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Modeling of Possible Processes for Origin of Life and Living Matter in Hot Mineral Water. Research of Physiological Processes of Bacterium *Bacillus Subtilis* in Hot Heavy Water

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Abstract

The physiological influence of deuterium on the Gram-positive chemoheterotrophic bacterium *Bacillus subtilis*, producer of inosine was studied on a heavy water (HW) medium with a maximal concentration of ^2H_2O. With the use of IR-spectroscopy (DNES-method) were studied samples of hot mineral water and sea water derived from different sources of Bulgaria. There are presented the data on the growth and the adaptation of *B. subtilis* in highly deuterated growth media with 2% (w/w) hydrolysate of deuterated biomass of the methylotrophic bacterium *Brevibacterium methylicum* as a source of ^{2}H-labeled growth substrates. It was studied the qualitative and quantitative composition of the cellular protein, amino acids and carbohydrates in the conditions of cellular adaptation to ^2H_2O. The taxonomy of the studied bacterium and the ability for assimilation of carbon substrates was also analyzed on an evolutionary level. It was shown on the example of chemoheterotrophic bacteria that hot mineral water with pH = 9–11 is more suitable for maintenance and origin of life than other water samples.

Keywords: heavy water, adaptation, biosynthesis, *Bacillus subtilis*, hot mineral water, origin of life and living mater

1. Introduction

One of the most interesting biological phenomena is the ability of some microorganisms to grow in heavy water (HW) media in which all hydrogen atoms are replaced with deuterium (^{2}H) (Mosin *et al*., 2000). The chemical structure of ^2H_2O molecule is analogous to that one for H_2O, with small differences in the length of the covalent H–O-bonds and the angles between them. The molecular mass of ^2H_2O exceeds on 10% that one for H_2O. That difference stipulates the isotopic effects, which may be sufficiently big for H/^{2}H pair (Lobishev & Kalinichenko, 1978). As a result, physical-chemical properties of ^2H_2O differ from H_2O: ^2H_2O boils at 101.44^{0}C, freezes at 3.82^{0}C, has maximal density at 11.2 ^{0}C (1.106 g/cm^3). The chemical reactions in ^2H_2O are somehow slower compared to H_2O. ^2H_2O is less ionized, the dissociation constant is smaller, and the solubility of the organic and inorganic substances in ^2H_2O is smaller compared to these ones in H_2O. Due to isotopic effects the hydrogen bonds with the participation of deuterium are slightly stronger

than those ones formed of hydrogen. According to the theory of chemical bond, breaking up of covalent H–O-bonds can occur faster, than ^{2}H–O-bonds, mobility of $^2H_3O^+$ ion is lower on 28.5% than H_3O^+ ion, and O^2H^- ion – on 39.8% than OH^- ion. The maximum kinetic isotopic effect, which can be observed at ordinary temperatures in chemical reactions leading to rupture of bonds involving H and ^{2}H lies in the range of 4 to 6 for C–H versus C–^{2}H, N–H versus N–^{2}H, and O–H versus O–^{2}H-bonds (Cleland *et al*., 1976).

The content of deuterium in nature makes up approx. 0.015 atom% (Vertes, 2003). In natural waters the ratio between ^2H_2O and H_2O compiles approx. 1:5700 (with the assumption that all deuterium presents in form of ^2H_2O) (Lis, 2007). In mixtures ^2H_2O with H_2O it is occurred with high speed the isotopic exchange with the formation of semi-heavy water (H^2HO): $^2H_2O + H_2O = H^2HO$. For this reason deuterium presents in smaller content in aqueous solutions in form of H^2HO, while in the higher content – in form of ^2H_2O.

For a long time it was considered that heavy water is incompatible with life. Experiments with the cultivation of cells of different organisms in ^2H_2O show toxic influence of deuterium. The high concentrations of ^2H_2O lead to the slowing down the cellular metabolism, mitotic inhibition in the prophase stage and in some cases – somatic mutations (Thomson, 1960). Experiments show that ^2H_2O influences negatively the different organisms. This is observed even while using natural water with an increased content of ^2H_2O or H^2HO (Bild *et al*., 2004). Bacteria can endure up to 90% (v/v) ^2H_2O (Mosin & Ignatov, 2012), plant cells can develop normally up to – 75% (v/v) ^2H_2O (Kushner *et al*., 1999), and animal cells – up to not more than 35% (v/v) ^2H_2O (Daboll *et al*., 1962). The decrease of the deuterium content in water to 25% (v/v) of the physiological level stimulates the cellular metabolism (Sinyak *et al*., 2003).

With the development of new biotechnological approaches, there appears an opportunity to use adapted to deuterium strain producers as amino acids and nucleosides. The traditional method for production of ^{2}H-labelled compounds consists in the growth in media containing maximal concentrations of ^2H_2O and deuterated substrates, e.g., [^{2}H]methanol, [^{2}H]glucose etc. (Mosin *et al*., 2013). The adaptation to ^2H_2O allows to obtain the unique biological material for the studying of molecular structure with NMR (Crespi, 1989). The recent advances in the technical and computing capabilities of analytical methods have allowed to considerable increase the efficiency of de novo biological studies, as well as to carry out structural-functional studies with deuterated molecules on a molecular level.

This study is a continuation of our research for the practical utilization of different microbial producers of natural compounds as nucleosides at the growth on ^2H_2O. They relate to different taxonomic groups of organisms having a chemoheterotrophic pathway of assimilation of carbon substrates. It is believed that the initial life forms on Earth had probably existed as heterotrophic bacteria that received food and energy from organic substrates (Baleux, 1977). That is why the chemoheterotrophic bacterium *Bacillus subtilis* was chosen as a model for our studies.

The purpose of our research was studying the physiological influence of deuterium on *B. subtilis* applicable to possible processes for origin of life and living matter.

2. Material and Methods

2.1. Bacterial Strains

The object of the research was an inosine producer strain of spore-forming aerobic Gram-positive chemoheterotrophic bacterium *B. subtilis* B-3157 polyauxotroph of histidine, tyrosine, adenine, and uracil

(demand, 10 mg/l), obtained from the Institute of Genetics and Selection of Industrial Microorganisms (Russian Federation). The initial strain was adapted to deuterium by plating individual colonies onto 2% (w/w) agarose with increasing ^2H_2O concentration and subsequent selection of colonies steady to the action of ^2H_2O.

