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Evaluation of Biosynthetic Pathways of ^{2}H- and ^{13}C-Labeled Amino Acids by an Obligate Methylotrophic Bacterium *Methylobacillus Flagellatum* and a Facultative Methylotrophic Bacterium *Brevibacterium Methylicum*

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Abstract

By the method of electron impact mass-spectrometry was studied the pathways of biosynthesis of ^{2}H, ^{13}C-labeled amino acids of a facultative methylotrophic bacterium *Brevibacterium methylicum* and an obligate methylotrophic bacterium *Methylobacillus flagellatum* obtained on growth media containing as a source of stable isotopes [^{2}H]methanol, [^{13}C]methanol and $^{2}H_2O$. For mass-spectrometric analysis the multicomponential mixtures of ^{2}H- and ^{13}C-labeled amino acids, derived from cultural media and protein hydrolysates after hydrolysis in 6 *M* ^{2}HCl (3 % phenol) and 2 *M* $Ba(OH)_2$ were modified into N-benzyloxycarbonyl-derivatives of amino acids as well as into methyl esters of N-5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl) derivatives of [^{2}H, ^{13}C]amino acids, which were preparative separated using a method of reverse-phase HCLP. Biosynthetically obtained ^{2}H- and ^{13}C-labeled amino acids represented the mixtures differing in quantities of isotopes incorporated into molecule. The levels of ^{2}H and ^{13}C enrichment of secreted amino acids and amino acid resigues of protein were found to vary from 20,0 atom % to *L*-leucine/isoleucine up to 97,5 atom % for *L*-alanine depending on concentration of ^{2}H- and ^{13}C-labelled substrates.

Keywords: stable isotopes, biosynthesis, methylotrophic bacteria, isotope labeled amino acids.

1. Introduction

The enrichment of molecules by stable isotopes (^{2}H, ^{13}C, ^{15}N, ^{18}O) is an important tool for a variety of biochemical and metabolic studies with amino acids and other biologically active substances (BAS) (Mosin, 1996). The preferential usage of stable isotopes as compared to their counterparts are stipulated by the lack of radioactive radiation hazards and determination of the localization of the label in the molecule by high resolution techniques, including NMR (LeMaster, 1990), IR spectroscopy [MacCarthy, 1986], and mass spectrometry (Mosin et al., 1996). The development of these methods for the detection of stable isotopes in biological probes in recent years has significantly increased the efficiency of biological research, as well as studies of the

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structure and mechanism of action of cellular BAS at the molecular level. In particular, ^{2}H- and ^{13}C-labeled amino acids are used for studying of the spatial structure and conformational changes of proteins, the interaction of protein molecules and in chemical syntheses of some isotope-labeled compounds based on them (Crespi, 1986). For example, isotopically labeled *L*-phenylalanine is used in the synthesis of peptide hormones and neurotransmitters (Mosin et al., 1996).

An important factor in studies using labeled amino acids is their accessibility. ^{2}H and ^{13}C-labeled amino acids can be prepared using chemical, enzymatic and microbiological methods. The chemical synthesis is multistage often requires a large expenditure of costly labeled reagents and substrates and leads to a racemic mixture of *D*- and *L*-enantiomers for separation of which is required the special separation methods (Matthews et al., 1977). Fine chemical synthesis of ^{2}H- and ^{13}C-labeled amino acids is linked with using a combination of chemical and enzymatic approaches (LeMaster, Cronan, 1982).

Microbiology provides an alternative to chemical synthesis a method for obtaining ^{2}H- and ^{13}C-labeled amino acids, which leads to high yields of the synthesized products, the effective incorporation of stable isotopes in the molecule, and preservation of the natural configuration of synthesized [^{2}H, ^{13}C] compounds. For the preparation of biosynthetic ^{2}H- and ^{13}C-labeled amino acids use several approaches, one of which is consisted in uniform enrichment of synthesized compounds at the carbon skeleton in the molecule due to using the bacterial strains growing on selective media containing as a source of stable isotopes [^{13}C] methanol, [^{2}H]methanol and $^{2}H_2O$ (Mosin, Ignatov, 2013, Mosin et al., 2012). This approach also involves the use of complex chemical components of biomass grown on [^{2}H, ^{13}C] growth substrates with further separating and fractionating of target ^{2}H- and ^{13}C- labeled compounds. Another approach is a site-specific enrichment of amino acids at certain positions of molecules due to assimilation by cell the isotopically labeled precursors such as [1,4-^{13}C]succinate, [1, 2-^{13}C]acetate and [1-^{13}C]lactate (Patel et al., 1993).

The research concerns the biosynthetic preparation of ^{2}H- and ^{13}C-labeled amino acids due to the disposal by the cell of low-molecular labeled substrates – [^{2}H]methanol, [^{13}C]methanol and $^{2}H_2O$ followed by the monitoring of the inclusion of stable isotopes of ^{2}H and ^{13}C into molecules of biosynthesized amino acids by electron impact mass spectrometry. The sensitivity of EI mass spectrometry is 10^{-9}–10^{-11} mol in samples, which is considerably higher than the IR and NMR spectroscopy. This method combined with RP-HPLC method has worked well for the study of the level of isotopic enrichment of [^{2}H, ^{13}C]amino acid molecules in the composition of their multi component mixtures as the samples of culture liquids of bacterial strains, producers of amino acids and hydrolysates of total protein of biomass obtained on minimal growth media M9 containing isotopic labeled substrates.

2. Material and methods

Objects of research

Investigations were carried out with genetically marked strains of bacteria obtained from the culture collection of the Russian National Collection of Industrial Microorganisms (PMBC) of State Research Institute of Genetics and Selection of Industrial Microorganisms:

1) *Brevibacterium methylicum VKPM B 5652* – *L*-leucine-dependent strain of facultative methylotrophic bacteria producing *L*-phenylalanine;

2) *Methylobacillus flagellatum KT* – *L*-isoleucine-dependent strain of obligate methylotrophic bacteria producing *L*-leucine.

Chemicals

In the research was used $^{2}H_2O$ (99,9 atom % ^{2}H), ^{2}HCl (95,6 atom % ^{2}H), [^{2}H]methanol (98,5 atom % ^{2}H) and [^{13}C]methanol (99,5 atom % ^{13}C) obtained from the Russian Scientific-technical Center "Isotope" (St. Petersburg, Russia). For the synthesis of the amino acids derivates was used N-5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride), chem. pur. ≥99,0 % (HPLC) (Sigma Aldrich, USA), benzyloxycarbonyl (CBz-chloride), ≥98,0 % (HPLC) (Sigma Aldrich, USA) and diazomethane, prepared from N-nitrosomethylurea, ≥99,0 % (HPLC) (Sigma Aldrich, USA).

Growth conditions of microorganisms and isolation of ^{2}H, ^{13}C-labeled proteins and amino acids

The cultivation of methylotrophic bacteria *B. methylicum* and *M. flagellatum* was performed in a mineral M9 medium in Erlenmeyer flasks with 250 ml volume filled up with 50 ml of the growth medium as described in (Mosin et al., 2013), using as a source of stable isotopes [^{2}H]methanol, [^{13}C]methanol and $^{2}H_2O$ in the presence of *L*-leucine for *B. methylicum* and *L*-isoleucine for *M. flagellatum* in concentrations of 10 mg/l. Cells were separated by centrifugation in the centrifuge T-24 ("Heraues Sepatech", Germany) (10000 g, 20 min). In the culture liquids were analyzed the secreted amino acids.

To isolate the protein fraction of the total biomass the cells were washed twice with distilled water followed by centrifugation (10000 g, 20 min), exposed to ultrasound at 40 kHz (3×15 min) and centrifuged. The resulting precipitate (10 mg) obtained after the separation of lipids and pigments by a mixture of organic solvents: chloroform–methanol–acetone (2 : 1 : 1) was used as the protein fraction of the total biomass.

Pigments and lipids extracted with chloroform–methanol–acetone (2 : 1 : 1) according to Bligh and Dyer method (Bligh et al., 1959).

Hydrolysis of total protein was performed with 6 *M* ^{2}HCl (3 % phenol in $^{2}H_2O$) or 2 *M* $Ba(OH)_2$ (+110 °C, 24 h).

Synthesis of N-Dns-[^{2}H, ^{13}C]amino acids

For the synthesis of N-Dns-[^{2}H, ^{13}C]amino acids to 4–5 mg of lyophilized samples of culture liquid and protein hydrolysates dissolved in 1 ml of 2 *M* $NaHCO_3$, pH = 9–10 was added portionwise with stirring 25,5 mg of dansyl chloride in 2 ml of acetone. The reaction mixture was kept under stirring for 1 hour at t = +40 °C, then acidified with 2 *M* HCl to pH = 3,0 and extracted with ethyl acetate (3×5 ml). The combined extract was washed with water until pH = 7,0, dried over anhydrous sodium sulfate, the solvent was removed at 10 mm. Hg.

Synthesis of methyl esters of N-Dns-[^{2}H, ^{13}C]amino acids

Synthesis of methyl esters of N-Dns-[^{2}H, ^{13}C]amino acids was carried out with using diazomethane. For obtaining of diazomethane to 20 ml of 40 % KOH dissolved in 40 ml of diethyl ether was added 3,0 g of wet nitrosomethylurea and stirred at ice-water bath for 15–20 min. After intensive gassing closure ether layer was separated, washed with ice water until pH = 7,0, dried over anhydrous sodium sulfate, and further used to treat N-[^{2}H, ^{13}C]-Dns-amino acids in composition of culture liquids and hydrolysates of total proteins of biomass.

Synthesis of N-Cbz-[^{2}H, ^{13}C]amino acids

For the synthesis of N-Cbz-[^{2}H, ^{13}C]amino acids to 1,5 ml cooled to 0 °C of culture liquid solution (50 mg) or protein hydrolyzate (5,4 mg) in 4 *M* NaOH were added in portions with stirring 2 ml of 4 *M* NaOH and 28,5 mg of benzyloxycarbonyl. The reaction mixture was kept at 0 °C, stirred for about 3 hours, acidified with 2 *M* HCl to pH = 3,0 and extracted with ethyl acetate (3×5 ml). The combined extract was washed with water until pH = 7,0, dried over anhydrous sodium sulfate, the solvent was removed at 10 mm. Hg.

Methods for analytical determination of ^{2}H- and ^{13}C-labeled amino acids and proteins

TLC of derivatives of ^{2}H- and ^{13}C-labeled amino acids was performed on plates with Silufol UV-254 ("Kavalier", Slovakia) in solvent systems: chloroform–methanol–acetic acid, 10 : 1 : 0,3, vol. % (A) for N-Cbz-[^{2}H, ^{13}C]amino-acids, and methanol–chloroform–acetone, 7 : 1 : 1, vol. % (B) for methyl esters of N-Dns-[^{2}H, ^{13}C] amino asids. N-Cbz-[^{2}H, ^{13}C]amino acids were detected by UV absorbance at λ = 254 nm. Methyl esters of N-Dns-[^{2}H, ^{13}C]amino acids were detected by their fluorescence in UV light. Secreted *L*-phenylalanine and *L*-leucine were determined with a spectrophotometer Beckman DU- 6 ("Beckman Coulter", USA) at λ = 540 nm in 10 ml samples of liquid culture (LC) after the treatment with 1 % of ninhydrin.

Analytical and preparative separation of methyl esters of N-Dns-[^{2}H, ^{13}C] amino acids

Analytical and preparative separation of the mixture of methyl esters of N-Dns-[^{2}H, ^{13}C] of amino acids from the culture liquid and protein hydrolysates was carried out at t = 20±25 ^{0}C by RP HPLC on a liquid chromatograph Knauer Smartline (Knauer, Germany) equipped with a UV detector UF-2563 and integrator-R 3A (Shimadzu, Japan) using 250×10 mm column with the stationary phase of Separon SGX C18, 7 μm (Kova, SK); mobile phase: (A) acetonitril–trifluoroacetic acid = 100 : 0,1–0,5 vol. % and (B) acetonitrile = 100 vol. % under the gradient elution conditions; sample volume – 50–100 μl; elution rate – 1,5 ml/min. The yield of methyl esters of the individual N-Dns-[^{2}H, ^{13}C] amino acids was 75–89 %; the chromatographic purity – 95–98 %.

Ion exchange chromatography of protein hydrolysates

Ion exchange chromatography of protein hydrolysates was performed on a Biotronic LC 5001 apparatus ("Eppendorf-Nethleler-Hinz", Germany) using a column with Biotronic resin BIC 2710; t = 20±25 ^{0}C; 3,2×230 mm; stationary phase: sulfonated styrene (7,25 % of cross-linking) resin UR-30 (Beckman Spinco, USA); mobile phase – 0,2 M Na-citrate buffer; operating pressure – 50–60 atm; feed rate of Na-citrate buffer – 18,5; ninhydrin – 9,25 ml/h; detection at λ = 570 nm and λ = 440 nm (for proline).

Mass spectrometry derivatives [^{2}H, ^{13}C]amino acids

Mass spectra of electron impact of [^{2}H, ^{13}C]amino acid derivatives were recorded on a MB-80 A (Hitachi, Japan) with a double focusing with ionizing voltage of 70 eV, an accelerating voltage of 8 kV and the temperature of the cathode source of 180–200 ^{0}C. The scanning of samples was analyzed at a resolution of 7500 arbitrary units using 10% image sharpness.

