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Microbiological Synthesis of ^{2}H-Labeled Phenylalanine, Alanine, Valine, and Leucine/Isoleucine with Different Degrees of Deuterium Enrichment by the Gram-Positive Facultative Methylotrophic Bacterium *Brevibacterium Methylicum*

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Abstract

The microbiological synthesis of [^{2}H]amino acids was performed by the conversion of low molecular weight substrates ([U-^{2}H] MeOH and ^2H_2O) using the Gram-positive aerobic facultative methylotrophic bacterium *Brevibacterium methylicum*, an L-phenylalanine producer, realizing the NAD^+ dependent methanol dehydrogenase (EC 1.6.99.3) variant of the ribulose-5-monophosphate (RuMP) cycle of carbon assimilation. In this process, the adapted cells of the methylotroph with enhanced growth characteristics were used on a minimal salt medium M9, supplemented with 2% (v/v) [U-^{2}H]MeOH and an increasing gradient of ^2H_2O concentration from 0; 24.5, 49.0; 73.5 up to 98% (v/v) ^2H_2O. Alanine, valine, and leucine/isoleucine were produced and accumulated exogeneously in quantities of 5–6 μmol, in addition to the main product of biosynthesis. This method enables the production of [^{2}H]amino acids with different degrees of deuterium enrichment, depending on the ^2H_2O concentration in the growth medium, from 17 at.% ^{2}H (on the growth medium with 24.5 % (v/v) ^2H_2O) up to 75 at.% ^{2}H (on the growth medium with 98 % (v/v) ^2H_2O). This has been confirmed with the data from the electron impact (EI) mass spectrometry analysis of the methyl ethers of N-dimethylamino(naphthalene)-5-sulfochloride [^{2}H]amino acids under these experimental conditions.

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

Introduction

Labeling the amino acid molecules with deuterium is becoming an essential step in the various biochemical studies using ^{2}H-labeled molecules and the investigation of certain aspects of their metabolism [1,2].

Chemical or biosynthetic methods can be used to introduce the deuterium into the amino acid molecules. The chemical synthesis of ^{2}H-labeled compounds is significantly limited, as it involves a very laborious and costly multistep process resulting in the production of a mixture of D,L-racemates [3]. Although the chemomicrobiological synthesis overcomes this limitation [4], the quantities of purified enzymes required for it are prohibitive [5]. The biosynthesis of ^{2}H-labeled amino acids usually involves the growth of an organism on specific growth media containing the labeled substrates: *e.g.*, the autotrophic growth of the algae on a medium containing 96% (v/v) ^2H_2O, is a well established method for the biosynthesis of highly deuterated biochemicals, including amino acids [6,7]. However, this method, while being generally applicable, is limited by the low resistance of the plant cells to ^2H_2O and the high costs of the mixture of ^{2}H-labeled amino acids isolated from the hydrolysates of the biomass [8,9].

Alternative and relatively inexpensive objects for the biosynthesis of ^{2}H-labeled amino acids appear to be certain auxotrophic mutants of methylotrophic bacteria, using MeOH as the main source of carbon and energy *via* the RuMP and serine cycle of carbon assimilation [10]. These bacteria reveal great practical advantage for industrial usage because of their ability to produce and accumulate gram quantities of ^{2}H-labeled amino acids during their growth on the medium containing ^2H_2O and [U-^{2}H]MeOH as well as the comparatively lower price of [U-^{2}H] MeOH [11].

It is only in recent years, however, that some progress has been made in the isolation of a number of versatile RuMP cycle methylotrophic bacteria, suitable for such studies, although the research that has been using methylotrophs has been limited and

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has suffered particularly from the low growth characteristics on the ^2H_2O-containing media. Thus, the production of ^2H-labeled amino acids by the obligate methylotroph *Methylobacillus flagellatus* realizing 2-keto 3-deoxy 6-phospho-6-gluconate aldolase/transaldolase (KDPGA/TA) variant of the RuMP cycle of carbon assimilation, involves the bacterial growth on the salt medium with approximately 75% (v/v) ^2H_2O [12]. We selected a mutant of Gram-positive aerobic facultative methylotroph *Brevibacterium methylicum*, realizing the NAD^+ dependent methanol dehydrogenase (EC 1.6.99.3) variant of the RuMP cycle of carbon assimilation, which appears more convenient for the preparation of ^2H-labeled amino acids than the obligate methylotroph *M. flagellatus* because of its ability to grow on the minimal salt medium M9 with 98% (v/v) of ^2H_2O [13].

