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Studying the Process of Formation of Precambrian Period Limestone Fossils of Stromatolites in Hot Mineral Water Interacting with Caco₃

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Abstract
We studied the conditions of formations of stromatolites in hot mineral water. Studying these formations are very important and interesting as stromatolites store information about nascent life on earth and organic part of the first living organisms - numerous colonies of cyanobacteria, blue-green algae and bacteria, encountered in strata of limestone and dolomite in the crater of extinct volcanoes and thermal sources. For this aim were performed experiments with hot mineral and seawater from Bulgaria using IR and DNES spectroscopy. There were discussed the reactions of condensation and dehydration occurring in alkaline aqueous solutions at $65–95$ °C and pH = $9–10$, resulting in synthesis from separate molecules larger organic molecules as polymers and short polipeptides. It was shown that hot alkaline mineral water with temperature from $+65$ °C to $+95$ °C and pH value from 9 to 11 is more suitable for the origination of life and living matter than other analyzed water samples. The pH value of seawater on contrary is limited to the range of 7,5 to 8,4 units. In hot mineral waters the local maximums in IR spectra are more manifested compared to the local maximums obtained in IR-spectra of the same water at a lower temperature. The difference in the local maximums from $+20$ °C to $+95$ °C at each $+5$ °C according to Student t-criterion – p < 0,05. These data indicate that the origination of first organic forms of life depends on the structure and physical chemical properties of water, as well as its temperature and pH value. Also it was demonstrated the role of limestone and silica in the formation of the first organic life forms.
Keywords: stromatolites, hot mineral water, origin of life, IR, DNES.

Introduction

Previous biological experiments with D_2O and structural-conformational studies with deuterated macromolecules, performed by us, enable to modeling conditions under which the first living forms of life might be evolved [1–3]. The content of deuterium in hot mineral water may be increased due to the physical chemical processes of the deuterium accumulation. It can be presumed that primary water might contain more deuterium at early stages of evolution of first living structures, and deuterium was distributed non-uniformly in the hydrosphere and atmosphere [4]. The primary reductive atmosphere of the Earth consisted basically of gas mixture CO, H_2, N_2, NH_3, CH_4, lacked O_2–O_3 layer protecting the Earth surface from rigid short-wave solar radiation carrying huge energy capable to cause radiolysis and photolysis of water. The processes accompanying accumulation of deuterium in the hydrosphere are solar radiation, volcanic geothermal processes and electric discharges in the atmosphere. These natural processes could lead to the enrichment of the hydrosphere by deuterium in the form of HDO which evaporates more slowly then H_2O, and condenses faster. If this is true, this is a significant fact regarding thermal stability of deuterated macromolecules in the preservation of life under thermal conditions, because chemical bonds with participation of deuterium are stronger than those ones formed of hydrogen.

The natural prevalence of deuterium makes up approximately 0,015–0,020 atom %, and depends strongly on the uniformity of substance and the total amount of matter formed in the course of early Galaxy evolution [5]. The average ratio of $D/^1H$ in nature makes up approximately 1:5700. In natural waters, the deuterium is distributed irregularly: from 0,02–0,03 mol. % for river water and sea water, to 0,015 mol. % for water of Antarctic ice – the most purified from deuterium natural water containing in 1,5 times less deuterium than that of seawater.

The constant sources of deuterium are explosions of nova stars and thermonuclear processes frequently occurring inside the stars. Probably, it could explain a known fact, why the amount of deuterium is slightly increased during the global changes of climate in warming conditions. The gravitational field of the Earth is insufficiently strong for the retaining of lighter hydrogen, and our planet is gradually losing hydrogen as a result of its dissociation into interplanetary space. Hydrogen evaporates faster than heavy deuterium, which can be collected by the hydrosphere. Therefore, as a result of this natural process of fractionation of H/D isotopes throughout the process of Earth evolution there should be an accumulation of deuterium in the hydrosphere and surface waters, while in the atmosphere and in water vapour deuterium content tends to be low. Thus, on the planet there occurs a natural process of separation of H and D isotopes, playing an essential role in the maintenance of life on the planet.

The second point regards the influence of temperature on the biochemical processes in living matter. Our recent studies have shown that the most favorable for the origin of life and living matter seem to be hot alkaline mineral waters interacting with $CaCO_3$ [6, 7]. According to the law for conservation of energy 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. According to J. Szostak, the accumulation of organic compounds in small isolated lakes is more possible compared to the ocean [8]. It is most likely that life originated near a hydrothermal vent: an underwater spout of hot water. Geothermal activity gives more opportunities for the origination of life. In 2009 A. Mulkidjanian and M. Galperin demonstrate that the cell cytoplasm contains potassium, zinc, manganese and phosphate ions, which are not particularly widespread in the sea aquatorium [9]. J. Trevors and G. Pollack proposed in 2005 that the first cells on the Earth assembled in a hydrogel environment [10]. Gel environments are capable of retaining water, oily hydrocarbons, solutes, and gas bubbles, and are capable of carrying out many functions, even in the absence of a membrane. Hydrocarbons are an organic compounds consisting entirely of hydrogen and carbon. The previous data showed that the origination of living matter most probably occurred in hot alkaline mineral water containing $CaCO_3$ at t = +75-(+95) °C and pH = 9-11 (Ignatov, 2012). This might occur in ponds and hydrothermal vents in seawater or hot mineral water. This might occurred in ponds and hydrothermal vents in seawater or hot mineral water. An indisputable proof of this is the presence of stromatolites fossils. They lived in warm and hot water in zones of volcanic activity, which could be heated by magma and seem to be more stable than other first sea organisms [11].

The purpose of the research was studying the process of formation of stromatolites in hot mineral water for possible processes for origin of life and living matter in hot mineral water. Within the frames of the research the various samples of water from Bulgaria were studied.

Material and methods

Biological Objects
The objects for the study were the cactus juice of *Echinopsis pachanoi* and the Mediterranean jellyfish *Cotylorhiza tuberculata* (Chalkida, Greece, Aegean Sea).

Water Samples
The samples of water were taken from various water springs of Bulgaria:
1 – Mineral water (Rupite, Bulgaria);
2 – Seawater (Varna resort, Bulgaria);
3 – Mountain water (Teteven, Bulgaria);
5 – Deionized water (the control).

IR-Spectroscopy
IR-spectra of water samples were registered on Brucker Vertex ("Brucker", Germany) Fourier-IR spectrometer (spectral range: average IR – 370–4000 cm^{-1}; visible – 2500–8000 cm^{-1}; permission – 0.5 cm^{-1}; accuracy of wave number – 0.1 cm^{-1} on 2000 cm^{-1}) and on Thermo Nicolet Avatar 360 Fourier-transform IR (M. Chakarova).

DNES-Spectral Analysis
The research was made with the method of differential non-equilibrium spectrum (DNES). The device measures the angle of evaporation of water drops from 72 º to 0 º. As the main estimation criterion was used the average energy ($\Delta E_{H...O}$) of hydrogen O...H-bonds between H_2O molecules in water's samples. The spectra of water were measured in the range of energy of hydrogen bonds 0,08–0,1387 eV with using a specially designed computer program.

High-Frequency Coronal Electric Discharge Experiments
A device for high-frequency coronal electric discharge was used in this study, constructed by I. Ignatov and Ch. Stoyanov. The frequency of the applied saw-tooth electric voltage was 15 kHz, and the electric voltage – 15 кV. The electric discharge was obtained using a transparent firm polymer electrode on which a liquid sample of water (2–3 mm) was placed. The spectral range of the photons released upon electric discharge was from $\lambda = 400$ to $\lambda = 490$ nm and from $\lambda = 560$ to $\lambda = 700$ nm.

Results and discussion

Studying Various Water Samples on the Feasibility for Origin of Life
We have carried out the research of various samples of mineral water obtained from mineral springs and seawater from Bulgaria (Fig. 1, curves *1–5*). For this aim we employed the IR-spectrometry and DNES method relative to the control – deionized water.

For calculation of the function *f(E)* represented the energy spectrum of water, the experimental dependence between the wetting angle (θ) and the energy of hydrogen bonds (E) is established:

$$f(E) = \frac{14{,}33 f(\theta)}{[1-(1+bE)^2]^2} , \qquad (1)$$

where *b* = 14,33 eV^{-1}
The relation between the wetting angle (*θ*) and the energy (*E*) of the hydrogen bonds between H_2O molecules is calculated by the formula:

$$\theta = arcos \ (\text{-}1 - 14{,}33E) \qquad (2)$$

Cactus juice was also investigated by the DNES method (Fig. 1, *curve 1*). The cactus was selected as a model system because this plant contains approximately 90 % of water. The closest to the spectrum of cactus juice was the spectrum of mineral water contacting with Ca^{2+} and HCO_3^- ions (Fig. 1, *curve 2*). DNES-spectra of cactus juice and mineral water have magnitudes of local maximums (*E*, eV) at −0,1112; -0,1187; -0,1262; -0,1287 and −0,1387 eV. The similar local maximums in the DNES-spectrum between the cactus juice and seawater were detected at -0,1362 eV. The DNES-spectrum of the control sample of deionized water (Fig. 1, *curve 5*) was substantially different from DNES-spectra of seawater and mineral water.
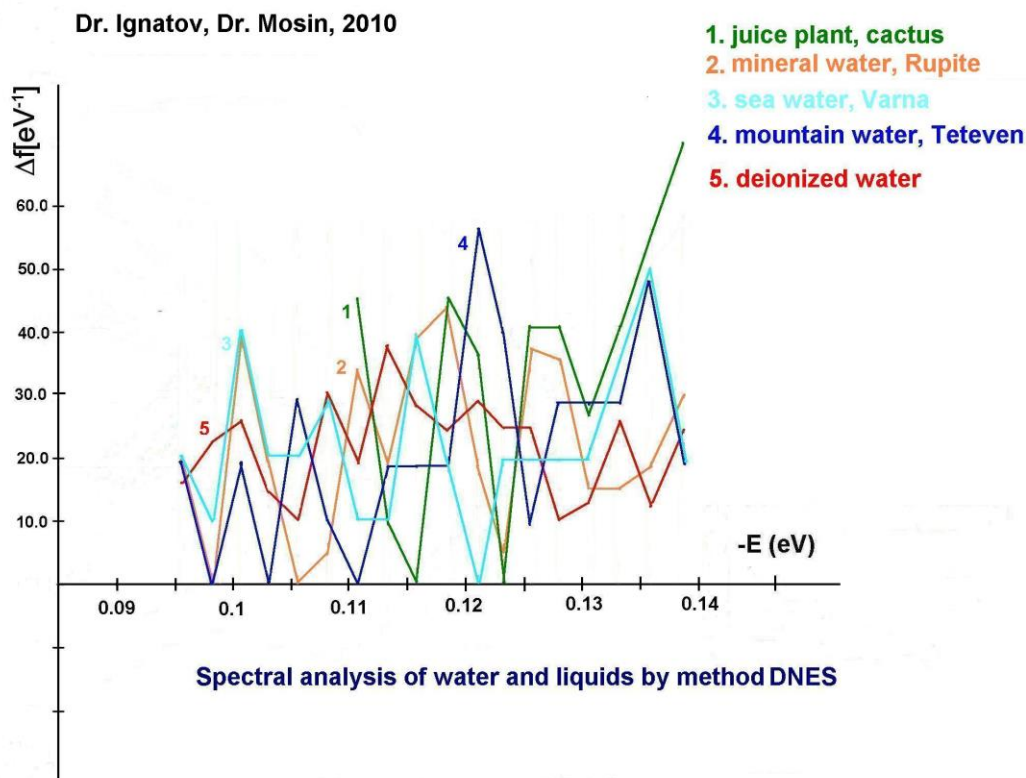
Fig. 1. DNES-spectra of water samples of various origin: 1 – the cactus juice; 2 – mineral water from Rupite village (Bulgaria); 3 – seawater (Varna, Bulgaria); 4 – mountain water (Teteven, Bulgaria); 5 – deionized water (the control)

