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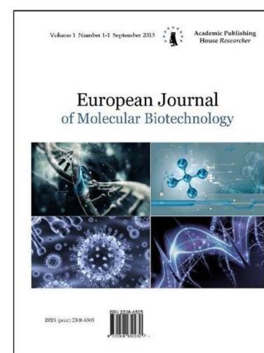

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Studying of Phototransformation of Light Signal by Photoreceptor Pigments – Rhodopsin, Iodopsin and Bacteriorhodopsin

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Abstract

This review article views predominately the structure and function of animal and bacterial photoreceptor pigments (rhodopsin, iodopsin, bacteriorhodopsin) and their aspects of nano- and biotechnological usage. On an example of bacteriorhodopsin is described the method of its isolation from purple membranes of photo-organotrophic halobacterium *Halobacterium halobium* by cellular autolysis by distilled water, processing of bacterial biomass by ultrasound at 22 KHz, alcohol extraction of low and high-weight molecular impurities, cellular RNA, carotenoids and lipids, the solubilization with 0,5 % (w/v) SDS-Na and subsequent fractionation by methanol and gel filtration chromatography on Sephadex G-200 Column balanced with 0.09 M Tris-borate buffer (*pH* = 8,35) with 0,1 % (w/v) SDS-Na and 2,5 mM EDTA. Within the framework of the research the mechanism of color perception by the visual analyzer having the ability to analyze certain ranges of the optical spectrum, as colors was studied along with an analysis of the additive mixing of two colors. It was shown that at the mixing of electromagnetic waves with different wavelengths, the visual analyzer perceive them as separate or average wave length corresponding to mix color.

Keywords: vision; rhodopsin; iodopsin; bacteriorhodopsin; additive color mixing.

Introduction

Vision (visual perception) is a process of psycho-physiological processing of the images of surrounding objects, carried out by the visual system, which allows to get an idea of the size, shape and color of surrounding objects, their relative position and distance between them. By means of this animals can receive 90 % of all incoming information to the brain.

The function of the visual system is carried out through various interrelated complex structures designated as visual analyzer, consisting of a peripheral part (retina, optic nerve, optic tract) and the central department of combining stem and subcortical centers of the midbrain, as

well as the visual cortex of the cerebral hemispheres. The human eye can perceive only light waves of a certain length – from λ = 380 to λ = 770 nm.

Light rays from treated subjects pass through the optical system of the eye (cornea, lens and vitreous body) and onto the retina, where the light-sensitive photoreceptor cells (rods and cones) are located. Light incidented on the photoreceptors, triggers a cascade of biochemical reactions of visual pigments (in particular, the most studied of them is rhodopsin responsible for the perception of electromagnetic radiation in the visible range), and in turn, – the occurrence of nerve impulses, which are transmitted through the following retinal neurons and further to the optic nerve. The optic nerve carries the nerve impulses into the lateral geniculate body – subcortical center of vision, and thence to the cortical center, located in the occipital lobe of the brain, where the visual image is formed.

Over the last decade have been obtained new data revealing the molecular basis of visual perception. There were identified visual molecules of eucariotes (rhodopsin, iodopsin) and procariots (bacteriorhodopsin) involved in light perception and cleared up the mechanism of their action.

The structural research of rhodopsin and its affiliated chromophore proteins (iodopsin, bacteriorhodopsin) and the analysis of their functions have been carried out in the Scientific Research Center of Medical Biophysics (Bulgaria) throughout the last 20 years. The purpose of the research was the studying of basic biochemical mechanisms associated with visual perception and some nano- and biotechnological applications of visual phototransforming pigments as trans membrane protein bacteriorhodopsin, extracted from purple membranes of halophilic bacterium *Halobacterium halobium*.

Materials and methods

As a producer of bacteriorhodopsin (BR) was used a carotenoid strain of extreme photo-organorotrophic halobacterium *Halobacterium halobium* ET 1001, obtained from Moscow State University (Russia). The strain was modified by selection of individual colonies on solid (2 % (w/v) agarose) media with peptone and 4,3 M NaCl. BR (yield 8–10 mg from 1 g biomass) was obtained in synthetic (SM) medium (g/l): D,L-alanine – 0,43; L-arginine – 0,4; D,L-aspartic acid – 0,45; L-cysteine – 0,05; L-glutamic acid – 1,3; L-lycine – 0,06; D,L-histidine – 0,3; D,L-isoleucine – 0,44; L-leucine – 0,8; L-lysine – 0,85; D,L-methionine – 0,37; D,L-phenylalanine – 0,26; L-proline – 0,05; D,L-serine – 0,61; D,L-threonine – 0,5; L-tyrosine – 0,2; D,L-tryptophan – 0,5; D,L-valine – 1,0; AMP – 0,1; UMP – 0,1; NaCl – 250; $MgSO_4 \cdot 7H_2O$ – 20; KCl – 2; NH_4Cl – 0,5; KNO_3 – 0,1; KH_2PO_4 – 0,05; K_2HPO_4 – 0,05; Na^+-citrate – 0,5; $MnSO_4 \cdot 2H_2O$ – 3 10^{-4}; $CaCl_2 \cdot 6H_2O$ – 0,065; $ZnSO_4 \cdot 7H_2O$ – $4 \cdot 10^{-5}$; $FeSO_4 \cdot 7H_2O$ – $5 \cdot 10^{-4}$; $CuSO_4 \cdot 5H_2O$ – $5 \cdot 10^{-5}$; glycerol – 1,0; biotin – $1 \cdot 10^{-4}$; folic acid – $1.5 \cdot 10^{-4}$; vitamin B_{12} – $2 \cdot 10^{-5}$. The growth medium was autoclaved for 30 min at 0,5 atm, the *pH* value was adjusted to 6,5–6,7 with 0,5 M KOH. Bacterial growth was performed in 500 ml Erlenmeyer flasks (volume of the reaction mixture 100 ml) for 4–5 days at 35 °C on Biorad shaker ("Birad Labs", Hungary) under intense aeration and monochromatic illumination (3 lamps × 1,5 lx). All further manipulations for BR isolation were carried out with the use of a photomask lamp equipped with an orange light filter. Biomass (1 g) was washed with distilled water and pelleted by centrifugation on T-24 centrifuge ("Carl Zeiss", Germany) (1500 g, 20 min). The precipitate was suspended in 100 ml of dist. H_2O and kept for 3 h at 4 °C. The reaction mixture was centrifuged (1500 g, 15 min), the pellet was resuspended in 20 ml dist. H_2O and disintegrated by infrasound sonication (22 kHz, 3 times × 5 min) in an ice bath (0 °C). The cell homogenate after washing with dist. H_2O was resuspended in 10 ml of buffer containing 125 mM NaCl, 20 mM $MgCl_2$, and 4 mM Tris-HCl (*pH* = 8,0), then 5 mg of RNA-ase (2–3 units of activity) was added. The mixture was incubated for 2 h at 37 °C. Then 10 ml of the same buffer was added and kept for 10–12 h at 4 °C. The aqueous fraction was separated by centrifugation (1500 g, 20 min), the PM precipitate was treated with 50 % (v/v) ethanol (5 times × 7 ml) at 4 °C followed by separation of the solvent. This procedure was repeated 6 times to give a colorless washings. The protein content in the samples was determined spectrophotometrically on DU-6 spectrophotometer ("Beckman Coulter", USA) by the ratio D_{280}/D_{568} (ε_{280} = $1,1 \cdot 10^5$; ε_{568} = $6,3 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) [1]. PM regeneration is performed as described in [2]. Yield of PM fraction – 120 mg (80–85%). Fraction PM (in H_2O) (1 mg/ml) was dissolved in 1 ml of 0,5 % (w/v) sodium dodecyl sulfate (SDS-Na), and incubated for 5–7 h at 37 °C

followed by centrifugation (1200 g, 15 min). The precipitate was separated, than methanol was added to the supernatant in divided portions (3 times × 100 ml) at 0 °C. The reaction mixture was kept for 14–15 h in ice bath at 4 °C and then centrifuged (1200 g, 15 min). Fractionation procedure was performed three times, reducing the concentration of 0,5 % SDS-Na to 0,2 and 0,1 %. Crystal protein (output 8–10 mg) was washed with cold ^2H_2O (2 times × 1 ml) and centrifuged (1200 g, 15 min). Protein sample (5 mg) was dissolved in 100 ml of buffer solution and placed on a column (150×10 mm), stationary phase – Sephadex G-200 ("Pharmasia", USA) (specific volume packed beads – 30–40 units per 1 g dry. Sephadex) equilibrated with buffer containing 0,1 % (w/v) SDS-Na and 2,5 mM ETDA. Elution proceeded by 0,09 M Tris-borate buffer containing 0,5 M NaCl, *pH* = 8,35 at a flow rate of 10 ml/cm^2 · h. Combined protein fraction was subjected to freeze-drying, in sealed glass ampoules (10 × 50 mm) and stored in frost camera at -10 °C.

Quantitative analysis of the protein was performed in 12,5% (w/v) polyacrylamide gel (PAAG) containing 0,1 % (w/v) SDS-Na. The samples were prepared for electrophoresis by standard procedures (LKB protocol, Sweden). Electrophoretic gel stained with Coomassie blue R-250 was scanned on a CDS-200 laser densitometer (Beckman, USA) for quantitative analysis of the protein.

Absorption spectra of pigments were recorded on programmed DU-6 spectrophotometer ("Beckman Coulter", USA) at λ = 280 nm and λ = 750 nm.

IR-spectra were registered on Brucker Vertex IR spectrometer ("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}) and Thermo Nicolet Avatar 360 Fourier-transform IR.

Colors were analyzed by using color analyzer "Tsvetan" ("Photopribor", Cherkassk, Ukraine). Operating relative absorbance, % from -80 to 70. Measurement error, ±5 %. Response time from 0,4 to 63 sec. Overall dimensions, 300 mm.

The structural studies were carried out with using scanning electrom microscopy (SEM) on JSM 35 CF (JEOL Ltd., Corea) device, equiped with X-ray microanalyzer "Tracor Northern TN", SE detector, thermomolecular pump, and tungsten electron gun (Harpin type W filament, DC heating); working pressure: 10^{-4} Pa (10^{-6} Torr); magnification: 300000, resolution: 3,0 nm, accelerating voltage: 1–30 kV; sample size: 60–130 mm.

Results and discussion

Molecular basis of vision

The process of perception of light has a definite localization in photoreceptor light-sensitive cells of the retina. The retina in its structure is a multilayer layer of nervous tissue that is sensitive to light, which lines the inside of the back of the eyeball. Pigmented retina located at the membrane referred to as retinal pigmented epithelium (RPE), which absorbs light passing through the retina. This prevents the reverse reflection of the light through the retina and does not allow the vision to disperse.

Light enters through the eye and creates a complex biochemical reaction in the photoreceptor cells of the retina. Photoreceptor cells are divided into two types that due to their characteristic form are designated as rods and cones [3]. Rods are receptors of light of low intensity; they arranged in a colored layer of the retina, in which is synthesized photochromic protein rhodopsin, responsible for color perception. Cones on the contrary contain a group of visual pigments (iodopsin), and adapted to distinguish different colors. Rods can persive black and white images in the dim light, cones – to carry out color vision in bright light. Human retina contains approximately 3 million of cones and 100 million of rods. Their dimensions are very small – the length of about 50 mm, the diameter from 1 to 4 μm.

Electrical signals generated by the rods and cones, are handled by other retinal cells – bipolar and ganglion cells before they are transmitted to the brain via the optic nerve [4]. Additionally, there are two intermediate layers of neurons. Horizontal cells transmit messages back and forth between the photoreceptor cells, bipolar cells and each other. Amacrine cells of the retina are linked to bipolar cells, ganglion cells, as well as with each other. Both types of these intermediate neurons play a major role in the processing of visual information at the level of the retina before it is transmitted to the brain for final processing.

Cones are approximately 100 times less sensitive to light than rods, but much better perceive the rapid movement. The wand can be stimulated by a single photon. Cascade of molecular interactions enhances this "quantum" of information into a chemical signal, which is then perceived by the nervous system. The degree of enhancement signal varies depending on ambient light: rods are more sensitive under low than inder bright light. As a result, they operate effectively in a wide range of ambient light. Sensory system of rods is packed up in clearly distinguishable cellular substructure that can be easily selected and investigated *in vitro* in isolated state. This property makes them as indispensable object for further structural-functional studies as well as studies of photoreceptor pigments (rhodopsin, iodopsin). These anumal photoreceptor pigments are used as models for studying of bacterial photoreceptor pigment bacteriorhodopsin (BR) from purple membranes of halobacterium *Halobacterium halobium*.

Rhodopsin and its structural and functional properties

Rhodopsin [5] is one of the most important integral photoreceptor proteins of rod cells, which absorbs a photon and creates a biochemical response constituting a first step in a chain of events that provide vision. Rhodopsin consists of two components – a colorless protein opsin and a chromophore component 11-*cis*-retinal residue, acted as the light acceptor (Fig. 1). The absorption of a light photon by 11-*cis*-retinal "turns on" the enzymatic activity of opsin and further photosensitive biochemical cascade of reactions that are responsible for vision [6].

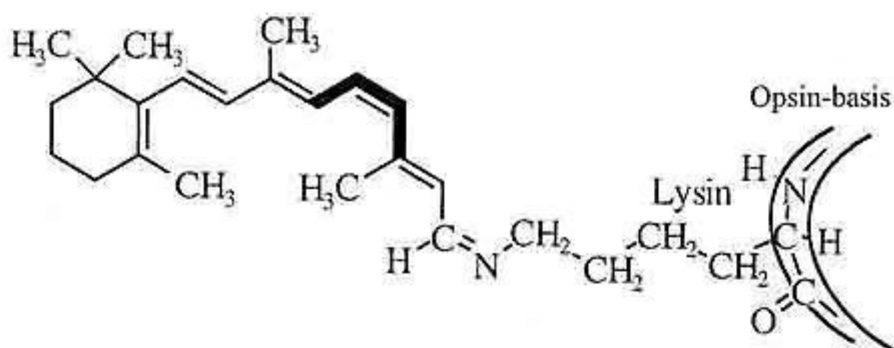

Figure 1. Configuration of photosensitive chromophore of rhodopsin in the basic (unexcited) phase (at the double bond is marked 11-cis-configuration)

Rhodopsin belongs to the group of the G-protein-coupled receptors (GPCR-receptors) of the retibylidene protein family responsible for transmembrane signaling mechanism based on the interaction with intracellular membrane G-proteins – universal intermediaries in the transmission of hormonal signals from the cell membrane receptors to effector proteins, causing the final cellular response. The establishment of the spatial structure of rhodopsin is so important because rhodopsin as the "originator" of the family of GPCR-receptors is a "model" for the structure and function of other receptors that it is extremely important from fundamental scientific and practical points of view [7].

Spatial structure of rhodopsin was long defied by the study of "direct" methods – X-ray diffraction and NMR spectroscopy, while the molecular structure of related to rhodopsin transmembrane chromoprotein bacteriorhodopsin [8] aving a similar structure, performing the functions of ATP-dependent translocase in the cell membranes of halophilic microorganisms pumped protons across the cytoplasmic membrane of the cell and is involved in the anaerobic photosynthetic phosphorylation (non-green synthesis), was determined as early as 1990. On the contrary the structure of rhodopsin remained unknown until 2003 [9]. The opsin fragment of the rhodopsin molecule has 348 amino acid residues in a polypeptide chain that is formed by seven transmembrane α-helix segments situated across the membrane and joined with short non-helix sections [10]. The N-terminus of α-helix is located in the extracellular region, while the C-terminus – in the cytoplasmic region. The 11-*cis*-retinal residue is connected to one of the α-helixes, located near the middle of the membrane, so that its long axis is parallel to the membrane surface (Fig. 2).

It was also determined the dislocation of 11-*cis*-retinal aldimine bond with ε-amino group of Lys-296 residue located in the seventh α-helix.

Thus, 11-*cis*-retinal is mounted in the center of a complex highly organized protein in the cellular membrane comprising rods. This structure provides a photochemical "adjustment" of retinal residue, affecting its absorption spectrum. The free 11-*cis*-retinal in a dissolved form has an absorption maximum in the ultraviolet region - at a wavelength of 380 nm, while rhodopsin absorbs green light at 500 nm [11]. This shift in the wavelength of light is important from a functional point of view; it is aligned with the spectrum of light that enters the retina.

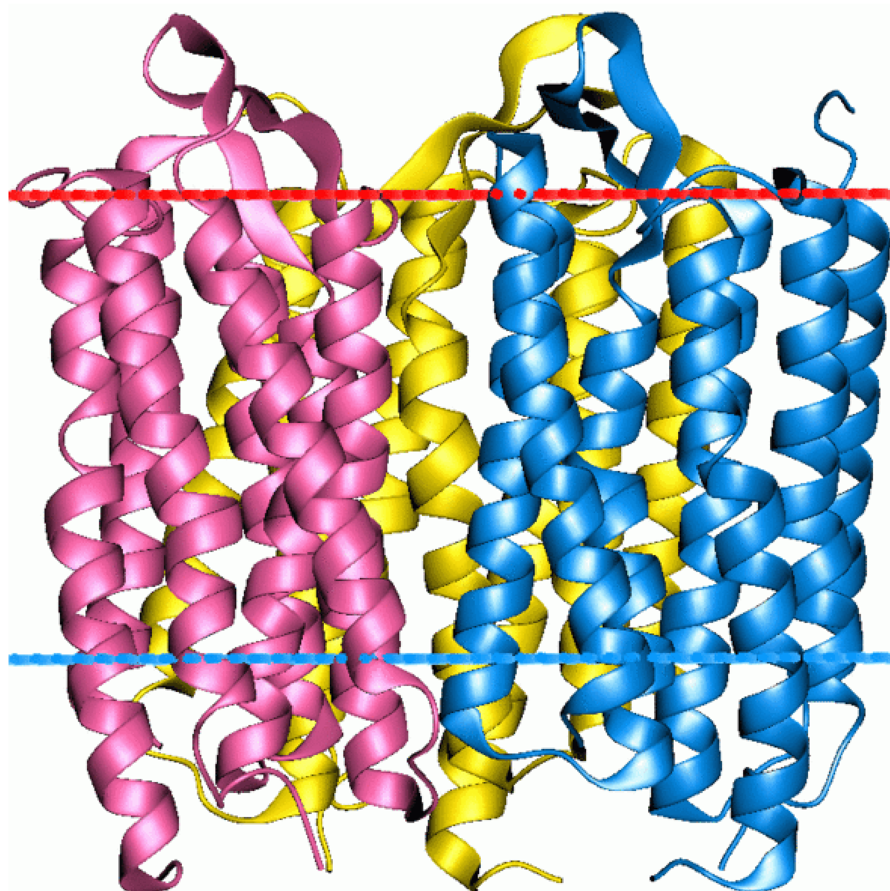

Figure 2. The structure of rhodopsin according to computer modeling data

The absorption spectrum of rhodopsin is defined by properties of the chromophore – 11-*cis*-retinal residue and opsin fragment. This range in vertebrates has two characteristic peaks – one in the ultraviolet (λ = 278 nm) due to the opsin fragment, and the other – in the visible region (λ = 500 nm) corresponds to absorption of the chromophore (Fig. 3). Further transformation of rhodopsin under the action of light to the final stable product consists of a series of very fast intermediate stages. Investigating intermediates absorption spectra of rhodopsin in extracts at low temperatures at which these products are stable, allows to describe in the detail the photochemical changes of rhodopsin [12].

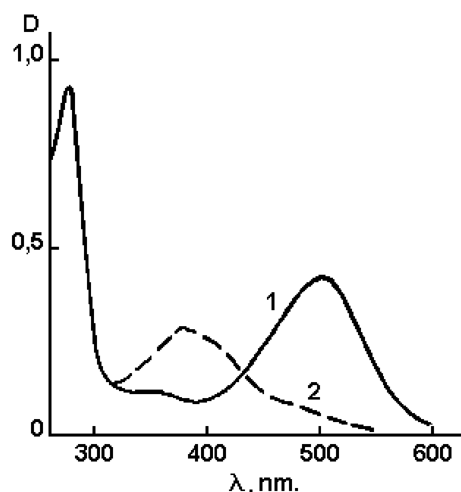

Figure 3. Absorption spectrum of rhodopsin from the frog Rana temporaria (in water extract): 1 – rhodopsin (restored pigment); 2 – yellow indicator (discolored pigment)

Upon absorption of light photon it is occurred isomerization of 11-*cis*-retinal into 11-*trans*-retinal (quantum yield, 0,67), that induces a conformational change in the protein and activates photopsin and promotes its binding to G protein transducin, which triggers a second messenger cascade [13]. Subsequent cycles of the photochemical reactions of rhodopsin lead to a local depolarization of the membrane and the stimulation of the nerve impulse propagates along the nerve fiber due to changes in ion transport in the photoreceptor (Fig. 4). Subsequently rhodopsin restored (regenerated) with participation of retinal isomerase through steps: 11-*trans*-retinal → 11-*trans*-retinol → 11-*cis*-retinol → 11-*cis*-retinal, the latter is connected with opsin to form rhodopsin.

