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Bioconversion of methylpyrazines and pyridines using novel pyrazines-degrading microorganisms

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Microorganisms capable of degrading methylpyrazines were enriched from soil using 2,5-dimethylpyrazine or 2,3,5,6-tetramethylpyrazine as a sole carbon source. Based on the comparison of 16S rRNA gene sequences, 2,5-dimethylpyrazine-degrading bacteria 25DMP1 and 25DOT1 were presumed to belong to two distinct species of *Arthrobacter* genus. 2,5-Dimethylpyrazine-induced intact cells of 25DMP1 and 25DOT1 strains were capable to transform both 2,3-dimethylpyrazine and 2,3,5-trimethylpyrazine. Two isolates capable to aerobically utilize pyridine and 2,3,5,6-tetramethylpyrazine were identified as *Rhodococcus jostii* TMP1 and *Rhodococcus wratislaviensis* TMP2, respectively. 2,3,5,6-tetramethylpyrazine-induced cells of TMP1 strain transformed a wide range of methylated pyrazines and pyridines. The resting cells of *Rhodococcus jostii* TMP1 could be applicable for the synthesis of hydroxypyridines such as 2,4,6-trimethylpyridin-3-ol under the mild conditions.

Key words: 2,5-dimethylpyrazine, 2,3,5,6-tetramethylpyrazine, 2,4,6-trimethylpyridin-3-ol, *Rhodococcus, Arthrobacter*

Abbreviations: 2,5-DMP – 2,5-dimethylpyrazine, 2,6-DMP – 2,6-dimethylpyrazine, TMP – 2,3,5-trimethylpyrazine, 2,3-DMP – 2,3-dimethylpyrazine, TTMP – 2,3,5,6-tetramethylpyrazine, DCPIP – 2,6-dichlorophenol-indophenol, NBT – nitro blue tetrazolium.

INTRODUCTION

In the past decades, biocatalysis has emerged as an important tool in the industrial synthesis of bulk and fine chemicals. Since hydroxylated aromatics are used as the precursors for pharmaceuticals, selective hydroxylation of aromatic compounds is among the most challenging chemical reactions in synthetic chemistry [1–4].

Recently, it has been proposed that substituted 3-pyridinol derivatives would possess reactivities toward the chain-carrying peroxyl radicals, but be much more resistant to air oxidation than currently used equivalently substituted phenols [5]. Moreover, it has been shown that 2,4,6-trimethylpyridin-3-ol exhibits not only antioxidative but also geroprotective and anti-ischemic activity [6].

The chemo- and regioselective hydroxylation of the pyridine ring has few analogues in non-enzymatic chemistry [7]. For example, the synthesis of 2,4,6-trimethylpyridin-3-ol requires a multiple step approach including expensive reagents and / or the reactions under harsh conditions. The classical procedure involves the nitration of 2,4,6-trimethylpyridine followed by the reduction (H_2, Pd/C, 50 bar) [8]. It has been shown that alkyl(aryl)-3-hydroxypyridines can be formed from 2-acylfurans in the presence of ammonia under pressure and at high temperature [9], but the problem of preparation of substituted furyl alkyl ketones arises. Recently,

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a procedure, which utilized a low-temperature bromide/lithium exchange, followed by the nitrobenzene quench [10, 11], has been used as a general way to prepare substituted 3-pyridinols from 3-bromo precursors [5].

The biocatalysis represents a potential new and mild synthetic route to substituted pyridinols. In the past decades, microbial bioconversion and degradation of hydroxypyridines have been investigated extensively [12–15]. Meanwhile, the microbial catabolism of pyrazine compounds has been studied to a lesser extent [16, 17]. Possible degradation pathways of 2,3-diethyl-5-methylpyrazine in *Mycobacterium* sp. strain DM-11 and 2,5-dimethylpyrazine (2,5-DMP) in *Rhodococcus erythropolis* strain DP-45 have been described [18, 19]. In addition to 2,5-DMP, strain DP-45 also grew on 2-methylpyrazine, 2,6-dimethylpyrazine (2,6-DMP), 2,3,5-trimethylpyrazine (TMP), 2-ethylpyrazine and 2-ethyl-5(6)-methylpyrazine. The co-metabolic degradation of other pyrazines, such as unsubstituted pyrazine, 2,3-diethylpyrazine, 2,3-dimethylpyrazine (2,3-DMP) and 2,3-diethyl-5-methylpyrazine, has also been observed [19]. Under aerobic conditions alkylated pyrazines are metabolized via the oxidative degradation leading to the hydroxylation of the ring at a free ring position [16, 17], therefore various catabolic oxidoreductases might be the enzymes applicable for regioselective hydroxylation of the substituted *N*-heterocycles.

