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Thermophilic Bacilli Isolated from Armenian Geothermal Springs and their Potential for production of Hydrolytic Enzymes

Hovik H. Panosyan*

Department of Biochemistry, Microbiology and Biotechnology, Yerevan State University, Yerevan Armenia

Corresponding author: Hovik H. Panosyan, Department of Biochemistry, Microbiology and Biotechnology, Yerevan State University, Yerevan Armenia.Tel: + 374 94 71 95 90, E-mail: hpanosyan@ysu.am

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Abstract:

The isolation and identification based on phenotypic and phylogenetic characteristics of the thermophilic bacteria from different geothermal springs (with temperature 27.5–70 °C) distributed on the territory of Armenia and Nagorno Karabakh were carried out. In total 135 thermophilic and thermotolerant bacilli strains were isolated under aerobic conditions at 55-65 °C and indentified based on 16S rRNA gene sequence analysis as representatives of genera Anoxybacillus, Bacillus, Brevibacillus, Geobacillus, Paenibacillus, Sporosarcina, Ureibacillus and Thermoactinomyces. These thermophilic bacilli were tested for enzyme production capacities such as lipase, protease, amylases and biotechnologically valuable enzyme producers were selected.

Keywords: Geothermal Springs, Thermophilic Bacilli, 16S rRNA Gene Sequencing, Hydrolytic Activity, Amylases, Lipases And Proteases

Introduction:

Thermophiles are naturally found in various geothermally heated regions of Earth such as hot springs and deep-sea hydrothermal vents. Particularly, geothermal springs offer a new source of fascinating microorganisms well adapted to these extreme environments (Hreggvidsson et al., 2012, Deepika, Satyanarayana, 2013). The adaptation to these harsh habitats explains the high genomic and metabolic flexibility of microbial communities in these ecosystems and makes thermophiles and their thermostable proteins very suitable for some industrial and biotechnological applications (Raddadi et al., 2015; DeCastro et al., 2016). The vast interest of scientists to these exotic niches was observed in the last decades and unknown bacteria were recently isolated from terrestrial hot springs in different parts of world such as USA (Bowen De León et al., 2013), Iceland (Krebs et al., 2014), Bulgaria (Derekova et al., 2008; Stefanova et al., 2015), China (Hedlund et al., 2012; Hou et al., 2013), Argentina (Urbieta et al., 2015), Turkay (Cihan et al., 2011), Russia (Kublanov et al., 2009), India (Saxena et al, 2017) and other parts of world.

Thermostable enzymes, which have been isolated mainly from thermophilic microorganisms, have found a number of commercial applications as they possess thermal stability to harsh industrial processes at high temperatures (DeCastro et al., 2016). The elevation of temperature is accompanied by a decrease in viscosity and an increase in the diffusion coefficient of organic compounds. Furthermore, by performing biological processes at high temperature the risk of contamination is reduced and controlled processes under strict conditions can be carried out (Antranikian, 2008; Kurosawa, 2013).

Thermophilic microorganisms are not grouped into a separate taxonomic unit, but appear in various taxonomic groups and at various phylogenetic distances throughout the taxonomic system (Horikoshi and Bull, 2011; Sharma et al., 2013). It was shown that representatives of the genus Bacillus and related genera to be the thermophilic aerobes most frequently iso¬lated from terrestrial geothermal water environments (Sharp et al., 1992). Typically, enzymes production in the course of Bacillus fermentation processes occurs during a relatively short period of time, with very low cost carbon and nitrogen sources. A great part of industrially valuable enzymes are mainly produced by bacilli (Schallmey et al., 2004). Therefore, screening for novel biocatalysts from thermophiles has become a very important field and can open a new horizon in biotechnology. Between not well known ecological zones of the Earth, thermal springs located in the Minor Caucasus still represent a challenge for searching of undescribed biotechnological resource. The geology of the region where Armenia and Nagorno-Karabakh are situated is complex, owing to accretion of terrains through plate-tectonic processes, and to ongoing tectonic activity and volcanism (Henneberger et al., 2000; Badalyan, 2000) Numerous geothermal springs of different geotectonic origin and with different physicochemical properties are found on the territory of

Armenia and Nagorno-Karabakh (Mkrtchyan, 1969). Recently microbiological investigations of some Armenian geothermal springs were carry out based on culture independent and molecular methods (Panosyan, 2010, Hedlund et al., 2013; Panosyan and Birkeland, 2014). Despite this progress very little is known about the diversity of biotechnologically valuable enzyme producers thriving in Armenian geothermal springs (Shahinyan et al., 2017).

