*Eco. Env. & Cons. 27 (4) : 2021; pp. (1748-1752)*
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*ISSN 0971–765X*

# The Solution of the Biosafety Problem of 2,4-dimethylpyridine by *Rhodococcus erythropolis* for the Environment

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(Received 6 May, 2021; Accepted 20 June, 2021)

**ABSTRACT**

The oxidation of 2,4-dimethylpyridine by the bacterium *Rhodococcus erythropolis* resulted in the formation of 4,6-dimethylpyridin-3-ol and pyridine-2,4-dicarboxylic acid, as well as the opening of the pyridine ring with the formation of (3*E*)-3-(formylimino)prop-1-ene-1,1,3-tricarboxylic acid and (3*E*)-3-(formylimino)-2-hydroxyprop-1-ene-1,1,3-tricarboxylic acid.

***Key words:*** *Biodegradation, 2,4-dimethylpyridine, Rhodococcus erythropolis, Bacteria*

## Introduction

The intensive development of the chemical industry has led to the continuous pollution of the biosphere with harmful emissions (Padoley *et al.,* 2008). Such substances are found in the wastewater of oil refineries and chemical plants, as well as factories for the production of synthetic rubber, plastics, and dyes (Sophonsiri and Morgenroth, 2004; Ronen *et al.,* 1997). Pyridines are widely used as solvents and starting reagents in organic and bioorganic chemistry (Kaiser *et al.,* 1996). Many works have been devoted for the degradation of pyridine (Padoley *et al.,* 2008). The microbial degradation of alkylpyridines has been less studied. Previously, we investigated the degradation of 4-methylpyridine (Khasaeva *et al.,* 2016a) and 2,6-dimethylpyridine (Khasaeva *et al.,* 2016b). This study is devoted to the microbial degradation of 2,4-dimethylpyridine.

## Materials and Methods

The bacterium *Rhodococcus erythropolis* VKM Ac-1164 was obtained from the All-Russian Microorganism Collection.

To study the degradation of 2,4-dimethylpyridine (**I**), a synthetic medium having the following composition (g/L): $Na_2HPO_4$ – 4.26; $KH_2PO_4$ – 2.65; $MgSO_4 \cdot 7H_2O$ – 0.2; $FeSO_4 \cdot 7H_2O$ – 0.01; $CaCl_2 \cdot 2H_2O$ – 0.02; $MnSO_4 \cdot H_2O$ – 0.002; $Na_2MoO_4$ – 0.001; deionised water – 1 L; pH 7.0 – 7.2; was used. Cultivation was carried out in flasks (750 ml) with 200 ml of this medium on a shaker (200 rpm/min) at 28-30°C.

As the source of carbon and nitrogen in the liquid medium, 2.0 g/l 2,4-dimethylpyridine was added. The degradation process was performed for 36 hours.

The degradation products were extracted with chloroform and after evaporation were dissolved in 0.5-1.0 ml of ethanol. They were separated on chromatographic plates of TLC Silica gel 60 $F_{254}$ (Merck, Germany). For chromatography, the following solvent systems were used:

1. chloroform - methanol (20:3);
2. chloroform - acetone - ethanol (7:2:2);

3. ethanol - ammonia - water (20:1:4);
4. ethyl acetate - petroleum ether (5:1).

Chromatograms were visualized under UV light or iodine vapor. For the preparative isolation of individual products, column chromatography with Silica gel 40 (Merck, Germany) was used in solvent system 3, and preparative thin layer chromatography was used in solvent systems 2, 3 and 4. Electron ionization (EI) mass spectrometry was performed at an electron energy of 70 eV on a Finnigan MAT-4615 instrument (Finnigan MAT, San Jose, CA, USA). $^1$H NMR spectra were recorded in a ÑDÑl$_3$ solution on a Bruker DPX 300 instrument (Bruker AG, Fällanden, Switzerland) operating at 400 MHz and 28°C.

## Results

### Detection and analysis of 4,6-dimethylpyridin-3-ol (II)

The study of the degradation pathways of 2,4-dimethylpyridine (**I**) by *R. erythropolis* VKM Ac-1164 was carried out during the growth of the organism on a mineral medium containing 2,4-dimethylpyridine as the only C and N source. As a result of the bioconversion (Figure 1), compound **II** was isolated and identified as 4,6-dimethylpyridin-3-ol.

