

Studying of the Biosynthesis of ^2H -Labeled Inosine by a Gram-positive Chemoheterotrophic Bacterium *Bacillus Subtilis* B-3157 on Heavy Water ($^2\text{H}_2\text{O}$) Medium

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Abstract

We studied the biosynthesis of ^2H -labeled purine ribonucleoside inosine (output, 3.9 g/l of liquid microbial culture (LC)) using a strain of the Gram-positive chemoheterotrophic bacterium *Bacillus subtilis* B-3157, adapted to deuterium. The strain was grown on heavy water (HW) medium with high degree of isotopic purity (99.9 atom% ^2H) containing 2% (v/v) hydrolysate of deuterated biomass of the methylotrophic bacterium *Brevibacterium methylicum* B-5662 as a source of ^2H -labeled growth substrates, obtained in minimal M9 growth medium with 98% (v/v) $^2\text{H}_2\text{O}$ and 2% (v/v) [^2H]methanol. Inosine was isolated from LC by adsorption/desorption on activated carbon with following extraction by 0.3 M ammonium-formate buffer (pH = 8.9), subsequent crystallization in 80% (v/v) ethanol, and ion exchange chromatography (IEC) on a column with AG50WX 4 cation exchange resin equilibrated with 0.3 M ammonium-formate buffer and 0.045 M NH_4Cl . The evaluation of the level of deuterium enrichment performed by fast atom bombardment (FAB) mass spectrometry demonstrated incorporation of 5 deuterium atoms into the inosine molecule (the total level of deuterium enrichment – 65.5 atom% ^2H); 3 deuterium atoms were included into the ribose and 2 deuterium atoms – into the hypoxanthine residue of the molecule.

Key words: ^2H -labeled inosine, biosynthesis, heavy water, *Bacillus subtilis*

1. Introduction

Natural nucleosides labeled with deuterium (^2H) are of considerable scientific and practical interest for various biochemical and diagnostic purposes (Andres, 2001), structure-function studies (Kundu *et al.*, 2001), and research of cell metabolism (Kushner *et al.*, 1999). Their usage is determined by the absence of radiation danger and the possibility of localizing the label in a molecule by ^2H -NMR (Crespi, 1989), IR spectroscopy (Caire *et al.*, 2002) and mass spectrometry (Mosin *et al.*, 1996). The recent advance in technical and computing capabilities of these analytical methods has allowed a considerable increase the efficiency of de novo biological studies with ^2H -labeled molecules, as well as to carry out the analysis of the structure and function of nucleosides and their analogs on a molecular level (Lukin & Santos, 2010). In particular, ^2H -labeled ribonucleosides and their analogs are used in template-directed syntheses of deuterated RNA molecules for studying their spatial structure and conformational changes (Chiraku *et al.*, 2001).

An important factor in studies with ^2H -labeled nucleosides and their analogs is their availability. ^2H -labeled nucleosides can be synthesized with using chemical, enzymatic, and microbiological methods (Chen *et al.*, 2002; Jung & Xu, 1998). Chemical synthesis is frequently multistage; requires expensive reagents and ^2H -labeled substrates, and eventually results to a racemic mixture of D- and L-enantiomers, requiring special methods for their separation (Daub, 1979). Finer chemical synthesis of [^2H]nucleosides combine both chemical and enzymatic approach (Huang *et al.*, 2006).

Microbiology proposes an alternative method for synthesis of [^2H]nucleosides, applicable for many scientific and applied purposes; characteristics of the method are high outputs of final products, efficient deuterium incorporation into synthesized molecules, and preservation of the natural L-configuration (Miroshnikov *et al.*, 2010). A traditional approach towards biosynthesis of ^2H -labeled natural compounds consists in growing of strains-producers on growth media with maximal concentrations of $^2\text{H}_2\text{O}$ and ^2H -labeled substrates (Mosin, 1996). However, the main obstacle seriously implementing this method is a deficiency in ^2H -labeled growth substrates with high deuterium content. First and foremost, this stems from a limited availability and high costs of highly purified deuterium itself, isolated from natural sources. The natural abundance of deuterium makes up 0.0015 atom%; however, despite a low deuterium content in specimens, recently developed methods for its enrichment and purification allow to produce ^2H -labeled substrates with high isotopic purity.

Starting from first experiments on the growth of biological objects in heavy water, the approach involving

hydrolysates of deuterated bacterial and micro algal biomass as growth substrates for growth of other bacterial strains-producers have been developed in this country (Den'ko, 1970). However, these experiments discovered a bacteriostatic effect of $^2\text{H}_2\text{O}$ consisted in inhibition of vitally important cell functions in $^2\text{H}_2\text{O}$; this effect on micro algal cells is caused by 70% (v/v) $^2\text{H}_2\text{O}$ and on protozoan and bacterial cells – 80–90% (v/v) $^2\text{H}_2\text{O}$ (Vertes, 2003). Attempts to use natural objects of various taxonomic accessory, including bacteria, micro algae, and yeasts (Mosin & Ignatov, 2012), for growth in $^2\text{H}_2\text{O}$ have not been widely used because of complexity of biosynthesis, consisted in need of complex growth media, applying intricate technological schemes, etc. That is why a number of applied items regarding the biosynthesis of natural ^2H -labeled compounds in $^2\text{H}_2\text{O}$ remain to be unstudied.

More promising seem the technological schemes involving as a source of ^2H -labeled growth substrates the biomass of methylotrophic bacteria, assimilating methanol via the ribulose-5-monophosphate (RMP) and serine pathways of carbon assimilation. The interest towards the using methylotrophic bacteria is steadily increasing owing to intensive development of chemical synthesis of methanol (Mosin *et al.*, 2013a). The assimilation rate of methylotrophic biomass by prokaryotic and eukaryotic cells makes up 85–98% (w/w), and their productivity calculated on the level of methanol bioconversion into cell components reaches 50–60% (w/w) (Trotsenko *et al.*, 1995). As we have earlier reported, methylotrophic bacteria are convenient objects able to grow in minimal media containing 2–4% (v/v) [^2H]methanol, where other bacteria are unable to reproduce, and may easily be adapted to maximal $^2\text{H}_2\text{O}$ concentrations, that is the most important for the biosynthesis of ^2H -labeled natural compounds (Skladnev & Tsygankov, 1991).

High scientific and applied interest towards the use of deuterated biomass of methylotrophic bacteria for the biosynthesis of ^2H -labeled ribonucleosides determined the direction of this research. The aim of this work was studying the possibility of using a strain of the Gram-positive chemoheterotrophic bacterium *Bacillus subtilis* B-3157 for the microbiological synthesis of [^2H]inosine on growth medium with maximal deuterium content with 98% (v/v) $^2\text{H}_2\text{O}$ and 2% (w/v) hydrolysate of deuterated biomass of the facultative methylotrophic bacterium *Brevibacterium methylicum* B-5662 as a source of deuterated substrates.

