VILNIUS UNIVERSITY

Laimonas Karvelis

INVESTIGATION OF THE DEGRADATION OF CARBOXYPYRIDINES IN BACTERIA

Summary of doctoral dissertation
Physical sciences, biochemistry (04 P)

Vilnius 2012

The research was carried out at the Department of Molecular Microbiology and Biotechnology, Institute of Biochemistry, Vilnius University, Vilnius, Lithuania, during 2006–2011.

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The dissertation will be defended publicly at the meeting of the Council of Biochemistry at 11 a.m. on September 28th, 2012, at the Institute of Biochemistry. Vilnius University, room No 522.
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The abstract of the dissertation has been sent on August 27, 2012.
The dissertation is available at the library of the Institute of Biochemistry ant the library of Vilnius University.

VILNIAUS UNIVERSITETAS

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KARBOKSIPIRIDINŲ DEGRADACIJOS BAKTERIJOSE TYRIMAS

Daktaro disertacijos santrauka
Fiziniai mokslai, biochemija (04 P)

Vilnius 2012

Disertacija rengta 2006–2011 metais Vilniaus universiteto Biochemijos institute.

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Disertacija bus ginama viešame Biochemijos mokslo krypties tarybos posėdyje 2012 m. rugsėjo 28 d. 11 val. Vilniaus universiteto Biochemijos institute, 522 kambarys.
Adresas: Mokslininkų g. 12, LT-08662 Vilnius, Lietuva

Disertacijos santrauka išsiuntinėta 2012 m. rugpjūčio 27 d.
Disertaciją galima peržiūrėti Biochemijos instituto ir Vilniaus universiteto bibliotekose.

Introduction

All renewable sources of carbon could be divided into three groups. About 75% of total mass is composed by hydrocarbons, 20% by lignin, and only 5% by proteins, nucleic acids, lipids, terpenoids, and alkaloids. Approximately 2/3rds of all known organic compounds are heterocyclic. *N*-heterocyclic compounds are widely distributed in the nature, and are involved in many important processes like transport of protons and electrons. Such heterocyclic compounds are parts of nucleic acids or high energy materials. The *N*-heterocyclic structure is found in the alkaloids, porphyrins, toxins and in some antibiotics.

In addition to conventional heterocyclic compounds, new *N*-heterocyclic compounds are formed and released into the environment by incomplete combustion of fossil fuels, in spills or effluents of industrial activities, by pesticide use, and so on. Due to high stability, *N*-heterocyclic compounds are among the most dangerous pollutants that have long-term negative consequences on the environment and disturb the ecological balance. Many of these *N*-heterocyclic compounds are never found in natural carbon and nitrogen cycles and, therefore, are called xenobiotics – materials that do not participate in the natural metabolism.

The soil bacteria play a key role in the biodegradation of xenobiotic compounds using such substances as a sole carbon, nitrogen, and energy source. The biodegradation of xenobiotics, despite their prevalence in nature and abundancy in industrial applications, is not well understood. Biological utilization of the pyridine compounds is not an exception. The identification of novel catabolic pathways, enzymes, and corresponding catabolic genes would improve our understanding of microbial biochemistry and genetics. Moreover, novel bacterial strains and enzymes involved in the biodegradation would provide new possibilities for the biosynthesis of valuable chemical compounds.

In a view of limited knowledge about the degradation of pyridine carboxylic acids via formation of non-traditional hydroxyl-intermediates during this process, **the aim** of the dissertation work was to investigate these biochemical pathways in bacteria.

The main tasks of this work were:

- to identify the novel bacterial strains capable of degradation of the pyridine monocarboxylic acids;
- to identify the enzymes involved in this process;
- to identify the genes encoding such enzymes.

Scientific novelty

Bacterial strain 5HP, which is the first bacterium described to be capable to degrade the 5-hydroxypicolinic acid, has been isolated. The genetic analysis demonstrated that 5HP strain belongs to the *Pusillimonas* genus. This is a first member of *Pusillimonas* genus, capable to utilize the *N*-heterocyclic compounds. Moreover, three different inducible metabolic pathways for degradation of 5-hidroxypicolinic acid, 3-hydroxypyridine ,and nicotinic acidhave been identified, all resulting in formation of the same metabolite, 2,5-dihydroxypyridine. The initial degradation step of 5-hydroxypicolinic acid is catalyzed by the 5-hydroxypicolinate 2-monooxygenase, discovered and partially purified during this work.

Achromobacter sp. JS18 is able to grow using the picolinic, nicotinic , and dipicolinic acids as a sole carbon and energy source. Each of these compounds induces different metabolic pathways. Our studies show that the JS18 strain is capable of degrading 3-hydroxypicolinic acid via the picolinic acid pathway. However, it has been found that 3-hydroxypicolinic acid is incapable to induce the transcription of genes required for its own degradation. Nevertheless, *Achromobacter* sp. JS18, due to its ability to transform a wide range of *N*-heterocyclic compounds, is very attractive for the biosynthesis of hydroxylated aromatic carboxylic acids.

A new bacterial strain *Sinorhizobium* sp. L1, capable to utilize 3-hydroxypyridine and nicotinic acid, has been isolated. In both cases, 2,5-dihydroxypyridine is formed, which is subsequently oxidized by different dioxygenase isoforms, depending on the

metabolic pathway. The gene, encoding 2,5-dihydroxypyridine dioxygenase in *Sinorhizobium* sp. L1, has been identified.

The statements to be defended:

- *Achromobacter* sp. JS18 can degrade picolinic, nicotinic, and dipicolinic acids.
- There exist at least three distinct metabolic pathways for degradation of pyridine compounds in *Pusillimonas* sp. 5HP.
- 5-Hydroxypicolinic acid induces the biosynthesis of 5-hydroxypicolinic acid 2-monooxygenase in *Pusillimonas* sp. strain 5HP.
- There are two isoforms of 2,5-dihydroxypyridine dioxygenase in *Sinorhizobium* sp. L1.

Contents of the doctoral thesis. The dissertation is written in Lithuanian and contains the following parts: Introduction, Overview of the literature, Materials and Methods, Results and Discussion, Conclusions, List of references (103 positions), List of publications, Tables (14) and Figures (59). 141 pages in total.

MATERIALS AND METHODS

Bacterial strains. *Pusillimonas* sp. 5HP capable to degrade 5-hydroxypyridine-2-carboxylic acid, 3-hydroxypyridine and pyridine-3-carboxylic acid, and *Sinorhizobium* sp. L1, capable to degrade 3-hydroxypyridine and pyridine-3-carboxylic acid were isolated during this work. *Achromobacter* sp. JS18, utilizing pyridine-2-carboxylic, pyridine-3-carboxylic, and pyridine-2,6-dicarboxylic acids, was from this laboratory collection. *Escherichia coli* DH5α (ϕ80dlacZΔM15 Δ(lacZY-argF) U169, deoR, recA1, endA1, hsdR17($r_K^-m_K^+$), dup E44, thi-1, gyrA96, relA1) was obtained from Pharmacia (part of GE Healthcare), *Escherichia coli* BL21 F^-, *omp*T, *gal*, *hsd*S_B ($r_B^-m_B^-$), *dcm*, *lon* (DE3) from Avidis.

Bacterial vectors. pUC19/PstI, pUC19/SalI, pUC19/HindIII, pUC19, and pTZ57R/T were obtained from Fermentas (part of Thermofisher); pET19 was obtained from Invitrogen.

Media and growth conditions: EFA1 medium, (g/l): K_2HPO_4 – 8.8, $(NH_4)_2SO_4$ – 1.0, NaCl – 2.35, yeast extract – 0.5, $MgSO_4$ x $7H_2O$ – 0.18, salt solution – 10 ml/l.; **EFA2 medium,** (g/l), the same as EFA1, but yeast extract – 0.9, peptone – 1.0, beef extract – 0.6; pH 7.2; **Salt solutions** (g/l):$CaCl_2$ x $2H_2O$ – 2.0, $MnSO_4$ x $4H_2O$ – 1.0, $FeSO_4$ x $7H_2O$ – 0.6, $NaMoO_4$ x $2H_2O$ – 0.5, all components were dissolved in 0.1N HCl and added in EFA1 or EFA2 media before cultivation. **NB** (nutrient broth) – 13 g/l. **NA** (nutrient agar) – 28 g/l.
All media and solutions were autoclaved at 1 atm for 30 min. The 5-hydroxypyridine-2-carboxylic acid containg media were autoclaved at 0,5 atm.
E. coli was cultivated at 37 °C. All other bacteria were cultivated at 30 °C. In liquid medium, bacteria were cultivated with aeration and shaking at 180 rpm.

Isolation of 3-hydroxypyridine-2-carboxylic acid degrading bacteria. The laboratory collection of bacterial strains was tested for ability to oxidate or/and growth on EFA1 agar plates containing 3-hydroxypyridine-2-carboxylic acid. The dark pigment producing bacteria were selected for the further work.

Isolation of 5-hydroxypyridine-2-carboxylic acid degrading bacteria. A small amount of soil was suspended in mineral medium 5 ml, containing 0,1% of 5-hydroxypyridine-2-carboxylic acid, and the suspension was incubated by shaking for 2–7 days. The aliquots were spread on the EFA1 agar plates supplemented with 5-hydroxypyridine-2-carboxylic acid. The pigment producing bacteria were selected for the further work.

DNA isolation. Plasmid DNA from *E. coli* was isolated by standard alkaline lysis (Sambrook et al., 1989). Genomic DNA was isolated as described by Woo and co-workers (Woo et al., 1992).

DNA digestion and ligation. DNA was digested with an appropriate DNA restriction endonucleases according to the recommendations of manufacturer. Ligation was performed using the T4 DNA ligase Fermentas (part of Thermofisher) overnight at 8 °C. The ligase was inactivated by heating the mixture for 10 min. at 65 °C.

DNA electrophoresis in agarose gel. Horizontal DNA electrophoresis was performed using 0.8–1.2% agarose gels in TAE buffer as described by Sambrook et al. (1989). Gels were stained with ethidium bromide and analyzed under UV using Transilluminator UVT-28ME, Herolab.

Preparation of electrocompetent cells and electroporation. *E. coli* competent cells were prepared by the method described by Sharma and Schimke (1996). Briefly, DNA was mixed with 100 μL of ice-cold competent cells. Later, transferred to the electroporation cuvette (capacity of 100 μL) and subjected to 20 kV/cm electric pulse using the Eppendorf electroporator. Cells were immediately diluted with 1 mL of NB medium and incubated for 30–45 min. at 37 °C. After the recovery, cells were spread on the plates containing an appropriate antibiotic or substrate.

