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Deuterium Labeled *L*-Phenylalanine and Microbiological Preparation for Application in Medical Diagnostics

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Abstract

It was carried out the microbiological preparation of deuterium labeled *L*-phenylalanine with using a Gram-positive aerobic facultative methylotrophic bacterium *Brevibacterium methylicum*, L-phenylalanine producer with ribulose-5-monophosphate (RuMP) cycle for carbon assimilation via conversion of low molecular weight substrates ([U-^{2}H]MeOH and ^2H_2O). For this purpose was the bacterium with improved growth characteristics on minimal salt media M9 supplemented with 2% (v/v) [U-^{2}H]MeOH and increasing gradient of ^2H_2O concentration from 0; 24.5; 49.0; 73.5 up to 98% (v/v) ^2H_2O. L-phenylalanine was isolated from the growth medium after adding 5 M ^{2}HCl (in ^2H_2O), pH = 2.0 by extraction with isopropanol and subsequent crystallization in ethanol (output 0.65 g/l). Alanine, valine, and leucine/isoleucine were produced and accumulated exogenously in amounts of 5–6 μmol in addition to the main product of biosynthesis. The method allows to obtain [^{2}H]phenylalanine with different levels of deuterium enrichment, depending on ^2H_2O concentration in growth media, from 17% ^{2}H (2 deuterium atoms) (on the growth medium with 24.5% (v/v) ^2H_2O) up to 75% ^{2}H (6 deuterium atoms) (on the growth medium with 98% (v/v) ^2H_2O) with introduction of deuterium to benzyl $C_6H_5CH_2$-fragment of molecule that is confirmed with the data of electron impact (EI) mass spectrometry analysis of methyl ethers of N-5-dimethylamino(naphthalene)-1-sulfochloride [^{2}H]amino acids after the separation by reverse-phase HPLC.

Keywords: *Brevibacterium methylicum*, [U-^{2}H]MeOH, heavy water, biosynthesis, [^{2}H]amino acids, EI mass spectrometry, HPLC.

1. Introduction

Labeling of amino acid molecules with deuterium is becoming an essential part for various biochemical and biomedical studies with ^{2}H-labeled molecules and investigation of certain aspects of their metabolism (LeMaster, 1990; Vértes, 2011). Tendencies to preferable using of stable isotopes in clinical diagnostics in comparison with their radioactive analogues are caused by absence of radiation danger and possibility of localization of a label in a molecule by highly resolution methods as NMR spectroscopy and mass spectrometry (Mosin et al., 1996). Development of these methods for detecting stable isotopes for last

years has allowed to improve considerably carrying out numerous biological studies with amino acids de novo, and also to study the pathways of their metabolism. ^{2}H-labeled amino acids are applied in medical diagnostics and biochemical research (Kushner et al, 1999), as well as in chemical synthesis of various isotopically labeled compounds on their basis, for example, [^{2}H]phenylalanine – in synthesis of peptide hormones and neurotransmitters. Isotopically labeled analogues of L-phenylalanine find the increasing application in diagnostic purposes, for example, for detecting hereditary phenylketonuria (PKU) – a metabolic genetic disorder characterized by a mutation in the gene of the hepatic enzyme phenylalanine hydrolase (PAH), making it nonfunctional and connected with disorders of phenylalanine metabolism in organism (DiLella et al., 1986; Surtees & Blau, 2000). For biomedical diagnostic of PKU is essential to study distribution of isotope label in end-products of metabolism of this amino acid by introducing isotopically labelled phenylalanin into organism (Dewick, 2009; Mosin, 1996). Therefore it is important to develop new biotechnological approaches for preparation of natural isotopically labelled analogues of phenylalanine, including its deuterated analogues. Advantages of biotechnological methods for synthesis of isotopically labelled amino acids in comparison with chemical synthesis consist in high exits of synthesised products and that they possess a natural L-configuration.

For introduction of deuterium into amino acid molecules either chemical or biosynthetic methods may be used. Chemical synthesis of ^{2}H-labeled compounds has one significant limitation; it is a very laborious and costly multistep process resulting in obtaining a racematic mixture of D,L- enantiomers (Blomquist et al., 1966). Although chemomicrobiological synthesis overcomes this problem (Faleev et al., 1989), the amounts of purified enzymes, required is prohibitive (Walker & Matheny, 1986). The biosynthesis of ^{2}H-labeled amino acids usually involves growth of an organism on selective growth media containing the labeled substrates: e.g., growth of algae autotrophically on media with 96% (v/v) $^{2}H_2O$, is a well established method for biosynthesis of highly deuterated biochemicals including amino acids (Cox & Kyli, 1988; Crespi, 1986). Though this method, being generally applicable, is still limited due to lower resistance of plant cells towards $^{2}H_2O$ along with the expense of the mixture of ^{2}H-labeled amino acids which is isolated from hydrolysates of biomass (Den'ko, 1970; Mosin & Ignatov, 2013).

Alternatively inexpensive sources for microbiological synthesis of [^{2}H]amino acids seem methylotrophic bacteria, capable to oxidize methanol (MeOH) and other one-carbon compounds containing methyl CH_3-group to formaldehyde via ribulose-5-monophosphate and serine pathways of carbon assimilation (Antony, 1986). Interest to use methylotrophic bacteria in biotechnology has recently increased owing to development of new perspective technologies for chemical synthesis of methanol. Owing to 50% level of bioconversion of methanol (at efficiency of methanol conversion 15,5–17,3 gram of dry biomass for 1 gram of consumed substrate) methlotrophic bacteria are considered as cheap sources of deuterated protein and irreplaceable amino acids, whose technological expenses for their preparation are defined, basically by cost of $^{2}H_2O$ and [^{2}H]methanol. These bacteria have shown a great practical advantage in terms of its industrial usage because of their ability to produce and accumulate in culture liquid large quantities of ^{2}H-labeled amino acids during its growth on media supplemented with $^{2}H_2O$ and [U-^{2}H]MeOH and is a cost effective process due to lower price of [U-^{2}H]MeOH (Karnaukhova et al., 1993). The traditional approach for this aim is cultivation of strain-producers of amino acids on growth media containing [^{2}H]methanol and heavy

water (^2H_2O) with subsequent fractionation of liquid cultural media for isolation of [^{2}H]amino acids (Mosin et al., 2012).

In the past few years, a certain progress was observed in terms of isolation of methylotrophs wherein RuMP cycle operates. However still for such studies, limited research is carried out with methylotrophs due to poor growth characteristics obtained on ^2H_2O containing media. Realizing this, the production of ^{2}H-labeled amino acids by obligate methylotrophs *Methylobacilus flaggellatum* for carbon assimilation was conducted by 2-keto 3-deoxy 6-phospho-6-gluconate aldolase/transaldolase (KDPGA/TA) variant of RuMP cycle which involves the bacterial growth on salt medium supplemented with approx. 75% (v/v) ^2H_2O (Skladnev et al., 1996). In the present study, a mutant strain of Gram-positive aerobic facultative methylotroph, *Brevibacterium methylicum*, with NAD^+ dependent methanol dehydrogenase (EC 1.6.99.3) variant of RuMP cycle for carbon assimilation was selected, which seems more convenient for the preparation of ^{2}H-labeled amino acids because of its ability to grow on minimal salt media M9 with 98% (v/v) ^2H_2O as compared to the obligate methylotroph *M. flagellatum* (Mosin et al., 1998).