2. Material and methods

2.1. Bacterial strains

The object of the research was a strain of inosine producer, spore-forming aerobic Gram-positive chemoheterotrophic bacterium *B. subtilis* B-3157, polyauxotrophic for histidine, tyrosine, adenine, and uracil (demand, 10 mg/l), obtained from Institute of Genetics and Selection of Industrial Microorganisms (Russia). The initial strain was adapted to deuterium by plating individual colonies onto 2% (w/v) agarose with stepwise increasing gradient of $^2\text{H}_2\text{O}$ concentration and subsequent selection of individual cell colonies stable to the action of $^2\text{H}_2\text{O}$.

2.2. Chemicals

Growth media were prepared using $^2\text{H}_2\text{O}$ (99.9 atom% ^2H), ^2HCl (95.5 atom% ^2H), and [^2H]methanol (97.5 atom% ^2H), purchased from JSC “Izotop” (St. Petersburg, Russia). Inorganic salts, D- and L-glucose (“Reanal”, Hungary) were preliminary crystallized in $^2\text{H}_2\text{O}$. $^2\text{H}_2\text{O}$ was distilled over KMnO_4 with subsequent control of the isotope purity by ^1H -NMR spectroscopy on a Bruker WM-250 (“Bruker Daltonics” Germany) with a working frequency 70 MHz (internal standard Me_4Si). According to ^1H -NMR, the level of isotopic purity of the growth medium was by 8–10 atom% lower than the isotope purity of the initial $^2\text{H}_2\text{O}$.

2.3. Biosynthesis of ^2H -Inosine

Biosynthetic [^2H]inosine was produced with an output 3.9 g/l in heavy water (HW) medium (89–90 atom% ^2H) using 2% (w/v) hydrolysate of deuterated biomass of the methanol assimilating strain of the facultative Gram-positive methylotrophic bacterium *Brevibacterium methylicum* B-5662 as a source of ^2H -labeled growth substrates. The strain was obtained by multistage adaptation on a solid (2% (w/v) agarose) minimal salt medium M9, containing 3 g/l KH_2PO_4 , 6 g/l Na_2HPO_4 , 0.5 g/l NaCl , 1 g/l NH_4Cl and 2% (v/v) [^2H]methanol with a stepwise increasing gradient of $^2\text{H}_2\text{O}$ concentration (0, 24.5, 73.5, and 98% (v/v) $^2\text{H}_2\text{O}$). Raw methylotrophic biomass (output, 200 g/l) was suspended in 100 ml of 0.5 N ^2HCl (in $^2\text{H}_2\text{O}$) and autoclaved for 30–40 min at 0.8 atm. The suspension was neutralized with 0.2 N KOH (in $^2\text{H}_2\text{O}$) to $\text{pH} = 7.0$ and used as a source of growth substrates while growing the inosine producer strain. For this purpose, an inoculum (5–6 % (w/v)) was added into HW medium with 99.8 atom% $^2\text{H}_2\text{O}$ containing 12% (w/v) glucose, 2% (w/v) hydrolysate of deuterated biomass of *B. methylicum*, 2% (w/v) NH_4NO_3 , 1% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2% (w/v) CaCO_3 , 0.01% (w/v) adenine, and 0.01% (w/v) uracil. As a control was used equivalent protonated medium containing 2% (w/v) yeast protein–vitamin concentrate (PVC).

2.4. Growth Conditions

Bacteria were grown in 500 ml Erlenmeyer flasks (containing 100 ml of the growth medium) for 3–4 days at 32°C under intensive aeration on a Biorad orbital shaker (“Biorad Labs”, Hungary). The bacterial growth was controlled on the ability to form individual colonies on the surface of solid (2% (w/v) agarose) media with the same ²H₂O-content, as well as on the optical density of the cell suspension measured on a Beckman DU-6 spectrophotometer (“Beckman Coulter”, USA) at $\lambda = 540$ nm in a quartz cuvette with an optical pathway length 10 mm.

2.5. Analytical Determination of [²H]Inosine

Inosine was analytically determined in 10 μ l of liquid culture (LC) samples on Silufol UV-254 chromatographic plates (150 \times 150 mm) (“Kavalier”, Czech Republic) using a standard set of ribonucleosides “Beckman-Spinco” (USA) in the solvent system: *n*-butanol–acetic acid–water (2 : 1 : 1, % (v/v)). Spots were eluted with 0.1 N HCl. The UV absorbance of eluates was recorded on a Beckman DU-6 spectrophotometer (“Beckman Coulter”, USA) using a standard calibration plot. The level of bioconversion of the carbon substrate was assessed using glucose oxidase (EC 1.1.3.4).

2.6. Isolation of [²H]Inosine from LC.

Samples of LC were separated on a T-26 centrifuge (“Carl Zeiss”, Germany) at 2000 g for 10 min, concentrated at 10 mm Hg in a RVO-6 rotor evaporator (“Microtechna”, Hungary) to half of their initial volume, and supplemented with acetone (3 \times 5 ml). The mixture was kept for ~10 h at 4°C, and the precipitate was separated by centrifugation at 1200 g for 5 min. The supernatant was supplemented with 20 g of activated carbon and kept for 24 h at 4°C. The water fraction was separated by filtration; the solid phase was supplemented with 20 ml 50% (v/v) ethanol solution in 25% (v/v) ammonia (1 : 1, (v/v)) and heated at 60°C with a reflux water condenser. After 2–3 h, the mixture was filtered and evaporated at 10 mm Hg. The product was extracted with 0.3 M ammonium–formate buffer (pH = 8.9), washed with acetone (2 \times 10 ml), and dried over anhydrous CaCl₂. Inosine was crystallized from 80% (v/v) ethanol ($[\alpha]_D^{20} = +1.61^0$; output, 3.1 g/l (80 %)). Inosine was finally purified by ion exchange chromatography using a calibrated column (150 \times 10 mm) with AG50WX 4 cation exchange resin (“Pharmacia”, USA). The column was equilibrated with 0.3 M ammonium–formate buffer (pH = 8.9) containing 0.045 M NH₄Cl and eluted with the same buffer under isocratic conditions (chromatographic purity, 92%). The eluate was dried in vacuum and stored in sealed ampoules at -14°C in frost camera. ²H-inosine: yield, 3.1 g/l (80%); $T_m = 68$ –70°C; $[\alpha]_D^{20} = 1.61$ (ethanol); $R_f = 0.5$; $pK_a = 1.2$ (phosphate buffer with pH = 6.87). UV-spectrum (0.1 N HCl): $\lambda_{max} = 249$ nm; $\epsilon_{249} = 7100$ M⁻¹ cm⁻¹. FAB mass spectrum (glycerol matrix, Cs⁺; accelerating voltage, 5 kV; ion current, 0.6–0.8 mA): $[M + H]^+ m/z$ (I, %) 273, 20% (4 atoms ²H); 274, 38 % (5 atoms ²H); 275, 28% (6 atoms ²H); 276, 14% (7 atoms ²H); $[A + H]^+ 136$, 46%; $[B + H]^+ 138$, 55%; $[B - HCN]^+ 111$, 49%; $[B - HCN]^+ 84$, 43%.

