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SYNTHESIS OF POLYHYDROXYLIC COMPOUNDS BY BIOCATALYTIC METHODS

Summary of doctoral thesis

Physical sciences, biochemistry (04 P)

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VILNIAUS UNIVERSITETAS

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INTRODUCTION

For many centuries biocatalysis has been used as a technology for producing various compounds (Roberts et al., 1995). However, the investigation of biocatalysts as tools for organic syntheses began only in 1980s. Many scientists attempted to apply enzymes or whole cells for the syntheses of chiral compounds. Much of the research was then focused on either the hydrolytic enzymes (e.g., lipases, proteases and acylases) or the redox enzymes (e.g., alcohol dehydrogenases and lyophilized yeasts) (Drauz et al., 2012).

Nowadays, a much wider range of biocatalysts is available, therefore unique decisions and new processes can have applications in pharmaceutical, pulp and paper industries, in the production of food and bulk chemicals as well as in agriculture or medicine (Fessner ir Anthonsen, 2009). The increase in the demand of chiral alcohols, especially compounds containing multiple chiral centers, has encouraged the development of new biocatalysts (Zeng ir Sabra, 2011). However, there is still a deficiency of enzymes performing efficient catalysis. The need to improve and develop new methods for the biosynthesis of alcohols is felt.

Selective hydroxylation of aromatic ring is a challenging task in organic synthesis (Ullrich ir Hofrichter, 2007). The hydroxylated pyridines are compounds of commercial interest because of their wide applications in the production of herbicides, insecticides, fungicides and plant growth regulators. Moreover, pyridine derivatives are attractive pharmaceutical synthons. Currently, pyridines are used either as biologically active substances or as building blocks for polymers with unique physical properties (Scriven ir Murugan, 2005).

Biocatalysis is an attractive tool for the syntheses of various hydroxylated pyridines. Under aerobic conditions in bacteria the metabolism of alkylated pyridines and pyrazines begins with hydroxylation of an aromatic ring at a free position. Such reactions are catalyzed by catabolic oxidoreductases, which could be applicable for regioselective hydroxylation of *N*-heterocycles (Drauz et al., 2012; Sun et al., 2014).

The amount of new regioselective biocatalysts – enzymes or whole cells – for the modification of pyridine derivatives in organic synthesis is insufficient. Although data

on the bacterial metabolism of pyridines is abundant, there is still a lack of enzymes with wide substrate specificity.

For the reasons mentioned above, **the objective of this study** was to investigate and evaluate the opportunities of synthesis of polyhydroxylated compounds using biocatalytic methods. The following tasks have been formulated to attain this aim:

- To develop a reactor for synthesis of dihydroxyacetone phosphate in which hydrogen peroxide is eliminated electrochemically.
- To determine the enantioselectivity of alcohol dehydrogenase IIG from *Pseudomonas putida* HK5 and to evaluate the applicability of this enzyme for the synthesis of chiral alcohols.
- To investigate substrate specificity of the recombinant protein TpdE from *Rhodococcus jostii* TMP1.
- To evaluate the synthesis potential of optically pure diols *in vitro* and *in vivo* using recombinant alcohol dehydrogenase TpdE.
- To evaluate the diversity of hydroxypyridines produced and the selectivity of hydroxylation using *Burkholderia* sp. MAK1 as a whole cell biocatalyst.

Scientific novelty:

Dihydroxyacetone phosphate (DHAP) is an important substrate in the biosynthesis of carbohydrates. Using L-α-glycerophosphate oxidase for the synthesis of DHAP, hydrogen peroxide is formed. This byproduct is detrimental to L-α-glycerophosphate oxidase. In this work we attempted to improve the efficiency of DHAP production. We have designed the DHAP bioreactor in which the working electrode consisted of a Prussian Blue-modified carbon fibre and L-α-glycerophosphate oxidase. In this unique reactor hydrogen peroxide was eliminated electrochemically. The main advantage of DHAP bioreactor is that there is no need for the additional enzyme that disrupts hydrogen peroxide. The results of this study open a possibility to apply the Prussian Blue-modified electrodes for optimization of other biocatalytic processes where hydrogen peroxide-producing oxidases are used.

The ability of enantioselective oxidation of various primary and secondary aliphatic and cyclic alcohols as well as amino alcohols by alcohol dehydrogenase IIG

from *Pseudomonas putida* HK5 has been shown for the first time. The study revealed that the enzyme prefers *(S)*-enantiomers. Thus, alcohol dehydrogenase IIG from *P. putida* HK5 could be applied for the production of enantiomerically pure alcohols using kinetic resolution of racemates.

It was demonstrated that alcohol dehydrogenase TpdE from *Rhodococcus jostii* TMP1 exhibits broad substrate specificity towards aliphatic 2,3-diketones, various acyloins and ketoesters, and an excellent enantioselectivity, which is a very important factor when determining the potential value of an enzyme. The GC-MS analysis of the reduction products of 2,3- and 3,4-diketones indicated that TpdE is able to reduce both keto groups in its substrate. Thus, two new chiral atoms in the product molecule are formed. Bioconversions of diketones to corresponding diols occurred using either pure enzyme or a whole *Escherichia coli* BL21 (DE3) cell biocatalyst with the recombinant TpdE. Because of its broad substrate specificity, stereoselectivity and biocatalysis *in vivo* or *in vitro* TpdE is an attractive tool for the asymmetric ketone reduction to corresponding chiral alcohols.

Oxyfunctionalization of aromatic compounds using a biocatalyst (enzyme or whole cell) is a convenient way for the production of desirable products. We have investigated the hydroxylation of pyridine derivatives by the cells of *Burkholderia* sp. MAK1. This whole cell biocatalyst oxidized 73 of the 110 tested aromatic compounds, mostly pyridines, pyrazines and pyrimidines. UV-VIS and HPLC-MS analyses revealed that hydroxylation occurs at 5-position of the ring of 2-hydroxy- and 2-aminopyridines containing methyl-, chloro-, bromo- and other substitutions. In order to prove the position of regioselctive hydroxylation several products have been purified and identified by ^{1}H and ^{13}C NMR spectroscopy. To our knowledge, biosynthesis of amino pyridinols has been shown for the first time. The newly synthesized 6-amino-4-chloro-3-hydroxypyridine, 6-amino-4-fluoro-3-hydroypyridine and 2-amino-4,5-dihydroxypirimidine are of great potential to be applied for the production of pharmaceuticals and antioxidants. In addition, it has been discovered that *Burkholderia* sp. MAK1 cells are capable of oxidizing pyridine, pyrazine and their methylated derivatives to corresponding N-oxides. The cells of *Burkholderia* sp. MAK1 can be immobilized in calcium alginate gel. This is beneficial because the process of biocatalysis becomes easier to manage, while the properties of the catalyst remain

unchanged. Thus, we have described a new whole cell biocatalyst which can be applied for the regioselective synthesis of hydroxypyridines.

