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EXPERIMENTAL ARTICLES

Study of the Initial Stages of 2-Methylpyridine Catabolism by *Arthrobacter* sp. Strain KM-2MP

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Abstract—Strain *Arthrobacter* sp. KM-2MP isolated from soil samples treated with pyridine and its derivatives is able to utilize 2-methylpyridine (2-MP) as the only source of carbon, nitrogen, and energy. According to the results of UV spectroscopy of the culture liquid, the substrate (2.5 g/l) is completely utilized within 24 h. Intermediate products of 2-MP degradation were detected and identified using gas chromatography–mass spectrometry analysis. On the basis of the obtained data, catabolic pathways of the substrate are proposed. The strain has successfully passed the tests and is recommended for 2-MP removal from industrial wastewater.

Keywords: biodegradation, 2-methylpyridine, hydroxypyridine, *Arthrobacter* sp. degrader strain, gas chromatography–mass spectrometry.

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As a result of massive industrial discharges, natural ecosystems are subjected to intense anthropogenic pressure. The most negative impact on the environment is produced by chemical plants as the sources of difficult-to-degrade organic heterocyclic compounds, with pyridine and its derivatives as an important class among them [1].

Sanitary legislation states the maximum permissible concentration (MPC) of pyridine vapors in air at 0.0015 mg/m^3; MPC in drinking water at 0.2 mg/l for pyridine and 0.05 mg/l for methyl- and dimethylpyridine, respectively [2]; and in the water of fish culture-related objects, at 0.01 and 0.001 mg/l, respectively [3]. These MPC values imply deep purification of wastewater. Various physicochemical methods of purification have been suggested, including adsorption [4], adsorption with electrosorption [5], ozone treatment, and ion exchange [6].

Microorganisms are promising for rational environmental management both in biotechnology for efficient waste processing at the stage of production and in detoxification of the contaminants already present in the environment [7, 8].

The ability of microorganisms to utilize alkylpyridines was initially demonstrated in the 1980s [9], and the area of research developed intensely ever since [10]. While the ability to cleave the methylpyridine (MP) ring is presently known to be inherent to a number of microorganisms, no clear understanding exists of the processes involved [11]. For example, one of *Arthrobacter* sp. strains utilizes 2-MP via reduction of the pyridine ring [9], while in the case of *A. globiformis* strain KM-1, decomposition starts with the ring oxidation [10].

The aim of the present work was to determine the kinetics of 2-MP utilization by the strain *Arthrobater* sp. KM-2MP, to detect and identify the intermediates of the degradation processes by means of gas chromatography–mass spectrometry (GC–MS), and to establish the pathways of 2-MP catabolism based on the structures of the sequentially formed intermediates.

MATERIALS AND METHODS

The subject of the study was a bacterial 2-MP-degrading strain isolated form enrichment cultures [12] inoculated with samples of soil exposed to pyridine and its derivatives (collected at the territory of the Akrikhin chemical pharmaceutical plant, Russia). Initially, the strain was identified as *Arthrobacter crystallopoietes* KM-4, patented [13], and deposited at the All-Russian Collection of Microorganisms, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, under the number VKM Ac-1098D [14]. Further analysis of 16S rRNA did not confirm its classification as *A. crystallopoietes*. At present, the strain is designated as *Arthrobacter* sp. KM-2MP.

Commercially available 2-MP pure reagent (Russia) was distilled immediately before its addition to the medium. Its purity was controlled by GC–MS. The witness standards of the expected intermediate compounds were 3-hydroxy-2-MP, 6-hydroxy-2-MP, 5-hydroxy-2-MP, and N-acetylpyrrole.

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Utilization of 2-MP in the growth medium was determined by measuring the optical density (OD) of the culture liquid at 262 nm [10] with a Hitachi-200-20 spectrophotometer (Japan). The culture liquid was acidified with HCl, the cells were removed by centrifugation, and 1 ml of the supernatant was added to 100 ml of 0.1 N HCl solution to adjust the concentration to the detection range of the equipment.

The measured values were compared to the calibration curve obtained from the optical densities of 2-MP standard solutions of varying concentrations in 0.1 N HCl.

