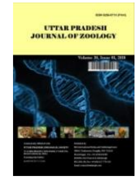
UTTAR PRADESH JOURNAL OF ZOOLOGY

41(24): 16-22, 2020
ISSN: 0256-971X (P)



PHYSICOCHEMICAL AND MICROBIOLOGICAL RESULTS OF HYPERTHERMAL (HOT) MINERAL WATER IN RUPITE, BULGARIA AS MODEL SYSTEM FOR ORIGIN OF LIFE

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Author II designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors NV and SM managed the analyses of the study. Author GD managed the literature searches. All authors read and approved the final manuscript.

Article Information

Editor(s):
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Reviewers:
(1) Maria De Sousa Brito Neta, Universidade De Brasilia, Brazil.
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Received: 25 October 2020
Accepted: 31 December 2020
Published: 23 January 2021

Original Research Article

ABSTRACT

The objective of current research is to prove the presence of bacteria genus *Bacillus* in hot mineral water from Rupite, Bulgaria. The water of Rupite is rich in hydrocarbon and calcium ions. The presence of bivalent Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) ions in warm and hot mineral waters take part in the activation of cortex-lytic enzymes during germination of *Bacillus subtilis* (Igura, 2003).

In 2010 Ignatov and Mosin indicate the possibility for origin of life and living matter in hot mineral water 3.5 billion years ago. Comparative analyses are conducted based on experiments for the similarities of spectra of hot mineral waters and cactus juice. The closest matches are the spectra of cactus juice and the water from Rupite, Bulgaria. Such compatibility is not observed between the cactus juice and sea water. In the cactus juice and the hot mineral water we observed five comparable local extremums (8.95; 9.67; 9.81; 10.47; 11.13 µm), whereas there was only one (9.10 µm) with the sea water. It is therefore justified to suggest that most probably life originated in hot mineral water. According to their temperature they can be cold (up to 37 °C), warm (from 37°C to 60 °C) and hot (over 60 °C). This is temperature standard in Bulgaria, European Union.

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Keywords: Mineral water; Rupite; physicochemical properties; microbiological indicators; bacteria.

1. INTRODUCTION

The first living organisms are stromatolites. They are organosedimentary structures dating back more than 3.5 billion years. 2.3 billion years ago commence cyanobacterial oxygenic photosynthesis and cyanobacteria participates in the formation of stromatolites [1]. The research indicates evidence that if there is presence of calcium and hydrocarbon ions, as well as *Bacillus licheniformis*, there is sedimentation of carbonate minerals. By changes of Mg/Ca molar ratios, pH increases [2]. It is a process of formation of microbialites (which include stromatolites). The presence of bivalent Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) ions in warm and hot mineral water takes part in the activation of cortex-lytic enzymes during germination of *Bacillus subtilis* [3]. At the current stage of evolution is observed biomineralization. Microorganisms were isolated and identified from speleothems at Khasi hill caves, Meghalaya, India. It is studied the biomineralization potential of *Bacillus subtilis*, *Rummeliibacillus Stabekisii* and *Staphylococcus Epidermidis* Strains [4]. During such biomineralization part of the modern stromatolites become structured [5]. Described are effects of *Bacillus subtilis* over stromatolites [6].

In this study, we identify the physicochemical and microbiological results of hyperthermal (hot) mineral water in source Rupite 1 and pond, District of Blagoevgrad, Bulgaria. As a result of conducted physicochemical and microbiological research we established the characteristics of the hyperthermal (hot) spring Rupite 1 with water t=74,3°C.

In 2010 Ignatov researched hot mineral waters in Bulgaria and conducted comparative spectrum analysis with cactus juice [7]. The closest match to the spectrum of cactus juice appears to be the water from Rupite [8]. In this water are contained the highest amounts of calcium (Ca^{2+}) and hydrogencarbonate (HCO_3^-) ions [9]. Ignatov and Mosin performed analyses with *Bacillus subtilis* with heavy water for analyses of the initial condition for origin of life in hot mineral water in primary atmosphere [10,11].