Thesis statements:

1. Synthesis of dihydroxyacetone phosphate occurs together with elimination of hydrogen peroxide when the electrocatalytic system consisting of L-α-glycerophosphate oxidase from *Enterococcus sp.* RL1 and Prussian Blue-modified carbon fiber is used.
2. Alcohol dehydrogenase IIG from *Pseudomonas putida* HK5 is an enantioselective enzyme and prefers (*S*)-(+) alcohols.
3. TpdE from *Rhodococcus jostii* TMP1 is a NADPH-dependent classic short chain dehydrogenase/reductase capable of reducing diketones.
4. TpdE catalyzes stereoselective reduction of diketones *in vivo* and *in vitro*.

Contents of the doctoral thesis

The dissertation is written in Lithuanian and contains the following parts: Introduction, Literature Review, Materials and Methods, Results and Discussion, Conclusions, List of References (176 positions), Tables (4), Figures (55). Total 134 pages.

MATERIALS AND METHODS

Reagents

Reagents and kits for molecular biology were from Thermo Fisher Scientific (Lithuania) or from Zymo Research Corporation (USA). PCR primers were purchased from Metabion (Germany). All other reagents were of analytical grade and were used as received without further purification.

Bacterial strains and plasmids

Escherichia coli DH5α [ϕ80dlacZΔM15 Δ(lacZY-argF)U169 deoR recA1 endA1 hsdR17($r_K^-m_K^+$) sup E44 thi-1 gyrA96 relA1] from Pharmacia (Sweden) was used for cloning experiments. pGPO and pTpdE were expressed in *Escherichia coli* BL21 (DE3) [F^- ompT gal $hsdS_B$ ($r_B^-m_B^-$) dcm lon (DE3)] Avidis (France). *Burkholderia* sp. MAK1 was previously isolated from soil samples (Gasparavičiūtė, 2008).

pTZ57R/T – Ap^r, oriColE1, lacZα, from Thermo Fisher Scientific (Lithuania). pET-28b(+) from Novagen (Germany). pET-*tpdE* – *tpdE* gene cloned into pET-21b(+) vector. pET-R19T – *tpdE* gene with a point mutation cloned into pET-28b(+) vector. pGPO – *gpo* gene cloned into pET-21d(+) vector.

Purification of the recombinant GPO from Enterococcus sp. RL1

Escherichia coli BL21 (DE3)/pGPO cells used for GPO expression were grown in the Brain Heart Infusion medium (Oxoid, UK) containing ampicillin (50 mg l^{-1}) and induced with isopropyl β-D-1-thiogalactopyranoside (0.05–0.5 mM). The GPO was purified on the HiTrap IMAC FF (GE, USA) column. For the elution of bound proteins, a linear gradient of imidazole (0–0.5 M) was used. The fractions containing GPO activity were pooled and dialyzed against 50 mM phosphate buffer solution, pH 7.2. The stock solution of GPO had an activity of 470 U ml^{-1} (16.6 mg protein ml^{-1}) in 50 mM phosphate buffer solution, pH 7.2. Protein concentration was measured routinely by the method of Lowry, using bovine serum albumin as a standard.

GPO assay procedures

GPO activity was assayed using the peroxidase/*o*-dianisidine method by measuring H_2O_2 formation. The reaction mixture contained 50 mM phosphate buffer solution, pH 7.2, 0.3 mM *o*-dianisidine, 5 U of horseradish peroxidase, 25 mM of GP, and an appropriate amount of the enzyme solution in a total volume of 1.0 ml. The reaction was started by adding the GPO, and an increase in the absorbance at 430 nm was recorded. One unit of GPO activity was defined as an amount of the enzyme that catalyzed the formation of 1 μmol of H_2O_2 per minute under the conditions of the assay.

Electrode pre-treatment and modification

Carbon fibre (CF) cloth was sonicated in the presence of non-ionic detergent for 10 min, rinsed in water and dried at room temperature. The CF cloth was then sealed into a plastic jacket with Cu wire contact to fabricate an electrode with working area of 20 cm^2. The mixture of 50 mM $K_3[Fe(CN)_6]$ and 50 mM $FeCl_3$ dissolved in 100 mM HCl solution was used to modify the CF cloth. CF electrode was immediately immersed in the mixture and incubated for 10 min at room temperature. The PB directly precipitated on the electrode surface. The PB modified CF (CF/PB) electrode was then washed with 100 mM HCl and dried at 80 °C for 90 min.

Reactor construction

CF/PB/GPO cloth (4 × 5 cm) for the reactor was prepared by covalent immobilization of GPO on CF/PB. In total, 200 μl of GPO stock solution was mixed with 200 μl of 5% (w/v) gelatine solution in phosphate buffer solution, pH 7.0, and 5 μL of 5% (v/v) glutaraldehyde. The mixture was deposited on the CF/PB surface immediately. A working electrode in reactor I was made of CF/PB (Fig. 1a), whilst in reactor II it was made of CF/PB/GPO (Fig. 1b). The working electrode was placed into the cell made of a plastic tube (7 cm in length, 2.5 cm in diameter). The stainless steel tube of 4 cm in length and 0.5 cm in diameter, connected to the outlet of the cell, was used as an auxiliary electrode. The cell was additionally equipped with an Ag/AgCl reference electrode.

The system was filled with 30 ml of 5 or 50 mM GP solution prepared in 50 mM phosphate buffer solution, pH 7.2. The substrate was pumped at a flow rate of

50 ml min^{-1}. The samples were taken periodically to determine the concentrations of GP, DHAP and H_2O_2.