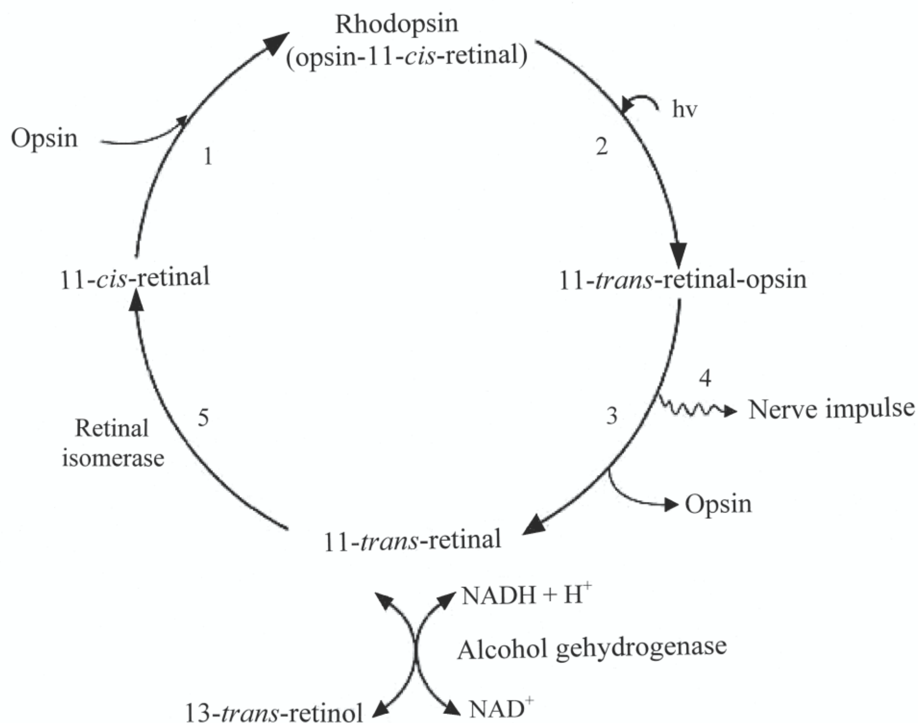

Figure 4. Photocycle scheme of rhodopsin: 1 – 11-cis-retinal in the dark links with protein opsin to form rhodopsin; 2 – under light illumination occurs photoisomerization of 11-cis-retinal into 11-trans-retinal; 3 – 11-trans-retinal-opsin complex splits onto 11-trans-retinal and opsin; 4 – local depolarization of the membrane and the occurrence of a nerve impulse propagates along the nerve fiber; 5 – regeneration of the original pigment

Bacteriorhodopsin and its applications

Bacteriorhodopsin (BR), named by analogy to the visual apparatus of mammalian chromoprotein rhodopsin, was isolated from the cell membrane of extreme photo-organo-heterotrophic halobacteria *Halobacterium halobium* in 1971 by D. Osterhelt and W. Stohenius. This photo-transforming trans-membrane chromo-protein with the molecular weight ~26,5 kDa is a chromoprotein determining the purple-red culour of halophilic bacteria, contained as chromophore group an equimolar mixture of 13-*cis*-and 13-*trans*-retinol C20-carotenoid, bound by Schiff base (as in the visual animal pigments) with Lys-216 residue of the protein.

In its structure and location in the cell membrane BR refers to integral transmembrane proteins, penetrating the cell membrane, which is divided into three fractions: yellow, red and purple. Purple fraction comprising on 75% (w/w) of cell membrane consists from carotenoids, phospholipids (mostly phosphoglycerol diesters with a small amount of nonpolar lipids and isoprenoids) forms a natural two-dimensional crystals which can be investigated using electron microscopy diffraction methods as X-ray scattering [15]. These methods have established the existence in the BR molecule seven α-helical protein segments, while in the middle are symmetrically located a retinal residue (Fig. 5).

Figure 5. The structure of BR from PM of halophilic bacterium H. halobium according to computer modeling data

Polypeptide chain of BR consists of 248 amino acid residues, 67% of which are hydrophobic, formed with the aromatic amino acids, and 33% – hydrophilic residues of aspartic and glutamic acids, arginine and lysine [16]. These residues play important structural and functional role in the spatial orientation of the α-helical segments of the BR molecule, arranged in PM in an orderly manner forming trimers with an average diameter ~0,5 μm and a thickness 5–6 nm; each trimmer is surrounded by six others so that to form a regular hexagonal lattice [17]. The BR molecule arranged in a direction perpendicular to the plane of the membrane. Hydrophobic domains

represent transmembrane segments and hydrophilic domains protruding from the membrane, connect the individual α-helical intramembranous segments of the BR molecules.

BR acts as a light-dependent proton pump, pumping protons across the cell membrane and generates an electrochemical gradient of H^+ on the surface of the cell membrane, which energy is used by the cell for the synthesis of ATP in the anaerobic photosynthetic phosphorylation. The mechanism of ATP synthesis is called "non-chlorophyll photosynthesis", in contrast to the plant photosynthesis with the participation of chlorophyll. In this mechanism, at absorbtion of a light photon BR molecule became decolorized by entering into the cycle of photochemical reactions, resulting in the release of a proton to the outside of the membrane, and the absorption of proton from intracellular space. By the absorption of a light photon is occured reversible isomerization of 13-*tras*-BR (λ_{max} = 548 nm) (the quantum yield 0,03 at 20 °C) in the 13-*cis*-BR (λ_{max} = 568 nm) [18], initiating a cascade of photochemical reactions lasting from 3 ms to 1 ps with the formation of transitional intermediates J, K, L, M, N, and O, followed by separation of H^+ from the retinal residue of BR and its connection from the side of cytoplasm (Fig. 6). As a result, between the internal and external surface of the membrane forms a concentration gradient of H^+, which leads that illuminated halobacteria cells begin to synthesize ATP, i.e. convert light energy into energy of chemical bonds. This process is reversible and in the dark flows in the opposite direction. In this way the BR molecule behaves as a photochromic carrier with a short relaxation time – the transition from the excited state to the ground state. Optical characteristics of BR vary depending on the method of preparation of PM and the polymer matrix.

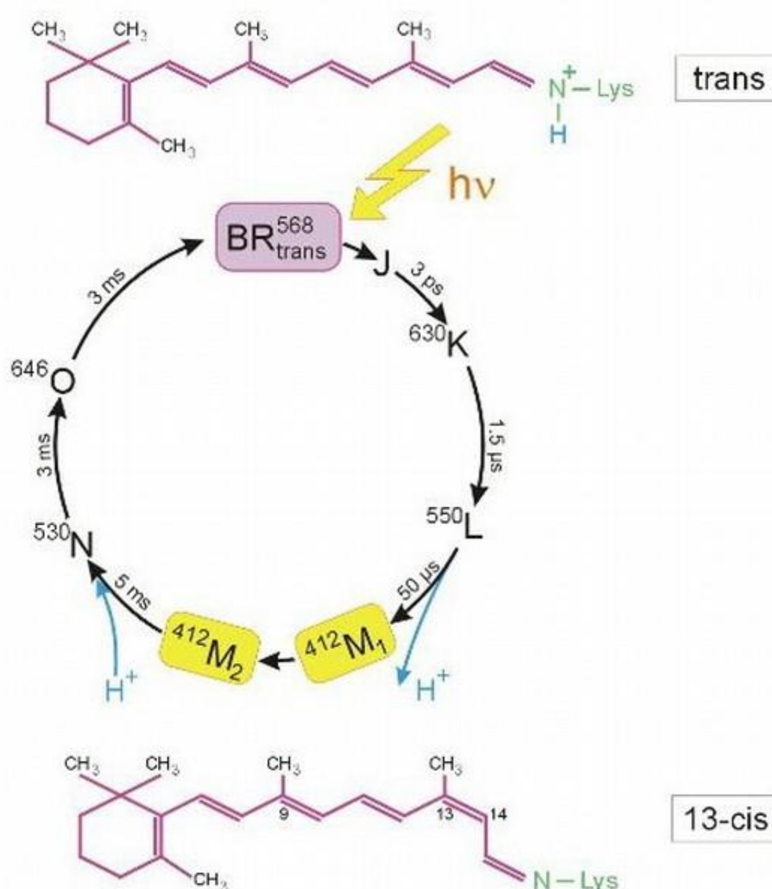

Figure 6. Photocycle scheme of BR (aqueous solution, pH = 7.2, t = 20 °C). Latin numbers J, K, L, M, N, O denote the spectral intermediates of BR. M_1 and M_2 represent spectral intermediants of meta- bacteriorhodopsin with the protonated and deprotonated aldimine bond. The superscripts correspond to the position of the absorption maximum of the photocycle intermediates (nm)

BR is the focus of bio-and nanotechnology because of its high sensitivity and resolution, and is used in molecular bioelectronics as natural photochromic material for light-controlled electrical regulated computer modules and optical systems [19, 20]. In addition, BR is very attractive as a model for studies related to the research of functional activity and structural properties of photo-transforming membrane proteins in the native and photo-converting membranes [21].

Nanofilms produced using the BR-containing purple membranes (PM) of halobacteria were first obtained and studied in this country in the framework of the project "Photochrome", when it was demonstrated effectiveness and prospects for the use of BR as photochromic material for holographic recording (Fig. 7). The main task for the manufacture of BR-containing nanofilms is the orientation of PM between the hydrophobic and hydrophilic media. Typically, to improve the characteristics of the BR-containing films use multiple layers of PM that are applied to the surface of the polymeric carrier and dried up, preserving their natural structure. The best results are achieved in the manufacture of nanofilms based on gelatin matrix [22]. This allows to achive high concentration of BR (up to 50 %) in nanofilms and avoid aggregation of membrane fragments and destruction of BR in the manufacturing process [23]. Embedded in a gelatin matrix PM fragments are durable (~10^4 h) and resistant to solar light, the effects of oxygen, temperatures greater than 80 °C (in water) and up to 140 °C (in air), *pH* = 1–12, and action of most proteases [24]. Dried PM are stacked on top of each other, focusing in the plane of the matrix, so that a layer with 1 μm thickness contains about 200 monolayers [25]. When illuminated such nanofilms exert the electric potential 100–200 mV, which coincides with the membrane potential of living cells [26]. These factors are of great practical importance for integration of PM into polymeric nanomatrix with keeping photochemical properties.

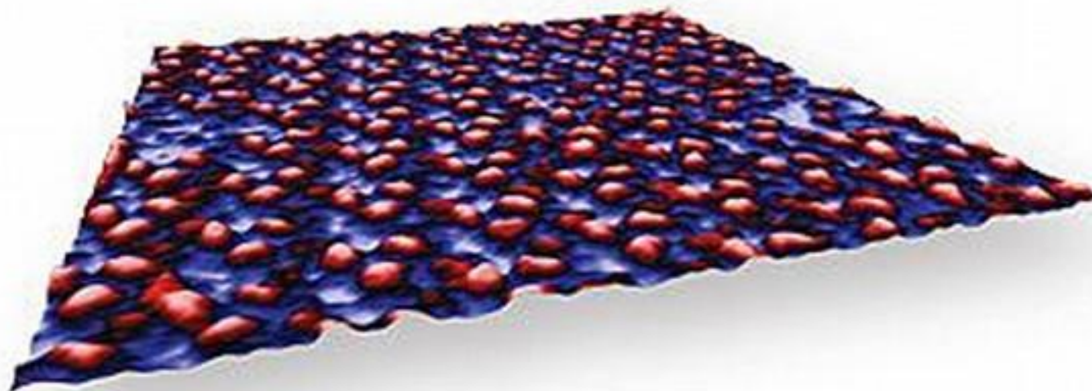

Figure 7. Artificial membrane from BR-containing PM in scanning electron microscope (SEM): scanning area – 100 × 100 mm, resolution – 50 nm, magnification – 100000 times. PM shown in purple, BR – in red color

Technology for preparation of BR consists in growing of halobacteria on liquid synthetic growth media (with 15–20 % (w/w) NaCl) with amino acids, or on natural growth media with peptons – mixtures of polypeptides and amino acids derived from the partial hydrolysis product or powdered milk, animal meat by proteolytic enzymes (pepsin, trypsin, chymotrypsin), or protein-vitamin concentrate of yeast [27]. The subsequent isolation of BR from purple membranes is carried out by a combination of physical, chemical and enzymatic methods [28]. Under optimal growing conditions (incubation period 4–5 days, temperature 35 °C, illumination with monochromatic light at λ = 560 nm) in cells are synthesized the purple carotenoid pigment, characterized as BR by the spectral ratio of protein and chromophore fragments D_{280}/D_{568} = 1,5:1,0 in the molecule.

Within the framework of the research we described an effective method for isolation of BR from PM of photo-organo-heterotrophic halobacterium *H. halobium* consisted by cellular autolysis by distilled water, processing of bacterial biomass by ultrasound at 22 KHz, llocation of PM fraction, purification of PM from low and high-molecular weight impurities, cellular RNA, carotenoids and lipids, PM solubilization in 0,5 % (w/v) solution of the ionic detergent SDS-Na to form a microemulsion with the subsequent fractionation of the protein by methanol [29]. The protein is localized in the PM; the release of low molecular weight impurities and intracellular contents is reached by osmotic shock of cells with distilled water in the cold after the removal of 4,3 M NaCl and the subsequent destruction of the cell membrane by ultrasound at 22 kHz. For the destruction of cellular RNA the cellular homogenate was treated with Rnase I. Fraction PM along with the desired protein in a complex with lipids and polysaccharides also contained impurity of related carotenoids and proteins. Therefore, it was necessary to use special methods of fractionation of the protein without damaging its native structure and dissociation.

BR being a transmembrane protein intricately penetrates bilipid layer in form of seven α-helices; the use of ammonium sulfate and other conventional agents to salting out did not give a positive result for isolation of the protein. The resolving was in the translation of the protein to a soluble form by the colloidal dissolution (solubilization) in an ionic detergent. Using as the ionic detergent SDS-Na was dictated by the need of solubilization of the protein in a native, biologically active form in complex with 13-*trans*-retinal, because BR solubilized in 0,5 % (v/v) SDS-Na retains a native α-helical configuration [30]. Therefore, there is no need the use organic solvents as acetone, methanol and chloroform for purification of lipids and protein, and precipitation and delipidization is combined in a single step, which significantly simplifies the further fractionation. A significant advantage of this method is that the isolated protein in complex with lipids and detergent molecules was distributed in the supernatant, and other high molecular weight impurities – in unreacted precipitate, easily separated by centrifugation. Fractionation of solubilized in 0,5 % (w/v) SDS-Na protein and its subsequent isolation in crystalline form was achieved at 4 ^{o}C in three steps precipitating procedure with methanol, reducing the concentration of detergent from 0,5; 0,25 and 0,1 % (w/v) respectively. The final stage of BR purification involved the separation of the protein from low-molecular-weight impurities by gel-permeation chromatography on dextran Sephadex G-200 Column balanced with 0,09 M Tris-borate buffer (*pH* = 8,35) with 0,1 % (w/v) SDS-Na and 2,5 mM EDTA (output of the protein 8–10 mg).

Absorption spectrum of PM purified from carotenoids (4) and (5) (chromatographic purity 80–85 %) is shown in Figure 8 at various processing stages (*b*) and (*c*) relative to the native BR (*a*). Formation of retinal-protein complex in the BR molecule leads to a bathochromic shift in the absorption spectrum of PM (Fig. 8*c*) – the main band with (1) with the absorption maximum at λ = 568 nm caused by the light isomerization of the chromophore by the C13=C14 bond is determined by the presence of 13-*trans*-retinal residue in BR^{568}; additional low-intensity band with (2) at λ = 412 nm characterizes a minor impurity of a spectral form of *meta*-bacteriorhodopsin (M^{412}) (formed in the light) with deprotonated aldimine bond between 13-*trans*-retinal residue and protein; the total bandwith (3) with λ = 280 nm is determined by the absorption of aromatic amino acids in the polypeptide chain of the protein (for native BR D_{280}/D_{568} = 1,5 : 1,0).

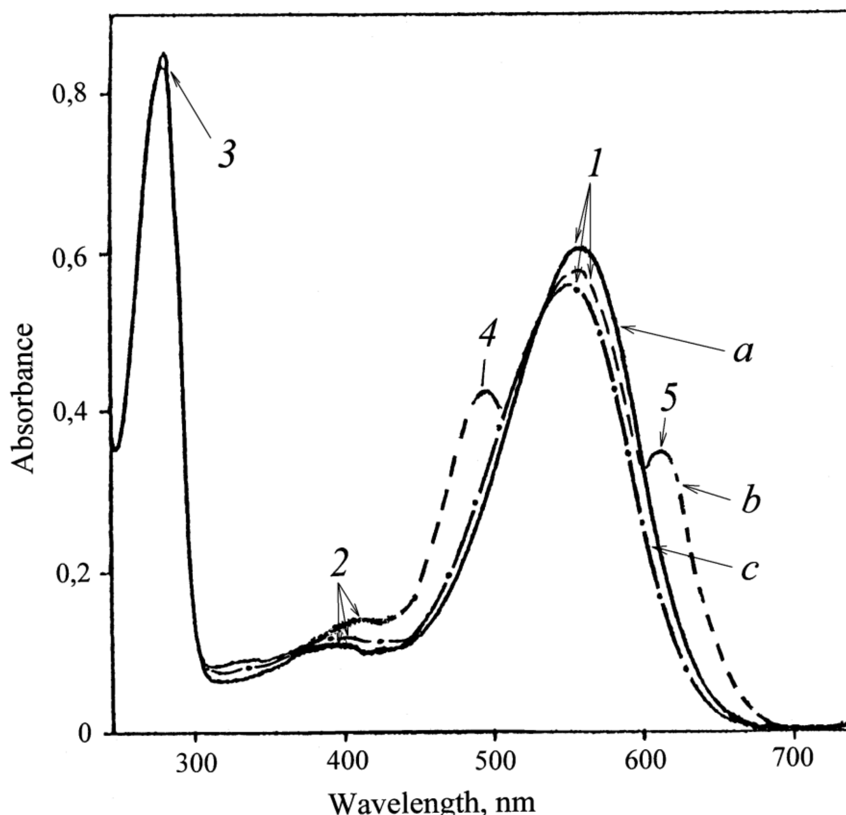

Figure 8. The absorption spectra of the PM (50% (v/v) ethanol) at various stages of processing: (a) – natural BR; (b) – PM after intermediate treatment; (c) – PM purified from carotenoids. The bandwith (1) is the spectral form of BR^{568}, (2) – impurity of spectral form of meta-bacteriorhodopsin (M^{412}), (3) – the total absorption bandwith of aromatic amino acids, (4) and (5) – extraneous carotenoids. As a control used the native BR

Iodopsin

Iodopsin is a violet, light-sensitive pigment of the retinal cone cells, responsible for color vision, and close analogue of rhodopsin. This pigment consists of a protein photopsin linked with a chromophore, retinal residue. According to the three-component theory of vision, it is believed that there have to be three types of this pigment and accordingly three types of cones that are sensitive to blue, green and red light. Iodopsin consists of three pigments – hlorolab, eritrolab and tsianolab. With the densitometry method W. Rushton studied the coefficient of light absorption in the photo layers of the retina with different wavelengths [31]. The hlorolab pigment absorbs the rays corresponding to yellow-green (450–630 nm absorption band), the eritrolab – yellow and red (λ = 500-700 nm), and the tsianolab – blue-green (λ = 500–700 nm) parts of the visible spectrum [32]. The different types of cones have not yet been found.

Color vision

The retina has three types of cone cells – S, M and L cells, having a different sensitivity to different parts of the visible range of the spectrum (Fig. 9). The cone cells of S type have a spectral range from λ = 400 to λ = 500 nm with a maximum peak at λ = 420–440 nm, the cone cells of M type – from λ = 450 to λ = 630 nm with a maximum peak at λ = 534–555 nm, while the cone cells of L type – from λ = 500 nm to λ = 700 nm with a maximum peak at λ = 564–580 nm. As the curves of the sensitivity of the cone cells overlap, it is impossible for monochromatic light to stimulate only one type of cone cells. The other types of cone cells react though to a lesser degree. The set of all possible values of the color combinations causing a visual reaction determines the human color space. Human brain generally can discern approximately 10 million of different colors.

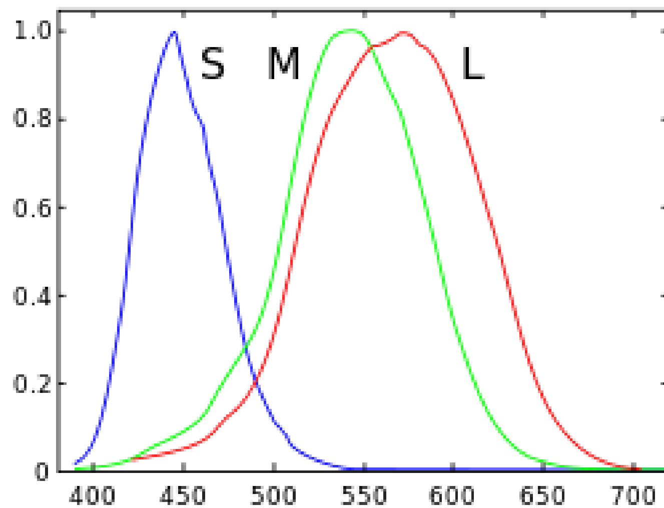

Figure 9. Spectral sensitivity of the different types of receptor cells (cones) in the retina

Additive mixing of colors

The electromagnetic waves spectrum stimulates the different types of cone cells from the three types S, L and M to a different degree. The red light stimulates the L cone cells more than the M cone cells. The blue light stimulates the S cone cells in the strongest way. The yellow-green light provides a strong stimulation to the L and M cone cells, and a weaker stimulation to the S cone cells. The brain then combines the information from all types of cone cells for different wavelengths and analyzes them as different colors.

