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MORPHO-CULTURAL AND PHYSIOLOGICAL CHARACTERISTICS OF BACTERIA ISOLATED FROM SOME THERMAL SPRINGS OF THE REPUBLIC OF AZERBAIJAN

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Abstract. In this work, some morpho-cultural and physiological properties of strains of thermophilic bacteria isolated from thermal springs of Azerbaijan were studied. Water samples from the "Babazyanan" springs in the Salyan region, as well as the "Ashagi Istisu" and "Yukhari Istisu" springs in the Kalbajar region were studied. A total of 11 strains of thermophilic bacteria were isolated. The isolated strains formed beige and light brown colonies of different sizes and consistency on agar media. The cells of the isolated strains were represented most of all by asporogenous, motile, gram-positive rods of various sizes. The strains grew within temperatures from 35° to 70°C, at a pH value of 5.0-11. Their identification was carried out and it was shown that the isolated strains were represented by bacteria of the genus Bacillus.

Keywords: *Thermophilic bacteria, thermal springs, Bacillus spp.*

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1. Introduction

Azerbaijan is rich in thermal springs that differ in their chemical composition and unique microbiota. An example is the thermal springs of the Greater Caucasus, the Lesser Caucasus, the thermal springs of the Talysh Mountains and the thermal water springs of Karabakh. For microorganisms, hot springs are their ecological poverty. The development of microorganisms in them depends on temperature, gas composition, salt composition and other factors (Ahmadova, 2007; 2003; Akhmedova, 2007).

The study of microorganisms in thermal habitats not only allows us to better understand the origin and evolution of early life, but also makes it possible to practically use the isolated strains and their enzymes in industry and biotechnology (Lebedeva & Kharitonova, 2020). Thermophilic microorganisms represent one of the most practically important groups of microorganisms, because they have a number of properties, such as heat resistance, fast cultivation times, wide distribution, etc. Therefore, the use of thermophilic microorganisms in various areas of human activity is a current trend in microbiology (Loginova *et al*., 1996; Shulgina, 2019).

In recent years, increasing attention has been paid to the use of bacteria in nanotechnology for the biological synthesis of metal nanoparticles (Zhang *et al*., 2011). The development of an environmentally friendly process for the synthesis of metal

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nanoparticles is an important step in the field of nanotechnology application in accordance with the principles of green chemistry. One possible option to achieve this goal is to use bacteria as reducing agents to convert the metal from its ionic form to its elemental form, forming a nanoparticle. Bacteria release enzymes and proteins into the environment, which can be used as reducing agents and to maintain particle stability (Pukhovskaya *et al*., 2021).

Many microorganisms can synthesise nanoparticles like silver, gold, magnesium and etc. Thermophilic microorganisms are widely used in the formation of metal nanoparticles. Since thermophilic microorganisms live at high temperatures, their metabolism proceeds at a high rate and the reaction product is obtained in a short period of time. This makes thermophilic microorganisms an indispencable object for the synthesis of metal nanoparticles (Deljou & Goudarzi, 2016; Gunashova *et al*., 2021; Gunashova, 2022).

2. Materials and methods

Water samples from the following thermal springs were studied: “Babazyanan” in the Salyan region, “Ashagi Istisu” and “Yukhari Istisu” in the Kelbajar region. The Babazyanan thermal spring is located in the Salyan region, in the Babazyanan mountains (Figure 1). The pH of the water is 7.0 and the temperature is 40-50°C depending on the depth of the water and the time of year.

Figure 1. Thermal spring Babazyanan

Figure 2. Thermal springs Ashagi Istisu and Yukhari Istisu

The water of the “Ashagi Istisu” and “Yukhari Istisu” springs in the Kelbajar region has healing properties and is distinguished by a unique microbiota, represented mainly by

thermophilic microorganisms. The water temperature of the “Ashagi Istisu” spring is 64°C and pH=8.0. The water temperature of Yukhari Istisu is 71°C and pH=9.0 (Figure 2).