Isolation of 2-MP degradation products from the culture liquid. In the process of growth, 200-ml samples were collected, acidified with HCl to pH 2.0–3.0, the cells were removed by centrifugation, and the supernatant (30 ml) was extracted three times with 10-ml portions of freshly distilled dichloromethane. The total acidic dichloromethane extract (~30 ml) was evaporated on a rotary evaporator to ~3 ml (extract 1). The remaining culture liquid (170 ml) was alkalized with a 40% NaOH solution to pH 8.0–9.00, and 30 ml of the alkaline solution was collected for the extraction procedure as described above (extract 2). To obtain the derivatized extracts, 30 ml of the culture liquid with pH 8.0–9.0 was dried on a rotary evaporator, the residue was dissolved in 2 ml of dichloromethane, and the solution was added to 1 ml of BF_3 methanol solution. The mixture was boiled for 2 min and washed with distilled water three times. The final volume was ~3 ml (extract 3). Derivatization of the culture liquid with pH 2.0–3.0 was performed in a similar manner yielding extract 4.

Silylation of the products of 2-MP degradation was performed in the culture liquid extracts by N,O-bis(trimethylsilyl)acetamide. The silylating agent (10 μl) was added to 1 ml of an extract. The process was carried out in an ultrasonic bath during 2 h at 60°C. Then, an aliquot of 100 μl was diluted with dichloromethane to 1 ml.

Identification of the individual products of 2-MP degradation. Analysis of the degradation products in the extracts was performed on a LECO Pegasus 4D (Germany) GC–MS apparatus equipped with an RTX-5MS silicone capillary column (30 m), under the electron energy of 70 eV. The range of the registered masses was from 29 to 500 Da. Chromatographic analysis of the extracts was carried out under the following temperature mode: 2 min at 50°C, 2 min at 280°C, and heating rate of 20°C/min. The injected volume was 0.1 μl.

The components of the analyzed mixtures were identified using the digital libraries of mass spectra of organic compounds of the National Institute of Science and Technology (NIST) (130 000 compounds) and the Wiley library (275 000) compounds, as well as manually, using the known directions of decomposition of the molecular ions of organic compounds [15].

Quantitative mass spectral analysis of the culture liquid was performed in samples collected during the culture growth (3, 6, 9, 12, 18, 20, and 22 h). Perdeuterated naphthalene was used as an internal standard in the amount of 10–100 μg, depending on the ratio between the analytical signals of the standard and analyzed components. Detection was performed by the total ion current (TIC) peak area and individual characteristic ions in the mass spectra using the internal standard technique. The quantity of the analyte was calculated according to the formula

$$m_x = S_x \times m_{st}/S_{st},$$

where S_x and S_{st} are the peak area values and m_x and m_{st} are the amount of the product and the internal standard, respectively [16].

Witness compound synthesis was carried out to confirm the structures of the intermediates discovered in the course of the study. N-acetylpyrrole and 3-hydroxy-2-MP were synthesized according to the methods proposed in [17] and [18], respectively.

RESULTS AND DISCUSSION

Growth of the 2-methylpyridine-degrading strain. The strain *Arthrobacter* sp. KM-2MP, degrader of 2-MP, was stored at the All-Russian Collection of Microorganisms in the lyophilized form. Horse serum with 5% *meso*-inositol was used as a protective medium. The initial cell density in the lyophilized suspension was 10^9 cell/ml [19].

Studies of the viability rate of *Arthrobacter* sp. KM-2MP after 20 years of storage indicated that the number of viable cells was 10^7 cell/ml, which was sufficient for the restoration of the bacterial population [20]. The activity of the strain in terms of 2-MP decomposition after long-term storage was quickly restored to the value of 2.0 g/l, which the strain exhibited prior to lyophilization. Sequential inoculations on a synthetic medium supplemented with the substrate increased the activity. UV spectroscopy of the culture liquid revealed that *Arthrobacter* sp. KM-2MP decomposed 2-MP at the concentration of 2.5 g/l over 24 h (Fig. 1).

In the liquid mineral medium containing 2-MP, the cells of *Arthrobacter* sp. KM-2MP grew as a homogeneous suspension generating a yellow pigment. The rate of the pigment synthesis depended upon the concentration of the introduced substrate. Pigment synthesis in the cells and its excretion into the medium started during the exponential growth phase (6 h) and continued until the stationary phase (18 h). Subsequently, the amount of the pigment decreased. The cells in the stationary phase culture were bright yellow, emitted yellow to green fluorescence under UV illumination, and precipitated rapidly, forming a dense sediment. The pale-yellow culture liquid exhibited very low fluorescence. The pigment is probably of azaquinone nature, although its structure and functional importance have not been studied yet.