The purpose of the present research was to study the microbiological synthesis of [^2H]amino acids with differing degrees of deuterium enrichment *via* the cultivation of the bacterium on the minimal growth media with increasing ^2H_2O content (from 24.5 % (v/v) up to 98 % (v/v) ^2H_2O).

Material and Methods

Chemicals

The ^2H_2O (99.9 at.% ^2H) was purchased from Russian Scientific Enterprises, Sankt Petersburg. [U-^2H]MeOH (97.5 at.% ^2H) was purchased from Biophysic Center (Pushino, Russia). The sequential grade dansyl chloride (DNSCl) was purchased 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 at.% ^2H_2O; the ^2H_2O was distilled over $KMnO_4$ with subsequent control of the isotopic enrichment by ^1H-NNR spectroscopy using the Brucker WM-250 device ("Brucker Corp.", USA) (working frequency – 70 MHz, internal standard – Me_4Si).

Bacterial strains

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

Media and growth conditions

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 the bacterial growth. In isotopic experiments, the minimal salt media M9 were enriched with [U-^2H]MeOH (2%,v/v) and an increasing gradient of ^2H_2O concentration from 0; 24.5; 49.0; 73.5 up to 98 % (v/v) ^2H_2O. The bacterial growth was performed under batch conditions in 250 ml Erlenmeyer flasks containing 20 ml of the growth medium at 32–34^{0}C and vigorously aerated with 100 rotations/min in an orbital shaker, Biorad 380-SW ("Biorad Labs", Poland). The exponentially growing cells (cell density 2.0 at absorbance 540 nm) were pelleted by centrifugation (1200 g for 15 min), the supernatant was lyophilized in vacuum and used for the chemical derivatization.

Determination of phenylalanine

Quantities 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 commercial available phenylalanine as a standard. The spots were treated by 0.1 % (w/v) ninhydrine solution (in acetone), eluted by 0.5 % (w/v) $CdCl_2$ solution 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 540 nm, the concentration of phenylalanine in samples was calculated using a standard curve.

Isolation of [^2H]phenylalanine from the growth medium

Deutero-biomass of *B. methylicum* obtained after growth in maximally deuterated medium M9 with 2 % (v/v) [U-^2H]MeOH and 98 % (v/v) ^2H_2O, was separated on centrifuge T-24 ("Heraues Sepatech", Germany) at 1200 g for 15 min. Supernatant was evaporated under vacuum. 30 ml of iso-PrOH was added to 5 g lyophilized growth medium M9, acidified to pH 2.0 using 5 *M* ^2HCl (in ^2H_2O) and keep at room temperature for 4 h. The salts were removed by centrifugation and the supernatant was evaporated. Phenylalanine (0.65 g/L) was recrystallised from EtOH: $[\alpha]_D^{20} = 35^0$ (EtOH); UV-spectrum (0.1 *M* HCl): λ_{max} = 257.5 nm, $\varepsilon_{257.5} = 1{,}97 \cdot 10^2\ M^{-1} \cdot sm^{-1}$.

Synthesis of methyl esters of N-DNS-[^2H]amino acids

320 mg (1.2 mol) DNSCl in 5 ml acetone was added to 200 mg of lyophilized growth media M9 in 5 ml 2 *M* $NaHCO_3$ (0.002 mol) solution, pH 9–10. The mixture was kept at 40 ^{0}C under vigorous steering 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-[^2H]amino acids was performed in a standard chemical procedure with using DZM. To 20 ml of 40 % (w/v) KOH in 40 ml of diethyl ether was added 3 g N-NMU and mixed on a water bath 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, then dried over sodium sulphate and processed with it N-DNS-[^2H]amino acids.

High-performance liquid chromatography

Analytical separation of the methyl esters of N-DNS-[^2H] amino acids was performed by a reversed-phase HPLC on liquid chromatograph Knauer ("Knauer", Germany), supplied with UV-detector and integrator C-R 3A ("Shimadzu", Japan). The methyl esters of N-DNS-[^2H]amino acids were detected at 254 nm by UV-absorbance . As a motionless phase was used Separon SGX C_{18}, 18 μm, 150 × 3.3 mm ("Kova", Czech Republik). The mobile phase was composed of the 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 5 min; from 100 % B to 20 % A for 2 min; at 20 % A 10 min.

EI mass spectrometry

EI mass spectrometry was performed to determine the deuterium enrichment levels by using methyl esters of N-DNS-

[^{2}H]amino acids on the Hitachi MB 80 mass spectrometer ("Hitachi", Japan) at ionizing energy of 70 eV, accelerating electrical voltage 8 kV, and ion source temperature 180–200^{O}C. Each measurement was repeated thrice. The deuterium enrichment of [^{2}H]amino acids was calculated on a parity of the contributions of the molecular ion ($M^{+\cdot}$) peaks of N-DNS-[^{2}H]amino acid methyl esters obtained in isotopic experiments relative to the control in a protonated minimal medium M9.