The present study focuses on isolation and identification of thermophilic bacilli from geothermal springs of Armenia and Nagorno- Karabakh as well as on screening of hydrolytic enzymes (amylase, lipase and protease) producers.

Materials and methods

Study sites and sampling

The location of geothermal mineral springs was determined using JPS. The samples were collected from Armenian (Akhurik, Arzakan, Bjni, Hankavan, Jermaxbjur, Jermuk, Tatev, Uys, Karvachar, Zuar) and Nagorno-Karabakhian (Karvachar, Zuar) geothermal springs of different geotectonic origin and with different physicochemical properties (Fig. 1). Temperature, pH and conductivity were determined in situ using a portable combined pH/EC/TDS Temperature tester (HANNA HI98129/HI98130). Water, mat and adjacent sediment samples were collected using sterile glass flasks and were maintained on ice until processed.

Fig. 1. Location of study sites. Maps of Armenia and Nagorno Karabakh showing the locations of studied geothermal springs. Close up photograph of hot springs.

Enrichment and isolation

To enrich aerobic endospore-forming thermophilic bacteria slurry water, mat and sediment samples (1g) were inoculated in Nutrient Broth (HiMedia) and incubated overnight at 55, 60 and 65 ºC with shaking at 150 rpm. Before inoculation all samples were treated at 80 oC for 10 min aiming to isolate only spore-forming microorganism (Netrusov, 2005). Cultures showing different colony morphology was further purified by streaking samples on the same medium supplemented with agar (2 %, w/v). All colonies obtained on plates were picked and purified by streaking onto same medium at least three times. The subcultures were considered pure after microscopic observation of a single morphological type per culture. The subcultures' purity, cell morphology and motility, endospore location and were determined by phase-contrast microscopy of freshly prepared wet mounts.

Phenotypic characterization of isolate. All isolates were tested for their colony morphology, Gram reaction and thermophilic growth using common methods (Netrusov, 2005). The temperature range for growth was determined after incubation of isolates at 5 to 80 ºC by 5 ºC intervals. The pH dependence of growth was tested at pH range of 5 to 12. The range of NaCl concentrations for growth was determined by adding 0 to 15 % NaCl to the incubation medium. Catalase activity was determined by bubble formation in a 3 % hydrogen peroxide solution. Biochemical tests such as nitrate reduction, gas production from D-glucose, formation of dihydroxyacetone and Voges-Proskauer test were carried out according to (Gordon et al., 1973).

DNA extraction, polymerase chain reaction (PCR) and sequencing

DNA was extracted from pure isolates using GenEluteTM Bacterial Genomic DNA Kit (Sigma) according to the manufacturer's recommendations and used as a template in the PCR assays. 16S rRNA genes were amplified using universal primer pairs 27f (5'-GAGTTTGATCCTGGCTCA-3') and 1525r (5'-GAAAGGAGGAGATCCAGCC-3') (Escherichia coli numbering). PCR mixtures used for amplification of sequences contained 10 ng DNA, 5 µl 10 X PCR buffer, 5 µl 10 mM dNTP (dATP, dGTP, dCTP and dTTP), 1 µl each primer (25 pmol/µl), 1,5 mM MgCl2, 0,2 µl Taq DNA polymerase, and sterile water up to the final vo¬lume of 50 µl. PCR amplification was completed using an DNA Engine thermocycler (BIO RAD). First, the templates were denaturized for 3 min at 96 ºC, then 30 cycles of the following steps were completed: denaturation for 30 at 96 ºC for, annealing for 30 s at 55 ºC, and extension at 2.5 min at 72 ºC. The 30 cycles were followed by a final 10 min extension at 72 ºC. PCR proucts were viewed under UV light after standard ethidium bromide gel electrophoresis. PCR products were purified with GenElute™ PCR Cleanup Kit (Sigma). Sequencing of bacterial 16S rDNA amplicons were performed on ABI PRISM capillary sequencer according to the protocol of the ABI Prism Big-Dye Terminator kit (Perkin Elmer) using above mentioned primers. Raw data of DNA sequences was analysed with Chromas and BioEdit software. A nucleotide BLAST search was performed in order to obtain information on the phylogenetically closest relative (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/Blast) (Altschul et al., 1997).