3. Results and discussions

Isolation of ^{2}H and ^{13}C -labeled amino acids from culture liquids and protein hydrolysates

The objects of the study were obtained by mutagenesis of *L*-phenylalanine-producing strain of the facultative methylotrophic bacteria *Brevibacterium methylicum*, assimilating methanol via xylulose-5-monophosphate cycle of carbon assimilation, and *L*-leucine-producing strain of obligate methylotrophic bacteria *Methylobacillus flagellatum*, implements a 2-keto-3 -deoxy-gluconate aldolase variant of ribulose-5-monophosphate cycle of carbon assimilation. To compensate auxotrophy for *L*-leucine and *L*-isoleucine, these amino acids were added into the growth medium in the protonated form. The levels of accumulation of *L*-phenylalanine and *L*-leucine in liquid cultures (LC) of these strains-producers reached values of 0,8 and 1,0 g/l respectively [14, 15]. The inclusion of deuterium into the molecules of secreted amino acids and total proteins was carried out via the cultivation of the strain of *B. methylicum* on mineral M9 medium with $^{2}H_2O$ and protonated methanol, as the level of inclusion of ^{2}H into the amino acid molecules due to assimilation of [^{2}H]methanol is negligible.

Since the cell assimilates hydrogen (deuterium) atoms from H_2O ($^{2}H_2O$) environment, we selected conditions of deuterium enrichment of amino acid molecules and proteins under a stepwise increase in concentration of $^{2}H_2O$ in growth media as shown in Table 1. The growth of microorganisms on $^{2}H_2O$ containing growth media was characterized by increasing the duration of the lag phase, the cell generation time, and the reduction of outputs of the microbial biomass (Table 1), so it was necessary to carry out the adaptation of cells to $^{2}H_2O$.

The method of the optional adaptation of the strain of methylotrophic bacteria *B. methylicum* to grow on $^{2}H_2O$ while maintaining the ability for the biosynthesis of *L*-phenylalanine was described in article (Mosin et al., 1998). In this research, were investigated samples of the culture liquids and biomass hydrolysates obtained during the multi-stage adaptation of *B. methylicum* to heavy water on minimal mineral M9 media with the different content of $^{2}H_2O$ (from 24,5 to 98,0 vol. % $^{2}H_2O$). Since this strain of methylotrophic bacteria was adapted to grow in $^{2}H_2O$, the study of inclusion levels of deuterium into the amino acid molecules was the most interesting.

Unlike the bacterial growth on $^{2}H_2O$ medium, wherein it was necessary to carry out the cell adaptation to deuterium, at the preparation of [^{13}C]amino acids via assimilation of $^{13}CH_3OH$ this stage was not required because this isotopic substrate does not exert the adverse biostatic effect on the growth characteristics of methylotrophs (Table 1). Therefore, in the case of the strain of obligate methylotrophic bacteria *M. flagellatum* the inclusion of ^{13}C into amino acid molecules was carried out in one step by growing the bacteria on minimal M9 media containing 1 % of [^{13}C]methanol as a source of carbon-13 isotope.

Table 1. The influence of the isotopic composition of growth media on the growth of strains of *B. methylicum* and *M. flagellatum*

Number of experiment	Growth media*	The value of lag-phase, h	The output of biomass, % from control	The generation time, h
1	0	24,0	100	2,2
2	24,5	32,1	90,6	2,4
3	49,0	40,5	70,1	3,0
4	73,5	45,8	56,4	3,5
5	98,0	60,5	32,9	4,4
6	CH_3OH	0	100	1,1
7	$^{13}CH_3OH$	0,1	72,0	1,0

Notes: * Data for experiments 1–5 were presented for *B. methylicum* while growing on aqueous M9 media containing 2 vol. % methanol and a specified amount (vol. %) of $^{2}H_2O$. Data for experiments 6–7 show for *M. flagellatum* when growing on aqueous M9 media containing 1% methanol (6) or 1 % [^{13}C]methanol.

The main stages for the isolation of [^{2}H, ^{13}C]amino acids involved the growing of respective strains producers on growth media containing labeled substrates – [^{2}H]methanol, [^{13}C]methanol and $^{2}H_2O$, isolation of culture liquids (CL) containing [^{2}H, ^{13}C]amino acids from microbial biomass, purification of lipids, cell disruption, derivatization of mixtures of amino acids by dansyl chloride, benzyloxycarbonyl chloride and diazomethane, separating of methyl esters of N-Dns-amino acid derivatives and N-Cbz-amino acid derivatives by reversed-phase HPLC and EI mass spectrometry of the obtained [^{2}H, ^{13}C]amino acid derivatives.

^{2}H and ^{13}C-labeled amino acids were isolated from lyophilized culture liquids of amino acid producing strains of *B. methylicum* and *M. flagellatum*, and as part of the total protein hydrolysates of microbial biomass. When isolating the total protein fraction it should be considered the presence of carbohydrates, lipids and pigments in samples. We used the protein rich bacterial strains with a relatively low content of carbohydrates in them. As a fraction of the total protein in the hydrolysis was subjected the residue obtained after the separation of exhaustive extraction of lipids and pigments by organic solvents (methanol–chloroform–acetone). In rare cases for the complete separation of the cellular components used the colloidal dissolution (solubilization) of proteins in 0,5 % of SDS, or the salting out by ammonium sulfate.

The hydrolysis of ^{2}H-labeled proteins was performed under conditions to prevent hydrogen isotopic exchange reactions with deuterium during the hydrolysis and preservation of the aromatic [^{2}H]amino acid residues in the protein. We considered two alternatives variants – the acid and alkaline hydrolysis. The acid hydrolysis of the protein under standard conditions (6 *M* HCl, 24 h, +110 ^{0}C), is known to induce complete degradation of tryptophan and partial degradation of serine, threonine, and several other amino acids in the protein (Cohen, Putter, 1970). Another significant drawback when carrying out the hydrolysis in HCl consists in the isotopic (^{1}H–^{2}H) exchange of aromatic protons (deuterons) in molecules of tryptophan, tyrosine and histidine, as well as protons (deuterons) at the C3 atom of aspartic and C4 glutamic acids (Penke et al., 1974). Therefore, to obtain the information about the real inclusion of deuterium into biosynthetically synthesized molecules of amino acids it was necessary to carry out the protein hydrolysis in deuterated reagents (6 *M* ^{2}HCl with 3 % phenol (in $^{2}H_2O$)).

Another variant of hydrolysis of the protein was consisted in using 2 *M* $Ba(OH)_2$ (+110 °C, 24 h). Under these conditions the reactions of isotopic ($^1H–^2H$) exchange at aromatic [^2H]amino acids – tyrosine and tryptophan do not occur, and tryptophan is not destroyed. Both these methods of hydrolysis showed good results for the conservation of aromatic [^2H]amino acids in the protein hydrolysate and the content of deuterium into the molecules of [^2H]amino acids. It must be emphasized, however, that for the preparative production of ^2H-labeled amino acids from the microbial protein is advisable to use the hydrolysis in ^2HCl in ^2H_2O (in the presence of phenol to maintain the aromatic amino acid) to prevent racemization. For studying the enrichment level of stable isotopes inclusion into residues of aromatic [^2H]amino acids and for analytical purposes it is better to use the hydrolysis of protein in the solution of $Ba(OH)_2$, in which there is no isotopic ($^1H–^2H$) exchange in amino acids and the residues of [^2H]phenylalanine, [^2H]tyrosine and [^2H]tryptophan are retained in the protein. The possible *D*,*L*-amino acid racemization by alkaline hydrolysis did not affect the result of the subsequent mass spectrometric study of the level of deuteration into [^2H]amino acid molecules.

For preparation of volatile derivatives the amino acids were converted into the methyl esters of N-Dns-[^2H, ^{13}C]amino acids or N-Cbz-[^2H, ^{13}C]amino acids, which were further separated by RP HPLC method. The conditions of N-derivatization of [^2H, ^{13}C]amino acids were practiced so as to obtain as much as possible intensive the molecular ion peaks ($M^{+\cdot}$) in EI mass spectra at the background level of the growth media metabolites. For this it was carried out the direct N-derivatization of [^2H, ^{13}C]amino acids in the composition of lyophilized culture liquids and total protein hydrolysates of biomass by 5-fold excess of dansyl chloride (in acetone) or benzyloxycarbonyl.

Under conditions of the reaction of N-derivatization for lysine, histidine, tyrosine, serine, threonine and cysteine along with monoderivatives were formed N-di-Dns and N-di-Cbz-derivatives. In addition, from arginine it was synthesized N-three-Dns-(Cbz)-arginine. Therefore, in mass-spectrometric studies the molecular ions ($M^{+\cdot}$) of these compounds were corresponded to di- or tri-derivatives.

The effectiveness of the use of N-Cbz- and N-Dns-amino acid derivatives in the RP-HPLC and EI mass spectrometric studies was demonstrated by us previously (Egorova et al., 1993). The volatility of N-derivatives of amino acids in EI mass spectrometric analysis can be further enhanced by the esterification of the carboxyl group, so that N-Dns-[^2H, ^{13}C]amino acids were converted into their methyl esters. To prevent the reverse isotopic exchange of aromatic protons (deuterons) in a process of esterification of ^2H-labeled amino acids, in this research we gave a preference to diazomethane. The freshly prepared solution of diazomethane in diethyl ether was treated with dry mixtures of amino acids residues. At derivatization of amino acids with diazomethane it was occurred an additional N-methylation at α-NH-(Dns)-group in [^2H]amino acids, leading to the appearance in the EI mass spectra of methyl esters of N-Dns-amino acids the additional peaks corresponding to compounds with a molecular mass on 14 mass units larger than the original compounds.

Study of levels of inclusion of stable isotopes of ^2H and ^{13}C into molecules of amino acids and hydrolysates

The levels of inclusion of stable isotopes of ^2H and ^{13}C into multicomponent mixtures of amino acid molecules of culture liquids and protein hydrolysates were determined analytically by EI mass spectrometry method. According to the mass spectrometric analysis the molecular ion peaks $[M]^+$ of methyl esters of N-Dns-[^2H]derivatives of aromatic [^2H]amino acids have a low intensity in EI mass spectra and were polymorphously split, so the areas of the molecular enrichment were strongly broadened. Moreover, mass spectra of the mixture components are additive, so the mixture can be analyzed only if the spectra of the various components are recorded under the same conditions. These calculations provide for the solution of the system of n equations in n unknowns for a mixture of n components. For components, the concentration of which exceeds 10 mol. %, the accuracy and reproducibility of the analysis makes up ±0,5 mol. % (at 90 % confidence probability). Therefore, to obtain reproducible results on deuteration levels it was necessary to chromatographically isolate individual derivatives of [^2H] amino acids from the protein hydrolyzate. Methyl esters of N-Dns-[^2H, ^{13}C]amino acid derivatives or N-Cbz-[^2H, ^{13}C]amino acid derivatives were separated by the method of preparative RP-HPLC on

octadecylsilane gel Separon SGX C18. The best result on separation was achieved by gradient elution of the methyl esters of N-Dns-[^{2}H, ^{13}C]amino acid derivatives with a mixture of solvents: (A) – acetonitril–trifluoroacetic acid = 100 : 0,1–0,5 vol. % and (B) – acetonitrile 100 vol. % in the gradient elution conditions by gradually increasing the concentration of component B in the mixture from 0 to 100 % (Table 2). In this case, each component of the mixture was separated in the most optimal composition of the eluent, thereby achieving their full separation quality in much less time than in isocratic mode. In addition, using the gradient was significantly increased the maximum number of peaks in the chromatogram can accommodate – a peak capacity, which is very important in the separation of complex multi-component mixtures, which are the protein hydrolysates. Thus, it was possible to separate the tryptophan and intractable pair of phenylalanine/tyrosine by this method. The degrees of chromatographic purity of ^{2}H- and ^{13}C-labeled amino acids, isolated from culture liquids of *B. methylicum* and *M. flagellatum* and protein hydrolysates in the form of their N-Cbz-[^{2}H, ^{13}C]amino acid derivatives comprised 96–98 % with yields – 67–89 %. For some [^{2}H, ^{13}C]amino acids was proved to be more convenient the separation as methyl esters of N-Dns-[^{2}H, ^{13}C] amino acid derivatives. The degrees of the chromatographic purity of methyl esters N-Dns-[^{2}H]phenylalanine, N-Dns-[^{2}H]tyrosine and N-Dns-[^{2}H]tryptophan obtained from the hydrolysates of bacteriorhodopsin were 96, 97 and 98 %, respectively. This result is important because of the chemical stability of methyl esters of N-Dns-amino acids; the presence of high-molecular ions ($M^{+\cdot}$) at higher molecular weights have proved to be very convenient for mass spectrometric investigations and enable to identify ^{2}H- and ^{13}C-labeled amino acids in the presence of low molecular weight metabolites in growth media and other products of derivatization. The latter fact is very important to study the composition of the pool of ^{2}H- and ^{13}C-labeled amino acids secreted into the culture liquids of amino acid producing strains and total protein hydrolysates.

Table 2. The results of one-step gradient separation of a mixture of methyl esters of N-Dns-[^{2}H, ^{13}C]amino acids from hydrolysates by RP HPLC, t = 20±25 ^{0}C on a 250×10 mm column with octadecylsilane gel Separon SGX C18, 7 μ (Kova, Slovakia)

Number of processing	Components of the mobile phase, vol. %		Elution time
	A*	B**	
1	90	10	10
2	80	20	10
3	60	40	10
5	50	50	10
6	30	60	5
8	20	80	5
9	10	90	5
10	0	100	5

Notes: * A – acetonitrile–trifluoroacetic acid = 100 : 0,1–0,5 vol. % ** B – acetonitrile = 100 vol. %

The fragmentation pathways of methyl esters of N-Dns-phenylalanine and N-Dns-leucine by the electron impact mass spectrometry lead to the formation of molecular ion peaks ($M^{+\cdot}$) at m/z = 412 and m/z = 378 and to the formation of dansyl fragments and products of the further decay to N-dimethylaminonaphthalene, and the formation of the amine fragment A^{+} and aminoacyl fragment B^{+} (Fig. 1). The fragmentation of methyl esters of N-Dns-phenylalanine and N-Dns-leucine shown on Fig. 1 is typical for these derivatives of all other amino acids, which allows carry out the mass spectrometric monitoring of ^{2}H- and ^{13}C-labeled amino acids in the intact culture liquids of producing strains containing in liquid media the amino acid mixtures and other metabolites of the growth medium until the stage of chromatographic separation, as well as explore the inclusion of stable isotopes of ^{2}H- and ^{13}C into the molecules of amino acids of protein hydrolysates.