The present work describes the isolation and characterization of novel methylpyrazines-degrading bacteria, which are capable to convert a wide range of methylpyridines and methylpyrazines. For the first time, biocatalytical synthesis of 2,4,6-trimethylpyridin-3-ol is presented.

EXPERIMENTAL

Chemicals

Chemicals were purchased from Sigma-Aldrich and Fluka and were of the highest purity available. Nutrient agar and yeast extract were purchased from Oxoid (Hampshire, UK).

Isolation of pyrazines utilizing microorganisms

Various soils and the enrichment culture technique were used to isolate 2,5-DMP- and 2,3,5,6-tetramethylpyrazine (TTMP)-degrading microorganisms. Samples of the soil (5 g) were suspended in 20 ml of KT medium containing 2,5-DMP or TTMP (final concentration 1 g/l) as a sole carbon source and cultivated for 1–3 weeks. KT medium consisted of 5.0 g NaCl, 1.0 g $NH_4H_2PO_4$, 1.0 g K_2HPO_4, 0.4 g $MgSO_4 \cdot 7H_2O$ in 1 l of distilled water (pH 7.2 with KOH). After cultivation the aliquots of the culture were diluted and spread on the agar plates containing KT medium supplemented with an appropriate substrate (1 g/l) and cultivated aerobically at 30 °C for 2–7 days. The largest colonies were selected and purified by repeated streaking first on the nutrient agar, and then on KT medium supplemented with an appropriate substrate (1 g/l). The organisms that demonstrated better growth on KT medium supplemented with a substrate compared to unsupplemented KT medium were selected as capable to utilize the substrate as a carbon source.

Preparation of resting cells and cell-free extracts

The isolates 25DOT1 and 25DMP1 were cultivated aerobically in KT medium supplemented with 2,5-DMP (0.5 g/l) at 30 °C for 2 and 3 days, respectively. The isolates TMP1 and TMP2 were cultivated aerobically in KT medium supplemented with TTMP (0.5 g/l) at 30 °C for 2 days. The biomass of all strains was aseptically collected (3,000 × g, 15 min) and washed twice with 0.9% NaCl. Succinic acid (0.5 g/l) was used as a control substrate for the preparation of resting cells.

For enzyme analysis bacteria were grown in KT medium supplemented with TTMP (0.5 g/l) or glucose (1 g/l). Biomass was collected by centrifugation, resuspended in 50 mM potassium phosphate buffer (pH 7.2) and disrupted by sonication. Cell-free extracts were obtained by collecting the supernatant after centrifugation at 10,000 × g for 30 min.

Characterisation of isolates

DNA was extracted according to Woo et al. [20]. 16S rRNA encoding genes were amplified using universal primers w001 and w002 [21]. The PCR products were purified with a DNA purification kit and cloned into the pTZ57R/T plasmid (Fermentas, Lithuania). The phylogenetic trees were created using MEGA4 [22]. The nucleotide sequences determined in this study were deposited in the EMBL-Bank under the accession numbers HE578012–HE578015.