Nucleotide sequence determination and analysis. Plasmid DNA was purified using the ZYMO Plasmid PREP kit. Concentration of DNA was determined by electrophoresis in agarose gel using the Mass Ruler™ DNA Ladder (High Range). The nucleotide sequences were determined at the Macrogen (South Korea). The chromatograms of sequencing were analyzed using Chromas 2.24 program and VectorNTI Advance™ 9.0 program (Gorelenkov et al., 2001). Comparisons of DNA and protein sequences were performed with BLAST(ref.?).
Phylogenetic analyses were conducted using MEGA 5.0 (Tamura et al., 2011). The evolutionary history was inferred using the Neighbour-joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The evolutionary distances were computed using Poisson correction method (Zuckerandl & Pauling, 1965), and are represented as the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

PCR reactions. 2x Maxima PCR Master Mix was used according to the recommendations of the manufacturer. PCR was performed with T-personal thermocycler (Biometra).

Resting cells reactions. The *Pusillimonas* sp. 5HP cells were grown with aeration in 5 mL of EFA2 media, containing corresponding pyridine compound(s) for 2–4 days at 30 °C. The *Achromobacter* sp. JS18 and *Sinorhizobium* sp. L1 were pre-grown in 20 mL of EFA medium, containing correspounding pyridine compound, at 30 °C overnight with aeration. Cells were transferred into the 50 mL of the same media, and the cultivation continued for next 24–48 hours. The cells were harvested by centrifugation at 10000 *g* for 20 minutes and washed twice with 50 mM potassium phosphate buffer (pH 7.2).
The washed cells were suspended in the 1 mL of the same buffer, and the 0.1–0.2 mM of substrate was added. Cells were resuspended and immediately harvested by

centrifugation. The supernatant was transferred to the quartz cuvette, and the primary spectrum was read over a range of 200 to 400 nm. Later, the supernatant and the harvested cells were transferred to the same reaction cell and further incubated. The spectra were recorded in appropriate time intervals after centrifugation of cells, using Helios γ spectrophotometer (Thermo).

Preparation of cell-free extracts. The cells grown in the liquid media were harvested by centrifugation and washed twice with 20 mM potassium phosphate buffer (pH 7.0), then resuspended in the same buffer, and disrupted by sonication at 22 kHz for 2–10 min. The cells debris was removed by centrifugation (12000 *g* for 20 min).

Determination of 2,5-dihydroxypyridine dioxygenase activity. The dioxygenase activity was measured in the presence of Fe(II) ions, according to (Gauthier & Rittenberg, 1971).

Determination of 5-hydroxypicolinate 2-monoxygenase activity. The monoxygenase activity was measured in the presence of NADH and FAD, in the same manner as the 6-hydroxynicotinate 3-monooxygenase, according to (Nakano et al., 1999).

Protein analysis by SDS-PAGE. Analysis was performed in the gels containing 9–14% acrylamide for the separation gel, and 4.5% acrylamide for the stacking gel. After protein migration, gel was stained with PageBlue™ Protein Staining Solution. Unstained Protein Molecular Weight Marker (14.4–116 kDa) was used as the standard. The procedure was performed following the manufacturer's recommendations.

Protein *de novo* sequencing. Proteins were extracted from the polyacrylamide gel after separation. Samples were prepared for the mass spectrometry analysis according to the specifications of Proteomics centre at the Institute of Biochemistry, Vilnius University. The Mass Spectra were analyzed using the GPS Explorer™ De Novo Explorer program.

The three dimensional structure prediction and analysis. The protein structures were predicted using the swiss-modelling. The three dimensional structures were analyzed by RasWin 2.6 program.

RESULTS AND DISCUSSION

Isolation of 3-hydroxypicolinic acid-degrading bacteria

The laboratory bacteria collection was tested for capability to degrade the 3-hydroxypicolinic acid. A *Achromobacter* sp. JS18 strain, producing a brown pigment during growth on 3-hydroxypicolinic acid containing medium, was selected. This strain was used for the further analysis.

Bioconversion of pyridine carboxylic acids by intact cells of *Achromobacter* sp. JS18

Achromobacter sp. JS18 was tested for ability to degrade various pyridine carboxylic acids. *Achromobacter* sp. JS18 cells could easily degrade the picolinic and the nicotinic acids in addition to 3-hydroxypicolinic acid, however, the other pyridine mono- and dicarboxylic acids were not metabolized.

The analysis of bioconversion of the pyridine carboxylic acids was carried out using *Achromobacter* sp. JS18 resting cells, pre-grown in the EFA1 medium with the appropriate substrates. The bioconversion of the picolinic and the nicotinic acids started by the hydroxylation at C6 (Fig. 1). These data are similar to published earlier, pointing to the classic pathways of pyridine ring metabolism. *Achromobacter* sp. JS18 cells, pre-cultivated in the presence of dipicolinic acid, were able to convert the dipicolinic acid, picolinic, and nicotinic acids. The changes in UV-VIS absorption spectra were similar.

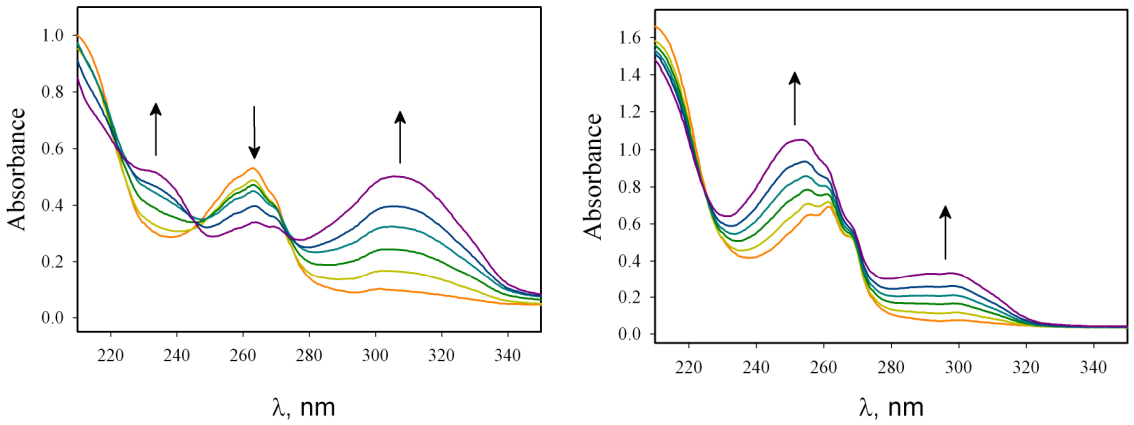

Fig. 1. Bioconversion of picolinic and nicotinic acids by *Achromobacter* sp. JS18 resting cells, pre-grown with respectively picolinic (left) and nicotinic acids (right). The UV-VIS spectra were recorded after 0, 10, 20, 30, 40 and 60 minutes of incubation.

The *Achromobacter* sp. JS18 resting cells could transform the pyrazine-2-carboxylic acid yielding a hydroxylated derivatives as the end product. The cells, pre-grown on the picolinic acid, produced a 6-hydroxypyrazine-2-carboxylic acid. However, the cells pre-grown on the nicotinic acid, formed a 5-hydroxypyrazine-2-carboxylic acid. Moreover, the dipicolinic acid pre-grown cells produced the mixture of 5- and 6-hydroxypyrazine-2-carboxylic acids (Fig. 2).

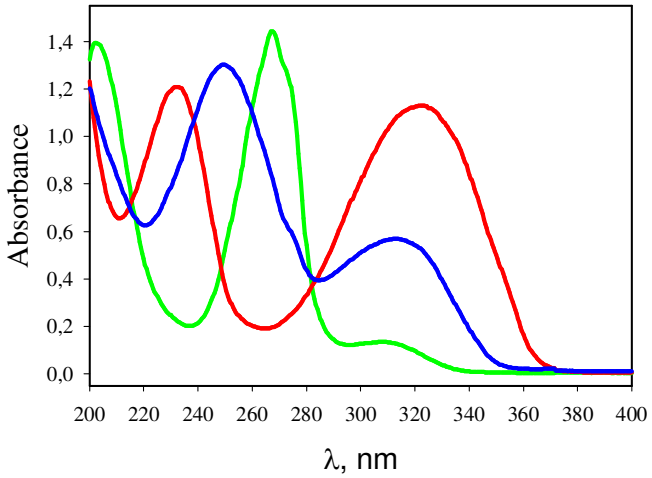

Fig. 2. Bioconversion of pyrazine-2-carboxylic acid by resting cells of *Achromobacter* sp. JS18. The spectrum of the obtained product after bioconversion with the cells pre-grown in the presence of respectively picolinic and nicotinic acids is marked in red and blue, accordingly. The spectrum of the substrate (pyrazine-2-carboxylic acid) is in green.

The *Achromobacter* sp.JS18 cells cultivated in the media containing nicotinic acid could hydroxylate pyridazine-3-carboxylic and 2-hydroxynicotinic acid at C6. The formed 2,6-dihydroxynicotinic acid is easily autooxidated yielding a blue pigment. It was proposed that quinonic dimer, in analogy with the product, obtained during the autooxidation of trihydroxypyridine, was formed (Gupta et al., 1975; Shukla et al., 1986; Holmes et al., 1972).

The *Achromobacter* sp.JS18 cells grown in the medium containing picolinic acid, could utilize 3-hydroxypicolinic acid much faster in comparison to the picolinic or 6-hydroxypicolinic acids since the slowest stage of bioconversion, hydroxylation at C3, was eliminated. The hydroxylation, catalyzed by picolinate dehydrogenase, took place at C6 that was confirmed by enzymatic analysis. In addition, the picolinate-induced cells could transform the 5-hydroxypicolinic acid. The final product was not identified, but it

was proposed that the 5,6-dihydroxypicolinic acid was formed, since absorbance at 260 nm, specific for the carboxyl group,did not disappear, however an absorbance near the 310 nm wavelength, has increased (Fig. 3, top).