Water samples were taken into 1000 ml glass bottles in triplicate, maintaining sterility conditions. The selected samples were stored in the refrigerator for no more than 12 hours, then analyzed in the laboratory.

Cultivation of water samples was carried out on a solid nutrient medium of the following composition: peptone-5 g/l, beef extract-1 g/l, yeast extract-2 g/l, sodium chloride-5 g/l, agar-15 g/l. Incubation was carried out for 3-5 days at a temperature of 55-60℃. The grown colonies were isolated into a pure culture using Koch's method. This method is carried out by dilution in proportions of 1:10, 1:100, 1:1000 and 1:10000, using test tubes containing 9 ml of sterile water. Add 0.1 ml of the last diluted suspension to the solid nutrient medium and distribute it evenly over the surface with a sterile spatula. Then, without touching any place, use the same spatula to treat the surface of the nutrient medium inside the 2nd, 3rd and 4th Petri dishes. After 3-5 days, the purity of the grown colonies is checked by microscopy and if they are clean, they are re-cultured and stored in fresh nutrient medium. The morphological properties of the isolated strains, their size, motility and sporulation were studied by microscopy using a light microscope (XSP 30-series microscope) (x1000) (Egorov, 1995; Netrusov, 2005). H_2S release was determined using TSI (Triple sugar iron agar) medium (Sigma, USA). The ability of the strains to grow at different temperatures and pH was studied by incubation in nutrient agar medium. To do this, bacteria were grown at different temperatures (+35℃, +44℃, +55℃, +60℃, +65℃, +70℃) in a thermostat for 24 hours. Various pH values were adjusted by adding a 10% hydrochloric acid solution or a 10% sodium hydroxide solution to the nutrient medium. The result was determined after days of incubation in a thermostat at 60℃. Identification of microorganisms to genus was carried out according to Bergey’s Manual (Hoult *et al*., 1997).

3. Results and discussion

Five different bacterial strains were isolated from a sample collected from the Babazyanan geothermal spring. They were assigned the following codes: Б1, Б2, Б3, Б4, Б5. Three bacterial strains were isolated from the Ashagi Istisu thermal spring in the Kalbajar (KA1, KA2, KA3) and three bacterial strains were also isolated from the Yukhari Istisu spring (KY1, KY2, KY3) (Figure 3).