A scientific group led by Koonin, the National Center for Biotechnology Information of the National Library of Medicine of the National Institutes of Health of the USA in Bethesda, USA, announced the possibility of the emergence of life in freshwater lakes that receive steam and hot water from geothermal sources [12].

In 2016 Bruce Damer and David Deamer suggested that the cell membrane cannot be formed in sea water. Before the continents of the Earth were formed there were volcanic islands. In the hot mineral water found on those volcanic islands, it is possible for lipids to have formed [13].

The team of Kambourova proved the presence of new Anoxybacillus Rupiences [14] and archaea in Rupite water [15]. Czech scientists proved the presence of cyanobacteria in the water of Rupite [16].

Analyses were conducted by Ignatov and Mosin for effects of heavy water with deuterium over *Bacillus subtilis* [17].

The purpose of the current research is to check the bacterial diversity specifically for Bacillus Subtilis in the water of Rupite, and confirm that mineral water as a favorable medium for living organisms.

Below is given a chemical reaction for structuring of the first living species on Earth 3.5 billion of years ago – stromatolites [18]:

$$2\ HCO_3^- + Ca^{2+} \rightarrow CaCO_3 + CO_2 + H_2O$$

It has been shown in experimental models that the uptake of hydrogen ions during reduction increases the pH and results in the release of carbonate ions in stromatolites [19, 20].

Ignatov shows the formation of nascent hydrogen in stromatolites [21].

The following reaction (Mehandjiev, Vassileva, Gluhchev et al., 2019) is possible for the structure of nascent hydrogen H* in electric discharge conditions in primary atmosphere and hydrosphere [22].

$$H_2O + e^- \rightarrow H^* + OH^-,$$

This reaction after recombination is formed:

$$H^* + H^* \rightarrow H_2$$

The nascent hydrogen is very active for chemical reactions in primary hydrosphere for origin of life with additional formation of H_2.

The authors of current study prove the presence of the genus *Bacillus* and its species in the water of Rupite. The research is connected with other bacteria also.

2. METHODS AND MATERIALS

2.1 Methods for Microbiological Research

Vitek® MS is an automated system for microbial identification of the company BioMerieux. The device is based on innovative technology mass spectrometry. MALDI-ToF MS [23] is an abbreviation for Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry. For the identification are needed microbial cultures. In the conducted analysis were used 48 h pure cultures over nutrient agar. Each culture was mixed with a matrix over a special plate, positioned in a preparatory station with a bacteria protocol, inserted in the device and influenced by a laser. To control the process was used a standard strain of Escherichia coli ATCC 8739, which has well known characteristics. As a result were generated the so called MALDI-ToF spectra that were analyzed by the means of a software, and compared to existing profiles in the database.

2.2 Methods for Physicochemical Analysis

Method for determination of color according to Rublyovska Scale – method by Bulgarian State Standard (BDS) 8451: 1977;

Method for determination of smell at 20°C – method BDS 8451: 1977 technical device – glass mercury thermometer, conditions No 21;

Method for determination of turbidity – EN ISO 7027, technical device turbidimeter type TURB 355 IR ID No 200807088;

Method for determination of pH – BDS 3424 : 1981, technical device pH meter type UB10 ID NoUB10128148;

Method for determination of oxidisability – BDS 3413: 1981;

Method for determination of chlorides – BDS 3414: 1980;

Method for determination of nitrates – Validated Laboratory Method (VLM) – NO_3 – No 2, technical device photometer "NOVA 60 A" ID No 08450505;

Method for determination of nitrites – VLM NO_2 – No 3, technical device photometer "NOVA 60 A" ID No 08450505;

Method for determination of ammonium ions – VLM – NH_4 – No 1, technical device photometer "NOVA 60 A" ID No 08450505;

Method for determination of general hardness – BDS ISO 6058;

Method for determination of sulphates – VLM – SO_4 – No 4, technical device photometer "NOVA 60 A" ID No 08450505;

Method for determination of calcium – BDS ISO 6058;