The main aim of the present study is to study the physiological adaptation through deuterated enrichment technique for Gram-positive aerobic facultative methylotrophic bacterium *B. methylicum*, L-phenylalanine producer, to high level of ^2H_2O in order to increase level of [^{2}H]amino acids by this selected bacterium.

2. Material and Methods

2.1. Chemicals

^2H_2O (99.9% (atom) ^{2}H) was purchased from JSC "Isotope" (Sankt Petersburg, Russia), [U-^{2}H]MeOH (97.5% (atom) ^{2}H) (Biophysics Center, Pushino, Russia). Dansylchloride (DNSCl) of sequential grade was from Sigma Chemicals Corp. (USA). Diazomethane (DZM) was prepared from N-nitroso-methylurea (N-NMU) ("Pierce Chemicals Corp.", USA). Inorganic salts were crystallized in 99.9% (atom) ^2H_2O; ^2H_2O was distilled over $KMnO_4$ with the subsequent control of isotopic enrichment by ^{1}H-NMR spectroscopy on Brucker WM-250 device ("Brucker Corp.", USA) (working frequency – 70 MHz, internal standard – Me_4Si).

2.2. Bacterial Strain

A Gram-positive parental strain of L-leucine auxotroph of facultative methylotrophic bacterium *Brevibacterium methylicum* # 5662, producer of L-phenylalanine used in this research was obtained from Russian State Scientific Center for Genetics and Selection of Industrial Microorganisms GNIIGENETIKA. The parental strain was modified by adaptation to ^2H_2O *via* plating the cells onto 2% (w/v) agarose media with an increasing gradient of ^2H_2O concentration from 0; 24.5; 49.0; 73.5 up to 98% (v/v) ^2H_2O and the subsequent selection of separate colonies resistant to ^2H_2O, capable to produce L-phenylalanine while grown on liquid growth media with the same isotopic content.

2.3. Adaptation and Growth Conditions

The parent strain was adapted to deuterium via plating the cells to individual colonies followed by

subsequent selection of cell colonies which were resistant to deuterium on solid 2% (w/v) agarose media M9 with 2% (v/v) MeOH/[U-^{2}H]MeOH containing a step-wise increasing gradient of ^2H_2O concentration from 0; 24.5; 49.0; 73.5 up to 98% (v/v) ^2H_2O. Minimal salt medium M9 (g/l): KH_2PO_4 – 3; Na_2HPO_4 – 6; NaCl – 0.5; NH_4Cl – 1, with MeOH (2%, v/v) as a carbon and energy source and L-Leu (100 mg/l) was used for bacterial growth. Selection of separate colonies was performed by their resistance to ^2H_2O. Cell colonies grown on the growth media M9 with a low gradient of ^2H_2O concentration were transferred onto the growth media with higher gradient of ^2H_2O concentration; this was repeated up to 98% (v/v) ^2H_2O. At a final stage of this technique on growth medium with 98% (v/v) ^2H_2O were isolated the separate cellular colonies representing posterity of one cell, resistant to the action of ^2H_2O. Then cells were transferred to the liquid growth medium M9 with the same deuterium content, and grew up during 3–4 days at temperature 34 ^{0}C.The adaptation was monitored by formation of individual colonies onto the surface of solid agarose media with ^2H_2O and also by values of optical density (OD) of cell suspension in liquid media, determined in a quartz cuvette (light path 10 mm) at λ = 620 nm using a Beckman DU-6 spectrophotometer ("Beckman Coulter", USA). The bacterium adapted to ^2H_2O was grown on the maximally deuterated M9 medium with 2% (v/v) [^{2}H]methanol and 98% (v/v) ^2H_2O in 250 ml Erlenmeyer flasks containing 50 ml of growth medium at 32–34 ^{0}C and vigorously aerated at 200 rpm on orbital shaker Biorad 380-SW ("Biorad Labs", Poland). The exponentially growing cells (cell density 2.0 at absorbance 620 nm) were pelleted by centrifugation (1200 g for 15 min), the supernatant was lyophilized in vacuum and used for chemical derivatization.

2.4. Determination of Phenylalanine

The amount of phenylalanine was determined for 10 μl aliquots of liquid minimal salt medium M9 by TLC method with solvent mixture iso-PrOH–ammonia (7 : 3, v/v) using pure commercially available phenylalanine as a standard. The spots were treated with 0.1% (w/v) ninhydrin solution (prepared in acetone), and was eluted with 0.5% (w/v) $CdCl_2$ solution prepared in 50% (v/v) EtOH (in H_2O) (2 ml). Absorbance of the eluates was measured on spectrophotometer Beckman DU-6 ("Beckman Coulter", USA) at λ = 540 nm, the concentration of phenylalanine in samples was calculated using a standard curve.

2.5. Isolation of [^{2}H]Phenylalanine from Growth Medium

Deutero-biomass of *B. methylicum* obtained after growth in maximally deuterated medium M9 with 2% (v/v) [U-^{2}H]MeOH and 98% (v/v) ^2H_2O, was separated using centrifugation ("Heraues Sepatech", Model T-24, Germany) at 1200 g for 15 min. Supernatant thus obtained was evaporated under vacuum. To 5 g of the lyophilized growth medium M9; 30 ml of iso-PrOH was added, and thus acidified to pH = 2.0 using 5 M ^{2}HCl (in ^2H_2O) and keep at room temperature for 4 h. The salts were removed through centrifugation and the supernatant was evaporated. Phenylalanine (0.65 g/l) was recrystallized from EtOH: $[\alpha]_D^{20}$ = 35^0 (EtOH); UV-spectrum (0.1 *M* HCl): λ_{max} = 257.5 nm, $\varepsilon_{257.5}$ = $1.97 \cdot 10^2$ $M^{-1} \cdot cm^{-1}$.

2.6. Synthesis of Methyl Esters of N-DNS-[^{2}H]Amino Acids

To 200 mg of lyophilized growth media M9 in 5 ml 2 *M* $NaHCO_3$ (0.002 mol) solution, pH = 9–10, 320 mg (1.2 mol) DNSCl in 5 ml of acetone was added. A mixture was kept at 40 ^{0}C under vigorous stirring for 1 h.

