animals and microorganisms [1]. Bacterial and fungal lipases are used in food, detergent, drug and cosmetics productions due to the unique ability of performing chemical biotransformation [2-4]. Bacterial lipases are stable and active in organic solutions, do not require co-enzymes, they have higher level of enantomeric excess and have wider range of substrate selection compared to inorganic catalyzers [5, 6].

Many representatives of the genus *Bacillus* and related genera isolated from diverse extreme environments like terrestrial geothermal springs and marine hydrothermal vents have been reported as the main source of thermostable lipases [7, 8]. Lipases from bacilli are easily produced and display high tolerance toward organic solvents, proving them useful in the synthesis of esters for food industry, cosmetics and biodiesel production. Many of them preserve their activity at extreme temperatures and pH, and therefore they can be applied in laundry formulations [9]. Thermostable enzymes are able to brave high temperature, thus endow longer half-life to the biocatalyst. Their ability to conduct various reactions to higher process rates because of increase in substrate diffusion coefficient and reduce viscosity at higher temperatures makes them a preferred choice over mesophilic sources [10]. Isolation of more thermostable lipase producers from high temperature environments such as hot springs is required in order to satisfy the need for thermozymes.

The aim of the presented work was the screening active lipase producers among the thermophilic bacilli strains isolated from different geothermal springs of Armenia, their identification based on phenotypic characteristics, as

well as the estimation of the effects of temperature and pH on their lipase production and activity.

Materials and methods. *Enrichment and selection of active lipase producers***.** The 72 thermophilic bacilli strains used in this study have been previously isolated from the geothermal springs distributed in Armenia [11].

All isolates were preliminary screened for their lipolytic activity in the solid medium containing Tween 80 (1%), $CaCl_2$ (0.1%), peptone (0.5%), NaCl (0.5%) and agar (1%), pH 7.0 [12]. The plates were incubating at 55°C. The lipase activity was measured by the diameter of the precipitated area formed around the colony after 72 h of incubation. The lipase activity index (LAI) value, which represents the ratio between the halo diameter and the microbial colony diameter, were used to evaluate the production level of lipase [7, 13].

Phenotypic characteristics of selected isolates. Microbial colonies were described by color, size, shape, surface and margins on nutrient broth (NB) agar. The cell morphology, sporulation and motility were determined by light microscope (Motic 10).

The temperature range for growth was determined after incubation of isolates at temperature from 25 to 65°C with 10°C intervals. The pH dependence of growth was tested at pH range from 5 to 10. The anaerobic growth, catalase and oxidase activity, reduction of nitrate to nitrite, Voges-Proskauer reaction, formation of dihydroxyacetone and indol were determined according to [14]. The utilization of citrate and different substrates as carbon sources (D-glucose, L-arabinose and D-mannitol) was determined using the Simmon's and Hugh Leyfsona's medium, correspondingly [12]. The casein, starch and gelatin hydrolyses were tested by streak flask technique [12].

The identification of microorganisms was performed according to the Bergey's manual [15].

Effect of temperature and pH on lipase production. The optimal conditions for enzyme production were tested in an inducing medium with pH from 5.0-10 containing 1% Tween 80 as a substrate [16]. The plates were incubating at temperatures from 25 to 65 °C with 10°C intervals. The lipase activity index (LAI) was collaborated to find the optimal conditions for lipase production.

Determination of crude lipase activity at different pH and temperature. Lipase activity was measured by titrating free fatty acids released by hydrolysis of tween [12, 17]. Bacteria were cultured in the enrichment medium continuing 1% tween 80 overnight with shaking (150 rpm) at 55°C. The cultures were centrifuged at 9000 rpm for 5 min and supernatant was used as crude enzyme source. Reaction mixture containing 1.0 ml tween 80, 5.0 ml buffer (50 mM sodium acetate buffer (pH 5), 50 mM phosphate buffer (pH 6.0 and 7.0), 50 mM Tris–HCl (pH 8.0 and 9.0) or 50 mM sodium bicarbonate buffer (pH 10)) and 1.0 ml of crude enzyme was incubated with shaking (150 rpm) at temperature ranging between 25°C and 65°C with 10°C intervals for 30 minutes. The reaction was terminated by adding 1 ml ethanol and titrated with 0.1 N potassium hydroxide using phenolphthalein (0.1%) as an indicator. The control contained the same ingredients, but the reaction was terminated prior to

addition of the crude enzyme. One unit of lipase activity (U) was defined as the release of 1 μmol of fatty acid per min under mentioned conditions.