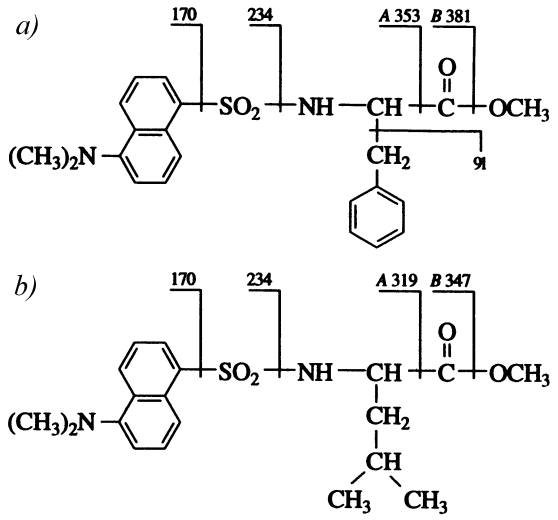
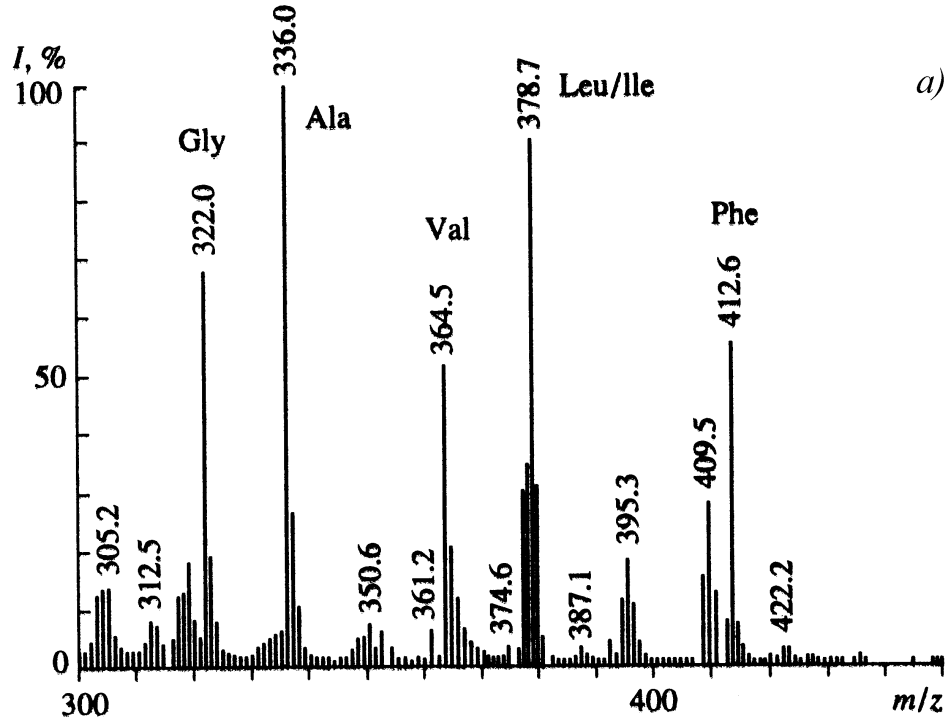


Fig. 1. Fragmentation of methyl esters of N-Dns-phenylalanine with M_r = 412 (*a*) and N-Dns-leucine M_r = 378 (*b*) in the electron impact mass spectrometry method



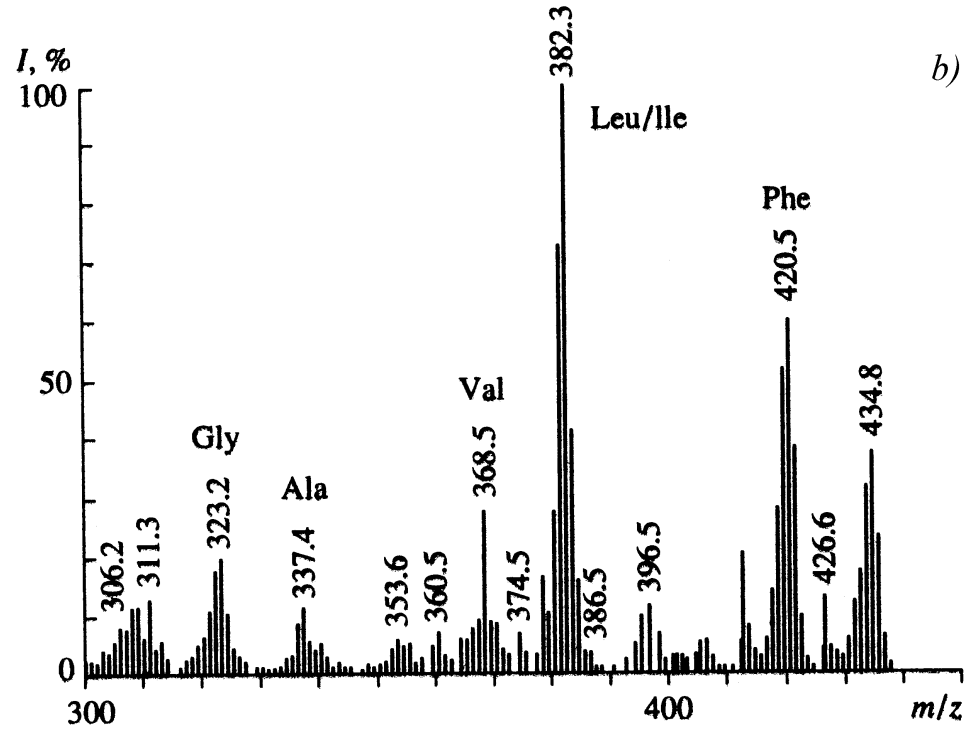


Fig. 2. EI mass spectra of methyl esters of N-Dns-[^{13}C]-amino acids from liquid culture of *M. flagellatum* after treatment with diazomethane and dansyl chloride: *a*) – 1 % methanol and H_2O (control); *b*) – 1 % [^{13}C] methanol and H_2O. Symbols of amino acids are marked peaks of molecular ions $[M]^+$ of methyl esters of N-Dns-[^{13}C] amino acids. The intensity of the peaks is given in %.

When using as a source of stable isotopes [^{13}C]methanol and ^2H_2O in the cell are synthesized the isotopically-substituted amino acids differ in the number of atoms substituted on isotopes of ^{13}C and ^{2}H. At the same time, the higher is the molecular weight of the amino acids, the larger is a set of possible molecular ions ($M^{+\cdot}$) corresponding to isotope-substituted forms. The peaks at *m/z* = 323,2; 337,4; 368,5; 382,3; 420,5 in the EI mass spectrum of [^{13}C]amino acid derivatives in the derivatized liquid culture (LC) of *M. flagellatum*, obtained on aqueous medium with 1% [^{13}C] methanol (Fig. 2b) correspond by the mass/charge ratios (*m/z*) to methyl esters of N-Dns-[^{13}C]glycine, N-Dns-[^{13}C]alanine, N-Dns-[^{13}C]valine, N-Dns-[^{13}C]leucine/[^{13}C]isoleucine and N-Dns-[^{13}C] phenylalanine. It should be emphasized that the value of *m/z* for the molecular ion ($M^{+\cdot}$) of methyl esters of N-Dns-[^{13}C]leucine and [^{13}C]isoleucine in the EI mass spectra is the same, so these amino acids could not be accurately identified by this method. The maximum levels of inclusion of ^{13}C isotope into amino acid molecules as measured by an increase of the averaged values of mass to charge ratio *m/z* for molecular ions ($M^{+\cdot}$) of isotopically-labeled sample in comparison with a molecular weight of a non-labeled natural amino acid are varied from 35 % for [^{13}C]alanine to 95 % for [^{13}C] phenylalanine (Fig. 2). Considering the auxotrophy of this strain for *L*-isoleucine, the variations in the range can be explained by the contribution of an exogenous isoleucine to the level of isotopic incorporation of [^{13}C]leucine, and other metabolically related amino acids – [^{13}C]alanine and [^{13}C]valine.

The mass spectrometry data on levels of inclusion of ^{13}C and ^{2}H into the molecule of N-Cbz-[^{2}H, ^{13}C]amino acid derivatives within various concentrations of ^2H_2O were not different from those of the methyl esters of N-Dns-[^{2}H, ^{13}C]amino acid derivatives (the accuracy of determination the levels of isotopic incorporation into amino acid molecules by this method comprises ± 5 %). As an example, Figure 3, b illustrates the mass spectrum of N-Cbz-[^{13}C]-Leu (relative to the unlabeled N-Cbz-Leu, Fig. 3, a) isolated by the RP HPLC method from LC of *M. flagellatum* after the treatment by benzyloxycarbonyl. The peak of molecular ion of N-Cbz- [^{13}C]-Leu corresponds the mean value of (M^+) at *m/z* = 269 (relative to the (M^+) at *m/z* = 265 under the control conditions), indicating that the inclusion of 4 atoms of isotope ^{13}C into the molecule of leucine (Fig. 3, Scheme). The specific fragmentation of N-benzyloxycarbonyl derivative of [^{13}C]leucine under electron impact

mass spectrometry makes it possible to localize the sites of incorporating of atoms of the isotope ^{13}C at the carbon skeleton of the molecule. As can be seen from Fig. 3, these are 4 carbon atoms in the positions (LeMaster, 1990; MacCarthy, 1986; Mosin et al., 1996; Crespi, 1986) of the carbon skeleton of the leucine molecule.

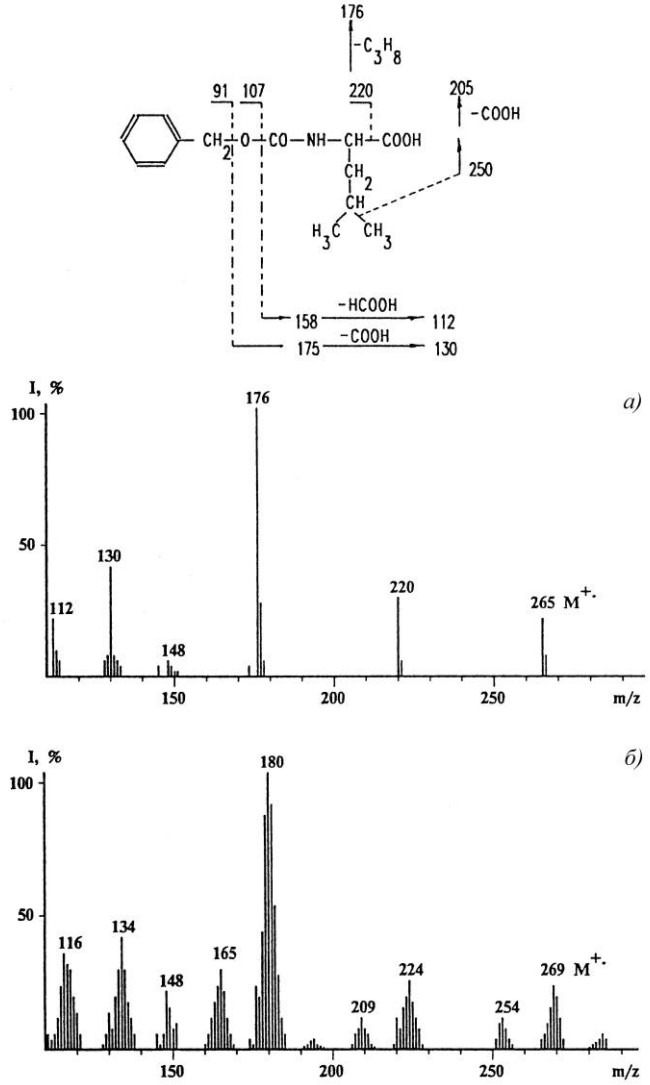


Fig. 3. EI mass spectra of N-Cbz-[^{13}C]-Leu, isolated from liquid culture of *M. flagellatum* after the treatment by benzyloxycarbonyl: a – 1 % methanol and H_2O (the control); b – 1 % [^{13}C] methanol and H_2O.

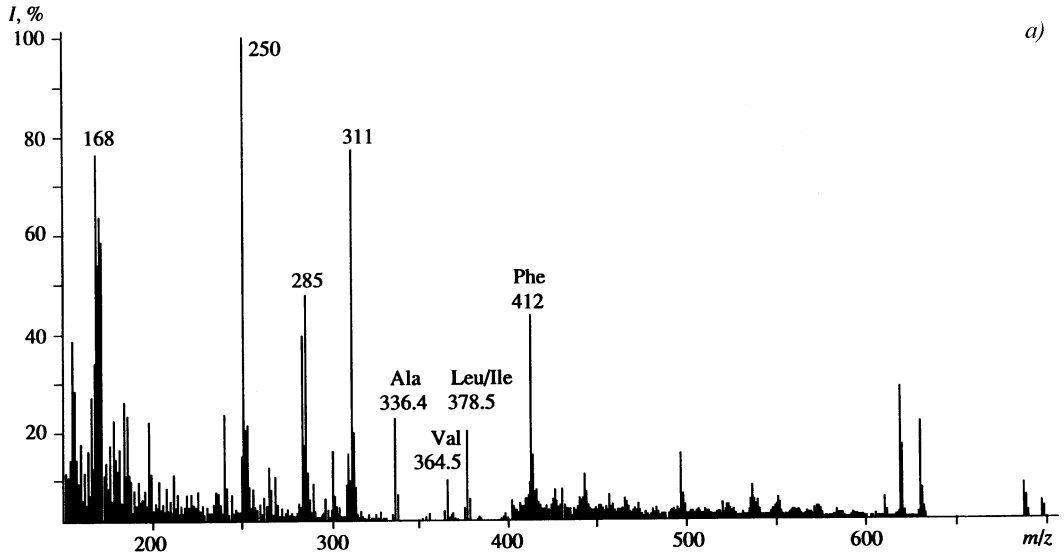
For the strain of methylotrophic bacteria *B. methylicum* there was a specific increase in the levels of isotopic incorporation of deuterium into molecules of individual [^{2}H]amino acids in the composition of culture liquids (Table 3) with stepwise increasing concentrations of $^{2}H_2O$ in growth medium. The inclusion levels of deuterium into molecules of different [^{2}H] amino acids under the same growing conditions are varied. In all experiments was observed the proportional increase in the levels of isotopic incorporation of ^{2}H into the molecules of metabolically related [^{2}H]amino acids with stepwise increasing concentrations of heavy water in the growth media (Table 3). This result was recorded in all experiments wherein as a source of stable isotopes was used $^{2}H_2O$.