As it was shown from these experimental data, the closest to the IR-spectrum of the cactus juice was mineral water from Rupite Village (Bulgaria), which DNES and IR spectrum is shown in Fig. 2 and Fig. 3 (Thermo Nicolet Avatar 360 Fourier-transform IR). IR-spectra of cactus juice and mineral water containing HCO_3^- (1320−1488 mg/l), Ca^{2+} (29−36 mg/l), pH (6,85−7,19), have local maximums at λ = 8,95; 9,67; 9,81; 10,47 and 11,12 μm (Fourier-IR spectrometer Brucker Vertex). Common local maximums in the IR-spectrum between the cactus juice and seawater are detected at λ = 9,10 μm. The local maximums obtained with the IR method at λ = 9,81 μm (*k* = 1019 cm⁻¹) and λ = 8,95 μm (*k* = 1117 cm⁻¹) (Thermo Nicolet Avatar 360 Fourier-transform IR) are located on the spectral curve of the local maximum at λ = 9,7 μm (*k* = 1031 cm⁻¹) (Fig. 3). With the DNES method were obtained the following results – (wave length λ, μm) 8,95; 9,10; 9,64; 9,83; 10,45 and 11,15 μm, or (wave numbers, *k*, cm⁻¹) 897; 957; 1017; 1037; 1099 and 1117 cm⁻¹ (Table).
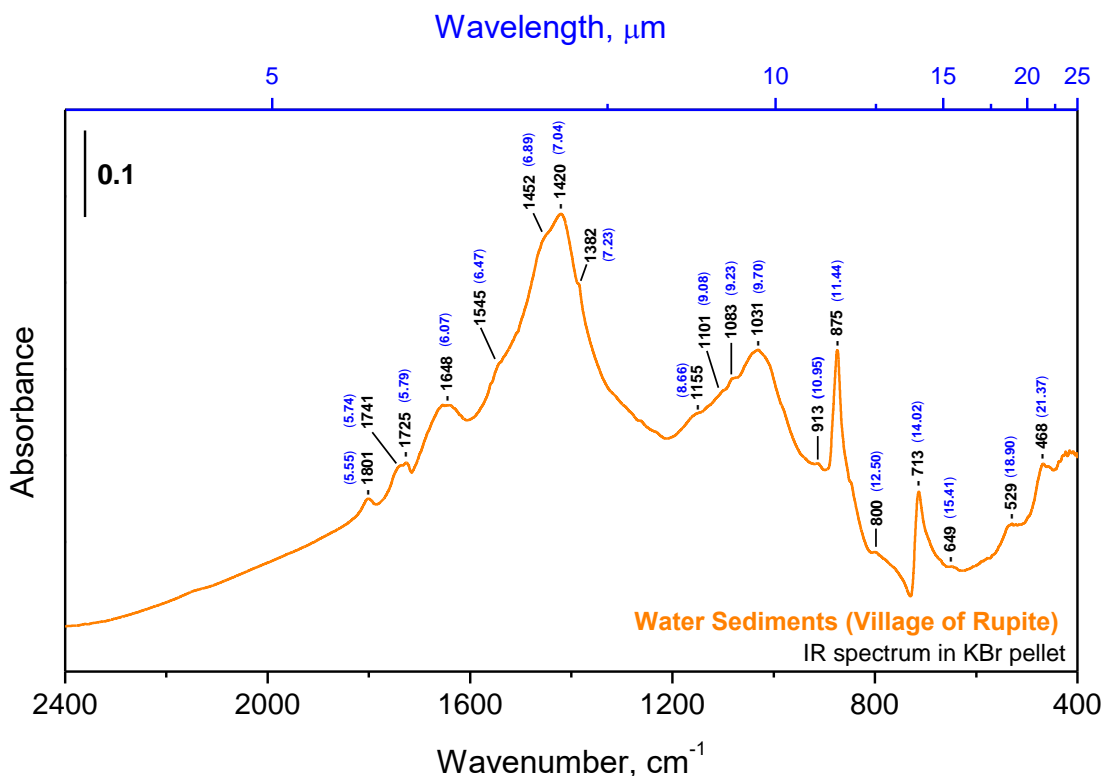
Fig. 2. IR-spectrum of water sediments obtained from Rupite Village (Bulgaria)

Table 1. Characteristics of spectra of water of various origin obtained by DNES-method*

-*E*, eV			λ, µm	k, cm⁻¹
Cactus juice	Mineral water from Rupite Village (Bulgaria)	Seawater		
0,1112	0,1112	–	11,15	897
0,1187	0,1187	–	10,45	957
0,1262	0,1262	–	9,83	1017
0,1287	0,1287	–	9,64	1037
0,1362	–	0,1362	9,10	1099
0,1387	0,1387	–	8,95	1117

Notes:

*The function of the distribution of energies Δf was measured in reciprocal electron volts (eV⁻¹). It is shown at which values of the spectrum -*E* (eV) were observed the biggest local maximums of this function; λ – wave length; *k* – wave number.

The results with the Mediterranean jellyfish *Cotylorhiza tuberculata* indicated that the jellyfish has local maximums in IR-spectra at λ = 8,98 µm and λ = 10,18 µm (Fig. 3). Before measurements the jellyfish was kept in seawater for several days. On comparison the seawater has a local maximum at λ = 8,93 µm in IR-spectra. These results were obtained with Thermo Nicolet Avatar 360 Fourier-transform IR. With DNES method the local maximums in spectra for jellyfish are detected at λ = 8,95 and 10,21 µm, and for seawater – at λ = 9,10 µm. A differential spectrum was recorded between jellyfish and seawater by using the Thermo Nicolet Avatar 360 Fourier-transform IR method. In the IR spectrum of jellyfish are observed more pronouncedly expressed local maximums, detected by Thermo Nicolet Avatar 360 Fourier-transform IR and DNES method. The measurements demonstrate that two common local maximums are observed in IR spectra of jellyfish and seawater. These maximums are not observed in the IR spectrum of cactus juice and

mineral water from Rupite (Bulgaria). Jellyfish contains approximately 97 (w/w) % of water and is more unstable living organism compared to those ones formed stromatolites. The explanation for this is the smaller concentration of salts and, therefore, the smaller number of local maximums in the IR spectrum in relation to seawater.
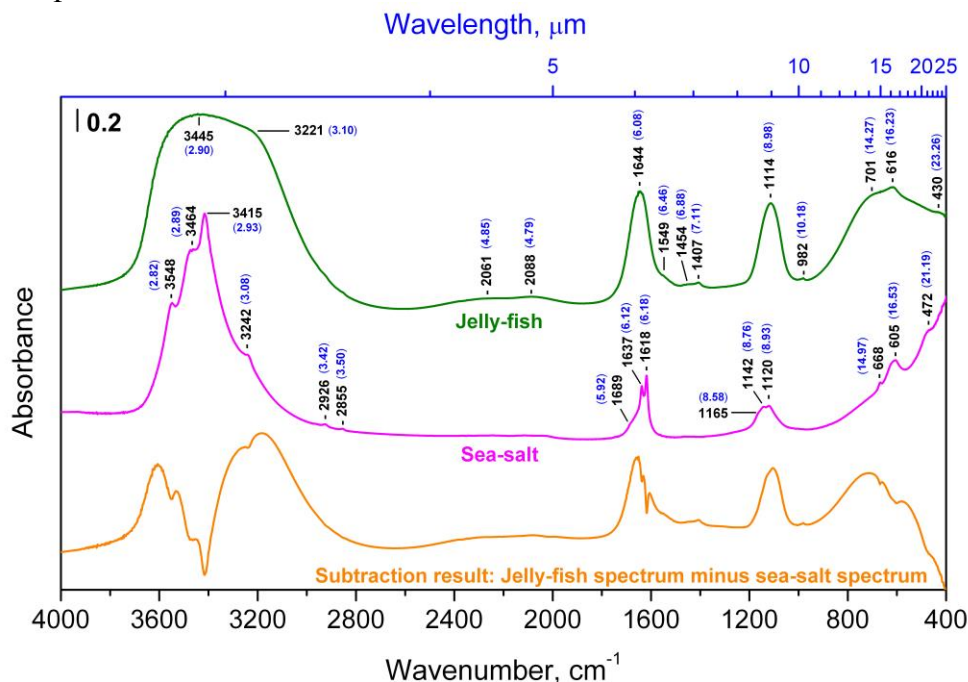
Fig. 3. IR spectrum of seawater obtained from Varna (Bulgaria) and jellyfish *Cotylorhiza tuberculata*, Chalkida (Greece), Aegean Sea

Such a character of IR- and DNES-spectra and distribution of local maximums may prove that hot mineral alkaline water is preferable for origin and maintenance of life compared to other types of water anal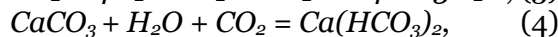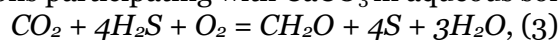yzed by these methods. Thus, in hot mineral waters the local maximums in the IR-spectrum are more manifested compared to the local maximums obtained in IR-spectrum of the same water at a lower temperature. The difference in the local maximums from t = +20 °C to t = +95 °C at each 5 °C according to the Student *t*-criterion makes up p < 0,05. These data indicate that the origination of life and living matter depends on the structure and physical chemical properties of water, as well as its temperature and pH value. The most closed to the IR- and DNES-spectrum of water, which contains bicarbonates and calcium ions typical for the formation of stromatolites is the IR-spectrum of cactus juice. For this reason cactus juice was applied as a model system. The most closed to local maximums in IR-spectrum of cactus juice are local maximums in IR-spectra of alkaline mineral water interacting with $CaCO_3$ and then seawater. In connection with these data the following reactions participating with $CaCO_3$ in aqueous solutions are important:

$$CO_2 + 4H_2S + O_2 = CH_2O + 4S + 3H_2O, \quad (3)$$
$$CaCO_3 + H_2O + CO_2 = Ca(HCO_3)_2, \quad (4)$$
$$CO_2 + OH^- = HCO_3^- \quad (5)$$
$$2HCO_3^- + Ca^{2+} = CaCO_3 + CO_2 + H_2O \quad (6)$$

The equation (3) shows how some chemosynthetic bacteria use energy from the oxidation of H_2S and CO_2 to S and formaldehyde (CH_2O). The equation (4) 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 (5)). Equation (6) is valid for the process of formation of the stromatolites – the limestone and dolomite layered acretionary structures (thin-reef columns or mounds of various shapes) formed in the presense of $CaCO_3$ in shallow seawater or in areas with a periodic change of fresh and salt water by colonies of cyanobacteria. Cyanobacteria release free oxygen into water, while chemically binding hydrogen and carbon. Cyanobacteria are remarkable in that that they are able to use atmospheric nitrogen and convert it into organic forms of nitrogen. During photosynthesis, they may use carbon dioxide

as the sole carbon source. In contrast to photosynthetic bacteria, cyanobacteria isolated molecular oxygen in the process of photosynthesis. During the past 3 billion years before the Cambrian they were basic, along with photochemical reactions in the upper atmosphere, free source of oxygen in the Earth's atmosphere.