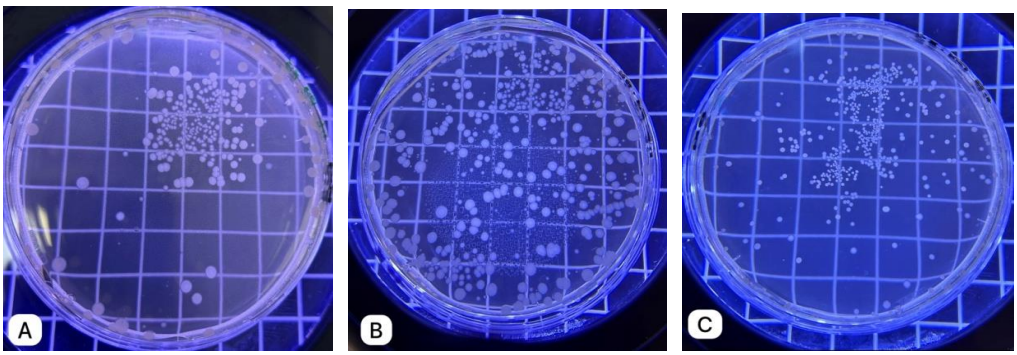
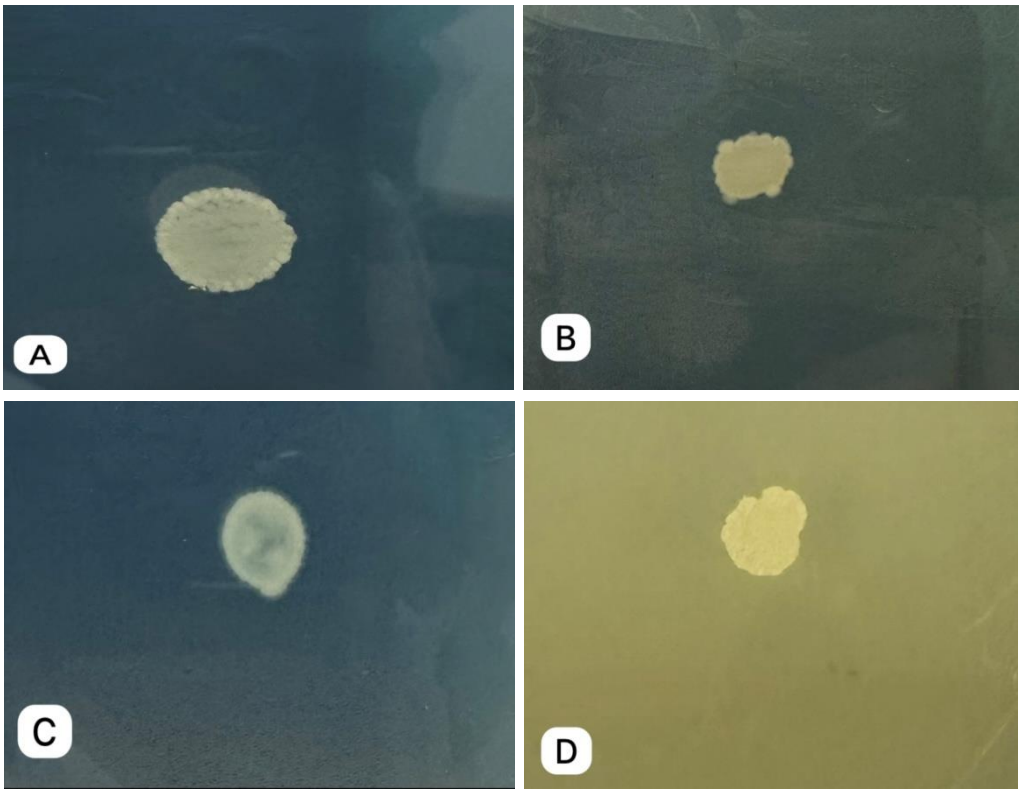


Figure 3. Bacterial colonies isolated from thermal springs (incubation period 3-5 days, at a temperature 55−60℃); **A**-from Babazyanan thermal spring; **B**-from Ashagi Istisu thermal spring; **C**-from Yukhari Istisu thermal spring

A number of different identification tests were performed on these strains, such as sporulation, morphology, motility, aerobic growth, sugar utilization and H_2S production. Morphologically, the colonies of the strains varied in shape, color, texture and consistency. After 24 hours of incubation, the studied strains formed round, convex colonies of yellow-cream color, with a wavy and smooth edge (Figure 4; Table 1).

Table 1. Cultural properties of bacterial strains on nutrient agar

Strains	Shape	Color	Margin	Elevation	Consistency
Б1	Round	Cream	Undulate	Raised	butyrous
Б2	Round	Brownish	Undulate	Raised	butyrous
Б3	Irregular	Cream	Undulate	Convex	butyrous
Б4	Round	Cream	Undulate	Convex	sticky
Б5	Round	Cream	Smooth	Convex	sticky
KA1	Round	Cream	Undulate	Raised	butyrous
KA2	Round	Cream	Undulate	Raised	butyrous
KA3	Rhizoid	Cream	Smooth	Flat	butyrous
KY1	Irregular	Beige	Undulate	Convex	butyrous
KY2	Round	Cream	Undulate	Flat	butyrous
KY3	Round	Cream	Undulate	Convex	butyrous



