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BIOCHEMICAL AND MOLECULAR-GENETIC CHARACTERIZATION OF A STRAIN ISOLATED FROM A THERMAL HEALING SPRING IN STAROZAGORSKI MINERALNI BANI, STARA ZAGORA REGION, BULGARIA

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Abstract: *Mineral waters of springs near the village of Starozagorski Mineralni Bani, Stara Zagora region, are well known for their healing effect in diseases of bones and joints, peripheral nervous system, gynecological, kidney and urological, gastro-intestinal and hepatic and biliary diseases. The physico-chemical and microbiological parameters of healing spring water in the village of Starozagorski Mineralni Bani, Stara Zagora region were determined. According to its physico-chemical and microbiological parameter, spring water meets the standard requirements for quality of drinking water. A strain was isolated from the examined healing spring as a pure culture and its colonial and morphological characteristics were examined. The isolated strain SMB was rod-shaped, Gram - positive, motile, sporeforming. It formed a precipitate on the surface of the liquid culture medium, did not turbidify the liquid culture medium and formed a precipitate on the bottom of the tube. Strain SMB was a facultative aerobe. It was identified by biochemical (API 50 CHB) and molecular - genetic methods (sequencing of the gene for the 16S rRNA) as a representative of the species Bacillus thuringiensis. After performing further examinations of its antimicrobial activity against phytopathogenic molds strain SMB would be incorporated in the composition of biological preparations against plant diseases.*

Keywords: *mineral water, physico-chemical, microbiological, identification, sequencing, 16S rRNA*

1. Introduction

Mineral water from springs near Starozagorski Mineralni Bani, Stara Zagora region, come to the surface with a temperature of about 40°C from about 1600 m in depth. They are known to have healing effect in diseases of bones and joints, peripheral nervous system, gynecological, kidney and urological, gastro-intestinal and hepatic and biliary diseases [1 - 8].

Mineral water is slightly mineralized, hyperthermal, with neutral pH (pH=6.9) due to the presence of 60 mg of carbon dioxide in it. It contains hydrocarbons, sulfates, calcium, magnesium, silicon, fluorine, and other trace elements; the mineral content is 0.498 g/l to 2 g/l; the content of metasilicic acid in colloidal state is 34 mg/l, which makes it effective in a number of diseases.

Potable water used in enterprises of the food and microbiological industry must meet the requirements for potable water.

Thus the total bacterial abundance (TBA), the number of coliform bacteria and pathogenic bacteria should be determined and monitored periodically. Pathogenic bacteria are controlled by hygienic - epidemiology laboratories.

In Bulgaria there are a number of mineral springs with unexamined microflora. Studies have shown that microorganisms with valuable properties can be isolated from healing and spring waters. The thermal healing spring with water temperature of 40°C in Starozagorski Mineralni Bani, Stara Zagora region has not been examined so far.

The purpose of the present research was the physico-chemical and microbiological analysis of the spring water from the thermal healing spring with water temperature of 40°C in Starozagorski Mineralni Bani, Stara Zagora region and the physiological, biochemical and molecular-genetic identification of a strain isolated from the spring.

2. Materials and Methods

2.1. Physico-chemical methods

The colour was determined according to the Rublyovska Scale - method BS 8451: 1977;

The odor was determined according to the the method for determination of the odor at 20°C - method BS 8451: 1977, technical means – Mercury thermometer, conditions № 21;

The turbidity was determined according to EN ISO 7027, technical means - turbidity meter type TURB 355 IR ID № 200807088;

The pH was determined according to BS 3424: 1981, technical means - pH meter type UB10 ID №UB10128148;

The oxidation was determined according to BS 3413: 1981;

Method for determination of chlorides - BS 3414: 1980;

The nitrates were determined according to VLM - NO_3 - №2, technical means – photometer "NOVA 60 A" ID № 08450505;

The nitrites were determined according to VLM - NO_2 - №3, technical means – photometer "NOVA 60 A" ID № 08450505;

The ammonium ions were determined according to VLM - NH_4 - №1, technical means – photometer "NOVA 60 A" ID № 08450505;