After the reaction was completed, the solution was acidified by 2 *M* HCl till pH = 3.0, and extracted with ethyl acetate (3 × 5 ml). The combined extracts were dried over sodium sulphate and evaporated under vacuum. The further derivatization to methyl esters of N-DNS-[^{2}H]amino acids was performed using a standard chemical procedure with DZM. To, 20 ml of 40% (w/v) KOH in 40 ml of diethyl ether 3 g of N-NMU was added and mixed at 120 rpm on a water bath cum stirrer at 4 ^{0}C for 15–20 min. After intensive evolution of gaseous DZM ether layer was separated and washed out by cold water till pH = 7.0 was achieved, then dried over sodium sulphate for further processing and analysis.

2.7. High-Performance Liquid Chromatography

Analytical separation of methyl esters of N-DNS-[^{2}H]amino acids was performed by a reversed-phase HPLC on liquid chromatograph Knauer ("Knauer GmbH", Germany), supplied with UV-detector and integrator C-R 3A ("Shimadzu", Japan). Methyl esters of N-DNS-[^{2}H]amino acids were detected at λ = 254 nm by UV-absorbance. As a motionless phase was used Separon SGX C_{18} ("Kova", Czech Republic), 22 ^{0}C, 18 μm, 150 × 3.3 mm. The mobile phase composed of a mixture of solvents: (A) – acetonitrile–trifluoroacetic acid (20: 80%, v/v) and (B) – acetonitrile (100%, v/v). The gradient started from 20% A to 100% B for 30 min; at 100% B for 5 min; from 100% B to 20% A for 2 min; at 20% A for 10 min.

2.8. EI Mass-Spectrometry

EI MS was performed for the determination of deuterium enrichment levels using methyl esters of N-DNS-[^{2}H] amino acids on Hitachi MB 80 mass spectrometer ("Hitachi", Japan) at an ionizing energy of 70 eV, with an accelerating electrical voltage of 8 kV, and an ion source temperature of 180–200 ^{O}C. Each measurement was repeated at least three times. Calculation of deuterium enrichment of [^{2}H]amino acids was performed on a parity of contributions of molecular ion ($M^{+\cdot}$) peaks of N-DNS-[^{2}H]amino acid methyl esters obtained in isotopic experiments relative to the control in protonated minimal medium M9. Statistical processing of experimental data was carried out with using the program of statistical package, STATISTICA 6.

3. Results and Discussion

3.1. Study of phenylalanine biosynthesis by the strain B. methylicum in ^2H_2O containing media

As is known, the majority of the microorganisms distributed in nature cannot serve as good producers of aromatic amino acids, owing to the presence in a cell effective mechanism of regulation of their biosynthesis, though this ability is known for a number of their mutant forms (Mosin et al., 1996). Effective microbial producers of *L*-phenylalanine are, as a rule, the mutants, which do not have negative control over such key enzymes of biosynthesis of this amino acid as prephenate dehydratase (EC 4.2.1.51), chorismate mutase (EC 5.4.99.5) and 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (EC 2.5.1.54). Phenylalanine is synthesized in most bacteria *via* shikimic acid pathway from prephenic acid, which through formation of phenylpyruvate that turns into phenylalanine under the influence of cellular

transaminases (Conn, 1986; Herrmann & Weaver, 1999). The precursors for the biosynthesis of phenylalanine are phosphoenolpyruvate (PRP) and erythrose-4-phosphate (ERP). The latter compound is an intermediate in the pentose phosphate (PenP) pathway and, in some methylotrophs, during the RuMP cycle of carbon assimilation (Antony & Williams, 2003; Lindstom & Stirling, 1990). The basic metabolic transformation of phenylalanine in animals is enzymatic hydroxilation of this amino acid with formation of tyrosine (Bohinski, 1983).

Phenylalanine is synthesized in most organisms *via* shikimic acid pathway from the common aromatic amino acid precursors as erythrose-4-phosphate (E4P) formed through formation stages of shikimic acid, chorismic acid and prephenic acid (Fig. 1). The basic intermediate products of phenylalanine catabolism and metabolically related tyrosine in various organisms are fumaric acid, pyruvate, succinic acid, acetaldehyde etc. At hereditary penylketonuria disease the hepatic enzyme phenylalanine hydroxylase (PAH), which is necessary to metabolize phenylalanine to tyrosine is nonfunctional, that caused accumulation of phenylalanine and its metabolites (phenylpiruvate, phenyllactate, phenylacetate, *ortho*-hydroxyphenyl acetate), superfluous amounts of which negatively affect development of nervous system (Michals & Matalon, 1985; Filiano, 2006).