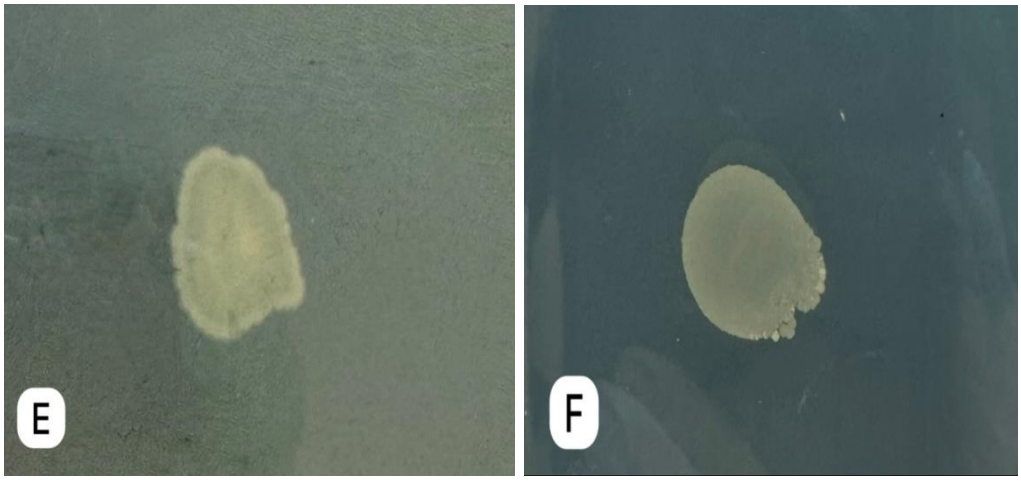


Figure 4. Colonies of bacterial strains on nutrient agar after 24 hours of incubation at a temperature of 55°C; **A**-КА1; **B** -КА2; **C**- Б1; **D**- Б2; **E**- КУ1; **F**- КУ2

The cells of all strains were gram-positive, rod-shaped, spore-forming, motile and nonmotile, obligate aerobes. The morphological characteristics of the strains were analysed using a light microscope. The resulting images of the isolates are presented in Figure 5.