The total hardness was determined according to ISO 6058;

The sulphates were determined according to VLM - SO_4 - №4, technical means – photometer "NOVA 60 A" ID № 08450505;

The calcium was determined according to ISO 6058;

The magnesium was determined according to BS 7211: 1982;

The phosphates were determined according to VLM - PO_4 - №5, technical means – photometer "NOVA 60 A" ID № 08450505;

The manganese was determined according to VLM - Mn - №7, technical means - photometer "NOVA 60 A" ID № 08450505;

The iron was determined according to VLM - Fe - № 6, technical means - photometer "NOVA 60 A" ID № 08450505;

The fluoride was determined according to VLM - F - № 8, technical means - photometer "NOVA 60 A" ID № 08450505;

The electrical conductivity was determined according to BS EN 27888, technical means - conductivity inoLab cond № 720 ID 11081137.

2.2. Microbiological methods

The applied methods for microbiological indicators were in accordance with Ordinance № 9/2001 Darjaven vestnik,

issue 30 and Decree № 178 / 23.07.2004 on the quality of water intended for drinking purposes.
The presence of *Escherichia coli* and coliform bacteria were determined according to BS EN ISO 9308-1: 2004;
The presence of enterococci were determined according to BS EN ISO 7899-2;
The presence of spores of sulfite reducing anaerobes was determined according to BS EN 26461-2: 2004;
The total number of aerobic and facultative anaerobic bacteria was determined according to BS EN ISO 6222: 2002;
The presence of *Pseudomonas aeruginosa* was determined according to BS EN ISO 16266: 2008;

2.3. Determination of the sulphytereducing anaerobic bacteria (Clostridium perfringens) in water

The samples were heated in a water bath at 80°C for 15 min and pour plated in tubes. The inoculated tubes were incubated at 37°C for 24 h until the appearance of black colonies and cavities in the medium. The amount of sulphytreducing sporeforming anaerobes (*Clostridium perfringens*) was determined by their titer (the smallest volume of water in which they were established) using standard tables.

2.4. Identification methods

Determination of the biochemical profile

The system API 50 CHB (BioMerieux SA, France) was used for the identification of the species of the genus *Bacillus* based on their ability to utilize 49 carbon sources. Fresh 24-hour culture of the studied strain was suspended in API 50 CHB medium, an integral part of the used kit. The API strips were placed in the incubation boxes, the microtubules were inoculated with the prepared cell suspension and sealed with sterile liquid paraffin. The results were reported on the 24th and the 48th hour of incubation at 37±1°C. Reporting was done, based on the colour change of each microtubule, compared to the colour of the control microtubule (microtubule 0). Positive results were recorded in the cases of color change from red to orange or bright yellow. The obtained results were processed with apiweb® identification software.

Molecular-genetic methods

Isolation of total DNA [1]

16S rDNA amplification

All PCR reactions were performed using the PCR kit – PCR VWR, in a volume of 25 µl in a Progene cycler (Techne, UK) according to the manufacturer's instructions. 50 ng total DNA of the studied strain and 10 pmol primers were mixed in each reaction.
DNA of the studied strain was amplified using universal primers for the 16S rDNA gene - 27f (5'AGAGTTTGATCMTGGCTCAG3') [2] and 1492r (5'ACCTTGTTACGACTT3') [2]. The amplification program included: denaturation - 95°C for 3 minutes, 40 cycles - 93°C for 30 s, 55°C for 60 s, 72°C for 2 min, final elongation - 72°C for 7 min. The resulting PCR product from the 16S rDNA amplification of the tested strain was visualized on a 2% agarose gel, stained with ethidium bromide solution (0.5 µg/ml), using an UVP Documentation System (UK) [3].

Purification of the product of the PCR-reaction – 16S rDNA – from TAEagarose Gel

The purification of 16S rDNA was conducted using DNA-purification kit (GFX Microspin™) according to the manufacturer's instructions.