Statistical analysis. The standard deviation, standard error and mean values were calculated using Microsoft Excel 2010, R-Project. The p values were calculated using the R-Project online resource for statistical computing (http://www.r-project.org/).

Results and discussion. *Detection of active lipase producers***.** In order to detect the lipase producing strains all bacilli cultures were cultivated on medium containing tween 80 as carbon and energy source. From 72 studied bacilli strains 20 were detected to be lipase producers. The corresponding results are represented in Table 1.

Table 1

Number of lipase producers among screened bacilli isolates in the media containing Tween 80

Origin (hot spring location)	*Number of screened isolates*	*Number of lipase producer strains*
Jermuk	15	4
Uyts	16	4
Akhurik	10	6
Gyl	3	2
Arzakan	10	1
Qarvachar	10	4
Hanqavan	6	2
Darayurd	1	1
Bjni	1	0

Two most active lipase producers designed as strains Akhurik 106 and Ackurik 107 were selected for further studies. The LAI values for Akhurik 106 and Akhurik 107 were 2.0 cm and 1 cm, respectively (Fig. 1).

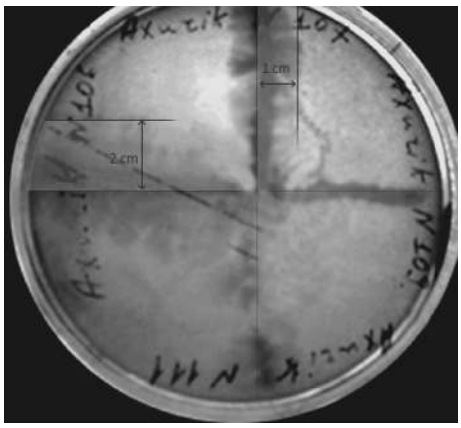

Fig. 1. Lipase production by the studied strains (Ca^{2+} salts of fatty acids precipitation zones around the colonies indicate lipase production). For the others to this and the other figures, see Materials and methods.

Phenotypic characteristics of selected isolates. On solid NB the strain Akhurik 106 formed irregular, white colored, opaque, flat, smooth surface, lobate margin, homogenous colonies, while the strain Akhurik 107 formed circular, milky colored, umbonate, homogenous colonies (Fig. 2).

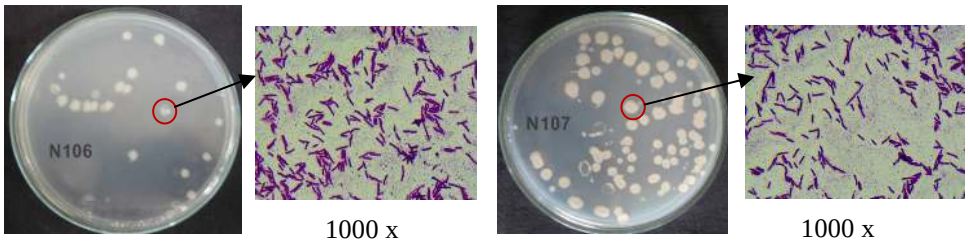

Fig. 2. Colonies and morphological characteristics of Akhurik 106 and Akhurik 107

Table 2

Some phenotypic characteristics of the studied isolates

Phenotypic characteristics	*Akhurik 106*	*Akhurik 107*	*B. subtilis**
***Cell size** [μm]*			
Width	1.5–2.0	1.0-2.0	0.7-0.8
Length	4.0–5.0	4.0-5.0	2.0-3.0
Motility	+	+	+
Endospore			
Form	Ellipisoidal	Ellipisoidal	Ellipsoidal
Location	Central	Central	Subterminal
Swell sporangia	-	-	-
Optimum temperature	55˚C	55˚C	28-30˚C
Optimum pH	6.5-7.0	7.0-8.0	5.5-8.5
Oxidase	+	+	+/-
Voges-Proskauer test	-	+	+
Nitrate reduction to nitrite	-	+	-
Acid from			
D-Glucose	+	+	+
L-Arabinose	+	+	+
D-Manitol	+	+	+
Gas from glucose	-	-	-
Hydrolysis of			
Casein	-	-	+
Gelatin	+	+	+
Starch	+	+	+
Utilization of citrate	-	-	+
Formation of			
Indole	-	-	NA
Dihydroxyacetone	-	+	NA

**B. subtilis* was chosen as a reference strain [Bergey's Manual of Systematic Bacteriology, 2009].

Cells of the strains were Gram positive, motile rod shaped varying in length between 4.0 to 5.0 μm and in width between 1.0 to 2.0 μm (Fig. 2).