Bioconversion of 2,4,6-trimethylpyridine

Rhodococcus jostii TMP1 was cultivated in KT medium supplemented with TTMP (0.5 g/l) and yeast extract (0.2 g/l). For the bioconversion reaction *Rhodococcus jostii* TMP1 was grown for 2 days in 1 l flasks containing 200 ml of medium. Then cells were collected by centrifugation, washed twice and resuspended in 10 mM potassium phosphate buffer (pH 7.2) at four-fold higher density. Bioconversion reactions were carried out in 200 ml volume at 30 °C with shaking at 180 rpm. 2,4,6-trimethylpyridine and glucose were added to the reaction mixture in portions of 20 mg and 125 mg, respectively, following the conversion by the changes in UV absorption spectrum in 200–320 nm range. The reaction was performed for 96 hours. Accumulation of the bioconversion product was monitored by thin layer chromatography in chlorophorm : methanol 9 : 1 and using substrate compound as a reference. To isolate the bioconversion product bacteria were removed from the reaction mixtures by centrifugation at 4,000 × g for 20 min and supernatant was evaporated under reduced pressure. The product was isolated by sequentially dissolving it in acetonitrile and chloroform. Total yield of 79.5% was achieved. Product structure was determined using ^{1}H NMR, ^{13}C NMR and MS analyses. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity INOVA 300 spectrometer (300 and 75 MHz, respectively). Spectra were calibrated with respect to the solvent signal ($CDCl_3$: ^{1}H δ = 7.26; ^{13}C δ = 77.2). MS was

recorded on a LC2020 mass spectrometer (Shimadzu) after the separation on the YMC-Pack Pro C18/S-0.3 μm column (150 × 3 mm) using a water : acetonitrile gradient.

RESULTS AND DISCUSSION

Isolation and identification of methylpyrazines-degrading bacteria

By the means of enrichment culture using pyrazine compounds, two isolates (25DMP1 and 25DOT1) degrading 2,5-DMP and two isolates (TMP1 and TMP2) degrading TTMP were screened. The ability of the isolated strains to grow on *N*-heterocyclic compounds as a sole source of carbon was tested using the following compounds: pyrazine, 2,5-, 2,3- and 2,6-DMP, TMP, TTMP, 2,3-diethyl-5-methylpyrazine, pyrazine-2-carboxylic acid, pyrazine-2,3-dicarboxylic acid, 5,6-dimethyl-2,3-pyrazinecarbonitrile, pyridine, 2,3-, 2,5-, 2,6-, 3,4- and 3,5-pyridinedicarboxylic acid, 2-, 3- and 4-carboxypyridine, 2- and 3-hidroxypyridine, 2-, 3-, and 4-methylpyridine, 2,3-, 2,4- 2,5-, 2,6-, 3,4- and 3,5-dimethylpyridine and 2,3,5-trimethylpyridine. None of the strains used the mentioned compounds, except for enrichment substrates and pyridine, which supported the growth of TMP1 and TMP2 strains. All strains utilized succinate, therefore it was further used as a control substrate in resting cells reactions.

Nucleotide sequences of 16S rDNA of all isolated strains were determined by sequencing cloned DNA fragments, which were obtained by PCR amplification. TMP1 and TMP2 strains showed the highest 16S rDNA sequence similarity to *Rhodococcus* spp.: TMP1 is related to *Rhodococcus jostii* and TMP2 belongs to *Rhodococcus wratislaviensis* (Fig. 1a). 25DMP1 and 25DOT1 strains are closely related to the members of *Arthrobacter* genus: 25DOT1 is most similar to *Arthrobacter nitroguajacolicus*, but 25DMP1 is probably a representative of a new species (Fig. 1b).

Bioconversion of alkylpyridines and alkylpyrazines using resting cells

The changes in the UV-VIS spectrum during the typical conversion of methylpyrazines by resting cells are illustrated in