The analyses for the activity of the three types of cones – S, L and M in the perception of colors also shows how the brain "deciphers" the colors. The foundation of this analysis, shown in Figure 10, was made by M. Marinov and I. Ignatov in 2008. However, it is not clear whether the green color we perceive is a combined effect of yellow and blue, or whether it corresponds to a wavelength of the green color from the visible spectrum. Our brain can register the colors, i.e. the green color as a spectrometer, with certain lengths of the electromagnetic waves. It can also register the green color as a mixture of yellow and blue. The full perception of colors by the visual analyzer cannot be defined by a spectrometer.

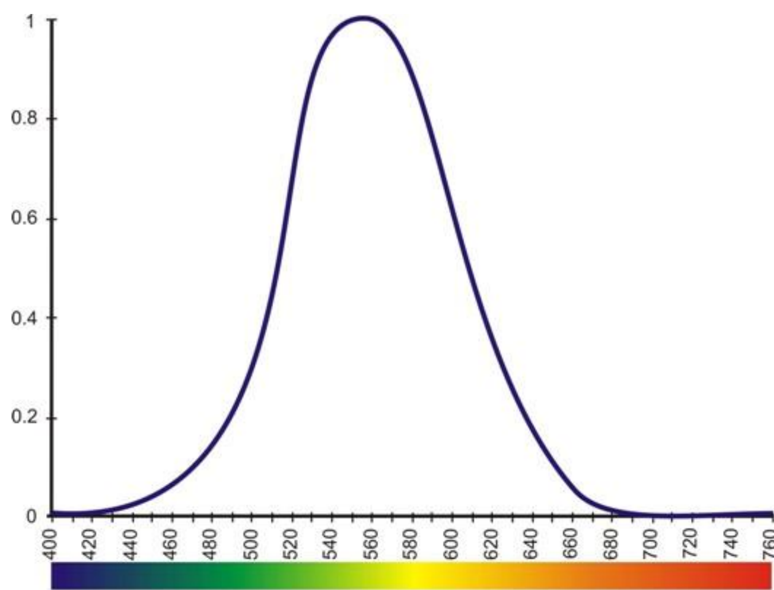

Figure 10. Spectral sensitivity of the visual analyzer

As an example in the mixing of electromagnetic waves that correspond to green and red color, yellow color is obtained. In the mixing of green and red, no medium color is obtained; the brain therefore perceives it as yellow color [33] (Ignatov, & Mosin, 2013). When there is an emission of electromagnetic waves that correspond to green and red color, the brain adopts an "average decision" – yellow (Fig. 11). Analogously, for the yellow and blue color, the brain adopts an "average decision" – green. This means that a spectral mixing of colors is observed between the blue-yellow and green-red pairs [34]. In its turn, green and blue color is perceived as cyan. Vision sensitivity is at its lowest for the violet, blue and red color. The mixing of electromagnetic waves that correspond to blue and red color is perceived as violet. In the mixing of electromagnetic waves that correspond to more colors, the brain does not perceive them as separate or average, but as a white color. Thus the notion of color is not determined solely by the wavelength. The analysis is performed by brain, and the notion of color is at its essence a product of our consciousness.

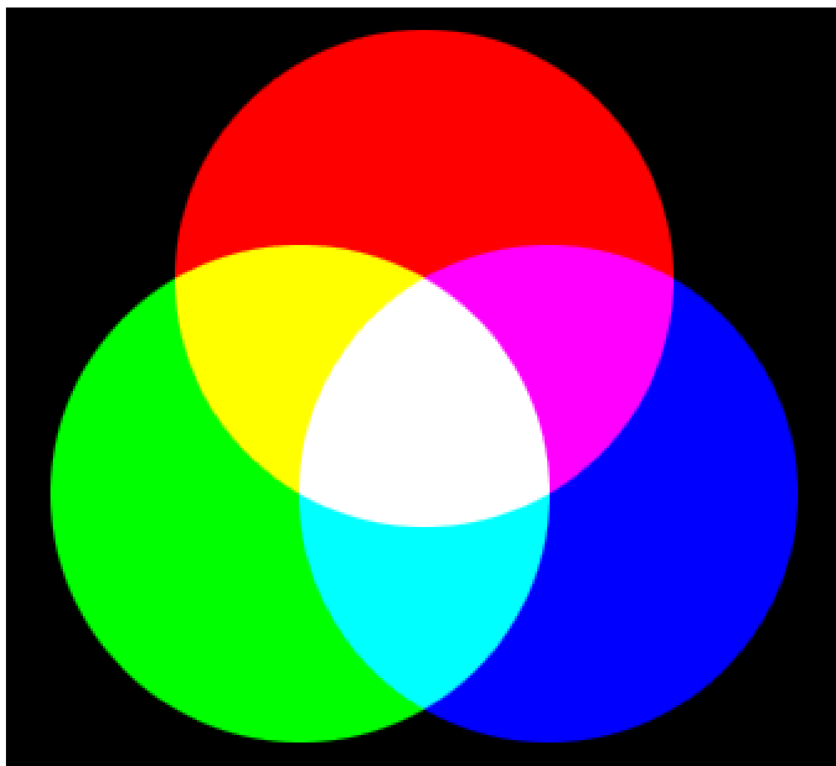

Figure 11. Additive mixing of colors

Conclusions

The mechanism of color perception by the visual analyzer have been carried out by the authors using photoreceptive chromo-protein rhodopsin as a model. A further research into the function of rhodopsin and other retina affiliated chromo-proteins as iodopsin will allow investigate in detail the mechanism of visual perception of light for better treatment of functional eye diseases in ophthalmology. It should be noted that rhodopsin up till now remains to be the most studied model chromoprotein of all GPCR-receptor family. This allowed us to better analyze the functional properties of another analogous trans membrane bacterial chromoprotein – bacteriorhodopsin isolated from purple membrane of halobacterium *H. halobium* in semi-preparative quantities, and study its application in nanotechnologies.

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Изучение фототрансформации светового сигнала фоторецепторными пигментами – родопсином, йодопсином и бактериородопсином

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Аннотация. В этой обзорной статье рассмотрены преимущественно структура и функции животных и бактериальных фоторецепторов пигментов (родопсин, йодопсин, бактериородопсин) и основные аспекты их нано- и биотехнологие использования. На примере бактериородопсина описан способ его выделения из пурпурных мембран аэробной экстремальной фотоорганотрофной галобактерии *Halobacterium halobium*, заключающийся в клеточном автолизе дистиллированной водой, обработкой бактериальной биомассы ультразвуком при 22 кГц, спиртовой экстракции низких и высокомолекулярных примесей,

клеточной РНК, каротиноидов и липидов, коллоидном растворении в 0,5 % SDS-Na, фракционированием белка метанолом и гель-фильтрационную хроматографию на колонке Sephadex G- 200, уравновешенной 0,09 М трис-боратным буфером (*pH* = 8,35) с 0,1 % SDS-Na и 2,5 мМ ЭДТА. В рамках работы был исследован механизма восприятия цвета зрительным аппаратом клетки, имеющим способность определять определенные диапазоны оптического спектра как цвета, а также был произведен анализ аддитивного смешения двух цветов. Было показано, что при смешении электромагнитных волн с различными длинами волн, зрительный анализатор воспринимает их в виде отдельной или средней длины волны, соответствующей смешанному цвету.

Ключевые слова: зрение; родопсин; йодопсин; бактериородопсин; адитивное смешение цвета.

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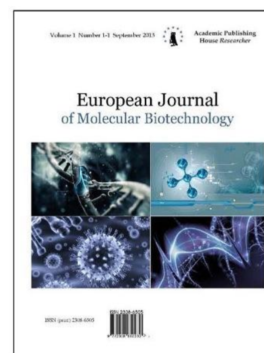

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The Research of Growth Capability Intensification and Lipolytic Activity of Fat Splitting Microbial Cultures by Influence of Natural Mineral Components

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Abstract

23 bacterial strains with lipase activity were separated from the waste water of the meat-processing enterprises and Volgograd water-handling facilities aeration tank. There were studied their morphological, cultural and biochemical properties and their animal and vegetable fat splitting ability. Comparative analysis of fat splitting ability has been carried out for searched strains and devices of enzyme activity increasing and growing process intensity of searched microorganisms were investigated. Mineral nature ingredients – the lake Elton brine, the bischofite, the Dead Sea salt – were added in the culture mediums for increasing of biomass accumulation. It has been investigated that addition of the brine into the culture medium in 5 % concentration increases the growth rate of producer lipase on 293 % in comparison with growth of the control medium without the brine. Addition of 0,25 % the Dead Sea salt concentration intensifies the growth on 317 % in comparison with the control medium. Laboratory simulation of fat waste water purification process has been realized with one of separated activity bacterial strains and it has been showed that addition of the 0,5 % bishofite into the fermenter stimulates lipase activity of the searched microorganisms.

Keywords: waste water; biological purification; fat biodegradation; lipase activity; bischofite; brine; the Dead Sea salt.

Введение

Развитие пищевых предприятий в России, повышение нагрузки на водоотводящие и очистные сооружения для промышленных и хозяйственно-бытовых стоков за последние годы, сделало особенно актуальным решение проблемы очистки сточных вод от масложировых загрязнений. Так, доля производственных сточных вод в общем объёме

стоков мясоперерабатывающего предприятия составляет 70–75 %. Из них около 50 % стоков содержат жировые загрязнения [1]. Недостаточная очистка стоков от жира приводит к «зарастанию» канализационных труб и коммуникаций, уменьшению их пропускной способности, появлению неприятных запахов вследствие гниения органических веществ, снижает эффективность работы очистных сооружений. Жиры представляют собой сложные органические вещества, зачастую поступающие в очистные сооружения в твердом состоянии и недоступные для усвоения активным илом аэротенков. Как показали исследования Московского государственного строительного университета, городские биологические очистные сооружения способны осуществлять биохимическую деструкцию жиров при совместном их присутствии в эмульгированном и растворенном виде, соответственно, в следующих концентрациях: 10 и 80 мг/л; 20 и 50 мг/л; 30 и 20 мг/л [1]. В связи с этим, сточные воды, поступающие на биологические очистные сооружения, должны иметь указанные показатели. Поэтому важнейшее значение приобретает локальная очистка стоков от масложировых примесей непосредственно на предприятиях перед сбросом их в городские коллекторы. В то же время механическое отделение твердого жира от стока значительно сокращает нагрузку на сооружения биологической очистки, но не приводит к решению проблемы утилизации жировой массы - забитые жиром колодцы, жироуловители требуют периодической откачки собранного жира и его переработки. Особенно серьезной проблемой является обработка осадков из отстойников — жиромассы и донных осадков [1]. Необходима разработка эффективных методов не только очистки сточной воды от жиров, но и утилизации и обезвреживания отделяемой от воды жировой массы.

Одним из перспективных способов решения указанных проблем является биоферментная технология разложения жировых загрязнений. Биоферментные технологии по разложению и утилизации жиров в сточных водах основаны на использовании микробных липаз и микроорганизмов, способных к их продуцированию. В настоящее время применение биопрепаратов, содержащих комплекс специально подобранных активных микроорганизмов-деструкторов, для очистки сточных вод становится все более масштабным. Однако, на рынке Российской Федерации представлены лишь несколько препаратов, в основном зарубежного происхождения [2]. Ассоциации микроорганизмов (грибов и бактерий), входящих в состав подобных биопрепаратов разлагают углеводороды, жиры, белки и углеводы, снижая ХПК и БПК воды, удаляют неприятные запахи, устраняют засоры в жироотводящих системах, поддерживая их длительное время в рабочем состоянии. Ферментные системы абсолютно безопасны для коммуникаций, арматуры, пластмассовых деталей, прокладок, не разрушают трубы. При регулярном и правильном применении они существенно продлевают срок эксплуатации системы сточной канализации. Использование биопрепаратов не требует применения дорогостоящего оборудования, отмечается сокращение объемов жировых отходов, образующихся при эксплуатации жироуловителей. Предприятиям, не имеющим очистных сооружений и сбрасывающим сточные воды в централизованную систему канализации, улучшение их качества позволяет уменьшить размеры штрафов [1].

Экспериментальные исследования свидетельствуют о том, что микробные липазы являются ферментами с широкой специфичностью и большим разнообразием свойств. Свойства липаз и характер липолитической активности даже у одного рода могут значительно варьировать. Изучение микробных липаз представляет большой теоретический и практический интерес, так как они могут быть использованы при гидролизе разнообразных жировых субстратов [3]. Липазы катализируют гидролиз жиров и масел с образованием диацилглицеридов, моноацилглицеридов, глицерина и жирной кислоты. Катаболизм включает три основных фазы превращения органических веществ органотрофами. В первой фазе с помощью экзоферментов бактерии гидролизуют липиды до жирных кислот и глицерина, которые могут легко транспортироваться в цитоплазму. Во второй фазе поступившие в цитоплазму органические вещества расщепляются до фрагментов, содержащих два-три углеродных атома. В третьей фазе эти соединения окисляются до углекислого газа и воды. [4]. Липазы можно разделить на две группы: специфичные и неспецифичные. Ферменты из первой группы гидролизуют сложноэфирные связи в первом или втором положении. Многие микробные липазы обычно гидролизуют первичные сложноэфирные связи (а-эфирные связи). В гидролизатах с участием таких

ферментов обычно обнаруживаются жирные кислоты, 2,3- и 1,2-диглицериды, 2-моноглицериды. При более длительных гидролизах жирнокислотный остаток из 2-моноглицерида мигрирует в первое положение с образованием 1-моноглицерида, который легко гидролизуется специфичной липазой с образованием глицерина и жирной кислоты. К этой группе относятся липазы из Rhizopus arrhizus, Rhizopus delemar, Rhizopus microsporus, Mucor miechei, Aspergillus niger, Pseudomonas sp. и т.д.

Липазы второй группы не различают эфирные связи во всех трех положениях триглицеридной молекулы и способны подвергать субстрат тотальному гидролизу. В гидролизатах триглицеридов с участием этих видов липаз обнаруживаются, как правило, остатки триглицеридов (негидролизованная часть), глицерин и жирные кислоты. Такие липазы были выделены из Geotrichum candidum, Oospora lactis, Humicola lanuginosa и т. д. Активность липаз зависит от длины цепочки и степени насыщенности жирной кислоты. Дженсон описал, что липаза Geotrichum candidum проявляла высокую специфичность к олеиновой и линолевой кислотам независимо от их положения в молекулах триглицеридов. Такими же свойствами обладают липазы из Achromobacter lipolyticum, тогда как липаза из Aspergillus niger проявляла большую специфичность к стеариновой кислоте и молекулам субстратов. [5]. Установлено также, что жирные кислоты, образующиеся в результате ферментативного гидролиза с участием Alcaligenes faecalis и Bacillus licheniformis, выделенных из сточных вод после процесса эмульсионного обезжиривания овчинно-шубного сырья, в дальнейшем вовлекаются в цикл трикарбоновых кислот и последующая их деструкция сопровождается уменьшением длины углеводородной цепочки и образованием более сильных кислот [6], [7].

Российскими учёными активно развивается данное направление исследований. Так, в работе [8] проведена сравнительная оценка 7 микроорганизмов: бактерий (Bacillus mesentericus, B.subtilis, Acinetobacter sp.), грибов (Aspergillus orysae, Penicillium orysae), дрожжей (Candida scottii, Yarrowia lipolytica), по показателям липолитической активности, содержанию клеточного белка и характеристикам роста на жиросодержащих питательных средах. Установлено, что дрожжи Y.lipolytica, обладающие лучшими ростовыми характеристиками, способны ассимилировать до 95 % жировых отходов от их общего содержания в среде, а образующаяся биомасса содержит не менее 42 % истинного белка. Кроме того, автором показано, что введение в питательную среду ионов калия и кальция оказывают стимулирующее действие на экзолипазу дрожжей, что позволяет повысить липолитическую активность на 4-5 %, а добавление перекиси водорода в концентрации 2,5 г/л к посевному материалу значительно ускорял процесс биоконверсии жировых отходов.

Современные биопрепараты для очистки сточных вод – это консорциумы микроорганизмов, выделенные методом накопительных культур обычно из активного ила аэротенков локальных или городских сооружений очистки сточных вод. Биопрепараты, содержащие ограниченное число видов микроорганизмов, по спектру разлагаемых веществ уступают свежему активному илу. Однако они содержат быстро растущие штаммы, которые инициируют процессы разложения органических загрязнений. В нестерильном процессе развиваются также микроорганизмы, содержащиеся в отходах, и в микробное сообщество включаются недостающие звенья [9].

Так, в работе [10] из образцов активного ила очистных сооружений мясокомбината выделена и идентифицирована группа новых штаммов бактерий рода Serratia, проявляющих высокую липолитическую активность. Автором установлено, что исследуемые микроорганизмы способны осуществлять 100% биодеградацию жиров растительного и животного происхождения. Показано также, что введение в питательную среду для культивирования липолитически активных микроорганизмов оптимальных концентраций соевой муки и автолизата дрожжей повышает липазную активность в 10–16 раз, а полная деструкция жировых загрязнений осуществляется за 96 ч. культивирования. Наиболее активный из изученных штаммов Serratia marcescens запатентован в качестве основы биопрепарата для очистки жиросодержащих сточных вод [11].

ООО «РСЭ-трейдинг» (г. Москва) разработан биопрепарат для очистки водоотводящих коммуникаций от жировых загрязнений, а также утилизации жиров, скопившихся в жироуловителях [12]. Биопрепарат Микрозим™ Гриз Трит производится промышленными

партиями в виде сухого спорового порошка на экологически чистом питающем носителе из кукурузной муки. Этот порошок и вносится в жироуловитель. Для удобства препарат можно вносить также через мойки и трапы. Биодеструктор жиров Микрозим™ Гриз Трит содержит от семи до 12 уникальных видов живых спорообразующих микроорганизмов, способных усваивать жиры, белки, углеводы и полисахариды и образовывать в качестве конечных продуктов воду, углекислый газ и легкий донный осадок. Препарат имеет рабочий диапазон pH от 4,25 до 10, диапазон рабочих температур 5...50˚ C (оптимально 10...40˚ C), одинаково эффективно действует как в анаэробных, так и в аэробных условиях.

Таким образом, биопрепараты, содержащие культуры липидорасщепляющих микроорганизмов могут найти широкое применение, как в бытовых условиях, так и в промышленности при локальной очистке сточных вод, содержащих разнообразные отходы жировой природы. Следовательно, поиск новых активных штаммов микроорганизмов, быстро и эффективно разлагающих различные жировые субстраты, в широких диапазонах температур, в условиях меняющегося химического состава сточных вод является весьма актуальной задачей.

Целью данной работы было выделение и изучение основных свойств микроорганизмов, способных утилизировать липиды, а также исследование возможности увеличения скорости роста липазопродуцентов под влиянием природных минеральных веществ.

Материалы и методы исследования

Липидорасщепляющие микроорганизмы выделяли из надосадочной жидкости активного ила городских очистных сооружений и смывов с технологического оборудования Волгоградского мясокомбината. Выделение жирорасщепляющих бактериальных штаммов производили на селективных плотных питательных средах, содержащих в качестве единственного источника углерода свиной или говяжий жир, а также минеральные соли [12].

Методика выделения микроорганизмов заключалась в высеве проб сточной воды и надосадочной жидкости активного ила в объеме 1 мл на приготовленные селективные среды. Посевы инкубировали в течение 24 ч при 27°С и 48 ч при комнатной температуре (18°С), после чего проводили визуальный анализ выросших колоний. Изолированные колонии бактериологической петлей были отсеяны на скошенный агар для выделения чистых культур микроорганизмов.

С целью исследования культуральных свойств из чистых культур с помощью бактериального стандарта мутности готовили взвеси с концентрацией 10^9 микробных клеток (м.к.) в 1 мл, которые были десятикратно разведены в физиологическом растворе и высеяны на пластинки питательного агара в чашки Петри для получения изолированных колоний. Культуральные свойства выделенных штаммов оценивали, анализируя внешний вид колоний (поверхность, размер, цвет, характер края, наличие складчатости).

Морфологические свойства культур определяли по результатам окраски по Грамму и микроскопирования в проходящем свете оптического микроскопа МЛ-1 (ЛОМО, г. Санкт-Петербург).

Липолитические свойства выделенных бактериальных штаммов подтверждали выращиванием на среде, содержащей твин 80 (полиоксиэтилен сорбитан моноолеат), и оценивали по результатам роста в бульоне Штерна, в котором в качестве единственного источника углерода использовали оливковое масло с концентрацией 1 мл в 100 мл бульона. Бактерии, обладающие липолитическими свойствами, при ферментации оливкового масла выделяют альдегиды, подкисляя среду. Стерильный бульон Штерна разливали в пробирки по 10 мл в каждую и вносили исследуемые культуры в объеме 0,1 мл с концентрацией 10^9 м.к. в 1 мл. Пробирки с микроорганизмами термостатировали при температуре 27°С в течение 120 ч и проводили наблюдение за изменением pH бульона Штерна с помощью pH-метра (pH/ORP Meter HI 2215). В качестве контрольного варианта использовали бульон Штерна без внесения в него микроорганизмов.