2.7. Protein Hydrolysis

Dry biomass (10 g) was treated with a chloroform–methanol–acetone mixture (2 : 1 : 1, % (v/v)), evaporated in vacuum, and supplemented with 5 ml 6 N ²HCl (in ²H₂O). The ampoules were kept at 110°C for ~24 h. Then the reaction mixture was suspended in hot ²H₂O and filtered. The hydrolysate was evaporated at 10 mm Hg. Residual ²HCl was removed in an exsiccator over solid NaOH. For preparation of ²H-labeled growth substrates 200 mg of raw deuterium-biomass was suspended in 200 ml 0.5 ²HCl (in ²H₂O) and autoclaved at 60°C for 1.5 h. The reaction mixture was neutralized with 0.5 N NaOH (in ²H₂O) till pH = 6.5–6.7, and evaporated at 10 mm Hg. The dry residue was used for preparation of growth media.

2.8. Hydrolysis of Intracellular Polycarbohydrates

Dry biomass (50 mg) was placed into a 250 ml round bottomed flask, supplemented with 50 ml distilled ²H₂O and 1.6 ml 25% (v/v) H₂SO₄ (in ²H₂O), and boiled in a reflux water evaporator for ~90 min. After cooling, the reaction mixture was suspended in one volume of hot distilled ²H₂O and neutralized with 1 N Ba(OH)₂ (in ²H₂O) to pH = 7.0. BaSO₄ was separated by centrifugation (1500 g, 5 min); the supernatant was decanted and evaporated at 10 mm Hg.

2.9. Amino Acid Analysis

The amino acids of the hydrolyzed biomass were analyzed on Biotronic LC-5001 (230 \times 3.2) column (“Eppendorf–Nethleler–Hinz”, Germany) with UR-30 sulfonated styrene resin (“Beckman–Spinco”, USA) as a stationary phase; the mobile phase, 0.2 N sodium–citrate buffer (pH = 2.5); the granule diameter, 25 μ m; working pressure, 50–60 atm; the eluent input rate, 18.5 ml/h; the ninhydrin input rate, 9.25 ml/h; detection at $\lambda = 570$ and $\lambda = 440$ nm (for proline).

2.10. Analysis of Carbohydrates

Carbohydrates were analyzed on Knauer Smartline chromatograph (“Knauer”, Germany) equipped with a Gilson pump (“Gilson Inc.”, Germany) and a Waters K 401 refractometer (“Water Associates”, Germany) using

Ultrasorb CN column (250 × 10 mm) as a stationary phase; the mobile phase, acetonitrile–water (75 : 25, % (v/v); the granule diameter, 10 μm; the input rate, 0.6 ml/min.

2.11. The UV Spectroscopy

The UV spectra were registered with Beckman DU-6 programmed spectrophotometer (“Beckman Coulter”, USA) at $\lambda = 220\text{--}280$ nm.

2.12. FAB Mass Spectrometry

FAB mass spectra were recorded on VG-70 SEQ chromatograph (“Fisons VG Analytical”, USA) equipped with a cesium source on a glycerol matrix with accelerating voltage 5 kV and ion current 0.6–0.8 mA.

2.12. EI Mass Spectrometry

EI mass spectra were recorded with MB-80A device (“Hitachi”, Japan) with double focusing (the energy of ionizing electrons, 70 eV; the accelerating voltage, 8 kV; the cathode temperature, 180–200°C) after amino acid modification into methyl esters of N-5-dimethylamino(naphthalene)-1-sulfonyl (dansyl) amino acid derivatives according to an earlier elaborated protocol (Mosin *et al.*, 1998).

2.13. IR-spectroscopy

IR-spectra of water samples were registered on Bruker Vertex (“Bruker”, Germany) spectrometer (spectral range: average IR – 500–4500 cm^{-1} ; permission – 0.5 cm^{-1} ; accuracy of wave number – 0.1 cm^{-1} on 2000 cm^{-1}).

2.14. Statistical Analysis

The experiments were repeated three times. Statistical data processing was performed using the software package Statistica 6.0. For data with normal distribution, inter-group comparisons were performed using Student’s t-test. P value less than 0.05 was considered significant.

3. Results and Discussion

3.1. Preparing Deutero-Biomass of *B. methylicum*.

A mutant strain of the Gram-positive chemoheterotrophic bacterium *B. subtilis* B-3157 polyauxotrophic for histidine, tyrosine, adenine, and uracil (preliminary adapted to deuterium by selection of individual colonies) was used as the inosine producer. Because of impaired metabolic pathways involved in the regulation of the biosynthesis of purine ribonucleosides, this strain under standard growth conditions (PVC medium, late exponential growth, 32°C) synthesizes 17–20 g of inosine per 1 liter of LC (Mosin *et al.*, 1999b).

The maximal yield of inosine was attained on a protonated medium with 12% (w/v) glucose as a source of carbon and energy and 2% (w/v) yeast PVC as a source of growth factors and amine nitrogen (Mosin *et al.*, 2013b). In our experiments it was necessary to replace the protonated growth substrates with their deuterated analogs, as well as to use $^2\text{H}_2\text{O}$ of high isotopic purity. For this purpose, we used autoclaved biomass of the Gram-positive facultative methylotrophic bacterium *Brevibacterium methylicum* B-5662, capable to assimilate methanol via the ribulose-5-monophosphate (RuMP) pathway of carbon assimilation. Owing to a 50–60% rate of methanol bioconversion (conversion efficiency, 15.5–17.3 gram dry biomass per 1 gram of assimilated substrate) and stable growth on deuterated minimal medium M9 with 98% (v/v) $^2\text{H}_2\text{O}$ and 2% (v/v) [^2H]methanol, this strain is the most convenient source for producing the deuterated biomass; moreover, the cost of bioconversion is mainly determined by the cost of $^2\text{H}_2\text{O}$ and [^2H]methanol (Mosin *et al.*, 2012).

Adaptation of *B. methylicum* was necessary to improve the growth characteristics of this strain and attain high output of microbial biomass on the maximally deuterated M9 medium. For this purpose, we used a stepwise increasing gradient of $^2\text{H}_2\text{O}$ -concentration in M9 growth media (from 24.5, 49.0, 73.5, up to 98% (v/v) $^2\text{H}_2\text{O}$) in the presence of 2% (v/v) methanol and its ^2H -labeled analog ([^2H]methanol), because we assumed that gradual cell adaptation to $^2\text{H}_2\text{O}$ would have a favorable effect on the growth parameters of the strain.