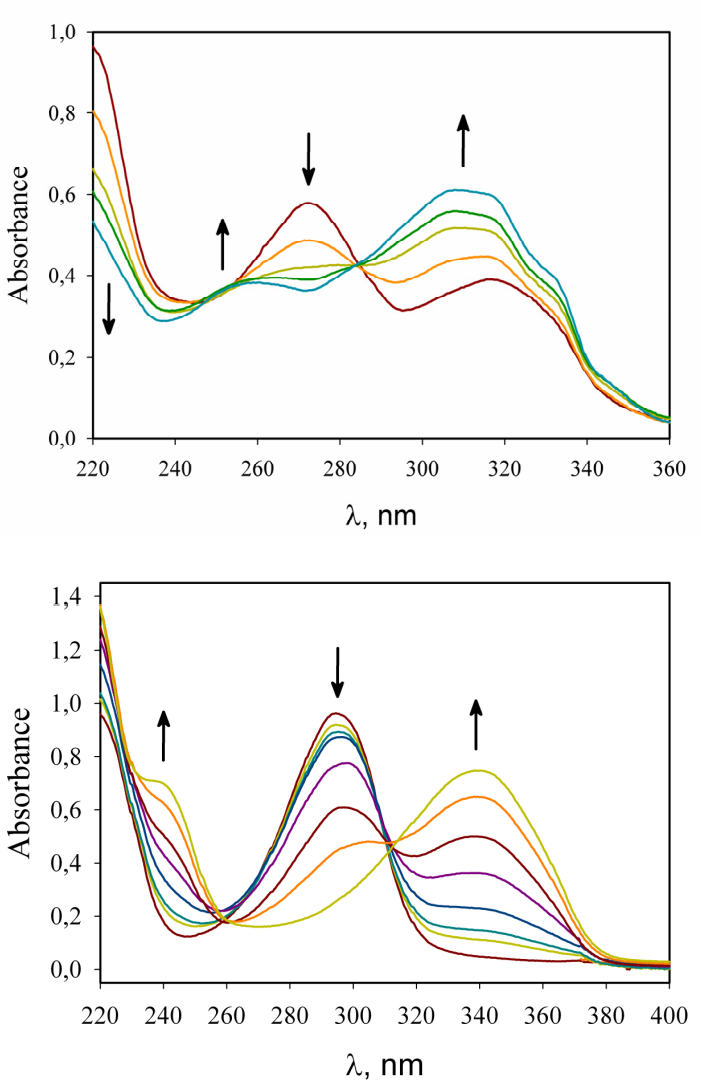

Fig. 3. Transformation of the 5-hydroxypicolinic (top) and the 3-hydroxypicolinic (bottom) acid by resting cells of *Achromobacter* sp. JS18, pre-grown in the presence of picolinic (top) and dipicolinic (bottom) acid.

Intermediates of the biodegradation of the dipicolinic acid, such as the 3-hydroxydipicolinic acid (Kobayashi et al., 1962), were not identified. The dipicolinic acid-induced cells were unable to consume the 5-hydroxypicolinic acid; but could transform the 3-hydroxypicolinic acid forming an unidentified product with the specific absorbance at 340 nm (Fig. 3, bottom), possibly the 3,6-dihydroxypicolinic acid.

Achromobacter sp. JS18 cells, which were pre-grown in the media containing the acetic acid, were unable to transform any of the described above pyridine compound.

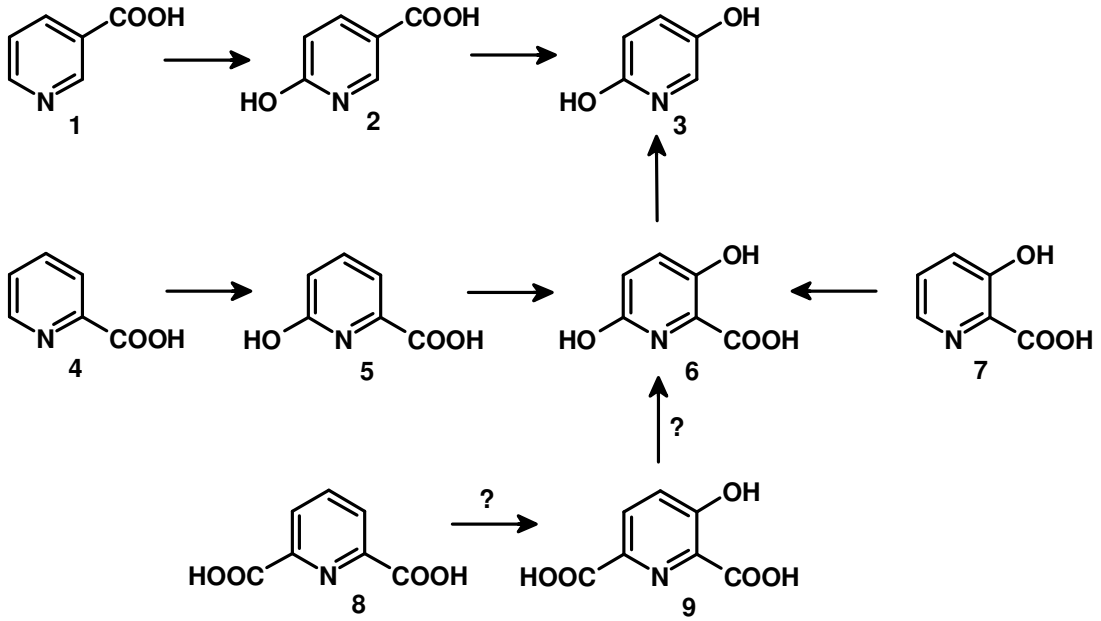

Fig. 4. The hypothetical pathway of degradation of nicotinic, picolinic and dipicolinic acids in *Achromobacter* sp. JS18. 1 – nicotinic acid, 2 – 6-hydroxynicotinic acid, 3 – 2,5-dihysroxypyridine, 4 – picolinic acid, 5 – 6-hydroxypicolinic acid, 6 – 3,6-dihydroxypicolinic acid, 7 – 3-hydroxypicolinic acid, 8 – dipicolinic acid, 9 – 3-hydroxydipicolinic acid.

The obtained data led us to propose a hypothetical pathway for degradation of the pyridine carboxylic acids in *Achromobacter* sp. JS18 cells (Fig. 4).

Investigation of the enzymes, catalyzing degradation of the carboxypyridines in *Achromobacter* sp. JS18

It is known that nicotinate and picolinate dehydrogenases usually are the molybdene containing enzymes, and their activity depends on the molybdenum or tungsten ions present in the growth media (Nagel et al., 1989; Siegmund et al., 1990). Moreover, these two enzymes are membrane-bound or membrane associated (Orpin et al., 1972). The nicotinate dehydrogenases could be divided into two subgroups depending on the electron acceptors, NAD(P)-dependent and the artificial electron acceptors-dependent ones. Only one picolinate dehydrogenase whose activity requires presence of NAD+ and Fe(II) ions (Orpin et al., 1972) is known.

The respective the picolinate and nicotinate dehydrogenases activities was detected in the *Achromobacter* sp. JS18 cells, harvested after cultivation in the presence either of picolinic and nicotinic acids. Both enzymes were active in the presence of the artificial electron acceptors. The picolinate dehydrogenase was specific for phenazine methosulphate (PMS), while nicotinate dehydrogenase activity was observed in the presence of ferricyanide, PMS, dichlorophenol indophenol (DCPIP), Nitro blue tetrazolium (NBT) and cytochrome *c*. The picolinate dehydrogenase was most stable and active at slightly basic pH (around 7.5), while higher stability and activity of nicotinate dehydrogenase was observed at acidic pH (around 5.8). The optimal pH for the stability and activity of the other known nicotinate dehydrogenases is from 7.0 to 8.5. The sedimentation analysis of *Achromobacter* sp. JS18 cell-free extracts showed that both picolinate and nicotinate dehydrogenases are the cytosolic enzymes.

The picolinate dehydrogenase from *Achromobacter* sp. JS18 cells was purified partially. The molecular mass of the enzyme determined by gel-filtration was around 295 kDa. The spectral analysis showed that partially purified enzyme absorbed at 320 and 435 nm, hence flavine group could be expected.

The nicotinate dehydrogenase from *Achromobacter* sp. JS18 was purified according the scheme described in the Materials and Methods. The purified enzyme consisted of three subunits (97, 58 and 33 kDa) according to the SDS-PAGE analysis (Fig. 5).

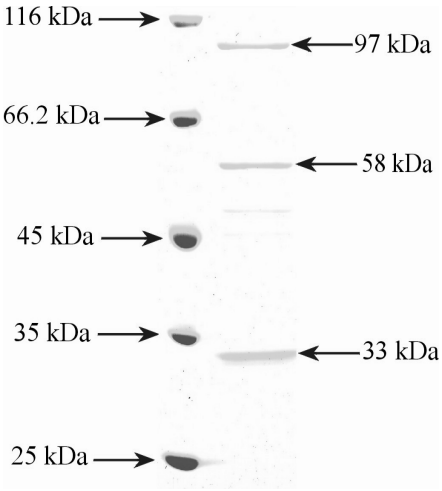

Fig. 5. The SDS-PAGE analysis of the purified nicotinate dehydrogenase from *Achromobacter* sp. JS18.

The *Achromobacter* sp. JS18 cell-free extract showed also a 2,5-dihydroxypyridine dioxygenase activity in the presence of Fe(II). Since the highest activity of the enzyme was found in the nicotinate pre-grown cells, these cells were used for the enzyme production and purification. The molecular mass of the purified enzyme determined by gel-filtration was 227±3 kDa. Under denaturing conditions, the enzyme broke down to monomers. Their molecular mass was around 38.7 kDa as determined by SDS-PAGE analysis. This molecular mass is similar to ones of the other 2,5-dihydroxypyridine dioxygenases (Gauthier et al., 1971; Jimenez et al., 2008). The purified enzyme was used for the *de novo* sequencing. Four peptides were identified, and their amino acid sequences were determined. All sequenced peptides were found to be presented in *Achromobacter piechaudii* ATCC 43553 single hypothetical protein (EFF78588.1). The primers for the PCR were created based on this protein encoding gene sequence. The gene fragment encoding dioxygenase from *Achromobacter* sp. JS18 was amplified by the PCR, using the created primers and *Achromobacter* sp. JS18 chromosomal DNA. The sequence of the amplified DNA fragment was found to be most homologous to 2,5-dihydroxypyridine dioxygenase (Fig. 6.).