Для изучения влияния природных минеральных материалов на интенсивность роста и накопления биомассы готовили питательные среды, содержащие в качестве источников углерода и азота 2% глюкозы и 2% нитрата аммония, соответственно, а также различные

концентрации рапы оз. Эльтон, бишофита Волгоградского месторождения и солей Мёртвого моря (Израиль). Среды автоклавировали при 0,5 атм. в течение 20 мин. Для оценки влияния минеральных добавок на интенсивность роста липазопродуцентов пользовались фотоколориметрическим методом, сравнивая оптические плотности суточных суспензий микроорганизмов в экспериментальных и контрольной средах на приборе КФК-2-УХЛ-4.2 при длине волны светофильтра 670 нм в кюветах с длиной оптического пути 5,065 *мм*. В качестве контрольной использовали питательную среду, содержащую только глюкозу и нитрат аммония.

Рапа озера Эльтон (Волгоградская область) относится к бромным крепким рассолам хлоридного и магниево-натриевого состава. В рапе преобладают галит – около 56 %, бишофит – около 29 %, содержатся также кизерит, карналлит и др. соли [13]. Из катионов в рапе содержатся: литий, аммоний, калий, натрий, магний, кальций. Из анионов присутствуют хлорид, бромид, сульфат, гидрокарбонат. Реакция среды рапы нейтральная (pH=7,1).

Волгоградский природный бишофит представляет собой спрессованный под высоким давлением вышележащих слоев камень. По химико-минеральному составу — это комплекс солей и микроэлементов (Табл. 1) [14].

Таблица 1

Химический состав Волгоградского природного бишофита

Наименование вещества	Химическая формула	Содержание, %масс.
Хлористый магний	$MgCl_2 \cdot 6H_2O$	90-96
Хлористый калий-магний	$KCl \cdot MgCl_2 \cdot 6H_2O$	0,1-5,5
Сернокислый магний	$MgSO_4 \cdot 6H_2O$	0,1-2,5
Бромистый магний	$MgBr_2$	0,4-0,95
Сернокислый кальций	$CaSO_4 \cdot 2H_2O$	0,1-0,7
Хлористый натрий	NaCl	0,1-0,4
Бор	B	0,002-0,08
Кальций	Ca	0,003-0,005
Висмут	Bi	0,0005-0,001
Молибден	Mo	0,005-0,001
Железо	Fe	0,003-0,005
Алюминий	Al	0,001-0,02
Титан	Ti	0,005-0,001
Медь	Cu	0,0001-0,003
Кремний	Si	0,02-0,2
Барий	Ba	0,0001-0,0006
Стронций	Sr	0,001-0,02
Рубидий	Rb	0,0001-0,002
Цезий	Cs	0,0001-0,001
Литий и др.	Li	0,0001-0,0003

Соли Мертвого моря добываются на северо-западной части акватории Мертвого моря, вблизи курорта Калья, государство Израиль. Соль получают из воды Мертвого моря путем выпаривания. Химический состав соли Мертвого моря включает 26 минералов и микроэлементов: NaCI 14–16 %, KC1 18–22%, $MgCI_2$ 25–31 %, $CaCI_2$ 0,5–1 %, бромиды 0,2–0,3 %, кристализационная вода 26—32 %, нерастворимые компоненты (железо, фтор) 0,2 % и такие микроэлементы, как медь, цинк, кобальт (их содержание менее 1 мг/кг).

Для глубинного культивирования липолитически активного бактериального штамма использовали лабораторный ферментёр марки LKB BIOTEK POLYFERM 1607 ёмкостью 1 л, куда вносили 150 мл питательной среды (контрольной или содержащей бишофит), 1,5 мл оливкового масла (1 % от объёма среды) и 5 мл бактериальной взвеси из суточной культуры, приготовленной по стандарту мутности на 10 единиц. Все компоненты заранее стерилизовали в автоклаве при 0,5 атм. Культивирование проводили в течение 3 суток при 20°C, постоянных аэрации и перемешивании. В ходе эксперимента ежедневно осуществляли

мониторинг pH культуральной жидкости, отбирая пробы и определяя величину водородного показателя.

Результаты и обсуждение

В результате первичного анализа культуральных и морфологических свойств микроорганизмов, выросших на экспериментальных селективных средах, было выделено 23 бактериальные культуры: 6 штаммов – из надосадочной жидкости активного ила городских очистных сооружений и 17 культур – из смыва с производственного оборудования Волгоградского мясокомбината. Из выделенных культур были отобраны штаммы, характеризующиеся максимальными скоростями роста на селективных средах при комнатной температуре, и изучена их липолитическая активность с использованием бульона Штерна. При культивировании изучаемых микроорганизмов в бульоне Штерна наблюдали изменение цвета культуральной жидкости и фиксировали изменение активной реакции среды. Полученные данные сведены в таблицу 2.

Таблица 2

Изменение pH культуральной жидкости при культивировании в бульоне Штерна микроорганизмов, выделенных на селективных жиросодержащих средах

Источник	№ штамма	Изменение pH	
		24 ч	48
Мясокомбинат	2	7,04	7,00
	4	7,04	7,06
	6	5,25	5,12
	8	7,07	7,15
	16	7,49	7,83
	17	7,21	7,28
Активный ил	1	5,82	5,68
	2	6,98	7,49
	3	6,02	5,76
	4	7,12	7,59
	5	7,15	7,31
	6	7,25	7,67

Как свидетельствуют данные, представленные в таблице 2, у всех штаммов микроорганизмов в процессе культивирования в бульоне Штерна наблюдалось изменение pH культуральной среды. При выращивании штаммов № 4, 8, 16, 17, выделенных из смывов на мясокомбинате и штаммов № 2, 4, 5, 6, выделенных из активного ила, наблюдали повышение кислотности среды в течение всего времени выращивания бактерий. Штаммы № 2, 6 (мясокомбинат) и № 1 и 3 (активный ил) в процессе культивирования в течение 2 суток снижали pH культуральной среды за счет образования жирных кислот и альдегидов в результате деструкции жиров. Полученные данные позволили выбрать наиболее активные деструкторы жира в каждом из источников выделения. Наиболее высокой липолитической активностью обладают штамм № 6, выделенный из смывов с оборудования мясокомбината, и штамм № 1, полученный из надосадочной жидкости активного ила городских очистных сооружений.

Культуральные и морфологические свойства наиболее активных бактерий-жиродеструкторов приведены в таблице 3.

Таблица 3

Культуральные и морфологические свойства липолитически активных бактериальных штаммов

Источник выделения	Культуральные свойства				Морфологические свойства
	цвет	край	поверхность	размер, мм	
Очистные сооружения	бежевый	ровный	выпуклая, слизистая	2-3	грам (–) палочки
Мясокомбинат	светло-бежевый	ровный	гладкая, блестящая	4-5	грам (+) короткие палочки

Для выбора липазопродуцента с наибольшими ростовыми характеристиками были приготовлены жидкие питательные среды, содержащие свиной жир в концентрациях (%) 0,5; 1,0; 1,5; 2,0 и минеральные соли. Стерилизацию сред проводили в автоклаве при 120°С в течение 15 мин. С целью получения мелкодисперсной жировой эмульсии, доступной для утилизации микробной клеткой, питательные среды подвергали ультразвуковому воздействию при частоте 44 кГц и силе тока 0,54 А. Полученные среды засевали взвесями наиболее липолитически активных микроорганизмов, посевы инкубировали в течение 24 ч при температуре 27 °С. Концентрацию биомассы определяли макрокультуральным методом. Результаты экспериментов представлены в таблице 4.

Таблица 4

Определение концентрации биомассы липолитических штаммов

Источник выделения	Содержание жира, %	Концентрация биомассы, м.к./мл
очистные сооружения	0,5	$17 \cdot 10^3$
	1,0	$181 \cdot 10^3$
	1,5	$176 \cdot 10^3$
	2,0	$1 \cdot 10^3$
мясокомбинат	0,5	$7 \cdot 10^3$
	1,0	$26 \cdot 10^3$
	1,5	$10 \cdot 10^3$
	2,0	$4 \cdot 10^3$

Данные, приведенные в таблице 4, свидетельствуют, что наибольший выход биомассы на селективной питательной среде, содержащей эмульгированный свиной жир, дает бактериальный штамм, выделенный из надосадочной жидкости аэротенка городских очистных сооружений Волгограда.

В ходе дальнейших исследований для изучения возможности интенсификации роста полученных бактериальных штаммов и повышения их липазной активности изучали влияние природных минеральных веществ на скорость роста и накопления биомассы штамма № 6, выделенного из смывов с технологического оборудования Волгоградского мясокомбината. Ранее нами были получены данные о стимулирующем влиянии бишофита и рапы оз. Эльтон на ростовые процессы ассоциации микроорганизмов активного ила городских очистных сооружений [14, 16]. Для решения поставленной задачи микроорганизмы культивировали на питательных средах, содержащих различные концентрации рапы оз. Эльтон, солей Мертвого моря и бишофита. Интенсивность накопления бактериальной массы в средах с различными концентрациями минеральных добавок сравнивали с интенсивностью накопления биомассы в контрольных питательных

средах фотоколориметрическим методом, рассчитывая коэффициент прироста биомассы по формуле:

$$K = \frac{D_{оп.}}{D_{контр.}} \cdot 100\%,$$

где $D_{оп.}$ – оптическая плотность суточной суспензии бактериальных клеток в среде с минеральной добавкой, усл. ед.; $D_{контр.}$ - оптическая плотность суточной суспензии бактериальных клеток в контрольной среде, усл. ед.

Полученные данные в виде графических зависимостей коэффициента прироста биомассы микроорганизмов (К) от концентрации минеральной добавки в питательной среде приведены ниже на рисунке.

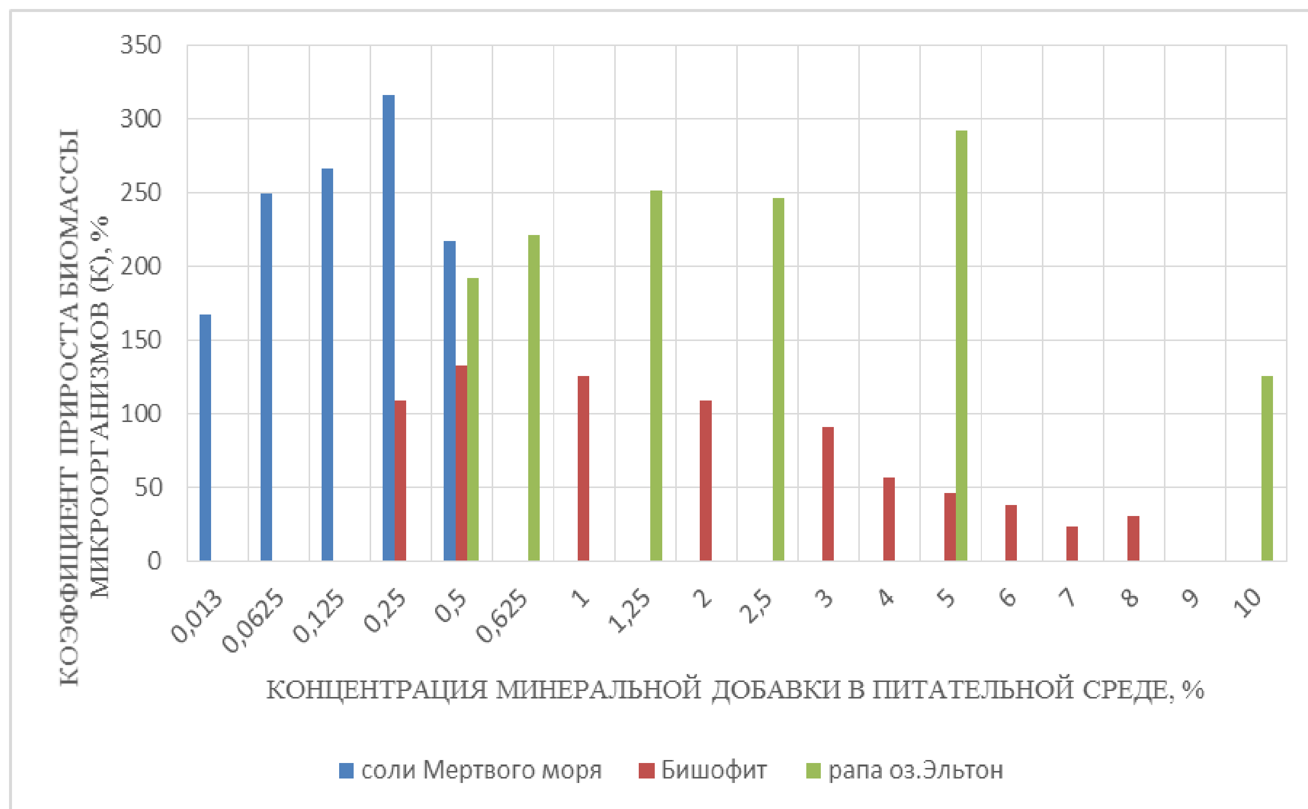

Рисунок. Зависимость коэффициента прироста биомассы липолитически активного бактериального штамма, выделенного из смывов мясокомбината, от концентрации минеральных добавок в питательных средах

Анализ полученных зависимостей показывает, что максимальный прирост биомассы изучаемых микроорганизмов наблюдается при введении в питательную среду солей Мёртвого моря в концентрации 0,25 % (вес.). В этих условиях коэффициент прироста бактериальной массы, по сравнению с контрольной средой, составляет 317 %. Введение в культуральную жидкость рапы озера Эльтон обеспечивает максимальный прирост биомассы (293 %) при концентрации рапы 5 % (вес.), а оптимальная концентрация бишофита составляет 0,5 % (об.), обеспечивая прирост бактериальной массы 133 % по сравнению с контрольной средой. Таким образом, все исследуемые природные минеральные вещества перспективны в качестве минеральных добавок к питательным средам для культивирования липидорасщепляющих бактерий. Однако рапа оз. Эльтон и бишофит существенно доступнее (оба ресурса добываются в Волгоградской области), чем соли Мёртвого моря.

На следующем этапе исследований была изучена возможность повышения липолитической активности штамма № 6 при глубинном культивировании бактерий на среде, содержащей оливковое масло в качестве единственного источника углерода, в присутствии оптимальной концентрации бишофита, установленной в ходе

предварительного эксперимента. Для оценки липолитической активности в ходе трёхсуточного культивирования микроорганизмов ежедневно производили измерения рН культуральной жидкости. Снижение величины водородного показателя свидетельствует о накоплении в среде культивирования жирных кислот, снижающих величину рН. Результаты, полученные в ходе данного эксперимента, сведены в таблицу 5.

Таблица 5

Изменение активной реакции культуральной жидкости в ходе культивирования липидорасщепляющих микроорганизмов на экспериментальной и контрольной средах

Время культивирования, ч	рН экспериментальной среды	рН контрольной среды
0	6,46	6,31
24	5,08	5,83
48	5,01	5,70
72	4,89	5,70

Приведенные в таблице 5 данные свидетельствуют о том, что при культивировании исследуемого микроорганизма на питательной среде, содержащей 0,5 % (об.) бишофита, в первые сутки рН культуральной жидкости снижается на 1,34 единицы, в то время как изменение рН контрольной среды составило 0,48 единиц. Полученные данные доказывают стимулирующее действие бишофита на липолитическую активность исследуемого бактериального штамма. Это подтверждается также дальнейшим (в течение 72 ч.), хотя и не таким значительным, как в первые 24 ч., снижением рН культуральной жидкости при выращивании исследуемых микроорганизмов на питательной среде с добавлением бишофита.

Таким образом, в ходе проведенных исследований доказан стимулирующий эффект, который оказывают природные минеральные вещества – рапа озера Эльтон, соли Мёртвого моря и бишофит – на скорость роста и накопления биомассы липидорасщепляющих бактерий, а также на их липолитическую активность при глубинном культивировании и оптимальном содержании бишофита в питательной среде. Выделенные бактериальные культуры являются перспективными для дальнейших исследований с целью создания бактериального препарата, эффективно разлагающего различные жировые загрязнения сточных вод. А природные минеральные вещества могут стать ценными компонентами готовых форм подобных бактериальных препаратов.

Заключение

Из надосадочной жидкости активного ила городских очистных сооружений и смыва с производственного оборудования Волгоградского мясокомбината выделены 23 бактериальные культуры, обладающие липидорасщепляющими свойствами. В результате исследования липолитической активности выделенных культур отобраны наиболее активные штаммы, изучены их культуральные и морфологические свойства. Изучено влияние добавок рапы озера Эльтон, солей Мёртвого моря, бишофита Волгоградского месторождения на скорость роста и накопления биомассы наиболее активных липидорасщепляющих штаммов. В ходе исследования были определены оптимальные концентрации природных минеральных веществ, стимулирующие рост изучаемых бактерий. Установлено, что введение в культуральную жидкость бишофита в концентрации 0,5 % (об.) при глубинном выращивании микроорганизмов существенно стимулирует липолитическую

активность бактериального штамма, выделенного из смывов с производственного оборудования мясокомбината.

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Исследование возможности интенсификации роста и липолитической активности липидорасщепляющих микробных культур под влиянием природных неорганических веществ

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Аннотация. Из сточных вод мясокомбината и аэротенка городских водоочистных сооружений выделено 23 бактериальные культуры, обладающие липазной активностью. Изучены их морфологические, культуральные, биохимические свойства, в том числе способность расщеплять животные жиры и растительные масла. Проведен сравнительный анализ способности выделенных бактерий разлагать жировые загрязнения сточных вод и изучены способы увеличения ферментативной активности и интенсивности ростовых процессов исследуемых микроорганизмов. Для повышения скорости накопления биомассы липазопродуцентов в питательные среды вводили добавки природных минеральных веществ (рапы оз. Эльтон, бишофита, солей Мёртвого моря). В ходе исследований установлено, что введение в питательную среду 0,5 % рапы озера Эльтон способствует повышению скорости роста липазопродуцента на 293 % по сравнению с ростом в контрольной среде, не содержащей рапы, а добавление 0,25 % солей Мёртвого моря интенсифицирует рост на 317 % по сравнению с контролем. Осуществлено лабораторное моделирование процесса очистки маслосодержащей воды с помощью одного из выделенных бактериальных штаммов и показано, что введение в ферментёр бишофита в концентрации 0,5 % (об.) стимулирует липазную активность исследуемых микроорганизмов.

Ключевые слова: сточные воды; биологическая очистка; биодеградация жиров; липазная активность; бишофит; рапа; соли Мертвого моря.

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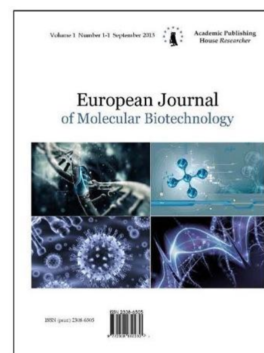

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Evolution, Metabolism and Biotechnological Usage of Methylotrophic Microorganisms

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Abstract

Methylotrophs – aerobic chemoheterotrophic microorganisms submitted by cocci and bacilli mobile forms, are inhabitants of reservoirs and soils of various type, where there are going on various processes of decomposition of organic substances with formation of the one-carbon C_1-compounds and some C_2-, and C_3-compounds, capable to be assimilated by methylotrophs. These microorganisms assimilating carbon on ribuloso-5-monophospate and serine pathways, are allocated from soil ground, the sewage containing decomposing vegetative remainss, from ruminant paunch and other sources. Methylotrophic bacteria recently draw the increasing attention of biotechnology as feasible sources of natural biologically active compounds – fodder fibers and irreplaceable amino acids, carotenoid pigments, lipids and policarbohydrates. For preparation of these compounds are used genetically marked strains of methylotrophic bacteria, obtained *via* genetic engineering approaches and selection. Recently developed gene-engineering methods of manipulation with the methylotrophic genom allow create on the basis of microbic DNA of methylotrophs expression vectors of eukaryotic proteins for medical and veterinary purposes, as human insulins. In this review article there are submitted data including the results of the authors' own research on evolution of methylotrophic bacteria, the metabolism and their biotechnological usage.

Keywords: methylotrophic microorganisms; evolution; metabolism.

Introduction

Methylotrophs – is a taxonomic heterogeneous group of microorganisms presented by chemoheterotrophic obligate and facultative methylotrophic bacteria and yeasts capable of assimilating carbon *via* ribulose-5-monophosphate and serine pathways of assimilation of more reduced than CO_2-carbon C_1-compounds – formaldehyde (HCOH), formic acid (HCOOH), and compounds containing either a methyl group (CH_3) or two or more methyl groups that are not directly connected with each other, as dimethyl ether CH_3-O-CH_3 [1]. In nature this class of

compounds is most widely occurred in natural gas, methane (CH_4), which is found in deposits of coal, oil and synthesized in large amounts by methane-forming bacteria under anaerobic conditions. The decay of pectins and other natural organic substances containing methyl esters producing methanol (CH_3OH) and ethanol (CH_3CH_2OH), which is also a substrate for growth of methylotrophic bacteria. The tissues of plants and animals contain other substrates for methylotrophic bacteria – methyl formate (CH_3COOH), methylamines (CH_3NH_2), dimethylamines $(CH_3)_2NH$, trimethylamines $(CH_3)_3N$) and their oxides.

Assimilation of C_1- compounds by microbial cell is almost always paired with breathing and, therefore is realized by strict aerobes. The only exception is the assimilation of methanol by methane-producing bacteria under anaerobic conditions. The ability to oxidize methane belongs mostly to prokaryotes. Among anaerobes such ability has methane-producing archaea, sulfate-reducing eubacteria, and some chemotrophic and phototrophic eubacteria. Methanol can also be used as a substrate for some methylotrophic yeasts.