The cells formed central ellipsoid endospores. The strain Akhurik 107 was positive according to Voges-Proskauer test, formed dihydroxyacetone and reduced nitrite from nitrate. The isolates hydrolyzed starch and gelatin, but did not use citrate and did not form gas from glucose. Some phenotypic characteristics of the isolates are displayed in the Table 2.

The both strains showed ability to growth in the different range of temperature. The optimal temperature value was 55°C. The pH range for growth of strains Akhurik 106 and Akhurik 107 was from 6.0 to 9.0 with the optimum pH at 6.5-7.0 and 7.0-8.0, correspondingly.

Following to the criteria of Bergey's manual the isolates were identified as *Bacillus* sp. (Akhurik 106) and *Bacillus licheniformis* (Akhurik 107). The identification of the studied isolates up to species should be confirmed by 16S rDNA analysis.

Effect of temperature and pH on lipase production. The enzyme production by microorganisms depends on environmental conditions such as temperature and pH, and presence of inductors or repressors [16]. The pH of the medium plays a critical role for the optimal physiological performances of the bacterial cell and the transport of various nutrient components across the cell membrane aiming at maximizing the enzyme yields. In an order to optimize the conditions for lipase production by selected bacilli the LAI were evaluated in Petri dishes containing the inducing medium with pH from 5.0-10. Incubation was performed at optimum growth temperature of the strains. The results indicated that the strains able to produce lipase from wide range of pH (from 5.0 to 10), but the highest lipase production (LAI 1.4) of the *Bacillus licheniformis* Akhurik 107 occurred in the medium with pH 7.0. For the strain *Bacillus* sp. Akhurik 106 the highest lipase production (LAI 1.5) observed at pH 6.0 and 7.0 (Fig. 3). Willerding A.L. et al. [16], showed the highest lipase production for 24 different bacterial strains observed at pH 8.0 and 30°C. The optimal production of lipase by *Bacillus* strain J33, *B. megaterium* AKG-1 and *B. thermocatenulatus* DSM 730 was also reported at pH 8.0, 7.0-8.0 and 7.4, respectively [18, 19]. Even though the activity values are comparable to the data from literature, the lipases produced by the studied strains remain active in wide pH range.

The lipase production levels at optimal pH varied depending on the temperature and incubation time. The lowest value of LAI was detected at 25°C temperature after 24 hours incubation. The LAI values reached its maximum (1.5 and 1.6 for *Bacillus* sp. Akhurik 106 and *Bacillus licheniformis* Akhurik 107, correspondingly) at 55°C temperature after 48 h of incubation (Fig. 4).

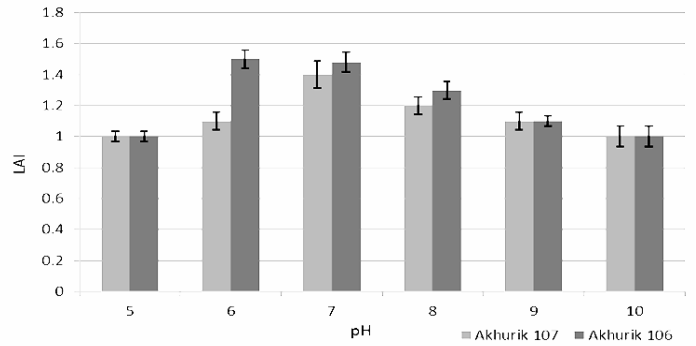

Fig. 3. Lipase production rates at different pH values by the studied bacilli expressed with LAI.