DNA-sequencing

Sequencing of the gene encoding the 16S rRNA was conducted by „Macrogen Europe Laboratory", the Netherlands using the Sanger method for DNA-sequencing. The obtained forward and reverse partial sequences of the two ends of the 16S rRNA gene were assembled using the software CLC Sequence Viewer. The total gene sequence of the 16S rRNA gene was compared with the sequences available in the online GenBank database through the online software BLASTn and the species identification was determined by the rate of correspondence between the squence of the studied strain and the reference strain from the online database [4].

3. Results and Discussion

Determination of the physicochemical characteristics of thermal healing spring in the village of Starozagorski Mineralni Bani, Stara Zagora region with water temperature of 40°C

The values of the main physico-chemical parameters of the healing spring in the village of Starozagorski Mineralni Bani, Stara Zagora region were compared with the requirements set by the standards (Table 1).

Table 1

Physico-chemical analysis of the healing spring water in the village of Starozagorski Mineralni Bani, Stara Zagora region

Controlled parameter	Unit	Maximum value	Result
Colour according to the Rublyovska scale	Colour degrees	Acceptable for the consumer	Acceptable
Odor at 20 °C	Total result	Acceptable for the consumer	Acceptable
Turbidity	NTU	Acceptable for the consumer	Acceptable
pH	pH units	≥ 6,5 and ≤ 9,5	6,9
Oxydation	mgO_2/dm^3	5,0	0,6
Chlorides	mg/dm^3	250	26
Nitrates	mg/dm^3	50	7
Nitrites	mg/dm^3	0,50	0,00
Ammonium ions	mg/dm^3	0,50	0,00
Total hardness	$mgekv/dm^3$	12	7
Sulphates	mg/dm^3	250	14
Calcium	mg/dm^3	150	72
Magnesium	mg/dm^3	80	30
Phosphates	mg/dm^3	0,5	0,0
Manganese	mg/dm^3	50	0
Iron	$\mu g/dm^3$	200	16
Fluorides	mg/dm^3	1,5	0.0
Conductivity	$\mu S/dm^3$	2000	750

Experimental data showed that the thermal healing spring waters met the required values for all controlled parameters of Ordinance № 9/2001 Dyrjaven vestnik, issue 30 and Decree № 178/23.07.2004 for the quality of potable water.

Determination of the microbiological safety of thermal healing spring in the village of Starozagorski Mineralni Bani, Stara Zagora region with water temperature of 40°C

The experimental data from the microbiological examination of the thermal healing spring water indicated that the water met the standard criteria for microbiological quality of mineral and potable water (Table 2).

Microflora of the thermal healing spring water from the spring in the village of Starozagorski Mineralni Bani, Stara Zagora region with water temperature of 40°C

One strain was isolated from the spring water. The colonial and morphological characteristics of the isolated bacterial strain were determined (Table 3). The isolated strain was rod-shaped, Gram - positive, motile, sporeforming. The strain formed a precipitate on the surface of the liquid culture medium, did not turbidify the liquid culture medium and formed a precipitate on the bottom of the tube. The strain was a facultative aerobe (Table 4).

The ability of the isolated strain to absorb the 49 carbon sources included in the kit system for rapid identification of bacilli API 50 CHB/E was examined. After processing of the test results with apiweb® the species identification of strains with the corresponding percentage of reliability was obtained (Table 5). The strain *Bacillus* SMB belongs to the species *Bacillus thuringiensis* with percentage of reliability of 99%.

Table 2

Microbiological analysis of healing spring water from the spring in the village of Starozagorski Mineralni Bani, Stara Zagora region with water temperature of 40°C

Controlled parameter	Norm, cfu/cm^3	Result, cfu/cm^3
Coliforms	0/100	0/100
Escherichia coli	0/100	0/100
Enterococci	0/100	0/100
Sulphyte reducing anaerobic bacteria *(Clostridium perfringens)*	0/100	0/100
TBA at 22 °C	100	0
TBA at 37 °C	20	0
Pseudomonas aeruginosa	0/250	0/250