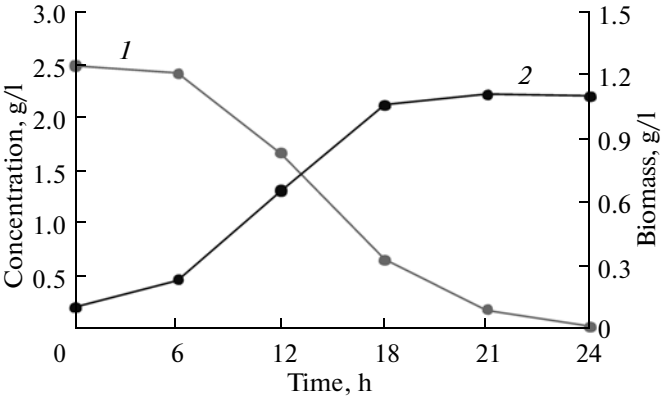

Fig. 1. Substrate consumption (*1*) and biomass accumulation (*2*) during the growth of *Arthrobacter* sp. KM-2MP (according to UV spectrometry of the culture liquid).

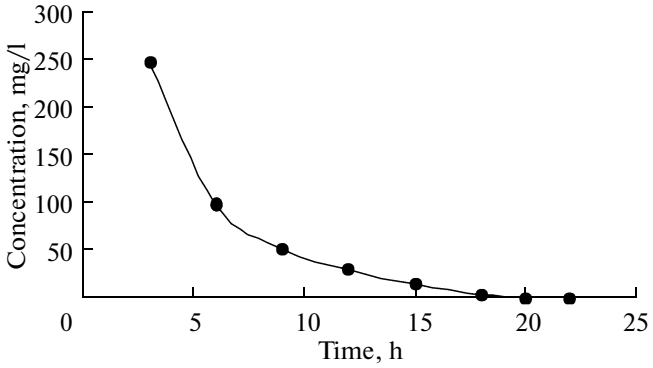

Fig. 2. Consumption of 2-MP depending on the cultivation time (GC-MS data).

Dynamics of 2-MP utilization. Utilization of 2-MP was studied during the growth of *Arthrobacter* sp. KM-2MP in a mineral medium supplemented with 2.5 g/l substrate as the single source of carbon, nitrogen, and energy. The rate of 2-MP utilization was determined by two independent methods, namely, UV spectrometry of culture liquid (using the calibration curves) and quantitative mass spectrometry analysis of dichloromethane extracts from the culture liquid. According to the UV spectra of the culture liquid, 2-MP consumption by the bacteria was almost complete within 24 h (Fig. 1). After the first 18 h, about 20% of the substrate remained in the medium, while during the following 4 h 2-MP concentration decreased down to 0.01 g/l. In parallel, biomass accumulation was observed from 0.2 g/l to the maximum value of 1.1 g/l.

The completion of 2-MP utilization by *Arthrobacter* sp. KM-2MP was confirmed by the results of the quantitative mass spectrometry analysis of the culture liquid. The dynamics of 2-MP consumption is presented on Fig. 2, where the concentrations obtained for the alkaline and acidic extracts are summed up.

According to the data of quantitative mass spectrometry analysis, 2-MP was almost entirely decomposed by the bacterial cells: after 3-h cultivation, 2-MP concentration in the extracts decreased to 0.25 g/l; after 22 h, it decreased to 2.5×10^{-4} g/l. Analysis of the 24-h culture revealed no 2-MP in the culture liquid.

Analysis and detection of the products of 2-MP detoxification. No reasonable precise data presently exist concerning the degradation pathways of xenobiotics of the pyridine family. This is partially due to the fact that one of the carbon atoms of the benzene ring is substituted by a more electronegative nitrogen atom, resulting in a considerable redistribution of the electron density in the core [21]. On the other hand, up to now no cell-free extract capable of transformation of pyridine or its derivatives was obtained. The enzymes of the pyridine ring biodegradation were neither identified nor characterized.

The study of 2-MP metabolic pathways in *Arthrobacter* sp. KM-2MP was therefore based on analysis of structures of the intermediate products of 2-MP degradation in the growing culture.

Analysis of the chromatograms of dichloromethane extracts obtained from the samples collected after 6, 12, 18, and 22 h of cultivation reflects the dynamics of changes in the composition of the products of 2-MP biodegradation (Fig. 3). A decrease in 2-MP content from the initial value to practically complete absence was observed (Fig. 3a, peak 1), which was paralleled by an increase in the content of 2-butanone (peak 2), 4-oxopentanoic acid (peak 9), and other products (peaks 3–8 and 10–15; see table).

Interpretation of the mass spectra turned out to be less straightforward. The mass spectra of only several products of 2-MP transformation were represented in the digital mass spectra databases, namely, those of hydroxy-2-MP, 4-oxopentanoic acid, and 2-butanone. The spectra of the rest of the compounds were identified according to the known directions of decomposition of organic compounds under the conditions of electron ionization (EI) [15] and are presented in the table.