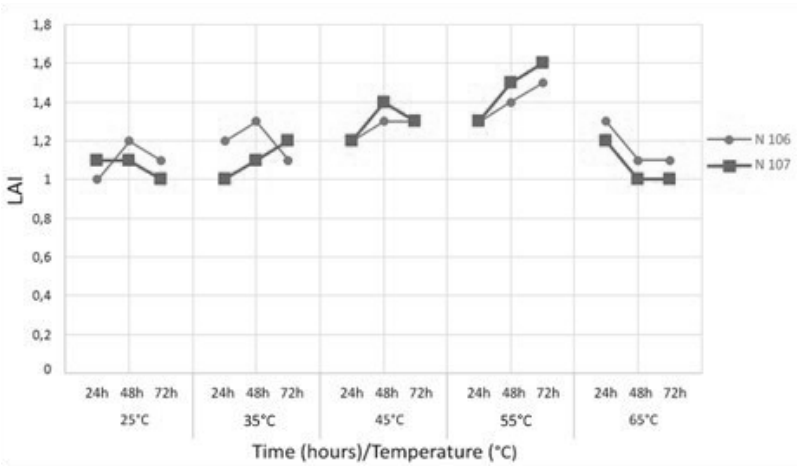

Fig. 4. Lipase activity index (LAI) up to 72 hours at different temperatures.

Sidhu et al. 1998 [20] showed that thermostabile lipase production by thermophilic *Bacillus* sp. RS-12 was growth-associated and at the medium contained 0.5% Tween 80 gave a maximal yield of the enzyme at 50°C cultivation temperature. *B. thermoleovorans* ID-1, isolated from hot springs in Indonesia, showed high extracellular lipase production at 65°C [12]. The results indicated that optimal conditions for lipase production of the strains *Bacillus* sp. Akhurik 106 and *Bacillus licheniformis* Akhurik 107 were pH 6.0-7.0 and 55°C cultivation temperature.

Effect of pH and temperature on lipase activity. In order to optimize the lipase activity of the strains the effect of the pH and temperature on lipase activity of the strains was determined. The crude lipase activity was studied at the different range of pH (5-10) and optimal growth temperature of the strains. The highest lipase activity levels (0.89 U/ml and 0.75 U/ml for the strains *Bacillus* sp. Akhurik 106 and *Bacillus licheniformis* Akhurik 107, respectively) were defined at pH 6.5 - 7.0 (Fig. 5).

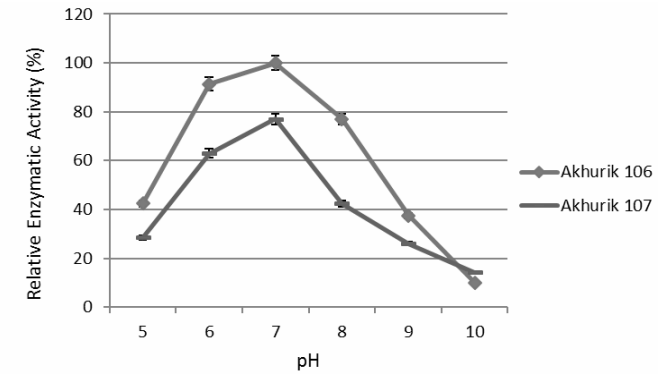

Fig. 5. Effect of the different pH on the lipase activity of studied strains. The maximal enzymatic activity (0.89 U/ml) at pH 7.0, for *Bacillus* sp. Akhurik 106 was defined as 100%.

Assuming that enzyme is stable at elevated temperatures, the productivity of the reaction can be enhanced greatly by operating at a relatively high temperature. Therefore, the optimal lipase activity temperature has been determined. The temperature optimum for lipase activity at defined optimal pH coincides with the growth optimal temperature of the strains. Thus, the highest lipase activity for both strains was displayed at 55°C (Fig. 6), but the crude enzyme remained active in temperature range of 25 to 65°C.

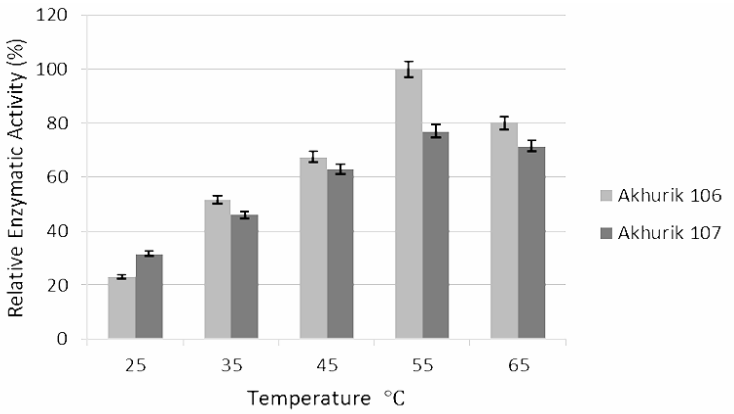

Fig. 6. Effect of the different temperature on the lipase activity of studied strains. The maximal enzymatic activity (0.89 U/ml) for *Bacillus* sp. Akhurik 106 at 55°C, pH 7.0 was defined as 100%.