Selection of prospective producers of hydrolytic enzymes

Starch hydrolysis were tested by streak plate technique using the medium containing soluble starch (2 % w/v), pepton (1 %, w/v) KH2PO4 (0,5 % w/v), agar (1,5 % w/v), pH 7.0. Amylolytic activity was determined after flooding the plates with Lugol's solution (0.5 % w/v I2 and 1.0 % KI w/v in distilled water), which turns non-degraded starch a dark color. Colonies that produced clear zones on this medium were identified as amylolytic strains (Dhawale et al., 1982).

The purified microbial isolates were screened for lipolytic activity on agar plates containing peptone (1 % w/v), NaCl (0.5 % w/v), CaCl2•2H2O (0.01 % w/v), agar (2 % w/v), and Tween-20 (1 % v/v). The presence of lipolytic activity was indicated by a visible precipitate resulting from the calcium salt formed by the fatty acid

from the hydrolysis reaction (Lee et al., 2015).

Proteolytic activity was detected by casein hydrolysis on agar plates with medium containing 0.5 % of skim milk powder, 0.5 % of glucose, 2 % of agar, pH 7.0. Enzyme activity was indicated by the formation of a clear zone around colonies after precipitation with 1 M HCl solution (Panda et al., 2013)

Results and Discussion

The temperature of water of studied geothermal springs in the outlet varied between 27-70 oC. Despite the varying phy¬sical-chemical properties of the thermal springs used for the sampling, they belong to the category of hot springs from low-tem¬perature fields and are characterized by neutral to alkaline pH and high concentration of dissolved minerals and gases. Studied mesothermal springs mainly are related to the hydrocarbonate sodium or hydrocarbonate-sulphate sodium-mag¬nesium classes. The location and physical-chemical parameters of geothermal springs of Armenia and Nagorno-Karabakh are shown on the Table 1.

Collected 52 water, mat and adjacent sediment samples were analyzed to evaluate the total thermophilic aerobic endospore-forming bacterial abundance. A total of 135 isolates with different colony morphologies were obtained from the samples from different geother-mal regions. All isolates were rod-shaped, gram-positive, endospore-forming, catalase-positive bacteria. Results of nitrate reduction, gas production from glucose, Voges-Proskauer test and some other properties are shown on the Table 2.

Isolates optimal growth temperature of which was between 60-65^{o}C were defined as oligate thermophilic, whereas the ones growing at 55 oC were taken as thermotolerant. Although 135 of 40 isolates exhibited thermophlic growth, 95 were found to be thermotolerant. Most of the isolates were able to grow with pH range 6.5-8.5 and 0-5 % of NaCl.

Table 1. The location and physical-chemical parameters of geothermal springs distributed on the territory of Armenia and Nagorno-Karabakh.

Geothermal spring	Spring location	Components containing >20% of total anions and >20% of total kations*		pH	Conductivity, µS/sm	Temperature of outlet water T, °C
Akhurik	40°44′34.04″N, 43°46′53.95″E	HCO_3-SO_4	Na-Mg	6.5	2490	28-32
Arzakan	40°26′902″N, 44°36′508″E	HCO_3	Na	7.2	4378.3	>42
Bjni	40°45′94.44″N, 44°64′86.11″E	HCO_3-Cl	Na-Ca-Mg	6.2-7.0	4138.3	30-37
Hankavan	40°37′57.55″N, 44°29′04. 65″E	Cl-HCO_3	Na-Ca	7.0-7.2	6722.9	42-44
Jemaxbjur	39°47′0.50″ N, 45°54′27.0″ E	HCO_3-SO_4-Cl	Na-Ca-Mg	6.28	1586	31.2
Jemuk	39°50′479″N, 45°40′067″E	HCO_3-SO_4	Na	7.5	4340	>53
Tatev	39°22′54″ N, 46°14′24″ E	HCO_3	Ca	6.55	1920	27
Uyc	39°31′00″ N, 46°03′09″ E	HCO_3-SO_4-Cl	Na-Ca-Mg	6.23	2700	25.8
Karvachar	40°02′570″N, 46°00′990″E	HCO_3-SO_4	Na-Ca	7.3	4600	70
Zuar	40°02′47.6″N 46°14′09.3″E	HCO_3-SO_4	Na-Ca	7.0	4300	42