Stromatolites mostly consist of calcium carbonate because the carbonate type of sedimentation in the sea is most common, but under other hydro-chemical conditions were formed phosphate, silica, glandular stromatolites. The multi-layer coloring of stromatolites can be varried, as the inhabitants of the lower layer can rise in dark time of the top and vice versa. Bacteria glide up and down at a speed of up to 2 cm per hour.

Formation of stromatolites can be represented as follows:

1. Capture of sediment particles;
2. Biomineralization of organic tissue;
3. The precipitation of minerals on the surface and the sludge organisms.

In 2010 D. Ward described fossilized stromatolites in the Glacier National Park (USA) [13]. Stromatolites aged 3,5 billion years had lived in warm and hot water in zones of volcanic activity, which could be heated by magma. This suggests that the first living forms evidently evolved in hot geysers [14].

In nature, there are places with a different composition and origin of water being under the same external conditions, but having different temperatures from 0°C to 10 °C and above 12,5 °C up to 100 ° C in geothermal sources. For example, in the town of Zlatna Panega, Teteven region, Bulgaria in the lake formed by the water spring with the river Vit is only a few kilometers away, the average temperature is 21 °C. The average river temperature is around 15 °C. The photos in Fig. 4 and 5 show the obvious difference between the fauna of karst water sources and rivers. This is proof of optimally favorable active living algae places with the same external conditions. The only difference is in the physical composition of water. The mineral water which interacts with calcium carbonate, as well as sea water is alkaline.

Fig. 4. Karst and mineral springs, vegetation of Zlatna Panega, Teteven region, Bulgaria. Photo Alexander Ignatov

Fig. 5. The river Vit, Teteven region, vegetation, 3 km from the source of Zlatna Panega, Bulgaria. Photo Alexander Ignatov

With calcium-silicate rocks are associated the development of the oldest forms of life on Earth. The earliest evidence for the existence of living organisms with calcareous structures layered on Earth date back 3,5 billion years. These ancient limestone fossils (dolomites) of Precambrian period – stromatolites, which built a skeleton of limestone and silica (SiO_2) (Fig. 6–8).
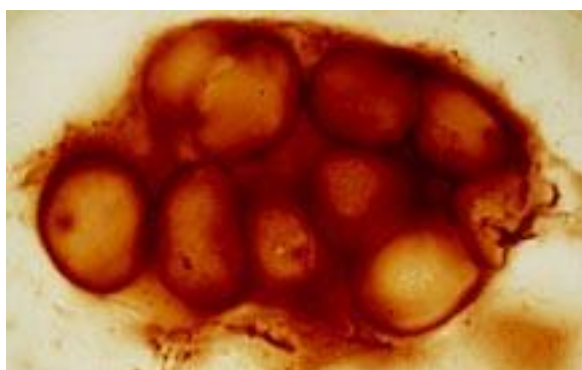
Fig. 6. Colonial (cocci) form of the late Proterozoic of Australia (850 million years ago)

Fig. 7. Filamentous forms of *Palaeo Lyngbya* from the same location

Fig. 8. Stromatolites (Australia)

Stromatolites consisting of calcium carbonate and sand-clay material, formed on the bottom of shallow ponds back in Archean in the most ancient geological era of the earth – 2,5–3,5 billion years ago. Studying these formations are very important and interesting as stromatolites store information about nascent life on earth and organic part of the first living organisms – numerous colonies of cyanobacteria, blue-green algae and bacteria, encountered in strata of limestone and dolomite in the crater of extinct volcanoes and thermal sources. As an example, the photograph in Fig. 9 shows the numerous colonies of blue-green algae in the Rupite, Bulgaria. The place is located in the crater of an ancient extinct volcano. At a temperature of +75 °C plant life is growing in full force.

Fig. 9. Algae in mineral water, +75 °C, Rupite, Vanga place, Bulgaria. Photo Alexander Ignatov

At the beginning of the evolution of life there has been an increasing volcanic activity on Earth, even at the bottom of the primary ocean. At that same time on the Earth there had been more silicon and silicon compounds, and they reacted wit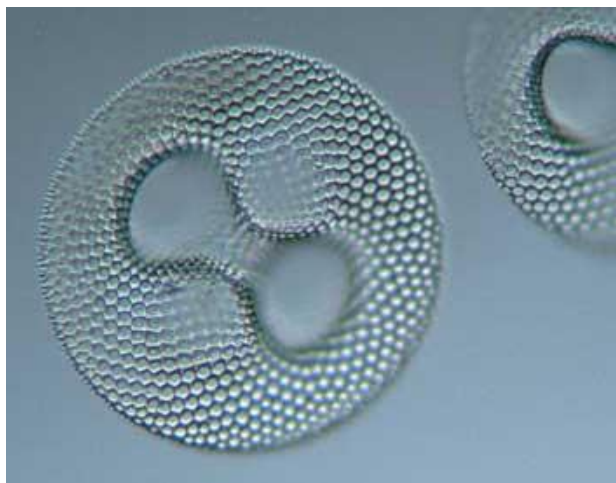h water. For this reason, they were absorbed in the early living organisms – diatoms and radiolarians. These planktonic forms of microorganisms are found in the upper layers of the sea water along with other organisms with calcareous (foraminifera) and chitin shells. The size of the skeleton of silicon organisms reaches several tens of micrometers (Fig. 10). After the death these organisms sank to the bottom of the sea, and their substance was chemically reacted with seawater. Calcium carbonate and chitin of foraminifera plankton organisms was dissolved in water better than silica of diatoms and radiolarians, forming silica precipitations. The siliceous shales with siliceous deposits of microorganisms were formed in the Phanerozoic era of deep ocean basins at depths of about 2-3 km. The heyday of the microorganisms with silica skeleton could lead to such an accumulation of silicon in the ocean, so that at the sea bottom could be formed the silica gel. Then the silica could

crystallize around scattered in the limestone crystallization centers, gradually replacing the molecules of calcium carbonate. Later, the organisms - foraminifera that have calcareous shells began to absorb calcium from the limestone rocks.

Fig. 10: Organism with a silicon skeleton

The most common mineral of the earth's crust quartz SiO_2, apparently played a large role in the origin of life. The quartz crystal has a tetrahedral structure, which is composed of chain silicate structures (Fig. 11–17). The uniqueness of quartz is that its crystals are optically active, e.g. they can affect the polarized light. But quartz crystals not merely affect the light passing through them, they also exert optically active properties on the surface of the crystal. Consequently, on the surface of *D*- and *L*- enantiomeric quartz crystals it was theoretically possible the selective absorption of *L*- and *D*-isomers of organic compounds.
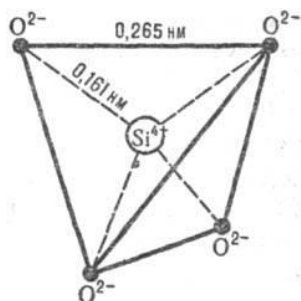
Fig. 11. Elementary correct silicon-oxygen tetrahedron SiO_4^{4-}
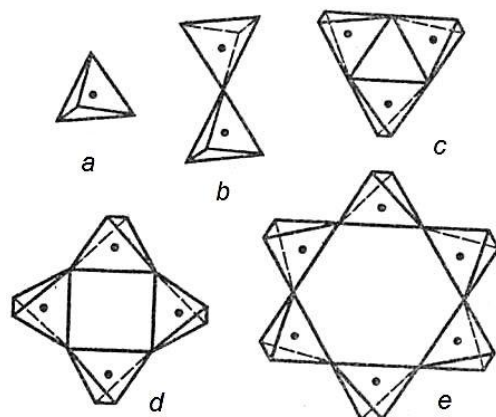
Fig. 12. The simplest types of silicon anionic groups: $a - SiO_4$; $b - Si_2O_7$; $c - Si_3O_9$; $d - Si_4O_{12}$; $e - Si_6O_{18}$
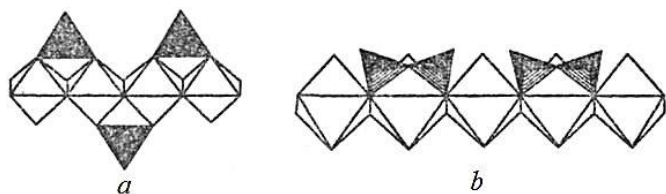
Fig. 13. Elementary-silicon-oxygen units of ortho-group SiO_4^{4-} in the structure of Mg-pyroxene enstatite (*a*) and diorto-group $Si_2O_7^{6-}$ in Ca-piroksenoide in wollastonite (*b*)
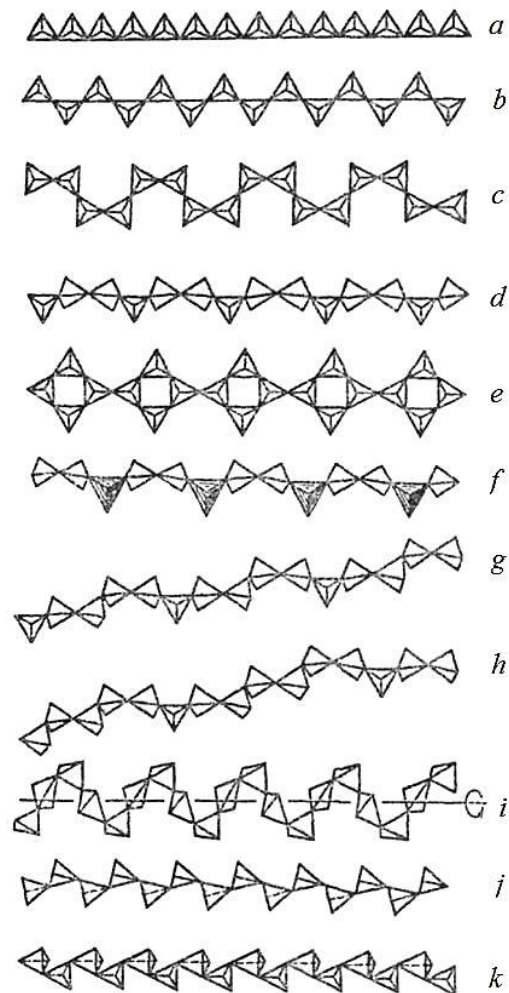
Fig. 14. The most important types of silicon-chain anionic groups: *a* – meta-germanate; *b* - pyroxene; *c* – batisite; *d* – wollastonite; *e* - vlasovite; *f* – melilite; *g* – rhodonite; *h* – piroksmangite; *i* – metaphosphate; *j* – fluoro-barite; *k* – barite.
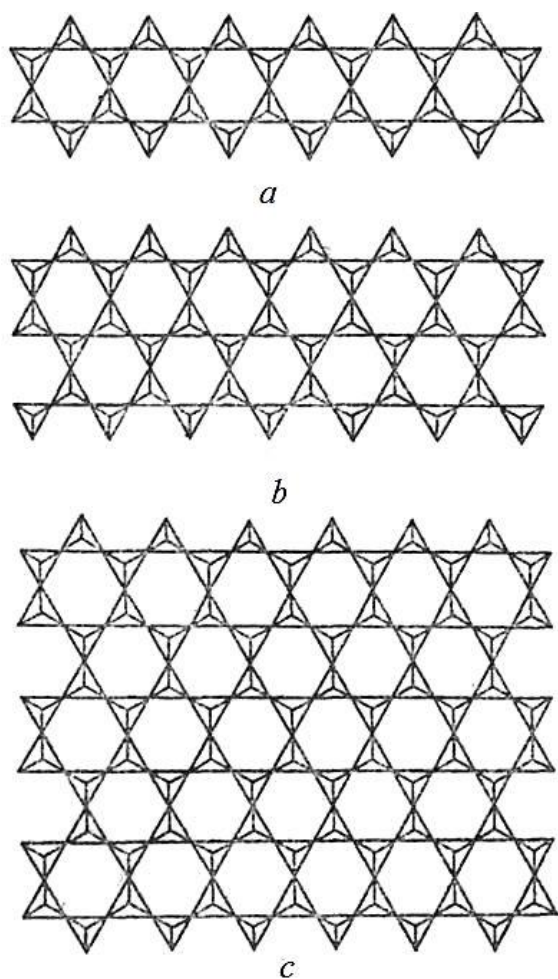
Fig. 15. Condensation of pyroxene silicon-oxygen anions in the belt double row amphibole (*a*), three-row amphibole (*b*), layered talc and anions close to them (*c*)
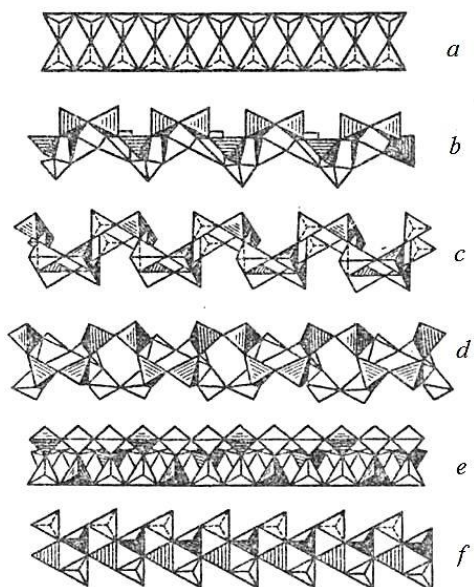
Fig. 16. The most important types of silicon band groups: *a* – sillimanite; *b* – epididymitis; *c* – orthoclase; *d* – narsarsukite; *e* – fenakite prismatic; *f* – evklazovaya inlaid.
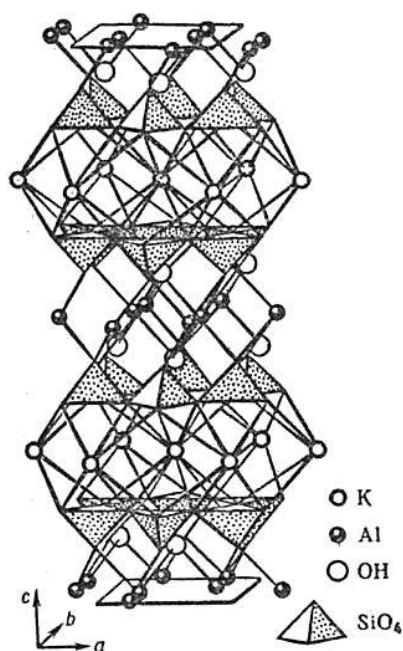
Fig. 17. A fragment (elementary packet) of the layered crystal structure of muscovite $KAl_2(AlSi_3O_{10}XOH)_2$, illustrating the alternation of aluminum-silicon-oxygen layers of polyhedral meshes with the large aluminum and potassium cations, reminiscent of the DNA chain