Compound **II** accumulated in the log phase (18-20 hours) and decreased in the stationary phase (30 hours). The structure of compound **II** was established on the basis of the $^1$H NMR spectrum. In the $^1$H NMR spectrum were observed a singlet of proton H-2 with a chemical shift of 7.63 ppm, a singlet of proton H-5 with a chemical shift of 7.00 ppm and two singlets of three protons (4-$CH_3$ and 6-$CH_3$ groups) each with chemical shifts of 2.30 ppm and 2.50 ppm, respectively. The spectrum also contained a singlet of the proton of the OH-group present as a broadened signal with a chemical shift of 6.20 ppm. The structure of compound **II** corresponded to the formula in Table 1.

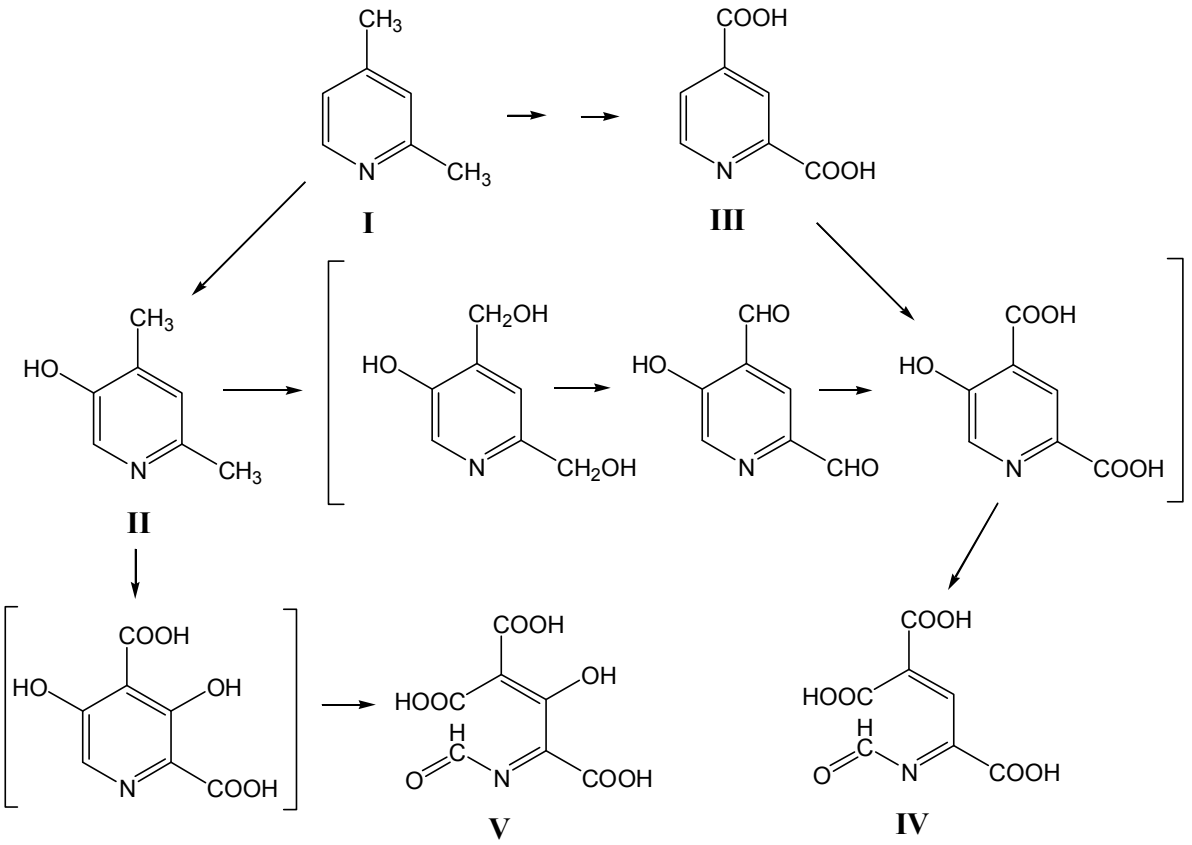



**Fig. 1.** The process of catabolism of 2,4-dimethylpyridine (**I**) by *R. erythropolis* VKM Ac-1164.
**II** - 4,6-dimethylpyridin-3-ol; **III** - pyridine-2,4-dicarboxylic acid; **IV** - (3*E*)-3-(formylimino)prop-1-ene-1,1,3-tricarboxylic acid; **V** - (3*E*)-3-(formylimino)-2-hydroxyprop-1-ene-1,1,3-tricarboxylic acid.

**Detection and analysis of pyridine-2,4-dicarboxylic acid (III)**

Chromatographic analysis of the extract of the culture liquid from the stationary growth phase showed the presence of pyridine-2,4-dicarboxylic acid (**III**), which was obtained as the dibutyl ester, and identified as dibutyl pyridine-2,4-dicarboxylate (**IIIa**, Table 2).