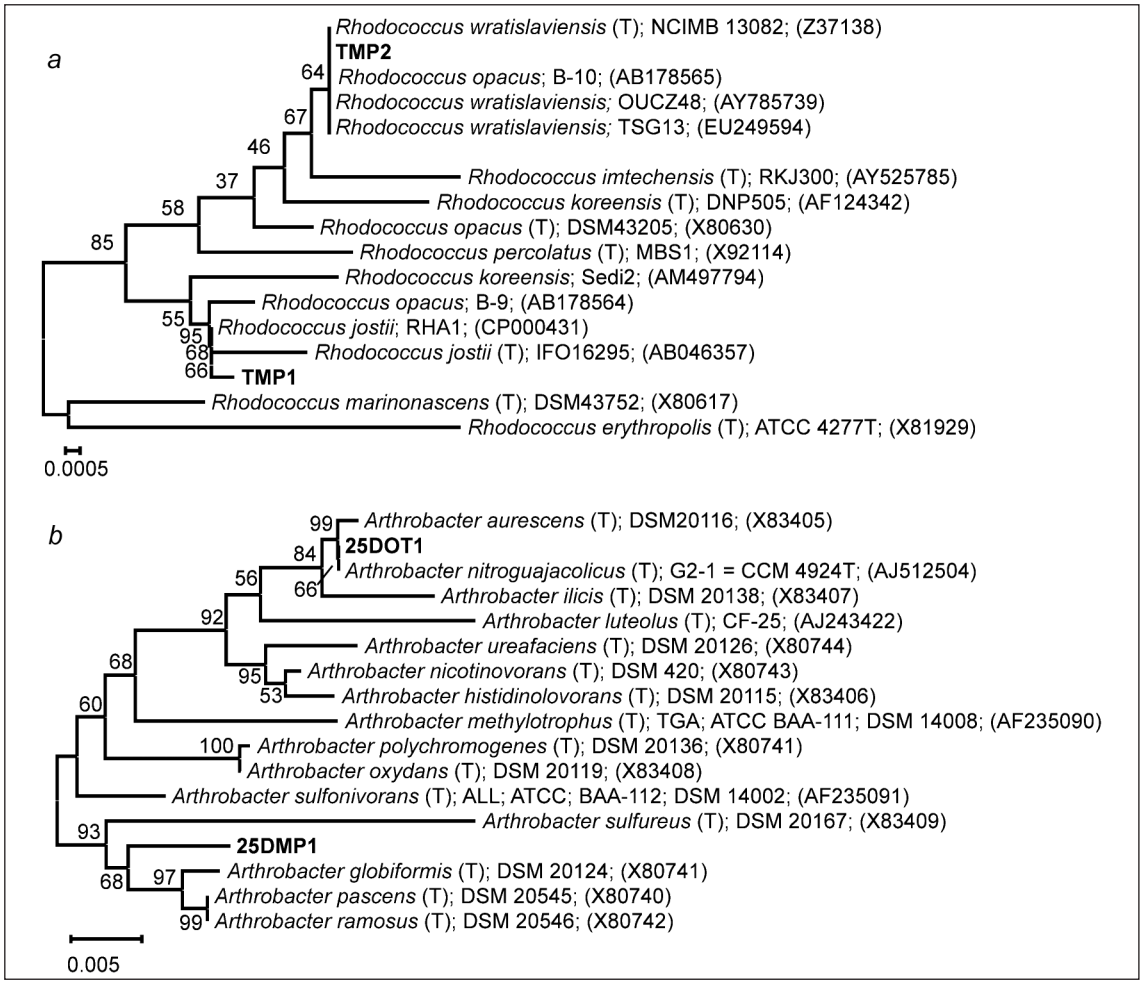

Fig. 1. Phylogenetic tree illustrating the relationship among partial 16S rDNA sequences of TTMP-degrading (*a*) and 2,5-DMP-degrading (*b*) bacteria. A phylogenetic tree was created as described in Experimental. The percentage of replicate trees, in which the associated taxa are clustered together in the bootstrap test (1 000 replicates), is shown next to the branches. Bars represent the number of base substitutions per site. T – type strain. Accession numbers are given in parentheses