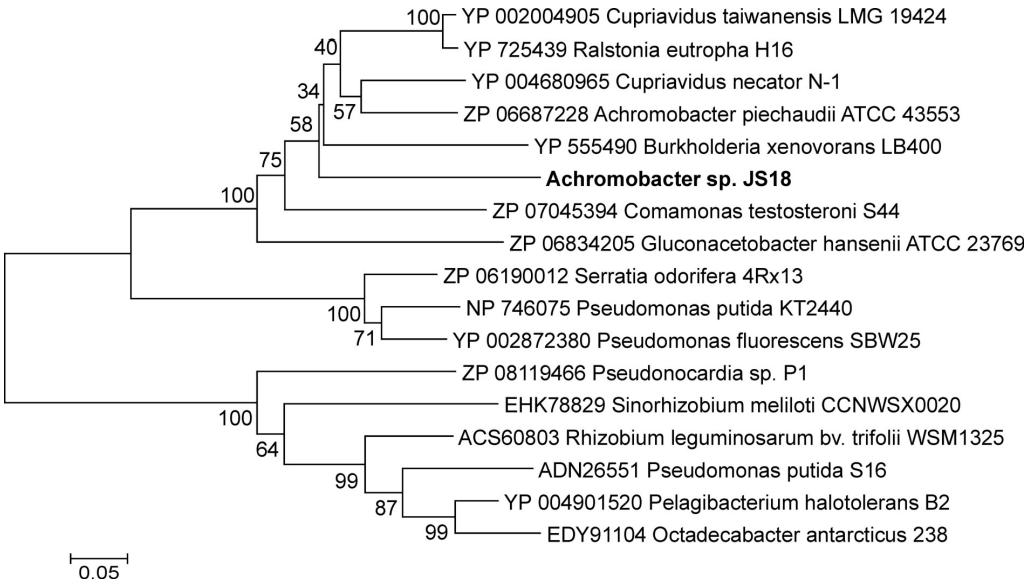

Fig. 6. The phylogenetic tree of 2,5-dihydroxypyridine dioxygenase from *Achromobacter* sp. JS18. The numbers at the branches show how many times the group to the right of the branch accured among the 100 tree generated in bootstrap analysis. Scale bar represents expected amino acid substitution per position. The GeneBank accession numbers are indicated for each protein. Details of phylogenetic method are given in Materials and Methods.

Isolation of the 5-hydroxypicolinic acid utilizing bacterium

Bacterium 5HP, capable to degrade 5-hydroxypicolinic acid was isolated from soil by the enrichment technique. The green pigment, which later turned to brown, was accumulated during growth in the medium, containing the 5-hydroxypicolinic acid. The 16S rRNA-based phylogenetic analysis indicated that the isolate 5HP belongs to the *Pusillimonas* genus and is closely related to *Pusillimonas noertemannii* strain BN9 (Fig. 7). Therefore, it was shown that *Pusillimonas* genus can utilize the pyridine compounds.

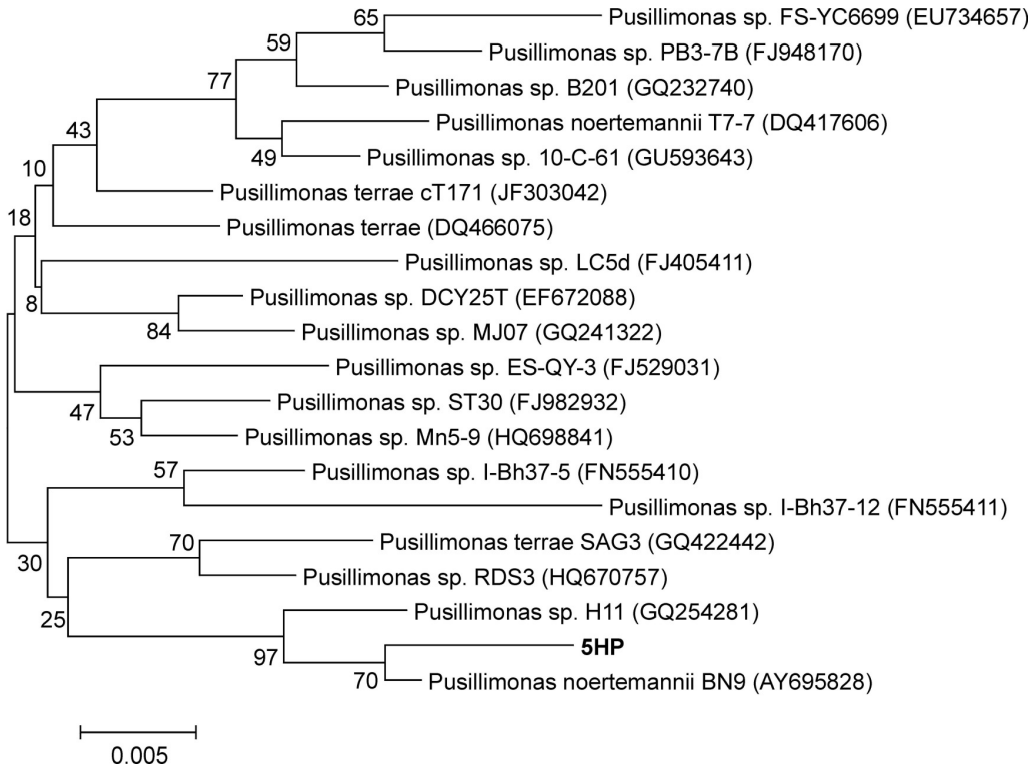

Fig. 7. The phylogenetic tree of the bacterial strain 5HPand its nearest relatives based on 16S rRNA gene sequence analysis. Neighbour-joining method was used. The numbers at the branches show how many times the group to the right of the branch occurred among 100 trees generated in a bootstrap analysis. Scale bar represents 5 expected substitutions per 1000 nucleotides. The culture collection accession number and the GenBank accession number is indicated for each strain.

Bioconversion of pyridine compounds by *Pusillimonas* sp. 5HP intact cells

Due to very rare prevalence of the 5-hydroxypicolinic acid in the nature (Moon et al., 2010), *Pusillimonas* sp. 5HP cells were tested for ability to produce this acid from the other compounds. Strain 5HP was analyzed for ability to utilize the 5-hydroxy-2-hydroxymethylpyridine, picolinic, dipicolinic, and isocinchomeronic acids.

Unfortunately, these compounds were neither metabolized nor oxidized. The analysis of the other potential substrates for bioconversion showed that *Pusillimonas* sp. 5HP cells could use 3-hydroxypyridine, 3-cyanopyridine, and nicotinic acid as a sole source of carbon and energy. When bacteria were grown in the liquid media containing the 5-hydroxypicolinic acid or 3-hydroxypyridine at concentrations greater than 0.2%, the green pigment was formed. The formation of the similar pigment allowed to assume that metabolic pathways for both compounds had the same intermediate – 2,5-dihydroxypyridine. This idea was tested by using the intact cells. The *Pusillimonas* sp. 5HP cells pre-grown in the presence of 5-hydroxypicolinic acid could consume only 5-hydroxypicolinic acid and 2,5-dihydroxypyridine (Fig. 8, 9). No transformation was observed with 3-hydroxy- and 6-hydroxypicolinic acid (data not shown).

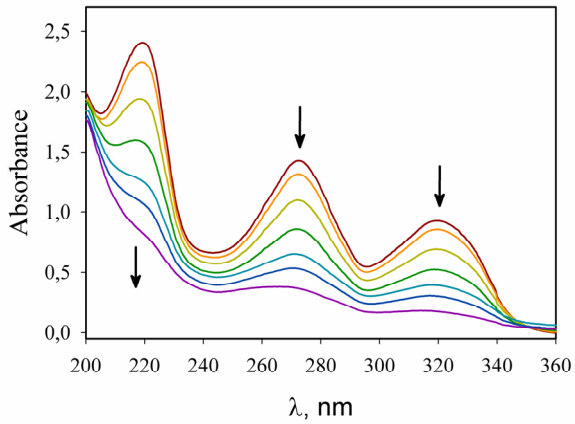

Fig. 8. Bioconversion of 5-hydroxypicolinic acid by *Pusillimonas* sp. 5HP resting cells, pre-grown with 5-hydroxypicolinic acid. The UV-VIS spectra were recorded after 0, 30, 60, 90, 120, 150, and 180 minutes incubation.

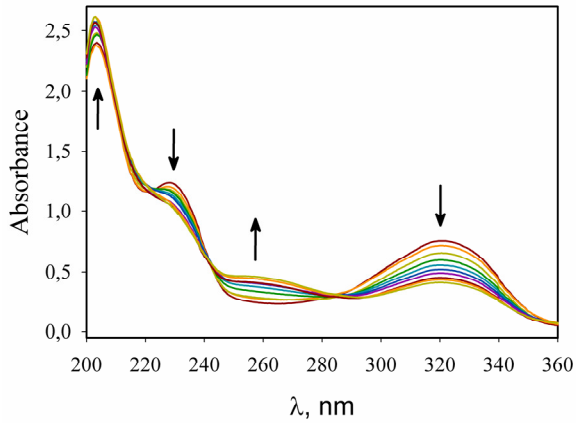

Fig. 9. Bioconversion of 2,5-dihydroxypyridine by *Pusillimonas* sp. 5HP resting cells, pre-grown with 5-hydroxypicolinic acid. The UV-VIS spectra were recorded after 0, 15, 45, 75, 105, 135, 165, 195, 240, and 315 minutes incubation.

3-Hydroxypyridine-induced *Pusillimonas* sp. 5HP cells could consume only 3-hydroxypyridine (Fig. 10), forming 2,5-dihydroxypyridine, which was further utilized.

The 3-cyanopyridine and the nicotinic acid-induced *Pusillimonas* sp. 5HP cells could consume nicotinic acid (Fig. 11), 6-hydroxynicotinic acid and 2,5-dihydroxypyridine, but could not transform either 3-hydroxypyridine or 5-hydroxypicolinic acid. These cells also could transform pyrazine-2-carboxylic acid to

yield 5-hydroxypyrazine-2-carboxylic acid. The 3-cyanopyridine pre-grown cells consumed 3-cyanopyridine (Fig. 12) and nicotinamide.

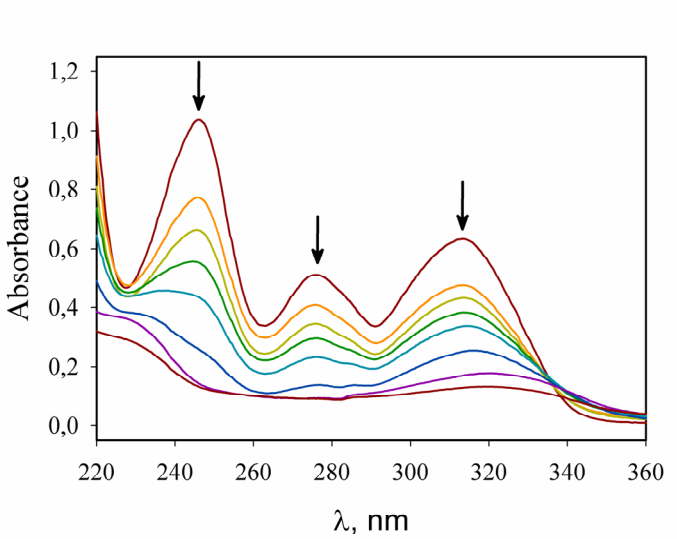

Fig. 10. Bioconversion of 3-hydroxypyridine by *Pusillimonas* sp. 5HP resting cells, pre-grown with 3-hydroxypyridine. The UV-VIS spectra were recorded after 0, 12, 20, 30, 45, 60, 75 and 90 minutes incubation.