To study the effect of the degree of carbon source deuteration on the growth parameters of the strain, in experiments 1, 3, 5, 7, and 9 was used protonated methanol, and [^2H]methanol in experiments 2, 4, 6, 8, and 10 (Table 1). The results demonstrated that the replacement of protonated methanol with its deuterated analog within the same concentration of $^2\text{H}_2\text{O}$ in the growth media slightly decreased the growth characteristics (Table 1, experiments 2, 4, 6, 8, and 10). Therefore, in further experiments were used M9 media with $^2\text{H}_2\text{O}$ and [^2H]methanol. When the initial strain of *B. methylicum* was cultivated on protonated M9 medium with water and methanol, the duration of lag-phase and cell generation time were 20 and 2.2 h, respectively, with an output of biomass 200 gram per 1 liter of LC (Table 1, experiment 1). In the intermediate experiments (2–10), these parameters varied proportionally to the $^2\text{H}_2\text{O}$ concentration (Table 1). The observed effect consisted in the increase in the lag-phase period and cell generation time with a simultaneous decrease in microbial biomass outputs on media with increasing $^2\text{H}_2\text{O}$ -content. The most remarkable values of this parameters were detected in experiment 10, in which was used the maximally deuterated medium with 98% (v/v) $^2\text{H}_2\text{O}$ and 2% (v/v) [^2H]methanol; the lag-phase and cell generation time in these conditions were increased in 3- and 2.2-fold times,

respectively, as compared to the control conditions (water and methanol; Table 1, experiment 1), and the biomass output decreased in 3.1-fold. The adaptation to deuterium (experiment 10', Table 1) permitted to improve essentially the growth characteristics of *B. methylicum* on maximally deuterated growth medium. The output of biomass produced by the adapted bacterium decreased by 13% as compared to the control with an increase in the generation time to 2.8 h and the lag phase to 40 h (experiment 10', Table 1).

Table 1. Isotopic components of growth media M9 and characteristics of bacterial growth of *B. methylicum**

Experiment number	Media components, % (v/v)				Lag-period (h)	Yield of biomass, gram from 1 l of LC	Generation time (h)
	H ₂ O	² H ₂ O	Methanol	[² H]methanol			
1	98.0	0	2	0	20±1.40	200.2±3.20	2.2±0.20
2	98.0	0	0	2	30±1.44	184.6±2.78	2.4±0.23
3	73.5	24.5	2	0	32±0.91	181.2±1.89	2.4±0.25
4	73.5	24.5	0	2	34±0.89	171.8±1.81	2.6±0.23
5	49.0	49.0	2	0	40±0.90	140.2±1.96	3.0±0.32
6	49.0	49.0	0	2	44±1.38	121.3±1.83	3.2±0.36
7	24.5	73.5	2	0	45±1.41	112.8±1.19	3.5±0.27
8	24.5	73.5	0	2	49±0.91	94.4±1.74	3.8±0.25
9	0	98.0	2	0	58±1.94	65.8±1.13	4.4±0.70
10	0	98.0	0	2	60±2.01	60.2±1.44	4.9±0.72
10'	0	98.0	0	2	40±0.88	174.0±1.83	2.8±0.30

Keys: * The data in Expts. 1–10 are submitted for *B. methylicum* at growing on growth media with 2% (v/v) methanol/[²H]methanol and specified amounts (% (v/v) ²H₂O).

The data in Expt. 10' are submitted for adapted for maximum content of deuterium in growth medium bacterium *B. methylicum* at the growing on growth media with 2% (v/v) [²H]methanol and 98% (v/v) ²H₂O.

As the control used experiment where used ordinary protonated water and methanol.

The adaptation was monitored by recording the growth dynamics of the initial *B. methylicum* (Figure 1, curve 1, control, protonated M9 medium) and adapted to deuterium (Figure 1, curve 3) strain in the maximally deuterated M9 medium with 98% (v/v) ²H₂O and 2% (v/v) [²H]methanol. Unlike the adapted strain (Figure 1, curve 3), the growth dynamics of the initial strain (Figure 1, curve 1) in the maximally deuterated medium were inhibited by deuterium. Being transferred to the protonated medium, the adapted strain returned to normal growth after a certain lag-phase period, that was characteristic for other adapted bacterial strains. The effect of growth reversion in protonated/deuterated media demonstrates that adaptation to ²H₂O is a phenotypic phenomenon, although it cannot be excluded that a certain genotype determined the manifestation of the same phenotypic attribute in media with high deuterium content. In general, the improved growth characteristics of the adapted bacterium significantly simplify the scheme for the production of deuterated biomass, the optimal conditions for which are satisfied the following: maximally deuterated M9 medium with 98% (v/v) ²H₂O and 2% (v/v) [²H]methanol, incubation period 3–4 days, and temperature 35°C.

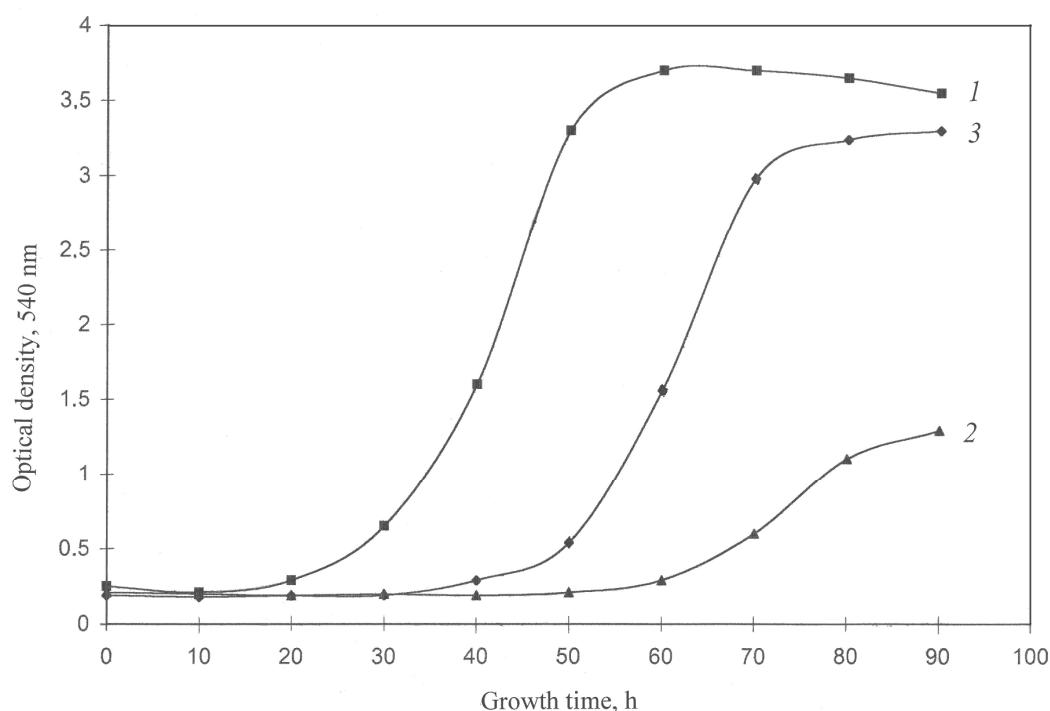


Figure 1. Growth dynamics of *B. methylicum* (1, 2, 3) on media M9 with various isotopic content: 1 – non-adapted bacterium on protonated medium M9 (Table 1, experiment 1); 2 – non-adapted bacterium on maximally deuterated medium M9 (Table 1, experiment 10); 3 – adapted to $^2\text{H}_2\text{O}$ bacterium on maximally deuterated medium M9 (Table 1, experiment 10')