Determination of hydrogen peroxide, DHAP and GP

Concentration of H_2O_2 was measured using a three-electrode cell (1 ml) equipped with a magnetic stirrer, using a working Pt electrode (diameter 1 mm) at 0.6 V vs. Ag/AgCl and Ti plate as an auxiliary electrode. The linear range of the assay was 0.01–10 mM of H_2O_2. DHAP was analyzed using glycerol-3-phosphate dehydrogenase. The standard reaction mixture contained 50 mM phosphate buffer solution, pH 7.2, 0.2 mM NADH, 2.8 U glycerol-3-phosphate dehydrogenase, and the sample from the reactor in a total volume of 1 ml. The assay was started by the addition of the sample and conducted at 25 °C. The change in absorbance at 340 nm resulting from NADH oxidation (ε_{340} = 6220 M^{-1} cm^{-1}) was recorded. After the removal of H_2O_2 from the samples with an excess of MnO_2, GP was determined spectrophotometrically by a GPO-peroxidase coupled assay. The standard reaction mixture contained 50 mM phosphate buffer solution, pH 7.2, 0.3 mM *o*-dianisidine, 5 U of horseradish peroxidase, 2.3 U of GPO, and the sample in a total volume of 1 ml. The assay was started by the addition of GPO and conducted at 25 °C. The absorbance at 420 nm was recorded. The GP standards from 0 to 0.25 mM of GP were analysed in parallel.

Electrochemical measurements were performed using a potentiostat Voltalab PGZ 402 (Radiometer).

Expression and purification of the recombinant TpdE from R. jostii TMP1

E. coli BL21 (DE3) was routinely grown in Nutrient Broth (NB) or Brain Heart Infusion (BHI) medium and was used as the TpdE expression host. Culture was grown at 30 ºC in the medium containing 50 mg ampicillin/l to maintain the plasmids. Growth was monitored from the OD_{600} values. TpdE expression was induced by the addition of IPTG at 30 ºC for 4–19 h. Cells were harvested by centrifugation, and the pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.2) containing 0.1 M NaCl. The cell suspension was disrupted by sonication, and the crude cell extract was centrifuged for 10 min at 4000 rpm. The TpdE tagged with C-$(His)_6$ was purified from clear supernatant by affinity chromatography using Ni^{2+}-chelating column and desalted by

dialysis in 50 mM potassium phosphate buffer (pH 7.2) or using HiTrap Desalting column.

Activity measurements of TpdE

The activity of TpdE was determined spectrophotometrically by monitoring the decrease in absorption at 340 nm resulting from the oxidation of NADPH at 30°C. The assay mixture consisted of 0.2 mM NADPH, 10 mM substrate and 50 mM phosphate buffer (pH 7.2). The reaction was started by the addition of the substrate.

Determination of optimal pH for TpdE

The pH optimum of the enzyme was determined using the following buffer systems: sodium citrate (pH 4.5–6.0), Tris-HCl (pH 5.7–8.5), sodium phosphate (pH 6–8), sodium succinate (pH 4–6), glycine-NaOH (pH 8.6–9.5) and MES (pH 5.3–6.8). The concentration of the buffers was 50 mM. The activity was assayed spectrophotometrically by measuring a decrease in absorption at 340 nm resulting from oxidation of NADPH at 30°C. In all reactions diacetyl was used as the second substrate.

Determination of thermostability of TpdE

The stability of the TpdE was investigated at 25°C–50°C. The enzyme solution was kept at different temperatures for 10 min in 50 mM phosphate buffer (pH 7.2) and then immediately cooled on ice. The residual activity was measured at 30 °C as described previously.

HPLC-MS analyses

HPLC-MS analyses were performed using a high performance liquid chromatography system (CBM-20A controller, two LC-2020AD pumps, SIL-30AC auto sampler and CTO-20AC column oven; Shimadzu, Japan) equipped with a photo diode array (PDA) detector (SPD-M20A Prominence diode array detector; Shimadzu, Japan) and a mass spectrometer (LCMS-2020, Shimadzu, Japan) equipped with an ESI source. The chromatographic separation was conducted using a Hydrosphere C18 column, 4 × 150 mm (YMC, Japan), or YMC Pack Pro column, 3× 150 mm (YMC, Japan) at 40 °C and a mobile phase that consisted of 0.1 % formic acid water solution (solvent A),

and acetonitrile (solvent B) delivered in the gradient elution mode. Mass scans were measured from m/z 10 up to m/z 500, at 350 °C interface temperature, 250 °C DL temperature, ±4,500 V interface voltage, neutral DL/Qarray, using N 2 as nebulizing and drying gas. Mass spectrometry data was acquired in both the positive and negative ionization mode. The data was analyzed using the LabSolutions LCMS software.

GC-MS analyses

GC-MS analyses were performed with a Shimadzu GCMS-QP2010Ultra Plus (Kyoto, Japan). Chromatographic separation was achieved on a Rtx®-1701 column (30 m × 0.25 mm I.D., 0.25 μm film thickness with 5m Integra-guard, Restek, USA) using helium as carrier gas at 40 cm/sec in a constant linear velocity mode. The GC oven temperature was initially increased from 80 °C to 250 °C at a rate of 15 °C/min and holding at 250 °C for 2.7 min, giving a total run time of 15 min. The temperatures of injector, interface, and Ion source were 250, 270, and 220 °C, respectively. Detection was operated by selected ion monitoring (SIM) mode (70 eV, electron impact mode).

In the SIM mode, the peaks were identified by matching the retention time and the abundant ions (m/z). The injection volume was 0.3 μL. Data was collected and analyzed using the GC-MS solution version 2.71 (Kyoto, Japan).

Metabolite structural analysis

Product structures were determined using ^{1}H NMR, ^{13}C NMR and MS analyses. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Ascend 400 spectrometer. The samples were dissolved in DMSO-d_6.

Bioconversion of pyridines, pyrimidines and pyrazines using the cells of Burkholderia sp. MAK1

0,05 g of wet biomass of *Burkholderia* sp. MAK1 cells was suspended in 1 ml of 10 mM potassium phosphate buffer, pH 7.2. The suspension was supplemented with 6 μl of 40 % glucose water solution and with 0.25 mM of corresponding substrate. The suspension was incubated at 30 °C. The process of the conversion was followed by HPLC-MS.