Table 3. Levels of ^{13}C and ^{2}H inclusion into molecules of amino acids (atom %), secreted into the culture liquid (CL) of *B. methylicum* and *M. flagellatum*, and into amino acid residues of protein

Amino acid	Content of $^{2}H_2O$ in the growth medium, %*								1% $^{13}CH_3OH$**	
	24,5		49,0		73,5		98,0			
	LC	Protein	LC	Protein	LC	Protein	LC	Protein	LC	Protein
Glycine	–	15,0	–	35,0	–	50,0	–	90,0	60,0	90,0
Alanine	24,5	20,0	50,0	45,0	50,0	62,5	55,0	97,5	35,0	95,0
Valine	20,0	15,0	50,0	46,0	50,0	50,0	55,8	50,0	50,0	50,0
Leucine /Isoleucine	20,0	15,0	50,0	42,0	50,0	50,0	50,0	50,0	40,0	49,0
Phenylalanine	15,0	24,5	27,5	37,5	51,2	50,0	75,0	95,0	95,0	80,5
Tyrosine	–	20,0	–	25,6	–	68,5	–	92,8	–	53,5
Serene	–	15,0	–	36,7	–	47,6	–	86,6	–	73,3
Aspartic acid	–	20,0	–	36,7	–	60,0	–	66,6	–	33,3
Glutamic acid	–	20,0	–	40,0	–	53,4	–	70,0	–	40,0
Lysine	–	10,0	–	35,3	–	40,0	–	58,9	–	54,4

Notes: * Data are submitted for inclusion of ^{2}H into the amino acid molecules when growing of *B. methylicum* on aqueous M9 media containing 2 % methanol and a specified amount (vol. %) of $^{2}H_2O$. ** Data are submitted for inclusion of ^{13}C when growing of *M. flagellatum* on aqueous M9 media containing 1 % [^{13}C]methanol.

From the mass spectrum of methyl esters of N-Dns-[^{2}H]amino asid derivatives of culture liquid of *B. methylicum*, obtained on the growth medium containing 49 % $^{2}H_2O$ (Fig. 4b) is shown that the phenylalanine molecule contains 6 isotopically-substituted forms with an average peak of molecular ion ($M^{+\cdot}$) with m/z = 414,2, which increases compared with the control conditions (m/z = 412,0, Fig. 4a) on 2,2 units, i.e. 27,5 atom.% of the total number of hydrogen atoms in the molecule are substituted with deuterium. The region in the mass spectrum with values m/z = 90–300 corresponds to relevant products of derivatization of metabolites in the growth medium. The peak with m/z = 431,0, recorded in the mass spectrum of the culture liquid manifested in all the experiments, corresponds to the product of additional methylation of the phenylalanine molecule at α-NH- (Dns)-group. The peak with m/z = 400 (Fig. 4b) corresponds to the product of cleavage of deuterated methyl group from the [^{2}H]phenylalanine derivative.



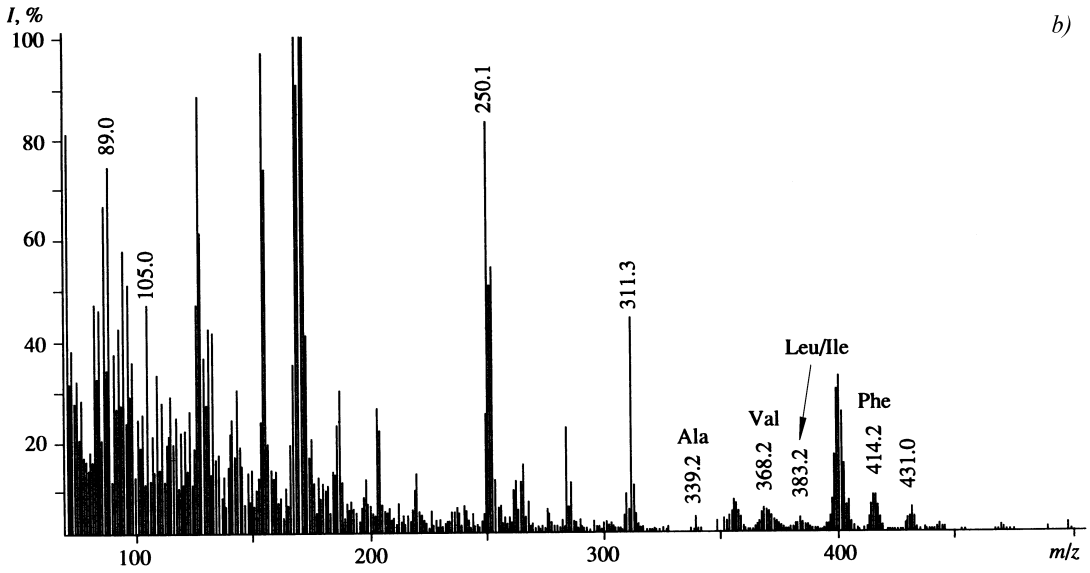


Fig. 4. EI mass spectrum of methyl esters of N-Dns-[^{2}H]-amino acids from liquid culture of *B. methylicum* after treatment with diazomethane and dansyl chloride: *a*) – 2 % methanol and 98,0 % H_2O (the control); *b*) – 2 % [^{2}H]methanol and 49,0 % of ^2H_2O

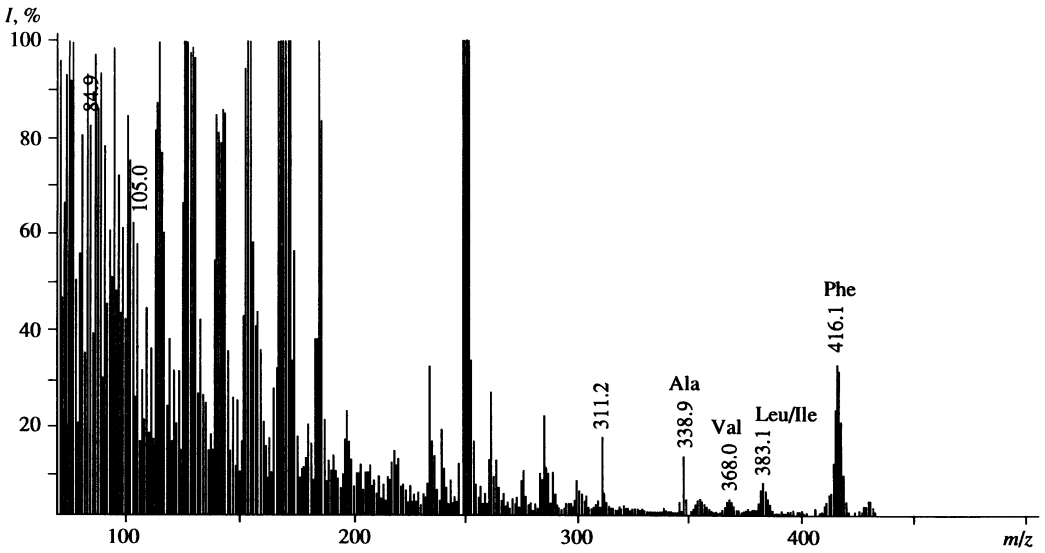


Fig. 5. EI mass spectrum of methyl ester of N-Dns-[^{2}H]-amino acids from liquid culture of *B. methylicum* when grown on growth medium containing 2 % [^{2}H]methanol and 73,5 % ^2H_2O

The presence in the EI mass spectrum of a sample of the liquid culture of *B. methylicum*, obtained on a medium containing 73,5 % ^2H_2O (Fig. 5) the molecular ion peak of the methyl ester of N-Dns-[^{2}H]phenylalanine ($M^{+\cdot}$) with m/z = 416,1 indicates on an increase in molecular weight of the [^{2}H]phenylalanine molecule on 4,1 unit i.e. 51,2 % of hydrogen atoms in the molecule of [^{2}H]phenylalanine in this case are replaced by deuterium. It is obvious that above mentioned deuterium atoms were entered into the [^{2}H]phenylalanine molecule through biosynthesis *de novo*, i.e. into the carbon skeleton of the molecule. The protons (deuterons) at heteroatoms in the NH_2- and COOH- groups of amino acids are appertained to the easily exchangeable ones, which are replaced by deuterium at the expense of their ease of the dissociation in H_2O (^2H_2O) solutions.

From Table 3 it is shown that in conditions of auxotrophy in *L*-leucine the levels of inclusion of ^{2}H into the molecules of [^{2}H]leucine/[^{2}H]isoleucine are lower than those ones for phenylalanine. This feature more clearly manifests in the medium with the highest concentration of ^2H_2O. Once again, this result is confirmed in Fig. 6 that shows the EI mass spectrum of methyl esters of

N-Dns-[^{2}H]amino acids of liquid culture after the growth of the bacteria *B. methylicum* under these conditions. Clearly, the molecular ion peak of methyl ester of N-Dns-[^{2}H]phenylalanyl ($M^{+\cdot}$) with *m/z* = 418,0 increases compared to control conditions for 6 units corresponding to the substitution of 75,0 atom % of the total number of hydrogen atoms in the molecule. Unlike [^{2}H] phenylalanine the inclusion level of deuterium enrichment in [^{2}H]leucine/[^{2}H]isoleucine was 50,0 atom %, and [^{2}H]valine – 58,8 atom %. The peak with *m/z* = 432, recorded in the EI mass spectrum of methyl esters of N-Dns-[^{2}H]amino acids of CL in Fig. 6 corresponds to the additional methylation product of [^{2}H]phenylalanine at α-NH_2 group. In addition, in the EI mass spectrum is recorded the peak of the enriched with deuterium the benzyl fragment $C_6H_5CH_2$ of [^{2}H]phenylalanine with *m/z* = 97 (instead of *m/z* = 91 in the control), indicating that the sites of localization of six deuterium atoms in the molecule of [^{2}H] phenylalanine are position of C1–C6 aromatic protons in the benzyl $C_6H_5CH_2$ fragment. From mass spectrometry data is demonstrated that at other concentrations of ^2H_2O in growth media deuterium is also included in the aromatic ring of [^{2}H]phenylalanine since the metabolism of the strain of *B. methylicum* adapted to ^2H_2O does not undergo significant changes in ^2H_2O.

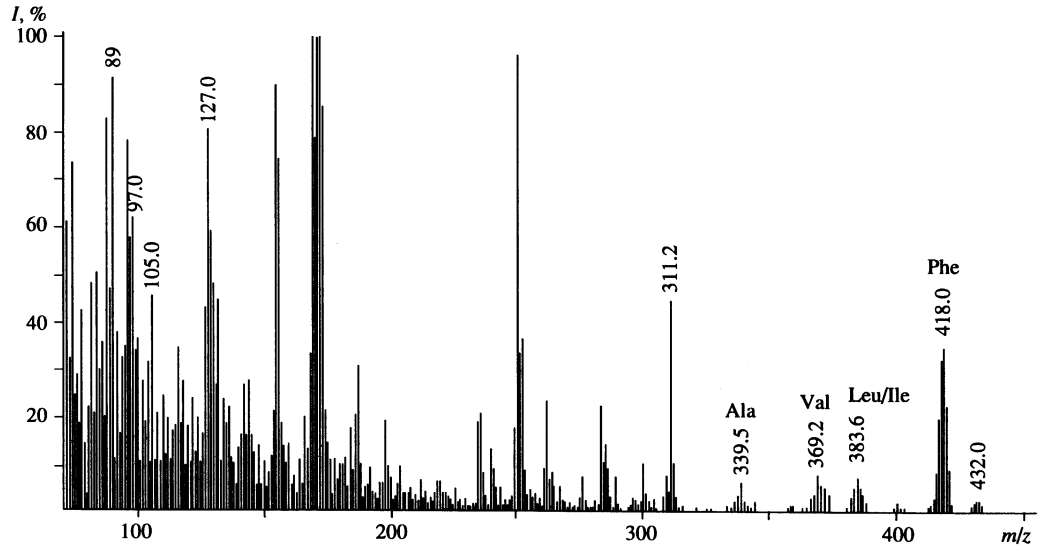


Fig. 6. EI mass spectrum of methyl esters N-Dns-[^{2}H]-amino acids from liquid culture of *B. methylicum* when growing on the growth medium containing 2 % [^{2}H]methanol and 98,0 % ^2H_2O (maximally deuterated M9-medium)

A similar pattern in inclusion levels of ^{13}C isotope into amino acid molecules associated with auxotrophic metabolism was manifested when growing the *L*-isoleucine-dependent strain of *M. flagellatum* on the growth medium with 1 % [^{13}C] methanol. As can be seen from Table 3, unlike that observed for [^{13}C]phenylalanine (the level of isotopic incorporation – 95,0 %), the level of incorporation of ^{13}C isotope into the molecules of [^{13}C]leucine/[^{13}C]isoleucine, [^{13}C]alanine and [^{13}C]valine were 38,0; 35,0 and 50,0 % respectively. The level of isotopic incorporation into [^{13}C]glycine (60 %), was although higher than that for the last three amino acids, but significantly lower than that of [^{13}C]phenylalanine.