**Detection and analysis of compounds (IV) and (V)**

Extracts of the culture liquid also contained carboxylic acids **IV** and **V**, which were found only in the logarithmic phase of growth. Compounds **IV** and **V** were not observed in culture fluids from the stationary phase.

From the mass spectral analysis of compound **IV,** in the form of its [(2E)-3-[2-(2,4-diaminophenyl) hydrazinyl]-2-(formylimino)-3-oxopropylidene] propanedioic acid (**IVa**) (Table 3), it was identified as the (3*E*)-3-(formylimino)prop-1-ene-1,1,3-tricarboxylic acid (**IV**).

From the mass spectral analysis of compound **V,** in the form of its [(2*E*)-3-[2-(2,4-diaminophenyl) hydrazinyl]-2-(formylimino)-1-hydroxy-3-oxopropylidene]propanedioic acid (**Va**) (Table 4), it was identified as the (3*E*)-3-(formylimino)-2-hydroxyprop-1-ene-1,1,3-tricarboxylic acid (**V**).

## Discussion

Further transformations of products (**IV**) and (**V**) can be compared with biotransformations of structurally related substances, for example, when the heterocyclic ring of pyridoxamine is opened by the bacterium *Pseudomonas* sp. MA-I with the formation of á-(*N*-acetylaminomethylene) succinic acid and when the heterocyclic ring of pyridoxine is opened by *Pseudomonas* sp. IA to form a-hydroxy-methyl-a2-(*N*-acetylaminomethylene) succinic acid (Huynh and Snell, 1985).

The structures of the two isolated tricarboxylic acids suggest that the two methyl groups of the hydroxylated pyridine ring (**II**) are oxidized to carbonyl groups between the C-5 and C-6 carbon atoms. The degradation of 2,4-dimethylpyridine (**I**) is shown in Figure 1. However, this study did not allow determination of which process is first: hydroxylation of the ring or oxidation of the methyl groups.

This study has shown that *R. erythropolis* VKM Ac-1164 has high destructive activity towards 2,4-dimethylpyridine (2.0 g/l added to the incubation medium was utilized in 36 h). The primary reaction of the bacterial enzyme system is the microbiological oxidation of 2,4-dimethylpyridine with the formation of substances **II** and **III**, the opening of the

**Table 1.** Mass spectrum of 4,6-dimethylpyridin-3-ol (**II**)

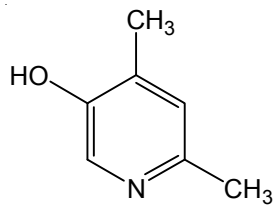
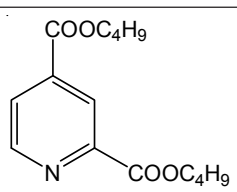
| Structure of compound **II** | *m/z* | Relative abundance, % | The formation of fragments |
|---|---|---|---|
|  | 123 | 100 | $M^+$ |
| | 122 | 7 | $M^+$ - H |
| | 107 | 7 | $M^+$ - H-CÍ$_3$ |
| | 104 | 7 | $M^+$- $H_2$-$C_2H_3$ |
| | 94 | 15 | $M^+$ - CHO |
| | 93 | 48 | $M^+$ - H-CHO |
| | 81 | 7 | $M^+$ -H-$CH_3$-CN |
| | 80 | 7 | $M^+$ - H-$CH_3$-HCN |

**Table 2.** Mass spectrum of dibutyl pyridine-2,4-dicarboxylate (**IIIa**)

| Structure of compound **IIIa** | *m/z* | Relative abundance, % | The formation of fragments |
|---|---|---|---|
|  | 279 | 5 | $M^+$ |
| | 206 | 12 | $M^+$ - $C_4H_9O$ |
| | 150 | 100 | $M^+$ - $C_4H_9O$-$C_4MH_8$ |
| | 133 | 8 | $M^+$ - $C_4H_9O$-$C_4H_9O$ |
| | 122 | 4 | $M^+$ - $H_4H_9O$-$C_4H_8$-CO |
| | 94 | 6 | $M^+$ - $C_4H_9O$-$C_4H_8$-2CO |

cyclic structure of which leads to the formation of such compounds as (3E)-3-(formylimino) prop-1-ene-1,1,3-tricarboxylic acid (**IV**) and (3E)-3-(formylimino)-2-hydroxyprop-1-ene-1,1,3-tricarboxylic acid (**V**). Alkylpyridines themselves are practically insoluble in water due to the chemical properties of the aromatic ring. After the opening of the pyridine ring, the products become soluble in water and thus would be convenient for further utilization by numerous other microorganisms in the environment.