RESULTS AND DISCUSSION

Bioreactor for DHAP production

Although a number of methods for DHAP synthesis have been already described (Charmantray et al., 2004; Enders et al., 2005; Schumperli et al., 2007), the efficient biocatalytic system, which could be successfully applied to the biotechnological production of DHAP, is yet to be developed. The aim of this work was the creation of the L-α-glycerophosphate oxidase bioreactor containing an integrated electrochemical module that removes hydrogen peroxide. For this, the reactor I was constructed. It consisted of two parts: one (carbon fibre/Prussian Blue/L-α-glycerophosphate oxidase abbreviated CF/PB/GPO) with immobilized enzyme and the other (carbon fibre/Prussian Blue – CF/PB) – as a working electrode for decomposition of hydrogen peroxide (Fig. 1a). After the reactor I was filled with 5 mM L-α-glycerophosphate solution, the production of DHAP and hydrogen peroxide was measured periodically.

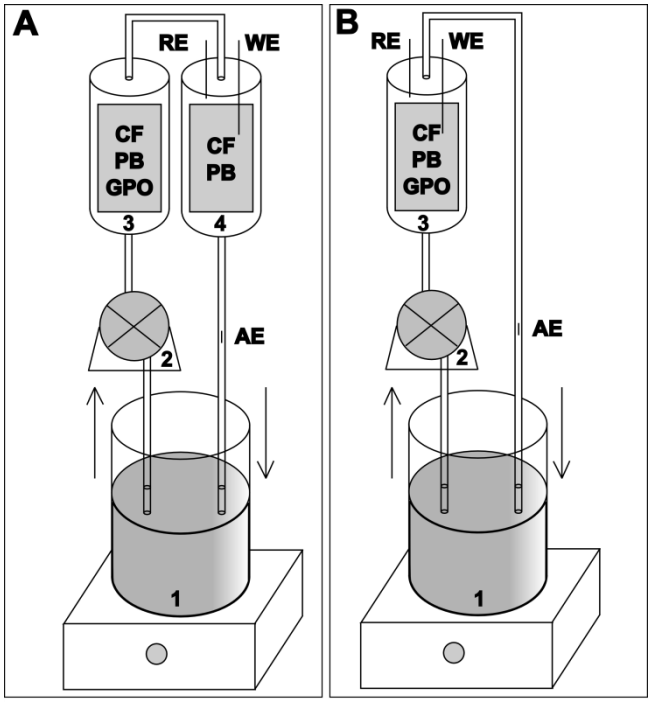


Fig. 1. The semi-continuously operating reactor for DHAP production. (A) reactor I, (B) reactor II. RE – reference electrode, WE – working electrode, AE – auxiliary electrode. 1 – substrate solution, 2 – peristaltic pump, 3 – CF/PB/GPO cloth (A) or electrode (B), 4 – CF/PB electrode.

Noteworthy, during the first 60 min of the experiment, the working electrode was turned off. As shown in Fig. 2a, during the first stage of bioconversion the amount of hydrogen peroxide produced was equal to that of synthesized DHAP. When the CF/PB electrode was turned on, hydrogen peroxide concentration stopped increasing while DHAP was further produced, indicating that the CF/PB electrode eliminates hydrogen peroxide.

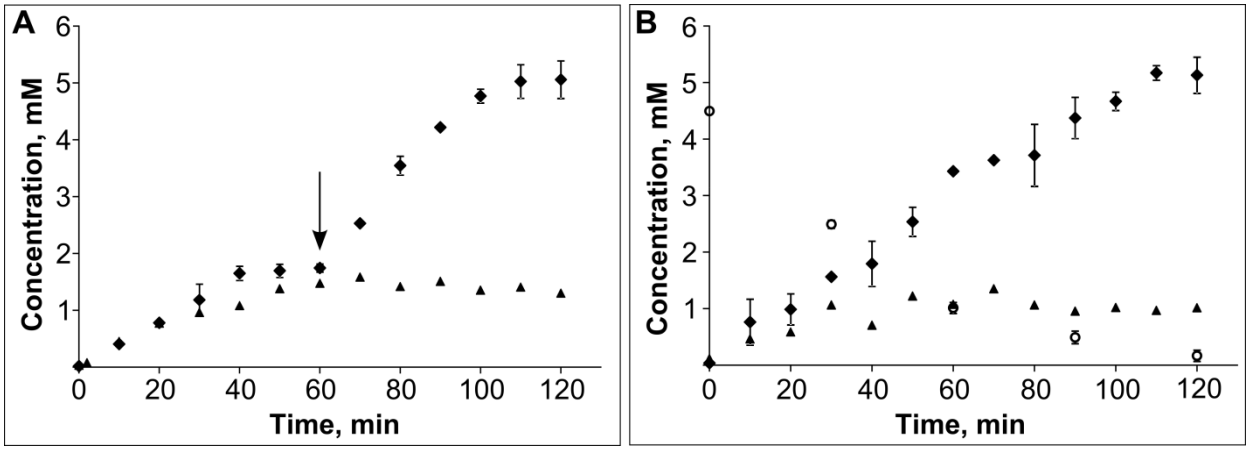


Fig. 2. Electrochemical conversion of L-α-glycerophosphate into DHAP in the reactor I. CF/PB electrode potential +500 mV; 30 ml of 5 mM L-α-glycerophosphate stock solution was pumped through the column at the rate of 50 ml min^{-1}. (A) DHAP and H_2O_2 production in the absence of the active CF/PB electrode during the first 60 min of the process; the arrow indicates the moment when the CF/PB electrode was turned on. (B) CF/PB electrode turned on throughout the conversion. Closed diamond – DHAP; open circle – L-α-glycerophosphate; closed triangle – H_2O_2.

When the working electrode was turned on at the beginning of the experiment (Fig. 2b), the rate of DHAP production was approximately 0.05 mM min^{-1}, and L-α-glycerophosphate was almost completely (>98 %) converted to DHAP in 2 hours. Within the first 30 min of the process the maximum of hydrogen peroxide concentration (1.2 ±0.3 mM) was observed, and it was stable throughout the bioconversion.