Summarizing the data on the level of incorporation of ^{13}C and ^{2}H isotopes into secreted molecules of amino acids, it can be concluded about the maintaining of minor metabolic pathways associated with the *de novo* biosynthesis of leucine and the metabolically related amino acids. Another logical explanation for the observed effect, if we take into account the origin of leucine and isoleucine due to biosynthesis in various pathways (leucine belongs to the family of pyruvate, while isoleucine – to the family of aspartate (Fig. 7) could be the assimilation by the cell of the unlabeled leucine from the growth media under the background biosynthesis of isotopic-labeled isoleucine de novo. Taking into account of these effects it should be emphasized that the use of auxotrophic forms of microorganisms for production of ^{13}C and ^{2}H-labeled amino acids could not be justified practically because of the multiple character of inclusion of isotopes into the molecule (Mosin,

Ignatov, 2014). On the contrary, the use for this purpose the prototrophic forms of microorganisms seem to be more promising for these aims.

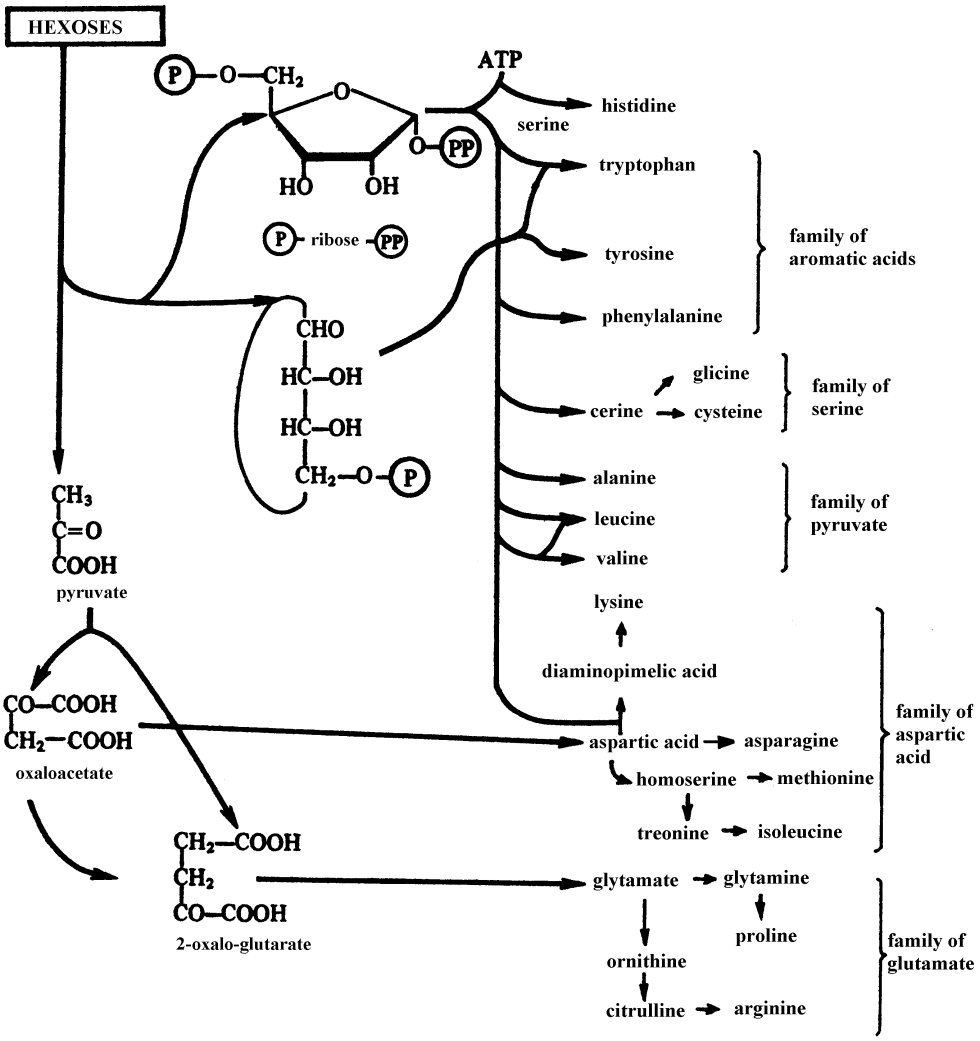


Fig. 7. The amino acids required for the synthesis of proteins produced in cells from precursors (by G. Schlegel (Schlegel, 1987))

The general principles for the study of levels of isotope inclusion of molecules of amino acids in this method of labeling were exemplified by the analysis of complex multicomponent mixtures obtained after total hydrolysis of proteins of biomass of methylotrophic bacteria As seen in Figure 8, up until 10 amino acids may be identified in the protein hydrolysate of *B. methylicum* by peaks of molecular ions ($M^{+\cdot}$) of corresponding methyl esters of N-Dns-[^{2}H]amino acid derivatives.

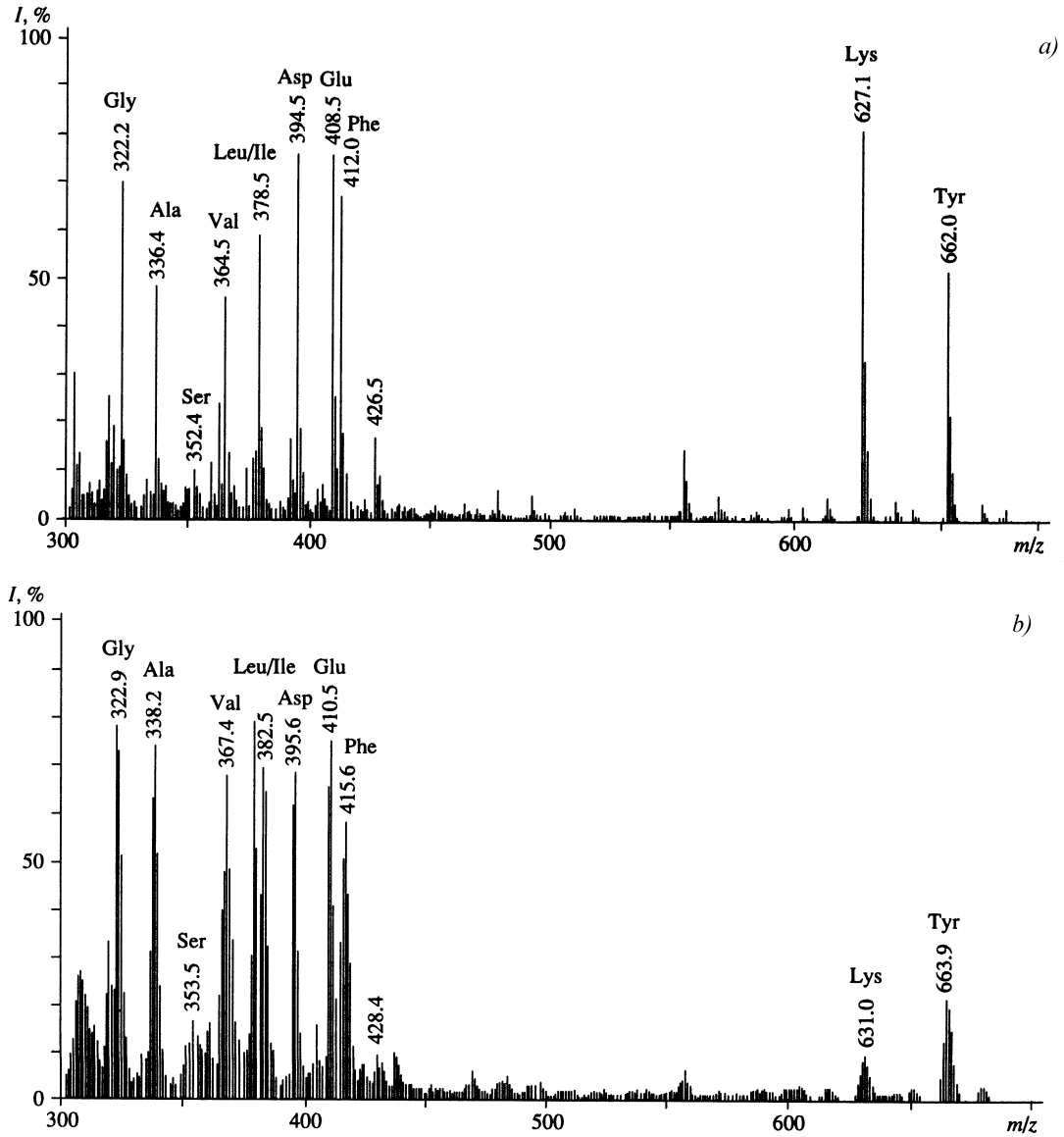


Fig. 8. EI mass spectrum of methyl ester of N-Dns-[^{2}H]-amino acids from hydrolysates of total protein of biomass of *B. methylicum* when growning on M9 medium, containing 2 % methanol and H_2O (the control) (*a*) and 2 % [^{2}H]methanol and 98,0 % ^2H_2O (*b*)

As in the case with secreted amino acids, the molecular ion peaks ($M^{+\cdot}$) were corresponded to isotopic mixtures of amino acid derivatives of isotopically substituted forms. For lysine and tyrosine the peaks ($M^{+\cdot}$) were corresponded to di-methyl esters of amino acid derivatives – α,ε-di-Dns-lysine [($M^{+\cdot}$) at m/z = 631,0) and O,N-di-Dns-tyrosine ($M^{+\cdot}$) at m/z = 663,9]. The levels of isotopic incorporation of deuterium into the molecules of [^{2}H]amino acids from the hydrolysate of total protein biomass at the ^2H_2O content in the growth medium from 49,0 % to 98,0 % were varied from 25,6 % for [^{2}H]tyrosine to 45,0 % for [^{2}H]alanine (Fig. 8b and Table 3). The levels of isotopic incorporation of deuterium into the molecules of [^{2}H]glycine, [^{2}H]valine, [^{2}H]phenylalanine, [^{2}H]serine, [^{2}H]lysine, [^{2}H]aspartic and [^{2}H]glutamic acid are raged within 35–46 %. As in the case with secreted amino acids, with the increase of ^2H_2O concentration in growth media, it was observed the proportional increase in the level of incorporation of ^{2}H isotope into amino acid molecules. With regard to other [^{2}H]amino acids not detectable by this method, it is obvious that the levels of isotope inclusion into the amino acid molecules are roughly the same. This is confirmed by data of separation of protein hydrolysates of methylotrophic bacteria by RP HPLC method as N-Cbz-[^{2}H]amino acid derivatives and methyl esters of N-Dns-[^{2}H]amino acid

derivatives and ion-exchange chromatography of protein hydrolysates, wherein it is detected 15 amino acids (Fig. 9, Table 4).

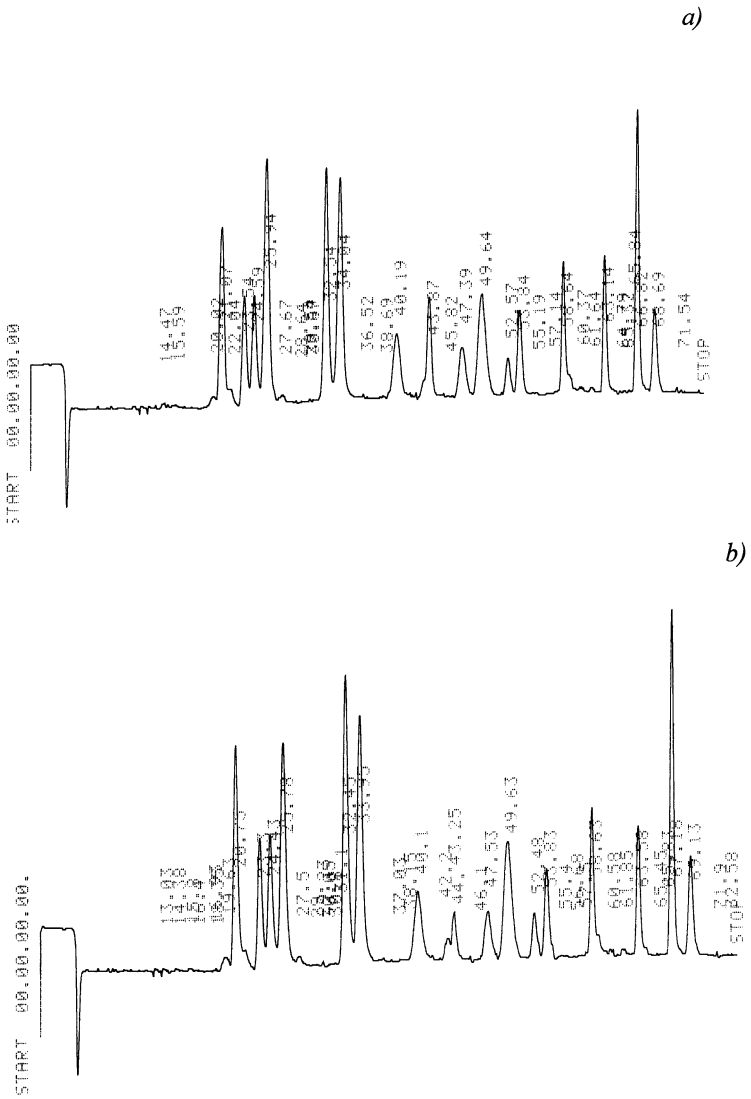


Fig. 9. Ion exchange chromatography of amino acids from hydrolysates of protonated (*a*) and deuterated (*b*) cells of *B. methylicum* on maximally deuterated M9 medium: Biotronic LC-5001 (230×3,2 mm) (“Eppendorf-Nethleler-Hinz”, Germany); mobile phase: UR-30 sulfonated styrene resin ("Beckman-Spinco", USA); pellet diameter – 25 mm; working pressure – 50–60 atm; mobile phase – 0,2 M Na-citrate buffer (pH = 2,5); eluent flow rate – 18,5 ml/h; ninhydrin – 9,25 ml/h; detection at λ = 570 and λ = 440 nm (for proline).