*Data obtained from Mkrtchyan (1969).

Table 2. General characteristics of the isolated thermophilic bacilli from geothermal springs.

Property	Characteristic	Positive Results (%)
Gram reaction	+	100
Cell shape	Rod	100
Motility	+	100
Catalase activity	+	100
Swollen endospore	+	12
Endospore location	Central	90
	Subterminal or Terminal	10
Nitrate reduction	+	85
Gas production from glucose	-	100
Voges-Proskauer test	+	40
Dihydroxyacetone formation	+	85

All thermophilic aerobic endospore-forming isolates were identified based on their 16S rRNA gene analysis. BLAST results for the isolates, based on 16S rRNA gene sequences for identifcation of the closest relatives in the GenBank database indicate that they all belong to Clostridium-Bacillus subphylum, group of Bacillus-like genera distributed in genera Anoxybacillus, Bacillus, Brevibacillus, Geobacillus, Paenibacillus, Sporosarcina, Ureibacillus and Thermoactinomyces (Table 3). Representatives of the genera Geobacillus and Anoxybacillus are the most distributed obligate thermophiles in the studied hot springs. All isolates from the hot springs that belonged to the genus Bacillus were thermotoleant microorganisms among which B. licheniformis appeared as the dominating species. Studied springs demonstrated significantly lower content of species belonged to genera Brevibacillus, Ureibacillus Paenibacillus, Thermoactinomyces and Sporosarcina. Some bacilli sequences shared less than 96-97% identity with their closest match in GenBank, indicating that the Armenian geothermal springs harbour novel bacilli species. While these results are important for further taxonomic work, positive results on hydrolitic activities are indicative of potential application of these bacterial cultures.

Table 3. Diversity of bacilli of studied Armenian geothermal springs.

Geothermal spring	Distributed genera (dominated species)
Akhurik	*Bacillus* (*B. licheniformis, B. pumilus, B. murimartini*), *Geobacillus* (*G. pallidus*), *Brevibacillus* (*B. borstelensis*), *Thermoactinomyces* (*Thermoactinomyces* sp.)
Arzakan	*Bacillus* (*B. licheniformis, B. simplex*), *Anoxybacillus* (*A. rupiensis*), *Geobacillus* (*G. toebii, G. thermodenitrificans, G. stearothermophilus, G. caldoxylosilyticus*), *Paenibacillus* (*Paenibacillus* sp.), *Sporosarcina* (*Sporosarcina* sp.)
Bjni	*Bacillus* (*B. licheniformis, B. aestuarii*), *Ureibacillus* (*U. thermosphaericus*), *Anoxybacillus* (*Anoxybacillus* sp.), *Geobacillus* (*G. toebii*)
Hankavan	*Bacillus* (*B. licheniformis*), *Brevibacillus* (*B. thermoruber*), *Geobacillus* (*G. stearothermophilus*), *Anoxybacillus* (*Anoxybacillus* sp.)
Jermaxbjur	*Bacillus* (*B. subterraneus*), *Anoxybacillus* (*Anoxybacillus* sp.)
Jermuk	*Bacillus* (*B. licheniformis, B. amyloliquefaciens*), *Anoxybacillus* (*A. gonensis, A. kestanbolensis*), *Geobacillus* (*G. stearothermophilus, G. caldoxylosilyticus*)
Tatev	*Bacillus* (*B. licheniformis*), *Geobacillus* (*G. toebii*), *Anoxybacillus* (*Anoxybacillus* sp.), *Thermoactinomyces* (*T. vulgaris*)
Uyts	*Bacillus* (*B. licheniformis*), *Ureibacillus* (*U. terrenus, U. thermosphaericus*), *Anoxybacillus* (*Anoxybacillus* sp.), *Geobacillus* (*G. toebii*)
Karvachar	*Anoxybacillus* (*A. flavithermus*), *Geobacillus* (*G. toebii*)
Zuar	*Bacillus* (*B. licheniformis*), *Anoxybacillus* (*Anoxybacillus* sp.), *Geobacillus* (*G. toebii*)