Another interesting property of quartz is that its structure resembles that of water. The discoverers of hydrogen bonds Bernal and Fowler in 1932, compared the structure of iquid water with the crystalline structure of quartz, and the associates of water were considered as tetramers $4H_2O$, in which four water molecules were joined in a compact tetrahedron with twelve internal hydrogen bonds. The result of the structure is a four-sided pyramid - the tetrahedron.

According to some researchers's opinions these hydrogen bonds can form tetramers of both right- and left-handed so as quartz crystals have right- and left-rotational crystalline forms (Fig. 18). As such each tetramer has four unused external hydrogen bonds, the tetramers can be connected to these external bonds to polymer chains, such as a DNA molecule. Since there are only four external bonds and internal – in 3 times more, this allows to tetramers in liquid water to bend, rotate, and even to break these loose by thermal vibrations the hydrogen bonds. According to this hypothesis, it causes the water flow.
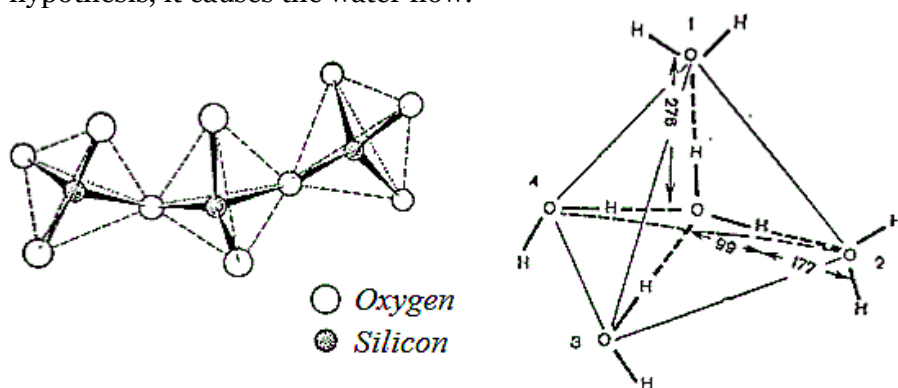
Fig. 18. The crystal structure of quartz (left) and the tetrahedral structure of water (right)

The estimated water structure could have been due to its rheological ancient bond with quartz and other silicon-oxygen minerals prevailing in the earth's crust, from the depths of which once there appeared water on the Earth. As a small crystal of salt makes the surrounding solution

to crystallize into the crystals like it, so quartz could initiate water molecules to line up into the tetrahedral structure, which is the most energetically favorable.

It is known that water in geysers is rich in carbonates, while the temperature is ranged from +100 °C to +150 °C. In 2011 a team of Japanese scientists under the leadership of T. Sugawara showed that life originated in warm or, more likely, in hot water [15]. From aqueous solution of organic molecules, DNA and synthetic enzymes were created proto cells. Under experimental conditions the initial solution was heated to a temperature close to the water's boiling point +95 °C. Then the temperature was lowered to +65 °C with formation of proto cells with primitive membrane. These experiments are excellent confirmation of the possibility that first organic forms of life originated in hot water.

The Reactions of Condensation-Dehydration in Hot Water

The prognosis was made to predict a possible transition from synthesis of small organic molecules under the temperatures +70–100 °C to more complex organic molecules as proteins. There are reactions of condensation-dehydration of amino acids into separate blocks of peptides that occur under t = +75-95 °C and alkaline conditions, with pH = 9–11. The important factor in reaction of condensation of two amino acid molecules into the dipeptide is allocation of H_2O molecule when a peptide chain is formed, as the reaction of polycondensation of amino acids is accompanied by dehydration, the H_2O removal from reaction mixture speeds up the reaction rates. This testifies that formation of early organic forms may have occured 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 small 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 a proton is removed, and from another – a hydroxyl group with the formation of H_2O molecule.

In 1969 the possibility of existence of condensation-dehydration reactions under conditions of primary hydrosphere was proven by M. Calvin [16]. From most chemical substances hydrocyanic acid (HCN) and its derivatives – cyanoamid (CH_2N_2) and dicyanoamid ($HN(CN)_2$) possess dehydration ability and the ability to catalyze the process of linkage of H_2O from primary hydrosphere [17]. The presence of HCN in primary hydrosphere was proven by S. Miller's early experiments [18]. Chemical reactions with HCN and its derivatives are complex with a 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 show the results of synthesis from separate smaller molecules to larger organic molecules of polymers, e.g. proteins, polycarboxydrates, lipids, and ribonucleic acids (Fig. 19). Furthermore, polycondensation reactions catalyzed by HCN and its derivatives depend on acidity of water solutions in which they proceed [19]. 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, that testifies our hypothesis.
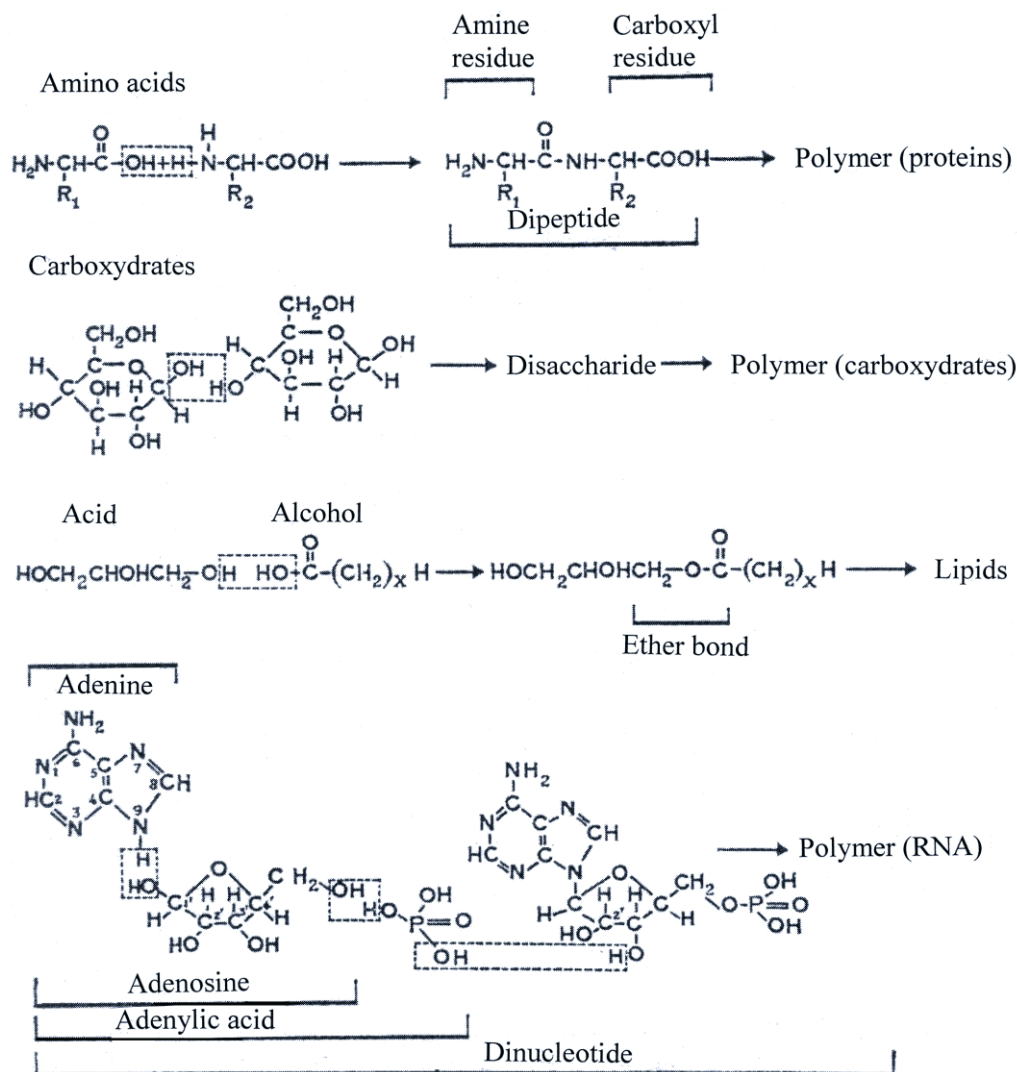
Fig. 19. Possible reactions of condensation and dehydration in alkaline conditions with pH = 9–10 catalyzed by HCN and its derivatives, resulting in synthesis from separate molecules larger organic molecules of polymers. The top three equations: condensation and the subsequent polymerization of amino acids in proteins; carbohydrates – in polycarboxydrates and acids and ethers – into lipids. The bottom equation – condensation of adenine with ribose and H_3PO_4, leading to formation of dinucleotide