The findings suggest about the possibility of achieving maximum levels of inclusion of stable isotopes ^{2}H and ^{13}C into the amino acid residues of the total protein biomass (except for alanine, valine and leucine/isoleucine, reduced levels of inclusion of which explains the effect of auxotrophy for *L*-leucine and *L*-isoleucine). For example, in the case of the deuterated amino acid substitution at full stable isotopes has been achieved by using as a source of deuterium 98,0 % $^{2}H_2O$ (Table 4). As can be seen from Table 4, when the growning of *B. methylicum* on growth medium with 98,0 % $^{2}H_2O$, the inclusion levels of ^{2}H into residues of glycine, alanine, phenylalanine and tyrosine constitute 90,0; 97,5; 95,0 and 92,8 atom %. In experiments on the inclusion of ^{13}C isotope into the total protein biomass due to the assimilation of [^{13}C]methanol by methylotrophic bacteria *M. flagellatum* were also observed high levels of isotopic incorporation in [^{13}C]glycine (90,0 %), [^{13}C] alanine (95,0 %) and [^{13}C]phenylalanine (80,5 %) (see Table 3). As in the case with secreted amino acids the reduced inclusion levels of stable isotopes into [^{13}C]leucine/isoleucine (49,0 %), as well as into the related metabolic [^{13}C]amino acids under these conditions could be explained by

the effect of auxotrophy of the strain in *L*-isoleucine, which was added to the growth medium in the protonated form.

Table 4. The amino acid composition of a protein hydrolyzate of total protein of biomass of *B. methylicum*, obtained in the maximum deuterated growth medium* and levels of deuterium enrichment of molecules **

Amino acid	Output, % of dry weight of biomass		The number of included deuterium atoms in carbon skeleton of the molecule	The deuterium enrichment level, % from a total number of hydrogen atoms***
	Protonated sample (control)	Deuterated sample obtained in 98,0% ^2H_2O		
Glycine	8,03	9,69	2	90,0
Alanine	12,95	13,98	4	97,5
Valine	3,54	3,74	4	50,0
Leucine	8,62	7,33	5	50,0
Isoleucine	4,14	3,64	5	50,0
Phenylalanine	3,88	3,94	8	95,0
Tyrosine	1,56	1,83	7	92,8
Serine	4,18	4,90	3	86,6
Threonine	4,81	5,51	–	–
Methionine	4,94	2,25	–	–
Aspartic acid	7,88	9,59	2	66,6
Glumatic acid	11,68	10,38	4	70,0
Lysine	4,34	3,98	5	58,9
Arginine	4,63	5,28	–	–
Histidine	3,43	3,73	–	–

Notes: * Data obtained on M9 medium with 98,0 % ^2H_2O and 2 % [^{2}H]methanol. ** When calculating the deuterium enrichment level protons (deuterons) at COOH and NH_2 groups of amino acid molecules are not taken into account because of the ease of their dissociation and isotopic exchange in $H_2O/^2H_2O$. *** A dash indicates no data.

In all isotopic experiments on the integration of stable isotopes ^{2}H and ^{13}C into the amino acid molecules the levels of inclusion of ^{2}H and ^{13}C into metabolically related amino acids found a certain correlation. Thus, the isotopic incorporation levels for alanine, valine and leucine (pyruvate family), phenylalanine and tyrosine (aromatic amino acid family, synthesized from shikimic acid) are correlated (see Table 3 and Table 4). At the same time the levels of isotope inclusion for alanine, valine and leucine/isoleucine are stable within a wide variation of ^2H_2O concentration due to the effect of auxotrophy on leucine. The levels of isotopic incorporation for glycine and serine (serine family), aspartic acid, and lysine (asparagines family) also have similar values and are in correlation. The levels of isotopic incorporation into secreted amino acids and corresponding amino acid residues in the total protein when growing on media with the same isotope content generally are well correlated. The reason for some of the observed differences in the level of inclusion of isotopes into amino acid molecules can be associated with the effect of auxotrophy of the used strains in leucine and isoleucine.

4. Conclusion

The research has demonstrated the effectiveness of this method of biosynthesis of ^{2}H- and ^{13}C-labeled amino acids with different isotope enrichment levels, and electron impact mass spectrometry of N-Cbz-amino acid derivatives and methyl esters of N-Dns-amino acid derivatives for the study of isotopic enrichment levels of [^{2}H, ^{13}C]amino acid molecules in composition of multicomponent mixtures obtained biosynthetically with using microorganisms. The method is indispensable for the study of a pool of amino acids, secreted into the culture liquid of producing

strains grown on media with stable isotopes and protein hydrolysates of microbial biomass and may find further use in diagnostic studies.

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Установление путей биосинтеза ^{2}H- и ^{13}C-меченых аминокислот облигатной метилотрофной бактерией *Methylobacillus flagellatum* и факультативной метилотрофной бактерией *Brevibacterium methylicum*

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Аннотация. Методом масс-спектрометрии электронного удара установлены пути биосинтеза ^{2}H- и ^{13}C-меченные аминокислот штамма факультативных метилотрофных бактерий *Brevibacterium methylicum* и штамма облигатных метилотрофных бактерий *Methylobacillus flagellatum* при выращивании бактерий на средах, содержащих в качестве источников стабильных изотопов [^{2}H]метанол, [^{13}C]метанол и $^{2}H_2O$. Для масс-спектрометрического анализа мультикомпонентные смеси ^{2}H- и ^{13}C-меченных аминокислот в составе культуральных жидкостей и белковых гидролизатов (гидролиз в 6 *M* ^{2}HCl (3 % фенол) и 2 *M* $Ba(OH)_2$), модифицировали в N-бензилоксикарбонил-производные аминокислот и метиловые эфиры N-5-(диметиламино)нафталин-1-сульфонил-хлоридных (дансил) производных [^{2}H, ^{13}C]аминокислот, которые препаративно разделяли методом ОФ ВЭЖХ. Синтезированные ^{2}H- и ^{13}C-меченные аминокислоты представляют собой смеси, различающиеся количеством включенных в молекулу изотопов. Уровни включения ^{2}H и ^{13}C в молекулы секретируемых аминокислот и аминокислотные остатки суммарных белков биомассы варьируют в зависимости от содержания ^{2}H- и ^{13}C-меченых субстратов в ростовых средах и различаются для разных аминокислот (до 20,0 атом. % для *L*-лейцина/изолейцина и до 97,5 атом. % для *L*-аланина).

Ключевые слова: стабильные изотопы, биосинтез, метилотрофные бактерии, изотопно-меченые аминокислоты.

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Production Technology and Physicochemical Properties of Composition Containing Surfactant Proteins

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Abstract

The article describes a production method of substance containing great amount of phospolipids (up to 36 %) and surfactant proteins (up to 2 %) in terms of lyophilisate composition. Basic physical and chemical characteristics of the substance (density, viscosity, surface tension and the coefficient of sliding friction) indicate a high lubricant capacity of the derived product. These properties are kept when mixed with native human synovial fluid in the ratio of 1 to 9 inclusive. The obtained data allows to consider the derived composition, containing surfactant proteins and phospholipids, a variety of bionic lubricant suitable for testing as a potential equivalent of synovial fluid which can be used in traumatology and orthopedics, a cosmetic component or agent which increases the stability of the cell suspension during culturing in bioreactors.

Keywords: pulmonary surfactant, surfactant proteins, phospholipids, boundary lubrication, viscosity, tribology, articular cartilage; cartilage tissue engineering, surface friction

1. Introduction

We are now actively developing technologies of the creation and production of synthetic lubricants and their modifications to apply in different lines of industry, agriculture, medicine and veterinary medicine. Talking about the necessity to provide the contact of lubricant with biological objects, we find the biomimetic approach most effective, as it involves the production and application of the lubricants, which properties are maximally close to natural (Wang, 2014; Hwang et al., 2015; Park et al., 2016). One of the most promising sources of natural lubricants are surfactant mixtures produced from mammals' lung containing specific surfactant proteins (SP) and phospholipids (Sarker et al., 2011; Casals, Cañadas, 2012; Schenck, Fiegel, 2016).

Directly in medicine, veterinary and pharmaceutical biotechnologies lubricants are used to reduce friction between the surfaces of the natural parts of the body and to improve the adaptation of the moving parts (most often - in pulmonology, traumatology and orthopedics) (McNary et al., 2012; Lopez-Rodriguez, Pérez-Gil, 2014; Sui et al., 2016). In particular, the bulk of hyaluronic acid (hyaluronan according to the IUPAK nomenclature) for biomedical use is used as a lubricant for cosmetic preparations and equivalents of synovial fluid (Ayhan et al., 2014; Zhu et al., 2015). Also it is necessary to use lubricants to reduce the friction losses of the cell mass in bioreactors which come as a result of mixing and aeration, etc. (Valentín-Vargas et al., 2012; Knöspel et al., 2016).

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Obviously, there is a constant need for new technologies of creation of new lubricants based on bionic principles. One option of such lubricant may be a composition based on natural surfactants of pulmonary origin.

Physical and chemical properties of lubricant composition, such as density, viscosity, surface tension force and the force of friction (Rantamäki et al., 2011; Hui et al., 2012; Antonov, 2013), form objective criteria to define its effectiveness. Other, but no less important characteristics of the lubricant composition - chemical storage stability, biocompatibility and biosecurity, including the ability of utilization by the natural biological system, in which this lubricant functions, determine the basic advantages of bionic materials (Dicker et al., 2014; Smith et al., 2013).

The goal of this work, based on the above, is an attempt to develop technology to produce compositions with high lubricating qualities from recyclable materials of meat-processing industry and to explore their physical and chemical properties.

2. Material and Methods

Obtaining substance containing surfactant proteins

The experiment involved 16 white male rats of Wistar line 6 months old weighing 180-240 g Protocol of experiments comply with the ethical standards set out in the "Code of Good Practice in Research involving animal experimentation" and Directive 2010/63 / EU of the European Parliament and of the Council of the European Union on the protection of animals used for scientific purposes.

We used bronchoalveolar lavage technique to obtain a substance containing surfactant proteins (SCSP) in the first stage (Blanco, Pérez-Gil, 2007). We used a special eluent to increase the proportion of lipophilic surfactant proteins in lavage fluid. The composition included 2% solution of a phospholipids mixture in a commercial preparation Verolec F-62 (E322) manufactured by Emulgrain SA (Argentina) and Trilon B (EDTA) in phosphate buffer (pH = 7,4). The derived lavage fluid was centrifuged at 500 g for 10 minutes in order to separate the large particles and then directly, with the addition of distilled water, at 12,000 g for 1 hour at 4-6 ° C. The precipitate received was lyophilized and stored at 4 ° C before use.

At the second phase we obtained the mixture enriched with surfactant proteins. Lyophilisate was resuspended in a borate buffer (pH = 8.6) with addition of 1% commercial phospholipids mixture Verolec F-62, it was centrifuged at 3500 g for 15 min. The supernatant was subjected to final purification by preparative column chromatography (gel filtration) on Sephadex G 75 (Nakahara et al., 2009). Such modification of the technology made it possible to lower the concentration of anti-inflammatory peptide impurities, and to ensure the presence in the mixture of two fractions - Water soluble SP-A and SP-D, and cationic amphipathic hydrophobic SP-B and SP-C, associate with surfactant's phospholipids in the composition of the final substance (Fig. 1).

The yield of resulting product was 1.8% -3.2% from the absolute weight of rat's lung mass, the part of SCSP was lyophilized, and then we analyzed the percentage content of lipids, proteins and minerals in it. A similar study was carried out for the commercial preparation "Surfactant BL" manufactured by OOO "Biosurf" (St. Petersburg, Russia). For final use, we resuspended SCSP in sterile saline to 2% concentration of protein and then dispensed it. The ampoules were stored in a refrigerator at 4 ° C.

Determination of physical and chemical properties of the liquids being tested

We defined density as the ratio of the gained weight "Ohaus DV214CD", Germany), of a plastic syringe filled up to 1 cm 3 SCSP in relation to this volume. The results were expressed in g / cm 3 , the calculated error, taking into account the passport characteristics of the products, was less than 2.0%. To determine the viscosity of the SCSP we used the rotational viscometer DV-II + (Brookfield, USA) with a special adapter for small samples. Index values are expressed in mPas.

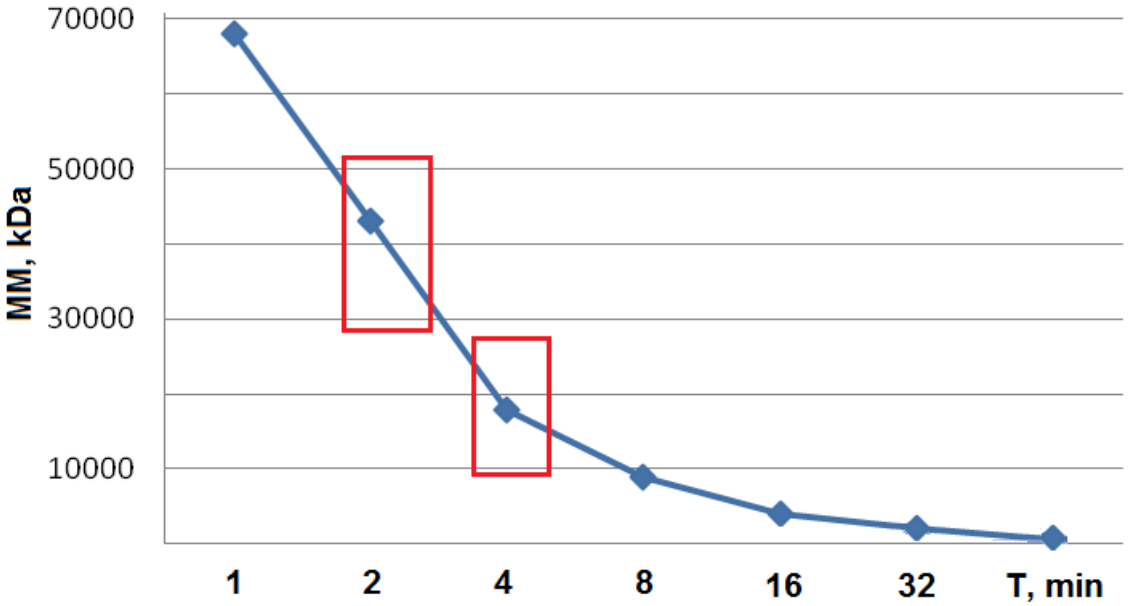

Fig. 1. Elution time needed for fractions of surfactant proteins within the evolution reaction performed by the preparative gel filtration with Sephadex G75. The time interval to collect the combined fractions, containing the surfactant proteins and not having the anti-inflammatory peptides, was defined.