Statistical data processing was performed using the software package Statistica 6. For data with normal distribution, inter-group comparisons were performed using Student's t-test. P value less than 0.05 was considered significant.

Results and Discussion

Phenylalanine is synthesized in most bacteria *via* the shikimic acid pathway [14,15]. 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, the RuMP cycle of carbon assimilation [16,17]. The native bacterial strains are not strong producers of phenylalanine owing to the effective mechanisms of its metabolic regulation, although certain bacterial mutants with mutations of prephenate dehydrogenase (EC 1.3.1.12), prephenate dehydratase (EC 4.2.1.51), chorismate mutase (EC 5.4.99.5) and several other enzymes have been proved to be active producers of this amino acid [18]. The best phenylalanine producing strains once selected were the mutants, partially or completely dependent on tyrosine or tryptophan for growth. The reports regarding other regulative mechanisms of the phenylalanine biosynthesis in the bacterial cell are quite uncommon, although it is known that there are a certain number of RuMP cycle auxotrophic mutants of the methylotrophs, entering several steps in the biosynthesis of aromatic amino acids [19,20].

A certain practical interest prompted this research regarding the ability of a leucine auxotroph of Gram-negative aerobic facultative methylotrophic bacterium *B. methylicum* to produce L-phenylalanine, realizing the NAD^+ dependent methanol dehydrogenase (EC 1.6.99.3) variant of the RuMP cycle of carbon assimilation. At best it remains a convenient, but insufficiently explored biotechnological tool. The initial stage of biochemical research on this strain of methylotrophic bacteria involved obtaining auxotrophic mutants, which in the majority of cases are characteristic of the limited spectrum of mutant phenotypes and, besides that, the high level of reversions. The initial L-leucine dependent strain *B. methylicum*, a producer of L-phenylalanine, was obtained *via* selection at an earlier stage in the research after processing the parental strain by *nitrosoguanidine.* Screening of the necessary cell colonies was conducted by their stability to the phenylalanine analogue – *meta*-fluoro-phenylalanine (50 mg/ml). The analogue-resistant mutants allocated on specific media were able to convert methanol and accumulate up to 1 gram per 1 liter of L-phenylalanine into the growth media. Comparative analyses (TLC, NMR) showed that the L-phenylalanine, produced by this strain of methylotrophic bacteria was absolutely identical to natural L-phenylalanine.

A further attempt was made to intensify the growth and biosynthetic parameters of this bacterium to grow on medium M9 with a high concentration of deuterated substrates, viz., [U-^{2}H]MeOH and ^2H_2O. To achieve this, a special adaptation method was employed *via* plating the cell colonies on 2% (w/v) agarose M9 medium, supplemented with 2% (v/v) [U-^{2}H]MeOH and increasing the ^2H_2O content from 0; 24.5; 49.0; 73.5 up to 98% (v/v) ^2H_2O, combined with the subsequent selection of deuterium-resistant cell colonies. Grown on the growth media with a low gradient of ^2H_2O concentration, the cell colonies were then transferred onto the growth medium with a higher ^2H_2O concentration gradient, up to 98% ^2H_2O. At the final stage of this adaptation technique, the separate cellular colonies representing the posterity of one cell, resistant to the action of ^2H_2O (degree of cell survival on the final deuterated medium was about 40 %), were allocated on the growth medium with 98% ^2H_2O. Then the cells were transferred to the liquid minimum growth medium M9 with the same deuterium content, and grown for 3 to 4 days at 34^{0}C.

Experimental conditions are listed in Table 1 (expts. 1–10) relative to the control (expt. 1) on the protonated medium and to the adapted bacterium (expt. 10'). Various combinations of [U-^{2}H]MeOH and ^2H_2O were added to the growth M9 medium as the 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 odd numbers of the experiment (Table 1, expts. 2, 4, 6, 8, and 10) were chosen to investigate the effect of substituting MeOH with its deuterated analogue on the growth characteristics, in the presence of ^2H_2O. This change caused only small alterations in the growth characteristics (Table 1, expts. 2, 4, 6, 8, and 10) relative to the experiments where protonated methanol was used (Table 1, expts. 3, 5, 7, and 9). Next, adding gradually increasing concentrations of ^2H_2O into the growth media was observed to cause a proportional decrease in the *lag*-period and yield of the microbial biomass in all the isotopic experiments. Thus, in the control (Table 1, expt. 1), the duration of the *lag*-period did not exceed 20.2 h, and the yield of the microbial biomass (wet weight) and production of phenylalanine were 200.2 and 0.95 gram per 1 liter of growth medium, respectively.