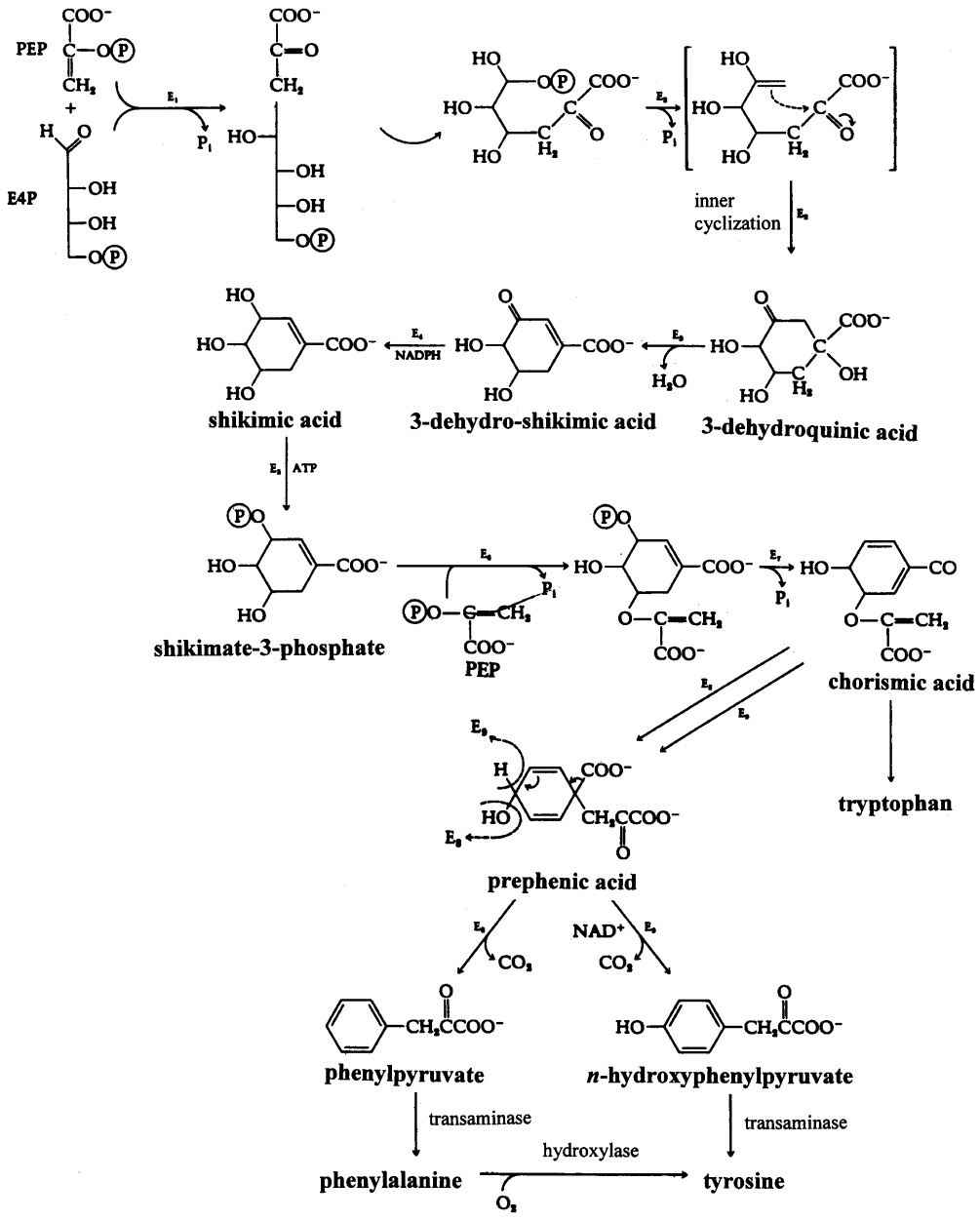

Figure 1. Scheme of biosynthesis of aromatic amino acids – phenylalanine, tyrosine and tryptophan from the general predecessors – phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) (Mosin & Ignatov, 2012)

The native bacterial strains can not be the potential producers of phenylalanine owing to their effective mechanisms in its metabolic regulation, although certain bacterial mutants with mutations in their metabolic machinery such as in their of prephenate dehydrogenase (EC 1.3.1.12), prephenate hydratase (EC

4.2.1.51), chorismate mutase (EC 5.4.99.5), 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (EC 2.5.1.54) and in other enzymes which thereby proves to be an active producers of this amino acid (Wendisch, 2007). The best phenylalanine producing strains once selected were the mutants partially or completely dependent on tyrosine or tryptophan for growth. The reports about other regulative mechanisms for phenylalanine biosynthesis in bacterial cells are quite uncommon, though it is known that RuMP cycle operates in certain number of auxotroph mutants of methylotrophs, covers numerous steps in aromatic amino acid biosynthesis (de Boer et al., 2007; Abou-Zeid et al., 1995).

A certain practical interest represents the research of the ability to produce L-phenylalanine by a leucine auxotroph of Gram-negative aerobic facultative methylotrophic bacterium *B. methylicum* realizing the NAD^+ dependent methanol dehydrogenase (EC 1.6.99.3) variant of the RuMP cycle of carbon assimilation – convenient, but insufficiently explored object for biotechnological usage. 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. Comparative analyses (TLC, NMR) showed, that L-phenylalanine, produced by this strain of methylotrophic bacteria is identical to the natural L-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 – [U-^{2}H]MeOH and ^2H_2O. For this, deuterium enrichment technique *via* plating cell colonies on 2% (w/v) agarose media M9 supplemented with 2% (v/v) [U-^{2}H]MeOH 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 Fig. 2. 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 (Fig. 2). 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.

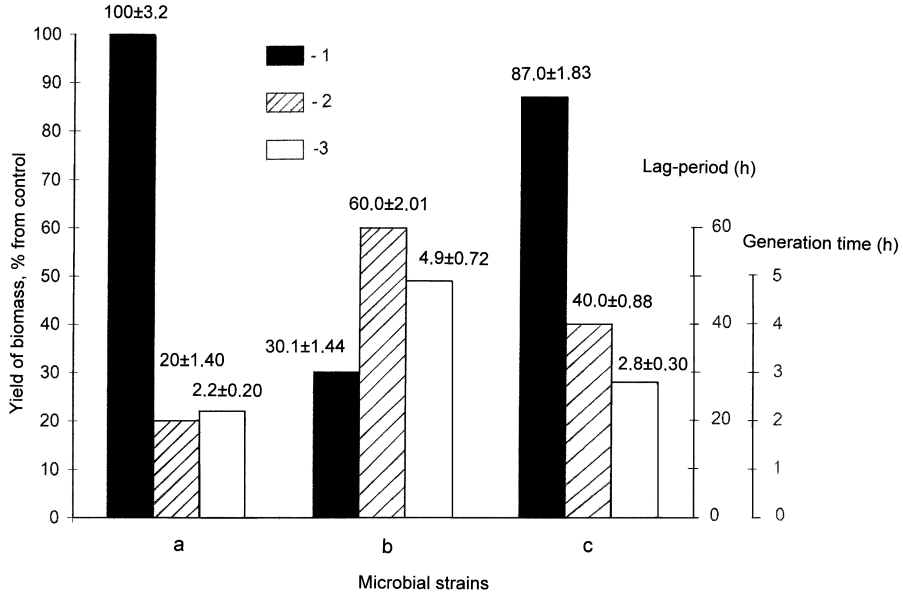

Figure 2. Yield of microbial biomass of *B. methylicum*, magnitude of lag-period and generation time in various experimental conditions: initial strain on protonated M9 medium (control) with water and methanol (*a*); initial strain on maximally deuterated M9 medium (b); adapted to deuterium strain on 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 1 (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 MeOH and H_2O. The maximum deuterium content was under conditions (10) and (10') in which we used 98% (v/v) ^2H_2O and 2% (v/v) [U-^{2}H]MeOH. The even numbers of experiment (Table 1, expts. 2, 4, 6, 8, 10) were chosen to investigate whether the replacement of MeOH by its deuterated analogue affected growth characteristics in presence of ^2H_2O. That caused small alterations in growth characteristics (Table 1, expts. 2, 4, 6, 8, 10) relative to experiments, where we used protonated methanol (Table 1, 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 1, 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 1, expts. 2–4) there was a small inhibition of bacterial growth compared with the control (Table 1, expt. 1). However, above 49% (v/v) ^2H_2O (Table 1, expts. 5–8), growth was markedly reduced, while at the upper content of ^2H_2O (Table 1, 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 1, expt. 10) with 98% (v/v) ^2H_2O and 2% (v/v) [U-^{2}H]MeOH, 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 1, expt. 1). While on comparing adapted bacterium on maximally deuterated growth medium (Table 1, expt. 10') containing 98% (v/v) ^2H_2O and 2% (v/v) [U-^{2}H]MeOH 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 1. 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