3.2. Biosynthesis of [^2H]Inosine.

The strategy for the biosynthesis of [^2H]inosine using biomass of *B. methylicum* as growth substrates was developed taking into account the ability of methylotrophic bacteria to synthesize large amounts of protein (output, 50% (w/w) of dry weight), 15–17% (w/w) of polysaccharides, 10–12% (w/w) of lipids (mainly, phospholipids), and 18% (w/w) of ash (Mosin & Ignatov, 2013). To provide high outputs of these compounds and minimize the isotopic exchange (^1H – ^2H) in amino acid residues of protein molecules, the biomass was hydrolyzed by autoclaving in 0.5 N ^2HCl (in $^2\text{H}_2\text{O}$). Since the *B. subtilis* inosine-producing strain is a polyauxotroph requiring tyrosine and histidine for its growth, we studied the qualitative and quantitative content of amino acids in the hydrolyzed methylotrophic biomass produced in the maximally deuterated medium M9 with 98% (v/v) $^2\text{H}_2\text{O}$ and 2% (v/v) [^2H]methanol, and the levels of their deuterium enrichment (Table 2). The methylotrophic hydrolysate contains 15 identified amino acids (except for proline detected at $\lambda = 440$ nm) with tyrosine and histidine content per 1 gram of dry methylotrophic hydrolysate 1.82% and 3.72% (w/w), thereby satisfying the auxotrophic requirements of the inosine producer strain for these amino acids. The content of other amino acids in the hydrolysate is also comparable with the needs of the strain in sources of carbon and amine nitrogen (Table 2).

The indicator determining the high efficiency of deuterium incorporation into the synthesized product is high levels of deuterium enrichment of amino acid molecules, varied from 49 atom% ^2H for leucine/isoleucine to 97.5 atom% ^2H for alanine (Table 2). This allowed using the hydrolysate of deuterated biomass of *B. methylicum* as a source of growth substrates for growing the *B. subtilis* inosine-producing strain.

Table 2. Amino acid composition of hydrolyzed biomass of the facultative methylotrophic bacterium *B. methylicum* obtained on maximally deuterated M9 medium with 98% (v/v) $^2\text{H}_2\text{O}$ and 2% (v/v) [^2H]methanol and levels of deuterium enrichment*

Amino acid	Yield, % (w/w) dry weight per 1 gram of biomass		Number of deuterium atoms incorporated into the carbon backbone of a molecule**	Level of deuterium enrichment of molecules, % of the total number of hydrogen atoms***
	Protonated sample (control)	Sample from deuterated M9 medium		
Glycine	8.03	9.69	2	90.0±1.86
Alanine	12.95	13.98	4	97.5±1.96
Valine	3.54	3.74	4	50.0±1.60
Leucine	8.62	7.33	5	49.0±1.52
Isoleucine	4.14	3.64	5	49.0±1.50
Phenylalanine	3.88	3.94	8	95.0±1.85
Tyrosine	1.56	1.83	7	92.8±1.80
Serine	4.18	4.90	3	86.6±1.56
Threonine	4.81	5.51	–	–
Methionine	4.94	2.25	–	–
Asparagine	7.88	9.59	2	66.6±1.62
Glutamic acid	11.68	10.38	4	70.0±1.64
Lysine	4.34	3.98	5	58.9±1.60
Arginine	4.63	5.28	–	–
Histidine	3.43	3.73	–	–

Keys: * The data were obtained for methyl esters of N-5-dimethylamino(naphthalene)-1-sulfonyl (dansyl) chloride amino acid derivatives.

** At calculation the level of deuterium enrichment, the protons (deuterons) at the carboxyl COOH^- and amino NH_2^- groups of amino acid molecules were not taken into account because of the dissociation in $\text{H}_2\text{O}/^2\text{H}_2\text{O}$.

*** A dash denotes the absence of data.

The growth and biosynthetic characteristics of inosine-producing strain *B. subtilis* were studied on protonated yeast PVC medium with H_2O and 2% (w/v) yeast PVC and on HW medium with 89% (v/v) $^2\text{H}_2\text{O}$ and 2% (w/w) of hydrolysate of deuterated biomass of *B. methylicum* (Figure 2). Experiments demonstrated a certain correlation between the changes of growth dynamics of *B. subtilis* (Fig. 2, curves 1, 1'), output of inosine (Figure 2, curves 2, 2'), and glucose assimilation (Figure 2, curves 3, 3'). The maximal output of inosine (17 g/l) was observed on protonated PVC medium at a glucose assimilation rate 10 g/l (Figure 2, curve 2). The output of inosine in the HW medium decreased in 4.4-fold, reaching 3.9 g/l (Figure 2, curve 2'), and the level of glucose assimilation – 4-fold, as testified by the remaining 40 g/l non-assimilated glucose in LC (Figure 2, curve 3'). The experimental data demonstrate that glucose is less efficiently assimilated during growth in the HW medium as compared to the control conditions in H_2O .