Methylotrophs family comprises obligate and facultative aerobic eubacteria, which possess the ability to use one-carbon compounds as a source of carbon and energy. Further enumeration of such microorganisms is wide. They are represented by various Gram-positive and Gram-negative forms – representatives of the families of *Pseudomonas*, *Bacillus*, *Hyphomicrobium*, *Protaminobacter*, *Arthrobacter*, *Nocardia* and others [2]. At present time methylotrophs are found among representatives of the following taxonomic genera: *Acidomonas, Afipia, Albibacter, Aminobacter, Amycolatopsis, Ancylobacter, Angulomicrobium, Arthrobacter, Bacillus, Burkholderia, Flavobacteria, Granulibacter, Hansschlegelia, Hyphomicrobium, Methylarcula, Methylibium, Methylobacillus, Methylobacterium, Methylohalomonas, Methyloligella, Methylonatrum, Methylophaga, Methylophilus, Methylopila, Methylorhabdus, Methylorosula, Methylotenera, Methyloversatilis, Methylovirgula, Methylovorus, Mycobacterium, Paracoccus, Pseudomonas, Xanthobacter*.

True methylotrophic bacteria belong to the family of *Methylococcaceae*, including genera of *Methylococcus*, represented by vegetative cells resembling cocci and coccobacilli (0,7–1,5×1,0–1,5 μm) and *Methylomonas*, submitted by monads and rod-shaped cells (0,5–1,0×0,7–2,0 μm). The main taxonomic character while allocating this family is the morphological characteristics of the cells and the ability to use methane as a sole source of carbon and energy under aerobic conditions [3]. Methylotrophs, assigned to the family of *Methylococcaceae*, are gram-negative eubacteria with different morphology and cell's size, which form moving or still forms. Some strains of this taxonomic family are able to form cysts.

Interest for the study of methylotrophs is associated not only with the characteristics of their metabolism, but also with the prospects of their practical use in biotechnology as producers of high-grade feed protein and essential amino acids, as leucine and phenylalanine [4], and their isotopically labeled analogues as well [5]. The digestibility of biomass of methylotrophic bacteria amounts 85–98 %, while the productivity measured by a conversion of methanol makes up 37,6–67,5 % [6] (Table 1). As we have shown, owing to strong growth in minimal salt media with methanol, high yields of biomass and bioconversion of methanol into the components of the cellular biomass (with conversion efficiency of 15,5–17,3 dry biomass per 1 g of consumed substrate) methylotrophic bacteria are considered as a cheap source of deuterated protein and amino acids [7]. The traditional approach for this is the growth of methylotrophic bacteria on minimal growth M9-media with 2 % (v/v) $C^2H_3O^2H$ and 98 % (v/v) ^2H_2O (Table 2). The profitability for obtaining the microbial protein is determined for methylotrophs, mainly by the cost of such inexpensive and available substrate, as is methanol.

Table 1: Growth parameters of different methylotrophic bacteria [6]

Bacterial strains	The molar yield of dry biomass, g/mol of methanol	The specific growth rate, h^{-1}	The level of carbon conversion of methanol, %	The quantity of consumed nitrogen,%
Ribulose-5-monophosphate pathway of carbon assimilation				
Pseudomonas C.	17,3	0,49	67,5	13,2

Pseudomonas methanolica	17,0	0,63	66,5	11,0
Methylomonas methanolica	15,7	0,52	62,0	11,7
Serine pathway of carbon assimilation				
Pseudomonas 1	12,1	0,17	47,5	11,37
Pseudomonas 135	12,1	0,14	47,5	11,48
Pseudomonas AM1	9,8	0,09	37,6	11,20
Pseudomonas M-27	13,1	0,11	51,0	9,40
Pseudomonas roseus	13,1	0,15	51,0	10,60

Table 2: Amino acid composition of the protein hydrolyzate of facultative methylotrophic bacteria *Brevibacterium methylicum 5652* obtained from maximally deuterated M9-medium with 2 % (v/v) $C^2H_3O^2H$ and 98 % (v/v) ^2H_2O and levels deuteration molecules [7]

Amino acid	Yield, % of dry weight of 1 kg of biomass of		Number of deuterium atoms incorporated into the carbon skeleton of the molecule *	The level of deuteration of molecules, % of the total number of hydrogen atoms **
	Protonated sample (control)	The sample obtained in 98 % (v/v) ^2H_2O		
Gly	8,03	9,69	2	90,0
Ala	12,95	13,98	4	97,5
Val	3,54	3,74	4	50,0
Leu	8,62	7,33	5	49,0
Ileu	4,14	3,64	5	49,0
Phe	3,88	3,94	8	95,0
Tyr	1,56	1,83	7	92,8
Ser	4,18	4,90	3	86,6
Thr	4,81	5,51	ND	ND
Met	4,94	2,25	ND	ND
Asp	7,88	9,59	2	66,6
Glu	11,68	10,38	4	70,0
Lys	4,34	3,98	5	58,9
Arg	4,63	5,28	ND	ND
His	3,43	3,73	ND	ND

** When calculating the level of deuteration protons (deuterons) at COOH- and NH_2-groups of amino acid molecules were not considered due to their easy dissociation and isotopic (^1H–^2H) exchange in H_2O/^2H_2O.
*** ND – no data.

Industrial value has also biotransformation carried out by methylotrophs: immobilized bacteria, cell extracts and purified enzymes for C_1-oxidation compounds, especially methanol dehydrogenase, catalyzing the oxidation of organic compounds with short chain aromatic and acyclic hydrocarbons, phenols, alcohols, and heterocyclic hydrocarbons may be used for biotransformation [8]. Biotransformation results in obtaining a product having commercial value, for example, for obtaining propylene oxide from propylene and the substrate for the synthesis of synthetic polymers. Under certain conditions of growing the methylotrophic bacteria up to 60 % of their biomas makes up of poly-β-hydroxybutyrate – a biopolymer having big commercial value as a substitute for plastics [9].

The content of phospholipids – cardiolipin, phosphatidylcholine and phosphatidylethanolamine in cell membranes of methylotrophic bacteria reaches 8–10 % (w/w) by weight of dry biomass, which makes it possible to use methylotrophic bacteria as inexpensive sources of phospholipids for the cosmetics industry, medicine and diagnostic purposes [10] Furthermore, some methylotrophs are sources of cytochrome C that allows to replace the precious drug used in cardiology – cytochrome C, the source of which is the mammalian cardiac muscle [11].

Methylotrophs can also serve as a basis for creating genetically engineered producer strains of eukaryotic proteins for medical and veterinary usage. Thus the levels of expression of some eukaryotic genes, e.g., interferon α_1 and α_f in methylotrophic cells are higher than those ones in *E. coli* [12].

In addition, methylotrophs are capable of synthesizing the various natural pigments (melanins, carotenoids, prodiginines) carrying out the various protective functions in the cell [13]. Pigments of methylotrophic bacteria are of interest in terms of their practical use: carotenoids are used as pro-vitamins as part of feed additives and natural food colors. Melanogenny methylotrophs can be used to produce dihydroxy-phenylalanine, anticancer, radioprotective and humic substances. Currently, interest for the using of methylotrophic bacteria and components of their cell biomass in biotechnology is increasing due to the development of new technologies for chemical synthesis of methanol.

The purpose of this review article is to examine the data on the evolution of methylotrophic bacteria, the metabolism and their biotechnological application.

Results and discussion

Obligate methylotrophs

On the ability to utilize carbon methylotrophs are divided into two major taxonomic sub-groups of microorganisms – obligate and facultative methylotrophs [14]. Obligate methylotrophs are able to grow only on methane and C_1-compounds; of other substrates their growth can maintain only methanol and dimethyl ether. On the contrary, facultative methylotrophs are able to grow not only on methanol and methylamine, but on methane and some polycarbon C_n-compounds. Often they also grow on formic acid and on a small number of simple C_2- and C_4-compounds (see Table 3).

Table 3: Substrates obligate and facultative methylotrophs
(for example, typical representatives of each group) [14]

Substrates		Obligate methylotrophs (*Methylomonas*)	Facultative methylotrophs (*Hyphomicrobium*)
C_1-compounds	Methan (CH_4)	+	–
	Dimethyl ether (CH_3–O–CH_3)	+	–
	Methanol (CH_3OH)	+	+
	Formic acid (HCOOH)	–	+
C_2-compounds	Ethanol (C_2H_5OH)	–	+
	Acetic acid (CH_3COOH)	–	+
C_4-compounds	β-hydroxybutyric acid (CH_3CH (OH) CH_2COOH)	–	+

The first studied obligate methylotrophic bacterium of *Methylomonas methanica* – the Gram-negative rods with polar flagella, was described almost 100 years ago, and for several decades had been the only known bacterium capable of oxidizing methane (Figure 1). Further development and improvement of the methods of accumulation and isolation of methane oxidizing bacteria in selective nutrient media recently led to the discovery of a large number of the novel microorganisms, which are similar in properties, but different in structure. Today obligate methylotrophic bacteria are classified into the genera: *Methylococcus*, *Methylomonas*, *Methylosinus*, *Methylocystis*, *Methylobacillus*, *Methylophilus*, *Methylophaga*, *Methylovorus* and *Methylobacterium*.

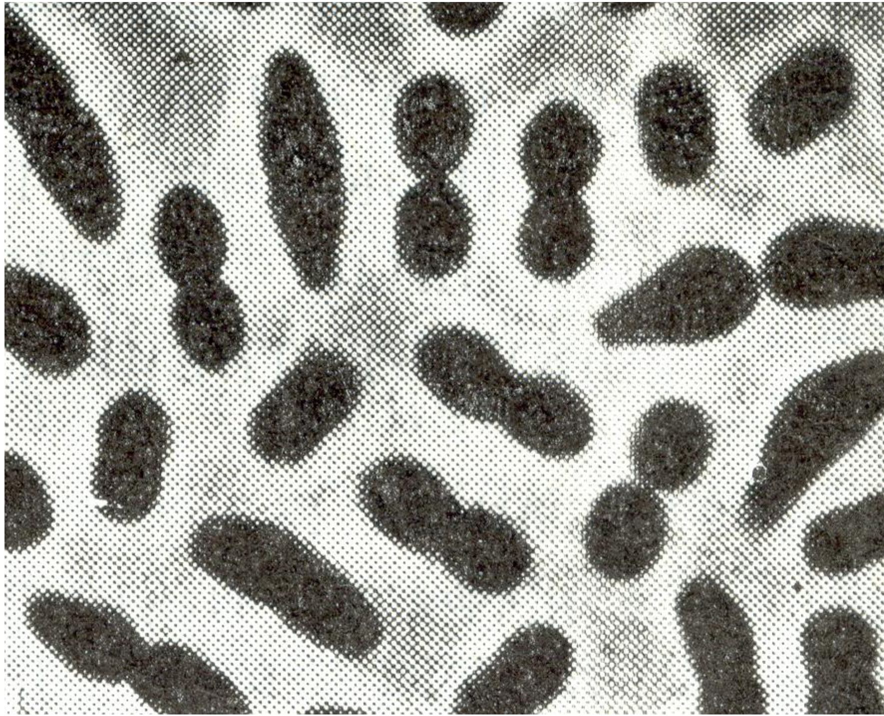


Figure 1. Electron micrograph of rod-shaped obligate methylotrophic bacterium *Methylomonas methanica* [15]

A characteristic feature of methylotrophs is the presence in their cells the developed system of intracytoplasmic membranes, which are divided into 2 types: intra-cytoplasmic membrane of type I and intra-cytoplasmic membrane of type II (Figure 2). Intra-cytoplasmic membrane of type I is presented by stacks of tightly packed vesicular discs distributed throughout the cytoplasm, while the intra-cytoplasmic membrane of type II has the form of lamellae – a system of intra-cytoplasmic membranes, derived from the cytoplasmic membrane and kept a distinct relationship with it. These membranes have the form of individual bubbles, tubes or plates (lamellae) arranged around the periphery of the cell cytoplasm [16]. On the topology and structure these membrane systems are reminiscent of the intra-cytoplasmic membrane of some nitrifying bacteria.

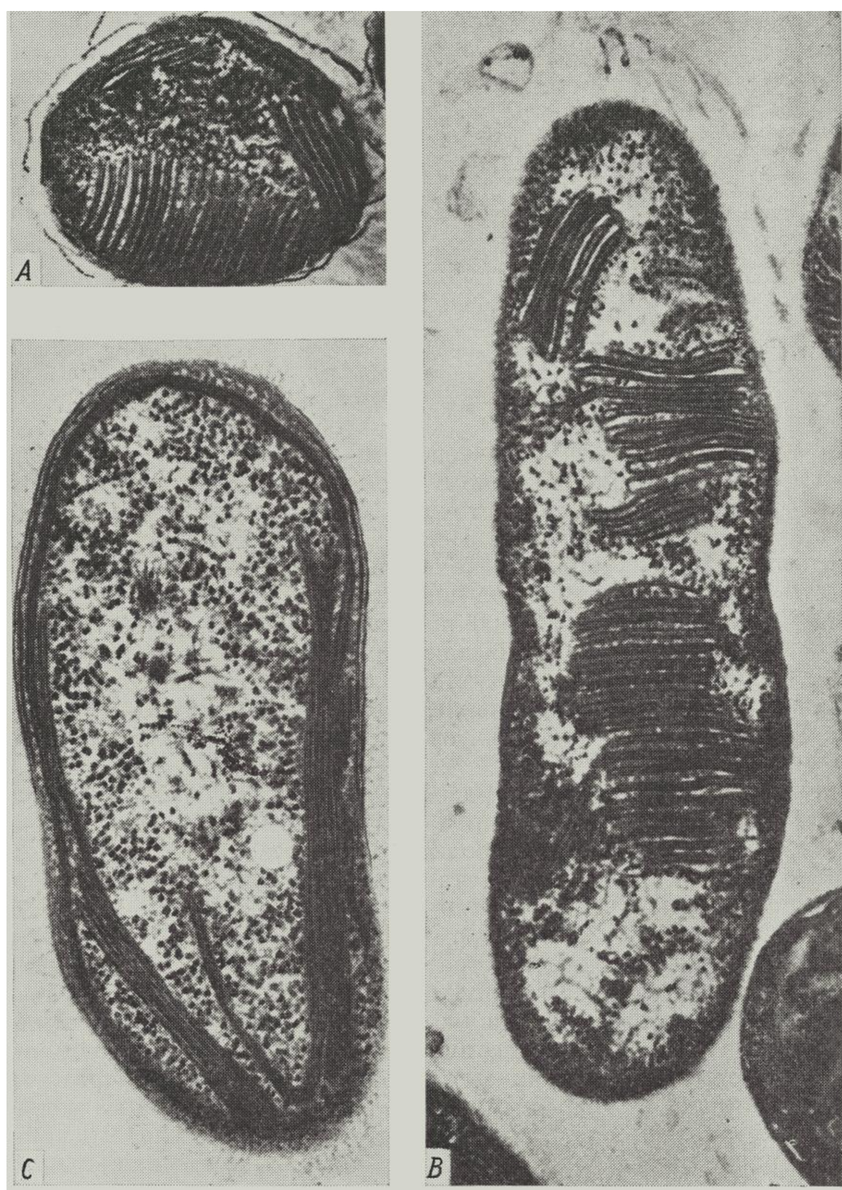


Figure 2. Electron micrographs of thin sections of cells of three obligate methylotrophs with intra-cytoplasmic membrane systems of two types: *A – Methylococcus*; membrane system of type I;
B – Methylomonas; membrane system of type I;
C – Methylosinus; membrane system of type II [16]

Based on the structural features of intra-cytoplasmic membranes, all obligate methylotrophic bacteria can be divided into two main groups – rod-shaped and coccoid bacteria [17]. Some of them form stable to drying resting cells, which in their structure resemble cysts similar to nitrogen-fixing bacteria *Azotobacter* and exospores, which are small spherical cells, spunning off from the poles of the parent cell (Figure 3).

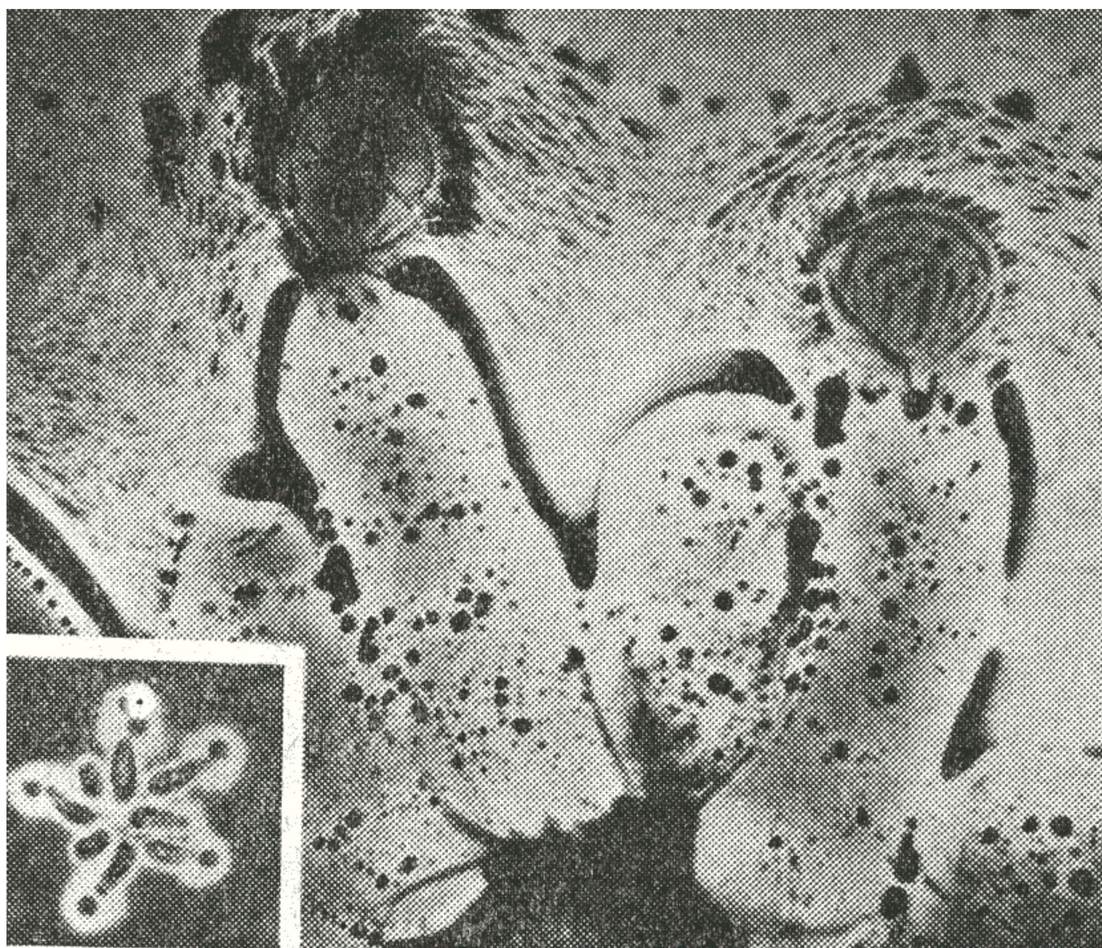


Figure 3. Electron micrograph of exospores of obligate methylotrophic bacterium *Methylosinus*. The inset at the bottom is shown the electron micrograph of budding exospores budding [17]

The best substrates for all obligate methylotrophic bacteria are methane and methanol. The rate of bacterial growth on methanol usually is not great. Obligate methylotrophs are able to oxidize only a few organic substrates that are unable to support their growth. Such compounds include formic acid (HCOOH), which they oxidized to CO_2, ethylene (C_2H_4), ethyl alcohol (CH_3CH_2OH), oxidized to acetaldehyde (CH_3CHO). As a nitrogen source, these bacteria can utilize both nitrate and ammonia. However, the ammonia as being the methane oxidation inhibitor reduces the rate of bacterial growth when its concentration in growth medium is greater than 0,05 %. As a rule in nutrient media containing ammonia are formed trace amounts of nitrate. Thus, methane oxidizing bacteria are nitrifying bacteria, although scientific evidences that they can produce energy at such a small ratio of ammonium oxidation, are not numerous.

Facultative methylotrophs

To facultative methylotrophs are included some of the genera *Pseudomonas*, *Arthrobacter*, *Mycobacterium*, *Bacillus*, *Acetobacter*, *Achromobacter*, *Nocardia*, *Hyphomicrobium*, *Brevibacterium* and others. Despite the fact that obligate methylotrophs are able to grow only *via* assimilation of methanol as a sole carbon source, cumulative cultures at the utilization of the substrates are enriched with microorganisms of other types, so called facultative methylotrophs. These bacteria, unlike obligate methylotrophs are capable along with methane and methanol to assimilate as carbon sources some other one-carbon and poly-carbon compounds. The best studied of the facultative methylotrophs is the budding bacterium *Hyphomicrobium*. This bacterium is known as a powerful denitrifier, and can be isolated from enrichment cultures by incubation of microorganisms in a medium containing methanol and NH_4^+ ions under anaerobic conditions.

