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Biological characterization of thermophilic bacterial strains from hot springs in the Republic of Azerbaijan

Gunay Abbasli*, Farayat Ahmadova

Baku State University, 33 Academician Zahid Khalilov Str., AZ1148, Baku, Azerbaijan

***For correspondence:** gunay_a-va1995@mail.ru*

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The work is devoted to the study of the biological properties of strains of thermophilic bacteria isolated from hot springs of Azerbaijan for their taxonomic identification. Strain B1 was isolated from the Babazanan hot spring in the Salyan district, strains KA2 and KY2, from the Ashagi Istisu and Yukhari Istisu springs in the Kalbajar district, respectively. As a result of studying the morphology of the strains, it was established that the bacterial cells are gram-positive rods that form endospores. The cultural properties of the strains were generally similar, characterized by a milky color, round shape, raised surface, wavy edges and soft consistency. Physiological studies have shown that the strains efficiently utilize organic acids, alcohols, and sugars as carbon sources. They also demonstrate effective utilization of both organic and inorganic nitrogen sources. The optimal temperature for the growth of strains was 55-60℃, and the optimal pH value was 7.0-9.0. According to biochemical characteristics, the strains were catalase-positive, while oxidase activity was absent. Proteolytic activity was observed in strains KA2 and KY2 but not in strainB1.The absence of hydrogen sulfide synthesis indicates that these strains are safe for use in biotechnological purposes. As a result of a general analysis of morphological, cultural, physiological and biochemical characteristics, the isolates were attributed to the genus *Bacillus*. The adaptation of these bacteria to extremophilic conditions, along with their non-pathogenic nature and metabolic flexibility, forms a solid foundation for their application in biotechnology and nanobiotechnology – particularly in the environmentally safe synthesis of metal nanoparticles.

Keywords: *Genus Bacillus, morphological characterization, thermophilic enzymatic activity, physiological traits of thermophiles, biochemical characterization*

INTRODUCTION

Thermophilic bacteria are garnering increasing attention from researchers due to their remarkable ability to thrive in extreme environmental conditions. These microorganisms are predominantly found in hot springs and have a number of unique physiological and biochemical adaptations (Aanniz et al., 2015; Marzban & Tesei, 2025).

One of the most promising areas of application of thermophilic bacteria is the biological synthesis of metal nanoparticles. Unlike traditional chemical and physical methods, biological synthesis allows obtaining nanoparticles without the use of toxic reagents and harsh physical conditions (Iravani, 2014; Deljou & Goudarzi, 2016). Even though there are other methods of biological synthesis of nanoparticles, such as their accumulation in various components of ecosystems, including plants, fish, mollusks and parasites (Hadjiyeva et al., 2024; Rzayev et al., 2022), the use of bacteria for these purposes is more promising due to their availability and wide distribution, high growth rate and large amount of biomass they form (Gunashova, 2022; Sulaiman et al., 2018).

Azerbaijan has a rich reservoir of thermal water springs, which are located in various regions of the Republic. Thermal springs such as Ashagi Istisu and Yukhari Istisu in the Kalbajar district, as well as Babazanan in the Salyan district, are characterized by high temperatures and rich mineral composition, which makes them a favorable environment for the development of thermophilic microflora (Ahmadova, 2007; Kamal, 2012; Gunashova et al., 2021).

This work aims to conduct a detailed investigation of the morphological, cultural, physiological and selected biochemical characteristics of thermophilic bacterial strains isolated from the aforementioned water sources in Azerbaijan, to identify them at the genus level.

MATERIALS AND METHODS

This paper will examine the morphological, cultural, physiological and biochemical properties of strains B1, KA2 and KY2. Methods for isolating pure cultures of these strains and assessing their ability to synthesize silver nanoparticles are described in detail in our previous publications (Gunashova et al., 2021; Gunashova et al., 2021; Gunashova, 2022).