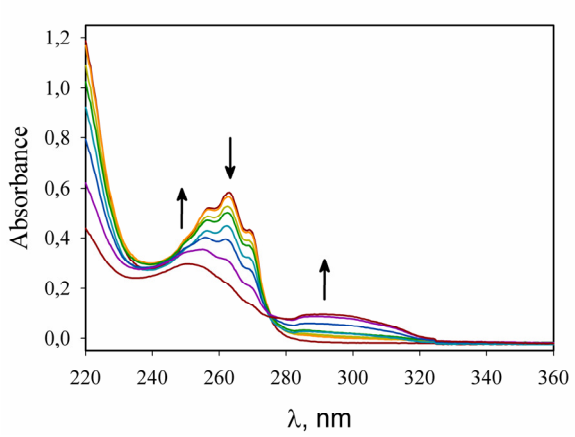

Fig. 11. Bioconversion of nicotinic acid by *Pusillimonas* sp. 5HP resting cells pre-grown with nicotinic acid. The UV-VIS spectra were recorded after 0, 30, 60, 90, 120 and 150 minutes incubation.

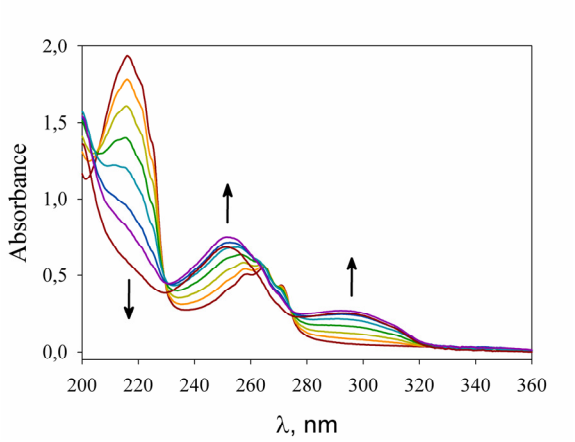

Fig. 12. Bioconversion of 3-cyanopyridine by *Pusillimonas* sp. 5HP resting cells, pre-grown with 3-cyanopyridine. The UV-VIS spectra were recorded after 0, 15, 35, 60, 90, 120, 150 and 180 minutes incubation

Since the nitrile group attacking enzymes usually are not specific for the compound, the *Pusillimonas* sp. 5HP cells cultivated in the presence of 3-cyanopyridine were able to transform the other nitriles such as 5-hydroxypyridine-2-carbonitrile, pyrazine-2-carbonitrile, 3-methyl- and 6-methylpyridine-2-carbonitriles, and pyridine-2,6-

dicarbonitrile to the corresponding acids or amides. The *Pusillimonas* sp. 5HP cells cultivated in the presence of acetic acid were not able to use any of the previously mentioned pyridine compounds (data not shown).

The investigations using the intact cells led to identification of all intermediates of 3-hydroxypyridine, 5-hydroxypicolinic, and nicotinic acid conversion. All these pathways have the same intermediate, 2,5-dihydroxypyridine (Fig. 13).

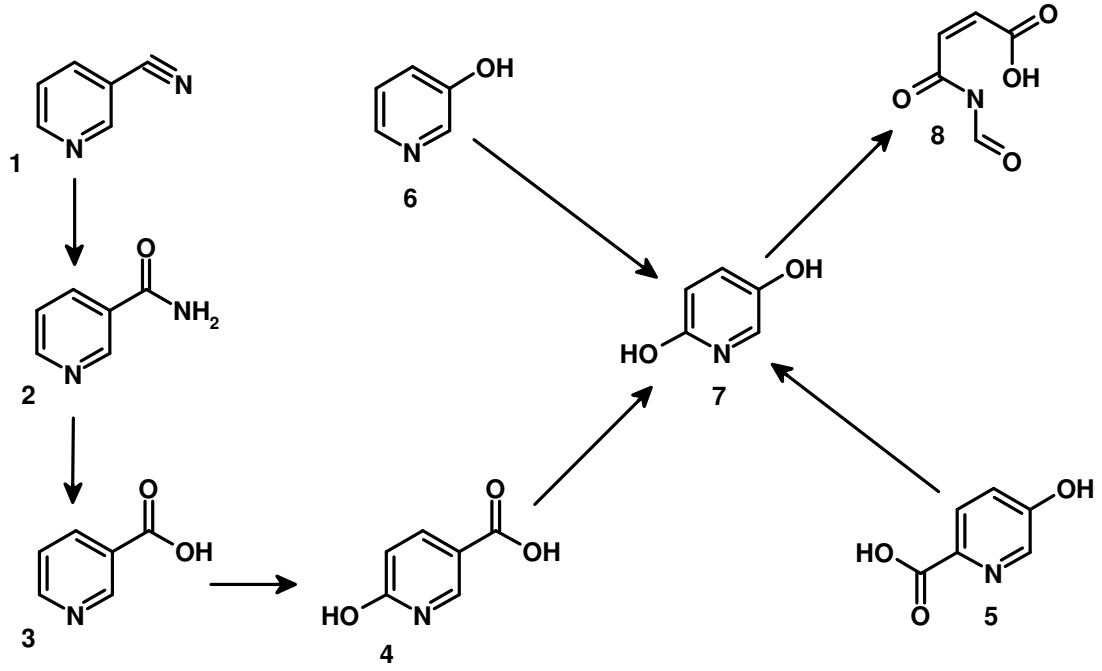

Fig. 13. The degradation pathways of 5-hydroxypicolinic acid, 3-hydroxypyridine, 3-cyanopyridine, and nicotinic acid in *Pusillimonas* sp. 5HP. 1 – 3-cyanopyridine, 2 – nicotinamide, 3 – nicotinic acid, 4 – 6-hydroxynicotinic acid, 5 – 5-hydroxypicolinic acid, 6 – 3-hydroxypyridine, 7 – 2,5-dihydroxypyridine, 8 – *N*-formylmaleamic acid.

Investigation of the 5-hydroxypicolinate 2-monooxygenase

The enzymatic conversion of 6-hydroxynicotinic acid into 2,5-dihydroxypyridine is known for more than decade (Nakano et al., 1999). The enzyme, catalyzing similar oxidative decarboxylation of 5-hydroxypicolinic acid, was unknown. The *Pusillimonas* sp. 5HP cell-free extract showed the expected enzymatic activity with 5-hydroxypicolinic acid in the presence of NADH. The formation of 2,5-dihydroxypyridine was confirmed experimentally by using 2,5-dihydroxypyridine dioxygenase from *Sinorhizobium* sp. L1. Addition of this enzyme to the reaction mixture decreased absorbance at 320 nm, as expected.

The known 6-hydroxynicotinate 3-monooxygenases require FAD for activity (Nakano et al., 1999), but 5-hydroxypicolinate 2-monooxygenase activity was stimulated by FAD only. No effect was observed with the other flavins. The 5-hydroxypicolinate 2-monooxygenase selectivity to FAD and NADH suggested that the protein could specifically bind to Cibacron Blue F3GA dye. The enzyme was partially purified using the affinity Blue (and anion exchange) resin. The molecular mass of partially purified enzyme was about 43 kDa, according to the SDS-PAGE analysis (Fig. 14).

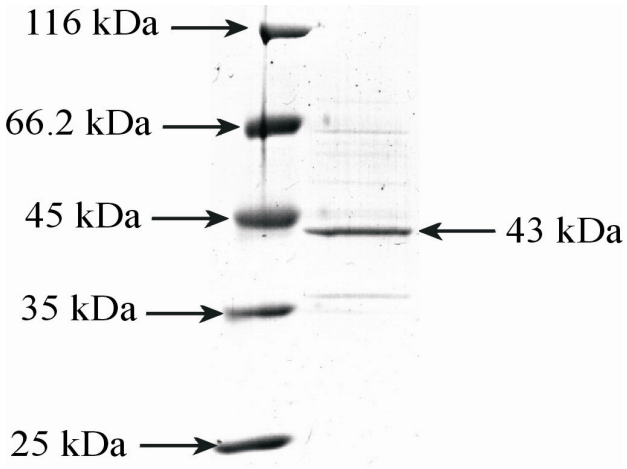

Fig. 14. SDS-PAGE analysis of the partially purified 5-hydroxypicolinate 2-monooxygenase from *Pusillimonas* sp. 5HP.

The partially purified enzyme was used for *de novo* sequencing. Only one peptide with amino acid sequence VGFIPEALVGR was identified. Since for the digestion was used trypsin, which hydrolyze peptide bonds between lysine (K), lower frequency between arginine (R), the identified sequence could be extended by these amino acids. Additionally, mass spectroscopy analysis had a disadvantage that it could not discriminate some amino acids. Hence the presence of L and I or K and Q were interpreted equally. Analysis of the resulting peptide by the BLAST program showed similarity to KVAFLPQAIVGR (E=0,004) peptide of salicylate 1-monooxygenase (YP_555487) from *Burkholderia xenovorans* LB400.

Investigation of enzymes involved in nicotinic acid biodegradation in *Sinorhizobium* sp. L1

The strain L1 was isolated as 3-hydroxypyridine utilizing bacterium. Its 16S rRNA analysis revealed that it belongs to the *Sinorhizobium* (*Ensifer*) genus (Fig. 15). The strain L1 also utilized the nicotinic acid, nicotinamide, and 3-hydroxymethylpyridine. The strain L1 degraded nicotinamide in a traditional manner: the amide was hydrolyzed to the corresponding acid, and the metabolism proceeded via nicotinic acid pathway by formation of 2,5-dihydroxypyridine.