2.2. Chemicals

Growth media were prepared using ^2H_2O (99.9 atom% ^2H), ^2HCl (95.5 atom% ^2H), and [^2H]methanol (97.5 atom% ^2H), purchased from JSC "Izotop" (St. Petersburg, Russian Federation). 5-dimethylamino(naphthalene)-1-sulfonyl (dansyl chloride) of analytical reagent grade was from Sigma-Aldrich Corporation (St. Louis, USA). Deionized water was provided by the Milli-Q integral water purification system ("Millipore", USA). Inorganic salts and D- and L-glucose ("Reanal", Hungary) were initially crystallized in 99.9 atom% ^2H_2O. ^2H_2O was distilled over $KMnO_4$ with subsequent control of the isotope purity by NMR spectroscopy on a Brucker WM-250 ("Brucker Corp.", USA) with a working frequency of 70 MHz (internal standard – Me_4Si).

2.3. Biosynthesis of ^2H-Inosine

[^2H]inosine was produced with an output 3.9 g/l in heavy water (HW) medium (89–90 atom% ^2H) with 2% (w/v) hydrolysate of deuterated biomass of methanol-assimilating strain of the facultative Gram-negative methylotrophic bacterium *Brevibacterium methylicum* as a source of ^2H-labeled growth substrates. The strain was obtained by multistage adaptation on a solid 2% (w/w) agarose M9 minimal medium containing 3 g/l KH_2PO_4, 6 g/l Na_2HPO_4, 0.5 g/l NaCl, and 1 g/l NH_4Cl with 2% (v/v) [^2H]methanol and a stepwise increasing ^2H_2O concentration gradient (0, 24.5, 73.5, and 98% (v/v) ^2H_2O). Raw methylotrophic biomass (yield, 200 g/l) was suspended in 100 ml 0.5 N ^2HCl (in ^2H_2O) and autoclaved for 30–40 min at 0.8 atm. The resulting suspension was neutralized with 0.2 N KOH (in ^2H_2O) to pH = 7.0, and used as a source of growth substrates when cultivating the inosine producer strain. For this purpose, an inoculum (5–6% (w/w)) was added to the HW medium with ^2H_2O containing 12% (w/w) glucose, 2% (w/w) hydrolysate of deuterated biomass *B. methylicum*, 2% (w/w) NH_4NO_3, 1% (w/w) $MgSO_4\ 7H_2O$, 2% (w/w) $CaCO_3$, 0.01% (w/w) adenine, and 0.01% (w/w) uracil. A protonated medium with 2% (w/w) yeast protein–vitamin concentrate (PVC) was used as a control.

2.4. Growth Conditions

Bacteria were grown in 500 ml Erlenmeyer flasks (containing 100 ml of the growth medium) for 3–4 days at 32°C under intensive aeration in a Biorad orbital shaker ("Biorad Labs", Hungary). The bacterial growth was controlled on the ability to form individual colonies on the surface of solid (2% (w/w) agarose) media, as well as the optical density of the cell suspension measured on a Beckman DU-6 spectrophotometer ("Beckman Coulter", USA) at $\lambda = 540$ nm in a quartz cuvette with an optical pathway length 10 mm.

2.5. Analytical Determination of [^2H]Inosine

Inosine was analytically determined in culture liquid samples with a volume of 10 μl on Silufol UV-254 chromatographic plates (150 × 150 mm) ("Kavalier", Czech Republic) using a standard set of ribonucleosides "Beckman-Spinco" (USA) in the solvent system: *n*-butanol–acetic acid–water (2:1:1, % (v/v)). Spots were eluted with 0.1 N HCl. The UV absorption of eluates was recorded on a Beckman DU-6 spectrophotometer ("Beckman Coulter", USA) using a standard calibration plot. The level of bioconversion of the carbon substrate was assessed using glucose oxidase (EC 1.1.3.4).

2.6. Protein Hydrolysis

Deuterated biomass (10 g) was treated with a chloroform–methanol–acetone mixture (2:1:1.5, % (v/v)) and supplemented with 5 ml 6 N ^{2}HCl (in ^2H_2O). The ampules were kept at 110°C for ~24 h. Then the reaction mixture was suspended in hot ^2H_2O and filtered. The hydrolysate was evaporated at 10 mm Hg. ^{2}HCl was removed in an exsiccator over solid NaOH.

2.7. Hydrolysis of Intracellular Polycarbohydrates

Dry deuterated biomass (50 mg) was placed into a 250 ml round bottomed flask, supplemented with 50 ml distilled ^2H_2O and 1.6 ml 25% (v/v) H_2SO_4 (in ^2H_2O), and boiled in a reflux water evaporator for ~90 min. After cooling, the reaction mixture was suspended in one volume of hot distilled ^2H_2O and neutralized with 1 N $Ba(OH)_2$ (in ^2H_2O) to pH = 7.0. $BaSO_4$ was separated by centrifugation on a T-24 centrifuge ("Heraues Separatech", Germany) (1500 g, 5 min); the supernatant was decanted and evaporated at 10 mm Hg.

2.8. Amino Acid Analysis

The amino acids of the hydrolyzed biomass were analyzed on a Biotronic LC-5001 (230 × 3.2) column ("Eppendorf-Nethleler-Hinz", Germany) with a UR-30 ("Beckman-Spinco", USA) sulfonated styrene (7.25% crosslinked) resin as a stationary phase; the granule diameter was 25 μm; 0.2 N sodium–citrate buffer (pH = 2.5) was used as an eluent; the working pressure – 50–60 atm; the eluent input rate – 18.5 ml/h; the ninhydrin input rate – 9.25 ml/h; detection at λ = 570 and λ = 440 nm (for proline).

2.9. Analysis of Carbohydrates

Carbohydrates were analyzed on a Knauer Smartline chromatograph ("Knauer", Germany) equipped with a Gilson pump ("Gilson Inc.", Germany) and Waters K 401 refractometer ("Water Associates", Germany) using Ultrasorb CN C18 as a stationary phase: the column size – 250 × 10mm; the granule diameter – 10 μm; the mobile phase – acetonitrile–water (75 : 25, % (v/v); the input rate – 0.6 ml/min.