Table 3

Colonial characteristics of the isolated strain

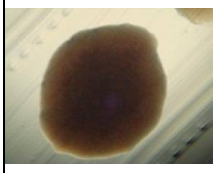
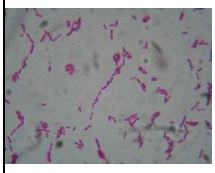
Strain	Description of the colonies	Visualization	Description of the cells	Visualization
SMB	Round, soft, smooth and shiny, whitish, drop-like colonies with wave-like edges, size – 2 – 3 mm		Gram-positive short, thick rods with round edges, motile, sporeforming, arranged singly, in pairs and in long chains	

Table 4

Growth of the isolate from the thermal healing spring water in the village of Starozagorski Mineralni Bani, Stara Zagora region in liquid medium for 24-48 h, at temperatures 3°C - 45°C (P=precipitate; T=turbidification; S=sludge)

Strain	3 °C			25 °C			30°C			37 °C			45 °C			Attitude to oxygen
	P	T	S	P	T	S	P	T	S	P	T	S	P	T	S	
SMB	–	–	–	+	–	+	+	–	+	+	–	+	–	–	–	Facultative aerobe

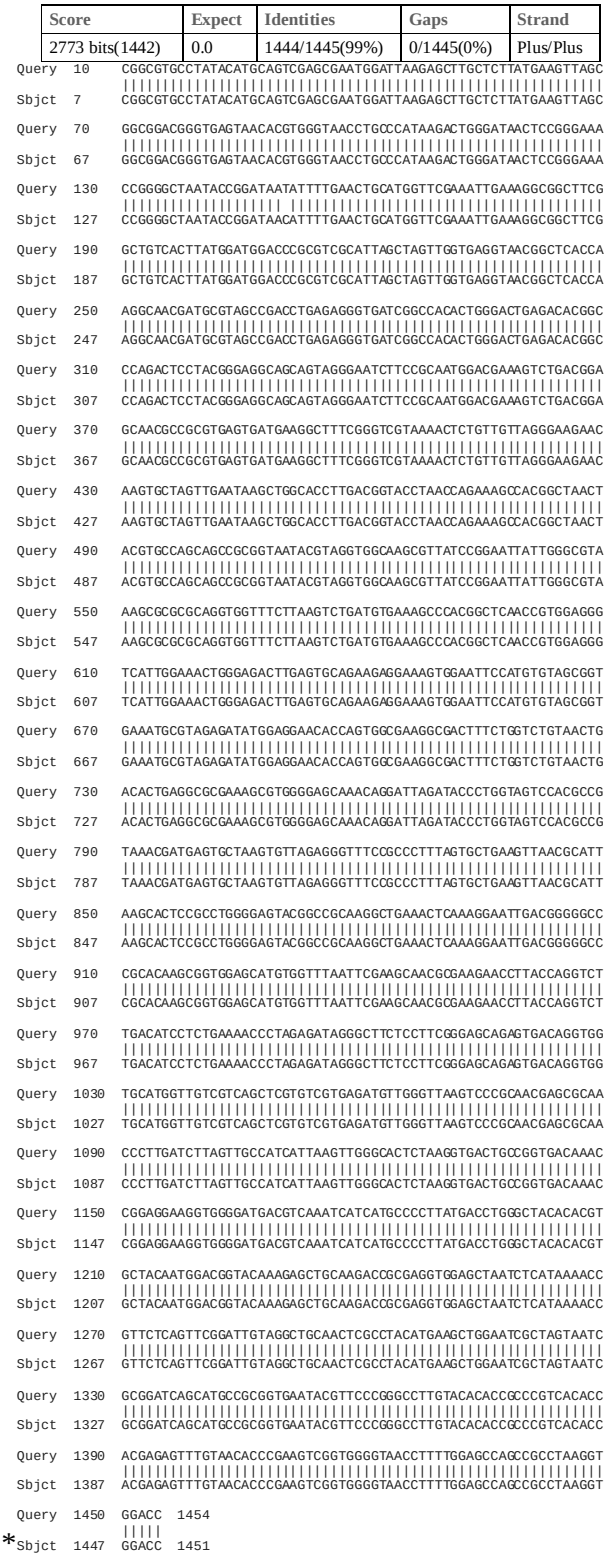
\+ - Occurance of Precipitate, Turbidity, Sludge;
– - Absence of Precipitate, Turbidity, Sludge.