Optimization of the DHAP bioreactor

Substrate concentration is a very important parameter in the economy of bioconversion process, since the higher the concentration of substrate, the lower the cost of the product separation. Therefore, the efficiency of the reactor I at higher initial concentration of substrate was examined. The system was filled with 50 mM L-α-

glycerophosphate solution. Such increase in the substrate concentration accelerated the rate of DHAP production 3 times.

To simplify the system, the reactor II consisting of CF/PB/GPO as a working electrode for decomposition of hydrogen peroxide was constructed (Fig. 1B). No operational differences between reactors I and II were observed. The amount of DHAP produced was equivalent to the amount of L-α-glycerophosphate consumed throughout the conversion (Fig. 3). Results from figure 3 demonstrate that 64 ±5 % of L-α-glycerophosphate was consumed for the production of DHAP. Moreover, the concentration of hydrogen peroxide did not increase above 2 mM. Thus, it can be concluded that catalase-independent DHAP production system presented here is both high-speed and immensely efficient.

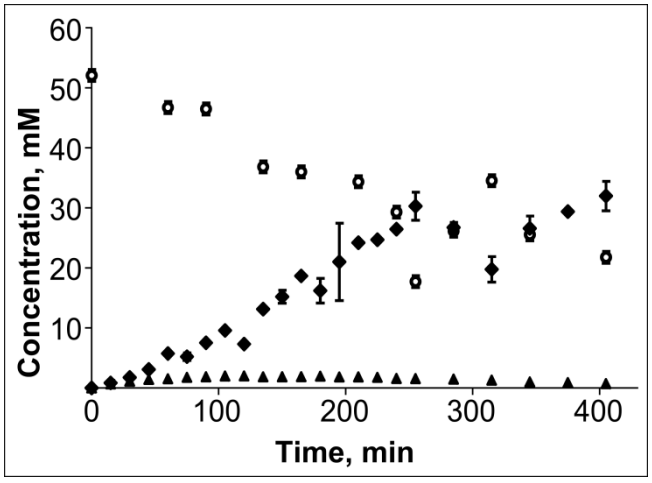


Fig. 3. Electrochemical conversion of L-α-glycerophosphate into DHAP in the reactor II. CF/PB/GPO electrode potential +500 mV; 30 ml of 50 mM L-α-glycerophosphate stock solution was pumped through the column at the rate of 50 ml min^{-1}. Closed diamond – DHAP; open circle – L-α-glycerophosphate; closed triangle – H_2O_2.

This is the first report that demonstrates the application of the Prussian Blue-modified carbon fibre electrode for the elimination of hydrogen peroxide. Such electrode with the immobilized L-α-glycerophosphate oxidase can be used as an efficient electrochemical reactor for DHAP production. Moreover, this study opens a possibility to apply the Prussian Blue-modified electrodes for optimization of other biocatalytic processes where hydrogen peroxide-producing oxidases are used.

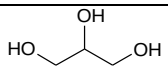
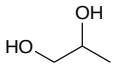
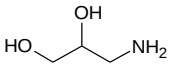
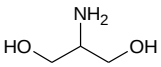
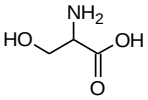
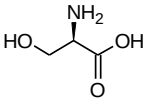
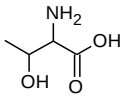
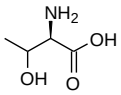
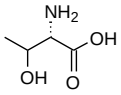
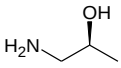
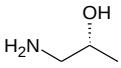
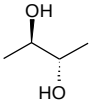
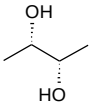
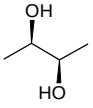
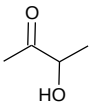
Oxidation of amino alcohols and vicinal diols by ADH IIG from *Pseudomonas putida* HK5

The potential of ADH IIG from *Pseudomonas putida* HK5 to oxidize amino alcohols and vicinal diols was investigated. The results in Table 1 demonstrate that the position of the amino group in propanediol is important to ADH IIG and the rate of oxidation differs more than three-fold between 2-amino-1,3-propanediol and (±)-3-amino-1,2-propanediol. The new and promising ability of ADH IIG to oxidize amino alcohols has been shown for the first time. Summarizing the results obtained from all tested amino alcohols, it is obvious that ADH IIG prefers 2-amino-1,3-propanediol as a substrate. In this case, 2-amino-3-hydroxypropionaldehyde should be formed as the product of oxidation by ADH IIG. Similar compounds are very unstable and can undergo self-condensation due to their structure. Self-condensation of two molecules of α-aminocarbonyl compounds is a well known process described in a classical scheme of pyrazine synthesis.

The results of HPLC-MS and GC-MS analyses have proved that ADH IIG from *P. putida* HK5 catalyzes the oxidation of 2-amino-1,3-propanediol to 2-amino-3-hydroxypropionaldehyde, which spontaneously undergoes self condensation and 2,5-dihydroxymethylpyrazine is formed. Hence, there is a good possibility of ADH IIG application for the synthesis of different pyrazines at appropriate conditions (Eicher and Hauptmann, 2003).

In parallel experiments enantioselectivity of ADH IIG was determined. The enantioselectivity of the enzyme was studied using 2,3-butanediol as a test substrate. As outlined in Table 1, ADH IIG preferably oxidized *(2S,3S)*-(+)-2,3-butanediol, whereas relative activity of the enzyme decreased more than five-fold using *meso*-2,3-butanediol as a substrate. Moreover, *(2R,3R)*-(–)-2,3-butanediol was a poor substrate for ADH IIG and relative activity of the enzyme differed more than twenty-fold when *(2S,3S)*-(+)- and *(2R,3R)*-(–)- butanediols were used. It should be mentioned that no ADH IIG activity was found in the reaction mixture with acetoin as a substrate. Previous studies of quinohemoprotein ADHs isolated from *Acetobacter*, *Gluconobacter* and *Comamonas* species elucidated an appreciable enantioselectivity in the oxidation of *(R)*- or *(S)*-secondary alcohols (Jongejan et al., 2000). During the oxidation of chiral 2,3-butanediol a preference of ADH IIG from *P. putida* HK5 for *(S)*-(+) alcohol was revealed. Because

Table 1. Substrate relative activity of free and immobilized ADH IIG. Activity was determined as described in Experimental section.