Number of thermophilic bacilli species belonging to the genera Bacillus, Geobacillus and Anoxybacillus have been isolated from different geothermal springs and reported as thermostable amylase, lipase and proteinase producers (Raddadi et al, 2015; DeCastro et al., 2016). Amylases are among the most important commercial enzymes, comprising 25 % of the total enzyme market and used in areas like manufacturing high fructose-containing syrups, fermentation of starch to ethanol, treatment of starch processing waste water (Elleuche, Antranikian, 2013). Lipases have a wide area of usage in detergent, food, bio-resolution of pharmaceuticals, agrochemicals, bioremediation, cosmetics and perfumery industry (Sharma et al., 2013). Proteases, which constitute the 60 % of the global enzyme market, have a wide area of usage in various sectors like detergents, leather, food, cosmetics, medicine and medical diagnosis (Sinha, Khare, 2013).

Therefore, the potential of bacilli species, as candidates for commercial production thermostable hydrolases should be considered. Taken into account this information, amylolytic, proteolytic, and lipolytic enzyme activities of the isolates were detected using a plating technique (Fig. 2). In total 65 strains (almost half of the isolates) showed good hydrolysis of the starch substrate, as indicated by the production of clear zones on the plate. In total 52 lipase and more than 30 thermophilic protease producers were selected among the obtained isolates. Some of isolates which were the members of the genus Bacillus, Anoxybacillus and Geobacillus were lipase and amylase producers. According to the petri dish enzyme assays, a large number of strains have the production capacity of more than one enzyme. Some of the isolates (mainly representative of genus Bacillus), were producers of the three enzymes together.

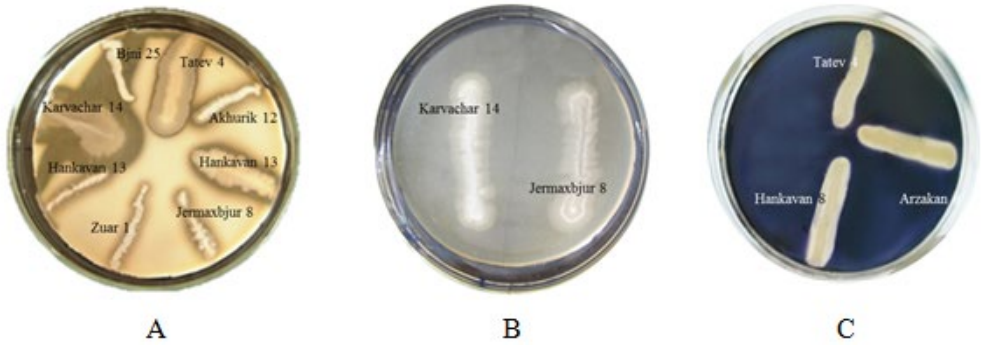

Fig. 2. Screening of microbial protease (A), lipase (B) and amilase (C) producing bacilli strains isolated from different geothermal springs on the agar plate.

Our results revealed the phylogenetic diversity of a large number of thermophilic bacilli in Armenian geothermal hot springs. As part of the microbiota, thermophilic bacilli presumably make significant contributions in the biogeochemical cycles of the springs under extreme temperature con-di¬tions. The results obtained show the importance of further investigation of the phy¬lo¬ge¬netic diversity of microbes in geothermal springs to discover and iso¬late new thermophilic species. The ability of amylase, lipase and protease production of isolates indicates their potential to use in biotechnology as valuable enzyme producers. In summary, obtained isolates from Armenian geothermal springs demonstrate the diversity of thermophilic bacilli with hydrolytic activity inhabiting these springs.

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