We isolated from aerobic enrichment cultures with methanol the leucine-dependent gram-positive rod-shaped facultative methylotrophic aerobic bacteria *Brevibacterium methylicum 5652*

implementing ribulose-5-monophosphate pathway of carbon assimilation, producer of phenylalanine and other metabolically related amino acids [18]. Unlike other traditional producers of *L*-phenylalanine, which do not have negative control of prephenate dehydratase (EC 4.2.1.51) or 3-deoxy-*D*-arabino-heptulosonate-7-phosphate synthase (EC 2.5.1.54), the uniqueness of this strain consists in that it requires *L*-leucine for growth. The initial stage of biochemical research with this strain of methylotrophic bacteria was connected with obtaining auxotrophic mutants, which in majority cases are characterized by the limited spectrum of mutant phenotypes and, besides that the high level of reversions. The initial *L*-leucine dependent strain *B. methylicum*, producer of *L*-phenylalanine was obtained *via* selection at previous stage of research after processing of parental strain by nitrozoguanidin. Screening for resistant cell colonies was carried out by their stability to the analogue of phenylalanine – *meta*-fluoro-phenylalanine (50 μg/ml). The analogue resistant mutants allocated on selective media were able to convert methanol and accumulate up to 1 gram per 1 liter of *L*-phenylalanine into growth media, which is important for biotechnological use of this strain in the production of phenylalanine.

Further attempts were made to intensify the growth and biosynthetic parameters of this bacterium in order to grow on media M9 with higher concentration of deuterated substrates – $C^2H_3O^2H$ and ^2H_2O. For this, was applied deuterium enrichment technique *via* plating cell colonies on 2 % (w/v) agarose media M9 supplemented with 2 % (v/v) $C^2H_3O^2H$ with an increase in the ^2H_2O content from 0; 24,5; 49,0; 73,5 up to 98 % (v/v) ^2H_2O, combined with subsequent selection of cell colonies which were resistant to deuterium. The degree of cell survive on maximum deuterated medium was approx. 40 %. The data on the yield of biomass of initial and adapted *B. methylicum*, magnitude of lag-period and generation time on protonated and maximum deuterated M9 medium are shown in Figure 4. The yield of biomass for adapted methylotroph (*c*) was decreased approx. on 13 % in comparison with control conditions (*a*) at an increase in the time of generation up to 2,8 h and the lag-period up to 40 h (Figure 4). As is shown from these data, as compared with the adapted strain, the growth characteristics of initial strain on maximally deuterated medium were inhibited by deuterium.

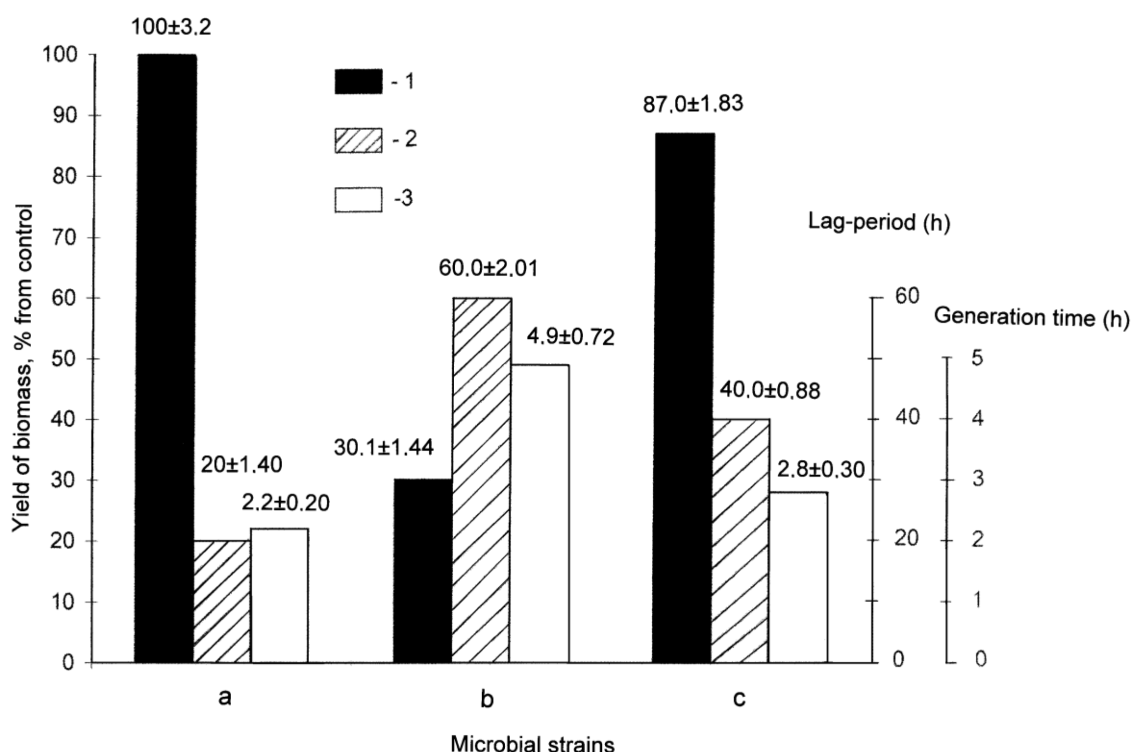


Figure 4. Yield of microbial biomass of *B. methylicum*, magnitude of lag-period and generation time in various experimental conditions: initial strain on protonated M9 medium (control) with water and methanol (*a*); initial strain on maximally deuterated M9 medium (*b*); adapted to deuterium strain on maximally deuterated M9 medium (*c*): 1 – yield of biomass, % from the control; 2 – duration of lag-period, h; 3 – generation time, h.

Experimental conditions are given in Table 4 (expts. 1–10) relative to the control (expt. 1) on protonated medium M9 and to the adapted bacterium (expt. 10'). Various compositions of [U-^{2}H]MeOH and ^2H_2O were added to growth media M9 as hydrogen/deuterium atoms could be assimilated both from $C^2H_3O^2H$ and ^2H_2O. The maximum deuterium content was under conditions (10) and (10') in which we used 98 % (v/v) ^2H_2O and 2 % (v/v) $C^2H_3O^2H$. The even numbers of experiment (Table 4, expts. 2, 4, 6, 8, 10) were chosen to investigate whether the replacement of CH_3OH by its deuterated analogue affected growth characteristics in presence of ^2H_2O. That caused small alterations in growth characteristics (Table 4, expts. 2, 4, 6, 8, 10) relative to experiments, where we used protonated methanol (Table 4, expts. 3, 5, 7, 9). The gradual increment in the concentration of ^2H_2O into growth medium caused the proportional increase in lag-period and yields microbial biomass in all isotopic experiments. Thus, in the control (Table 4, expt. 1), the duration of lag-period did not exceed 20,2 h, the yield of microbial biomass (wet weight) and production of phenylalanine were 200,2 and 0,95 gram per 1 liter of growth medium. The results suggested, that below 49 % (v/v) ^2H_2O (Table 4, expts. 2–4) there was a small inhibition of bacterial growth compared with the control (Table 4, expt. 1). However, above 49 % (v/v) ^2H_2O (Table 4, expts. 5–8), growth was markedly reduced, while at the upper content of ^2H_2O (Table 4, expts. 9–10) growth got 3,3-fold reduced. With increasing content of ^2H_2O in growth media there was a simultaneous increase both of lag-period and generation time. Thus, on maximally deuterated growth medium (Table 4, expt. 10) with 98 % (v/v) ^2H_2O and 2 % (v/v) $C^2H_3O^2H$, lag-period was 3 fold higher with an increased generation time to 2,2 fold as compared to protonated growth medium with protonated water and methanol which serve as control (Table 4, expt. 1). While on comparing adapted bacterium on maximally deuterated growth medium (Table 4, expt. 10') containing 98 % (v/v) ^2H_2O and 2 % (v/v) $C^2H_3O^2H$ with non adapted bacterium at similar concentration showed 2,10 and 2,89 fold increase in terms of phenylalanine production and biomass yield due to deuterium enrichment technique, while, the lag phase as well as generation time also got reduced to 1,5 fold and 1,75 fold in case of adapted bacterium.

Table 4: Effect of variation in isotopic content (0–98 % ^2H_2O, v/v) present in growth medium M9 on bacterial growth of *B. methylicum* and phenylalanine production

Exp. number	Media components, % (v/v)				Lag-period (h)	Yield in terms of wet biomass (g/l)	Generation time (h)	Phenylalanine production (g/l)
	H_2O	^2H_2O	CH_3OH	$C^2H_3O^2H$				
1 (control)	98,0	0	2	0	20,2±1,40	200,2±3,20	2,2±0,20	0,95±0,12
2	98,0	0	0	2	20,3±1,44	184,6±2,78	2,4±0,23	0,92±0,10
3	73,5	24,5	2	0	20,5±0,91	181,2±1,89	2,4±0,25	0,90±0,10
4	73,5	24,5	0	2	34,6±0,89	171,8±1,81	2,6±0,23	0,90±0,08
5	49,0	49,0	2	0	40,1±0,90	140,2±1,96	3,0±0,32	0,86±0,10
6	49,0	49,0	0	2	44,2±1,38	121,0±1,83	3,2±0,36	0,81±0,09
7	24,5	73,5	2	0	45,4±1,41	112,8±1,19	3,5±0,27	0,69±0,08
8	24,5	73,5	0	2	49,3±0,91	94,4±1,74	3.8±0.25	0,67±0,08
9	98,0	0	2	0	58,5±1,94	65,8±1,13	4,4±0,70	0,37±0,06
10	98,0	0	0	2	60.1±2,01	60,2±1,44	4,9±0,72	0,39±0,05
10'	98,0	0	0	2	40,2±0,88	174,0±1,83	2,8±0,30	0,82±0,08

* The date in expts. 1–10 described the growth characteristics for non-adapted bacteria in growth media, containing 2 % (v/v) $CH_3OH/C^2H_3O^2H$ and specified amounts (%, v/v) of ^2H_2O.
** The date in expt. 10' described the growth characteristics for bacteria adapted to maximum content of deuterium in growth medium.
***As the control used exprt. 1 where used ordinary protonated water and methanol

The adapted *B. methylicum* eventually came back to normal growth at placing over in protonated growth medium after some lag-period that proves phenotypical nature of a phenomenon of adaptation that was observed for others adapted by us strains of methylotrophic bacteria and representatives of other taxonomic groups of microorganisms [19, 20]. The effect of reversion of

growth in protonated/deuterated growth media proves that adaptation to ^2H_2O is rather a phenotypical phenomenon, although it is not excluded that a certain genotype determines the manifestation of the same phenotypic trait in the growth media with different isotopic compositions. On the whole, improved growth characteristics of adapted methylotroph essentially simplify the scheme of obtaining the deutero-biomass which optimum conditions are M9 growth medium with 98 % (v/v) ^2H_2O and 2 % (v/v) $C^2H_3O^2H$ with incubation period 3–4 days at temperature 35 °C.

Literature reports clearly reveal that the transfer of deuterated cells to protonated medium M9 eventually after some lag period results in normal growth that could be due to the phenomenon of adaptation wherein phenotypic variation was observed by the strain of methylotrophic bacteria. The effect of reversion of growth in protonated/deuterated growth media proves that adaptation to ^2H_2O is a phenotypical phenomenon, although it cannot be excluded that a certain genotype determined the manifestation of the same phenotypic attribute in media with high deuterium content. The improved growth characteristics of the adapted bacterium essentially simplify the obtaining of deutero-biomass in growth medium M9 with 98 % (v/v) ^2H_2O and 2 % (v/v) $C^2H_3O^2H$.

Adaptation, which conditions are shown in experiment 10' (Table 4) was observed by investigating of growth dynamics (expts. 1*a,* 1*b,* 1*c*) and accumulation of *L*-phenylalanine into growth media (expts. 2*a,* 2*b,* 2*c*) by initial (*a*) and adapted to deuterium (*c*) strain *B. methylicum* in maximum deuterated growth medium M9 (Figure 5, the control (*b*) is obtained on protonated growth medium M9). In the present study, the production of phenylalanine (Fig. 5, expts. 1b, 2b, 3b) was studied and was found to show a close linear extrapolation with respect to the time up to exponential growth dynamics (Fig. 5, expts. 1a, 2a, 3a). The level of phenylalanine production for non-adapted bacterium on maximally deuterated medium M9 was 0,39 g/liter after 80 hours of growth (Fig. 5, expt. 2b). The level of phenylalanine production by adapted bacterium under those growth conditions was 0,82 g/liter (Fig. 5, expt. 3b). Unlike to the adapted strain the growth of initial strain and production of phenylalanine in maximum deuterated growth medium were inhibited. The important feature of adapted to ^2H_2O strain *B. methylicum* was that it has kept its ability to synthesize and exogenously produce *L*-phenylalanine into growth medium. Thus, the use of adapted bacterium enabled to improve the level of phenylalanine production on maximally deuterated medium by 2,1 times with the reduction in the lag phase up to 20 h. This is an essential achievement for this strain of methylotrophic bacteria, because up till today there have not been any reports about production of phenylalanine by leucine auxotrophic methylotrophs with the NAD^+ dependent methanol dehydrogenase (EC 1.6.99.3) variant of the RuMP cycle of carbon assimilation. This makes this isolated strain unique for production of phenylalanine.

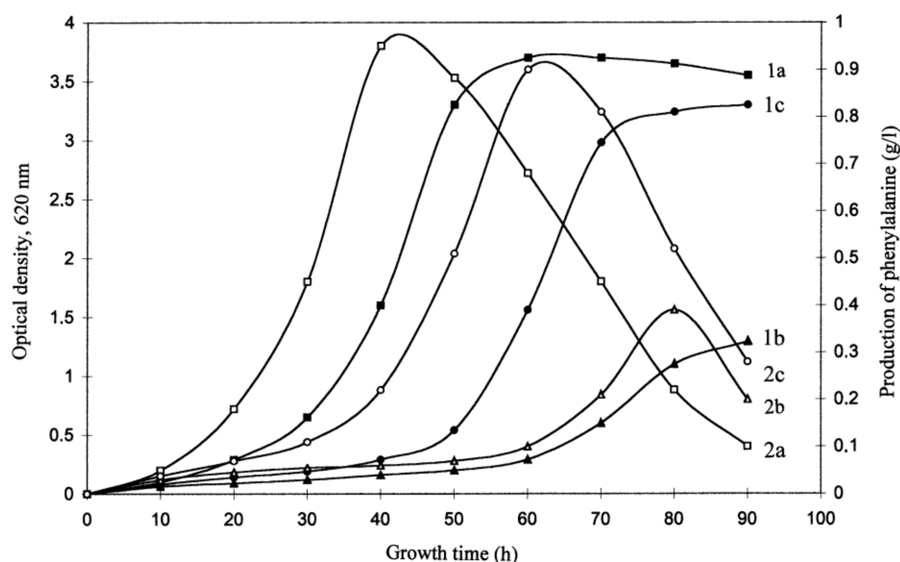


Figure 5. Growth dynamics of *B. methylicum* (1a, 2a, 3a) and production of phenylalanine (1b, 2b, 3b) on media M9 with various isotopic content: 1a, 1b – non-adapted bacterium on protonated medium (Table 1, expt. 1); 2a, 2b – non-adapted bacterium on maximally deuterated medium (Table 1, expt. 10); 3a, 3b – adapted bacterium on maximally deuterated medium (Table 1, expt. 10')

The general feature of phenylalanine biosynthesis in H_2O/^2H_2O-media was increase of its production at early exponential phase of growth when outputs of a microbial biomass were insignificant (Figure 5). In all the experiments it was observed that there was a decrease in phenylalanine accumulation in growth media at the late exponential phase of growth. Microscopic research of growing population of microorganisms showed that the character of phenylalanine accumulation in growth media did not correlate with morphological changes at various stages of the cellular growth. Most likely that phenylalanine, accumulated in growth media, inhibited enzymes of its biosynthetic pathways, or it later may be transformed into intermediate compounds of its biosynthesis, e.g. phenylpyruvate. Phenylalanine is synthesised in cells of microorganisms from prephenic acid, which through a formation stage of phenylpiruvate turns into phenylalanine under the influence of cellular transaminases. However, phenylalanine was not the only product of biosynthesis; other metabolically related amino acids (alanine, valine, and leucine/isoleucine) were also produced and accumulated into growth media in amounts of 5–6 μmol in addition to phenylalanine.

With increasing of ^2H_2O content in growth media, the levels of deuterium enrichment in [^{2}H]amino acid molecules were varried propotionaly. The similar result on proportional specific increase of levels of deuterium enrichment into [^{2}H]phenylalanine and other metabolically related [^{2}H]amino acids (alanine, valine and leucine/isoleucine) was observed in all isotopic experiments where used increasing concentration ^2H_2O in growth media (Table 5). Predictably, enrichment levels of [^{2}H]phenylalanine related to the family of aromatic amino acids synthesised from shikimic acid and metabolically related [^{2}H]amino acids of pyruvic acid family – alanine, valine and leucine at identical ^2H_2O concentration in growth media are correlated among themselves. Such result is fixed in all isotope experiments with ^2H_2O (Table 5). Unlike [^{2}H]phenylalanine, deuterium enrichment levels in accompanying [^{2}H]amino acids – Ala, Val and Leu/Ile keep a stable constancy within a wide interval of ^2H_2O concentration: from 49 % (v/v) to 98 % (v/v) ^2H_2O (Table 5). Summarizing these data, it is possible to draw a conclusion on preservation of minor pathways of the metabolism connected with biosynthesis of leucine and metabolic related amino acids of pyruvic acid family – alanine and valine, which enrichment levels were in correlation within identical concentration of H_2O in growth media (phenylalanine is related to the family of aromatic amino acids synthesized from shikimic acid). Since leucine was added into growth media in protonated form, another explanation of this effect, taking into consideration the various biosynthetic pathways of Leu and Ileu (Ileu belongs to the family of aspartic acid, while Leu belongs to the pyruvic acid family), could be cell assimilation of protonated leucine from growth media. Since Leu and Ileu could not be clearly estimated by EI MS method, nothing could be said about possible biosynthesis of [^{2}H]isoleucine. Evidently, higher levels of deuterium enrichment can be achieved by replacement of protonated leucine on its deuterated analogue, which may be isolated from hydrolysates of deuterated biomass of this methylotrophic bacterium.

Table 5: Effect of deuterium enrichment levels (atom%) in the molecules of [^{2}H]amino acids excreted by *B. methylicum**

[^{2}H]amino acid	Concentration of ^2H_2O in growth media, % (v/v)**			
	24,5	49,0	73,5	98,0
Ala	24,0±0,70	50,0±0,89	50,0±0,83	50,0±1,13
Val	20,0±0,72	50,0±0,88	50,0±0,72	62,5±1,40
Leu/Ileu	20,0±0,90	50,0±1,38	50,0±1,37	50,0±1,25
Phe	17,0±1,13	27,5±0,88	50,0±1,12	75,0±1,40

* At calculation of enrichment levels protons (deuterons) at COOH- and NH_2-groups of amino acids were not considered because of dissociation in H_2O (^2H_2O).

** The data on enrichment levels described bacteria grown on minimal growth media M9 containing 2 % (v/v) $C^2H_3O^2H$ and specified amounts (%, v/v) of ^2H_2O.

Metabolism of methyl compounds

The process of enzymatic oxidation of methane in the cells of methanotrophs can be represented schematically in Figure 6.

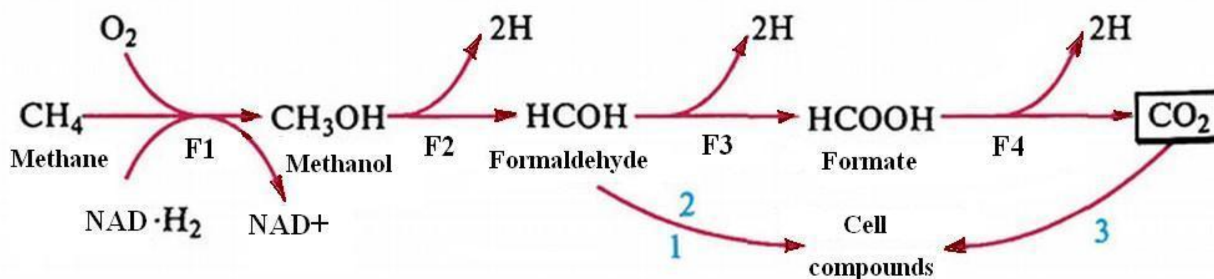


Figure 6. Diagram of the enzymatic oxidation of methane: F1 – methane monooxygenase; F2 – methanol dehydrogenase; F3 – formaldehyde dehydrogenase; F4 – formate dehydrogenase

The initial stage of the oxidation of methane to methanol is catalyzed by $NAD{\cdot}H_2$-dependent methane monooxygenase – a key enzyme of aerobic methanotrophs; other enzymes are present in other members of methylotrophs. The literature describes two forms of this enzyme: associated with intra-cytoplasmic membranes and soluble methane monooxygenase [21]. Electron donor for the first form of the enzyme can be the reduced cytochrome (Cyt) or $NAD{\cdot}H_2$, forming as a result of the reversed electron transport; for the second form of the enzyme – only $NAD(P){\cdot}H_2$ or compounds which are oxidized with its formation. Carbon are fixed by the cell at the stage of carbon dioxide (3) or formaldehyde formation (2) (Figure 6).

Subsequent stages of the enzymatic oxidation of methanol are catalyzed by relevant dehydrogenases – CytC dependent methanol dehydrogenase, CytC-dependent formaldehyde dehydrogenase (or its function is performed by methanol dehydrogenase) and NAD^+-dependent formate dehydrogenase, differing by the structure, the nature of electron acceptors, and other parameters.