The results suggested that below 49% (v/v) ^2H_2O (Table 1, expts. 2-4), a small inhibition of bacterial growth was recorded compared with the control (Table 1, expt. 1). Above 49% (v/v) 2H2O (Table 1, expts. 5–8), however, the growth was markedly reduced, while at the upper content of ^2H_2O (Table 1, expts. 9–10) the growth was extremely small. With every increase in the content of ^2H_2O in the growth medium, there was a simultaneous increase both in the *lag*-period and generation time. Thus, on a maximally deuterated growth medium (Table 1, expt. 10) with 98% (v/v) ^2H_2O and 2% (v/v) [U-^{2}H]MeOH, the *lag*-period was more than 3 and generation time more than 2.2 times than on the protonated growth medium (Table 1, expt. 1). In contrast to the adapted bacterium (Table 1, 10'), the production of phenylalanine and the yield of biomass for non-adapted bacterium on the maximally deuterated growth medium (Table 1, expt. 10) with 98% (v/v) ^2H_2O and 2% (v/v) [U-^{2}H]MeOH were decreased by 2.7 and 3.3 times, respectively. The growth rate and generation time for the adapted bacterium were approximately the same as for the control, despite a two-fold increasing in the *lag*-period (Table 1, expt. 10'), while the level of phenylalanine production was two-fold more, than for the non-adapted bacterium. The transfer of deuterated cells to the protonated medium M9 eventually resulted in normal growth.

Table 1.

*Isotopic components of growth media M9, characteristics of bacterial growth of B. methylicum * ** *** and production of phenylalanine*

Experiment number	Media components, % (v/v)				*L a g* – period (h)	Yield of biomass, gram of wet weight from 1 liter of growth medium	Generation time (h)	Production of phenylalanine, gram from 1 liter of growth medium
	H_2O	^2H_2O	MeOH	[U-^{2}H] MeOH				
1	98.0	0	2	0	20.2±1.40	200.2±3.20	2.2±0.20	0.95±0.12
2	98.0	0	0	2	20.3±1.44	184.6±2.78	2.4±0.23	0.92±0.10
3	73.5	24.5	2	0	20.5±0.91	181.2±1.89	2.4±0.25	0.90±0.10
4	73.5	24.5	0	2	34.6±0.89	171.8±1.81	2.6±0.23	0.90±0.08
5	49.0	49,0	2	0	40.1±0.90	140.2±1.96	3.0±0,32	0.86±0.10
6	49.0	49.0	0	2	44.2±1.38	121.0±1.83	3.2±0.36	0.81±0.09
7	24.5	73.5	2	0	45.4±1.41	112.8±1.19	3.5±0.27	0.69±0.08
8	24.5	73.5	0	2	49.3±0.91	94.4±1.74	3.8±0.25	0.67±0.08
9	0	98.0	2	0	58.5±1.94	65.8±1.13	4.4±0.70	0.37±0.06
10	0	98.0	0	2	60.1±2.01	60.2±1.44	4.9±0.72	0.39±0.05
10'	0	98.0	0	2	40.2±0.88	174.0±1.83	2.8±0.30	0,82±0.08

Note: ** 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) ^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.*

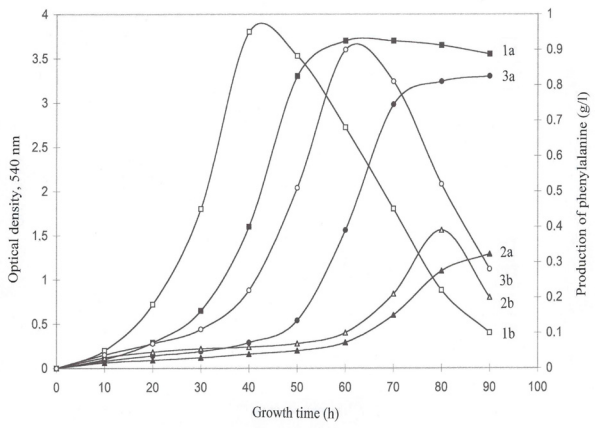

Figure 1.