2.9. FAB Mass Spectrometry

FAB mass spectra were recorded on a VG-70 SEQ chromatograph ("Fisons VG Analytical", USA) equipped with a cesium source on a glycerol matrix with accelerating voltage 5 kV and ion current 0.6–0.8 mA.

2.10. EI Mass Spectrometry

EI mass spectra were recorded with an MB-80A device (Hitachi, Japan) with double focusing (the energy of ionizing electrons – 70 eV; the accelerating voltage – 8 kV; the cathode temperature – 180–200 ℃) after amino acid modification into methyl esters of N-5-dimethylamino(naphthalene)-1-sulfonyl (dansyl) amino acid derivatives according to an earlier elaborated protocol (Mosin & Ignatov, 2013).

2.11. IR Spectroscopy

Samples of water for the research by the IR-spectroscopy method were taken from various sources of Bulgaria: 1 – hot mineral water (75 ℃) from Rupite village (Bulgaria); 2 – sea water (Varna, Bulgaria); 3 – cactus juice of *Echinopsis pachanoi*. IR-spectra were registered by Dr. Kristina Chakarova (Bulgarian Academy of Sciences, Sofia, Bulgaria) on Fourier-IR spectrometer Brucker Vertex ("Brucker", Germany) (a spectral range: average IR – 370–7800 cm^{-1}; visible – 2500–8000 cm^{-1}; the permission – 0.5 cm^{-1}; accuracy of wave number – 0.1 cm^{-1} on 2000 cm^{-1}).

3. Results and Discussion

3.1. Adaptation of B. Subtilis to Deuterium.

We have investigated isotopic effects of deuterium in prokaryotic cells of various taxonomic groups of microorganisms including chemoheterotrophic bacteria, which are believed to be at early stages of evolution. As a model for our experiments was used an inosine producer mutant strain of the Gram-positive chemoheterotrophic bacterium *B. subtilis* VKPM B-3157 (Mosin *et al*., 1999) polyauxotrophic for histidine, tyrosine, adenine, and uracil (preliminary adapted to deuterium by selection of individual colonies on solid 2% (w/v) agarose growth media with 99.9 atom% D_2O). Because of impaired metabolic pathways involved in the regulation of the biosynthesis of purine ribonucleosides, this strain under standard growth conditions (PVC medium, late exponential growth, 32°C) synthesizes 17–20 gram of inosine per 1 liter of cultural medium (Mosin *et al*., 2013). The maximal inosine yield for this strain was reached on a protonated growth medium with 12% (w/v) glucose as a source of carbon and energy and 2% (w/v) yeast PVC as a source of growth factors and amine nitrogen. In our experiments it was necessary to replace the protonated growth substrates with their deuterated analogs, as well as to use ^2H_2O of high isotopic purity. For this purpose, we used autoclaved biomass of the Gram-negative facultative methylotrophic bacterium *Brevibacterium methylicum* B-5662 strain adapted to deuterium capable to assimilate methanol via the ribulose-5-monophosphate (RuMP) pathway of carbon assimilation. Owing to a 50–60% rate of methanol bioconversion (conversion efficiency, 15.5–17.3 gram of dry biomass per 1 gram of assimilated substrate) and stable growth in a deuterated M9 minimal medium with 98% (v/v) ^2H_2O and 2% (v/v) [^{2}H]methanol, this strain is the most convenient source for producing deuterated biomass; moreover, the cost of bioconversion is mainly determined by the cost of ^2H_2O and [^{2}H]methanol.

The strategy for the biosynthesis of [^{2}H]inosine using biomass of *B. methylicum* as growth substrates was developed taking into account the ability of methylotrophic bacteria to synthesize large amounts of proteins (output, 50% (w/w) of dry weight), 15–17% (w/w) of polysaccharides, 10–12% (w/w) of lipids (mainly, phospholipids), and 18% (w/w) of ash (Mosin *et al*., 1998). To provide high outputs of these compounds and minimize the isotopic exchange (^{1}H–^{2}H) in amino acid residues of protein molecules, the biomass was hydrolyzed by autoclaving in 0.5 N ^{2}HCl (in ^2H_2O). Since the *B. subtilis* inosine-producing strain is a polyauxotroph requiring tyrosine and histidine for its growth, we studied the qualitative and quantitative compositions of the amino acids in the hydrolyzed methylotrophic biomass produced in the maximally deuterated medium M9 (98% (v/v) ^2H_2O and 2% (v/v) [^{2}H]methanol), and the enrichment levels (Table 1). The methylotrophic hydrolysate contains 15 identified amino acids (except for proline, detectable at λ = 440 nm) with tyrosine and histidine contents per 1 gram of dry methylotrophic hydrolysate 1.82% and 3.72% (w/w), respectively, thereby surrisfying the auxotrophic requirements of the inosine producer strain for these amino acids. The contents of other amino acids in the hydrolysate are also comparable with the needs of the strain in sources of carbon and amine nitrogen (Table 1). The indicator determining the high efficiency of deuterium incorporation into the synthesized product is high degrees of deuterium enrichment of amino acid molecules, which vary from 49 atom% ^{2}H for leucine/isoleucine to 97.5 atom% ^{2}H for alanine (Table 1). This allowed to use the hydrolysate of deuterated *B. methylicum* biomass as a source of growth substrates for cultivating the *B. subtilis* inosine-producing strain.

Table 1. Amino acid composition of hydrolyzed biomass of the facultative methylotrophic bacterium *B.*

methylicum obtained on a maximally deuterated M9 medium with 98% (v/v) ^2H_2O and 2% (v/v) [^{2}H]methanol and levels of deuterium enrichment*

Amino acid	Yield, % (w/w) dry weight per 1 gram of biomass		Number of deuterium atoms incorporated into the carbon backbone of a molecule**	Level of deuterium enrichment of molecules, % of the total number of hydrogen atoms***
	Protonated sample (control)	Sample from deuterated M9 medium		
Glycine	8.03	9.69	2	90.0
Alanine	12.95	13.98	4	97.5
Valine	3.54	3.74	4	50.0
Leucine	8.62	7.33	5	49.0
Isoleucine	4.14	3.64	5	49.0
Phenylalanine	3.88	3.94	8	95.0
Tyrosine	1.56	1.83	7	92.8
Serine	4.18	4.90	3	86.6
Threonine	4.81	5.51	–	–
Methionine	4.94	2.25	–	–
Asparagine	7.88	9.59	2	66.6
Glutamic acid	11.68	10.38	4	70.0
Lysine	4.34	3.98	5	58.9
Arginine	4.63	5.28	–	–
Histidine	3.43	3.73	–	–

Keys: * The data were obtained for methyl esters of N-5-dimethylamino(naphthalene)-1-sulfonyl (dansyl) chloride amino acid derivatives.