Morphological characteristics. The morphological features of the studied strains were studied using light microscopy (XSP-30 series, China). The bacterial cells were observed at 100× magnification using oil immersion. Observations were made on fixed, Gram-stained slides, as well as using the hanging drop method to assess cell motility. Samples were prepared from fresh cultures grown on nutrient agar at 60°C for 24 hours.

Cultural characteristics. Cultural characteristics were observed on colonies on the surface of nutrient agar and in nutrient broth. Cultures were incubated at 60°C for 24–48 hours. The growth pattern, colony morphology and pigmentation, consistency, sediment formation, surface film, and turbidity of the medium were carefully assessed.

Physiological and biochemical characteristics. The ability of the strains to utilize various carbon and nitrogen sources was assessed using Smith medium, supplemented with different substrates as the sole carbon or nitrogen source.

The names of the sources are given in Tables 2 and 3. Smith's medium without the addition of any carbon or nitrogen source was used as a control to assess the baseline optical density (OD). After inoculation of the strains into the appropriate medium, incubation was carried out at 60℃ for 24 hours. The OD of the bacterial suspensions was measured both before incubation, to determine the initial turbidity, and after incubation, to estimate biomass accumulation depending on the carbon and nitrogen sources. The measurements were carried out in triplicate using a Jenway 7315 spectrophotometer (UK) at a wavelength of 600 nm and the results are presented as mean values.

Catalase activity was tested by adding a drop of 10% hydrogen peroxide solution to the bacterial biomass. The appearance of oxygen bubbles indicated a positive reaction.

Oxidase activity was determined using test strips impregnated with tetramethyl-p-phenylenediamine. A color change to blue or violet within 30 seconds indicated a positive result.

Proteolytic activity was assessed by the ability to hydrolyze casein on milk-casein agar. After incubation at 60°C for 24 hours, the formation of transparent zones around the colonies proved the cleavage of casein.

Carbohydrate fermentation was also tested on triple sugar iron (TSI) agar containing glucose, lactose, sucrose, phenol red indicator and sodium thiosulfate. After inoculation and incubation at 60°C for 24-48 h, colour change, gas formation and formation of a black precipitate (H_2S) were recorded.

The ability to utilize L-rhamnose, D-xylose, and lysine was assessed using differential media containing each compound as the sole carbon source (L-rhamnose, D-xylose) or sole amino acid source (lysine). The criteria for a positive reaction were bacterial growth and a change in indicator color. To determine the optimal conditions for the strains, the temperature and pH range of growth were studied. To determine the pH range, the strains were inoculated into nutrient broths with different pH values (5.0-11.0) and incubated at 60℃ for 24 hours. The temperature range was determined by inoculating the strains into a nutrient broth with a neutral pH of 7.0. Incubation was carried out at different temperatures in the range from 35℃ to 75℃. The growth of strains at different temperatures and pH values was determined by spectrophotometric measurement of the OD of bacterial suspensions.

RESULTS

Morphological characteristics. Microscopic analyses of the morphology of bacterial strain cells showed that the cells of all strains were gram-positive rods, the sizes of which varied from 0.9 to 4.5 μm in length and from 0.6 to 1.2 μm in width. The cells were mostly single rods and were predominantly motile (Fig. 1, Table 1). Endospore formation was observed in older cultures or under conditions unfavorable for thermophilic bacteria.

Cultural characteristics. The cultural properties of the strains were studied both on nutrient agar and in nutrient broth. Milky, beige colonies were observed on nutrient agar with a round or irregular shape, slightly convex profile, wavy edges and soft consistency. In nutrient broth, growth was manifested as moderate turbidity and visible sediment formation (Table 1).

Physiological and biochemical studies. Among all the studied substrates, the greatest bacterial growth was observed when using organic acids. Pyruvic acid provided the highest OD values: 1.52 for strain KA2, 1.44 for KY2 and 1.12 for B1. High utilization was also observed with the addition of citric acid and oxalic acid. Among sugars, good utilization was observed for sucrose and glucose, while lactose was not assimilated by any of the strains. Strain KY2 demonstrated active utilization of galactose (OD - 1.00), which is characterized by the specific enzymatic activity of the strain. Interesting results were

obtained in the analysis of alcohols: glycerol and ethanol were actively metabolized by all strains, especially strain B1. This may be an indicator of the presence of thermostable dehydrogenase systems in these bacteria. The ability of strains to utilize various carbon-containing substrates is presented in Table 2.