Substrate		Relative activity of free ADH IIG, %	Relative activity of ADH IIG-GA-SS, %
Glycerol		100	100
1,2-Propanediol		119	na*
(±)-3-Amino-1,2-propanediol		23.8	34
2-Amino-1,3-propanediol		74.4	83
rac-Serine		0	na
(*R*)-Serine		0	na
rac-Threonine		0	na
(*R*)-Threonine		0	na
(*S*)-Threonine		0	na
(*S*)-(+)-1-Amino-2-propanol		0.8	0.2
(*R*)-(–)-1-Amino-2-propanol		0	0
meso-2,3-Butanediol		19.6	na
(*2S,3S*)-(+)-2,3-Butanediol		106.8	115
(*2R,3R*)-(–)-2,3-Butanediol		5.2	na
Acetoin		0	na

*na – not analysed

of the evident enantio preference of the enzyme, ADH IIG can become an attractive tool for the enantiospecific oxidation.

Bioinformatic analysis of TpdE protein

The amino acid sequence of TpdE was compared to its closest relatives by sequence and/or by function. The analysis of amino acid sequence of TpdE revealed that the protein has the highest similarity to 3-oxoacyl-[acyl-carrier proteins] reductases (Fig. 4). Therefore, it can be stated that TpdE is a 3-oxoacyl-[acyl-carrier protein] reductase and belongs to the short chain dehydrogenase/reductase (SDR) family. However, the functions of the proteins with homologous sequences are experimentally unverified. The TpdE sequence analysis showed that the protein has a NAD(P)H/NAD(P)$^+$-binding domain – Rossmann fold – which is common for SDR superfamily.

The comparison of TpdE with the other SDRs showed that amino acids Ser148, Tyr161 and Lys165 are involved in the catalytic triad of TpdE. Usually, in catalytic triad Tyr accepts a proton by acting as a base, Ser stabilizes a substrate, and Lys interacts with the ribose of nicotinamide and lowers the pKa value of Tyr (Oppermann et al., 2003). According to the length of the TpdE chain, the conservative motifs of cofactor binding and active site, this enzyme can be assigned to the classic family of SDRs.

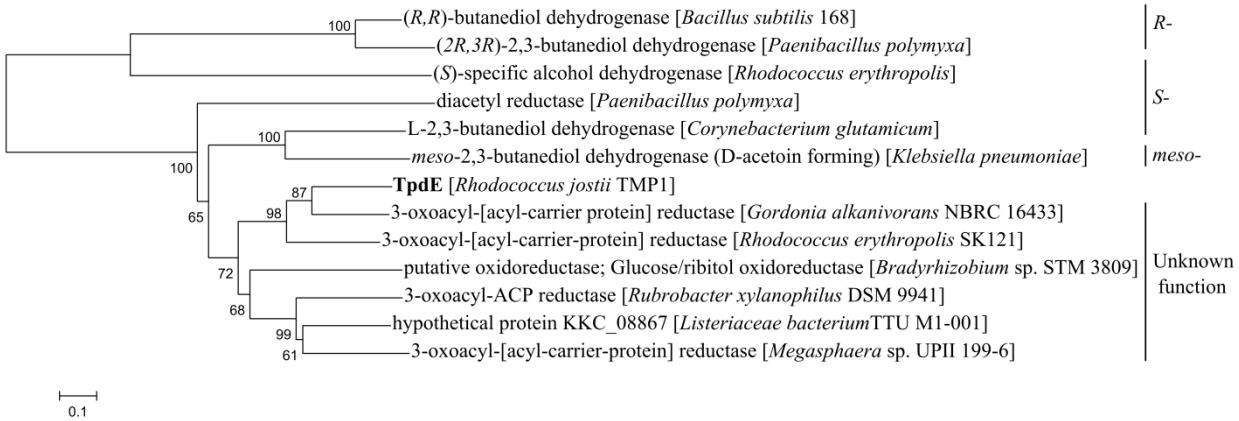


Fig. 4. Phylogenetic analysis of amino acid sequence of TpdE from *Rhodococcus jostii* TMP1. The tree was constructed by the program MEGA 4 using the neighbor-joining method.

TpdE showed a low pairwise sequence identity (<37 %) to SDRs with known functions.

Expression and purification of TpdE

In order to obtain a large amount of the TpdE protein, the study of the optimal conditions for the expression of the gene was performed. The largest amount of the active enzyme was produced by *E. coli* BL21 (DE3) pET-*tpdE* cells in BHI medium when synthesis of the protein was induced with 0.1 mM IPTG at OD_{600} 0.8 for 17 hours at 30 °C. C-terminal $(His)_6$-tagged TpdE protein was purified by affinity chromatography using Ni^{2+}-chelating column. After a one step procedure, TpdE was purified to near homogeneity.

The study of the progress of TpdE-catalyzed reaction and biochemical characterization of the enzyme

At first it was demonstrated that TpdE is capable of reducing diacetyl when NADPH is used as co-substrate. Then the progress of the reaction was investigated using diacetyl or acetoin as a substrate. It was shown that TpdE catalyzes diacetyl reduction to acetoin and then acetoin is converted to 2,3-butanediol (Fig. 5).

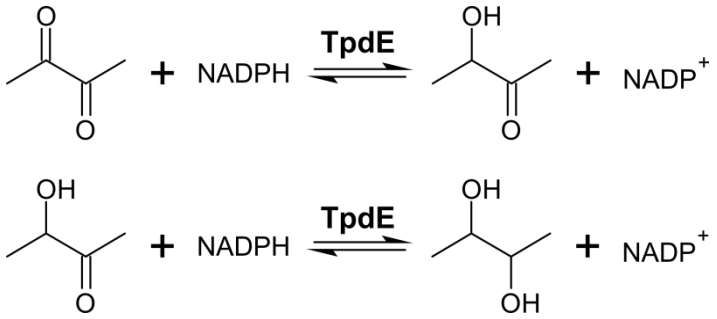


Fig. 5. The two step diacetyl conversion to 2,3-butanediol catalyzed by TpdE.