Bacterial strains	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	MeOH	[U-^{2}H] MeOH				
Non adapted	1(control)	98.0	0	2	0	20.2 ±1.40	200.2 ±3.20	2.2 ±0.20	0.95 ±0.12
Non adapted	2	98.0	0	0	2	20.3 ±1.44	184.6 ±2.78	2.4 ±0.23	0.92 ±0.10
Non adapted	3	73.5	24.5	2	0	20.5 ±0.91	181.2 ±1.89	2.4 ±0.25	0.90 ±0.10
Non adapted	4	73.5	24.5	0	2	34.6 ±0.89	171.8 ±1.81	2.6 ±0.23	0.90 ±0.08
Non adapted	5	49.0	49.0	2	0	40.1 ±0.90	140.2 ±1.96	3.0 ±0.32	0.86 ±0.10
Non adapted	6	49.0	49.0	0	2	44.2 ±1.38	121.0 ±1.83	3.2 ±0.36	0.81 ±0.09
Non adapted	7	24.5	73.5	2	0	45.4 ±1.41	112.8 ±1.19	3.5 ±0.27	0.69 ±0.08
Non adapted	8	24.5	73.5	0	2	49.3 ±0.91	94.4 ±1.74	3.8 ±0.25	0.67 ±0.08
Non adapted	9	98.0	0	2	0	58.5 ±1.94	65.8 ±1.13	4.4 ±0.70	0.37 ±0.06
Non adapted	10	98.0	0	0	2	60.1 ±2.01	60.2 ±1.44	4.9 ±0.72	0.39 ±0.05
Adapted	10'	98.0	0	0	2	40.2 ±0.88	174.0 ±1.83	2.8 ±0.30	0.82 ±0.08

Notes:

* The date in expts. 1–10 described the growth characteristics for non-adapted bacteria in growth media, containing 2 % (v/v) MeOH/[U-^{2}H]MeOH 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 strain of *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 (Mosin & Ignatov, 2012; Mosin & Ignatov, 2013a, Ignatov & Mosin, 2013a; Ignatov & Mosin, 2013b) The effect of reversion of growth in protonated/deuterated growth media proves that adaptation to $^{2}H_2O$ 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% $^{2}H_2O$ and 2% [^{2}H]methanol with incubation period 3–4 days at temperature 35 ^{0}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 (Mosin & Ignatov, 2013b; Mosin et al., 2013). The effect of reversion of growth in protonated/deuterated growth media proves that adaptation to $^{2}H_2O$ 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) $^{2}H_2O$ and 2% (v/v) [U-^{2}H]methanol.
Adaptation, which conditions are shown in experiment 10' (Table 1) 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 (Fig. 3, the control (*b*) is obtained on protonated growth medium M9). In the present study, the production of phenylalanine (Fig. 3, 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. 3, 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. 3, expt. 2b). The level of phenylalanine production by adapted bacterium under those growth conditions was 0.82 g/liter (Fig. 3, 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 $^{2}H_2O$ 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.

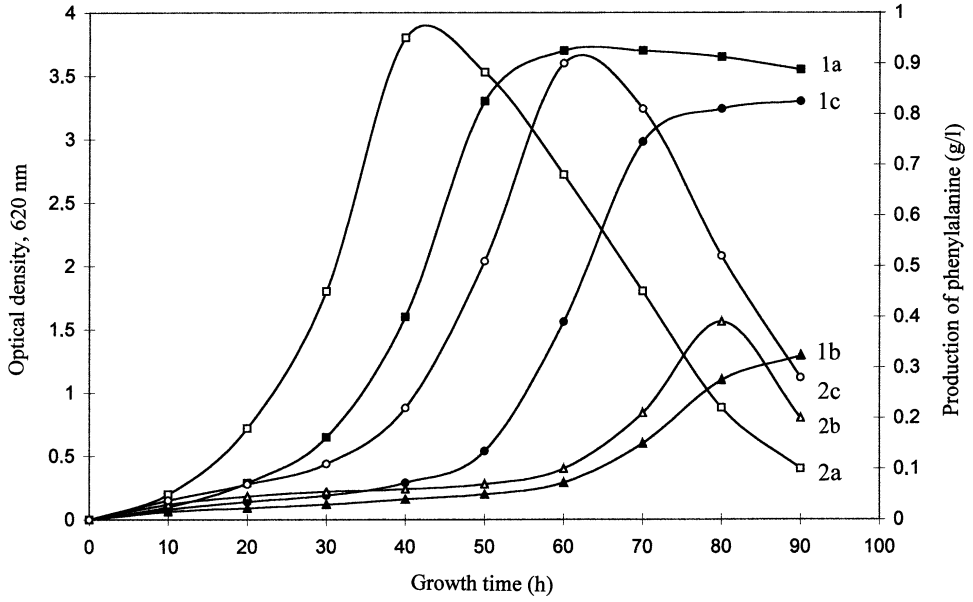

Figure 3. Growth dynamics of *B. methylicum* (1a, 2a, 3a) and production of phenylalanine (1b, 2b, 3b) on media M9 with various isotopic content: 1a, 1b – non-adapted bacterium on protonated medium (Table 1, expt. 1); 2a, 2b – non-adapted bacterium on maximally deuterated medium (Table 1, expt. 10); 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 (Fig. 3). 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 (Maksimova et al., 1990). It is necessary to notice, that 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. This fact required isolation of [^{2}H]phenylalanine from growth medium, which was carried out by extraction of lyophilized LC with iso-PrOH and the subsequent crystallization of [^{2}H]phenylalanine in EtOH. Analytical separation of [^{2}H]phenylalanine and metabolically related [^{2}H]amino acids was performed using a reversed-phase HPLC on Separon SGX C_{18} Column, developed for methyl esters of N-DNS-[^{2}H]amino acids with chromatographic purity of 96–98% and yield of 67–89%.

3.2. Study of deuterium inclusion into molecules of secreted amino acids

For evaluation of deuterium enrichment by EI MS method, methyl esters of N-DNS-[^{2}H]amino acids were applied because the peaks of molecular ions ($M^{+\cdot}$) allow to monitor the enrichment of multicomponential mixtures of [^{2}H]amino acids in composition with growth media metabolites. Furthermore, EI MS allows detect amino acid samples with concentrations 10^{-9}–10^{-10} mol (Mosin, 1996). This method in combination with reversed-phase HPLC has proved to be better for research studies on deuterium enrichment levels of [^{2}H]amino acids in composition of their multicomponental mixtures of lyophilized growth media, and can be used for the analysis of amino acids of various natural sources. N-DNS-amino acids were obtained through the derivatization of lyophilized growth media with DNSCl. The reaction was carried out in the alkaline environment in the presence of 2 M $NaHCO_3$ (pH = 9–10) in water-organic solvent (acetone) in the mass ratio of DNSCl – the amino acid, (2: 1 w/w). The volatility of N-DNS-amino acids in the mass spectrometry analysis may be increased by additional derivatization on carboxyl group (esterification) by diazomethane. The choice of DZM as esterification reagent was caused by the necessity of carrying out the reaction of esterification in soft conditions excluding isotopic (^{1}H–^{2}H) exchange in an aromatic fragment of the phenylalanine molecule. However, with DZM treatment derivatization occurs on αNH_2-group in molecules, so that their N-methylated derivatives could be formed in addition to the methyl ester of N-DNS-amino acids.