As a result of studying the ability of three thermophilic bacterial strains KA2, KY2 and B1 to use various nitrogen sources, it was found that among all the studied compounds, the greatest bacterial growth was observed when using peptone. Peptone provided the maximum OD values: 1.88 for KA2, 1.44 for KY2 and 1.76 for B1. High nitrogen utilization rates were also recorded for ammonium sulphate and potassium nitrate, demonstrating efficient uptake of both inorganic and organic nitrogen sources. Moderate growth was observed with tryptone and asparagine, while methionine supported comparatively lower growth, indicating the strains' specific requirements for nitrogen sources. The control variant showed the lowest OD (0.32), indicating no growth in the absence of nitrogen sources. Data reflecting the response of the strains to various nitrogen sources are presented in Table 3.

Biochemical analysis of three bacterial strains (B1, KA2 and KY2) showed that all of them exhibited catalase-positive activity, while none of the strains exhibited oxidase activity, which was confirmed using a positive control (*Pseudomonas aeruginosa*) and a negative control (*Escherichia coli*). Protease activity, assessed using milk agar, was observed in strains KA2 and KY2, which was manifested by the formation of clear lysis zones around their colonies. In contrast, strain B1 did not have such zones, indicating the absence of proteolytic activity.

When cultured on triple sugar iron agar (TSI), all three strains produced yellow coloration at both the bottom and top of the agar, indicating fermentation of glucose and sucrose. No black precipitate was found in the tube, indicating a negative result for hydrogen sulfide (H_2S) production in all tested strains. Further testing on media containing D-xylose and L-rhamnose revealed no colour change, demonstrating the inability of the strains to ferment these sugars. This indicates the absence of the necessary enzymatic pathways to metabolise D-xylose and L-rhamnose and therefore the inability to use them as carbon sources.

Lysine decarboxylase activity was assessed using lysine broth initially stained purple. Strains B1 and KA2 retained the purple color, indicating a positive decarboxylation reaction, while KY2 showed a color change to yellow, indicating a negative result. The accuracy of these observations was confirmed using control microorganisms: *Escherichia coli* (positive control) and *Staphylococcus aureus* (negative control).

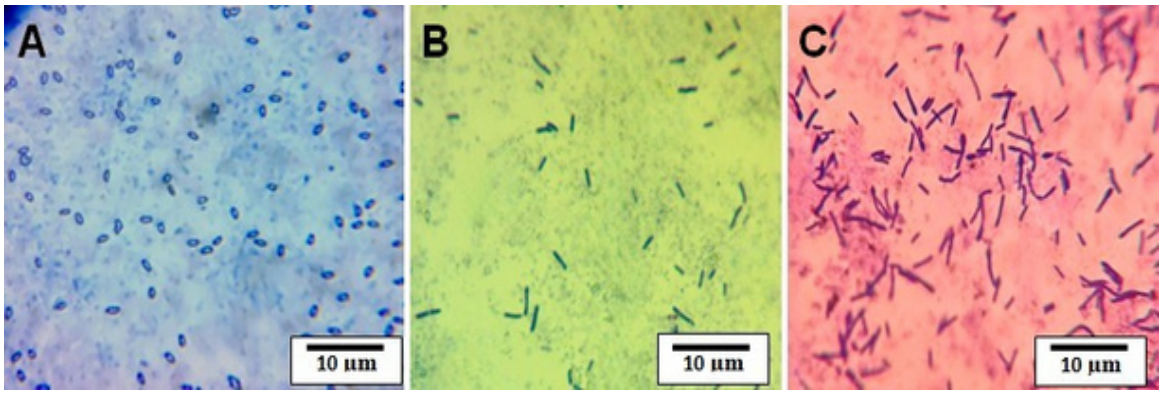

Fig. 1. Cells of 24-hour bacterial strains under a light microscope (scale bar=10 µm; 100×)A) cells of strain B1 B) cells of strain KA2 C) cells of strain KY2.