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 production of phenylalanine (Fig.1, expts. 1b, 2b, 3b) was close to a linear extrapolation with respect to the time up to exponential growth dynamics (Fig.1, expts. 1a, 2a, 3a). The quantity of phenylalanine production for the non-adapted bacterium on the maximally deuterated medium M9 was 0.39 g/liter after 80 hours of growth (Fig.1, expt. 2b). The level of phenylalanine production for the adapted bacterium under those growth conditions was 0.82 g/liter (Fig.1, expt. 3b). Thus, the use of the adapted bacterium enabled an improvement in the level of phenylalanine production on the maximally deuterated medium by 2.3 times. The general feature of phenylalanine biosynthesis in the $H_2O/^2H_2O$-media was the increase of its production at an early exponential growth phase when the outputs of a microbial biomass were insignificant. In all the experiments a decrease in the quantity of phenylalanine in the growth media was observed during a late exponential growth phase. Microscopic research of the growing population of the microorganisms showed that the character of the phenylalanine accumulation in the growth medium did not correlate with the morphological changes at various cellular growth stages. It was most likely that the phenylalanine, accumulated in the growth medium, inhibited the enzymes of its biosynthetic pathways, or perhaps it was later on transformed into the intermediate compounds of its biosynthesis, *e.g.* phenylpyruvate [21]. As for phenylalanine, it was synthesized in the bacterial cell from prephenic acid, which through the formation of phenylpyruvate was converted into phenylalanine under the influence of the cellular transaminases. However, phenylalanine was not the only product of biosynthesis; other metabolically related amino acids were also produced and accumulated into the growth medium in quantities of 5–6 μmol, in addition to the phenylalanine. This required the isolation of the [^{2}H]phenylalanine, which was carried out by extraction using iso-PrOH and the subsequent crystallization in ETOH. The analytical separation of [^{2}H]phenylalanine and the metabolically related [^{2}H]amino acids was performed using the reversed-phase HPLC method on Separon SGX C_{18} Column, developed for the methyl esters of N-DNS-[^{2}H]amino acids with chromatographic purity of 96–98% and yield of 67–89%.

For evaluation of the deuterium enrichment by the EI MS method, the methyl esters of N-DNS-[^{2}H]amino acids were utilized because the peaks of the molecular ions ($M^{+\cdot}$) permitted monitoring of the enrichment of the multicomponent mixtures of [^{2}H]amino acids in the composition with the growth medium metabolites. Furthermore, EI-MS allowed the detection of amino acid samples with concentrations of 10^{-9}–10^{-10} mol [22]. This method, combined with the reversed-phase HPLC has proved good in the research study of deuterium enrichment levels of the [^{2}H]amino acids in the composition of their multicomponent combinations of lyophilized growth media, and can be used for the analysis of amino acids of various natural substances. The N-DNS-amino acids were obtained by derivatization of the 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 a water-organic solvent (acetone) in the ratio of DNSCl to amino acid, equal 2:1, % (w/w). The volatility of the N-DNS-amino acids during the mass spectrometry analysis was increased through additional derivatization on the carboxyl group (esterification) by the diazomethane. The choice of DZM as an esterification reagent was arrived at because of the need to conduct the esterification reaction under soft conditions excluding isotopic (^{1}H–^{2}H) exchange in an aromatic fragment of the phenylalanine molecule. However, with DZM treatment the derivatization on the aNH_2-group in the molecules occurred, so that their N-methylated derivatives were formed after the addition of the methyl ester of the N-DNS-amino acids.

The control over the inclusion of deuterium into the phenylalanine molecule by the conversion of the [U-^{2}H]MeOH during the growth of the bacterium on medium M9, containing H_2O and 2% (v/v) [U-^{2}H]MeOH (Table 1, expt. 2) has detected an insignificant amount of deuterium in the molecule. The degree of enrichment was calculated using the intensity of the peak ($M^{+\cdot}$) at *m/z* 413 minus the contribution of the peak of an impurity of a natural isotope (no more than 5 %). This testified to the dilution of the deuterium label *via* the biochemical processes connected with the disintegration of the [U-^{2}H]MeOH during its assimilation by

the cell, as well as the reactions of the isotopic (^{1}H-^{2}H) exchange and dissociation in ^2H_2O. Thus, from 4 deuterium atoms in one molecule of $C^2H_3O^2H$, only the deuterium at the hydroxyl group has the most mobility and easily dissociates one deuterium atom in water, to form C^2H_3OH. The three remaining deuterium atoms in the C^2H_3OH are included into the cycle of biochemical oxidation of methanol leading to the formation of substances more oxidized than methanol, *e.g*. formaldehyde. In particular, this confirms the classic scheme of the biochemical oxidations of methanol until the formation of formaldehyde by the methylotrophic bacteria, which is then assimilated by this strain of methylotrophic bacteria *via* the RuMP cycle of carbon assimilation.