To measure the surface tension k_σ we used the principle of the drop (stalagmometric) method. At first we sequentially recorded the process of formation and free-fall of three drops of the biological fluid from the plastic capillary with set point inner diameter D_j = 1 mm recording everything on digital video camera Nikon D3200 (Japan) We picked the drops into a plastic mini-cell of the specified weight, then we measured the weight gain with torsion balance, then calculated the average weight of 1 drop (m_d mg). During the separation of the drop of liquid from the lower end of the vertical tube its weight $m_d g$ was balanced by the surface tension force F, which is equal to the multiplication σ on the drop waist perimeter πD_j and prevents its separation.

$$m_d g = \pi D_j \sigma \quad (1).$$

To improve the accuracy of the correction we set up the adjustment factor k_σ, which can be defined as the ratio of the reference (table) value σ for glycerine to the value obtained in the pilot plant (it was 1.025). Thus, according to the known values of k_σ and the inner diameter of the capillary, the final formula for calculating σ (mN / m) becomes:

$$\sigma = k_\sigma m_\kappa g / \pi D_j = 3199\, m_\kappa \quad (2).$$

We used the evaluation formula adapted to the comparative method of Poiseuille's law to measure biological fluids. After substituting the values for the calibration liquid, the calculation formula determining the viscosity of the studied biological fluid (Pa · s) had acquired the form:

$$\eta = 0{,}689\, \rho t \quad (3).$$

The coefficient of sliding friction force M (dimensionless) was determined on a flat surface by fixing the maximum readings of traction micro dynamometer DEP1-1D-0,1R-1 (Pet-Ves, St. Petersburg, Russia) at the beginning of the motion of the reference load weighting 10 g and 1cm base area ² in conditions of contact "glass to glass" with complete wetting by the liquid under study. We referred this value (sliding friction force F_f) to the weight of the control weight mg in H:

$$\mu = F_f / mg \quad (4)$$

The described investigative techniques have been used to study the physical and chemical characteristics of the properties of the obtained SCSP, synovial fluid samples of five patients with the second stage of osteoarthritis, which were taken right before Viscosupplementation sessions, as well as their model mixtures in the ratio 1: 1, 1: 2,1: 4 and 1: 9.

Statistical analysis of the results

Quantitative data were processed using Statistica 10.0 (StatSoft Inc., USA) with the calculation of the indices adopted to characterize the non-parametric samples in biomedical research. Results were shown as Median [1st quartile ÷ 3rd quartile]. To prove the validity of differences the non-parametric Friedman criterion for multiple groups was applied. P values less than 0.05 were considered significant.

3. Results

The composition of SCSP according to the results of the definition included lipids (mostly - phospholipids), fractions of proteins with mass 16-24 kD (including the subunit SP - B, SP - C) and mass32-48 kD (including SP - D and some of SP - A), a small amount of impurity protein fractions and mineral salts. Quantitative results of the composition definition of freeze-dried SCSP are shown in Figure 2.

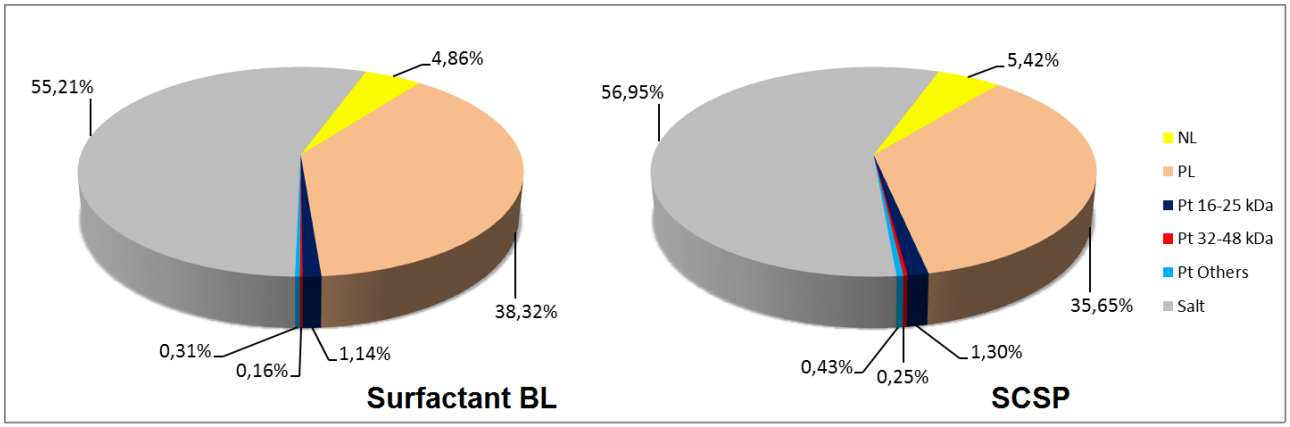

Fig. 2. The chemical composition of the market image drug Surfactant BL manufactured by Biosurf (St. Petersburg, Russia) and the original substance containing surfactant proteins (%).

Quantitative analysis of the selected SCSP showed that the resulting mixture contains more neutral lipids by 10%, but the content of the phospholipids fraction is reduced by 7% in comparison with commercial preparation "Surfactant BL». The concentration of proteins weighing 16-24 kD was bigger by 12.3% and of proteins weighing 32-48 kDa, was respectively, bigger by 36% in comparison with commercial preparation "Surfactant BL", the content of other associated proteins was also increased by 27.9%. Such differences may be caused by the difference in phases and the quality of purification of proteins. Salts in SCSP and commercial preparation "Surfactant BL" had no any significant differences and amounted to 3% only.

Figure 3 summarizes our findings in physicochemical properties of the biological fluids under study.

The obtained results of physical properties of synovial fluid and SCSP showed that synovial fluid has higher viscosity, but the friction force and surface tension is less by 15-20%. These figures are provided by key components, due to them synovial fluid performs its main function during the articular surfaces motion.

Mixing SCSP and synovial fluid in various ratios showed that different concentrations of certain components significantly alter the biological fluid properties. The best result was achieved by mixing in ratio of 1 to 4. The viscosity index decreased by 45% in comparison to synovial fluid, this result can be affected by the effective dilution of SCSP of synovial fluid. The surface tension decreased with adding of SCSP to synovial fluid, this reduction is caused by surfactant proteins SP-B and SP-C content in SCSP, the maximum reduction was achieved with ratio of 1 to 1. (Fig. 3).

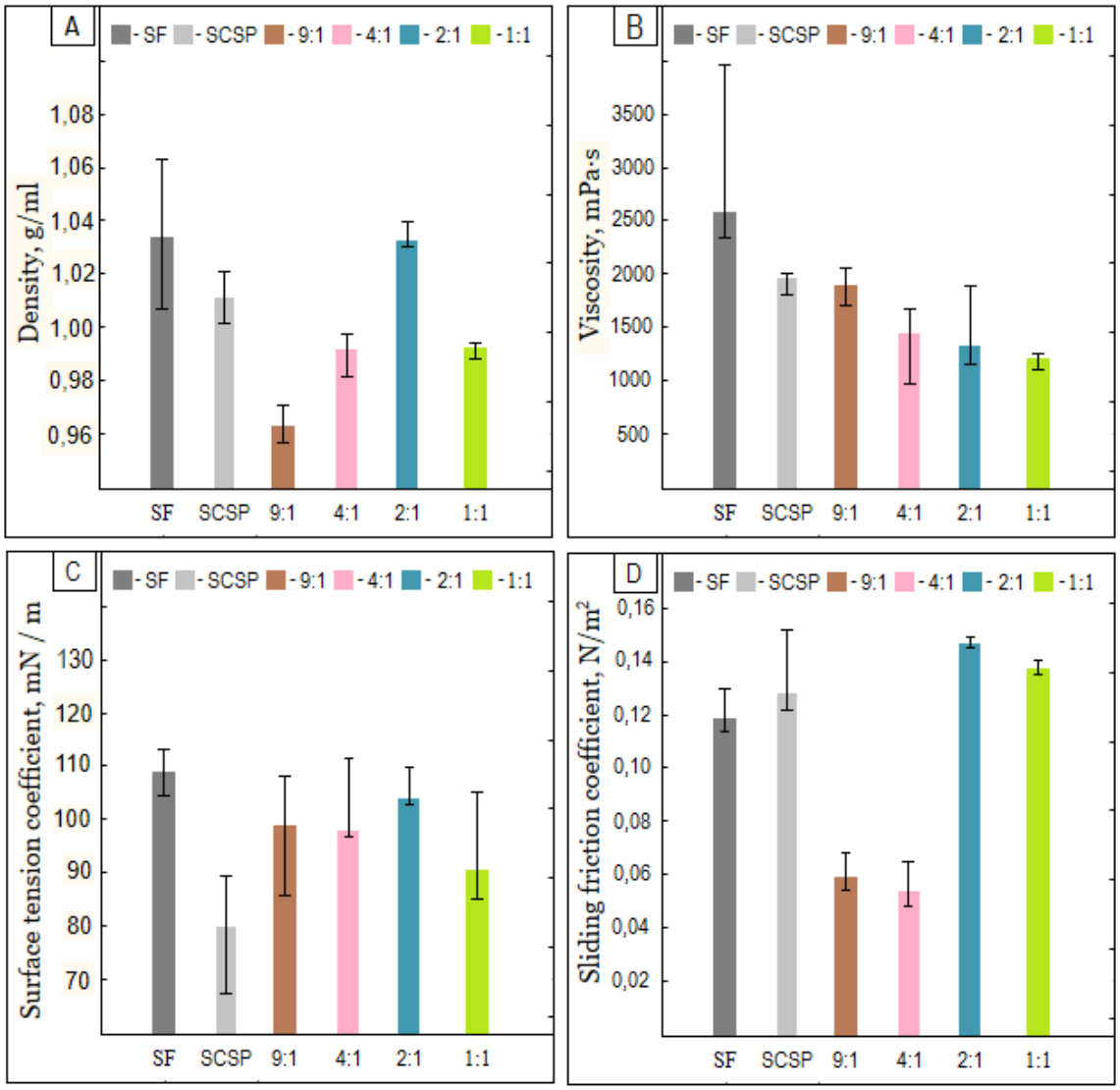

Fig. 3. The physicochemical properties of synovial fluid, substance containing surfactant proteins, and its mixtures in model suspensions. A. Density, g/ml. B. Viscosity, mPa s. C. Tension coefficient the Surface, mN/m. D. Sliding friction coefficient, N/m² . Quantitative results are represented in the form of Me [Q1 ÷ Q3].

The coefficient of sliding friction decreased with ratio of 1 to 9 and 1 to 4, when SCSP had been added to synovial fluid, and started to rise with ratio of 1 to 2 and 1 to 1, hypothetically the friction coefficient may have been affected by rising concentration of phospholipids, as adhesion of sliding surfaces takes place, and their movement is possible only when the force exerted on the load increases.

4. Discussion

Protein-containing bionic lubricants are able to provide a so-called boundary lubrication that means the lubrication, which is effective at high pressures and minimum layer of lubricating substance (McNary et al., 2012; Lu, 2009; Greene et al., 2011). Thus, hyaluronic acid is responsible for hydrodynamic lubrication at moderate pressures and the sufficient layer of the synovial fluid layer between the joint surfaces, and in case of direct contact of cartilage and high pressure of the contact - specific glycoprotein lubricin weighting 227 kDa (Flannery et al., 2009; Novochadov et al., 2014; Ludwig et al., 2015). Similar mechanisms are required in terms of maintaining the elasticity of the alveoli, which is provided by the surfactant lung system having a high grade of homology with lubricin, primarily due to the presence of mucin domain fragment (Blanco, Pérez-Gil, 2007, Fathi-Azarbayjani, Jouyban, 2015).

The Data, Obtained by us at study of SCSP , indicates that surfactant-associated proteins content in conjunction with phospholipids provides a biological fluid with high rate of force coefficients of surface-tension and sliding frictional force, having reduced viscosity at the same time. That in turn can allow using of SCSP as improved lubricating composition.

Known physical and chemical characteristics of the resulting substance (alone or in various dilutions with human synovial fluid) are comparable by their values to the characteristics of drugs currently used as a synovial fluid prosthetic in joint diseases (Shen, Chen, 2014; Zhang et al., 2016). One of the SCSP's advantages is that it has functionally active association of phospholipids and proteins, which is, in modern concepts, necessary for mediating not only frictional, but also signal anti-inflammatory properties of natural lubricants of a human body (Andrades et al., 2012; Kosinska et al., 2013). The surfactant proteins listed above, as well as their association with phospholipids, of course, fulfills the function of reducing the surface tension and the coefficient of sliding friction. Therefore, the resulting lubricant composition can be used as an additive for cosmetic products industry, as well as for bioreactors to reduce the cell loss in cultivation them in the form of suspensions. This technology can be successfully scaled to manufacture the product from the arm animal's lungs saving the ability to use the remaining by-product as a raw material for the food industry and other biotechnologies.

5. Conclusion

The resulting product has composition with basic properties of a lubricating composite. The main components of SCSP are surfactant-associated proteins and phospholipids. Physical and chemical properties correspond to the characteristics of natural lubricant – the synovial fluid of articular cartilage. Therefore, this product can be tested as a potential component of synovial fluid prosthetic and other biomedical products.