The control over deuterium inclusion into phenylalanine molecule due to the conversion of [U-^{2}H]MeOH at growth of the bacterium on medium M9, containing H_2O and 2% (v/v) [U-^{2}H]MeOH (Table 1, expt. 2) has shown insignificant amount of deuterium detected in molecule. The enrichment level was calculated on intensity of a peak ($M^{+\cdot}$) at *m/z* 413 minus the contribution of peak of an impurity of a natural isotope (no more than 5%). This testified about delution of deuterium label *via* biochemical processes connected with disintegration of [U-^{2}H]MeOH during its assimilation by a cell, as well as reactions of isotopic (^{1}H–^{2}H) exchange and dissociation in ^2H_2O. Thus, from 4 deuterium atoms in molecule $C^2H_3O^2H$, only deuterium at hydroxyl O^2H-group is the most mobile and easily dissociates one deuterium atom in water with formation of C^2H_3OH. Three remained deuterium atoms in C^2H_3OH are included into a cycle of biochemical oxidation of methanol leading to formation of substances more oxidized than methanol, *e.g*. formaldehyde. In particular, this confirms the classical scheme of biochemical oxidations of methanol to formaldehyde by methylotrophic bacteria, after that formaldehyde assimilates by this strain of methylotrophic bacteria *via* RuMP cycle of carbon assimilation.

EI mass spectra of methyl esters of N-DNS-amino acids obtained from growth media M9 where used 0; 49.0; 73.5 and 98% (v/v) ^2H_2O with 2% (v/v) [U-^{2}H]MeOH (Table 1, expts. 1, 6, 8, 10) are shown in consecutive order in Fig. 4–7. The fragmentation pathways of methyl esters of N-DNS-amino acids by EI MS method lead to the formation of distinguished peaks of molecular ions ($M^{+\cdot}$) from which the fragments with smaller *m/z* ratio further are formed. The peak of amino fragment A at *m/z* 353 generally has a low intensity, while and peak of aminoacyl fragment B at *m/z* 381 has the lowest intensity in EI mass spectra, or ever absent (see as example Fig. 4 corresponds to expt. 1, Table 1). A high continuous left background region in EI mass spectrum at *m/z* 100–200 is associated with peaks of contaminant metabolites and products of derivatization of metabolites of growth medium with DNSCl and DZM, and peaks at *m/z* 250, 234, 170 are fragments of further decay of dansyl fragment to N-(dimethylamino)naphthalene. A right

region in EI mass spectra contains four peaks of molecular ions ($M^{+\cdot}$) of N-DNS-amino acid methyl esters: Phe at *m/z* 412; Leu/Ile at *m/z* 378; Val at *m/z* 364; Ala at *m/z* 336 (Fig. 4), which fragmentation by the method of EI mass spectrometry will allow to carry out mass spectrometry monitoring of [^{2}H]amino acids in composition of intact LC of the strain-producer, containing mixes of amino acids and other metabolites of growth media before their chromatographic separation. However, since the value of ($M^{+\cdot}$) for Leu is the same as for Ile, these two amino acids could not be clearly estimated by EI MS method.

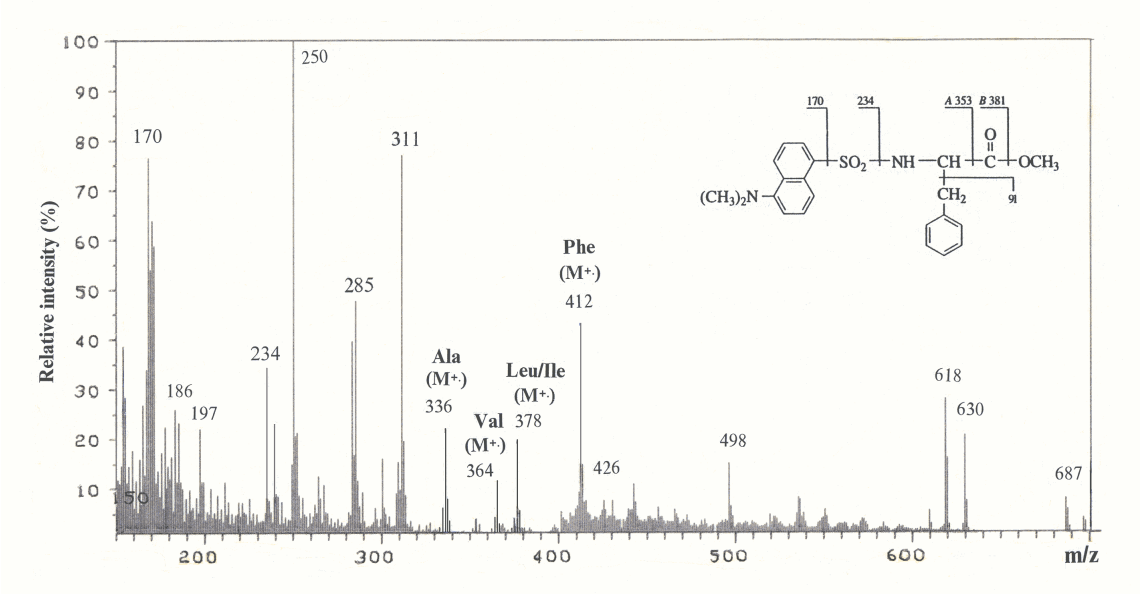

Figure 4. EI Mass spectrum of methyl esters of N-DNS-amino acids from protonated growth medium M9 (expt. 1, Table 1) after processing by DNSCl and DZM, and fragmentation of methyl ester of N-DNS-phenylalanine by EI MS method. Symbols of amino acids refer to the peaks of molecular ions ($M^{+\cdot}$) of methyl esters of N-DNS-amino acids.

The results confirmed the character of labeling of [^{2}H]amino acids as heterogeneous, judging by the presence of clusters of adduct peaks in molecular ions ($M^{+\cdot}$) in EI mass spectra; the species of molecules with different numbers of deuterium atoms were detected. Therefore, the peaks of a molecular ion of amino acid derivatives ($M^{+\cdot}$) were split polymorphously on separate clusters with an impurity of molecules having a statistical set of mass numbers *m/z* with the various contributions to a total level of deuterium enrichment of the molecule. The most abundant peak ($M^{+\cdot}$) in each cluster (the peak with the greatest contribution to level of deuterium enrichment), was a peak with an average *m/z* ratio registered by mass spectrometer in each experimental condition, relative to which the deuterium enrichment of each individual [^{2}H]amino acid was calculated. Thus, in experiment (expt. 6, Table 1) shown in Fig. 5 where was used 49% (v/v) ^2H_2O deuterium enrichment of Phe molecule was 2 (27.5% ^{2}H), calculated by ($M^{+\cdot}$) at *m/z* 414 (instead of ($M^{+\cdot}$) at *m/z* 412 for non-labeled compound); Leu/Ile – 5 (50% ^{2}H) (($M^{+\cdot}$) at *m/z* 383 instead of ($M^{+\cdot}$) at *m/z* 378)); Val – 4 (50% ^{2}H) (($M^{+\cdot}$) at *m/z* 368 instead of *m/z* ($M^{+\cdot}$) at 364)); Ala – 3 (50% ^{2}H) deuterium atoms (($M^{+\cdot}$) at *m/z* 339.2 instead of ($M^{+\cdot}$) at *m/z* 336)). The area of EI mass spectrum with values *m/z* 90–300

corresponds to products of derivatization of metabolites of growth media by dansylchloride and diazomethane. The law intensity peak at *m/z* 431 detected in EI mass spectra in all isotopic experiments, corresponds to a product of additional methylation of [^{2}H]phenylalanine on α-NH-(Dns)-group. The peak at *m/z* 400 (Fig. 5) corresponds to a product of chip off methyl CH_3-group from deuterated methyl ester of N-Dns-[^{2}H]phenylalanine.