The EI mass spectra of the methyl esters of N-DNS-amino acids obtained from the growth medium M9 which were used - 0; 49.0; 73.5 and 98% (v/v) ^2H_2O with 2% (v/v) [^{2}H]MeOH (Table 1, expts. 1, 6, 8, and 10) are shown in consecutive order in Figs. 2-5. The fragmentation pathways of the methyl esters of N-DNS-amino acids by the EI mass spectrometry method lead to the formation of distinguished peaks of the molecular ions ($M^{+\cdot}$) from which further fragments with a smaller *m/z* ratio are formed. The peak of the amino fragment A at *m/z* 353 generally has a low intensity, while the peak of the aminocyl fragment B at *m/z* 381 reveals the least or no intensity at all in the EI mass spectra (see as example Fig. 2 corresponds to expt. 1, Table 1). A high continuous left background region in the EI mass spectrum at *m/z* 100–200 is associated with peaks of the concomitant metabolites and products of derivation of the metabolites of the growth medium with DNSCl and DZM, and peaks at *m/z* 250, 234, 170 are fragments of further decay of the dansyl fragment to N-(dimethylamino) naphthalene. The right region in the EI mass spectra contains four peaks of the molecular ions ($M^{+\cdot}$) of the 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. 2). As the value of ($M^{+\cdot}$) for Leu is the same as for Ile, these two amino acids could not be clearly estimated by the EI mass spectrometry method.

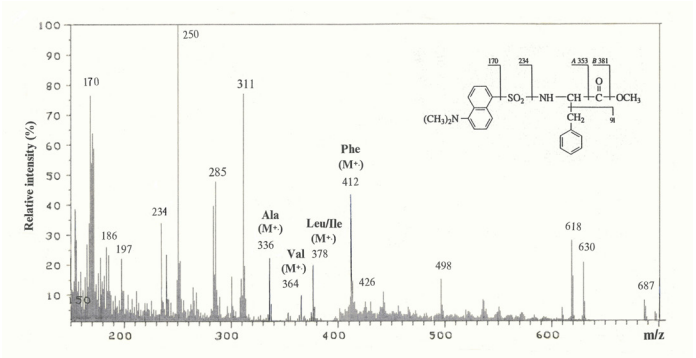

Figure 2.
EI mass spectrum of methyl esters of N-DNS-amino acids from the protonated growth medium M9 (expt. 1, Table 1) after processing by DNSCl and DZM, and fragmentation of methyl ester of N-DNS-phenylalanine by EI MS method. Symbols of amino acids refer to the peaks of molecular ions ($M^{+\cdot}$) of methyl esters of N-DNS-amino acids.

The results confirmed the character of labeling the [^{2}H]amino acids as heterogeneous, judging by the presence of the clusters of adduct peaks in the molecular ions ($M^{+\cdot}$) in the EI mass spectra; species of molecules possessing different numbers of deuterium atoms were also detected. The most abundant peak ($M^{+\cdot}$) in each cluster was a peak with an average *m/z* ratio registered by the mass spectrometer, relative to which the deuterium enrichment of each individual [^{2}H]amino acid was calculated. Thus, in the experiment (expt. 6, Table 1) as shown in Fig. 3 where 49% (v/v) ^2H_2O were used, the deuterium enrichment of Phe was 2 (27.5 at.% ^{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 at.% ^{2}H) (($M^{+\cdot}$) at *m/z* 383 instead of ($M^{+\cdot}$) at *m/z* 378)); Val – 4 (50 at.% ^{2}H) (($M^{+\cdot}$) at *m/z* 368 instead of *m/z* ($M^{+\cdot}$) at 364)); Ala – 3 (50 at.% ^{2}H) deuterium atoms (($M^{+\cdot}$) at *m/z* 339.2 instead of ($M^{+\cdot}$) at *m/z* 336)). The peak at *m/z* 431.0 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. 3) corresponds to a product of chip off methyl CH_3-group from deuterated methyl ester of N-DNS-[^{2}H]phenylalanine.

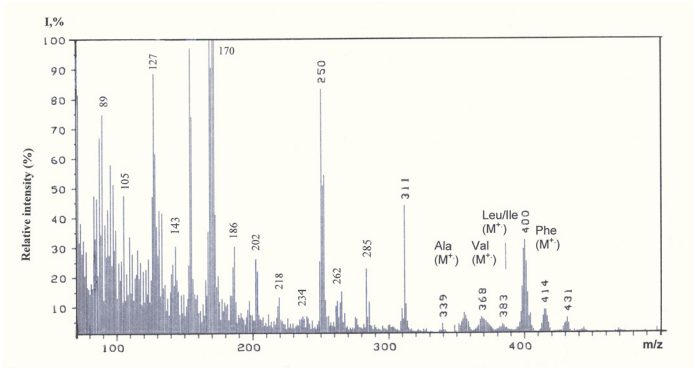

Figure 3.
EI mass spectrum of methyl esters of N-DNS-[^{2}H]amino acids from the growth medium M9 containing 2 % (v/v) [U-^{2}H]MeOH and 49.0 % (v/v) ^2H_2O (expt. 6, Table 1).