The effect of pH on the activity of TpdE was investigated within a range of 4.0-9.5 at 30 °C. The TpdE retained more than 50% of its activity between pH 6.0 and 8.0. Maximum activity for ketone reduction was observed at pH 7.5. In addition, the activity of TpdE reached the highest level when Na-phosphate and Tris-HCl buffer solutions were used.

An important parameter for characterization of enzymes is their optimal temperature. Therefore, the effect of temperature on the activity and stability of TpdE was examined. Maximum activity of TpdE was observed at 35–40 °C. Further increase in temperature caused a sharp reduction of the activity of the enzyme, which is most

likely associated with the loss of native protein structure. Termostability of TpdE was investigated in the range of 25–50 °C. After incubation at 40 °C, 71 % of the activity was lost. Moreover, the incubation at 45 °C and higher temperatures led to near complete inactivation of the enzyme. These results suggest that irreversible denaturation of the protein occurs at temperature higher than 35 °C. It can be summarized that optimal temperature for the action of TpdE is 30–35 °C. At higher temperatures, the reaction is faster, however the enzyme becomes unstable. Thus, higher reaction rate does not compensate for the loss of activity resulting from protein denaturation.

TpdE – a promising tool for the production of chiral alcohols

To determine the substrate specificity of TpdE, various ketones, acyloins and ketoesters were tested as potential substrates (Fig. 6). The study revealed that TpdE is able to reduce a variety of acyloins, diverse ketoesters and 2,3- and 3,4-diketones, differing in the length of the chain (Fig. 7).

In addition, the kinetic parameters of TpdE were determined. The highest value of specificity constant k_{cat}/K_M was defined for butan-3-one-2-yl-butanoate as a substrate. K_M values of 2,3-diketones ranged depending on chain lengths: longer chain of the substrate caused an increase in K_M value.

NADPH is a co-substrate of TpdE. That means that the constant supply of NADPH must be ensured for successful biocatalysis. Using a whole cell catalyst could be implemented as an efficient regeneration system for NADPH. Therefore, the bioconversion of diacetyl to 2,3-butanediol by *E. coli* BL21 (DE3) pTpdE cells was examined. As shown in Fig. 8, diacetyl was almost completely consumed after the 18 h of incubation. Thus, *E. coli* BL21 (DE3) pTpdE cells can act as a whole cell biocatalyst for production of 2,3-butanediol. In parallel experiments it was demonstrated that the same cells are capable of reducing 2,3-pentanedione, 2,3-hexanedione and 2,3-heptanedione.

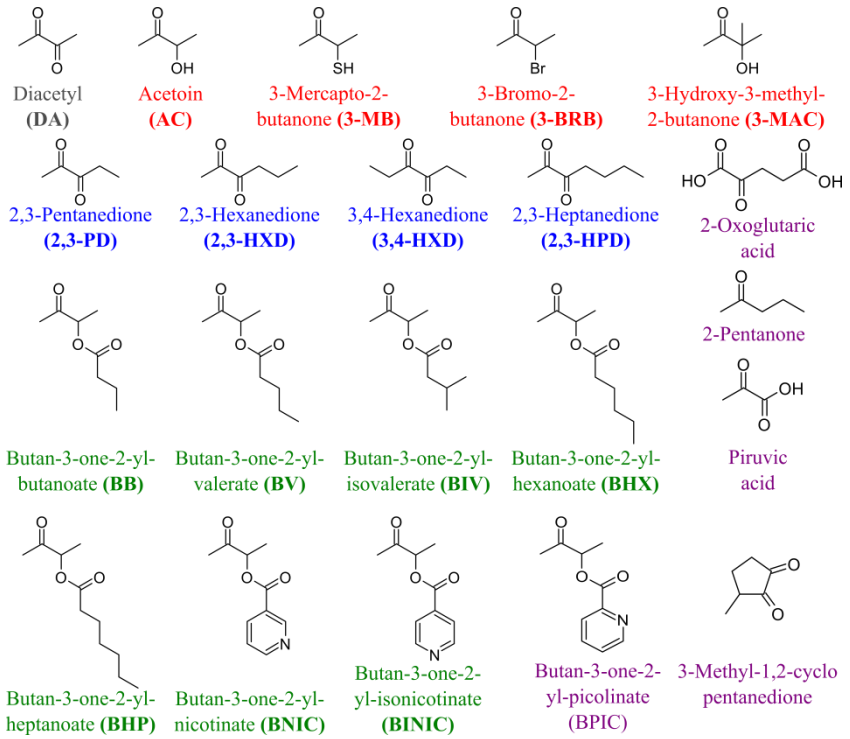


Fig. 6. The compounds used as potential TpdE substrates. Red are acyloins, blue – diketones, green – ketoesters, grey – diacetyl, purple are compounds that have not been reduced by TpdE.

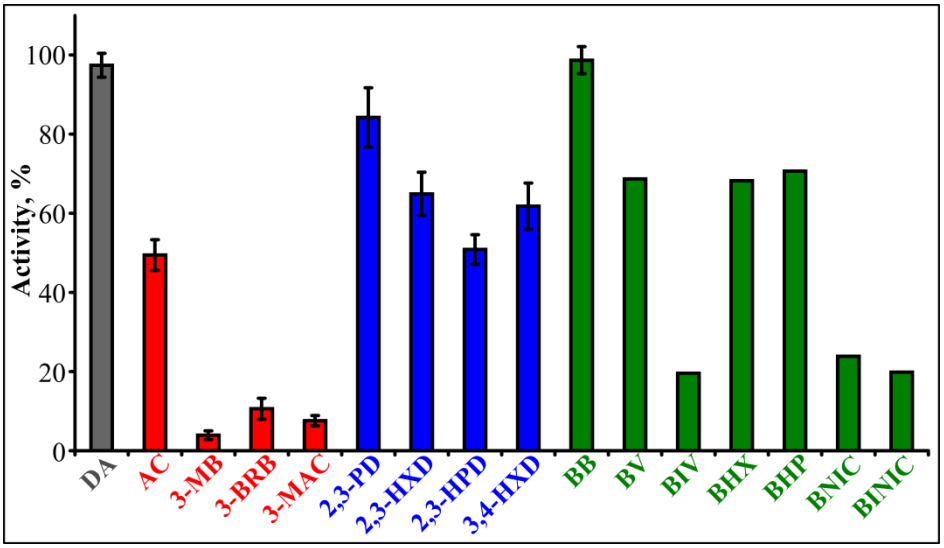


Fig. 7. The dependence of TpdE activity on the substrate used. TpdE activity was measured in phosphate buffer (50 mM, pH 7.2) containing 0.2 mM NADPH and 10 mM of corresponding substrate. 100 % is equal to the activity of TpdE using diacetyl as substrate.