** When calculating the level of deuterium enrichment, the protons(deuterons) at the carboxyl COOH- and amino NH_2- groups of amino acid molecules were not taken into account because of the dissociation in $H_2O/^2H_2O$.

*** A dash denotes the absence of data.

The growth and biosynthetic characteristics of inosine-producing strain *B. subtilis* were studied on a protonated yeast PVC medium with H_2O and 2% (w/w) yeast PVC and on an HW medium with 89% (v/v) ^2H_2O and 2% (w/w) hydrolysate of deuterated biomass of *B. methylicum* (Figure 1). Experiments demonstrated a certain correlation between the changes of growth dynamics of *B. subtilis* (Fig. 1, curves *1*, *1*'), output of inosine (Fig. 1, curves *2*, *2*'), and glucose assimilation (Fig. 3, curves *3*, *3*'). The maximal output of inosine (17 g/l) was observed on protonated PVC medium at a glucose assimilation rate 10 g/l (Fig. 1, curve *2*). The output of inosine in the HW medium decreased 4.4-fold, reaching 3.9 g/l (Fig. 1, curve *2*'), and the level of glucose assimilation, 4-fold, as suggested by the remaining 40 g/l unassimilated

glucose in cultural medium (Fig. 1, curve *3*'). The experimental data demonstrate that glucose is less efficiently assimilated during the growth in the HW medium as compared to the control conditions. This result demanded the examination of the contents of glucose and other intracellular carbohydrates in the biomass of the *B. subtilis* producer strain, which was performed by reverse phase HPLC on an Ultrasorb CN C18 column (10 μm, 10 × 250 mm) with acetonitrile and water (75 : 25, % (v/v)) as a mobile phase (Table 2). The fraction of intracellular carbohydrates in Table 2 (numbered according to the sequence of their elution from the column) comprises monosaccharides (glucose, fructose, rhamnose, and arabinose), disaccharides (maltose and sucrose), and four unidentified carbohydrates with retention times of 3.08 (15.63% (w/w)), 4.26 (7.46% (w/w)), 7.23 (11.72% (w/w)), and 9.14 (7.95% (w/w) min (not shown). As was expected, the output of glucose in the deuterated hydrolysate was 21.4% (w/w) of dry weight, that is, higher than the outputs of fructose (6.82% (w/w)), rhamnose (3.47% (w/w)), arabinose (3.69% (w/w)), and maltose (11.62% (w/w)) (Table 2). Their outputs did not differ considerably related to the control in H_2O except for sucrose, which is undetectable in the deuterated hydrolysate. The levels of deuterium enrichment in carbohydrates varied from 90.7 atom% ^2H for arabinose to 80.6 atom% ^2H for glucose.

The using of a combination of physical-chemical methods for isolating [^2H]inosine from the cultural medium of the inosine producer strain was determined by the need for preparing inosine of a high chromatographic purity (no less than 95%). Since cultural medium contains inorganic salts, proteins, and polysaccharides, along with inosine, as well as accompanying secondary metabolites of nucleic nature (adenosine and guanosine) and unreacted substrates (glucose and amino acids), the cultural medium was fractionated in a stepwise manner for isolating [^2H]inosine. The fractionation consisted in low-temperature precipitation of high molecular weight impurities with organic solvents (acetone and methanol), adsorption/desorption on the surface of activated carbon, extraction of the end product, crystallization, and ion exchange chromatography. The proteins and polysaccharides were removed by low temperature precipitation with acetone at 4 ℃ with subsequent adsorption of total ribonucleosides on activated carbon in the cold. The desorbed ribonucleosides were extracted from the reacted solid phase by eluting with EtOH–NH_3-solution at 60°C; inosine – by extracting with 0.3 M ammonium–formate buffer (pH = 8.9) with subsequent crystallization in 80% (v/v) of ethanol. The final purification consisted in column ion exchange chromatography on AG50WX 4 cation exchange resin equilibrated with 0.3 M ammonium–formate buffer containing 0.045 M NH_4Cl with collection of fractions at R_f = 0.5. Figure 2 (curves *1*–*3*) shows UV-absorption spectra of inosine isolated from the cultural medium. The presence of major absorption band I, corresponding to natural inosine (λ_{max} = 249 nm, ε_{249} = 7100 M^{-1} cm^{-1}), as well as the absence of secondary metabolites II and III in the obtained sample (Fig. 2, curve *3*), demonstrates its uniformity and the efficiency of the isolation method.