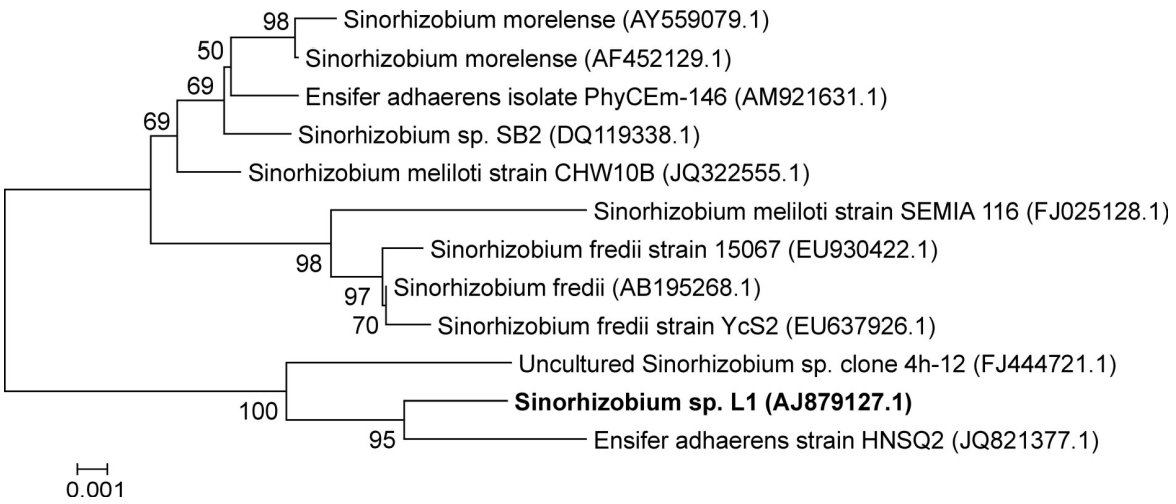

Fig. 15. The phylogenetic tree of the bacterial strain L1 and its nearest relatives based on the 16S rRNA gene sequence analysis. For details see Fig. 7.

According to literature, the 3-methylpyridine could be oxidized to the nicotinic acid via formation of 3-hydroxymethylpyridine (Korosteleva et al., 1981). It was also reported that there was a direct biogenic relationship between the nicotinic acid and 3-hydroxymethylpyridine (Gross et al., 1968). The bioconversion experiments with resting *Sinorhizobium* sp. L1 cells harvested from the medium containing 3-hydroxymethylpyridine did demonstrated the expected results. The cells showed negligible bioconversion activity, cell-free extract showed the same enzymatic activity as the nicotinic acid pre-grown cells (Table 1).

These investigations led to the assignment of 3-hydroxymethylpyridine degradation to the nicotinic acid pathway and place the 3-hydroxypyridine degradation into the separate pathway.

Table 1. The detected enzymes in the *Sinorhizobium* sp. L1 cell-free extracts.

Enzyme activity	Inducer (0.2%), present in the growth medium					
	Succinic acid	Nicotinamide	6-Hydroxynicotinic acid	Nicotinic acid	3-Hydroxypyridine	3-Hydroxymethyl pyridine
Nicotinamide deaminase	–	+	–	–	–	–
Nicotinate dehydrogenase	–	+	+	+	–	+
6-Hydroxynicotinate 3-monooxygenase	–	+	+	+	–	+/–
2,5-Dihydroxypyridine 5,6-dioxygenase	+	+	+	+	+	+
Maleamic acid deaminase	+	+	+	+	+	+

("+" – activity was detected; "–„ – no activity was detected)

The nicotinate dehydrogenase from *Sinorhizobium* sp. L1 was purified by the scheme described in the Materials and Methods. The purified enzyme consisted of three different subunits (85, 28 and 17.7 kDa) according to the SDS-PAGE analysis (Fig. 16).

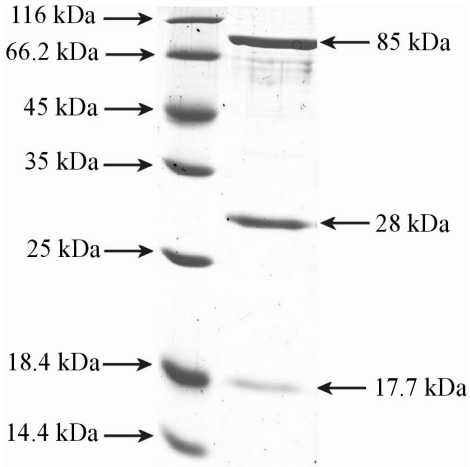

Fig. 16. The SDS-PAGE analysis of the purified nicotinate dehydrogenase from *Sinorhizobium* sp. L1.

The nicotinate dehydrogenase from *Sinorhizobium* sp. L1 could use not only artificial electron acceptors, like ferricyanide, PMS, DCPIP, NBT, but also the citochrome *c*. The determined K_M values for nicotinate dehydrogenase from *Sinorhizobium* sp. L1 in 20 mM potassium phosphate, pH 7.0 were 47 μM for ferricyanide, 225 μM for citochrome *c*, and approx 120 μM for the nicotinic acid (Fig. 17).

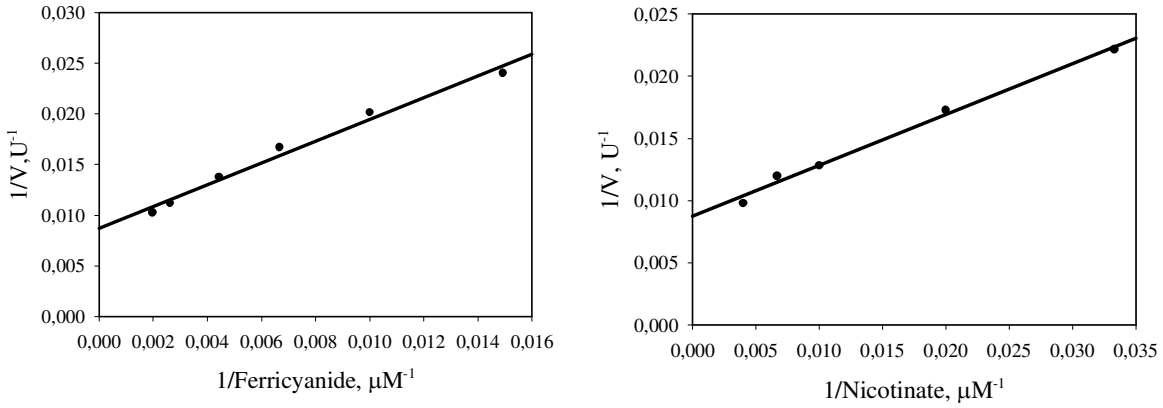

Fig. 17. The dependence of the reaction velocity on the concentration of nicotinate dehydrogenase from *Sinorhizobium* sp. L1. K_M values were determined using the double-reciprocal plot.

Another enzyme, detected in the *Sinorhizobium* sp. L1 cells, was the 2,5-dihydroxypyridine dioxygenase. It was found that *Sinorhizobium* sp. L1 strainproduced two isoforms of the enzyme depending on the inducing substance. 3-Hydroxypyridine-induced cells produced the 39.8 kDa isoform A, and nicotinate-induced cells produced the 38.3 kDa isoform B (Fig. 18). The molecular masses of the native enzymes determined by gel-filtration were 230±2 kDa and 214±2 kDa, respectively. These data are close to the molecular mases 2,5-dihydroxypyridine dioxygenases from *Pseudomonas putida* N-9, KT2440 and S16 (Gauthier et al., 1971; Jimenez et al., 2008; Tang et al., 2012).

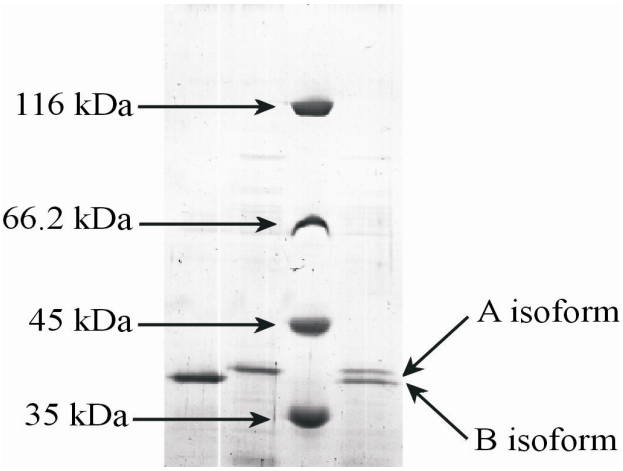

Fig. 18. The SDS-PAGE analysis of 2,5-dihydroxypyridine dioxygenases produced by *Sinorhizobium* sp. L1 cells. From left to right: 2,5-dihydroxypyridine dioxygenase purified from nicotinate-induced cells; 2,5-dihydroxypyridine dioxygenase purified from 3-Hydroxypyridine induced cells; molecular mass marker; a mixture of both isoforms of 2,5-dihydroxypyridine dioxygenase.

Both isoforms showed the similar enzymatic properties: optimal pH 7.5–7.75 for A isoform and 7.25–7.5 for B isoform. The K_M values at pH 7.2 were 175 and 150 μM for A and B isoforms, respectively (Fig. 19).

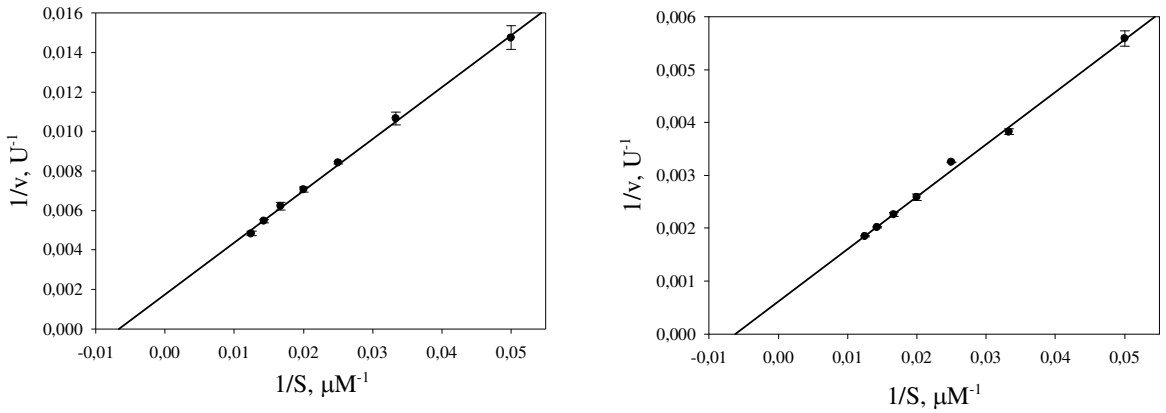

Fig. 19. The dependence of the reaction velocity on the concentration of 2,5-dihydroxypyridine dioxygenase isoforms from *Sinorhizobium* sp. L1. 2,5-Dihydroxypyridine dioxygenase from 3-hydroxypyridine-induced cells on the left, 2,5-Dihydroxypyridine dioxygenase from nicotinate-induced cells on the right.

Both enzyme isoforms were used for *de novo* sequencing. Four peptides were identified from the B isoform protein digest. Three of them were found to be homologous to the hypothetical protein (YP_002978554.1) from *Rhizobium leguminosarum bv. trifolii* WSM1325. The primers for the identified peptides were created based on this protein encoding gene sequence. The DNA fragment encoding dioxygenase from *Sinorhizobium* sp. L1 was amplified by PCR. The amplified DNA sequence was found to be the most homologous to 2,5-dihydroxypyridine dioxygenase. The A isoform protein digest analysis gave three peptides, one of them was found to be homologous to the same protein from *Rhizobium leguminosarum*. The full gene, encoding 2,5-dihydroxypyridine dioxygenase was identified by screening the gene library of *Sinorhizobium* sp. L1. pL1Pst25, harboring the corresponding gene, was detected by PCR with specific 2,5-dihydroxypyridine dioxygenase primers.