Table 1. Morphological and cultural characteristics of thermophilic bacterial strains.

Morphological characteristics of thermophilic bacterial strains

Bacterial strains	Cell shape	Cell size, µm	Cell motility	Types of flagella
B1	Rods	0.9-1.8x0.6-1	+	Peritrichous
KA2	Rods	1-2.8x0.5-0.8	-	-
KY2	Rods	1.7-3.8x0.6-0.9	+	Peritrichous

Cultural characteristics of thermophilic bacterial strains

Bacterial strains	Colony color	Colony shape	Colony margin	Colony elevation	Colony consistency
B1	Milky	Round	Wavy	Raised	Soft
KA2	Milky	Round	Wavy	Raised	Soft
KY2	Milky	Round	Wavy	Flat	Soft

Table 2. Utilization of carbon sources by thermophilic bacterial strains isolated from hot springs of Kalbajar and Babazan.

Carbon source, 0.5%	Optical density of strains, UV spectrophotometer Jenway 7315, in (Smith) medium		
	KA2	KУ2	B1
Sugars			
Control variant	0.36	0.36	0.36
Glucose	0.64	0.76	0.92
Sucrose	0.76	0.84	0.92
Galactose	0.60	1.00	0.76
Lactose	0.36	0.36	0.36
Alcohols			
Ethanol	1.04	0.96	0.80
Mannitol	0.56	0.72	0.88
Glycerol	0.88	0.80	1.04
Salts of organic acids			
Oxalic acid	1.44	1.00	1.20
Malic acid	1.12	0.96	1.28
Citric acid	1.36	1.48	1.00
Pyruvic acid	1.52	1.44	1.12
Acetic acid	1.28	1.12	1.20

Table 3. Utilization of nitrogen sources by thermophilic bacterial strains isolated from hot springs of Kalbajar and Babazan.

Nitrogen source. 0.5%	Optical density of strains. UV spectrophotometer Jenway 7315, in (Smith) medium		
	KA2	KY2	B1
Control (negative) variant	0.32	0.32	0.32
KNO3	1.08	1.20	1.36
(NH4)2SO4	1.60	1.52	1.68
Peptone	1.88	1.44	1.76
Methionine	0.80	0.96	1.24
Asparagine	1.00	1.16	1.44
Tryptone	1.20	1.28	1.52

Experimental data demonstrated a clear dependence of the growth of bacterial strains on the pH of the incubation medium. At extreme pH values of 5.0 and 11.0, the growth of all strains studied was significantly reduced or completely absent, indicating that these conditions are outside the physiologically acceptable range for maintaining the metabolic activity of thermophiles. However, when the pH increased to 10.0, growth decreased significantly (OD: B1 – 1.1, KY2 – 0.7), indicating the onset of the negative impact of excessive alkalinity. In contrast, strain KA2 showed maximum growth at pH 7.0 (OD – 1.75), although significant growth was also observed at pH 8.0 and 9.0 (1.4 and 1.1, respectively). At an acidic pH of 5.0, KA2 showed no growth, and at pH 6.0, only minimal growth (OD – 0.7) was observed, confirming its limited tolerance to acidic environments. The effect of pH on the growth of strains at a constant incubation temperature of 60°C, based on OD measurements, is shown in Fig. 2.

As a result of the analysis of the dependence of the OD of bacterial suspensions on temperature, it was established that the optimal temperature for the growth of all three strains was 60°C, at which the highest values of OD were observed. These results indicate that 60°C represents the optimal growth temperature for all three strains, with increased metabolic activity and biomass accumulation. At 35°C, only strain B1 showed moderate growth, while KY2 and KA2 showed no growth. At 75°C, growth was completely inhibited for strains B1 and KA2, and only minimal growth was detected for KY2, indicating that these temperatures are beyond the physiological capabilities of the strains. Significant growth was also observed at 55°C, especially for strain B1, although slightly lower growth values were recorded for KY2 and KA2 compared to their peak at 60°C. At intermediate temperatures (40–45°C), all strains showed moderate growth. Thus, the optimal growth temperature range of all thermophilic strains studied was between 55°C and 60°C, where the maximum growth rates were observed. Growth was significantly slower at both lower and higher temperatures, reflecting the temperature limits of their physiological adaptation (Fig.3).