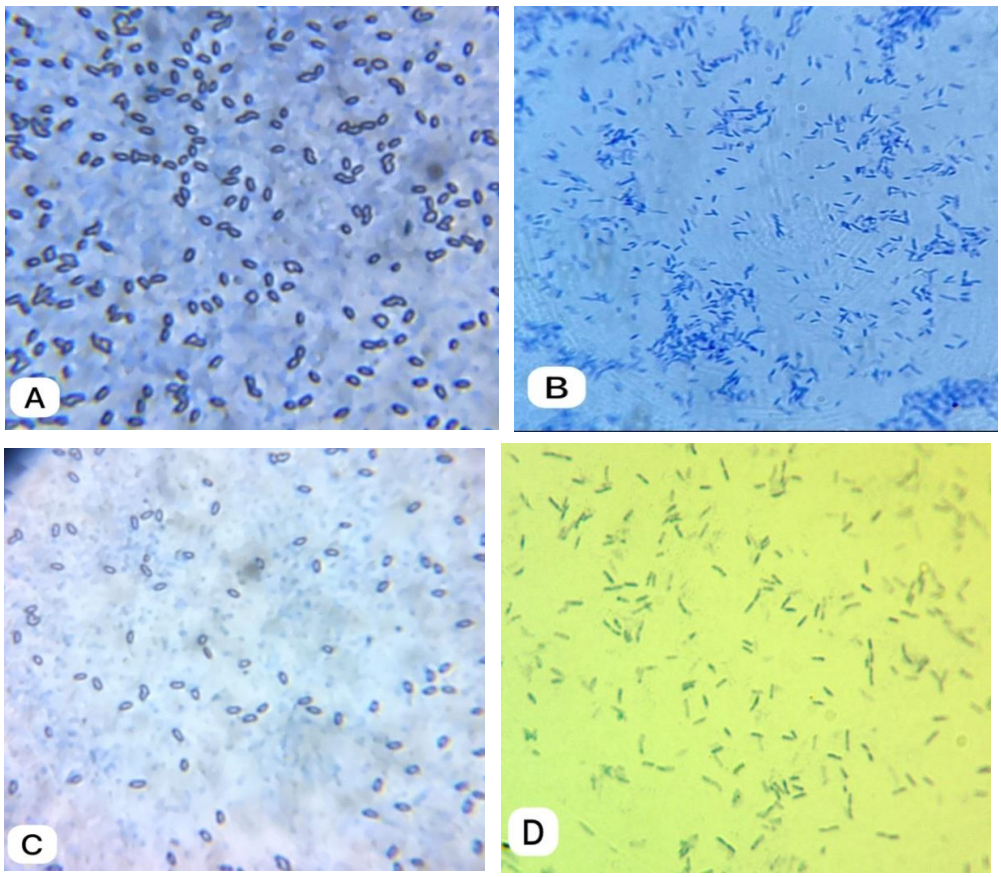


Figure 5. Microscopic images of bacterial strains (x1000)
A-Б1; **B**-Б2; **C**-КА1; **D**-КА2

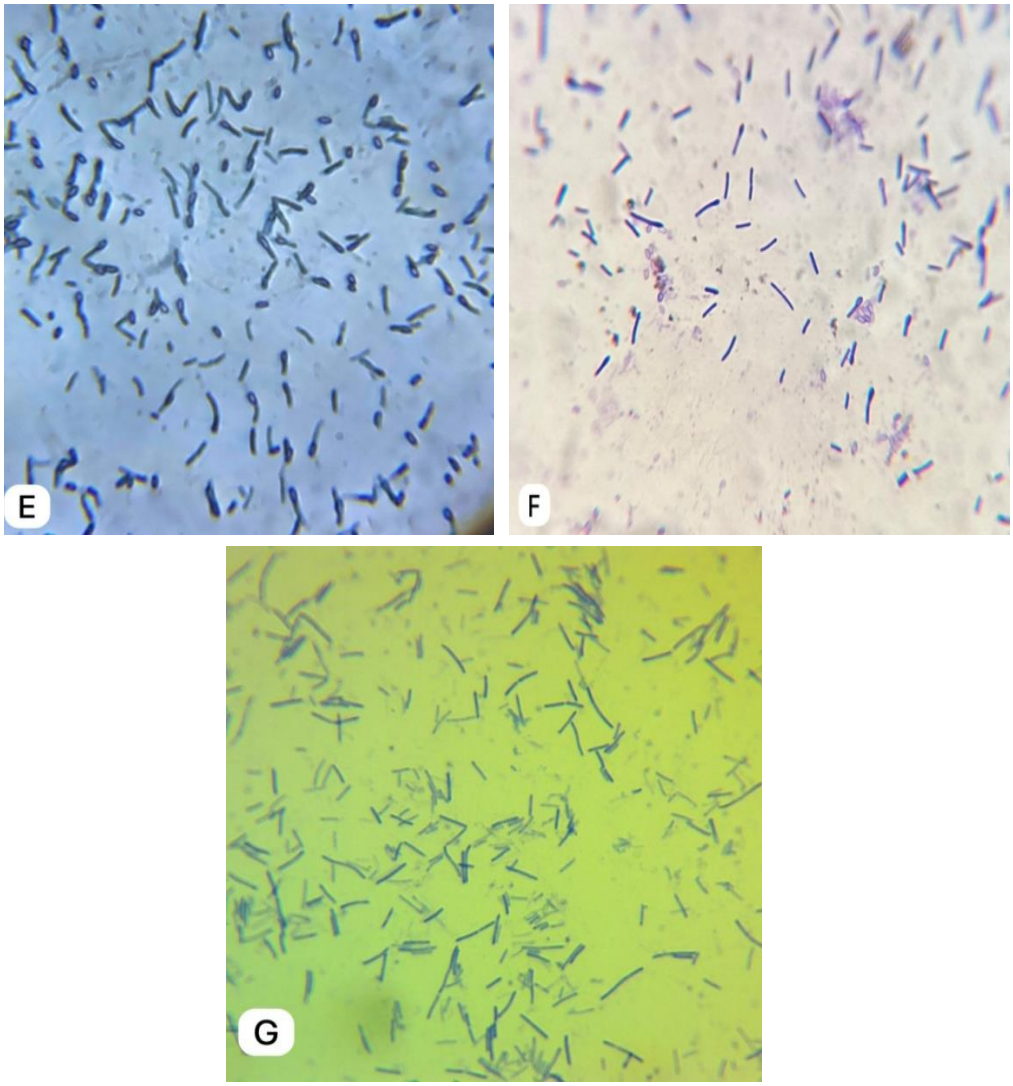


Figure 6. Microscopic images of bacterial strains (x1000)
E-KY1; **F**-KY2; **G**-KY3