The high destructive activity and completeness of substrate **I** consumption suggest the potential use of *R. erythropolis* VKM Ac-1164 in the purification of industrial wastewater containing alkylpyridines.

## Acknowledgements

We thank Dr. Vitaly Lashin (ACD/Labs, Moscow)

**Table 3.** Mass spectrum of [(2*E*)-3-[2-(2,4-diaminophenyl)hydrazinyl]-2-(formylimino)-3-oxopropylidene]propanedioic acid (**IVa**)

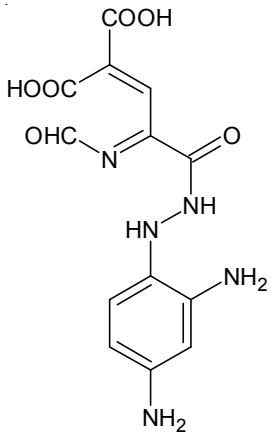
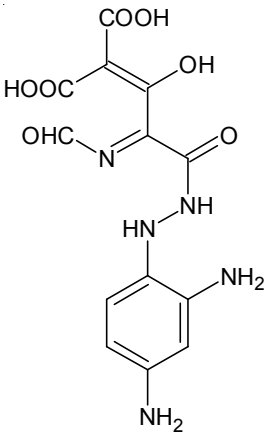
| Structure of compound **IVa** | *m/z* | Relative abundance, % | The formation of fragments |
|---|---|---|---|
|  | 412 | 100 | $M^+ + NH_3$ |
| | 395 | 54 | $M^+$ |
| | 394 | 53 | $M^+ - H$ |
| | 378 | 29 | $M^+ - OH$ |
| | 377 | 67 | $M^+ - H - OH$ |
| | 366 | 16 | $M^+ - CHO$ |
| | 351 | 80 | $M^+ - CO_2$ |
| | 349 | 33 | $M^+ - NO_2$ |
| | 347 | 20 | $M^+ - H - OH - NO$ |
| | 30 | 86 | $M^+ - C - 2OH - NO$ |
| | 317 | 30 | $M^+ - C - OH - 2NO - CO$ |
| | 289 | 32 | $M^+ - C - OH - NO - CO$ |
| | 232 | 9 | $M^+ - H - OH - NO - CH{=}C(COOH_2)_2$ |
| | 229 | 8 | $M^+ - C_6H_2(NO_2)_2$ |
| | 214 | 6 | $M^+ - NC_6H_3(NO_2)_2$ |
| | 170 | 8 | $M^+ - CONHNHC_6H_3(NO_2)_2$ |

**Table 4.** Mass spectrum of [(2*E*)-3-[2-(2,4-diaminophenyl)hydrazinyl]-2-(formylimino)-1-hydroxy-3-oxopropylidene] propanedioic acid (**Va**)

| Structure of compound **IVa** | *m/z* | Relative abundance, % | The formation of fragments |
|---|---|---|---|
|  | 428 | 100 | $M^+ + NH_3$ |
| | 411 | 5 | $M^+$ |
| | 410 | 5 | $M^+ - H$ |
| | 394 | 15 | $M^+ - OH$ |
| | 393 | 5 | $M^+ - H - OH$ |
| | 367 | 7 | $M^+ - CO_2$ |
| | 366 | 15 | $M^+ - ÑOOÍ$ |
| | 365 | 10 | $M^+ - NO_2$ |
| | 363 | 6 | $M^+ - H - OH - NO_2$ |
| | 351 | 24 | $M^+ - H - OH - NCO$ |
| | 347 | 17 | $M^+ - H - OH - NO_2$ |
| | 332 | 10 | $M^+ - H - OH - 2NO$ |
| | 304 | 20 | $M^+ - H - H_2O - 2NO\ CO$ |
| | 232 | 95 | $M^+ - H - OH - NO - C(OH){=}C(COOH)_2$ |
| | 230 | 16 | $M^+ - NC_6H_3(NO_2)_2$ |
| | 214 | 62 | $M^+ - H - OH - NO - C{=}C(COOH)_2 - H_2O$ |
| | 168 | 15 | $(C_6H_4((NO_2)_2)^{+\cdot}$ |

for consultations in organic chemistry and Dr. John B. Sutherland (U.S. Food and Drug Administration) for help with English.

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