It should be noted, that geothermal sources might be used for synthesis of various organic molecules. Thus, amino acids were detected in solutions of formaldehyde CH_2O with hydroxylamine NH_2OH, formaldehyde with hydrazine (N_2H_4) in water solutions with HCN, after heating of a reactionary mixture to +95 °C [20]. In model experiments the reaction products were polymerized into peptide chains that are the important stage towards inorganic synthesis of protein. In a reactionary mixture with a $HCN–NH_3$ solution in water were formed purines and pyrimidines (Fig. 20). In other experiments amino acid mixtures were subjected to influence of temperatures from +60 °C up to +170 °C with formation of short protein-like molecules resembling early evolutionary forms of proteins subsequently designated as thermal proteinoids. They consisted of 18 amino acids usually occurring in protein hydrolyzates. The synthesized proteinoids are similar to natural proteins on a number of other important properties, e. g. on linkage by nucleobases and ability to cause the reactions similar to those catalyzed by enzymes in living organisms as decarboxylation, amination, deamination, and oxidoreduction. Proteinoids are

capable to catalytically decompose glucose [21] and to have an effect similar to the action of α-melanocyte-stimulating hormone [22]. The best results on polycondensation were achieved with the mixes of amino acids containing aspartic and glutamic acids, which are essential amino acids occurring in all modern living organisms.
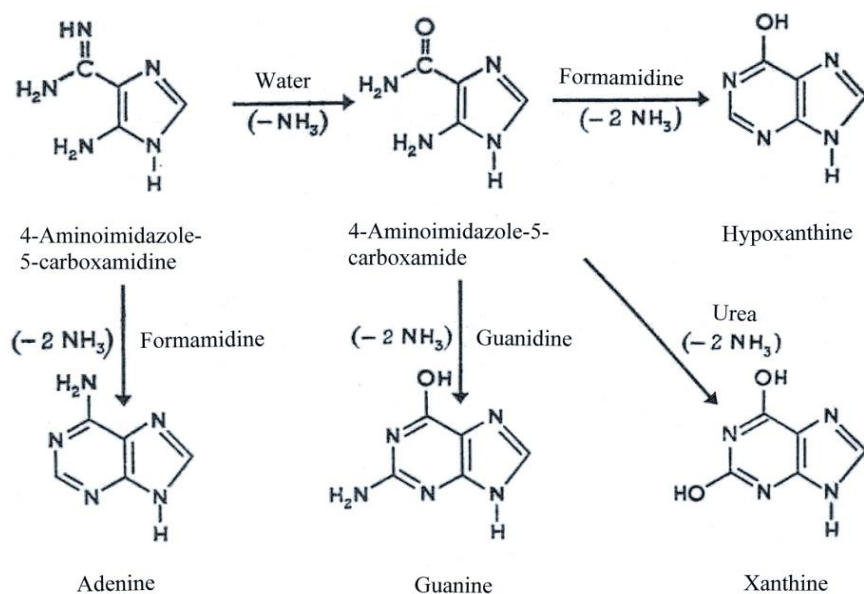
a)
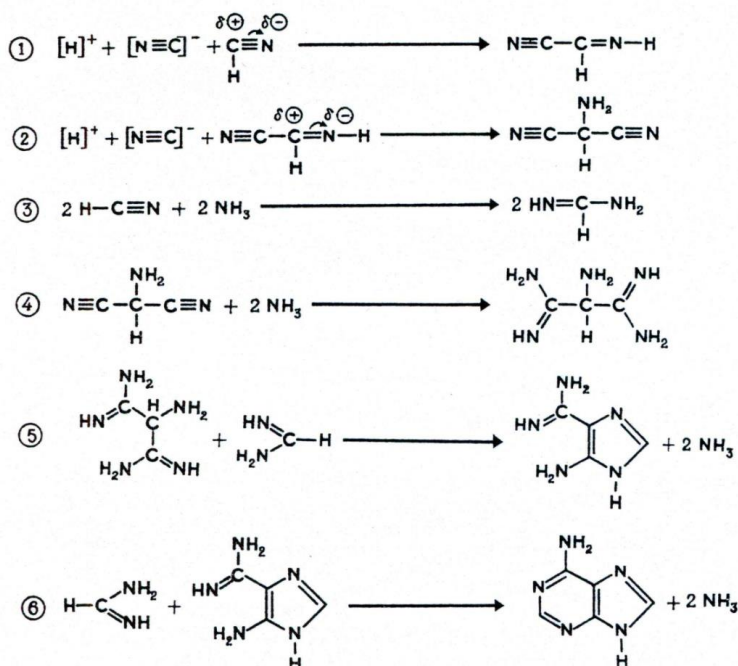
b)

Fig. 20. Prospective mechanisms of thermal (+95 °C) synthesis of purines in aqueous solutions: *a)* – synthesis of hypoxanthine, adenine, guanine and xanthine from 4-aminoimidazole-5-carboxamidine, 4-aminoimidazole-5-carboxamide, water, NH_3, formamidine and urea; *b)* – synthesis of adenine from NH_3 and HCN (total reaction: 5HCN = adenine)

Under certain conditions (temperature, pH) in hot mixture of thermal proteinoids in water solutions are formed elementary structures like proteinoid microspheres with diameter 5–10 μm [23]. The best results on polycondensation were achieved with the mixes of amino acids containing aspartic and glutamic acids, which are essential amino acids occurring in all modern living organisms. By morphological features the proteinoid microspheres with a diameter ~5–10 μm resemble cell membrane, which in certain conditions (pH = 4–5) may be double. The catalyst for their formation could serve sulfur and its derivatives which were found in ancient rocks in the form of grains of sulfides, as well as pyrite sands. Synthesis of protenoid microspheres from a mixture of thermal proteinoids is important because it provides material for the next stage of the evolution of life. This is the stage from disparate organic molecules to organized proteinoid molecules having organized structure and separated from the surrounding environment by the primitive membrane.

In further experiments was applied gas electric discharge analogous to S. Miller's experiments [24]. The first experiments on the modeling of non-equilibrium conditions with gas electric discharge simulating primary atmosphere and electrosynthesis of organic substances from anorganic ones under the energy of the electric field in a primary oxygen-free atmosphere were held in 1953 by S. Miller (USA) [24]. For this aim the mixture of water and gases consisted of hydrogen (H_2), methane (CH_4), ammonia (NH_3) and carbon monoxide (CO) was placed into a closed glass container being exposed by pulsating electrical spark discharges at the temperature of boiling water; oxygen was not allowed into the unit. After processing the reaction mixure by the electric discharge it was found that from the initial inorganic substance was synthesized organic compounds – aldehydes and amino acids. Experiments found that approximately ~10–15% of carbon was transferred into an organic form. However, about ~2% of carbon was detected in the amino acids, the most common of which was glycine. Initial analysis showed the presence in the reaction mixture obtained after the processing by spark electric discharge 5 amino acids. A more complete analysis carried out in 2008 [25], showed the formation by electrosynthesis in the reaction mixture 22 amino acids having from 5 to 20 carbon atoms in the molecule. Interestingly is that along with the amino acids in the reaction mixture after the treatment with electric spark discharges were detected trace amounts of nucleic acid precursors – nucleosides.

It should be noted that in the implementation of the gas discharge effect as well as in experiments of S. Miller are modeled extreme non-equilibrium conditions with gas electric discharge, resulting that in a thin layer of air gap with thickness ~100 μm are formed reactive radicals reacting with each other to form new compounds (electrosynthesis). Such extreme conditions are thought to have occurred in the primary oxygen-free atmosphere of the Earth, which supposedly consisted of a mixture of water and gases – H_2, CH_4, NH_3 and CO, subjected to spark electrical discharges (lightning) under the conditions of high solar (UV) and geothermal activity.

The analogous experiment was conducted by the authors under laboratory conditions. According to our previous experiments, the first living structures may have evolved in warm and hot mineral water with a high content of bicarbonate (HCO_3^-) anions, cations of alkali metals (Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}) and deuterium in the form of HDO [26]. There occurred gas electric discharge (lightning) in the primordial atmosphere close to the water surface. In the course of experiment was used the similar gas electric discharge on water drops placed on the electrode of the device for gas electric discharge formation. The similar composition and water temperature were modeled on the electrode of the gas discharge device made of hostafan, with electric voltage – 15 kV, electric impulse duration – 10 μs; electric current frequency – 15 kHz, wherein the air gap layer on the boundary with water sample was formed the electrical discharge, similar to plasma phenomena (lightning) and the electrostatic discharge on the surface of organic and inorganic samples of various kinds. Water drops were heated up to the boiling point in an electric field of high frequency and the electric discharge was applied, analogious as that in the primordial atmosphere. As a result, an organized structure with a size of 1,2–1,4 mm was formed in interelectrode space (Fig. 21). It was formed as a result of accretion of smaller elementary structures sized up 5–10 μm into the biggest structure having the size 1,2–1,4 mm and concentrated in the spase where the electric field is applied. It should be noted that no structure was organized in a control sample of water placed on the electrode. Before its placement on the electrode, the water was heated to boiling point and then cooled down. The structure organization increased with the increase of the duration of the gas electric discharge. Moreover, in experiments was observed the formation of

small structures and their further "adjoining" to the larger structure. The large structure was preserved the original size for some time in the absence of the electric discharge.

Fig. 21. The organized structure in water sample subjected to the temperature +100 ^{o}C in the electric field of high voltage and frequency (I. Ignatov, 2014). The material of the electrode – hostafan; the electric voltage – 15 kV, the electric impulse duration – 10 µs; the electric current frequency – 15 kHz.

This experiment shows that self-organization in water under certain external termal conditions may take place. In natural conditions water is heated up to +100 ^{o}C by the magma. The structure formed from heated water was evidently a result of self-organization. Living organisms are complex self-organizing systems. Thermodynamically they belong to the open systems because they constantly exchange substances and energy with the environment. The changes in the open systems are relatively stable in time. The stable correlation between components in an open system is called a dissipative structure. According to I. Prigozhin, the formation of dissipative structures and the elaboration to living cells is related to changes in entropy [27].

Taking into account these views it may be concluded that the initial stage of evolution, apparently, was connected with formation at high temperature of the mixtures of amino acids and nitrogenous substances – analogues of nucleic acids. Such synthesis is possible in aqueous solutions under thermal conditions in the presence of H_3PO_4. The next stage is polycondensation of amino acids into thermal proteinoids at temperatures 65–95 ^{o}C. After that in a mix of thermal proteinoids in hot water solutions were formed membrane like structures.

Our data are confirmed by experiments of T. Sugawara (Japan), who in 2011 created the membrane like proto cells from aqueous solution of organic molecules, DNA and synthetic enzymes under temperature close to the water boiling point +95 ^{o}C [28]. This data confirm the possibility that first organic forms of life originated in hot water.