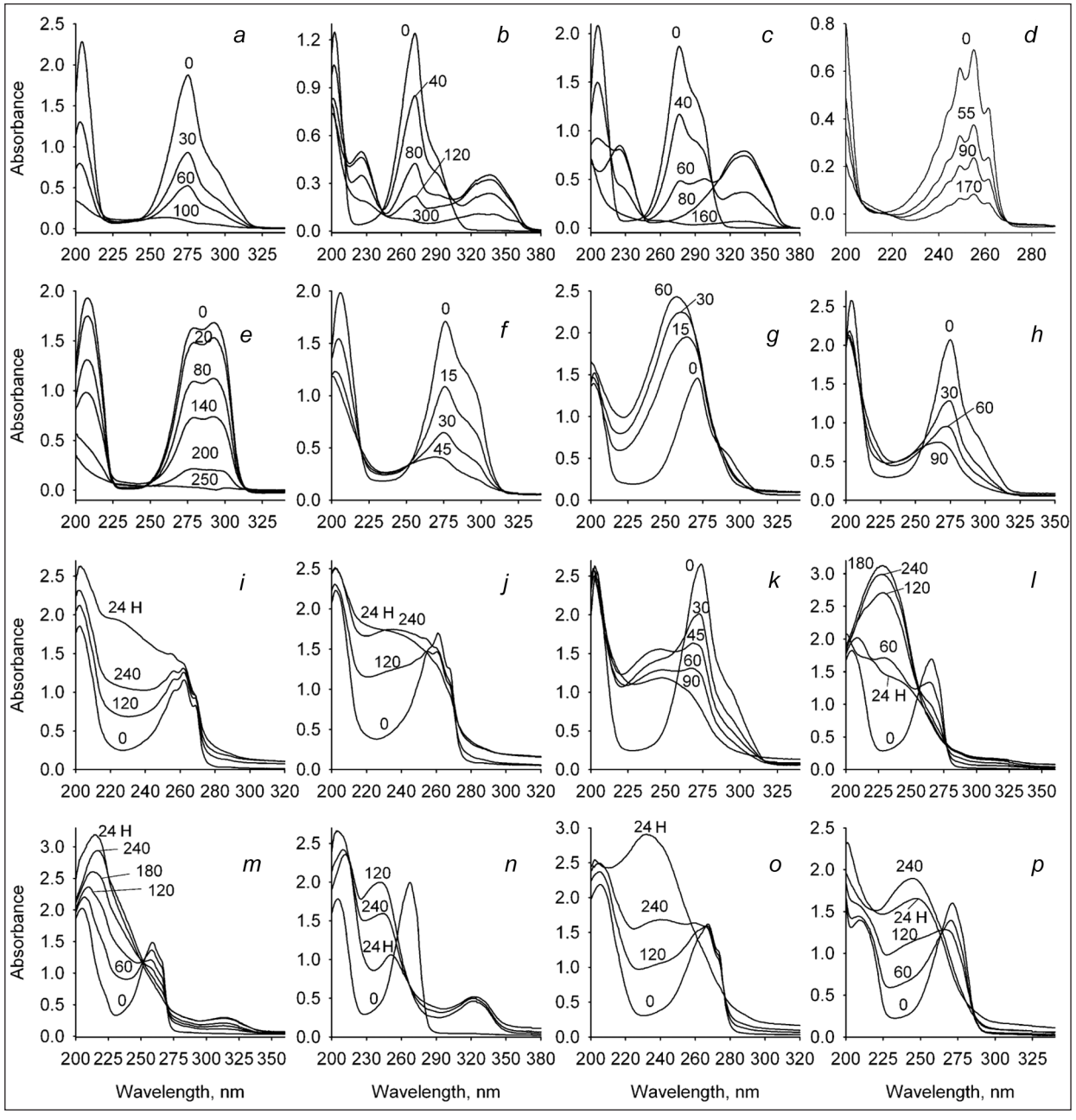

Fig. 2. Spectral changes during aerobic conversion of pyrazine and pyridine compounds by resting cells. *Arthrobacter nitroguajacolicus* 25DOT1 (*a*–*c*) and *Rhodococcus jostii* TMP1 (*d*–*p*) in 50 mM potassium phosphate buffer (pH 7.2) at 20 °C. Cells of *Arthrobacter nitroguajacolicus* 25DOT1 were induced with 2,5-DMP and cells of *Rhodococcus jostii* TMP1 were cultivated in the presence of pyridine (*d*) or TTMP (*e*–*p*). The reaction mixture was scanned after centrifugation (16,000 × g, 1 min). Incubation time is shown in min. 24 H – incubation for 24 hours. *a* – 2,5-DMP, *b* – 2,3-DMP, *c* – TMP, *d* – pyridine, *e* – TTMP, *f* – TMP, *g* – 2,3-DMP, *h* – 2,5-DMP, *i* – 3-methylpyridine, *j* – 3-ethylpyridine, *k* – 2,6-DMP, *l* – 2,3-dimethylpyridine, *m* – 2,4-dimethylpyridine, *n* – 2,6-dimethylpyridine, *o* – 3,5-dimethylpyridine, *p* – 2,3,5-trimethylpyridine

Fig. 2. *Arthrobacter* sp. 25DMP1 and *Arthrobacter nitroguajacolicus* 25DOT1 consumed 2,5-DMP only when it was previously induced by this substrate (Fig. 2a). Uninduced cells grown in the presence of succinic acid did not catalyze any conversion. The same results were observed for *Rhodococcus jostii* TMP1 and *Rhodococcus wratislaviensis* TMP2. Moreover, the cells pre-cultivated in the presence of pyridine did not consume TTMP and *vice versa*.