Method for determination of magnesium – BDS 7211: 1982;

Method for determination of phosphates – VLM - PO_4 – No 5, technical device photometer "NOVA 60 A" ID No 08450505;

Method for determination of manganese – VLM – Mn – No 7, technical device photometer "NOVA 60 A" ID № 08450505;

Method for determination of iron – VLM – Fe – No 6, technical device photometer "NOVA 60 A" ID No 08450505;

Method for determination of fluorides – VLM – F – No 8, technical device photometer "NOVA 60 A" ID No 08450505;

Method for determination of electrical conductivity – BDS EN 27888, technical device – conductivity meter inoLab cond 720 ID No 11081137.

3. RESULTS AND DISCUSSION

3.1 Hyperthermal (hot) Spring Rupite1 and Pond

The total mineralization of the mineral water from the spring Rupite 1, municipality of Petrich, District of Blagoevgrad is characterized as highly mineralized, hyperthermal, hydrocarbon-sodium, fluoride and silicon-rich water.

In the study: The contents of studied micro components are within the norms for mineral waters. The values of radiological indicator “gross beta activity” are above the control levels, but the results from the performed expert evaluation of the total indicative dose correspond to the requirements. The water has a stable physico-chemical composition and properties, and it corresponds to the requirements of Decree No 14 regarding the resort resources, resort localities and resorts (Official State Gazette, issue 79 from 1987, and amendment published in issue 70 from 2004).

3.2 Physicochemical Analyses of Water from Hyperthermal (hot) Spring Rupite 1

A comparative physicochemical analysis of hot mineral spring water Rupite 1, Blagoevgrad District is performed using the main indicators. The results from these examinations are given in Table 1.

The total mineralization of the mineral water from hyperthermal spring Rupite 1 is 2230 mg/ dm^3. It is characterized as highly mineralized, hyperthermal, hydrocarbon-sodium, fluoride and silicon-rich water. The protocol for research is from Regional Health Inspectorate, Haskovo, Bulgaria, 1.03.2020 –Rupite 1 [24].

3.3 Radiological indicators of hyperthermal spring Rupite 1 are:

Total a-activity 0,40±0,15 Bq/LRadon- 222 2,62±0,35 Bq/L

Total p-activity 2,44±0,35 Bq/LNatural uranium 0,000069±0,000017 mg/1

Radium-226 0,308±0,072 Bq/LTotal indicative dose 0,063±0,0147 mSv/year

3.4 Microbiological results of hyperthermal (hot) spring Rupite 1

The microbiological indicators for the water from Rupite were determined by the membrane method. The experimental studies from the determination of total number of mesophilicaerobic and facultative anaerobic bacteria are shown in Table 2.

According to the standard requirements from the examined water samples from the spring Rupite 1, the water is clean.

In Table 3 are given results for presence of bacteria with MALDI-ToF MS identification.

The results are consistent with microbiological proofs for the following bacteria – *Bacillus subtilis, Bacillus vallismortis, Bacillus amyloliquefaciens, Bacillus megaterium, Bacillus simplex* and *Stenotrophomonas maltophilia*.

They live in hot mineral water with temperature 73.4 °C and they are thermophiles.

Table 1. Comparison of the examined hyperthermal spring water in spring Rupite 1, Municipality of Petrich, District of Blagoevgard, Bulgaria according to their physicochemical properties