Conclusion

The data obtained testify that origination of first organic forms of life depends on physical-chemical properties of water and external factors – temperature, pH, electric discharges and isotopic composition. Hot mineral alkaline water interacting with $CaCO_3$ is most closed to these conditions. Next in line with regard to its quality is seawater. For chemical reaction of dehydration-condensation to occure in hot mineral water, water is required to be alkaline with the pH ranged 9–11. In warm and hot mineral waters the local maximums in IR-spectra from 8 to 14 µm were more expressed in comparison with the local maximums measured in the same water samples with lower temperature. The most common minerals of the earth's crust quartz SiO_2 and limestone, apparently

played a large role in the origin of life. At the beginning of the evolution of life there has been an increasing volcanic activity on Earth, even at the bottom of the primary ocean. At that same time on the Earth there had been more silicon and silicon compounds, and they reacted with water. For this reason, they were absorbed in the early living organisms - diatoms and radiolarians. After the death these organisms sank to the bottom of the sea, and their substance was chemically reacted with seawater. Calcium carbonate and chitin of foraminifera plankton organisms was dissolved in water better than silica of diatoms and radiolarians, forming silica precipitations. The siliceous shales with siliceous deposits of microorganisms were formed in the Phanerozoic era of deep ocean basins at depths of about 2-3 km. The heyday of the microorganisms with silica skeleton could lead to such an accumulation of silicon in the ocean, so that at the sea bottom could be formed the silica gel. Then the silica could crystallize around scattered in the limestone crystallization centers, gradually replacing the molecules of calcium carbonate. Later, the organisms - foraminifera that have calcareous shells began to absorb calcium from the limestone rocks.

Acknowledgements

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Исследование процесса формирования известковых испопаемых прекамбрийского периода – строматолитов в горячей минеральной воде взаимодействующей с CaCO₃

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Аннотация. Исследованы условия формирования строматолитов в горячей минеральной воде. Изучать эти образования важно и интересно, так как строматолиты

заключают информацию о зарождающейся жизни на Земле и органическом составе первых живых организмов – многочисленных колоний цианобактерий, сине-зелёных водорослей и бактерий, возникающих в толщах известняков и доломитов в жерлах погасших вулканов и термических источников. Для этой цели проведены исследования горячей минеральной и морской воды из Болгарских источников методами ИК и ДНЭС спектроскопии. В качестве модельных систем использовали сок кактуса *Echinopsis pachanoi* и средиземноморскую медузу *Cotylorhiza tuberculata*. Также рассмотрены реакции конденсации-дегидратации в щелочных водных растворах со значением pH = 9–11 и температурах t = 65–95 ºC, результатом которых является синтез из мелких молекул более крупных органических молекул полимеров как короткие полипептиды в процессе моделирования первичной гидросферы. Показано, что горячие минеральные воды с температурой от +65 ºC до +95 ºC и значением pH от 9 до 11 более пригодны для возникновения жизни, чем другие исследованные образцы воды. В горячей минеральной воде локальные максимумы в ИК спектре проявлялись больше всего, по сравнению с локальными максимумами в ИК спектре той же воды при более низкой температуре. Разница в локальных максимумах от +20 ºC до +95 ºC при увеличении температуры на каждый +5 ºC составила в соответствии с *t*-критерием Стьюдента p < 0,05. Также показана роль известняка и кремния в формировании первых органических форм.

Ключевые слова: строматолиты, горячая минеральная вода, происхождение жизни, ИК, ДНЭС.

UDC 579.6; 582.28(063)

Optimization of Process Parameters for Effective Bioremediation of Chromium Contaminated Soil by *Trichoderma Pseudokoningii*

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Abstract
Release of hexavalent chromium in environment is usually the outcome of anthropogenic activities. Apart from its carcinogenicity, hexavalent chromium also contributes damage to the gastrointestinal, respiratory, reproductive and immunological systems. Hence removal of this toxic metal is very essential for the safety of both humans and animals. Microbial bioreduction of Cr (VI) to less toxic Cr (III) has proved to be an ecological and economical option for chromate detoxification. In this paper, we report the conversion of hexavalent chromium by the chromium reducing fungal strain of *Trichoderma pseudokoningii*, isolated from tannery effluent enriched soil near Kolkata. The study reveals that the isolated strain could grow well at a concentration of 1000 mg/L chromium, but spore formation became scanty as the concentration increased. Removal of hexavalent chromium was found to be accomplished through bio reduction rather than biosorption or bioaccumulation since no membrane bound and intracellular fraction bound Cr (VI) could be traced. Extra cellular chromium reduction was found to be highest when the culture medium (pH = 7) was supplemented with 0.5 % (w/v) pure dextrose and 0.09 % peptone as sole carbon and nitrogen source respectively. Reduction of potassium dichromate Cr (VI) at a concentration of 220 mg/L was highest after 144 hours of inoculation, i.e. at the stationary phase of growth of the strain. The reduction rate was enhanced in presence of cystine and DTT which might be due to the increased rate of activity of chromium reductase enzyme having thiol groups at its active site. Addition of acid treated human hair and feather in the culture medium boosted the power of chromate reduction by the strain. The gradual chromate reduction by the strain in soil kept in near natural conditions was confirmed by the data of atomic absorption spectroscopy which indicated the prospective role of the strain in successful bioremediation.
Keywords: hexavalent chromium, chromate reduction, bioremediation, *Trichoderma pseudokoningii*.

Introduction
Chromium (Cr) can exist in nine valence states (Smith *et al.*, 2002), of which only trivalent chromium Cr (III) and hexavalent chromium Cr (VI) are stable in natural environment and hence are ecologically important. Studies have revealed that Cr (VI) is approximately 100 times more toxic (Beszedits, 1988) and 1000 times more mutagenic than Cr (III) (Lofroth *et al.*, 1978).

Hexavalent chromium polluted soils and sediments are usually the result of sewage sludge disposal or dumping of chromate wastes from industrial and manufacturing activities (McGrath and Smith, 1990). Chromium contamination of environment is of concern because of the mobility and toxicity of Cr (VI) and is recognized to be highly toxic, carcinogenic, mutagenic and teratogenic for mammals including humans (Flores *et al.,* 1999). Cr (VI) exposure in humans can induce allergies, irritations, eczema, ulceration, nasal and skin irritations, perforation of eardrum, respiratory track disorders and lung carcinoma (Poopal *et al*, 2009; Gibb *et al*, 2000a, Gibb *et al*, 2000b). Moreover, Cr (VI) shows the capability to accumulate in the placenta, damaging fatal development (Saxena *et al,* 1990).

Cr (VI) in soil and water also alters the structure of soil microbial communities leading to the reduction of microbial growth and related enzymatic activities, with a consequent persistence of organic matter in soils and accumulation of Cr (VI) (Shi *et al*, 2002).

In various states of India, mainly in Tamilnadu, Uttar Pradesh and West Bengal (Vijayanand and Hemapriya**,** 2014), tanneries are still employing the chrome tanning processes and release effluents into the environment without proper pre-treatment. It causes uncontrolled enrichment of Cr (VI) in nature. As "the maximum acceptable concentration of 0.05 mg/L for chromium in drinking water has been established on the basis of health considerations" (Sterrett, 1978), removal of excess Cr (VI) becomes the need of the hour.

Since the removal of hexavalent chromium by conventional processes is expensive and lacks specificity (Katiyar and Katiyar, 1997), bioremediation with the help of suitable bacteria and fungi is the best option left. A number of microbes are reported to remove hexavalent chromium (Ahluwalia, 2014), of which most were able to adsorb the toxic metal. Fungi, in general, are well known for their ability to biosorb and bioaccumulate metals but few reports about biotransformation, especially on chromium reduction (Das and Santra, 2012) are available.

The present research deals with the optimization of the parameters for bioreduction of hexavalent chromium by a chromate tolerant fungal strain and elucidation of its prospective application in bioremediation of chromium contaminated soil.

Materials and methods
Microorganism and its cultivation

A chromium tolerant fungal strain, *Trichoderma pseudokoningii,* isolated from tannery effluent rich soil in West Bengal, India (Ray *et al*, 2013) was used throughout this study. The fungal strain was cultivated in 500 mL Erlenmeyer flasks each containing 100 mL of Basal Medium (BM) composed of (g·l^{-1}): peptone 0.9; $(NH_4)_2HPO_4$ 0.4; KCl 0.1; $MgSO_4·7H_2O$ 0.1, and glucose 0.5 (pH: 7) for 48 hours.

Measurement of growth

Growth of the mycelial type culture was measured by weighing the dried (at 80°C for 120 min) mycelial mat on dried pre weighed filter paper (Whatman No 1).

Tolerance to Cr (VI)

The efficacy of the strain to tolerate hexavalent chromium was tested by supplementing the medium with different concentrations of Cr (VI), namely, 0-1000 mg·l^{-1}. The flasks were incubated for 0-12 days followed by an assessment of their growth by measuring the mycelial dry weight.

Studies on Chromium removal by the selected strain

The fungal strain was grown overnight in 500 ml flasks each containing 100 mL of chromium enriched medium. For determination of chromium concentration of the extracellular fraction, the fungal culture was centrifuged at 10.000 RPM for 5 minutes and the supernatant was used as the sample. To determine the intracellular chromium concentration, the harvested mycelium was washed twice with 0.1 M phosphate buffer (pH = 7), mechanically disrupted in a Sonicator (Rivotek, India) and the cell mass was extracted with 20 ml phosphate buffer (pH = 7). The supernatant obtained after removing the cell debris by centrifugation at 10.000 RPM for 20 min was used as the intracellular content. The separated pellet, after washing with 0.1 M phosphate buffer (pH = 7) was used as membrane fraction.

The chromium reducing activity of the three fractions thus obtained (the extracellular, the intracellular or cytoplasmic and the membrane fraction) were estimated by the decrease in chromium concentration in the sample with time using Cr (VI) specific colorimetric reagent S-diphenyl carbazide (DPC) 0.25% (w/v) prepared in acetone (AR). The reaction mixture containing 200 μl sample and 330 μL of 6 M H_2SO_4 was mixed with 400 μL of freshly prepared diphenylcarbazide solution and final volume was made to 10 ml using glass distilled water (Thacker *et al.*, 2006). The residual chromium was measured at 540 nm by a spectrophotometer (Shimadzu, Japan) and the Cr (VI) concentration was calculated from the standard curve of $K_2Cr_2O_7$.

Measurement of total Chromium

The total Cr content was determined by AAS (Varian spectra AA 220) after acid digestion with concentrated HNO_3, with the addition of 33% H_2O_2 (Page *et al.*, 1982).

Optimization of parameters for chromate reduction by the strain

The strain was grown in different flasks containing Cr (VI) supplemented media with a range of initial pH (4-9) at a fixed temperature and with a fixed pH at various temperatures (7°-37°C) to determine the most suitable pH and temperature respectively for achieving the maximum efficacy of the strain for chromate reduction.

Effect of exogenous additives on chromate removal

The Cr (VI) enriched growth medium was supplemented with untreated and acid treated indigenous sources of cysteine namely feathers, human hair, dried onion scales, Indian gooseberry fruit (*Phyllanthus emblica*) followed by a measurement of the residual chromium in the culture medium. For acid treatments, feathers and hairs were incubated with 50 mL of 1.0 N analytical-grade HCl for 24 h at 37°C followed by thorough rinsing with distilled water and total drying.

Assessment of chromate reduction efficacy of the strain from chromium rich soil samples

Soil samples (20 g), were sterilised and mixed with various concentrations (160-40mg·l^{-1}) of Cr (VI) and sprinkled with the culture of the working strain (10 ml) and kept at a near natural environment in earthen pots for 10 days with proper control of non-inoculated soil for each set. After acid digestion (USEPA, 1996), total chromium of all the soil samples was measured by atomic absorption spectroscopy.

Chemicals

All chemicals used were of analytical grade purchased from Sigma chemicals Co. (St. Louis, USA), Merck, Germany and Himedia, India.

Each experiment was done in triplicate and their values were averaged.