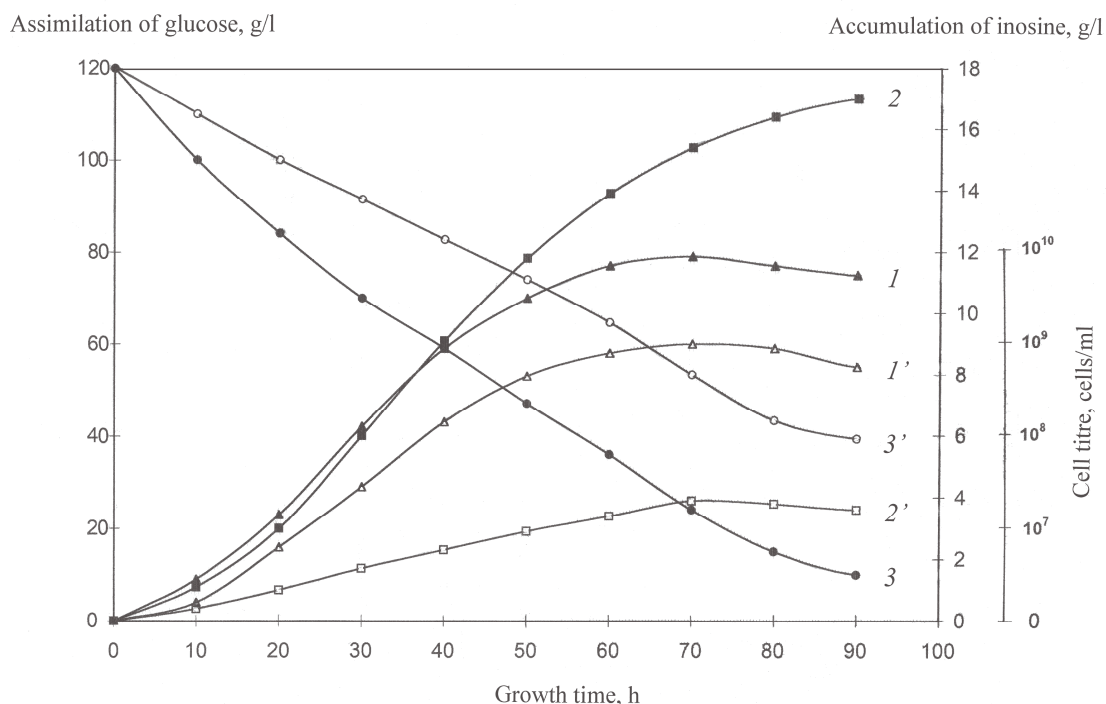


Figure 2. Growth dynamics of *B. subtilis* (1, 1') (cells/ml), inosine accumulation in LC (2, 2') (g/l), and glucose assimilation (3, 3') (g/l) under different experimental conditions: (1–3) – protonated yeast PVC medium; (1'–3') – HW medium with 2% (w/v) hydrolysate of deuterated biomass of *B. methylicum*.

This result demanded the examination of the content of glucose and other intracellular carbohydrates in the biomass of the *B. subtilis* inosine-producer strain, which was performed by reverse phase HPLC on an Ultrasorb CN column (10 μ m, 10 \times 250 mm) with acetonitrile and water (75 : 25, % (v/v)) as a mobile phase (Table 3). The fraction of intracellular carbohydrates in Table 3 (numbered according to the sequence of their elution from the column) comprises monosaccharides (glucose, fructose, rhamnose, and arabinose), disaccharides (maltose and sucrose), and four unidentified carbohydrates with retention times of 3.08 (15.63% (w/w)), 4.26 (7.46% (w/w)), 7.23 (11.72% (w/w)), and 9.14 (7.95% (w/w)) min (not shown). As was expected, the output of glucose in the deuterated hydrolysate was 21.4% (w/w) of dry weight, that is, higher than the outputs of fructose (6.82% (w/w)), rhamnose (3.47% (w/w)), arabinose (3.69% (w/w)), and maltose (11.62% (w/w)) (Table 3). Their outputs in microbial biomass did not differ considerably related to the control in H₂O except for sucrose, which is undetectable in the deuterated hydrolysate. The levels of deuterium enrichment in carbohydrates varied from 90.7 atom% ²H for arabinose to 80.6 atom% ²H for glucose.

Table 3. Qualitative and quantitative compositions of intracellular carbohydrates isolated from *B. subtilis* after growing on HW-medium and levels of the deuterium enrichment*

Carbohydrate	Content in biomass, % (w/w) of 1 g of dry biomass		Level of deuterium enrichment of molecules, %**
	Protonated sample (control)	Sample from the HW medium	
Glucose	20.01	21.40	80.6 \pm 1.86
Fructose	6.12	6.82	85.5 \pm 1.92
Rhamnose	2.91	3.47	90.3 \pm 2.12
Arabinose	3.26	3.69	90.7 \pm 3.10
Maltose	15.30	11.62	–
Sucrose	8.62	ND**	–

Keys: * The data were obtained by IR-spectroscopy.

** ND – not detected.

*** A dash denotes the absence of data.

3.3. Isolation of [²H]Inosine from LC.

The use of a combination of physical-chemical methods for isolating [²H]inosine from LC was determined by the need for preparing inosine of a high chromatographic purity (no less than 95%). Since LC along with inosine contains inorganic salts, proteins, and polysaccharides, as well as accompanying secondary nucleic metabolites (adenosine and guanosine) and non-reacted substrates (glucose and amino acids), LC was fractionated in a stepwise manner for isolating of [²H]inosine. The high sensitivity of inosine to acids and alkali and its instability during isolation required applying diluted acid and alkaline solutions with low concentration, as well as carrying out the isolation procedure at low temperature, thus avoiding long heating of the reaction mixture. The fractionation of LC consisted in low-temperature precipitation of high molecular weight impurities with organic solvents (acetone and methanol), adsorption/desorption on the surface of activated carbon, extraction of the end product, crystallization, and ion exchange chromatography. The proteins and polysaccharides were removed from LC by precipitation with acetone at 4°C with subsequent adsorption/desorption of total ribonucleosides on activated carbon. The desorbed ribonucleosides were extracted from the reacted solid phase by eluting with EtOH-NH₃-solution at 60°C; inosine – by extracting with 0.3 M ammonium-formate buffer (pH = 8.9) and subsequent crystallization in 80% (v/v) ethanol. The final purification consisted in column ion exchange chromatography on AG50WX 4 cation exchange resin equilibrated with 0.3 M ammonium-formate buffer containing 0.045 M NH₄Cl with collection of fractions at $R_f = 0.5$. The curves 1–3 in Figure 3 shows UV-absorption spectra of inosine isolated from the LC at various stages of isolation procedure. The presence of major absorbance band I, corresponding to natural inosine ($\lambda_{max} = 249$ nm, $\epsilon_{249} = 7100$ M⁻¹ cm⁻¹), and the absence of secondary metabolites II and III in the analyzed sample (Figure 3, curve 3), demonstrates the homogeneity of isolated product and the efficiency of the isolation method.