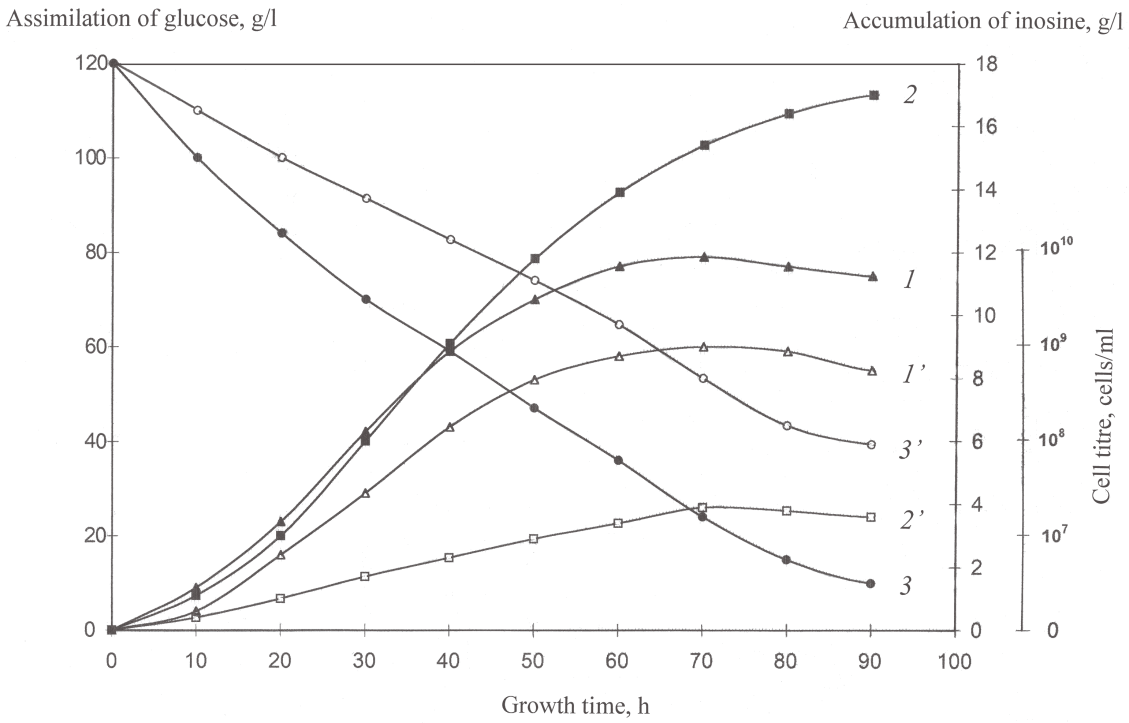

Figure 1. Growth dynamics of *B. subtilis* (*1*, *1*') (cells/ml), (*2*, *2*') inosine accumulation in cultural medium (g/l), and (*3*, *3*') glucose assimilation (g/l) under different experimental conditions: (*1*–*3*) – a protonated yeast PVC medium and (*1*'–*3*') – HW medium with 2% (w/w) hydrolysate of deuterated biomass of *B. methylicum*.

The level of deuterium enrichment of [^{2}H]inosine was determined by FAB mass spectrometry, the high sensitivity of which makes it possible to detect 10^{-8} to 10^{-10} moles of a substance in a sample, being considerably higher as compared to NMR. For this purpose, FAB spectra were recorded for the deuterated and protonated inosine, according to the difference in the molecular ion peaks for which the level of deuterium enrichment was calculated. The formation of a molecular ion peak for inosine in FAB mass spectrometry was accompanied by the migration of H^+. The level of deuterium enrichment of deuterated sample obtained from HW-medium was determined by a peak (I) of a molecular ion of inosine $[M + H]^+$ at *m*/*z* 274 with 38% (instead of $[M + H]^+$ at *m*/*z* 269 with 42% in the control conditions in H_2O) (Figure 3). Consequently, the presence of two "heavy" peaks in the FAB mass spectrum of inosine fragments of ribose II $(C_5H_9O_4)^+$ at *m*/*z* 136 (46%) (instead of *m*/*z* 133 (41%) in the control) and hypoxanthine III $(C_5H_4ON_4)^+$ at *m*/*z* 138 (55%) (instead of *m*/*z* 136 (48%) in the control), as well as the peaks of low molecular weight shelter fragments formed from FAB-decomposition of hypoxanthine fragment at *m*/*z* 111 (49%) (instead of *m*/*z* 109 (45%) in the control) and *m*/*z* 84 (43%) (instead of 82 (41%) in the control) suggests that three deuterium atoms are incorporated into the ribose residue of the inosine molecule, and two other deuterium atoms – into the hypoxanthine residue. Since the protons (deuterons) at positions of the ribose resudue in the inosine molecule could have been originated from glucose, the character of deuterium inclusion into the ribose residue is mainly determined by hexose-5-monophosphate (HMP) shunt, associated with the

assimilation of glucose and other carbohydrates. Since glucose in our experiments was used in a protonated form, its contribution to the level of deuteration enrichment of the ribose residue was neglected. However, deuterium was incorporated into the ribose residue of the inosine molecule owing to the preservation of the minor pathways of de novo glucose biosynthesis. Numerous isotopic $^{1}H–^{2}H$ exchange processes could also have led to specific incorporation of deuterium atoms at certain positions in the inosine molecule. Such accessible positions in the inosine molecule are hydroxyl (OH^-)- and imidazole protons at NH^+ heteroatoms, which can be easily exchanged on deuterium in $^{2}H_2O$ via keto–enol tautomerism. Three deuterium atoms in the ribose residue of inosine could have been originated from HMP shunt reactions, while two other deuterium atoms in the hypoxanthine residue could be synthesized de novo at the expense of [^{2}H]amino acids that originated from methylotrophic hydrolysate. In particular, the glycoside proton at position of the ribose residue could be replaced with deuterium in the reaction of CO_2 elimination at the stage of ribulose-5-monophosphate formation from 3-keto-6-phosphogluconic acid with subsequent proton(deuteron) attachment at the C_1 position of ribulose-5-monophosphate. In general, our studies confirm this scheme. However, it should be noted that the level of deuterium enrichment of inosine molecule is determined by isotopic purity of $^{2}H_2O$ and deuterated substrates.

Table 2. Qualitative and quantitative compositions of intracellular carbohydrates isolated from *B. subtilis* after cultivation in HW-medium and levels of the deuterium enrichment*

Carbohydrate	Content in biomass, % (w/w) of 1 g of dry biomass		Level of deuterium enrichment of molecules, %***
	Protonated sample (control)	Sample from the HW medium**	
Glucose	20.01	21.40	80.6
Fructose	6.12	6.82	85.5
Rhamnose	2.91	3.47	90.3
Arabinose	3.26	3.69	90.7
Maltose	15.30	11.62	–
Sucrose	8.62	ND	–

Keys: * The data were obtained by IR-spectroscopy.