The obtained results confirm that strains B1, KY2 and KA2 are true thermophiles, since their optimal growth and metabolic activity occur at elevated temperatures, which is a distinctive feature of thermophilic microorganisms. True thermophiles are adapted to exist at temperatures significantly higher than those optimal for most other microorganisms. The highest growth rate of the studied strains was observed at a temperature of 60°C, which is typical for thermophilic organisms, since their enzymatic and biochemical processes occur most effectively in this temperature range. Thus, the conducted analysis of the physiological and biochemical properties of the isolated thermophilic bacteria showed that the identified strains have pronounced thermotolerance, the ability to utilize various carbohydrate substrates and specific enzymatic activity.

DISCUSSION

This paper presents the characteristics of three thermophilic bacterial strains – B1, KA2 and KY2, isolated from thermal springs in Azerbaijan. Previously, these strains had already been the objects of research, where the ability of these strains to synthesize silver nanoparticles was studied, but at this stage, it became possible to conduct a detailed physiological-biochemical and morphocultural study.

As a result of studying the morphology of the cells, typical features and characteristics of the genus *Bacillus* were identified: gram-positive staining, the ability to form spores, and rod-shaped cells. Colonies on nutrient agar were round in shape with a raised surface, soft in consistency and milky-cream in color, with distinctly wavy edges. Similar phenotypic characteristics have been described for thermophilic bacilli such as *Bacillus licheniformis* and *B. stearothermophilus* (Hoult et al., 1997; Logan & De Vos, 2009; Sonenshein et al., 1993).

Catalase activity, along with the absence of oxidase activity found in all strains, is consistent with the characteristics described by Logan (2012), indicating a typical enzymatic profile for most members of the genus *Bacillus*. Proteolytic activity was observed in strains KA2 and KY2, but was absent in B1, demonstrating interstrain differences typical for this genus. Similar differences were noted in lysine decarboxylase activity, which may reflect high metabolic flexibility and the ability to adapt to different ecological niches (Gudzenko et al., 2023).

As a result of studying the ability of strains to utilize different sources of carbon and nitrogen, it was found that the greatest growth was observed when using organic acids such as pyruvic, citric and oxalic. This is consistent with existing knowledge of thermophilic bacteria that are adapted to efficiently utilize organic acids as carbon and energy sources (Brock, 1978; Libor et al., 1978).

The efficient use of sucrose and glucose, along with the absence of lactose, D-xylose and L-rhamnose fermentation in all strains, confirms the specificity of their enzymatic apparatus and suggests the absence or low activity of lactase, which is characteristic of many thermophilic bacteria.