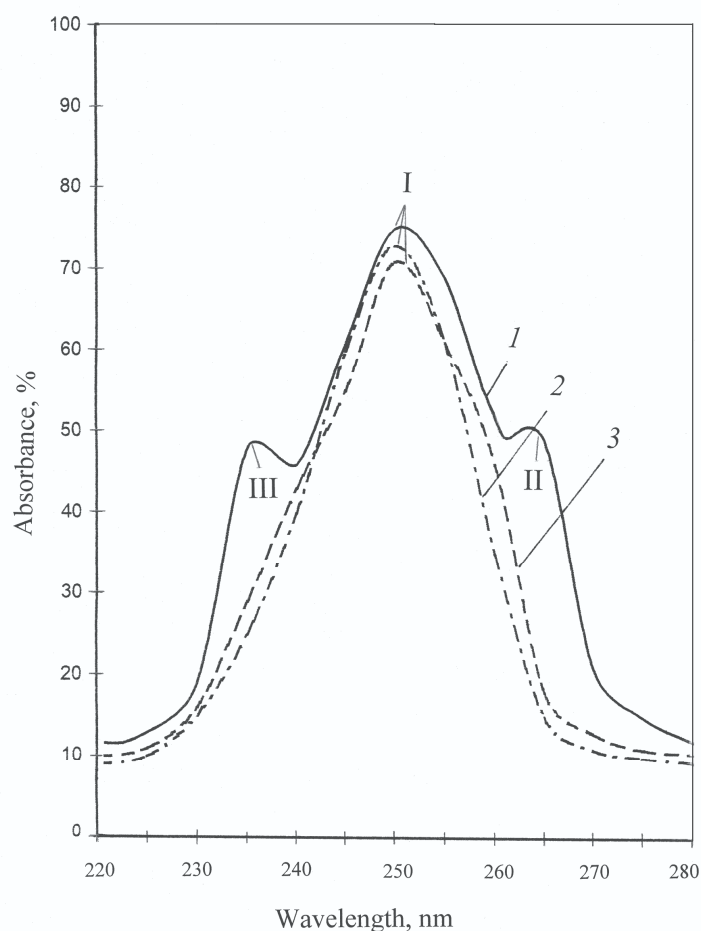


Figure 3. UV-absorption spectra of inosine (0.1 N HCl): (1) – initial LC after the growth of *B. subtilis* on HW medium; (2) – natural inosine, and (3) – inosine extracted from the LC. Natural inosine (2) was used as a control: (I) – inosine, (II, III) – secondary metabolites.

3.4. The Studying of the Level of Deuterium Enrichment of [²H]Inosine.

The level of deuterium enrichment of the [²H]inosine molecule was determined by FAB mass spectrometry, the high sensitivity of which allows to detect 10⁻⁸ to 10⁻¹⁰ moles of a substance in a sample (Caprioli, 1990). The formation of a molecular ion peak for inosine in FAB mass spectrometry was accompanied by the migration of H⁺. Biosynthetically ²H-labeled inosine, which FAB mass-spectrum represented in Figure 4b regarding the control (natural protonated inosine, Figure 4a), represented a mixture of isotope-substituted molecules with different numbers of hydrogen atoms replaced by deuterium. Correspondingly, the molecular ion peak of inosine [M + H]⁺, was polymorphically splintered into individual clusters with admixtures of molecules with statistical set of mass numbers *m/z* and different contributions to the total level of deuterium enrichment of the molecule. It was calculated according to the most intensive molecular ion peak (the peak with the largest contribution to the level of deuterium enrichment) recorded by a mass spectrometer under the same experimental conditions. These conditions are satisfied the most intensive molecular ion peak [M + H]⁺ at *m/z* 274 with 38% (instead of [M + H]⁺ at *m/z* 269 with 42% under the control conditions; Figure 4a). That result corresponds to five deuterium atoms incorporated into the inosine molecule (Figure 4b). The molecular ion peak of inosine also contained less intensive peaks with admixtures of molecules containing four (*m/z* 273, 20%), five (*m/z* 274, 38%), six (*m/z* 275, 28%), and seven (*m/z* 276, 14%) deuterium atoms (Table 4).

Table 4. Values of peaks [M + H]⁺ in the FAB mass spectra and levels of deuterium enrichment of riboxine isolated from HW-medium

Value of peak [M + H] ⁺	Contribution to the level of deuterium enrichment, mol.%	The number of deuterium atoms	Level of deuterium enrichment of molecules, % of the total number of hydrogen atoms*
273	20	4	20.0±0.60
274	38	5	62.5±1.80
275	28	6	72.5±1.96
276	14	7	87.5±2.98

Keys: *At calculation of the level of deuterium enrichment, the protons(deuterons) at the hydroxyl (OH⁻) and imidazole protons at NH⁺ heteroatoms were not taken into account because of keto–enol tautomerism in H₂O²H₂O.

Taking into account the contribution of the molecular ion peaks, the total level of deuterium enrichment (TLDE) of the inosine molecule calculated using the below equation was 65.5% of the total number of hydrogen atoms in the carbon backbone of the molecule:

$$TLDE = \frac{[M]_{r1}^+ C_1 + [M]_{r2}^+ C_2 + \dots + [M]_m^+ C_n}{\Sigma C_n}$$

where [M]_r⁺ - the values of the molecular ion peaks of inosine.
 C_n - the contribution of the molecular ion peaks to TLDE (mol %).

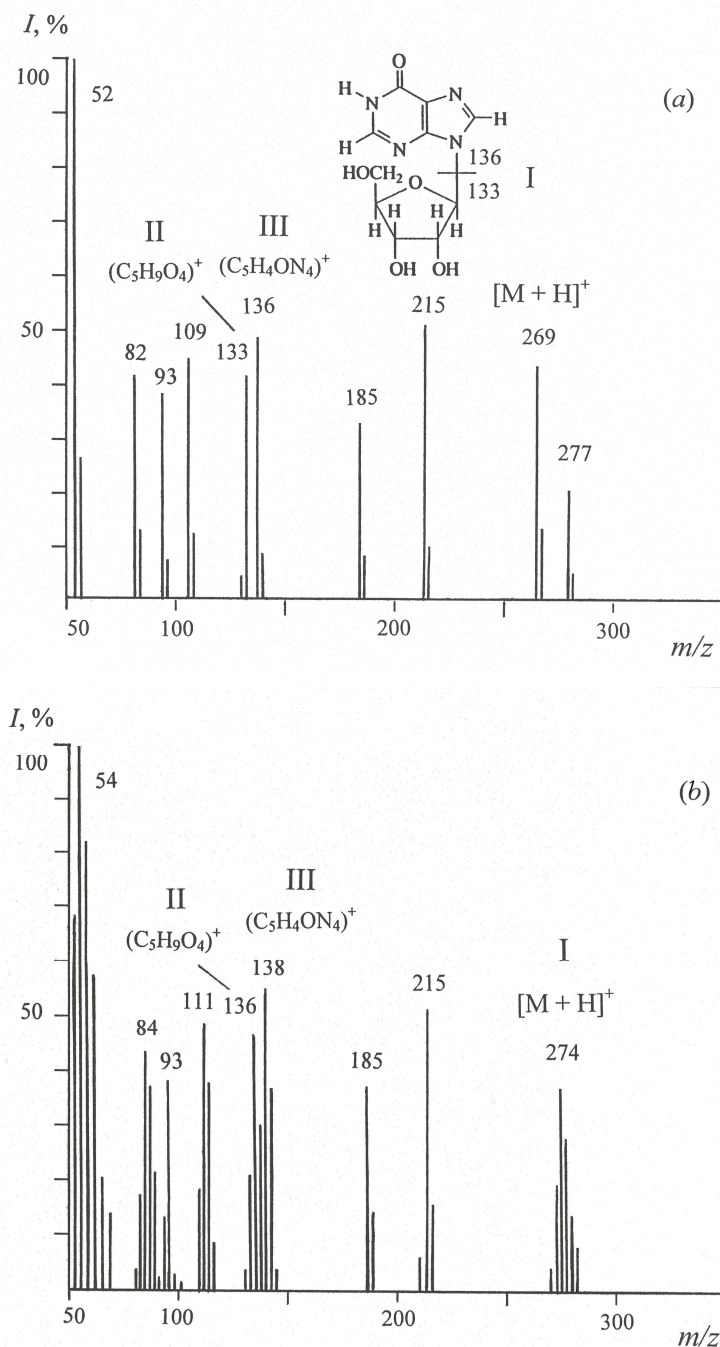


Figure 4. FAB mass spectra of inosine (glycerol as a matrix) under different experimental conditions: (a) – natural inosine; (b) – $[^2\text{H}]$ inosine isolated from HW medium (scanning interval at m/z 50–350; major peaks with a relative intensity of 100% at m/z 52 and m/z 54; ionization conditions: cesium source; accelerating voltage, 5 kV; ion current, 0.6–0.8 mA; resolution, 7500 arbitrary units): I – relative intensity of peaks (%); (I) – inosine; (II) – ribose fragment; (III) – hypoxanthine fragment.