Peak 7 with the retention time t_r of 6.52 min was the most intense in the chromatograms (Fig. 3). The molecular mass of the corresponding substance, 109 Da, matches the expected mass of hydroxy-2-MP as the primary oxidation product of the initial substrate, although retention times of such derivatives are typically higher. The mass spectra databases did not offer any acceptable solution for the problem.

The mass spectrum of this compound was characterized by an intense peak of the molecular ion at m/z value of 109. An ion of m/z 67 was formed upon elimination of a neutral 42 Da-particle from the M^+. The most probable neutral particle is a ketene molecule, which may be eliminated if an acetyl fragment is present in a molecule. Further decomposition of the ion of m/z 67 corresponded to pyrrole fragmentation. Therefore, the peak 7 compound was tentatively iden-

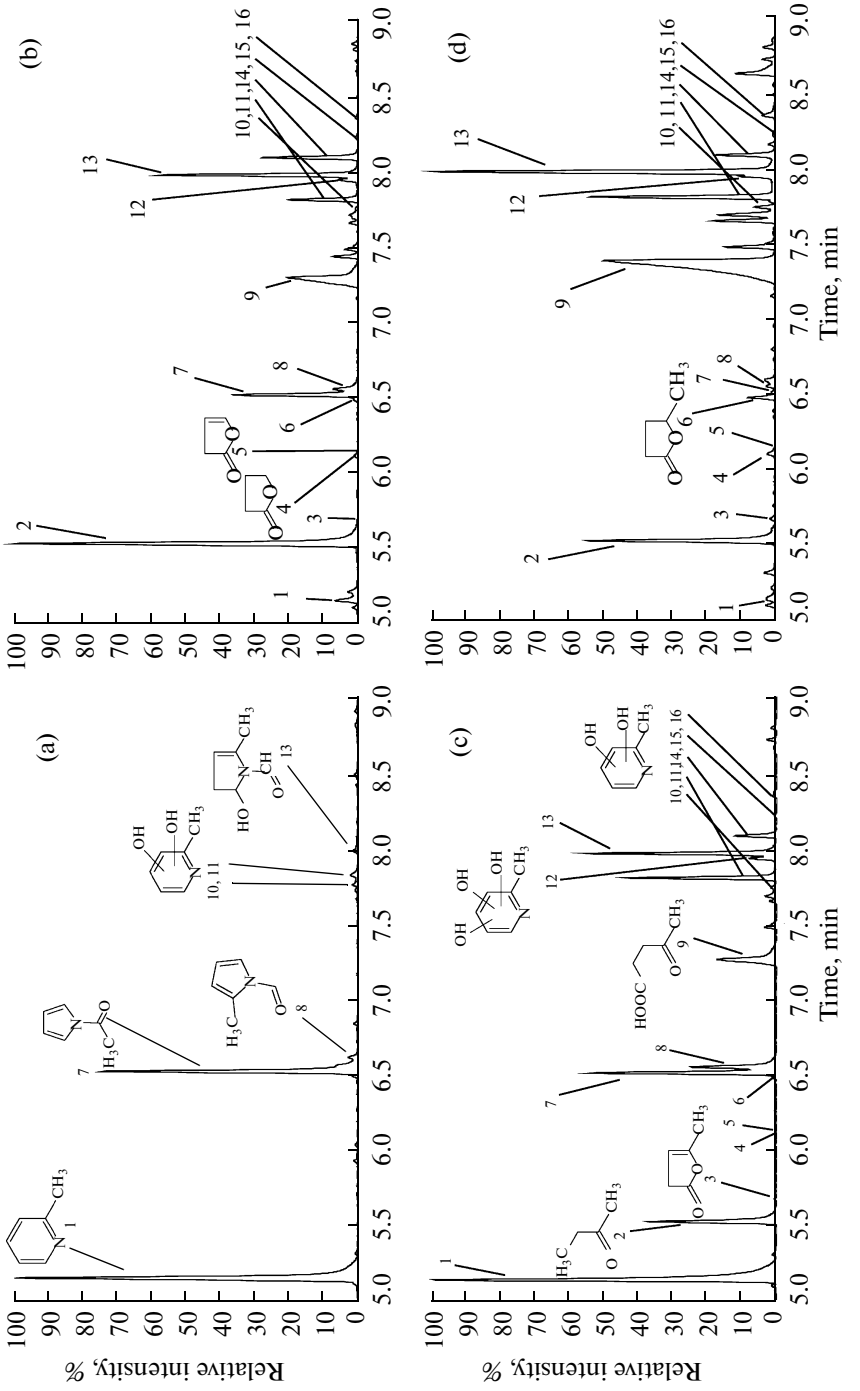

Fig. 3. Chromatograms (GC-MS) of acidic extracts of the culture liquid in the process of *Arthrobacter* s. KM-2MP growth in the presence of 2-MP: 6 (a), 12 (b), 18 (c), and 22 h (d).