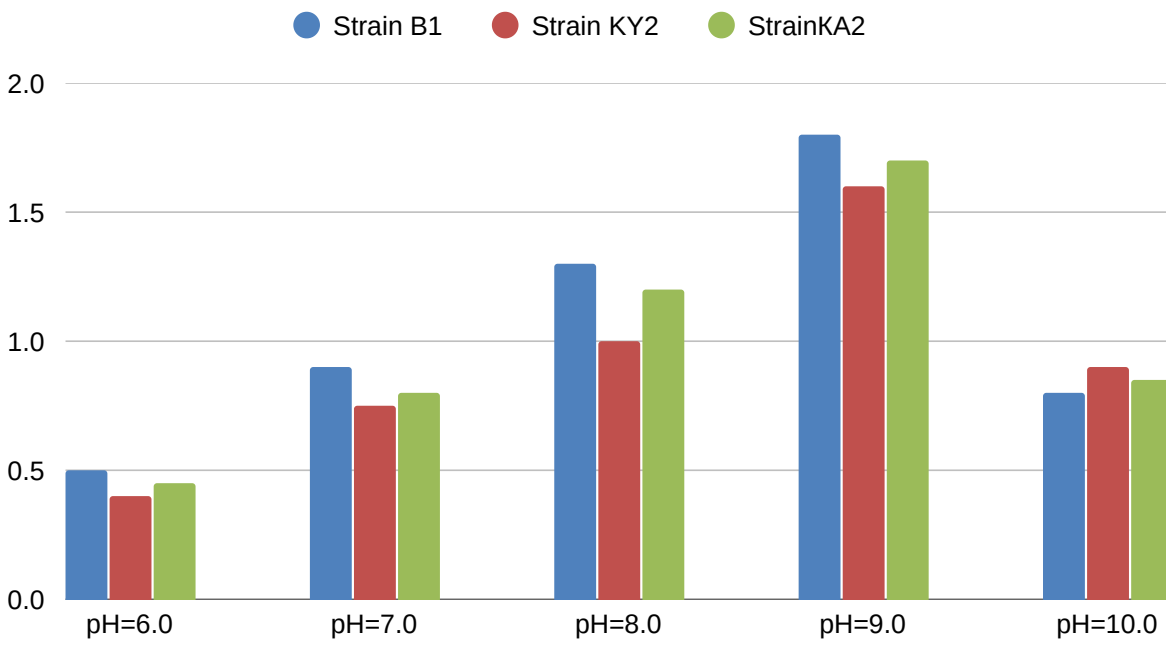

Fig. 2. Optical density of strain suspensions measured at different pH values

afterincubation at 60°C.

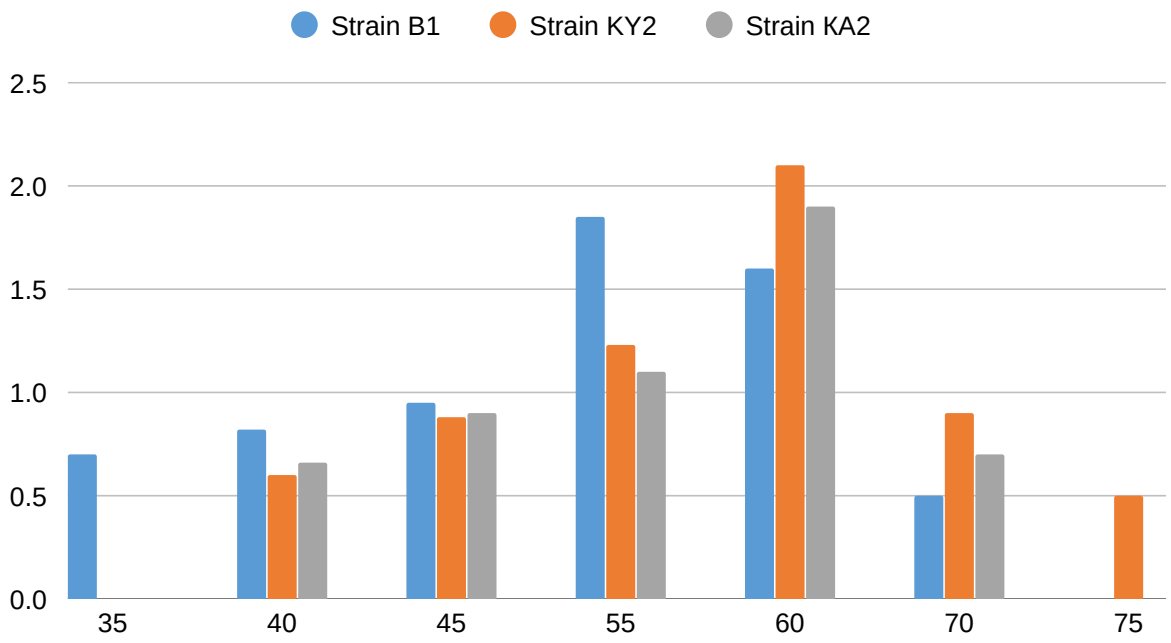

Fig. 3. Optical density of strain suspensions at different temperatures

with a nutrient medium pH of 7.0.