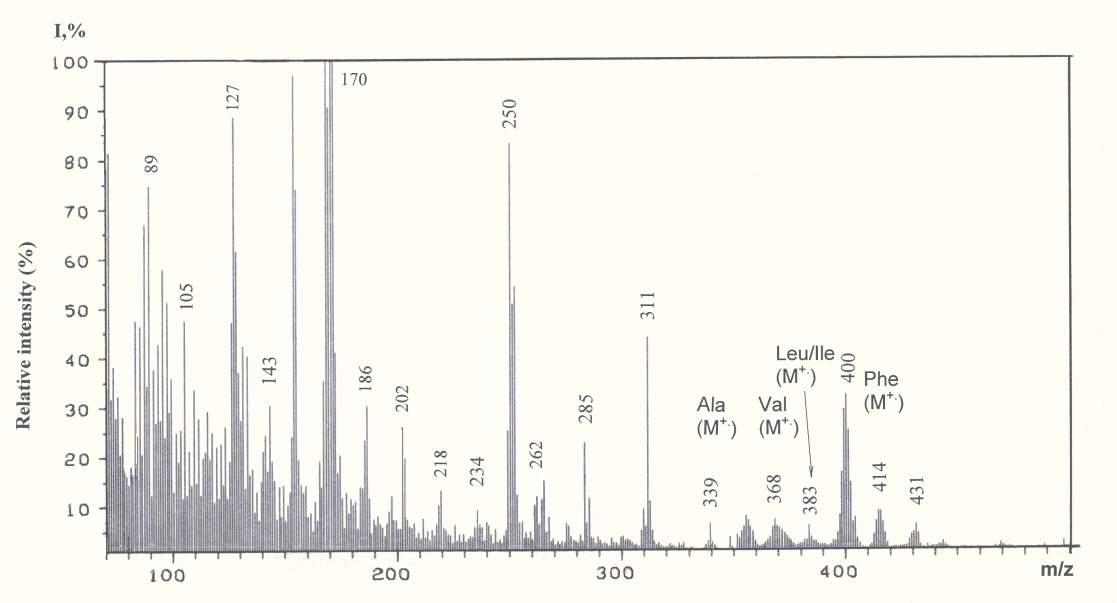

Figure 5. EI mass spectrum of methyl esters of N-DNS-[^{2}H]amino acids from growth medium M9 containing 2% (v/v) [U-^{2}H]MeOH and 49.0% (v/v) ^2H_2O (expt. 6, Table 1).

The similar result on proportional specific increase of levels of deuterium enrichment in [^{2}H]phenylalanine and other metabolically related [^{2}H]amino acids was observed in all isotopic experiments wherein used increasing concentration ^2H_2O in growth media. With increasing ^2H_2O content in growth media M9, the levels of deuterium enrichment in [^{2}H]amino acids varied proportionally. As shown in Fig. 6 in experiment (expt. 8, Table 1) where in 73.5% (v/v) of ^2H_2O, the deuterium enrichment of Phe was 4 (50% ^{2}H) (($M^{+\cdot}$) at *m/z* 416 instead of *m/z* 412 ($M^{+\cdot}$)); Leu/Ile – 5 (50% ^{2}H) (($M^{+\cdot}$) at *m/z* 383 instead of *m/z* at 378 ($M^{+\cdot}$)); Val – 4 (50% ^{2}H) (($M^{+\cdot}$) at *m/z* 368 instead of *m/z* at 364 ($M^{+\cdot}$)); Ala – 3 (50% ^{2}H) deuterium atoms (($M^{+\cdot}$) at *m/z* 339 instead of *m/z* at 336 ($M^{+\cdot}$)). Evidently, the deuterium atoms at carbon backbone of [^{2}H]amino acid molecules were synthesized *de novo* from deuterated substrates. Easily exchanged protons (deuterons), *e.g.* protons at heteroatoms in NH_2- and COOH-groups of amino acids could be replaced by deuterium due to dissociation in $H_2O/^2H_2O$.

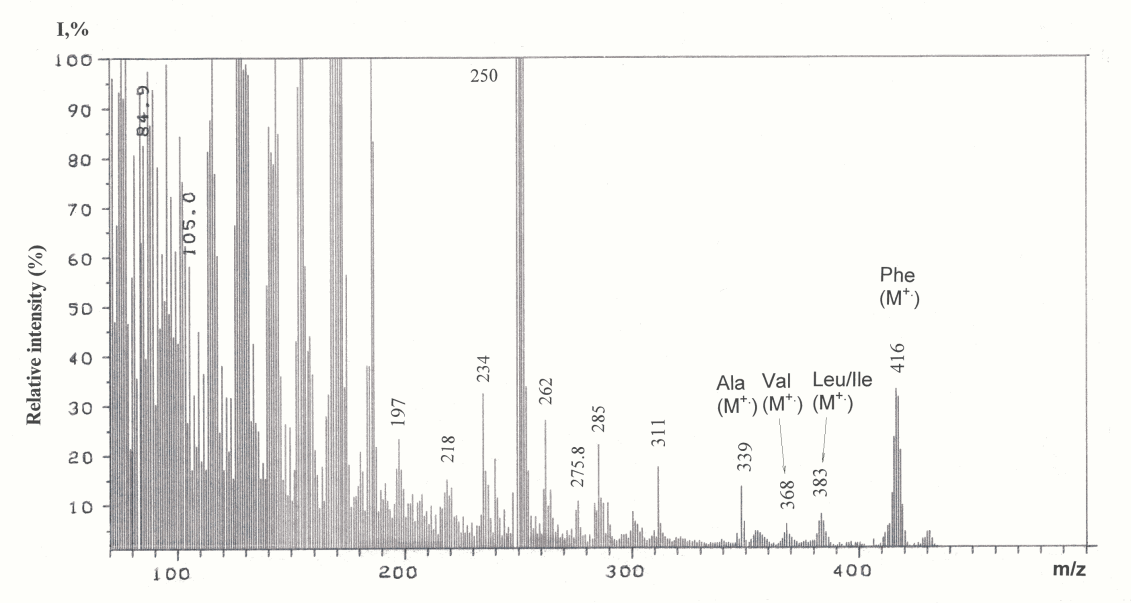

Figure 6. EI mass spectrum of methyl esters of N-DNS-[^{2}H]amino acids from growth medium M9 containing 2% (v/v) [U-^{2}H]MeOH and 73.5% (v/v) ^2H_2O (expt. 8, Table 1).