Controlled parameter	Measuring unit	Maximum Limit Value	Result
1. Color Rublyovska Scale	Chromaticity Values	Acceptable	Acceptable
2. Smell at 20°C	Rating	Acceptable	Unacceptable to consumers (with a slight smell of hydrogen sulphide)
3. Turbidity	NTU	Acceptable	Acceptable
4. pH	pH values	≥ 6,5 и ≤ 9,5	7.06±0.1
5. Oxidisability	mgO_2/dm^3	5,0	1.2
6. General hardness	mgekv/ dm^3	12	4±0.2
7. Calcium (Ca)	mg/ dm^3	150	31.66±1.6
8. Sodium (Na)	mg/ dm^3	150	542.00±54
9. Magnesium (Mg)	mg/ dm^3	80	14.35±0.7
10. Phosphates (PO_4)	mg/ dm^3	0,5	<0.02
11. Manganese (Mn)	mg/ dm^3	50	0.02
12. Iron (Fe)	µg/ dm^3	200	0.41
13. Hydrocarbonates (HCO_3)	mg/ dm^3	250	1526±76
14. Chlorides (Cl)			36.45±0.36
15. Sulphates (SO_4)	mg/ dm^3	250	80.24±4.0
16. Nitrates (NO_3)	mg/ dm^3	50	<1.00
17. Nitrites (NO_2)	mg/ dm^3	0.5	<0.05
18. Ammonium (NH_4)	mg/ dm^3	0.5	<0.05
19. Fluorides (F)	mg/ dm^3	1.5	6.09±0.08
20. Electrical conductivity	µS/ dm^3	2000	2230±67

Table 2. Determination of total number of mesophilic aerobic and facultative anaerobic bacteria in hyperthermal (hot) spring water in Rupite 1

Examined water source	Indicator, cfu/cm^3
Hyperthermal spring Rupite 1 with water t= 74,3°C	20± 1

Table 3. Microbiological indicators of hyperthermal (hot) spring water in Rupite 1, Municipality of Petrich, District of Blagoevgrad

Species of genus Subtilis in the examination of water from Rupite, Bulgaria	Bacterial Profile	Result with MALDI-ToF MS identification (Percent (%) of reliable identification)	Result with MALDI-ToF MS identification (Percent (%) of reliable identification) (after 24 hours)	Result with MALDI-ToF MS identification (Percent (%) of reliable identification) (after 72 hours)
1. Bacillus subtilis	3-2-2	33.3	33.3	33.3
2. Bacillus vallismortis	3-2-1	33.3	33.3	33.3
3. Bacillus amyloliquefaciens	3-2-3	33.3	33.3	33.3
4. Bacillus megaterium	2-3	99.9	99.9	99.9
5. Bacillus simplex	2-6	99.9	99.9	99.9
Genus Pseudomonas in the examination of water from Rupite, Bulgaria				
1. Pseudomonas fluorescens	3-8	99.9	99.9	99.9
Genus Stenotrophomonas in the examination of water from Rupite, Bulgaria				
1. Stenotrophomonas maltophilia	1-1	99.9	99.9	99.9

In the world there are places with thermophiles Bacillus – India (Leh and Ladakh Region) [25], Morocco [26] etc. *Bacillus altitudinis*was isolated in Yellowstone National Park, USA [27].

Szostak suggested that geothermal activity provides greater opportunities for the origination of life in open lakes (compared with seas), where there is a buildup of minerals. Ignatov and Mosin have shown that water with such spectrum is bio medium for origin of life in hot mineral water [28-30].

4. CONCLUSION

The research shows the following strains – *Bacillus subtilis, Bacillus vallismortis, Bacillus amyloliquefaciens, Bacillus megaterium, Bacillus simplex* and *Stenotrophomonas maltophilia*.

The presence of the highest number of strains in the waters of the springs in Rupite, in comparison with over 30 examined mineral springs in Bulgaria, confirms that their water is a favorable medium for activity of living organisms.

In 2012 *Bacillus subtilis* was used by Ignatov and Mosin as a model system for studying the possible effects for origin of life with different concentration of deuterium in the water.

The main conclusion is the large amount of calcium and hydrocarbonate ions in the water of Rupite. They are an integral part of the first living organisms – stromatolites.
There is a great variety of bacteria in the water of Rupite, which is consistent with the idea of hot spring water as a medium for origination of life and living matter.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research, and in ourcountry. There is absolutely no conflict of interest between the authors and manufacturers of the products, because we do not intend to use these products as an avenue for any litigation, but for the advancement of knowledge. Also, the research was not funded by the producing company but rather it was funded by private means of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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