** ND – not detected.

*** A dash denotes the absence of data.

Our experiments demonstrated, that the effects observed at the cellular growth on $^{2}H_2O$ possess complex multifactorial character connected to changes of morphological, cytological and physiological parameters – magnitude of the log-period, time of cellular generation, outputs of biomass, a ratio of amino acids, carbohydrates and lipids synthesized in $^{2}H_2O$, and with an evolutionary level of organization of investigated object as well. The general feature of bacterial growth in $^{2}H_2O$ was the proportional increase in duration of the log-period and time of cellular generation and simultaneous reduction of outputs of microbial biomass. The experimental data testify that cells realize the special adaptive mechanisms promoting functional

reorganization of work of the vital systems in the presence of ^2H_2O. Thus, the most sensitive to replacement of H on ^2H are the apparatus of biosynthesis of macromolecules and a respiratory chain, i.e., those cellular systems using high mobility of protons and high speed of breaking up of hydrogen bonds. Last fact allows consider adaptation to ^2H_2O as adaptation to the nonspecific factor affecting simultaneously the functional condition of several numbers of cellular systems: metabolism, ways of assimilation of carbon substrates, biosynthetic processes, and transport function, structure and functions of macromolecules.

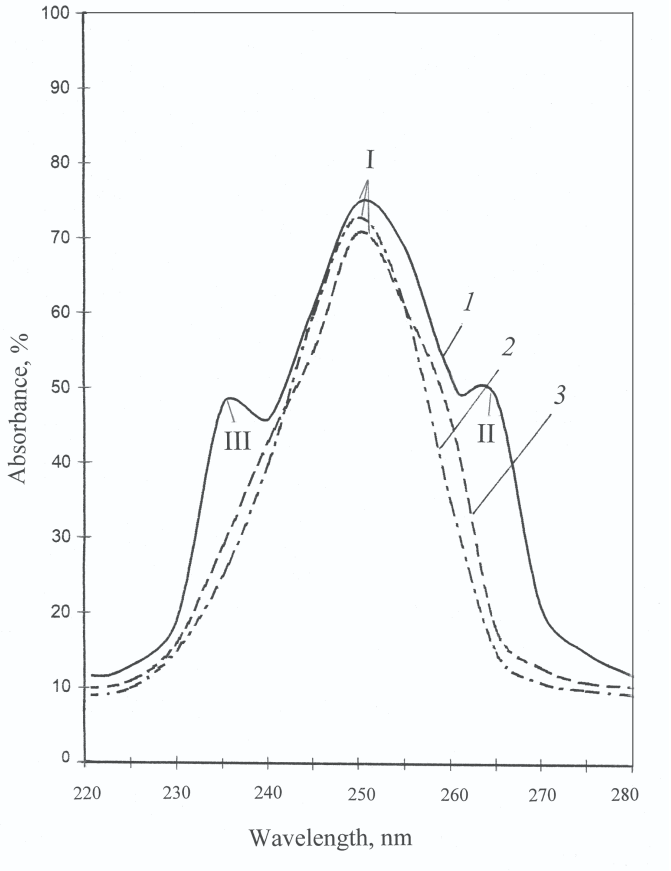

Figure 2. UV-absorption spectra of inosine (0.1 N HCl solution): (*1*) – initial cultural medium after growth *B. subtilis* in HW medium; (*2*) – natural inosine, and (*3*) – inosine extracted from the cultural medium. Natural inosine (*2*) was used as a control: (I) – inosine, (II, III) – secondary metabolites.

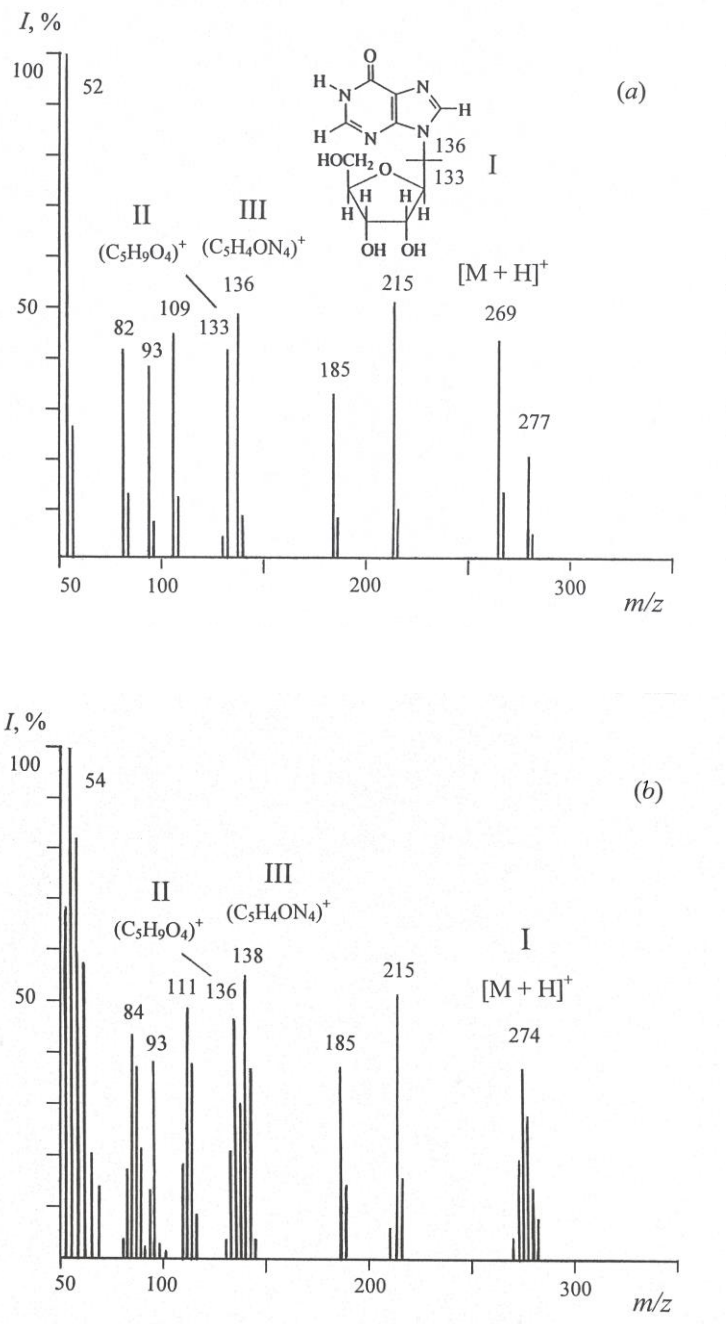

Figure 3. FAB mass spectra of inosine (glycerol as a matrix) under different experimental conditions: (*a*) – natural inosine; (*b*) – [^{2}H]inosine isolated from HW medium (scanning interval at *m*/*z* 50–350; major peaks with a relative intensity of 100% at *m*/*z* 52 and *m*/*z* 54; ionization conditions: cesium source; accelerating voltage – 5 kV; ion current – 0.6–0.8 mA; resolution – 7500 arbitrary units): *I* relative intensity of peaks (%); (I) inosine; (II) ribose fragment; (III) hypoxanthine fragment.