The morphology of these strains is typical of bacteria belonging to the genus Bacillus. The cells of the strains were represented by rods of different sizes. Cell sizes varied from 0.9 to 4.5 μm in length and from 0.5 to 1.2 μm in width.

Strains isolated from the Babazyanan thermal spring were capable of growth on nutrient media at pH 5.0-10 and at temperatures from 35 to 65℃ and under optimal conditions at pH 7.0-9.0 and temperature 55-60℃. For strains from the Ashaghi Istisu hot spring, the optimal pH was 7.0-9.0, with a temperature ranging from 55 to 60℃. And for Yukhari Istisu strains the optimal pH is 7.0-9.0, with an optimal temperature of 60-65℃, and the maximum and minimum growth temperatures were 70 and 40℃, respectively.

All bacterial strains did not produce H_2S (Figure 7).

A negative result for H_2S production is determined by the absence of a black color when the strain grows on Triple Sugar Iron agar (TSI) medium. An acid/acid (yellow slant/ yellow butt) reaction of indicates the fermentation of glucose and sucrose. This reaction was observed in all bacterial strains isolated from thermal springs.

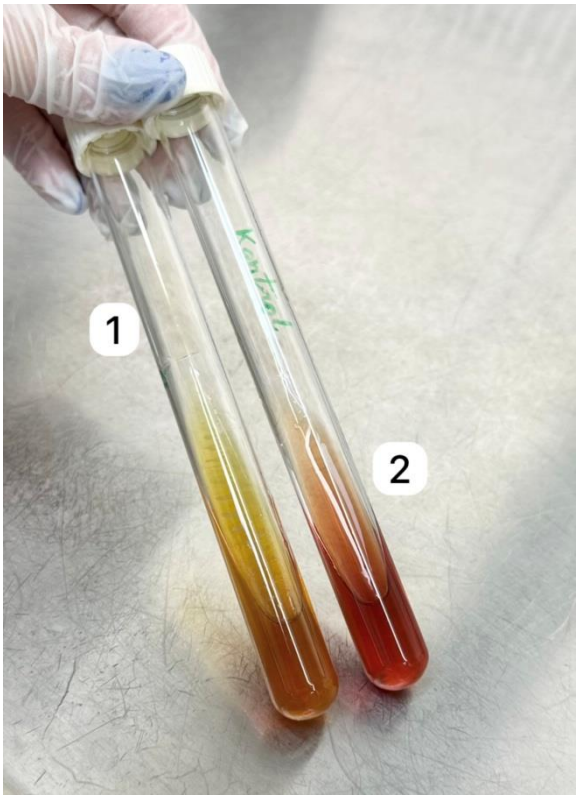


Figure 7. 1-KY2 strain on TSI medium; 2-Control TSI medium

The use of sugars by the isolated bacterial strains was also studied. It was found that all 11 strains isolated from three thermal springs are capable of producing acid from glucose, fructose, sucrose and maltose. None of the strains were able to utilize D-xylose, lactose, L-rhamnose (Figure 8).