Identification of the degradation genes

The restriction analysis of pL1Pst25 plasmid showed that it carries a 10.5-kb insert. The insert analysis revealed the presence of 11 open reading frames (Fig. 20). The putative functions of predicted proteins are listed in Table 2.

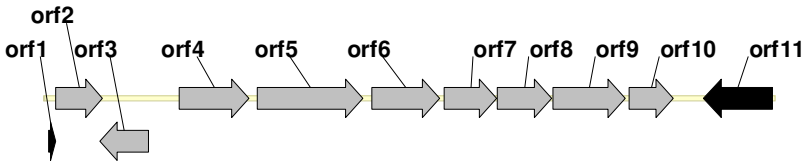

Fig. 20. Schematic representation of the *Sinorhizobium* sp. L1 genes predicted to be involved in degradation of 2,5-dihydroxypyridine.

Table 2. Summary of ORFs identified by significant homology in L1Pst25 plasmid.

ORF position	Length (a.a.)	Nearest homolog	GenBank Accession No.	Identity (%)	E value
Orf1 16-140	35	carbon-monoxide dehydrogenase, *Rhodopseudomonas palustris* HaA2	ABD07809	13/28 (46%)	6E-06
Orf2 137-811	224	hypothetical protein CTS44_14338 *Comamonas testosteroni* S44	EFI60974	119/215 (55%)	9E-81
Orf3 767-1468	233	GntR family transcriptional regulator *Comamonas testosteroni* S44	EFI60973	84/194 (43%)	6E-32
Orf4 1912-2904	329	hypothetical periplasmic protein TorT *Marinomonas* sp. MED121	EAQ65112	142/329 (43%)	3E-86
Orf5 3023-4534	503	sugar ABC transporter ATP-binding protein *Marinomonas* sp. MED121	EAQ65113	242/492 (49%)	3E-172
Orf6 4666-5630	290	Ribose/xylose/arabinose/galactoside ABC-type transport systems, permease component *Marinomonas* sp. MED121	EAQ65114	143/294 (49%)	6E-90
Orf7 5702-6451	249	maleate cis-trans isomerase *Pseudomonas putida* S16	ADN26549	197/249 (79%)	2E-148
Orf8 6461-7234	257	hydrolase, alpha/beta fold family *Octadecabacter antarcticus* 238	EDY88475	179/258 (69%)	7E-137
Orf9 7258-8289	343	leucyl aminopeptidase (aminopeptidase T) *Pelagibacterium halotolerans* B2	AEQ53770	304/342 (89%)	0.0
Orf10 8350-8976	208	N-carbamoylsarcosine amidase *Pelagibacterium halotolerans* B2	AEQ53771	153/203 (75%)	7E-110
Orf11 9411-10394	327	putative formaldehyde dehydrogenase, glutathione-independent *Dermacoccus* sp. Ellin185	EFP57883	166/271 (61%)	2E-104

The comparison of L1Pst25 fragment gene cluster organization and the similar gene clusters from the other bacteria are given in Fig. 21. The gene cluster encoding the degradation of 2,5-dihydroxypyridine from pL1Pst25 differed from the well characterized *nic* operon from *Pseudomonas putida* KT2440 (Jimenez et al., 2008). Although, the transport system proteins are rarely presented in the *nic* related gene clusters, these clusters usually contain 6-hydroxynicotinate 3-monooxygenase gene near the core quartet consisting of genes, encoding the maleate isomerase, *N*-formylmaleamate deformylase, 2,5-dihydroxypyridine dioxygenase, and maleamate amidase.

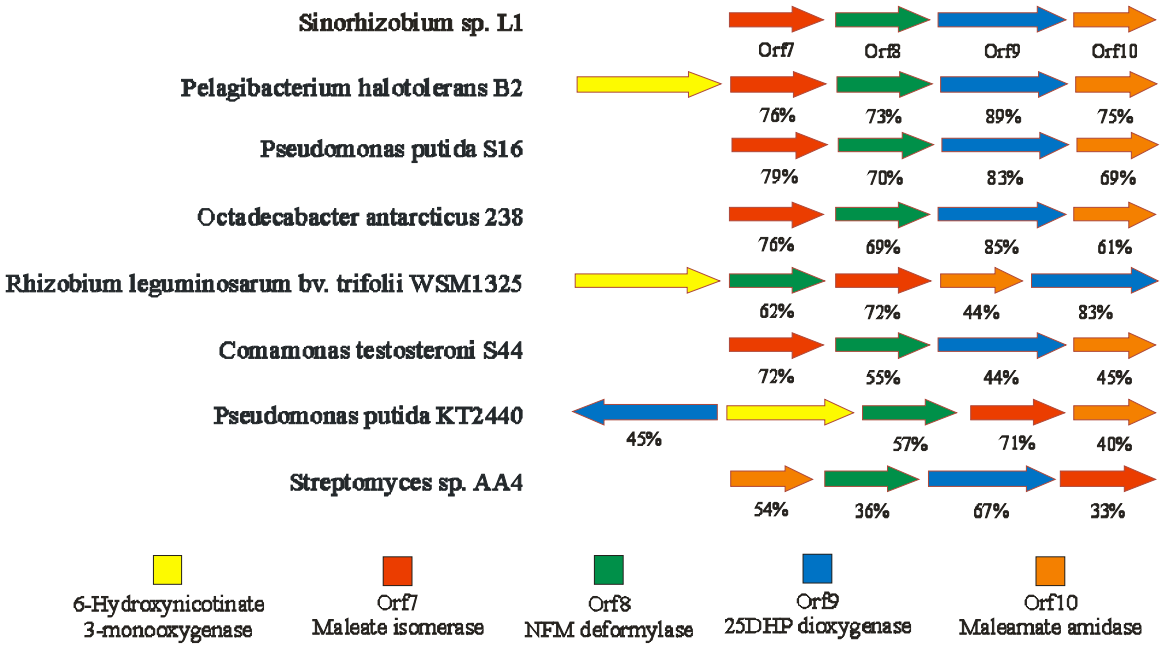

Fig. 21. Genentic organization of the L1Pst25 gene cluster of *Sinorhizobium* sp. L1 as compared with similar gene clusters from other bacteria. Orf7, maleate isomerase (red); Odf8, *N*-formylmaleamate deformylase (green); Orf9, 2,5-dihydroxypyridine dioxygenase (blue); Orf10, maleamate amidase (orange); 6-hydroxynicotinate 3-monooxygenase (yellow). The percentages represent amino acid identity of related enzymes.

The L1Pst25 DNR fragment gene cluster was similar to about 20-kb DNA fragment of *Comamonas testosteroni* S44. In both cases, transport system genes were located upstream the "gene quartet". Both fragments had a GntR type transcription regulators.

Expression of the recombinant 2,5-dihydroxypyridine dioxygenase

The *orf9* sequence was cloned into the pET19 vector, and the protein synthesis was induced in *E. coli* BL21 (DE3) cells (Fig. 22), using techniques described in Materials and Methods.

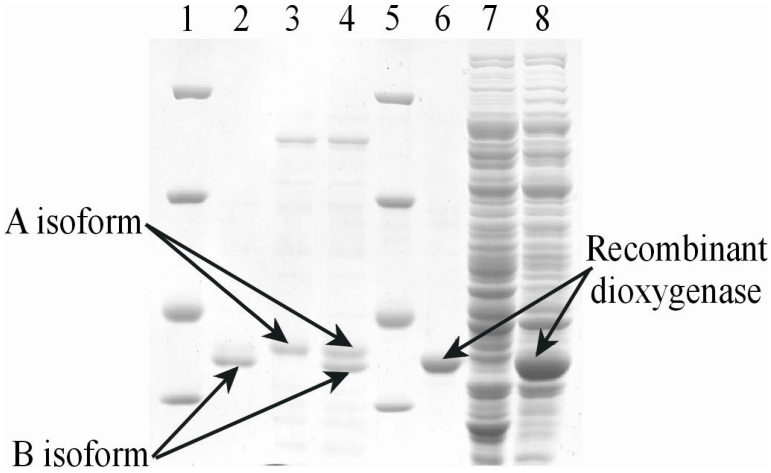

Fig. 22. The SDS-PAGE analysis of the expression of 2,5-dihydroxypyridine dioxygenase. 1) and 5) protein ladder (116, 66.2, 45 and 35 kDa); 2) the dioxygenase from 3-hydroxypyridine-induced cells of *Sinorhizobium* sp. L1; 3) the dioxygenase from nicotinate-induced cells of *Sinorhizobium* sp. L1; 4) the mixture of samples 2 and 3; 6) the purified recombinant 2,5-dihydroxypyridine dioxygenase from cells of *E. coli* BL21(DE3); 7) the control cell-free extract of *E. coli* BL21(DE3); 8) the cell-free extract of *E. coli* BL21(DE3)/ pET19orf9.

Under these conditions, the most of protein was produced in the inclusion bodies, but part of the recombinant 2,5-dihydroxypyridine dioxygenase remained soluble and showed an expected activity in the presence of 2,5-dihydroxypyridine. Moreover, the SDS-PAGE analysis of the recombinant 2,5-dihydroxypyridine dioxygenase led to conclusion that the protein is in the isoform B. This observation strengthens conclusion that the cloned DNA fragment encodes the part of nicotinate degradation operon in *Sinorhizobium* sp. L1.

CONCLUSIONS

1. The picolinic, nicotinic, and dipicolinic acids induce three distinct metabolic pathways in *Achromobactr* sp. JS18.
2. Bacteria, utilizing 5-hydroxypicolinic acid as the sole source of carbon and energy, has been isolated. The isolate 5HP belongs to *Pusillimonas* genus.
3. In *Pusillimonas* sp. strain 5HP, nicotinic acid, 5-hydroxypicolinic acid, and 3-hydroxypyridine induce three different metabolic pathways resulting in the formation of the same intermediate, 2,5-dihydroxypyridine.
4. 5-hydroxypicolinate 2-monooxygenase, which catalyzes oxidative decarboxylation of 5-hydroxypicolinic acid, has been detected and partially purified.
5. Two distinct isoforms of 2,5-dihydroxypyridine dioxygenase in *Sinorhizobium* sp. L1 are induced in the presence of 3-hydroxypyridine or nicotinic acid in the growth medium.
6. The identified gene cluster from *Sinorhizobium* sp. L1 encodes a part of nicotinic acid degradation pathway.