3.2. Possible Conditions for Origin of Life and Living Matter.

Biological experiments with ^2H_2O allow the better prognostication of the conditions under which life and

living matter had evolved (Ignatov, 2010; Ignatov, 2012). Circulating in bowels of cracks, crevices, channels and caves karst waters are enriched with $Ca(HCO_3)_2$ and other minerals, actively cooperating with live matter. Once appeared in these waters the process of self-organization of primary organic forms in water solutions may be supported by thermal energy of magma, volcanic activity and solar radiation. We have conducted experiments for the testing of various samples of mineral water from karst springs and sea water from Bulgaria and the cactus juice of *Echinopsis pachanoi* with IR-spectroscopy and Differential Non-equilibrium Energy Spectrum (DNES) method relative to the control – deionized water. The cactus is chosen as a model system because it contains approximately 90% (w/w) H_2O (Table 3).

Table 3. The local maximums of various water samples measured by IR-spectroscopy*.

Cactus juice, cm^{-1}	Mineral water (Rupite, Bulgaria), cm^{-1}	Sea water, cm^{-1}	Cactus juice, μm
898	897	–	11.13
955	953	–	10.47
1019	1018	–	9.81
1034	1037	–	9.67
1099	–	1099	9.10
1117	1113	–	8.95

Key: *A dash denotes non-maximums.

As shown from these data the closest to the spectrum of cactus juice is the IR-spectrum of the mineral waters of Rupite village (Bulgaria). IR spectra of the plant juice and mineral water with HCO_3^- (1320–1488 mg/l), Ca^{2+} (29–36 mg/l), pH (6.85–7.19), have local maximums at 8.95, 9.67, 9.81, 10.47 and 11.12 μm. Common local maximums in the IR-spectrum between the plant juice and the mineral water are detected at 9.10 μm. The local maximums obtained with IR method at 9.81 μm (1019 cm^{-1}) and 8.95μm (1117 cm^{-1}) (Table 3) are located on the spectral curve of the local maximum 9.7 μm (1031 cm^{-1}) (Figure 4). With the DNES-method were obtained the following results – 8.95, 9.10, 9.64, 9.83, 10.45 and 11.15 μm, or 897, 957, 1017, 1037, 1099 and 1117 wave numbers (cm^{-1}).

Another important parameter measured by the DNES method was the average energy ($\Delta E_{H\ldots O}$) of hydrogen H…O-bonds between individual molecules H_2O to be at 0.1067 ± 0.0011 eV. When the water temperature is changed, the average energy of the hydrogen H...O-bonds is changed. There is a restructuring of energies between H_2O molecules with a statistically reliable increase of local maximums in spectra.

It was observed that in the hot mineral waters the local maximums in the IR-spectrum are more manifested compared to the local maximums obtained in the same water at a lower temperature. The difference in the local maximums from +20 to +80°C at each +10°C according to Students' t-criterion – $p < 0.05$. Such a character of the IR- and DNES-spectrum, and distribution of local peaks may prove that hot mineral alkaline water is preferable for origin and maintenance of life than other types of water analyzed by these methods. These data indicate that the origination of life and living matter depends on the structure and physical -chemical properties of water. The most closed to each other were IR-peaks of alkaline mineral

water interacting with $CaCO_3$ and then sea water..

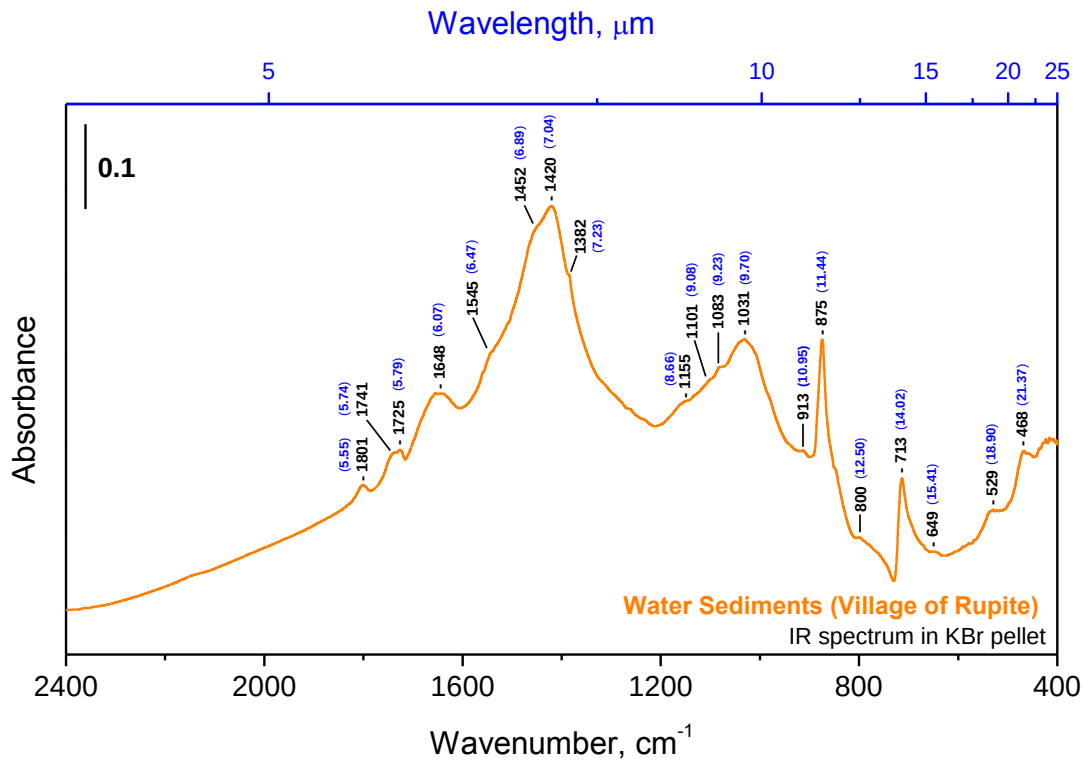

Figure 4. The IR-spectrum of water from Rupite village (Bulgaria).