The maximum values of crude enzyme activity were 0.89 U/ml and 0.75 U/ml for *Bacillus* sp. Akhurik 106 and *Bacillus licheniformis* Akhurik 107, respectively in temperature of 55°C and pH 7.0.

The lipase activity of different bacterial groups at wide range of pH and temperature has been reported [7]. Lee et al. 1999 [12] determined optimal lipase activity of *B. thermolevorans* ID-1 at pH 7.5. The thermostabile lipase produced by *Bacillus* sp. J 33 had a high activity at pH 7.6 and 55°C [21]. One

of the comparably notable thermostabile lipase was isolated by Wang et al. 1995 [22] from a *Bacillus* strain. This enzyme had maximum activity at 60°C. An extracellular *Bacillus* lipase isolated by Sidhu et al. 1998 [19] had an activity optimum at 50°C. The crude lipase from the studied strains, compared to the literature data, has an advantage of operating at wide range of pH and temperature.

The results indicated that studied *Bacillus* strains might be prospective for various biotechnological applications and industry as lipases producers. Purification and more detail investigation of the isolated bacilli lipase are planning to be performed.

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Characterization of Lipase Producing Thermophilic Bacilli Isolated from Armenian Geothermal Springs

72 bacilli strains previously isolated from different geothermal springs of Armenia were screened for their lipase production. Two most active lipase producing bacilli strains designed as *Bacillus* sp. Akhurik 106 and *Bacillus licheniformis* Akhurik 107 were selected and characterized based on morphological, cultural, biochemical and physiological properties. The temperature of 55°C and pH 6.5–7.0 were defined to be the optimal conditions for lipase production. The activities of crude enzymes of these strains were 0.89 U/ml and 0.75 U/ml at the optimum pH and temperature, respectively. These strains might be used as lipase producers.

Գ. Ս. Շահինյան, Հ. Հ. Փանոսյան, ՀՀ ԳԱԱ թղթակից անդամ Ա. Հ.Թռչունյան

Հայաստանի երկրաջերմային աղբյուրներից անջատված լիպազ արտադրիչ ջերմասեր բացիլների շտամների ուսումնասիրությունը

Հայաստանի տարածքում գտնվող տարբեր երկրաջերմային աղբյուրներից նախապես անջատված բացիլների 72 շտամներում ուսումնասիրվել է լիպազ սինթեզելու ունակությունը: Երկու, առավել ակտիվ լիպազ արտադրիչ շտամներն ընտրվել են հետագա ուսումնասիրությունների նպատակով: Վերջիններս ըստ ձևաբանական, կուլտուրային, կենսաքիմիական և ֆիզիոլոգիական հատկանիշների նույնականացվել են որպես *Bacillus* sp. Akhurik 106 և *Bacillus licheniformis* Akhurik 107. Պարզվել է, որ լիպազի սինթեզի համար ջերմաստիճանային օպտիմումը 55°C է, իսկ pH-ի՝ 6.5–7.0. Այդ պայմաններում չմաքրված ֆերմենտների ակտիվությունները *Bacillus* sp. Akhurik 106 և *Bacillus licheniformis* Akhurik 107 շտամների համար կազմել են համապատաս-

խանաբար 0.89 Մ/մլ և 0.75 Մ/մլ: Այդ շտամները կարող են կիրառվել որպես լիպազների արտադրիչներ:

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Изучение липаз-продуцирующих термофильных штаммов бацилл, изолированных из геотермальных источников Армении

Исследованы 72 штамма бацилл, ранее изолированных из разных геотермальных источников Армении, на способность синтезирования липаз. Были отобраны два наиболее активных липаз-продуцирующих штамма. По морфологическим, культуральным, биохимическим и физиологическим свойствам штаммы были идентифицированы как *Bacillus* sp. Akhurik 106 и *Bacillus licheniformis* Akhurik 107. Оптимальная температура синтеза липаз была 55°C, а pH 6.5–7.0. При этих условиях активность неочищенных ферментов штаммов *Bacillus* sp. Akhurik 106 и *Bacillus licheniformis* Akhurik 107 составила 0.89 и 0.75 Е/мл, соответственно. Эти штаммы могут быть использованы как продуценты липаз.

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