With each increase in the ^2H_2O content in the growth medium M9, the degree of deuterium enrichment in the [^{2}H] amino acids varied proportionately. As shown in Fig. 4 in the experiment (expt. 8, Table 1) where 73.5% (v/v) of ^2H_2O were used, the deuterium enrichment of Phe was 4 (40 at.% ^{2}H) (($M^{+\cdot}$) at *m/z* 416 instead of *m/z* 412 ($M^{+\cdot}$)); Leu/Ile – 5 (50 at.% ^{2}H) (($M^{+\cdot}$) at *m/z* 383 instead of *m/z* at 378 ($M^{+\cdot}$)); Val – 4 (50 at.% ^{2}H) (($M^{+\cdot}$) at *m/z* 368 instead of *m/z* at 364 ($M^{+\cdot}$)); Ala – 3 (50 at.% ^{2}H) deuterium atoms (($M^{+\cdot}$) at *m/z* 339 instead of *m/z* at 336 ($M^{+\cdot}$)). Evidently, the deuterium atoms in the carbon skeletons of [^{2}H]amino acid molecules were synthesized *de novo* from the deuterated substrates. The easily exchanged protons (deuterons), *e.g*. protons in the heteroatoms in the NH_2- and COOH- groups of the amino acids could be replaced by the deuterium due to dissociation in the H_2O (^2H_2O).

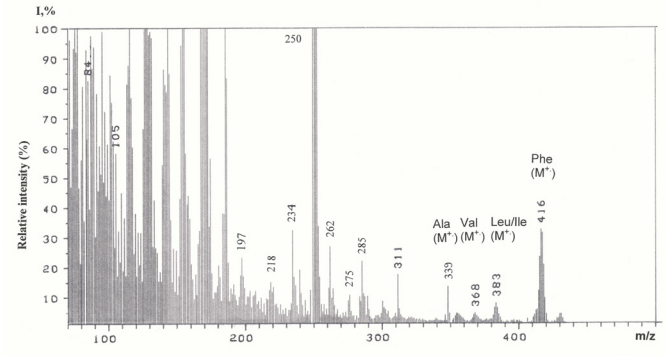

Figure 4.
EI mass spectrum of methyl esters of N-DNS--[^{2}H]amino acids from the growth medium M9 containing 2 % (v/v) [U-^{2}H]MeOH and 73.5 % (v/v) ^2H_2O (expt. 8, Table 1).

As the methylotrophic bacterium used was a leucine auxotroph, and leucine was added to the growth medium in protonated form, the degrees of deuterium enrichment in the [^{2}H] amino acids of the pyruvic acid family as alanine, valine and leucine were less than for the phenylalanine (phenylalanine related to the family of the aromatic amino acids synthesized from the shikimic acid). This is distinctly visible on the maximally deuterated M9 medium. As shown in Fig. 5 in the experiment (expt. 10, Table 1) where 98% (v/v) ^2H_2O was used, the deuterium enrichment of Phe was 6 (75 at.% ^{2}H) (($M^{+\cdot}$) at *m/z* 418 instead of *m/z* 412 ($M^{+\cdot}$)); Leu/Ile – 5 (50 at.% ^{2}H) (($M^{+\cdot}$) at *m/z* 383 instead of *m/z* 378 ($M^{+\cdot}$)); Val – 5 (62.5 at.% ^{2}H) (($M^{+\cdot}$) at *m/z* 369 instead of *m/z* 364 ($M^{+\cdot}$)); Ala – 3 (50 at.% ^{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. 5 corresponds to a product of additional methylation of [^{2}H]phenylalanine on a-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 localisation of 6 deuterium atoms in [^{2}H]phenylalanine at positions C1–C6 of aromatic protons in benzyl $C_6H_5CH_2$-fragment..