Interestingly, strain KY2 demonstrated active galactose metabolism, suggesting the presence of additional specific enzymes that expand the spectrum of its carbohydrate metabolism. However, the inability to utilize D-xylose and L-rhamnose indicates a limited capacity for pentose metabolism. Active metabolism of glycerol and ethanol, especially pronounced in strain B1, demonstrates metabolic flexibility, allowing the use of various alcohols as carbon sources. Similar abilities have also been described for thermophilic bacteria of the genus *Geobacillus*, which are adapted to various ecological niches (Brumm et al., 2010; Madigan et al., 2019).

Nitrogen source studies showed that peptone supported the highest growth of all strains, indicating a preference for organic nitrogen compounds. The high utilization of ammonium sulfate and potassium nitrate further indicates the ability to efficiently utilize both organic and inorganic forms of nitrogen, a characteristic of many thermophiles adapted to changing environmental conditions. Moderate growth on tryptone and asparagine, with relatively low growth on methionine, may indicate special biosynthetic requirements and methionine limitations in these strains (Sreekanth et al., 2013; Madigan et al., 2019; Liu et al., 2025). The control variant with the minimum OD confirms the necessity of available nitrogen sources for active growth and metabolism of thermophilic strains. It is also important to note the absence of hydrogen sulfide production, which may serve as a potential biosafety marker of these strains for future biotechnological and medical applications.

A study of the dependence of growth on temperature and pH of the environment confirmed that the isolated strains can develop in a wide range of conditions: at temperatures from 35°C to 75°C with an optimum of 55–60°C and at pH from 6.0 to 10.0 with an optimum of 7.0–9.0. This tolerance indicates a high degree of adaptation to extreme environmental conditions. Similar growth parameters and physiological resistance were found for thermophilic *Bacillus* species in the studies of Zeigler (2011). In conclusion, it should be noted that the combination of morphological, physiological and biochemical data convincingly confirms that strains B1, KA2 and KY2 belong to the genus *Bacillus*. Further study of the genomic and proteomic features of these microorganisms may contribute to a deeper understanding of the mechanisms of thermal tolerance and metal reduction, as well as optimization of their cultivation for applied purposes.

CONCLUSION

In the present study, three thermophilic bacterial strains, B1, KA2 and KY2, isolated from thermal springs in Kalbajar district, were taxonomically identified and comprehensively characterized. Detailed morphological, cultural and physiological-biochemical analysis allowed them to be classified as belonging to the genus *Bacillus*. All three isolates were gram-positive spore-forming rods, varying in size and forming milky in color, round colonies. Optimum growth was observed at temperatures from 55°C to 60°C and pH values from 7.0 to 9.0, although strains were tolerant over a wider range of temperatures from 35°C to 75°C and pH from 6.0 to 10.0. Physiological studies have shown the ability of the strains to utilize a number of carbohydrates, including glucose, fructose, sucrose and maltose, whereas the enzymatic

activity of lactose, D-xylose and L-rhamnose was not detected. The obtained data confirm that strains B1, KA2 and KY2 are thermophilic *Bacillus* species with a high degree of adaptation to extreme environmental conditions.

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ETHICAL CONSIDERATIONS

Sample collection was conducted in accordance with local environmental regulations and with respect for natural habitats. All laboratory procedures complied with standard microbiological safety and biosafety guidelines. The studied bacterial strains were confirmed to be non-pathogenic, and no genetically modified organisms were produced during the course of this research.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest related to this study. The authors confirm that they have no financial or personal relationships that could have influenced the research outcomes or interpretation of the results.

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ORCIDs:

Gunay Abbasli:https://orcid.org/0000-0003-2015-4720

Farayat Ahmadova: https://orcid.org/0009-0003-5834-5386