The used methylotrophic bacterium was leucine auxotroph with leucine being added to growth media in protonated form; therefore the levels of deuterium enrichment in [^{2}H]amino acids of pyruvic acid family as alanine, valine and leucine were less than for phenylalanine (phenylalanine related to the family of the aromatic amino acids synthesized from shikimic acid). This is distinctly visible on the maximally deuterated M9 medium. As shown in Fig. 7 in experiment (expt. 10, Table 1) wherein 98% (v/v) ^2H_2O was used the deuterium enrichment of Phe molecule was 6 (75 at.% ^{2}H) (($M^{+\cdot}$) at *m/z* 418 instead of *m/z* 412 ($M^{+\cdot}$)); Leu/Ile – 5 (50% ^{2}H) (($M^{+\cdot}$) at *m/z* 383 instead of *m/z* 378 ($M^{+\cdot}$)); Val – 5 (62.5% ^{2}H) (($M^{+\cdot}$) at *m/z* 369 instead of *m/z* 364 ($M^{+\cdot}$)); Ala – 3 (50% ^{2}H) deuterium atoms (($M^{+\cdot}$) at *m/z* 339 instead of *m/z* 336 ($M^{+\cdot}$)). The peak at *m/z* 432, detected in EI mass spectrum of methyl esters of N-DNS-amino acids in Fig. 7 corresponds to a product of additional methylation of [^{2}H]phenylalanine on α-NH_2-group. Additionally, in EI mass spectrum is detected a peak of deuterium enriched benzyl $C_6H_5CH_2$-fragment of phenylalanine molecule at *m/z* 97 (instead of *m/z* 91 in the control), that specifies sites of localization of 6 deuterium atoms in [^{2}H]phenylalanine at positions C1–C6 of aromatic protons in benzyl $C_6H_5CH_2$-fragment. EI MS data suggest that at other concentration of ^2H_2O deuterium atoms also incorporated into the aromatic ring of the [^{2}H]phenylalanine molecule as metabolism of adapted to ^2H_2O bacterium does not undergo essential changes in ^2H_2O. The obtained result on distribution of atoms of deuterium into [^{2}H]phenylalanine molecule is important for its further use in medical diagnostics where it is necessary to use [^{2}H]amino acids with high levels of isotope enrichment.

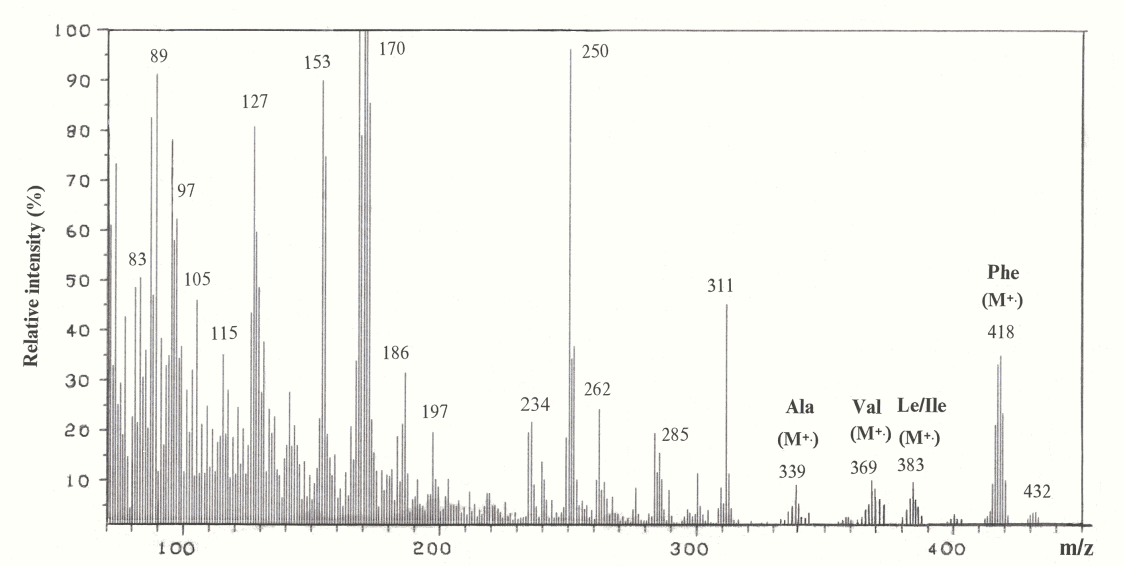

Figure 7. EI mass spectrum of methyl esters of N-DNS-[^{2}H]amino acids from growth medium M9 containing 2% (v/v) [U-^{2}H]MeOH and 98% (v/v) ^2H_2O (expt. 10, Table 1).

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 2). 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 2). 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 2). 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 2. 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
Alanine	24.0 ±0.70	50.0 ±0.89	50.0 ±0.83	50.0 ±1.13
Valine	20.0 ±0.72	50.0 ±0.88	50.0 ±0.72	62.5 ±1.40
Leucine/isoleucine	20.0 ±0.90	50.0 ±1.38	50.0 ±1.37	50.0 ±1.25
Phenylalanine	17.0 ±1.13	27.5 ±0.88	50.0 ±1.12	75.0 ±1.40

Notes:

* 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) [U-^{2}H]MeOH and specified amounts (%, v/v) of ^2H_2O.

4. Conclusions

As a result of the selection approach used in this research it was possible to adapt L-phenylalanine producer strain of aerobic Gram-positive facultative methylotrophic bacterium *B. methylicum* to maximal concentration of deuterated substrates for microbiological preparation of [^{2}H]phenylalanine and other metabolically related [^{2}H]amino acids (alanine, valine and leucine/isoleucine) with various levels of deuterium enrichment. Advantages of this methylotroph for synthesis of [^{2}H]amino acids are improved growth and biosynthetic characteristics on maximally deuterated growth medium, which was achieved by adaptation to ^2H_2O. By using the adapted methylotroph it was possible to obtain 0.82 g/liter of [^{2}H]phenylalanine (75% ^{2}H), which was isolated from growth medium by extraction with iso-PrOH and the subsequent crystallization in EtOH. [^{2}H]phenylalanine was also isolated from growth medium by reversed-phase HPLC as methyl ester of N-DNS-[^{2}H]phenylalanine with a yield of 85% and a purity of 97%. The method is suitable for preparation of other [^{2}H]amino acids produced by methylotrophic bacteria. It should be noted, however, that higher levels of deuterium enrichment into [^{2}H]amino acid molecule can be achieved via replacement of protonated leucine on its deuterated analogue.

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