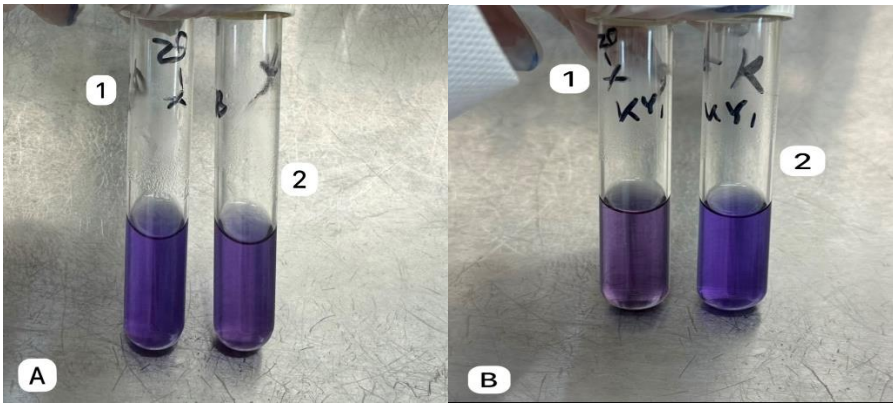


Figure 8. 1-Negative result for D-xylose, no color change; 2-negative result for L-rhamnose, no color change; **A**-Б1 strain; **B**-KY1 strain

All characteristics indicated above are described in Table 2.

Table 2. Characteristics of bacterial strain isolated from thermal springs

Characteristic	Б1	Б2	Б3	Б4	Б5	КА1	КА2	КА3	КУ1	КУ2	КУ3
Motility	+	+	+	+	+	+	-	-	+	+	+
Cell length (μm)	0.9-1.8	1.2-2	1-1.5	0.9-1.7	1-1.6	0.9-1.5	1-1.8	1.4-2	1.5-4	1.7-3.8	1.5-4.5
Cell width (μm)	0.6-1	0.5-0.7	0.4-0.6	0.5-0.8	0.3-0.6	0.5-0.6	0.5-0.8	0.4-0.8	0.3-1.2	0.6-0.9	0.6-1
H_2S production	-	-	-	-	-	-	-	-	-	-	-
Spores	+	+	+	+	+	+	+	-	+	+	+
Temperature range for growth ℃	35-65	35-65	35-65	35-65	35-65	40-70	40-70	40-70	40-75	40-75	40-75
Optimal temperature for growth ℃	55	55	55	55	55	60	60	60	60	60	60
pH range for growth	5.0-11	5.0-11	5.0-11	5.0-11	5.0-11	6.0-11	6.0-10	6.0-11	5.0-9.0	5.0-9.0	5.0-10
Glucose fermentation	+	+	+	+	+	+	+	+	+	+	+
Fructose fermentation	+	+	+	+	+	+	+	+	+	+	+
Sucrose fermentation	+	+	+	+	+	+	+	+	+	+	+
Maltose fermentation	+	+	+	+	+	+	+	+	+	+	+
D-xylose	-	-	-	-	-	-	-	-	-	-	-
Lactose fermentation	-	-	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-	-	-

Note: "+" means the presence of a certain characteristic; "-" indicates a negative test result

As can be seen from the table, the morphological and physiological characteristics of the isolated strains were studied, which is very important when selecting more effective strains for further research. Based on the above data, the strains of thermophilic bacteria we isolated were assigned to the genus Bacillus. The data obtained are consistent with the results of a number of studies on the composition of the microflora of thermal springs (Adiguzel *et al*., 2009; Coorevits *et al*., 2012; Gupta *et al*., 2014; Lopez *et al*., 2013; Nazina *et al*., 2001; Novik, 2018; Shubhrima *et al*., 2021; Verma *et al*., 2014; Zeigler, 2014).

3. Conclusion

Thus, as a result of the work done, a total of 11 bacterial strains, differing in their characteristics, were isolated from three thermal springs. It turned out that the cells of these bacteria are gram-positive rods that form spores and are obligate aerobes. The temperature range for growth for these bacteria is 35-70℃ and the pH value is `5.0-11. The use of sugars by these strains has also been studied. Based on the study of the morpho-cultural and physiological characteristics of these strains, they were assigned to the genus Bacillus.

This study makes a new and valuable contribution to the literature of microbial diversity of thermophiles in Azerbaijan. The data obtained allow us to assert that the thermal springs of Babazyanan in the Salyan region, Ashaghi Istisu and Yukhara Istisu in the Kalbajar zone, have a unique community of thermophilic microorganisms.

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