Major intermediates of 2-methylpyridine degradation (% of the introduced substrate mass)

No.	Name	Retention time, min	Molecular mass	Cultivation time			
				6 h	12 h	18 h	22 h
1	2-methylpyridine	5.40	93	78.9	63.6	9.69	0.57
2	2-butanone	5.52	72	0.0	7.55	46.5	12.3
3	5-methyl-2(3H)-furanone	5.65	98	0.0	0.38	0.28	0.34
4	butyrolactone	6.10	86	0.0	0.06	0.64	0.64
5	2(3H)-furanon	6.15	84	0.0	0.15	0.43	0.0
6	5-methyl-2(3H)-dihydrofuranone	6.45	100	0.09	0.04	0.11	1.44
7	N-acetylpyrrole	6.52	109	18.2	12.1	10.6	0.01
8	N-formyl-2-methylpyrrole	6.57	109	1.86	6.44	5.32	0.13
9	4-oxopentanoic acid	7.25	116	0.0	0.8	2.11	39.0
10	Dihydroxy-2-methylpyridine	7.75	125	0.32	0.11	0.45	0.0
11	Dihydroxy-2-methylpyridine	7.81	125	0.36	2.01	2.04	10.7
12	trihydroxy-2-methylpyridine	7.93	141	0.18	1.9	1.92	1.05
13	N-formyl-2-dihdroxy-2,3-dihydro-4-methylpyrrole	7.95	127	0.02	3.05	1.15	21.2
14	Dihydroxy-2-methylpyridine	8.05	125	0.0	1.28	6.27	3.32
15	Dihydroxy-2-methylpyridine	8.30	125	0.0	0.05	0.3	1.26
16	Dihydroxy-2-methylpyridine	8.35	125	0.0	0.25	0.17	0.51

tified as N-acetylpyrrole (Fig. 4a). To confirm the formation of N-acetylpyrrole as one of the intermediates in 2-MP degradation, the former one was synthesized. The mass spectra of the synthesized compound and of the substance revealed in the culture liquid were identical (Fig. 4b).

Therefore, the presence of N-acetylpyrrole among the products of 2-MP biodegradation by the *Arthrobacter* sp. KM-2MP was confirmed. Supposedly, N-acetylpyrrole is formed via the opening of the hydroxylated pyridine ring between C2 and C3 atoms with subsequent condensation between C and N atoms. Although such bond breaking in a chemical reaction is hindered, an enzymatic reaction is rather probable.

Peak 8 of a compound with retention time of 6.57 min and molecular mass of 109 Da could be one of the isomers of monohydroxy-2-MP (Fig. 3, peak 8). Since it is not possible to determine the precise structure of hydroxy-2-MP isomers by the mass-spectrometry decomposition, the intermediate was identified by mass spectrometry analysis of the commercial reagents (6-hydroxy-2-MP and 5-hydroxy-2MP) and the synthesized 3-hydroxy-2-MP. The determined retention times, t_r, were as follows: 3-hydroxy-2-MP, 10.00; 5-hydroxy-2-MP, 10.40; and 6-hydroxy-2-MP, 10.58 min, that is, they were 3–4 min higher than the retention time of the compound responsible for peak 8 in the culture liquid.

Analysis of the compound of peak 8 allowed the structure of N-formyl-2-methylpyrrole to be suggested. The mass spectrum of this compound (Fig. 5) was absent from the computer databases or literature. Under the conditions of electron ionization, a CO molecule is eliminated from N-formyl-2-methylpyrrole and $M^{+\bullet}$ of 2-methylpyrrole ($m/z = 8$) is formed. Further decomposition is associated with progressive elimination of a hydrogen atom ($m/z = 80$) and an HCN molecule ($m/z = 53$).

Another indirect proof of N-formyl-2-methylpyrrole structure was the fact that its retention time, $t_r = 6.57$, was close to that of N-acetylpyrrole, $t_r = 6.52$.