In connection with these data are important the following reactions participating with $CaCO_3$ in aqueous solutions:

(1) $CO_2 + 4H_2S + O_2 = CH_2O + 4S + 3H_2O$

(2) $CaCO_3 + H_2O + CO_2 = Ca(HCO_3)_2$

(3) $CO_2 + OH^- = HCO_3^-$

(4) $2HCO_3^- + Ca^{2+} = CaCO_3 + CO_2 + H_2O$

The equation (1) shows how some chemosynthetic bacteria use energy from the oxidation of H_2S and CO_2 to S and formaldehyde (CH_2O). The equation (2) is related to one of the most common processes in nature: in the presence of H_2O and CO_2, $CaCO_3$ transforms into $Ca(HCO_3)_2$. In the presence of hydroxyl OH^- ions CO_2 transforms into HCO_3^- (equation (3)). Equation (4) is valid for the process of formation of the stromatolites – the dolomite layered acretionary structures formed in shallow sea water by colonies of cyanobacteria.

In 2010 D. Ward described fossilized stromatolites in the Glacier National Park (USA) (Ward, 2010). Stromatolites aged 3.5 billion years had lived in warm and hot water in zones of volcanic activity, which could be heated by magma. It was previously thought that the first living forms evolved in hot geysers (Pons *et al.*, 2011). The water in geysers is rich in carbonates, while the temperature is ranged from +100 to +150 ℃. In 2011 a team of Japanese scientists under the leadership of T. Sugawara showed that life originated in warm or, more likely, hot water (Kurihara *et al.*, 2011). From aqueous solution of organic

molecules, DNA and synthetic enzymes were created a proto cells. For this the initial solution was heated to a temperature close to water's boiling point +95 ℃. Then its temperature was lowered to +65 ℃ with formation of proto cells with primitive membrane. This laboratory experiment is an excellent confirmation of the possibility that life originated in hot water.

The above mentioned data can predict a possible way of transition from synthesis of small organic molecules under high temperatures to more complex organic molecules as proteins. There are the reactions of condensation-dehydration of amino acids and nucleotides in separate blocks of peptides occur under alkaline conditions, with pH = 9–11. The important factor in the reaction of condensation of two amino acid molecules is allocation of H_2O molecule when peptide chain is formed (Miller, 1968). As reaction of amino acid polycondensation is accompanied by dehydratation, the H_2O removal from reactional mixture speeds up the reaction rates. This testifies that formation of organic forms may occur nearby active volcanoes, because at early periods of geological history volcanic activity occurred more actively than during subsequent geological times. However, dehydratation accompanies not only amino acid polymerization, but also association of other blocks into larger organic molecules, and also polymerization of nucleotides into nucleic acids. Such association is connected with the reaction of condensation, at which from one block removes H^+, and from another – hydroxyl group with formation of H_2O molecule.

The possibility of existence of condensation-dehydration reactions under conditions of primary hydrosphere was proven by M. Calvin (Calvin, 1969). From most chemical substances hydrocyanic acid (HCN) and its derivatives – cyanoamid (CH_2N_2) and di-cyanoamid ($HN(CN)_2$) possess dehydration ability and the ability to catalyze the process of linkage of H_2O from primary hydrosphere (Mathews & Moser, 1968). The presence of HCN in primary hydrosphere was proven by Miller's early experiments. Chemical reactions with HCN and its derivatives are complex with chemical point of view; in the presence of HCN, CH_2N_2 and $HN(CN)_2$ the condensation of separate blocks of amino acids accompanied by dehydration, can proceed at normal temperatures in strongly diluted H_2O-solutions. These reactions result in synthesis from separate smaller molecules larger organic molecules of polymers, e.g. proteins, polycarboxydrates, lipids, and ribonucleic acids. Furthermore, polycondensation reactions catalyzed by HCN, and its derivatives depend on acidity of water solutions in which they proceed (Abelson, 1966). In acid aqueous solutions with pH = 4–6 these reactions do not occur, whereas alkaline conditions with pH = 9–10 promote their course. There has not been unequivocal opinion, whether primary water was alkaline, but it is probable, that such pH value possessed mineral waters adjoining with basalts, i.e. these reactions could occur at the contact of water with basalt rocks.

It may be supposed that primary water might contain more deuterium at early stages of life evolution, and deuterium was distributed non-uniformly in hydrosphere and atmosphere (Ignatov & Mosin, 2012). The reason of this is that the primary atmosphere of the Earth was reductive, without O_2–O_3 layer protecting the Earth surface from rigid short-wave solar radiation carrying huge energy. This simplifies radiation to freely pass through O_2-free atmosphere and reaching hydrosphere, may be the cause of further radiolysis and photolysis of water. Energy of radiation, volcanic geothermal processes on a hot Earth surface and electric discharges in atmosphere, could lead to the accumulation of deuterium in hydrosphere in the form of H^2HO that evaporates more slowly then H_2O, but condenses faster. This fact may make inprint on thermostability of deuterated macromolecules as the covalent bonds formed with ^2H are more stronger than those ones formed with hydrogen.

4. Conclusions

The experimental data indicate that the phenomenon of cellular adaptation to $^{2}H_2O$ and origination of life have a complex multifactorial nature. These data have proved that life originated in hot mineral alkaline water. The evidence indicates that the emergence of life depends on the physical-chemical properties of water (temperature, pH value) and its composition. Mineral water interacting with $CaCO_3$ is closest to these conditions. Next in the line with regard to quality are sea and mountain water. For chemical reaction of dehydration-condensation to occur in hot mineral water, water is required to be alkaline in the pH range 9–11. The content of deuterium in hot mineral water may be increased due to the physical-chemical processes of the deuterium accumulation. These are solar radiation, causing radiolysis and photolysis of water, geothermal activity and electrical discharges in the atmosphere devoid of the protective ozone layer. We had a perspective selection of heterotrophic bacteria for our research as they are the microorganisms located on the lower stage of evolutionary development, and quickly adapt to changing environmental factors.

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