Results and discussion

The chromate tolerant fungal strain *Trichoderma pseudokoningii* (Ray *et al*, 2013) was found to grow both on solid state fermentation (PDA plate) and in submerged culture supplemented with up to 1000 mg·l^{-1} of Cr (VI). In submerged culture, around 40% growth was observed up to a concentration of 720 mg·l^{-1}, above which, the growth of the strain was severely hindered. (Fig. 1). Hence, for better expression of its chromate removal efficacy, further experiments were designed with the cultures supplemented with a sub lethal concentration of hexavalent chromium (220 mg·l^{-1}) allowing 80% growth of the strain. Similar level of tolerance to Cr (VI) was reported from a yeast strain DBVPG 6502 (Baldi *et al*, 1990), whereas two strains namely *Aspergillus parasiticus* and *Aspergillus niger* were cultivated in a medium supplemented with only 20 mg·l^{-1} of Cr (VI) (Shugaba *et al*, 2012).
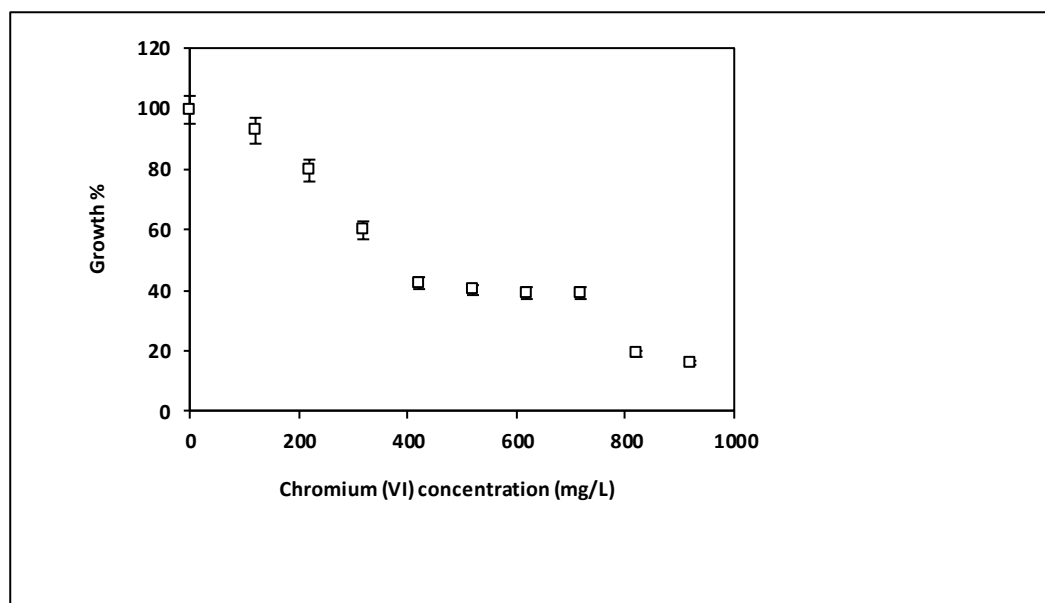
Fig. 1. Growth (dry weight) of *Trichoderma pseudokoningii* with different concentrations of Cr (VI)

Estimation of both the extra and intercellular chromate content indicated that about 68% of extra cellular hexavalent chromium was reduced within 4 days of incubation. Although a little amount of hexavalent chromium could be detected in the intracellular fluid only after 2 days of cultivation, the total hexavalent chromium was disappeared within 8 days of cultivation (Fig. 2). The existence of a small amount of intercellular chromate and absence of detectable Cr (VI) in the membrane fraction indicated the chromate removal process involved in the present strain was not through biosorption, as found in other fungal strains (Pal and Vimala, 2011; Khambhaty *et al*, 2009, Kumar *et al*, 2008; Mungasavalli *et al*, 2007). (Table 1). The total disappearance of hexavalent chromium from the medium and cellular environment indicated the chromate removal was not accomplished by bioaccumulation; instead a bio reduction method was adopted by the strain to convert the hexavalent into trivalent form.

Table 1. Comparative account of hexavalent chromium removing fungal strains

| Fungi	Initial concentration of Cr(VI) (mgL^{-1})	Mechanism involved	References
Aspergillus niger	400	Biosorption	Khambhaty *et al*, 2009
Aspergillus niger	30	Biosorption	Kumar *et al*, 2008
Aspergillus niger	10	Biosorption	Mungasavalli *et al*.2007
Aspergillus niger	125	Bioaccumulation	Holda *et al*, 2013
Aspergillus parasiticus	20		Shugaba *et al*, 2012
Aspergillus niger			Shugaba *et al*, 2012
Aspergillus sp. N3 and N5	60		Fukuda *et al*, 2008
Aspergillus foetidus	5		Prasenjit and Sumathi 2005
Aspergillus oryzae	5	Biosorption	Reya Issac *et al*, 2012
Aspergillus flavus, Aspergillus sp,A. niger	600	Reduction-coupled biotransformation	Bennett *et al*, 2013
Aspergillus sojae	5	Biosorption	Reya Issac *et al*, 2012

Aspergillus flavus *Aspergillus clavatus*	300	Biosorption and biotransformation	Das, 2014
Candida sp	500	Biosorption	Baldi *et al*, 1990
Mucor hiemalis	50	Biosorption	Pillicsanmer *et al*, 1995.
Rhizopus oryzae	400	Reduction	Sukumar, 2010
Rhizopus nigricans	100	Biosorption	Sudha and Abraham 2001
Rhizopus arrhizus	200	Biosorption	Preetha and Viruthagiri 2007.
Rhizopus arrhizus	50	Biosorption	Shroff and Vaidya, 2012
Rhizopus arrhizus	100	Biosorption	Prakasham *et al*, 1999.
Phanerochaete chrysosporium	100		Pal and Vimala .2011.
Penicillium sp	60		Kumar *et al,* 2008
Penicillium janthinellum	30		Kumar *et al,* 2008
Penicillium citrinum	125	Bioaccumulation	Holda *et al,* 2013
Halo tolerant *Penicillium*	400	Biosorption	Patel and Power, 2014
Paecilomyces sp,	1000	Biosorption	Juan Fernando Cárdenas and Ismael Acosta ,2011.
Fusarium solani	500	Biosorption	Sen and Ghosh Dastidar,2011
Fusarium solani	500	Bioreduction	Sen *et al,* 2013
Gloeophyllum sepoarium			Achal *et al,* 2011
Ganoderma lucidum and *Mucor hiemalis*	20	Biosorption	Verma *et al*, 2014
Trichoderma viride	125	Bioaccumulation	Holda *et al,* 2013
Trichoderma harzianum	40	reduction	Sarkar *et al*, 2013
Trichoderma harzianum	30	Biosorption	Sarkar *et al*, 2010
Trichoderma pseudokoningii	220	Reduction	Present work
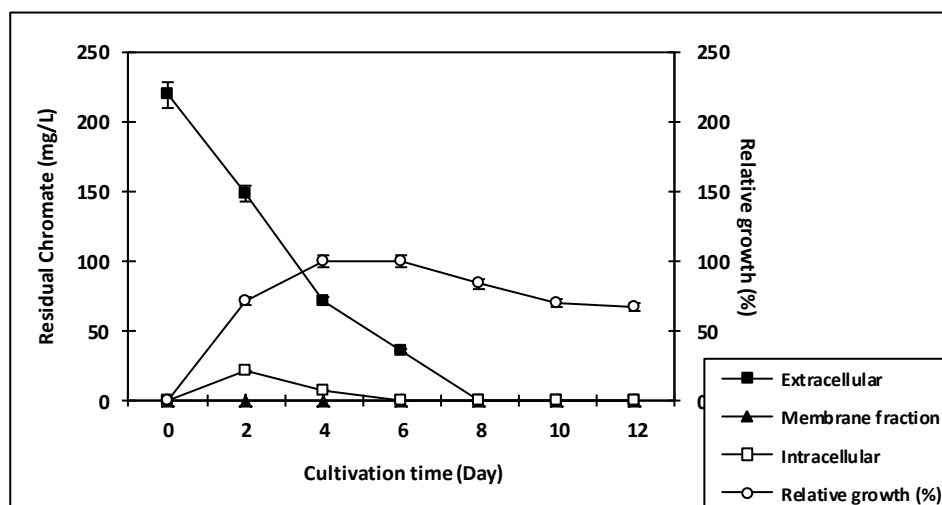
Fig. 2. Kinetics of extra and intra cellular chromate reduction by *Trichoderma pseudokoningii*

Initial Cr (VI) concentration: 220 ml/L, 100% growth = 150 mg

The Cr (VI) reduction was found to achieve its highest level at a neutral pH (Fig. 3) and the efficacy of chromate reduction was positively correlated with growth pattern of the strain. The preference of neutral pH for chromate removal was also found in *Aspergillus* sp N2 and *Penicillium* sp N3 (Fukuda *et al*, 2008) and in *Gloeophyllum sepoarium* (Achal *et al*, 2011).
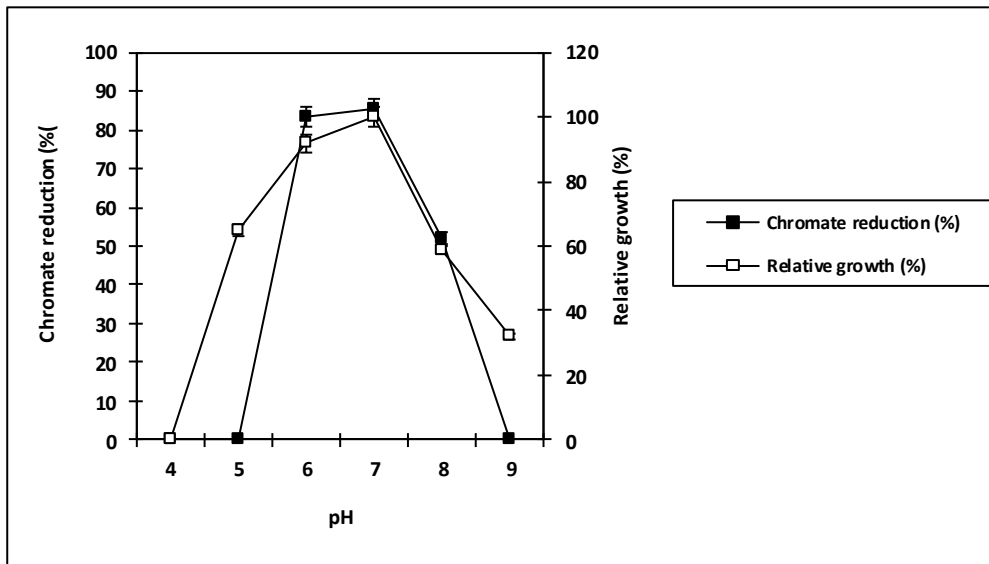
Fig. 3. Effect of pH on growth and chromate reduction by *Trichoderma pseudokoningii*