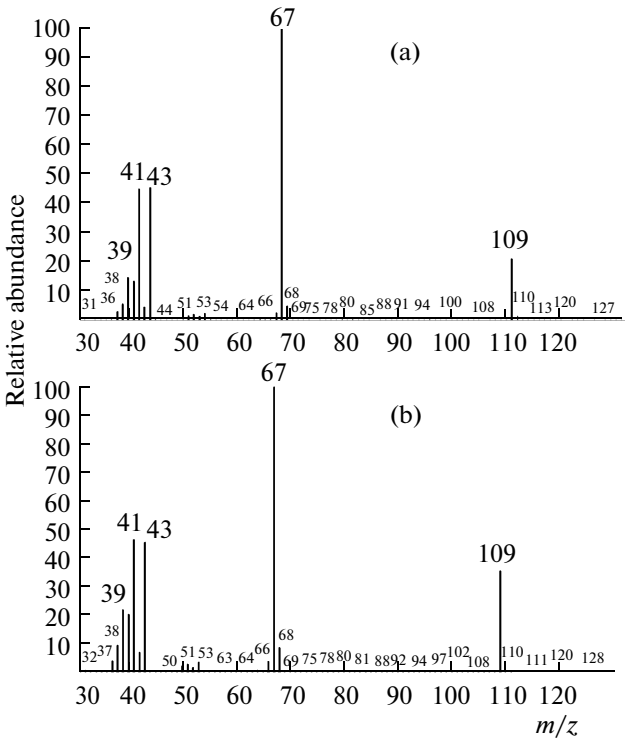

Fig. 4. Mass spectra of N-acetylpyrrole isolated from the culture liquid (a) and the synthesized compound (b).

The formation of N-formyl-2-methylpyrrole during biodegradation of 2-MP may occur according to the mechanism similar to that proposed for N-acetylpyrrole formation but with the enzymatic cleavage of the pyridine ring between C5 and C6 atoms and subsequent condensation between C and N atoms.

Upon further analysis of acidic extracts of the culture liquid (Fig. 3, peaks 10, 11, 14–16), a series of isomers of 125 Da and traces of a compound with the molecular mass of 141 Da (peak 12) were discovered. In the spectra of compounds with m/z of 125 Da, peaks of fragmented ions indicating the presence of phenolic hydroxyls were identified. This finding suggested that the compounds were isomers of dihydroxy-2-MP. The content of two of the isomers with m/z of 125 Da in the mixtures was high. They were detected in all acidic extracts (3–22 h). The content of the other three isomers was low. They were detected only in 12-, 15-, and 18-h extracts. These compounds possibly are hydroxyl-containing pyrroles, which, upon elimination of a water molecule, transform into the corresponding pyrrole structures being registered.

Analysis of the mass spectrum of compound 12 (Fig. 3) with t_r of 7.93 min (141 Da), the traces of which were detected in 12-, 18-, and 22-h extracts of the culture liquid, suggested its tentative identification as trihydroxy-2-MP (Fig. 6).

To confirm the proposed structures and identify other intermediates containing OH-groups, they were silylated in dichloromethane extracts. Trimethylsilyl derivatives of the compounds under study have characteristic peaks simplifying their identification by mass spectra. In particular, the approach made it possible to detect the compound with a molecular ion with m/z of 181 and retention time of 10.12 min. The mass spectrum of this compound was found in the database and corresponded to a trimethylsilyl derivative of hydroxy-2-methylpyridine. However, mass spectrometry decomposition does not allow classing the discovered substance unambiguously as either 3-hydroxy-2-MP or 5-hydroxy-2-MP.

Although we did not unambiguously detect 5-hydroxy-2-MP as an intermediate, the presence of 4-oxopentanoic acid and N-formyl-2-methylpyrrole confirms that this compound was formed in the course of 2-methylpyridine degradation.

As follows from the results described above, we presented mainly the compounds involved in the initial stages of 2-MP metabolism, namely, N-acetylpyrrole, 4-oxopentanoic acid, N-formyl-2-methylpyrrole, N-formyl-2-hydroxy-2,3-dihydro-4-methylpyrrole, isomers of dihydroxy-2-methylpyridine, and trihydroxy-2-methylpyridine.

The pyrrole derivatives are subsequently decomposed with the formation of pyrrole and further ring opening with elimination of an ammonia molecule and generation of 1,4-butanediol, which yields CO_2 after a number of oxidation stages. The 4-oxopentanoic acid is decarboxylated generating 2-butanone, which in turn is oxidized to 2-oxopropanoic and then to oxalic acid. Most of dihydroxy-2-MP is subjected to further hydroxylation with formation of trihydroxy-2-MP, which in turn is a substrate in the reaction of formation of a pigment of azaquinone structure as it has been previously demonstrated for 4-MP degradation by *A. crystallopoietes* KM-4 [22].