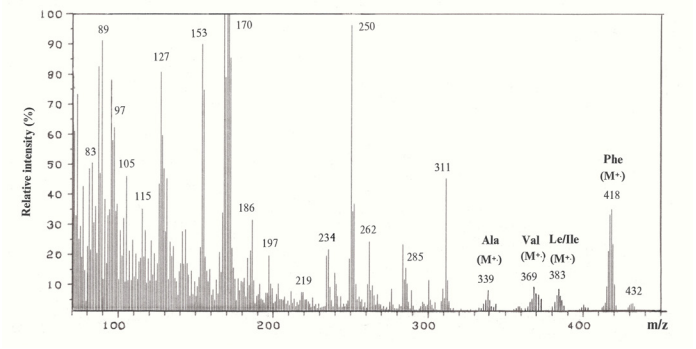

Figure 5.
EI mass spectrum of methyl esters of N-DNS--[^{2}H]amino acids from the growth medium M9 containing 2 % (v/v) [U-^{2}H]MeOH and 98 % (v/v) ^2H_2O (expt. 10, Table 1).

From the data recorded from the EI mass spectrometry it follows, that at other concentrations of ^2H_2O the deuterium atoms also get into the aromatic ring of the [^{2}H] phenylalanine molecule as the metabolism adapted to the ^2H_2O bacterium does not essentially undergo changes in ^2H_2O.

A similar result on the proportional specific increase in the degree of deuterium enrichment into the [^{2}H]phenylalanine and other metabolically related [^{2}H]amino acids was observed in all the isotopic experiments where increasing concentrations of ^2H_2O were used in the growth medium (**Table 2**). Predictably, the degrees of enrichment of [^{2}H]phenylalanine related to the family of the aromatic amino acids synthesized from the shikimic acid and metabolically related [^{2}H]amino acids of the pyruvic acid family – alanine, valine and leucine at identical ^2H_2O concentrations in the growth media were correlated among themselves. Such a result is fixed in all the isotope experiments with ^2H_2O (Table 2). The deuterium enrichment levels accompanying the [^{2}H] amino acids – Ala, Val and Leu/Ile, maintain a stable constancy within a wide range of ^2H_2O concentration, from 49% (v/v) to 98% (v/v) ^2H_2O (**Table 2**). Summarizing these data, it is possible to draw conclusions on the preservation of the minor pathways of the metabolism connected with the biosynthesis of leucine and the metabolically related amino acids of the pyruvic acid family – alanine and valine, the degrees of enrichment which correlated within the identical concentration of H_2O in the growth medium. As leucine was added into the growth medium in protonated form, another explanation of this effect included 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), which could involve the cell assimilation of the protonated leucine from the growth medium. As Leu and Ileu could not be clearly estimated by the EI MS method, no conclusions could be drawn about the possible biosynthesis of [^{2}H]isoleucine. Evidently, higher degrees of deuterium enrichment can be achieved by replacing the protonated leucine on its deuterated analogue, which may be isolated from the hydrolyzates of the deuterated biomass of this methylotrophic bacterium.

Table 2.
*Deuterium enrichment levels (at. % ^{2}H) in molecules of [^{2}H]amino acids excreted by B. methylicum**

[^{2}H]amino acid	Concentration of ^2H_2O in growth media, % (v/v)**			
	24.5	**49.0**	**73.5**	**98.0**
Ala	24.0±0.70	50.0±0.89	50.0±0.83	50.0±1.13
Val	20.0±0.72	50.0±0.88	50.0±0.72	62.5±1.40
Leu/ileu	20.0±0.90	50.0±1.38	50.0±1.37	50.0±1.25
Phe	17.0±1.13	27.5±0.88	50.0±1.12	75.0±1.40

***Note:** * 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 (%, v/v) ^2H_2O.*

Conclusions

Depending upon the approach selected for this research it was possible to adapt the L-phenylalanine producer strain of the aerobic Gram-positive facultative methylotrophic bacterium *B. methylicum* accordingly, to the maximal concentration of the deuterated substrates for the microbiological synthesis of [^{2}H] phenylalanine and other metabolically related [^{2}H]amino acids (alanine, valine and leucine/isoleucine) with different degrees of deuterium enrichment. The advantages of this methylotroph for the synthesis of the [^{2}H]amino acids are improved growth and the biosynthetic characteristics on the maximally deuterated growth medium, which was achieved by adaptation to the ^2H_2O. By using the adapted methylotroph it was possible to obtain 0.65 g/liter of [^{2}H]phenylalanine (75 at.% ^{2}H), which was isolated from the growth medium by extraction with iso-PrOH and the subsequent crystallization in ETOH. The [^{2}H]phenylalanine was also isolated from the growth medium by reversed-phase HPLC as a methyl ester of N-DNS-[^{2}H]phenylalanine with a yield of 85% and a purity of 97%. This method also is suitable for the preparation of other [^{2}H]amino acids produced by the methylotrophic bacteria. It should be noted, however, that higher degrees of deuterium enrichment into the [^{2}H]amino acid molecule can be achieved by the replacement of the protonated leucine on its deuterated analogue.

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