Generally, in similar biotransformations UV absorption maxima shift to the longer wavelength area, indicating the introduction of a hydroxyl group [23]. However, the formation of hydroxylated compounds was not observed during the conversion of TTMP (Fig. 2e), probably due to the fast degradation of intermediates or catabolic pathways not involving the hydroxylation. *Arthrobacter* sp. 25DMP1 and *Arthrobacter nitroguajacolicus* 25DOT1 cells induced in the presence of

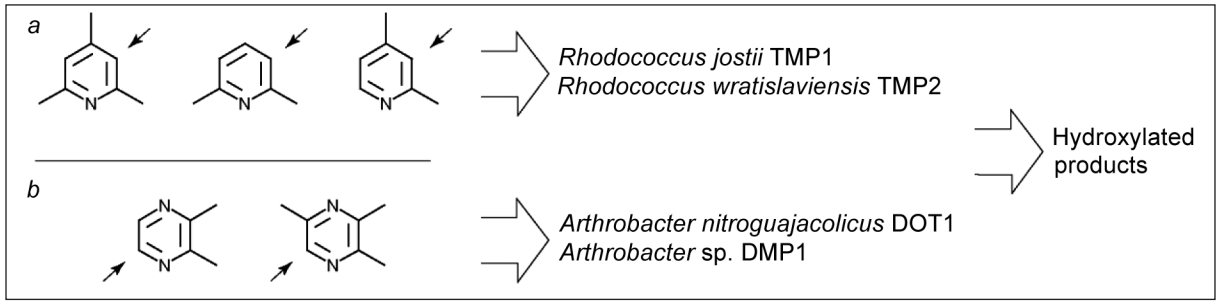

Fig. 3. Spectral changes during aerobic conversion of 2,4,6-trimethylpyridine by resting cells of *Rhodococcus jostii* TMP1. Incubation time is shown in min. 20 h and 44 h – 20 and 44 hours incubation, respectively

2,5-DMP converted both 2,3-DMP and TMP (Fig. 2b, c). New maxima were observed at 225 nm and 340 nm. The latter indicated the formation of hydroxylated intermediates. The initial steps of degradation of 2,5-DMP by *Arthrobacter* sp. 25DMP1 and *Arthrobacter nitroguajacolicus* 25DOT1 may be analogous to the pathway found in *Rhodococcus erythropolis* strain DP-45 [19].

Rhodococcus jostii TMP1 cells induced in the presence of TTMP also consumed or converted 2,3-DMP, 2,5-DMP and 2,6-DMP, and TMP (Fig. 2f–h, k). Compared with *Arthrobacter* sp. 25DMP1 and *Arthrobacter nitroguajacolicus* 25DOT1 the formation of hydroxylated intermediates was not observed, but the new maximum in the shorter wavelength area occurred in the case of 2,3-DMP and 2,6-DMP (Fig. 2g, k). That may indicate the formation of reduced intermediates or aromatic ring opening without a hydroxylation step. Moreover, for the first time the similar conversion of various methyl- and ethylpyridines by the TMP1 cells induced in the presence of TTMP was observed (Fig. 2i, j, l–p). The formation of hydroxylated intermediates might be expected in the case of 2,4- and 2,6-dimethylpyridines (Fig. 2m, n). All compounds that demonstrated hydroxylated intermediates in bioconversion experiments are summarized in Fig. 3.