Such compounds as 1,4-butanediol, 2-butanone, 2-oxopropanoic acid, and oxalic acid were detected in all acidic extracts after 15 h of cultivation with 2-MP. This fact confirms complete degradation of 2-MP by *Arthrobacter* sp. KM-2MP.

Formation of pigments of diazadiphenoquinone nature upon oxidation of compounds of the pyridine family, have been described in the literature, for example, in the course of 2-hydroxypyridine metabolism by *A. crystallopoietes* [23]. The pigment was formed upon autooxidation of 2,3,6-trihydroxypyridine. The compound is considered to be both the intermediate in the pyridine ring degradation and the pigment precursor [24].

Proposed pathways of 2-MP catabolism in strain *Arthrobacter* sp. KM-2MP. Summing up the obtained data on the structure of the intermediates (Fig. 7), it may be concluded that the strain degrades 2-MP through the following reactions.

1. Catabolism of 2-methylpyridine by *Arthrobacter* sp. KM-2MP starts with hydroxylation of the pyridine

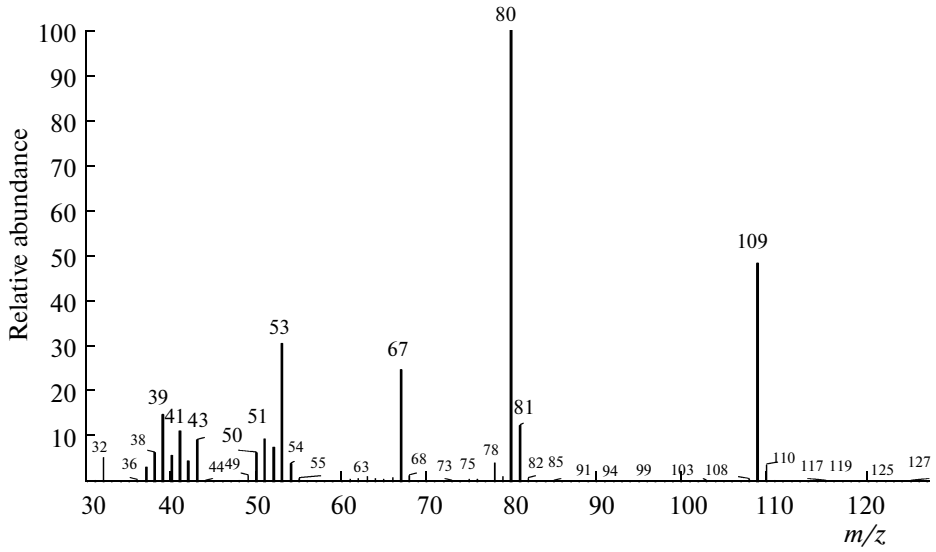

Fig. 5. EI mass spectrum of N-formyl-2-methylpyrrole.

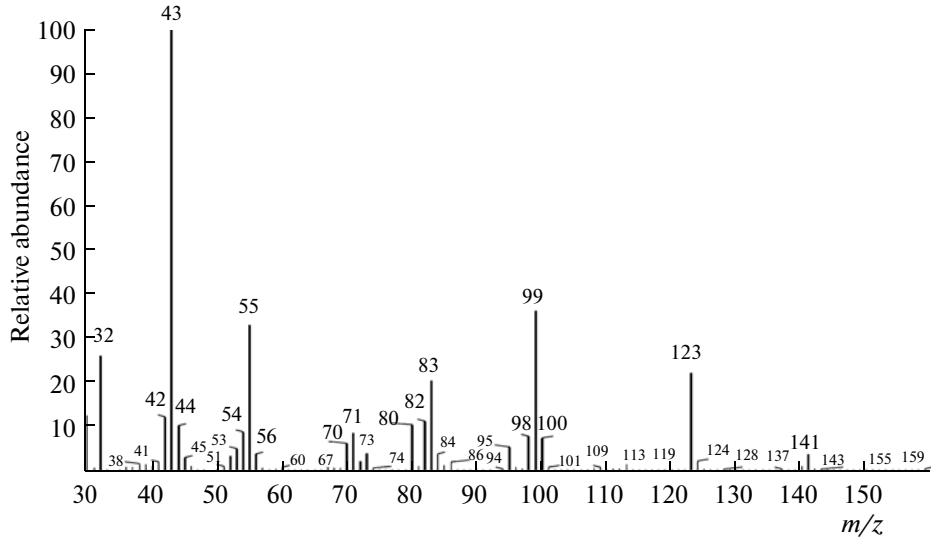

Fig. 6. EI mass spectrum of trihydroxy-2-methylpyridine.

ring at positions 3 and 5 yielding hydroxy-2-methylpyridines.