The energy efficiency of the oxidation of C_1-compounds by relevant dehydrogenases is determined by the place of receipt of the electrons along the respiratory chain, which in composition of transporters and their localization on the membrane is similar to those ones typical for most aerobic eubacteria. In the oxidative metabolism of C_1-compounds are involved $NAD{\cdot}H^+$, flavins, quinones, cytochromes *a*, *b*, *c*. Oxidation of methanol to formaldehyde, catalyzed by methanol dehydrogenase containing as prosthetic group the residue of pyrrolo-quinoline quinone (coenzyme PQQ), is accompanied by the transfer of electrons in the mitochondrial respiratory chain at the level of cytochrome c. This process leads to the synthesis of ATP molecule (Figure 7).

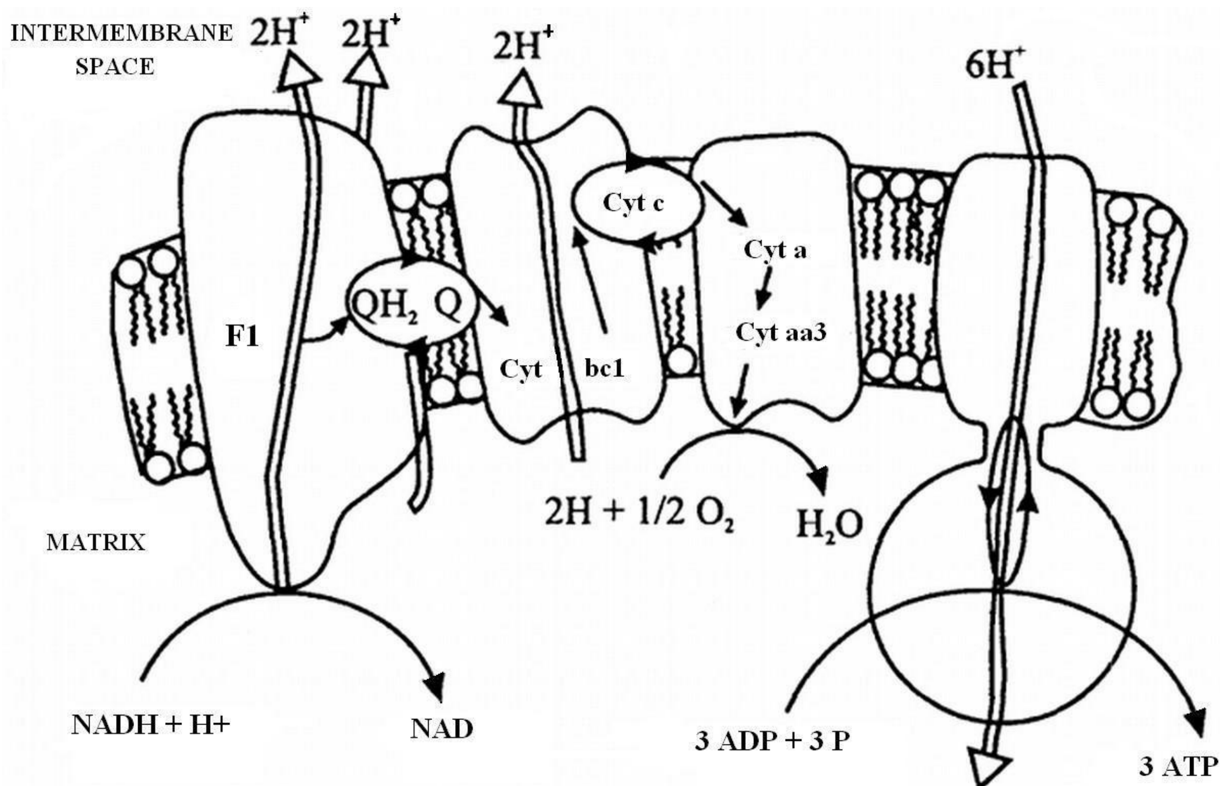
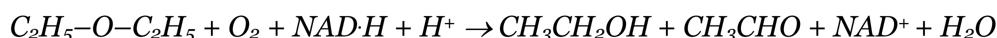


Figure 7. The respiratory electron transport chain: Cyt – cytochrome, Q – quinone, Fl – flavoprotein

Formaldehyde in methylotrophs is a key metabolite, at which diverge the constructive and energy pathways [22]. Part amount of formaldehyde is converted into cell substances *via* the specific to methylotrophic bacteria assimilation cyclic pathways, while most part of formaldehyde is oxidized with NAD^+-dependent formaldehyde dehydrogenase to formate, which is further cleaved to CO_2 with using formate dehydrogenase.

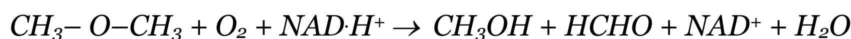
The NAD^+-dependent oxidation of formaldehyde and formate suggesting that the transfer of an electron pair can be linked to the transmembrane movement of protons involving ATP. The experimental data indicate, however, for smaller outputs of ATP. However, the question on the level at which the electrons are transferred from formaldehyde and formate into the respiratory chain is not entirely clear.

Some Gram-positive methylotrophic bacteria can utilize as a substrate for their growth diethyl ether ($C_2H_5–O–C_2H_5$). This compound is cleaved at oxidation by oxygenation, resulting in formation of ethanol (CH_3CH_2OH) and acetaldehyde (CH_3CHO):



$$C_2H_5–O–C_2H_5 + O_2 + NAD{\cdot}H + H^+ \rightarrow CH_3CH_2OH + CH_3CHO + NAD^+ + H_2O$$

A similar oxidation of dimethyl ether to methane in bacteria may result in formation of methanol and formaldehyde, although the mechanism of the reaction is not completely understood:



$$CH_3– O–CH_3 + O_2 + NAD{\cdot}H^+ \rightarrow CH_3OH + HCHO + NAD^+ + H_2O$$

In contrast to the oxidation of methanol occurring with the participation of methanol dehydrogenase, the oxidation of primary alcohols is carried out by pyridine-dependent dehydrogenases.

Experiments carried out by us on the study of the incorporation of deuterium into the components of the cellular biomass by assimilation [U-^{2}H]MeOH at the growth of methylotrophic bacteria *Methylobacillus flagelatum KT* and *Brevibacterium methylicum 5652* on the growth

media with 2 % (v/v) $C^2H_3O^2H$, showed a small amount of deuterium entering into the molecules with carbon of $C^2H_3O^2H$ (not more than 5 %) [23]. This result is explained by dilution of the deuterium label at the expense of biochemical processes associated with the decomposition of $C^2H_3O^2H$ at its assimilation by the cell, and the isotopic exchange reaction and dissociation in aqueous media. Thus, of four deuterium atoms in the molecule of $C^2H_3O^2H$, only one deuterium atom at the hydroxy group $-O^2H$ is most mobile and therefore readily dissociates in an aqueous medium to form C^2H_3OH. The three remaining deuterium atoms in the molecule of $C^2H_3O^2H$ are entered into the cycle of enzymatic oxidation of methanol that leads to loss of deuterium label due to the formation of compounds being more oxidized than methanol. In particular, such incorporation of deuterium into the components of the cellular biomass confirms the classical scheme of the enzymatic oxidation of methanol to formaldehyde in methylotrophs cells, which then utilized by ribulose-5-monophosphate or serine pathway of carbon assimilation.

Assimilation of carbon by methylotrophic bacteria

Methylotrophs are able to form carbon in cells from C_1-compounds, as from organic substrate, as well as due to the assimilation of CO_2, formed at oxidation of C_1-compounds in the r**educed** oxidative **pentose phosphate pathway** (Calvin cycle), resulting in fixation of carbon dioxide and formation from it the hexose molecule [24]. Experiments with using ^{13}C-labeled carbon substrates showed that the bulk of carbon in the cell was derived from oxidized substrate, instead of CO_2. The tricarboxylic acid cycle (TCA cycle) also plays no significant role in the catabolic pathways of methylotrophs, because TCA-cycle enzyme activity in the cells of methylotrophs is relatively low. In fact, the carbon source is an intermediate – formaldehyde, which in methylotrophs is a key metabolite, at which diverge structural and energy pathways leading to the two main pathways of assimilation of C_1-compounds by the cell – ribulose-5-monophosphate and serine pathways of carbon assimilation [25]. The enzymes which catalyze biochemical reactions are specific for each cycle.

The ribulose-5-monophosphate (RMP) pathway in many respects is similar to the Calvin cycle with CO_2 assimilation with the difference that in this cycle as an acceptor of CO_2 acts the pentose molecule. The key reaction of RMP cycle is the addition of formaldehyde to ribulose-5-phosphate catalyzed by hexoso phosphate synthase with forming a phosphorylated sugar – hexulose-6-phosphate, which is then isomerized to fructose-6-phosphate with participation of phospho hexulose isomerase (Figure 8, *A*). Then fructose-6-phosphate further is subjected to phosphorylation with phosphofructokinase. The resulting fructose-1,6-diphosphate is splitted into two molecules of trioses: 3-phosphoglyceraldehyde (3-PGA) and phospho-dioxyacetone, which are used for further enzymatic reactions. 3-PGA and fructose-6-phosphate is involved in a series of reactions leading to the regeneration of the acceptor of formaldehyde – ribulose-5-phosphate. These reactions are similar to those ones for the reduced oxidative pentose phosphate pathway, in which there occurs the catalyzed by the ribulose diphosphate carboxylase the acceptance by the ribulose-1,5-bisphosphate a molecule of CO_2 and subsequent hydrolytic cleavage of the resulting hexoses into 2 molecules of 3-phosphoglyceric acid (3-PG), subjected to a series of sequential enzymatic reactions leading to the formation of a molecule of glucose. However, the reduced oxidative pentose phosphate pathway is not widespread in methylotrophs and is found only in their individual representatives, which are able to grow autotrophically as well as those ones which can utilize formic acid (HCOOH) due to CO_2 assimilation. A prerequisite for the growth of methylotrophs on formic acid is the ability to synthesize the two key enzymes of the pathway – phospho ribulokinase and ribulose diphosphate carboxylase.

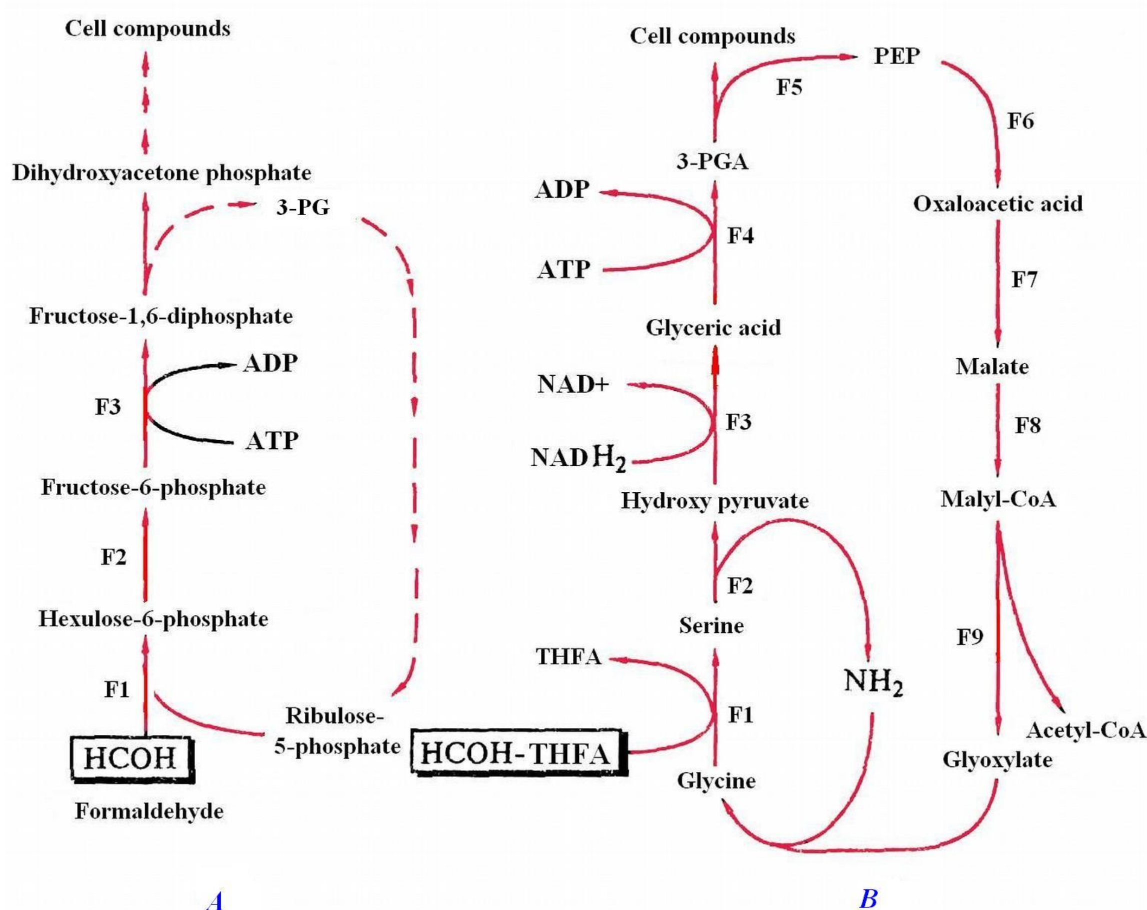
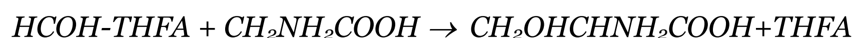


Figure 8. The pathways of assimilation of formaldehyde by methylotrophs. *A* – The ribulose-5-monophosphate pathway: F1 – hexose phosphate synthase; F2 – phospho hexulose isomerase; F3 – phosphofructokinase; F4 – fructose-biphosphate aldolase; dashed line indicates the reactions of regeneration of ribulose-5-phosphate, similar to the corresponding reduced oxidative pentose phosphate pathway. *B* – The serine pathway: F1 – serine hydroxymethyltransferase; F2 – serine glyoxylate aminotransferase; F3 – hydroxy pyruvate reductase; F4 – glycerate kinase; F5 – enolase; F6 – PEP carboxylase; F7 – malate dehydrogenase; F8 – malate tiokinaze; F9 – malyl-CoA lyase

The serine pathway differs significantly from the RMP pathway by the nature of formed intermediates and enzymes (Figure 8, *B*). The key enzyme in this pathway is serine hydroxymethyltransferase which catalyzes the formation of serine from glycine and formaldehyde presented in the form of a derivative of tetrahydrofolic acid (THFA):



$$HCOH\text{-}THFA + CH_2NH_2COOH \rightarrow CH_2OHCHNH_2COOH + THFA$$

Then through a chain of consecutive reactions of transamination, the consistent recovery and phosphorylation of which leads to the formation of 3-phosphoglyceric acid (3-PGA) (Figure 8, *B*). One part of 3-PGA is used for regeneration of glycine, the primary acceptor of C_1; another portion of 3-PGA under the influence of the enzyme enolase is converted into phosphoenolpyruvic acid (PEP) and then participates in subsequent reactions. Subsequent carboxylation of PEP with PEP-carboxylase results in the synthesis of the molecule of oxaloacetic acid (OAA). This reaction is notable because at this stage into the serine cycle CO_2 is introduced. Then OAA with involving the malate dehydrogenase is converted to malate, which in turn under the influence of malil-CoA lyase splits into glyoxylic acid and acetyl-CoA. Subsequent series of reactions lead to regeneratation of glycine, and thus, the cycle is closed (Figure 8, *B*).

Investigation of the distribution of two cyclic pathways of carbon assimilation in facultative and obligate methylotrophs – RMP and serine pathways has led to the establishment of interesting

features on the structure of the intra-cellular membrane [26]. Thus, the assimilation of formaldehyde through the RMP pathway is characteristic for methylotrophs with type I of membrane organization, and through the serine pathway – for methylotrophs with a system of intracytoplasmic membranes of type II. Another distinctive feature is that in the facultative methylotrophs the serine pathway is more common. Among the obligate methylotrophs the serine pathway functions only in those microorganisms which have membrane system of type II (*Methylosinus*, *Methylocystis*), and RMP pathway – in methylotrophs with a membrane system of type I (*Methylomonas*, *Methylobacter*, *Methylococcus*).

Evolution of methylotrophic bacteria

Since the nutritional needs of chemoautotrophs and methylotrophs are very simple, they were considered to be primitive organisms belonging perhaps to the earliest forms of life on Earth [27, 28]. Lately the submission of their place in the evolution of living organisms have changed. Biochemical apparatus of methylotrophic bacteria is just as complicated as that one for most of chemo-heterotrophs [29]. It is supposed that the first living organisms on Earth originated in anaerobic conditions, when primitive ocean was rich in organic matter formed at earlier stades of evolution. Oxygen-rich biosphere arose much later, about 2 billion years. ago. This important geochemical revolution in evolution is explained by photosynthesis. With such a character of the evolution the aerobic chemo-autotrophs and methylotrophs could appear only after oxygenic photosynthesis had been evolved. It can be assumed that chemo-autotrophs and methylotrophs could evolved from common prokaryotic microorganisms precursors that carry out photosynthesis, but lost the photosynthesis apparatus, and their electron transport chain functioning in photosynthesis, began to carry out a new feature of assimilation of C_1-compounds. Some contemporary representatives of the two major groups of prokaryotes, photosynthetic and non-photosynthetic ones, have very interesting properties. These ones include the existence of several complex characteristic of these types of systems of internal membranes; absence of a functioning of tricarboxylic acid cycle; availability of the Calvin cycle, or its analogue, the pentose phosphate cycle; localization in carboxysomes the key enzyme of the Calvin cycle (ribulose diphosphate carboxylase). Recent studies suggest the role of methylotrophs in the evolution of microorganisms. Methylotrophs play a crucial role in the circulation of methane and other C_1-compounds in the biosphere, which are maintained at a constant level, mainly due to the activity of methylotrophs.

Conclusions

Methylotrophs – taxonomycally heterogeneous group of microorganisms presented by chemoteterotrophic obligate and facultative methylotrophic bacteria and yeasts, capable of assimilating carbon from more reduced than CO_2 C_1-compounds. Practical interest to this taxonomic group of methylotrophs is associated with the peculiarities of their metabolism and with the prospects of their practical use in biotechnology as producers of high-grade feed protein and essential amino acids, and other important natural biologically active compounds, such as pigments, carbohydrates and lipids. Digestibility of biomass of methylotrophic bacteria eukaryotes makes up 85–98 %, and the productivity, as measured by a conversion of methanol amountds 37,6–67,5 %. Due to good growth in minimal media with methanol, high yields of biomass and bioconversion level of methanol into the components of the cell biomass (with conversion efficiency 15,5–17,3 g dry biomass per 1 g of consumed substrate) methylotrophic bacteria are regarded as cheaper sources of protein and essential amino acids. The profitability for obtaining the microbial protein is determined for methylotrophs mainly by the cost of such inexpensive and available substrate, as is methanol.

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Эволюция, метаболизм и биотехнологическое использование метилотрофных микроорганизмов

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Аннотация. Метилотрофы – аэробные хемогетеротрофные микроорганизмы, представленные кокковидными и палочковидными подвижными формами, являются обитателями водоемов и почв различного типа, протекают процессы разложения органических веществ с образованием одноуглеродных C_1-соединений и некоторых C_2- и C_3-соединений, способных ассимилироваться метилотрофами. Эти микроорганизмы, ассимилирующие углерод по рибулозомонофосфатному и сериновому циклам, выделяют из почвы, сточных вод, содержащих гниющие растительные остатки, из рубца жвачных животных и других источников. Метилотрофные бактерии в последнее время привлекают все большее внимание биотехнологии как удобные источники многочисленных природных биологически активных соединений – кормового белка и незаменимых аминокислот, каротиноидных пигментов, липидов и полисахаридов. Для получения этих соединений используют генетически маркированные метилотрофные штаммы продуценты, полученные в результате генно-инженерных подходов и селекции. Разработанные за последнее время генно-инженерные методы манипулирования геномом метилотрофов позволяют создавать векторы экспрессии эукариотических белков медицинского и ветеринарного назначения, прежде всего человеческих инсулинов на основе микробной ДНК метилотрофных бактерий. В данной обзорной статье приводятся данные, включая результаты собственных исследований авторов, по эволюции метилотрофных бактерий, метаболизму и их биотехнологическому использованию.

Ключевые слова: метилотрофные микроорганизмы; эволюция; метаболизм.

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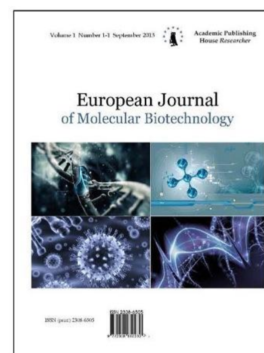

UDC 57

Effect of Climate Change on Plant-Microbe Interaction: An Overview

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Abstract

Climate change is one of the major issues affecting all of us on our planet. Predicted increase in temperature and decrease in precipitation due to climate change may add complexity and uncertainty to plant and agricultural systems and threaten their sustainable management. It is well known that beneficial plant-associated microorganisms may stimulate plant growth and enhance resistance to disease and abiotic stresses. Climate change will also influence crop quality and the dynamics of the relationships between pests/diseases and crops. Changes in climatic factors like temperature, solar radiation and precipitation have potentials to influence crop production. This now makes it possible to test whether some general patterns occur and whether different groups of plant-associated microorganisms respond differently or in the same way to climate change.