LIST OF PUBLICATIONS

Articles

1. Karvelis L., Meškys R. New *Rhizobium* strain degrading 3-hydroxypyridine. *Biologija* **2004** (2): 98–101.
2. Stanislauskienė R., Rudenkov M., Karvelis L., Gasparavičiūtė R., Meškienė R., Časaitė V., Meškys R. Analysis of phthalate degradation operon from *Arthrobacter* sp. 68b. *Biologija* **2011** 57: 45–54.
3. Karvelis L. Gasparavičiūtė R., Meškys R. Characterization of 2,5-dihydroxypyridine dioxygenases from *Sinorhizobium* sp. L1. *Biologija* **2012** (Submitted).
4. Karvelis L., Klimavičius A., Gasparavičiūtė R., Jančienė R, Meškys R. New *Pusillimonas* sp. strain, degrading 5-hydroxypicolinic acid. (Submitted).

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1. Razumienė, J., L. Karvelis, V. Gurevičienė, M. Rozgaitė, R. Meškys. Fermentinis nikotino rūgšties nustatymo metodas. *Chemija ir cheminė technologija: tarptautinė studentų mokslinė konferencija*. Vilnius, balandžio 27, 2007.
2. Razumiene J., L. Karvelis, V. Gureviciene, R. Meskys. Application of nicotinic acid 6-hydroxylase from *Sinorhizobium* sp. L-1 for amperometric determination of nicotinic acid and nicotinamide. *The 59th Annual Meeting of the International Society of Electrochemistry. Electrochemistry Down to the Molecular Level: Interfacial Science for Life and Technology.* September 7 to 12, 2008 Seville, Spain.
3. Kutanovas S., L. Karvelis, K. Michailova, V. Časaitė, J. Stankevičiūtė, R. Meškys. Biodegradation of carboxypyridines and carboxypyrazines. *COST Action CM00701 "Cascade Chemoenzymatic Processes – New Synergies Between Chemistry and Biochemistry" Joint Meeting-Conference*, Vilnius, Lithuania, 8-11 September, 2010.

ACKNOWLEDGEMENTS

I am very grateful to my scientific supervisor Dr. Rolandas Meškys for the opportunity to perform this work, for his supervision and advices. I would also like to thank my colleagues at the Department of Molecular Microbiology and Biotechnology, especially Virginia Dzekevičienė and Algimantas Krutkis. I express gratitude to Dr. R. Jančienė, Dr. A. Klimavičius, R. Rozenbergas and J Meškauskas for synthesis of chemical compounds used in this work. I give my thanks to Dr. Marija Ger for the peptide MALDI analysis. I sincerely thank to the reviewers Dr. Regina Vidžiūnaitė and Dr. Jaunius Urbonavičius for suggestions how to improved the thesis. I am very grateful to my family and my friends.

SANTRAUKA

Šio darbo metu buvo ieškoma bakterijų galinčių skaidyti piridino monokarboksirūgštis. Netradicinėse vietose hidroksilinančių ir/ar hidroksilintų piridino karboksirūgščių skaidyme dalyvaujančių mikroorganizmų atranka leido pakartotinai atrinkti laboratorinį *Achromobacter* sp. JS18 kamieną, galintį skaidyti 3-hidroksipiridino-2-karboksirūgštį bei iš dirvožemio išskirti naują 5HP kamieną galintį vieninteliu anglies ir energijos šaltiniu panaudoti 5-hidroksipiridino-2-karboksirūgštį. 16S rRNR geno analizės duomenimis 5HP kamienas priklauso *Pusillimonas* genčiai. Tai pirmas žinomas mikroorganizmas galintis skaidyti 5-hidroksipiridino-2-karboksirūgštį.

Tiriant *Pusillimonas* sp. 5HP ląsteles buvo nustatyta, kad šios ląstelės gali įsisavinti ne tik 5-hidroksipiridino-2-karboksirūgštį, bet ir 3-hidroksipiridiną, piridino-3-karbonitrilą ir/ar piridino-3-karboksirūgštį (nikotino rūgštį). Piridino-3-karbonitrilo skaidymas vyksta nikotino rūgšties keliu. Visi šie junginiai *Pusillimonas* sp. 5HP bakterijose indukuoja tris skirtingus katabolinius kelius, susidarant bendram metabolitui – 2,5-dihidroksipiridinui. Tiriant beląstelinį *Pusillimonas* sp. 5HP ekstraktą buvo pirmą kartą nustatyta ir dalinai išgryninta 5-hidroksipikolinato 2-monooksigenazė, katalizuojanti 5-hidroksipiridino-2-karboksirūgšties oksidacinį dekarboksilinimą, susidarant 2,5-dihidroksipiridinui. Tokiu būdu buvo parodytas dar neaprašytas degradacijos kelias, susidarant 2,5-dihidroksipiridinui iš 5-hidroksipiridino-2-karboksirūgšties.

Tiriant *Achromobacter* sp. JS18 ląstelių substratinį specifiškumą buvo nustatyta, kad bakterijose yra indukuojami trys skirtingi: piridino-2-karboksirūgšties, piridino-3-karboksirūgšties ir piridino-2,6-dikarboksirūgšties skaidymo keliai. Remiantis gautais tyrimų rezultatais, buvo pasiūlytas galimas minėtų piridino karboksirūgščių, įskaitant ir 3-hidroksipiridino-2-karboksirūgšties, skaidymo kelias. Visi skaidymo keliai veda į bendro metabolito – 2,5-dihidroksipiridino – susidarymą.

Tiriant *Sinorhizobium* sp. L1 ląstelės, buvo nustatyta, kad 3-hidroksipiridino ir piridino-3-karboksirūgšties skaidymai yra indukuojami procesai ir vyksta skirtingais keliais. 3-Hidroksimetilpiridinas *Sinorhizobium* sp. L1 ląstelėse yra oksiduojamas iki piridino-3-karboksirūgšties (nikotino rūgties). Tiek 3-hidroksipiridino, tiek ir piridino-3-karboksirūgšties skaidymas vyksta susidarant bendram metabolitui – 2,5-

dihidroksipiridinui. Tyrimų eigoje buvo pastebėta, kad *Sinorhizobium* sp. L1 ląstelėse 3-hidroksipiridinas ir piridino-3-karboksirūgštis indukuoja skirtingas 2,5-dihidroksipiridino dioksigenazės izoformas. Analizuojant *Sinorhizobium* sp. L1 genų biblioteką, buvo atrinktas DNR fragmentas, koduojantis dalį nikotino rūgšties katabolizme dalyvaujančių baltymų.

Šio darbo metu buvo išgrynintos trys 2,5-dihidroksipiridino 5,6-dioksigenazės, dvi nikotinato ir viena pikolinato dehidrogenazės, kurios yra palygintos su žinomais analogiškais baltymais 1-oje lentelėje.

1 lentelė. 2,5-Dihidroksipiridino 5,6-dioksigenazės, nikotinato dehidrogenazės, pikolinato dehidrogenazės palyginimas su žinomais analogiškais baltymais.

Šaltinis	K_M, mM	Specifinis aktyvumas, U/mg	pH optimumas	Subvienetų molekulinė masė, kDa	Natyvaus fermento molekulinė masė, kDa	Literatūros šaltinis
2,5-Dihidroksipiridino 5,6-dioksigenazės						
***Sinorhizobium* sp. L1 (A)**	**0,15**	**60**	**7,5-7,6**	**39,8**	**230**	Šis darbas
***Sinorhizobium* sp. L1 (B)**	**0,175**	**500**	**7,4-7,5**	**38,3**	**214**	Šis darbas
***Achromobacter* sp. JS18**	–	**0,3**	**7,5**	**38,7**	**227**	Šis darbas
Pseudomonas putida KT2440	0,07	2,3	8,0	39	243-244	Jimenez ir kt., 2008
Pseudomonas putida N9	–	38,3	8,0	38,5	242	Gauthier ir kt., 1971
Pseudomonas putida S16	0,168	7,0	6,5	40	–	Tang ir kt., 2012
Achromobacter sp. 2L	–	15,3	–	–	340	Cain ir kt., 1974
Nikotinato dehidrogenazės						
***Sinorhizobium* sp. L1**	**0,12**	**198**	**7,0**	**85; 28; 17,7**	–	Šis darbas
***Achromobacter* sp. JS18**	–	**0,79**	**5,6**	**97, 58, 33**	295	Šis darbas
Pseudomonas putida KT2440	–	1,18		128; 23,8	130	Jimenez ir kt., 2008
Comamonas testosteroni JA1	–	–	7,0	82,4; 46; 21	–	Jang ir kt., 2010
Pseudomonas fluorescens TN5	0,11	672	8,3	80; X	100	Hurh ir kt., 1994
Clostridium sp.	0,11	28,8	7,5-8,5	–	300	Holcenberg ir kt., 1969
Eubacterium barkeri	–	18	7,5-8,0	50; 37; 33; 23	160	Gladyshev ir kt., 1996
Bacillus niacini	1,0	0,54	7,5	85; 34; 20	300	Nagel ir kt., 1990
Bacillus sp.	0,07	0,83	7,4	–	400	Hirschenber ir kt., 1971
Pikolinato dehidrogenazės						
***Achromobacter* sp. JS18**	–	**0,9**	**7,5**	**85; 43; 30**	**295**	Šis darbas
Arthrobacter picolinophilus	–	0,636	7,5	–	230	Tate ir kt., 1974

Apibendrinant išgrynintų fermentų palyginimą galima teigti, kad išgrynintos ir ištirtos 2,5-dihidroksipiridino dioksigenazės yra panašios į žinomas, tačiau šio fermento A izoforma iš *Sinorhizobium* sp. L1 ląstelių išsiskiria specifiniu aktyvumu, kuris daugiau nei dešimt kartų didesnis už kitų analogiškų fermentų. Nikotinato dehidrogenazė iš *Achromobacter* sp. JS18 ląstelių skiriasi nuo žinomų subvienetų molekuline mase, optimalaus veikimo pH ir savo lokalizacija ląstelėje. Nikotinato dehidrogenazė iš *Sinorhizobium* sp. L1 ląstelių priešingai – yra panaši į žinomas, tačiau nuo kitų analogiškų fermentų skiriasi labai siauru specifiškumu substratams – galėjo oksiduoti tik nikotino rūgštį. Šiuo darbu buvo papildytos žinios ir apie pikolinato dehidrogenazes. Identifikuotas ir dalinai charakterizuotas fermentas skyrėsi nuo žinomo savo substratiniu specifiškumu bei lokalizacija ląstelėje.

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Scientific publications:

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