Table 5

API 50 CHB/E of the strain *Bacillus* SMB

#	Carbohydrates	SMB
1	Glycerol	-
2	Erythriol	-
3	D-arabinose	-
4	L-arabinose	-
5	Ribose	+ (90-100%)
6	D-xylose	-
7	L-xylose	-
8	Adonitol	-
9	β-metil-D-xyloside	-
10	Galactose	-
11	D-glucose	+ (50%)
12	D-fructose	+ (50%)
13	D-mannose	-
14	L-sorbose	-
15	Rhamnose	-
16	Dulcitol	-
17	Inositol	-
18	Manitol	-
19	Sorbitol	-
20	α-methyl-D-mannoside	-
21	α-methyl-D-glucoside	-
22	N-acetyl-glucosamine	+ (90-100%)
23	Amigdalin	-
24	Arbutin	+ (90-100%)
25	Esculin	+ (90-100%)
26	Salicin	+ (90-100%)
27	Cellobiose	-
28	Maltose	+ (90-100%)
29	Lactose	-
30	Melibiose	-
31	Saccharose	+ (90-100%)
32	Trehalose	+ (90-100%)
33	Inulin	-
34	Melezitose	-
35	D-raffinose	-
36	Amidon	+ (50%)
37	Glycogen	+ (90-100%)
38	Xylitol	-
39	β-gentiobiose	-
40	D-turanose	-
41	D-lyxose	-
42	D-tagarose	-
43	D-fuccose	-
44	L-fuccose	-
45	D-arabitol	-
46	L-arabitol	-
47	Gluconate	-
48	2-keto-gluconate	-
49	5-keto-gluconate	-
	Identification	*Bacillus thuringiensis*
	% of reliability	99

Bacillus thuringiensis B62, 16S ribosomal RNA gene, partial sequence; Sequence ID: gb|JX010983.1| Length: 1455 Number of Matches: 1



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Sbjct  7     CGGCGTGCCTATACATGCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGC  66

Query  70    GGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAA  129
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Query  130   CCGGGGCTAATACCGGATAATATTTTGAACTGCATGGTTCGAAATTGAAAGGCGGCTTCG  189
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Sbjct  307   CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGA  366

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Fig. 1. Comparison of the nucleotide sequence of the 16S rRNA gene of the strain *Bacillus* SMB and the partial sequence of the 16S rRNA gene of *Bacillus thuringiensis* B62.

Nedyalka VYLCHEVA-ZHEKOVA, Zapryana DENKOVA, Radosveta NIKOLOVA *Biochemical and molecular-genetic characterization of a strain isolated from a thermal healing spring in Starozagorski Mineralni Bani, Stara Zagora region, Bulgaria,* Food and Environment Safety, Volume XIV, Issue 1 – 2015, pag. 37 - 44

The 16S rRNA gene was sequenced and the resulting sequence was processed using the software BLASTn and it was confirmed that the studied strain belongs to the species *Bacillus thuringiensis* (Fig. 1).

4. Conclusion

The physicochemical and microbiological parameters of thermal healing spring water in the village of Starozagorski Mineralni Bani, Stara Zagora region were examined. The spring water met the requirements laid down in the Ordinance № 9/2001 Dyrjaven vestnik, issue 30 and Decree № 178/23.07.2004 for the quality of potable water when it comes to its physicochemical parameters. From a microbiological point of view the spring water was safe to use. The strain SMB isolated from the spring was identified using physiochemical, biochemical (API 50 CHB) and molecular-genetic (sequencing of the gene for the 16S rRNA) methods as a representative of the species *Bacillus thuringiensis*. After further examinations of its antimicrobial activity against phytopathogenic molds strain SMB would be incorporated in the composition of biological preparations against plant diseases.

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