Here, we review and discuss how the climatic parameters including atmospheric CO_2 and temperature influence the plant–microbe interaction in polluted soils. This review shows that predicting how plant–microbes interaction responds to altering climatic change is critical to select suitable crop plants that would be able to produce more yields and may tolerate multi-stress conditions.

Keywords: Climate change; Agriculture; Plant – Microbe interaction and Microorganisms.

Introduction

The soil is the third largest global stockpile of carbon and, together with plants, contains around 2.7 times more carbon than the atmosphere. As a result, there is much concern that climate change will augment the decomposition of this carbon, potentially shifting soils from being carbon sinks to sources of atmospheric carbon dioxide and thereby accelerating climate change—the so-called carbon cycle feedback. On the contrary, there is much current debate about the potential to increase the capacity of soils to sequester carbon from the atmosphere and hence mitigate climate change. Recent studies reveal that both of these processes, namely the loss and gain of carbon in soil, are strongly regulated by plant–microbial–soil interactions.

Soil is as an excellent medium for the growth and development of plants as well as microbes and plant-microbe interaction in soil is either beneficial or harmful. The beneficial plant-microbe interactions are caused by symbiotic or non-symbiotic bacteria and a highly specialized group of fungi (mycorrhizal fungi). Beneficial plant-associated microbes are known to stimulate the plant growth and enhance their resistance to degenerative diseases and abiotic stresses. Bacterial genera such as *Azospirillum*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Stenotrophomonas* and *Streptomyces* fall under this category. These are popularly known as plant growth promoting rhizobacteria (PGPR).

Growth promoting substances are produced in large quantities by these soil microorganisms that influence indirectly on the overall morphology of the plants. Mycorrhizal fungi, on the other hand are known for its symbiotic associations with the roots of many different plants ranging from garden vegetables up to the trees of old growth forests..

World population continues to grow, resulting in significant increases in urban development and agricultural, economic and industrial activities. Deforestation and habitat destruction are accelerating rapidly to accommodate the need for open space to support increasing population growth. Accompanying this are increased emissions of gases from agriculture, combustion of fossil fuels, and industrial processes. This has resulted in changes in the chemical composition of the atmosphere. Concern about increased emission of gases into the atmosphere focuses on the possible or potential effects of accumulation of these gases above levels that can be tolerated and balanced by the self-regulating processes and dynamics of the atmosphere.

Carbon is the key source for the growth of many microorganisms and change in climate has severe affect on the growth of different microorganism through different ways likely wise either of direct or indirect path. In regard of direct effects, recent studies show that even subtle warming (by approximately 1°C) can directly stimulate microbial activity causing an increase in ecosystem respiration rates in subarctic Pearland.

Fungi and bacteria play crucial roles in ecosystem function including decomposition of dead biological material, mineral nutrient cycling and as pathogens of plants and animals. In the last few years, more attention has been paid to direct climate change on these microbes if they are exposed to sunlight (such as on foliage surfaces or litter). Changes in species composition and biodiversity of these microbes in response to climate change have been documented and many of these changes appear to be related to how well species and strains of these fungi and bacteria tolerate [1-3].

Beneficial fungi that infect plant roots and assist in absorption of nutrients (termed mycorrhizae), although not exposed to solar radiation, might be indirectly affected by UV-B exposure of the host plant shoots [4-5]. Bacteria and fungi can also be pathogenic for both plants and animals, although Beneficial microorganisms and plant pathogens have received more attention than animal pathogens with respect to climate change [6-8].

Plant growth, disease incidence, productivity can be increased or reduced by several environmental factors. Increasing disease severity is thought to primarily involve modifications in the host plant tissues, while decreased severity appears due either to host plant changes or direct damage to the pathogen [8].

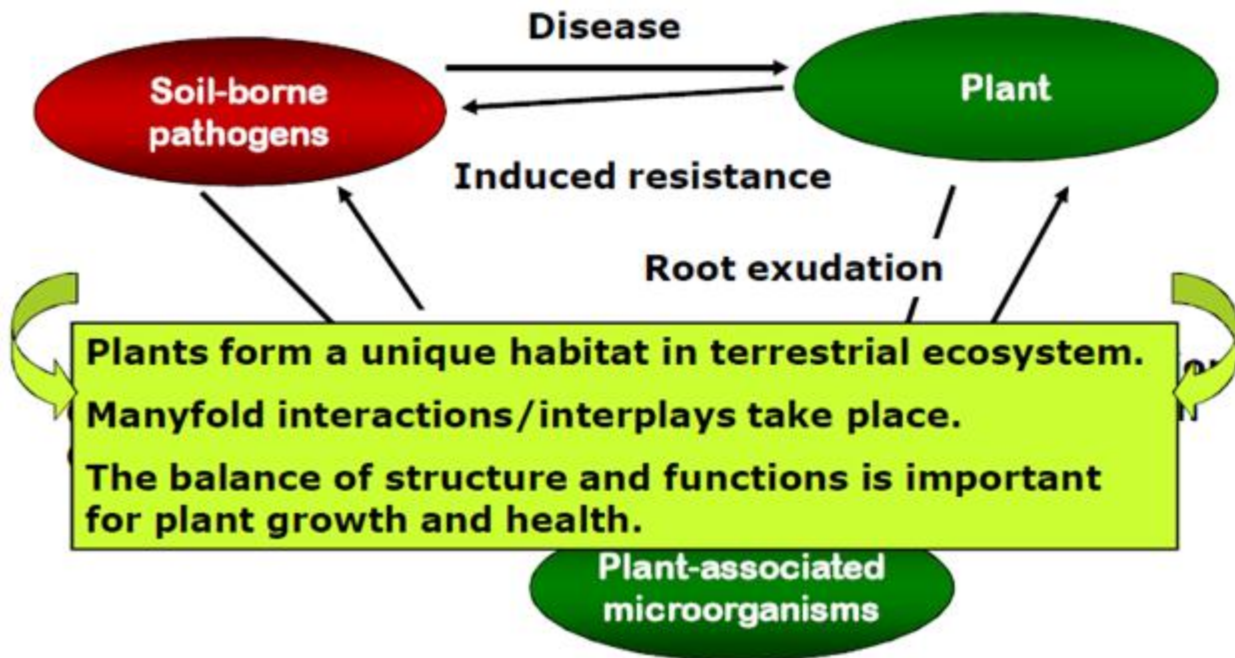


Figure 1. Plant – microbe interaction

Pathogens of insects and other animals may also be influenced by climate change. Studies involving biological control of insect pests using pathogens provide some indication of how change in climatic factors like UV rays, green house gas emission, water supply etc. may affect pathogens.

In general, there remains much uncertainty about how soil organisms directly respond to warming. For instance, it is unclear whether increases in microbial activity and carbon cycling in response to warming will be sustained due to short-term depletion of fast-cycling soil carbon pools, or whether soil communities will adapt to a warmer world [9].

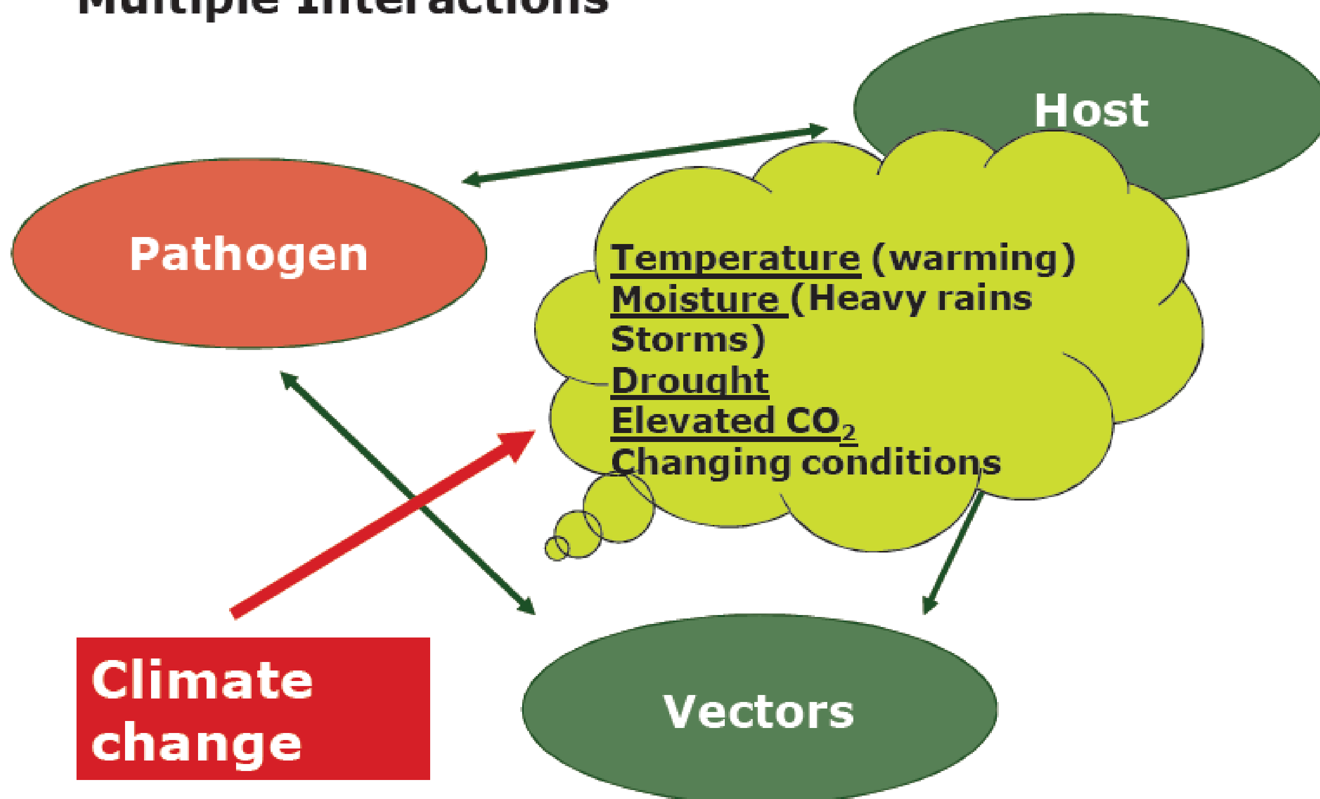


Figure 2. Interaction among climate change –pathogen and plants

Here, we review and discuss how the climatic parameters including atmospheric CO_2, temperature and drought influence the plant–microbe interaction in polluted soils. This review shows that predicting how plant–microbes interaction responds to altering climatic change is critical to select suitable crop plants that would be able to produce more yields and may tolerate multi-stress conditions.

Effect of carbon dioxide (CO_2)

Tropospheric concentration of CO_2 continuously projected to increase from 355 ppm (v/v) to 710 ppm, by the year 2050. Enormous studies have been done on the beneficial effects of elevated CO_2, concentrations on biomass production, probably due to increased water use efficiency. There is a relatively large number of studies on the beneficial effect of increased concentration of atmospheric CO2 on plant growth. Much less is known about CO_2, effects on the incidence and severity of biotic diseases of plants.

In the last few years, approximately 3,000 reports have been published on the subject (Jones & Curtis, 2000; Loladze, 2002). High CO_2 concentration results in benefits for plant growth, although there might be differences among species to species. Several authors reached the same conclusions with different crops, natural ecosystems and forest species.

Most soil-inhabiting fungi tolerate more than 10–20-fold increases in atmospheric CO, concentration. Some typical soil-borne plant pathogens like species of ***Phytophthora, Aphanomyces, Sclerotium*** and different pathotypes of ***Fusarium oxysporum*** have been found to be well adapted to and even multiply better at high CO* and low O2 levels.

CO2 enrichment promotes changes in plant metabolism, growth and physiological processes. There is a significant increase in the photosynthetic rate and a decrease in the transpiration rate per unit leaf area, while total plant transpiration sometimes increases, due to the larger leaf area (Jwa & Walling, 2001; Li et al., 2003). Stimulation of growth by carbon dioxide has been attributed to CO, fixation by the fungi. Carbon dioxide can be used as additional C-source by some fungi and

incorporated into organic acids, like oxaloacetic acid, fumaric or citric acid, thus entering the Krebs cycle to be utilized for energy supply and growth (Tabak & Cooke, 1968; Wells & Uota, 1970). Isolates of ***Rhizoctonia solani*** and ***Pythium irregulare*** were inhibited by CO2 concentrations exceeding 510 %. Griffin and Nair (1968), however, reported that an isolate of ***Sclerotium rolfsii*** had reduced mycelial growth at near ambient CO ***Rhizopus stolontfer, Cladosporium herbarum, Botrytis cinerea, Aspergillus niger*** and ***Alternaria tenuis*** were inhibited at CO, concentrations exceeding 5–10 %.

Soilborne diseases have been studied either by fumigating the soil containing inoculums or by incubating plants in enriched CO* atmospheres while growing in infested soil. In most cases, however, experiments were made with realistic CO* concentrations only for soil air composition, but far too high compared with the atmospheric CO_2. Carbon dioxide favors soil bome infections by ***Fusarium*** spp., especially the incitant of snow mold of cereals, and the members of the ***F. oxysporum*** group.

In a study it was found atmospheric concentration of O2 normally inhibits CO2 absorption by plants, and triggers photorespiration. With a rise in CO2 concentration, the inhibition of photosynthesis by O2 tends to decrease due to an increase in the CO2:O2 ratio. The number of infected barley seedlings grown in that soil was significantly greater than those from soil fumigated with normal air.

Other root diseases caused by ***Pythium splendens*** or ***Thielaviopsis basicola*** on poinsettia were not affected by elevated CO, atmospheres in the greenhouse (Zornbach & Schickedanz, 1987). Undoubtedly, the prevalent effect of a global rise of CO, on biotic diseases will be exerted via changes in the physiological and morphological status of the host plant.

Few studies were conducted in controlled conditions, which might not reflect plant responses in the field, where there are variations and interactions among temperature, precipitation, and other factors.

The increase in plant biomass production, i.e., the increase in production of shoots, leaves, flowers and fruit, represents more tissue that can be infected by pathogens. Increased carbohydrate contents can stimulate the development of sugar-dependent pathogens, such as rusts and powdery mildews. Increases in canopy density and plant size can promote higher growth, sporulation and spread of leaf infecting fungi, which require high air humidity, but not rain, as rusts, powdery mildews and leaf necrotrophs. The increase in crop residues can represent better survival conditions for necrotrophic pathogens. The reduction in stomatal opening can inhibit stomata invading pathogens, such as rusts, downy mildews and some necrotrophs. The shortened growth period and accelerated ripening and senescence can reduce the infection period for biotrophic pathogens, and increase the necrotrophic pathogen populations. The increase in root biomass increases the amount of tissue that could be infected by mycorrhyza or soilborne pathogens, but can compensate the losses inflicted by the pathogens. Higher root exudation can stimulate both pathogens and antagonistic microbiota in the rhizosphere (plant growth promoters).

Effects of temperature

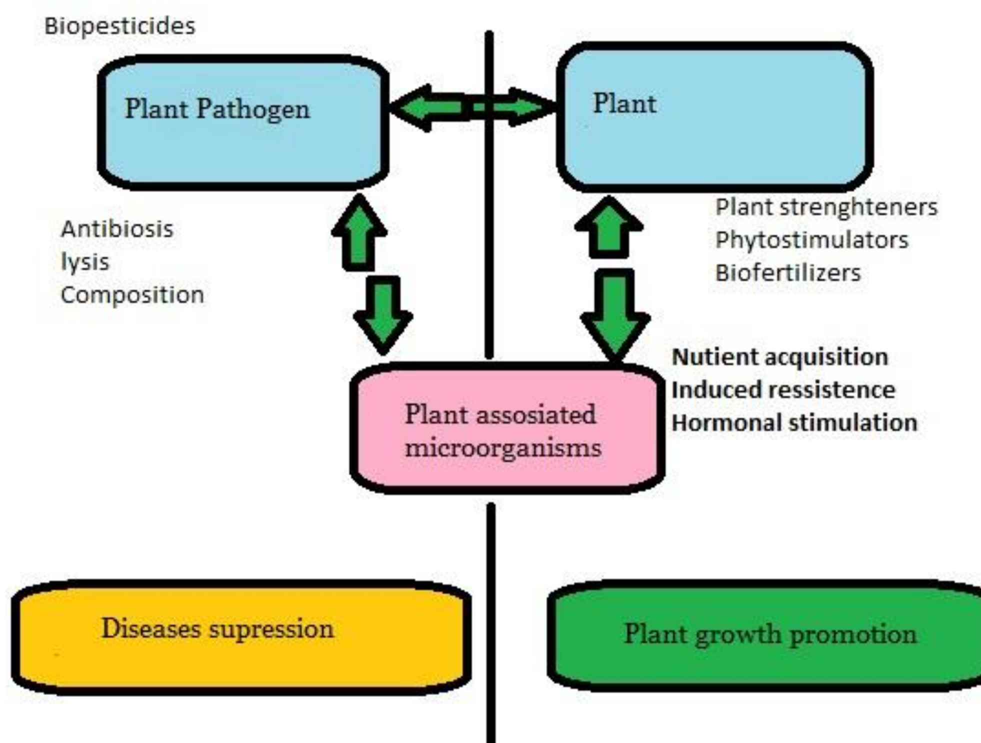
Global warming, a gradual increase in planet-wide temperatures due to CO2 and other greenhouse gasses has long been known to affects the physiology, development, growth and productivity of plants. It is well known that the temperature that is higher than the ideal increases the transpiration and stomatal conductance, but decreases the photosynthesis resulting in significant reduction in the plant biomass yield (Djanaguiraman et al., 2010; Qaderi et al., 2012).

Studies have considered the effects of temperature on plants as well as on microorganisms and it was found that only few studies that have deal with the interactive effects of higher temperature on plants in soils (Li et al., 2012). In general soil warming can affect the nutrient through altering release of soluble metal ions in to soil solution via decomposition of SOM, lysis of microbial cell and the destruction of soil aggregates, thereby changing metal bioavailability, its uptake and distribution in plant tissues.

It has also been reported that elevated temperature can increase the release of trace elements from organic to exchangeable complex through enhancing the soil enzymatic activity (Sardans and Penuelas, 2006) and thereby increasing plant metal uptake.

It is known that the elevated temperature increase the active sites on the root surface and/or change the lipid composition of the plasma membrane (Lynch and Steponkus, 1987) and thereby

its fluidity, and so facilitate both passive and active metal flux through the membrane (Fritioff et al., 2005). Since trace elements (e.g., Cu, Zn and Fe) have important roles in a large number of enzymes regulating many physiological processes, the higher enzyme activity and protein syntheses in temperature-increased environments may allow for greater metal uptake at additional uptake sites on membranes or an increased release of molecules facilitating metal uptake. However, it is also found decreased Cd, Pb, Fe, Zn and Cu concentrations in tubers on increased temperature and suggested which was related to dilution effect due to temperature induced growth rate of tubers. This result indicates that higher temperature greatly influences the element uptake or its accumulation (e.g., Cu, Zn and Fe) in plants through enhancing physiological processes and consequently the nutrient demand.



Conclusions and recommendations

The effects of climate change on plant growth in metal polluted soils will be complex, particularly plant species with narrow ranges of tolerance to various stress factors may have difficulty adapting to future climatic conditions. Since the direct and/or indirect effects of climate change on heavy metal mobility in soils may further hinder the ability of plants to adapt and make them more susceptible, further research is required to assess and predict how both climate change and heavy metals will influence the biomass production, metal accumulation and eco-physiological response in plants. The outcome of such studies will improve our understanding of interactions between external stress factors and biological processes and provide a stronger scientific background to counteract negative consequences of climatic changes on plants growing in metal polluted soils.

Several plant-associated microbes which are tolerant to various stress conditions including drought, heavy metals and temperature, were identified. Moreover, these microbes might have various plant growth promoting traits necessary to establish the plants under the conditions prevailing in metal polluted soils. In general, the climatic changes particularly the CO2 increases plant growth and improve plant–microbe interactions. It is likely these benefits will also occur in plants growing in metal polluted soils, but data to support this are lacking. Moreover, it is yet to be determined whether plants growing in metal contaminated soils under altered environmental conditions release more root exudates and thereby alter colonization/ survival potential of specific stress tolerant and/or plant beneficial microbes.

Thus, attempts should be made to assess and predict how the future climate change will influence the diversity, distribution, and activity of soil microbes and their capable of contributing to the overall plant growth and/or phytoremediation potential in metal polluted soils. This will provide not only an improved knowledge on biodiversity and microbial community structure, but also how climate change influences the plant–microbe–metal interactions in polluted soils.

Recent experiments have demonstrated that the e[CO_2] and/or inoculation of plant-beneficial microbes is a successful tool to improve the plant growth and heavy metal phytoextraction process in polluted soils. However, the issues of the harmful effects of climate change on the production and quality of food and feed are scarcely considered. Thus, determining the impacts of climate change on various factors (metal bio-availability, microbial diversity, etc.) potentially altering the biomass production and heavy metal accumulation in crop plants is of critical importance. Moreover, such knowledge is required to develop soil management strategies for food crops to adapt future climatic change as well as to reduce the entry of heavy metals into the food chain.

Finally, though the evidence gathered so far demonstrates that climate change is likely to have a significant impact on plant growth, the exact consequences of future climate change on plant–mictrobe interaction are difficult to predict due to the complex interactions between various climate parameters (e.g., CO_2, temperature) and soil physic biochemical properties (soil nutrition, microbial diversity, heavy metal concentration, etc.,). Therefore, research involving the interactive effects of various environmental factors on plant response to climate change on different type of soils is required before generalizations can be made.

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