Bioconversion of 2,4,6-trimethylpyridine

A capability of *Rhodococcus jostii* TMP1 to hydroxylate methylpyridines was further analysed using 2,4,6-trimethylpyridine as a substrate. The time-course analysis of bioconversion of this compound showed UV absorption maxima shift to the longer wavelength area. A new maximum at 317 nm was observed indicating the formation of a hydroxylated intermediate (Fig. 4). To elucidate the structure of the formed compound, the product of 2,4,6-trimethylpyridine bioconversion was purified as described in the part of experimental methods. In total, 200 mg of 2,4,6-trimethylpyridine was converted using the resting cells of *Rhodococcus jostii* TMP1. After purification 159 mg of product were obtained, resulting in bioconversion yield of 79.5%. The ^{1}H NMR ($CDCl_3$, 300 MHz) analysis of the obtained compound showed five peaks with δ 6.90 (br. s, 1H, OH), 6.78 (s, 1H, C*H*), 2.36 (s, 3H, CH_3), 2.32 (s, 3H, CH_3), 2.21 (s, 3H, CH_3); and eight peaks in ^{13}C NMR ($CDCl_3$, 75 MHz) spectrum: δ 148.6, 147.4, 144.7, 135.8, 123.9, 22.5, 18.6, 16.3. MS analysis confirmed the molecular formula of the compound as $C_8H_{11}NO$ (calculated $[M+H]^+$ mass was 138.08, found mass was 138.15). The analysis confirmed that *Rhodococcus jostii* TMP1 metabolised 2,4,6-trimethylpyridine to 2,4,6-trimethylpyridin-3-ol (Fig. 5).

It was earlier shown that 2-, and 4-substituted pyridines were metabolized by cells of *Pseudomonas putida* UV4, which contains a toluene dioxygenase enzyme [24]. The regioselectivity of the biotransformation in each case was determined by the position of the substituent. 4-Alkylpyridines were

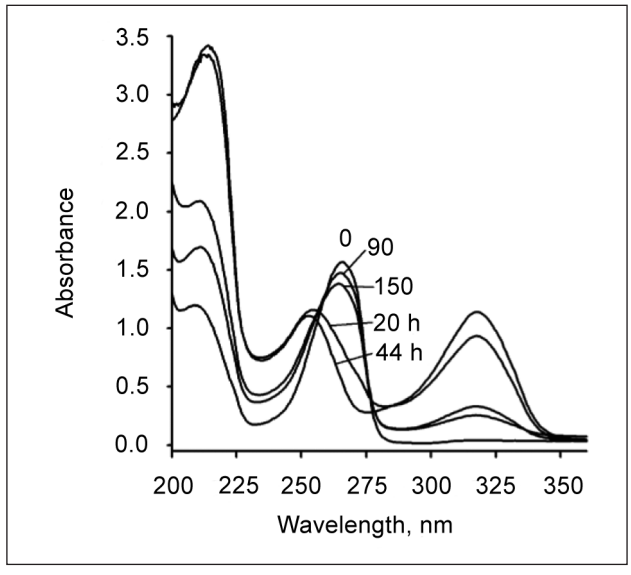

Fig. 4. Hydroxylation of *N*-heterocycles by TTMP-degrading (*a*) or 2,5-DMP-degrading bacteria (*b*). Arrows indicate potential hydroxylation sites

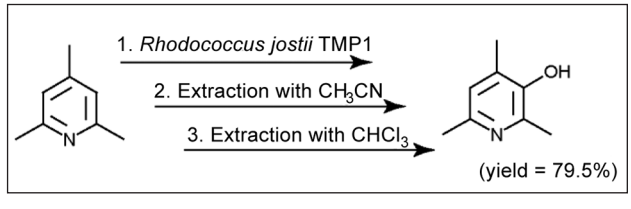

Fig. 5. 2,4,6-trimethylpyridin-3-ol synthesis from 2,4,6-trimethylpyridine using resting cells of *Rhodococcus jostii* TMP1

hydroxylated exclusively on the ring to give the corresponding 4-substituted 3-hydroxypyridines, while 2-alkylpyridines gave both ring and side-chain hydroxylation products [24]. However, the chemical yields of these biotransformations were generally poor (7–15%).