The fragmentation of the inosine molecule, shown in Figure 5, gives more precise information on the deuterium distribution in the molecule. The FAB fragmentation pathways of the inosine molecule (I) lead to formation of ribose $(\text{C}_5\text{H}_9\text{O}_4)^+$ fragment (II) at m/z 133 and hypoxanthine $(\text{C}_5\text{H}_4\text{ON}_4)^+$ fragment (III) at m/z 136 (their fragmentation is accompanied by the migration of H^+), which in turn, disintegrated into several low-molecular-weight splinter fragments at m/z 109, 108, 82, 81, and 54 due to HCN and CO elimination from hypoxanthine (Figure 5). Consequently, the presence of two “heavy” fragments of ribose II $(\text{C}_5\text{H}_9\text{O}_4)^+$ at m/z 136 (46%) (instead of m/z 133 (41%) in the control) and hypoxanthine III $(\text{C}_5\text{H}_4\text{ON}_4)^+$ at m/z 138 (55%) (instead of m/z 136

(48%) in the control), as well as the peaks of low molecular weight splinter fragments formed from FAB-decomposition of hypoxanthine fragment at m/z 111 (49%) (instead of m/z 109 (45%) in the control) and m/z 84 (43%) (instead of 82 (41%) in the control) suggests that three deuterium atoms are incorporated into the ribose residue, and two other deuterium atoms – into the hypoxanthine residue of the inosine molecule (Figure 5).

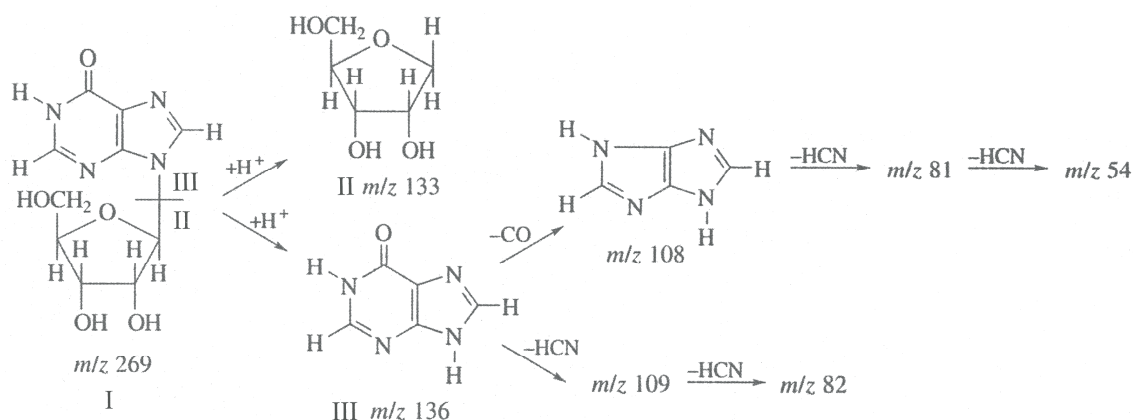


Figure 5. The fragmentation pathways of the inosine molecule by the FAB-method

When analyzing the level of deuterium enrichment of the inosine molecule we took into account the fact that the character of deuterium incorporation into the molecule determined by the pathways of carbon assimilation. Since the protons (deuterons) at positions of the ribose residue in the inosine molecule could have been originated from glucose, the character of deuterium inclusion into the ribose residue is mainly determined by hexose-5-monophosphate (HMP) shunt, associated with the assimilation of glucose and other carbohydrates. Since glucose in our experiments was used in a protonated form, its contribution to the level of deuterium enrichment of the ribose residue was neglected. However, deuterium was incorporated into the ribose residue of the inosine molecule owing to the preservation of the minor pathways of de novo glucose biosynthesis in $^2\text{H}_2\text{O}$ -medium. Numerous isotopic ^1H - ^2H exchange processes could also have led to specific incorporation of deuterium atoms at certain positions in the inosine molecule. Such accessible positions in the inosine molecule are hydroxyl (OH^-)- and imidazole protons at NH^+ heteroatoms, which can be easily exchanged on deuterium in $^2\text{H}_2\text{O}$ via keto-enol tautomerism. Three non-exchangeable deuterium atoms in the ribose residue of inosine could have been originated from HMP shunt reactions, while two other deuterium atoms at C2,C8-positions in the hypoxanthine residue could be synthesized de novo at the expense of [^2H]amino acids that originated from deuterated methylotrophic hydrolysate. In particular, the glycoside proton at β - N_9 -glycosidic bond could be replaced with deuterium in the reaction of CO_2 elimination at the stage of ribulose-5-monophosphate formation from 3-keto-6-phosphogluconic acid with subsequent proton (deuteron) attachment at the C1-position of ribulose-5-monophosphate. Two other protons at C2(C3) and C4 positions in ribose residue could be replaced with deuterium via further enzymatic isomerization of ribulose-5-monophosphate into ribose-5-monophosphate. In general, our studies confirmed this scheme (Ignatov & Mosin, 2013). However, it should be noted that the level of deuterium enrichment of inosine molecule is determined by isotopic purity of $^2\text{H}_2\text{O}$ and deuterated substrates.

4. Conclusion

We have demonstrated the feasibility of preparative microbiological synthesis of [^2H]inosine by a strain of the Gram-positive chemoheterotrophic bacterium *B. subtilis* using as a source of growth substrates hydrolyzed biomass of the facultative methylotrophic bacterium *B. methylicum* produced on maximally deuterated M9 growth medium. The output of [^2H]inosine in the maximally deuterated medium with 2% (w/v) hydrolysate of ^2H -labeled biomass of *B. methylicum* amounted to 3.9 g/l, and the total level of deuterium enrichment – 5 deuterium atoms (65.5 atom% ^2H). From total 5 deuterium atoms in the inosine molecule, 3 deuterium atoms were localized in the ribose residue, while 2 deuterium atoms – in the hypoxanthine residue of the molecule. To attain higher deuterium enrichment level of the final product, it is necessary to *thoroughly* control the isotope composition of the growth medium and exclude any possible sources of additional protons, in particular, to use [^2H]glucose, which may be isolated from deuterated biomass of the methylotrophic bacterium *B. methylicum*.

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