The growth pattern of the strain was closely related to chromium reduction and as the strain showed highest growth at 27°C (Fig. 4), maximum amount of hexavalent chromium was found to be reduced at 27°C, above which the reduction rate declined. Similar temperature preference was reported from *Gloeophyllum sepoarium* (Achal *et al*, 2011) and *Paecilomyces sp* (Cárdenas-González and Acosta-Rodríguez, 2011) and *Trichoderma harzianum* (Sarkar *et al*, 2013).
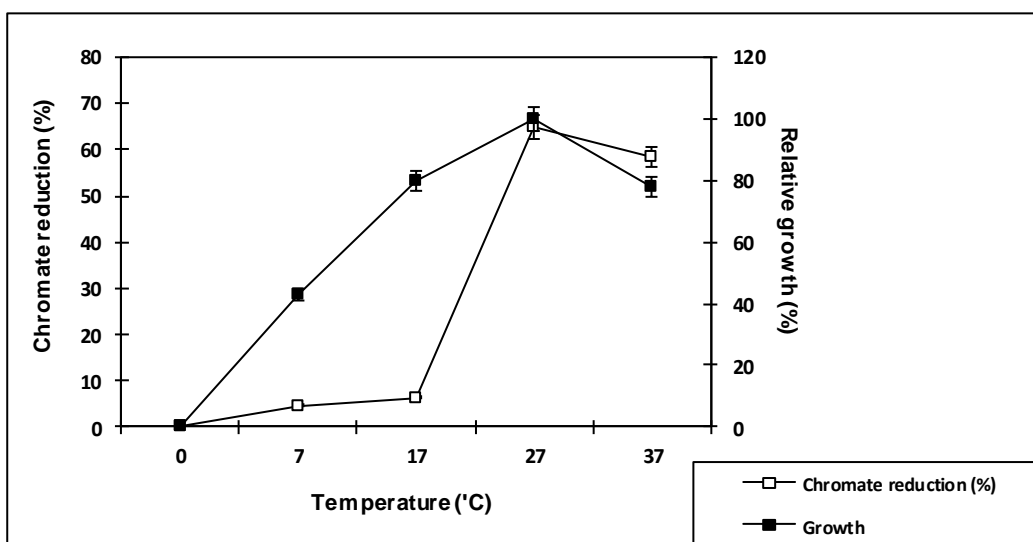

Fig. 4. Effect of temperature on growth and chromate reduction by *Trichoderma pseudokoningii*

The growth and bio reduction efficacy of the working strain was increased in presence of glucose Similar report was obtained from Cr (VI) removal by *Serratia* sp (González *et al,* 2014). The most preferred concentration of glucose was found to be 0.5 % (w/v) (Fig. 5).
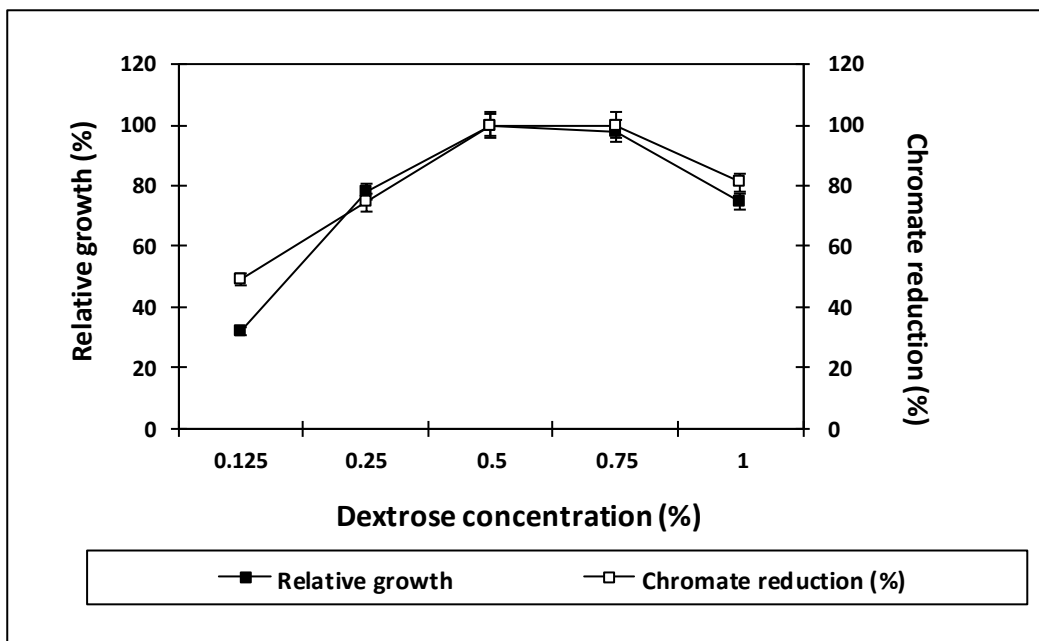
Fig. 5. Effect of dextrose concentration on growth and chromate reduction by *Trichoderma pseudokoningii*

Amongst the nitrogen sources tested peptone was found to promote growth as well as chromate reduction efficiency of the strain (Fig. 6).
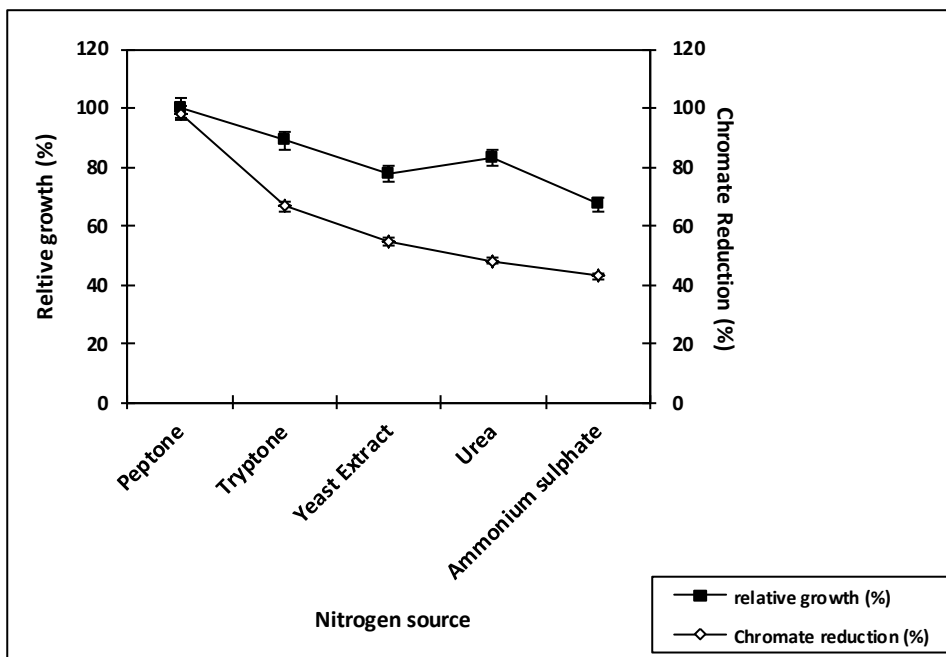
Fig. 6. Effect of nitrogen source on growth and chromate reduction by *Trichodermapseudokoningii*

Chromate reduction after 5 days of cultivation (with 80% chromate reduction without additive) in presence of various additives indicated that exogenous thiols like cysteine and DTT could enhance the chromate reducing activity of the strain (Fig. 7). This could be due to the increased activity of the chromate reductase through the activation of the catalytic site by the exogenous thiols. On the other hand the surfactants and chelators remarkably affected the efficacy.
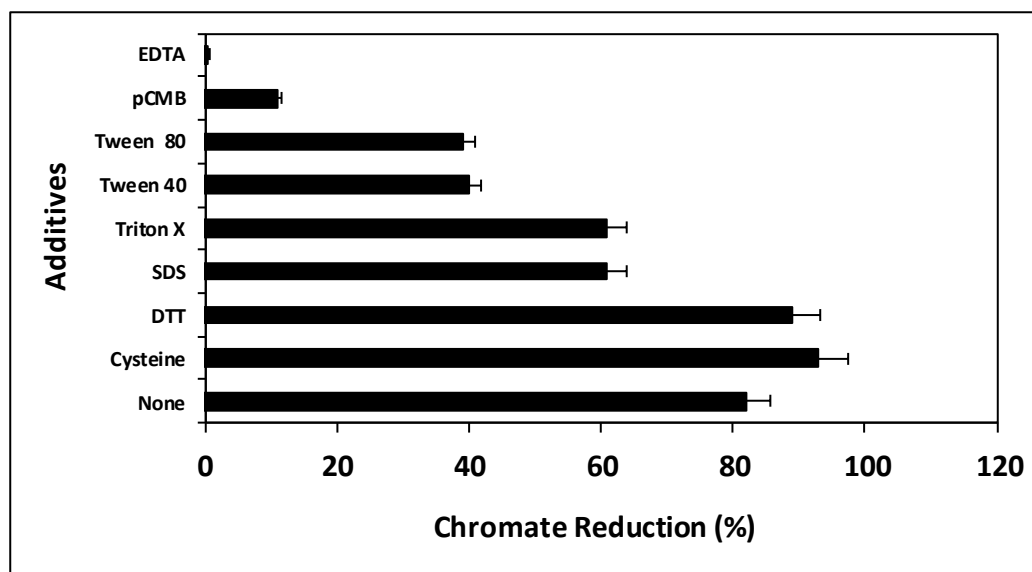
Fig. 7. Effect of additives on growth and chromate reduction by *Trichoderma pseudokoningii*

Since exogenous cysteine enhanced chromate reduction, some natural sources of cysteine were tried (Table 2), of which human hair and feather itself could reduce the chromate content even in the absence of the fungal spores. Acid treatment of human hair and feather gave remarkable results in chromate reduction and could be effectively used for bioremediation of polluted soil along with the fungal spores.

Table 2. Effect of natural additives on chromate reduction

Medium supplement	Residual concentration of Cr (VI) (mg/L)*	
	Non inoculated	**Inoculated with fungal spores**
Medium supplemented with Cr (VI)	212	130
Medium supplemented with Cr (VI) and untreated human hair	200	125
Medium supplemented with Cr (VI) and acid treated human hair	126	42.6
Medium supplemented with Cr (VI) and untreated feather	210	129
Medium supplemented with Cr (VI) and acid treated feather	170	119
Medium supplemented with Cr (VI) and dried onion scales	200	112
Medium supplemented with Cr (VI) and vitamin C.	200	110
Medium supplemented with Cr (VI) and dried Indian gooseberry fruit.	200	110

*Initial Cr (VI) concentration: 212 mg/L, cultivation time: 72 hrs.

Removal of Cr (VI) from soil

The soil samples treated with increasing concentration of hexavalent chromium and kept in near natural conditions for 10 days showed almost no change in total chromium content, whereas chromium infested soil samples with fungal spores showed remarkable reduction in chromate content (Table 3).

Although the chromate removal by the fungal strain was more effective in batch culture than that of the soil, which might be due to the presence of some natural constraints existing in the

microenvironment of soil. However 97-99% chromate removal within 10 days confirmed the fact that the strain could be used as successful bioremediation of hexavalent chromium pollution.

Table 3: Change in chromium content in the soil (as estimated by Atomic absorption spectrometry)*

Cr (VI) concentration (mg/L)	Total Chromium content (mg/L)	
	Non inoculated soil	**Soil inoculated with fungal culture**
400	397± 0.26	10.58±0.31
320	318.8± 0.21	5.23±0.30
240	237.2 ± 0.36	5.02±0.23
160	157.6± 0.18	2.36±0.26

*Time of exposure: 10 days.

Conclusion

The present study concludes that the presently described fungal strain of *Trichoderma pseudokoningii,* isolated from tannery effluent showed the ability to tolerate remarkably high concentrations of hexavalent chromium which was probably acquired as an adaptive feature for surviving in chromate infested environment. Further the increased rate of chromate bioreduction in presence of natural ingredients like human hair, feather, onion scales, and gooseberry fruits opens up a promising way of chromate detoxification of the polluted soil near industries of tanning, plating and pigment manufacture.

Acknowledgments

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