2. As a result of the 3-hydroxy-2-MP ring opening (Fig. 7, II) at the C2–C3 bond, an intermediate is formed containing a carbonyl group in the fourth position starting from the nitrogen atom, which allows a five-membered ring to close generating N-acetylpyrrole (Fig. 7, IX). Similarly, opening of the ring of 5-hydroxy-2-MP between the atoms C5 and C6 results in formation of N-formyl-2-methylpyrrole (Fig. 7, VIII). Therefore, N-carbonylpyrrole derivatives are formed and are further oxidized or reduced. This pathway has not been previously reported.

3. Hydroxy-2-MPs (Fig. 7, II and II) are oxidized to dihydroxy-2-MPs (Fig. 7, IV and V). Upon the ring opening at the stage of dihydroxy derivatives, 4-oxopentanoic acid is formed (Fig. 7, X). Further oxidation of dihydroxy-2-MP leads to the formation of trihydroxy-2-MP (Fig. 7, VI), which in turn is rapidly condensed into a pigment of azaquinone structure (Fig. 7, VII).

Thus, in the present work the strain *Arthrobacter* sp. KM-2MP was demonstrated to be capable of complete utilization of 2-MP at a concentration of 2.5 g/l and belongs to the best degrader strains described in the literature. Using the method of GC–MS, the

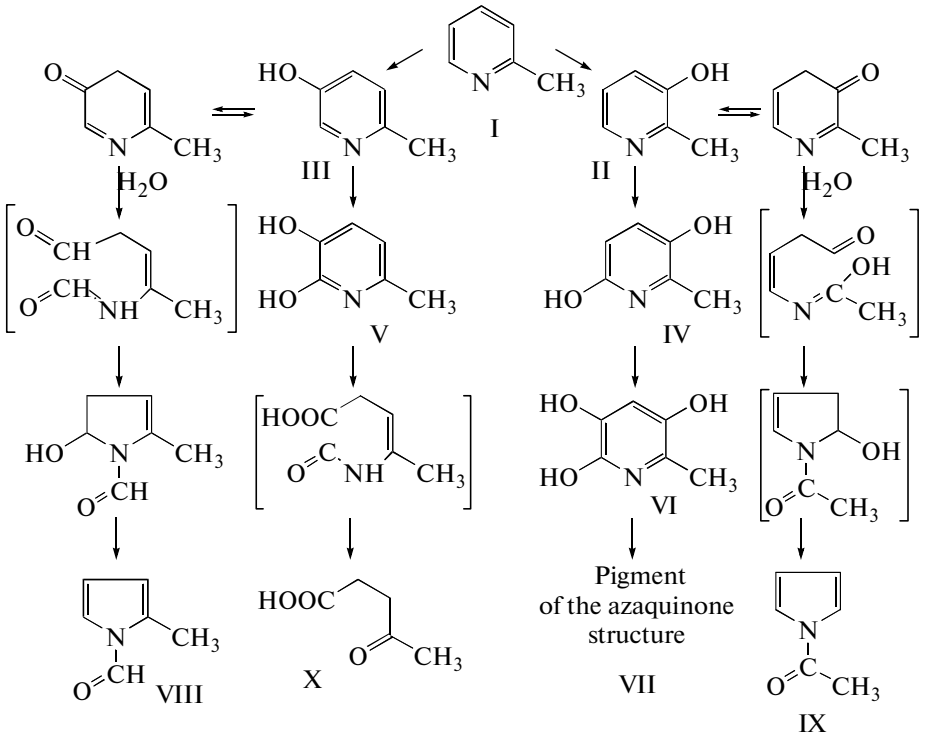

Fig. 7. 2-Methylpyridine catabolism by *Arthrobacter* sp. KM-2MP. I, 2-MP, II, 3-hydroxy-2-MP, III, 5-hydroxy-2-MP, IV, 3,6-dihydroxy-2-MP; V, 5,6-dihydroxy-2-MP; VI, 3,5,6-trihydroxy-2-MP; VII, pigment of azaquinone nature; VIII, N-formyl-2-methylpyrrole; IX, N-acetylpyrrole; and X, 4-oxopentanoic acid.

intermediates of the process of degradation were detected and identified. For the first time, catabolic pathways of 2-MP are proposed.

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