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Технология получения и физико-химические свойства композиции, содержащей белки сурфактанта

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Аннотация. В работе описана методика получения из легких млекопитающих композиции с высоким содержанием фосфолипидов (до 36 %) и белков сурфактанта (до 2 %) в пересчете на лиофилизат. Основные физико-химические характеристики композиции (плотность, вязкость, коэффициент поверхностного натяжения и коэффициент трения скольжения) свидетельствуют о высокой лубрикативной способности полученного продукта. Эти свойства сохраняются при смешивании с нативной синовиальной жидкостью человека в разведении до 1: 9 включительно. Полученные данные позволяют считать полученную композицию, содержащую фосфолипиды и белки сурфактанта, вариантом бионического лубриканта, пригодного для проведения испытаний в качестве потенциального протеза синовиальной жидкости в травматологии и ортопедии, компонента косметических средств или средства, увеличивающую стабильность клеточной суспензии при культивировании в биореакторах.

Ключевые слова: легочный сурфактант, белки сурфактанта, фосфолипиды, граничная смазка, вязкость, трение скольжения, трибология, суставной хрящ; тканевая инженерия хряща.

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Results of the Study of Mutagenic Effects of Microbial Polysaccharides

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Abstract

The article presents the results of a study of mutagenic effects of *Pseudomonas alcaligenes* polysaccharides. Pseudomonas genus – non-fermentative ubiquitous bacteria, having specific metabolic cycles and unique physical, chemical and biological properties was used as a producer of natural exopolysaccharides. In an experiment using the Ames test, three variants of test compounds were studied: 1. a compound of the *Pseudomonas alcaligenes* biofilm, 2. exopolysaccharide matrix and the microorganism cell wall compound, and 3. actually the microbial exopolysaccharide. In all cases the lack of mutagen action of polysaccharides of *Pseudomonas alcaligenes* is proved that make them perspective for use as nanomaterials of new generation – alternative wound coverings.

Keywords: *Pseudomonas alcaligenes*, polysaccharides, exopolysaccharides, Ames test, mutagenic effect, wound coverings.

1. Introduction

The majority of microbic polysaccharides have the unique specific structure or serological group and, as a rule, represent mix of molecules of different molecular weight, but of an identical chemical structure (Belyaev, 2000). They are divided on intracellular, localized in cytoplasm, and extracellular – an extracellular layer, capsules or covers. Free hydroxyl groups of exopolysaccharides (EPS) are acylated and alkylated, glycosidic bonds can be hydrolyzed by acids and specific enzymes (Arkadieva et el., 1989). Besides, in microbic glycans earlier unknown monosaccharides which do not occur neither in animals, nor in plants often are found (Malashenko et al., 2001; Kumar et al., 2007). Unique chemical, biological and rheological properties of microbial EPS long ago attracted attention of experts in various branches of science and engineering as the promising biologically active compounds important for the solution of a number of practical and theoretical tasks in the field of biomedicine and biotechnology. Following companies are considered to be full-fledged leaders in their study and production: «Rhone Poulenc» (France), «Statoil» (Norway), «Kelco» (USA). Through their activity the annual increase in production of polysaccharides of microbial origin in the world is on average 10 % (Sutherland, 2009). Microbial EPS are widely used in the oil and mining industries, in paints, in medicine - as a matrix for tissue engineering, producing surgical and non-woven materials, elements for osteosynthesis, drug delivery systems, the preparation of food technology and more (Greenberg,

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1991; Obraztcova, Sidorova, 2014; Perok et al., 2002). At the Microbiology Department, Institute of Medicine of Petrozavodsk State University the possibility of using *Pseudomonas alcaligenes* exopolysaccharide to develop measures to prevent infection of primary and secondary tension wounds was successfully investigated. For this physicochemical and biological properties of pseudomonads exopolysaccharides were studied (Voronov, Sidorova 2015). Opposing activity of lytic enzymes and microbial cellular wall components was investigated (Sidorova, Komkova, 2015). In *Pseudomonas alcaligens* hydrolysates 5 compounds of monosaccharide structure were revealed: galactose, arabinose, xylose, rhamnose, galacturonic acid (GA); in the structure of core oligosaccharides, except rhamnose, α-alanine is revealed, being typical aliphatic amino acid. Thanks to joint cultivation of *Pseudomonas alcaligens* with laboratory cultures of *Staphylococcus aureus* and *Escherichia coli* data on antagonistic properties of polysaccharides are obtained. By means of methods of direct antagonism it is established that variability on percentage suppression of test cultures determined by antibiotic effects of EPS. The listed biological properties of exopolysaccharides of pseudomonads do them perspective for development of an alternative wound covering capable not only to restore structure of a tissue, but also to use the antimicrobial potential of microbial polymer for prevention of infections and associated with them complications of various genesis. In the context of the above, the purpose of the present work was to study the mutagenic effect of *Pseudomonas alcaligenes* exopolysaccharide.

2. Materials and methods

Mutagenic activity of biological products based on *P. alcaligenes* metabolites was investigated according to the Ames test, described in the «Manual on experimental (preclinical) study of new pharmacological substances» (Khabriev, 2005) and recommended by Pharmacological State Committee of the Russian Ministry of Health for testing products at the stage of pre-clinical toxicological study. The test is used to evaluate the mutagenic activity of drugs on the basis of *Pseudomonas* and consists of two components: the registering and activating. The recording part is a set of indicator strains of *Salmonella typhimurium*, w~~hose~~hich characteristics are given in Table 1.

Table 1. Characteristics of indicator strains of *Salmonella typhimurium*

Strains	Mutations			Plasmid pKM 101	Type of registered mutations
	histidine auxotrophy	rfa	uvrB		
TA 100	G-46	+	+	+	Bases replacement
TA 98	D-3552	+	+	+	Frameshift

Indicator strains are able to record the action of mutagens that cause a replacement of base pairs in DNA molecule (TA 100), and frameshift mutations (TA 98). Under the mutagen action his gene is able to revert to the wild type, and the appearing revertant bacteria become histidine prototrophs. For the analysis of the obtained results mutagenic index (MI) is used, which is calculated as the ratio of the number of colonies of *Salmonella* in the experiment in relation to the control cultures grown in the presence of a mutagen.

To increase mutants sensitivity to mutagens action in indicator bacteria genome additional mutations are introduced which allow to obtain widely the used strains. Deletion of galbiouvrB captures biotin operon, the part of the galactose operon and uvrB gene. Last defect causes a disturbance of the excision repair system, which further enhances the sensitivity of the test strains to the action of a number of mutagens. Rfa mutation increases the permeability of the cell wall due to defects in the polysaccharide layer. The strains TA 100 and TA 98 carry pKM 101 plasmid, through which these strains are more sensitive to the action of a number of substances than the original plasmidless strains. The activating part consists of postmitochondrial supernatant of rat liver homogenate (S-9 fraction) and cofactors that are used for the normal functioning of microsomal oxidation systems. For forming activation mixture usually C-9 fraction is taken, NADP and glucose 6-phosphate. The latter are NADPH-generating system: NADPH serves as an electron donor, which is transferred to the cytochrome P-450. Rat liver S9 was used, which had been previously pretreated with inducers of microsomes. As inductor is used phenobarbital, 3-methylcholanthrene, polychlorinated biphenyls, Aroclor 1254 or sovol. To determine the mutagenic activity of the drug

into the molten semisolid starvation agar at 43–45°C 0,1 ml of the *Salmonella typhimurium* (2×10^8 cells) suspension is put in, 0.1 ml and 0.5 ml of a biological product activation mixture, after it the tube contents is rapidly mixed and layered on the lower selective agar. Plates are incubated at 37°C for 48 hours, the number of colonies are counted – histidine auxotrophy revertants to prototrophy. If a substance exhibits mutagenic activity, the number of revertants in the experimental plates exceeds the number of revertants in the control. During the experiment formulation positive and negative controls were considered. As a negative control, sterile water was used in an amount of 0.5 ml and 0.1 ml of an overnight culture of *Salmonella typhimurium* TA 100 with the addition of 0,1 ml of molten agar and histidine. For negative control spontaneous mutation in bacterial strains was used – separately for each version of the experiment. As positive control was used 0,1 ml of sodium azide, which was added to 0,1 ml of an overnight culture of *Salmonella typhimurium* TA 100 with 0,1 ml of histidine and molten agar solution. The experiment was performed times. The experimental results are accounted in accordance with the guidelines. As a positive, the result was considered, when there was an increase in the frequency of mutations in more than 2 times compared to the control.

3. Results and discussion

There were three variants of biological specimens used in this work:

1. Exopolysaccharide native biofilms *Pseudomonas alcaligenes* and in dilution $10^{-1} – 10^{-4}$ (n = 3);

2. Exopolysaccharide fractions and components *Pseudomonas alcaligenes* native cell wall and in dilution $10^{-1} – 10^{-4}$ (n = 3);

3. *Pseudomonas alcaligenes* native exopolysaccharides and in dilution $10^{-1} – 10^{-4}$ (n = 3).

For each experimental variant there was found a rate of geometric mean number excess of revertants in experimental and control values and then compared with critical value given in the guidelines.

Table 2. Evaluation of the potential mutagenic activity of *Pseudomonas alcaligenes* EPS and their complexes using the Ames test without metabolic activation

Bio-preparations variants	Dilutions	TA 100		
		$\bar{x}$	σ	$\overline{x0}/\overline{xk}$
1	native	104,2	6,3	1,50
	10^{-1}	93,1	5,4	1,28
	10^{-2}	101,9	6,9	1,64
	10^{-3}	98,4	5,1	1,21
	10^{-4}	92,1	6,4	1,52
«+» control		907,8	11,4	
«–» control		32,1	4,2	
2	native	108,4	6,7	2,16
	10^{-1}	98,5	5,9	1,90
	10^{-2}	98,2	5,9	1,90
	10^{-3}	101,1	6,8	2,19
	10^{-4}	98,7	5,2	1,67
«+» control		1010,5	10,3	
«–» control		24,9	3,1	
3	native	101,2	6,7	2,31
	10^{-1}	97,3	5,9	2,03
	10^{-2}	98,4	5,2	1,79
	10^{-3}	90,1	5,6	1,93
	10^{-4}	98,1	5,7	1,96
«+» control		1009,2	10,1	
«–» control		21,2	2,9	

Notes: $\bar{x}$ – the arithmetic average of the number of colonies, σ – the standard deviation,

$\overline{x0}/\overline{xk}$ - the ratio of the average number of revertants in an experiment to «–» control.

As can be seen from the data presented in Table 2, bacterial strains reduced the number of revertants under the action of substances taken as negative controls without metabolic activation by increasing dilution. Thus, the experimental data the potential mutagenic activity of EPS *Pseudomonas alcaligenes* and their complexes, in Ames test are objective.

Table 3. Evaluation of potential mutagenic activity of *Pseudomonas alcaligenes* EPS and their complexes with Ames test at metabolic activation

Bio-preparations variants	Dilutions	TA 100		
		$\bar{x}$	σ	$\overline{x0}/\overline{xk}$
1	native	108,6	6,7	1,76
	10^{-1}	98,9	5,9	1,55
	10^{-2}	96,4	5,4	1,42
	10^{-3}	102,1	6,1	1,60
	10^{-4}	98,7	5,8	1,52
«+» control		1020,6	10,4	
«–» control		52,3	3,8	
2	native	106,3	6,3	1,70
	10^{-1}	102,1	6,1	1,64
	10^{-2}	101,2	6,8	1,83
	10^{-3}	98,9	5,9	1,59
	10^{-4}	95,4	5,7	1,54
«+» control		1010,5	10,1	
«–» control		35,2	3,7	
3	native	104,9	6,2	1,44
	10^{-1}	97,7	6,0	1,39
	10^{-2}	101,2	5,8	1,34
	10^{-3}	100,1	6,3	1,46
	10^{-4}	99,1	6,2	1,44
«+» control		1017,4	10,4	
«–» control		61,6	4,3	

When analyzing the potential mutagenic activity of *Pseudomonas alcaligenes* EPS and their complexes with metabolic activation (Table 3), it was found that the ratio of the mean number of revertants to the negative control is lower compared with the results obtained in the experiment of evaluating the potential mutagenic activity of *Pseudomonas alcaligenes* EPS and complexes without metabolic activation (Table 2).

4. Conclusion

From the above data it can be concluded that *Pseudomonas alcaligenes* EPS and their complexes in concentrations of 0.1 - 1000 μg/plate causes no increase in the number of revertants among strains of *Salmonella typhimurium* TA98, TA100, and thus does not exhibit mutagenic effects and hence bio-inert wound covering being developed based on *Pseudomonas alcaligenes* EPS can be recommended for clinical trials as an alternative for the treatment of wounds of various etiologies.

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Результаты исследования мутагенного эффекта полисахаридов микробного происхождения

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Аннотация. В статье приводятся результаты исследования мутагенного эффекта полисахаридов *Pseudomonas alcaligens*. В качестве природных продуцентов экзополисахаридов использованы представители рода *Pseudomonas* – неферментирующие убиквитарные бактерии, обладающие специфическими метаболическими циклами и

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уникальными физико-химическими и биологическими свойствами. В эксперименте с использованием теста Эймса изучено 3 варианта тестируемых соединений: 1. в составе биопленки *Pseudomonas alcaligenes,* 2. в составе экзополисахаридного матрикса и компоненты клеточной стенки микроорганизма и 3. собственно – микробный экзополисахарид. Во всех случаях доказано отсутствие мутагенного действия полисахаридов *Pseudomonas alcaligenes,* что делает их перспективными для использования в качестве наноматериалов нового поколения – альтернативных раневых покрытий.

Ключевые слова: *Pseudomonas alcaligenes*, полисахариды, экзополисахариды, тест Эймса, мутагенный эффект, раневые покрытия.