The characterization of bioconversion products is not sufficient to determine the mechanism of substrate ring hydroxylation. While enzyme-catalyzed aromatic ring hydroxylation is well-known for carbocyclic arenes, the data about the direct hydroxylation of pyridine ring is limited [15, 25–27]. Electron-rich rings, such as thiophenes and furans, are good substrates for dioxygenases; however, electron-deficient rings, especially pyridines, are either poor substrates or completely unreactive. It was proposed that initial degradation of pyridine [12] or pyrazine-2-carboxylate [28] could proceed via reduction stage. The pyridine ring opening was suggested to take place between N-C1 or C2-C3 atoms after the reduction step [12]. The ring opening or formation of reduced intermediates might be expected in bacteria studied in this work according to the changes in UV-VIS spectra during the bioconversion (Fig. 2). In contrast, the degradation of 2,5-dimethylpyrazine by *Rhodococcus erythropolis* DP-45 proceeded to the formation of 2-hydroxy-3,6-dimethylpyrazine catalyzed by a flavin-dependent monooxygenase or a cytochrome P450-dependent monooxygenase [19]. Moreover, 5,6-diethyl-2-hydroxy-3-methylpyrazine was detected during the oxidation of 2,3-diethyl-5-methylpyrazine by *Mycobacterium* sp. DM-11 [18]. The formation of hydroxylated products was also observed throughout this study; hence mono- or dioxygenases might be involved in the catabolism of methylpyridines and methylpyrazines by *Arthrobacter* sp. 25DMP1, *Arthrobacter* sp. 25DOT1, *Rhodococcus jostii* TMP1 and *Rhodococcus wratislaviensis* TMP2. Repeated attempts to register oxidation or reduction of TTMP in the cell-free extracts from both TMP1 and TMP2 strains in the presence of NADP(H), NAD(H), methylene blue, DCPIP, NBT, FAD, FMN or their combination were unsuccessful. More detailed studies are required to elucidate the enzymes involved in TTMP catabolism.

CONCLUSIONS

Arthrobacter spp. 25DMP1 and 25DOT1 as well as *Rhodococcus jostii* TMP1 and *Rhodococcus wratislaviensis* TMP2 are capable to utilize various methylpyrazines and methylpyridines. These bacteria show a wide substrate specificity and biocatalytic activity including the regioselective hydroxylation of pyridine compounds (Fig. 4). Since chemo- and regioselective hydroxylation of the 3-position of the pyridine ring has few analogues in non-enzymatic chemistry, the resting cells of pyrazines-degrading bacteria are very promising for chemical synthesis of hydroxypyridines, in particular 2,4,6-trimethylpyridin-3-ol, under mild conditions and at a high bioconversion yield.

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NAUJAI IŠSKIRTŲ, METILPIRAZINUS SKAIDANČIŲ BAKTERIJŲ TAIKYMAS METILPIRAZINŲ IR PIRIDINŲ BIOTRANSFORMACIJAI

S a n t r a u k a

Hidroksilinti *N*-heterocikliniai junginiai yra potencialūs antioksidantai ir patrauklūs pirmtakai vaistų sintezėje, tačiau hidroksi grupės įvedimas į *N*-heterociklinį žiedą cheminiais metodais yra komplikuotas. Vienas iš šios problemos sprendimo būdų – *N*-heterociklinio žiedo hidroksilinimas biokataliziniais metodais. *N*-heterociklinius junginius skaidančios bakterijos dažnai pradiniame skaidymo etape regiospecifiškai hidroksilina žiedą, todėl tokias bakterijas ar jų gaminamus katabolinius fermentus galima pritaikyti biokatalizinei hidroksilintų *N*-heterociklų sintezei.

Darbe buvo išskirtos keturios naujos bakterijos, skaidančios metilintus pirazino darinius: 2,5-dimetilpiraziną arba 2,3,5,6-tetrametilpiraziną. Parodyta, kad 2,3,5,6-tetrametilpiraziną skaidančios *Rhodococcus jostii* TMP1 bakterijos gali būti panaudotos 2,4,6-trimetilpiridin-3-olio biosintezei iš 2,4,6-trimetilpiridino. Taip pat buvo nustatyta, kad pirazinus skaidančios bakterijos gali modifikuoti įvairius alkilpiridinus ir alkilpirazinus bei yra potencialiai panaudotinos alkilintų *N*-heterociklų hidroksilinimui.