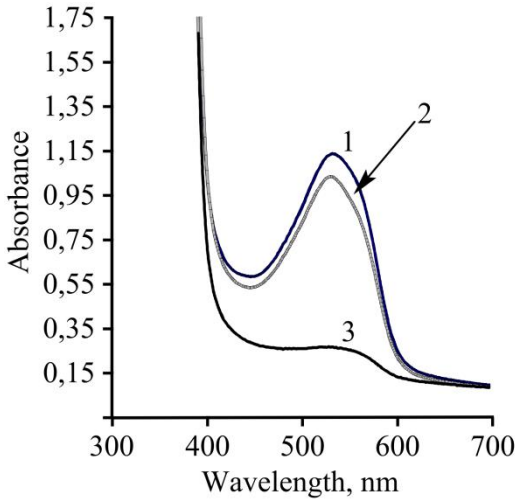


Fig. 8. The consumption of diacetyl in *E. coli* BL21 (DE3) pTpdE cells. Diacetyl was derivatized with α-naphthol. 1 – initial absorption of diacetyl, 2 – control sample, diacetyl was incubated for 18 h in 50 mM phosphate buffer, pH=7.2, without cells, 3 – diacetyl together with the cells was incubated for 18 h.

Previously, we showed that diacetyl – a symmetric diketone – is reduced to 2,3-butanediol by a two step reaction. Therefore, the next stage of our research was to study a process of reduction of various 2,3- and 3,4-diketones. GC-MS analysis revealed that one dominant product was formed after the reduction of 2,3-pentanedione using TpdE as catalyst. Two main fragments of the product were formed: *m/z* 45 and *m/z* 59 (Fig. 9).

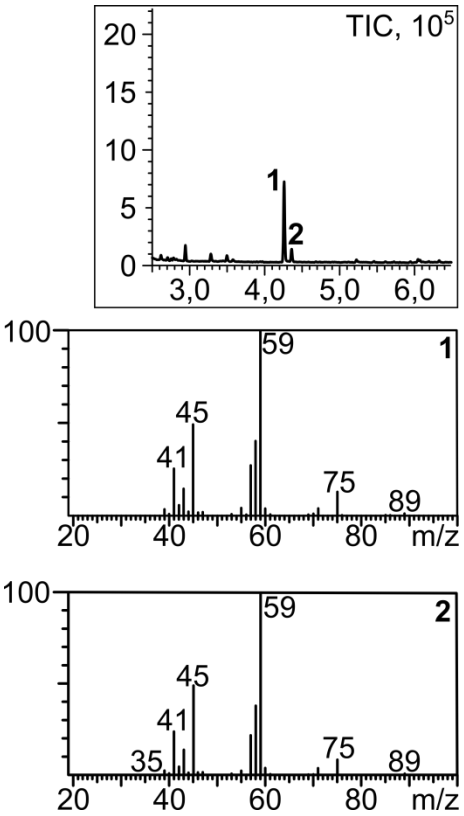


Fig. 9. Analysis of the products of 2,3-pentanedione reduction by TpdE. Chromatogram and masses of the molecule fragments are presented. Analysis performed by GC-MS.

The probable scheme of fragmentation pattern of 2,3-pentanediol as a potential product was designed (Fig 10). Comparison of GC-MS analysis data with this scheme proved that both keto groups of 2,3-pentanedione are reduced by TpdE. Similar analyses of 2,3-hexanedione, 3,4-hexanedione and 2,3-heptanedione reduction by TpdE supported the hypothesis that TpdE is capable of reducing both keto groups of various diketones.

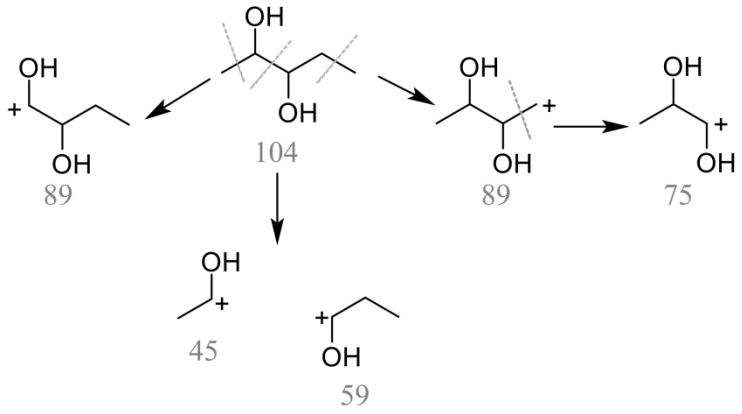


Fig. 10. The scheme of fragmentation of 2,3-pentanediol. Numbers below the fragments indicate a ratio of their mass and charge (*m/z*).

The stereoselectivity of TpdE was demonstrated with GC-MS analysis of the products formed. Moreover, the data was compared with the results of reverse reaction. These results revealed that TpdE catalyzes stereoselective reduction of diketones to corresponding (*2S,3S*)-2,3-butanediols.

The study of *Burkholderia* sp. MAK1 as a whole cell biocatalyst for the production of hydroxylated *N*-heterocyclic compounds.

Burkholderia sp. MAK1 cells were studied as a potential biocatalyst for regioselective biosynthesis of pyridinols. 110 different compounds (various pyridines, pyrimidines and pyrazines) were tested as potential substrates. Using HPLC-MS analysis it was demonstrated that 73 of them were hydroxylated selectively. Summarizing the results of the bioconversions, a pattern of hydroxylation of *N*-heterocycles by the cells of *Burkholderia* sp. MAK1 was noticed. It was demonstrated that 2-hydroxy- and 2-amino-pyridines or pyrimidines were hydroxylated regioselectively at the fifth position of the aromatic ring (Fig. 11), whereas pyridine, pyrazine and their methylated derivatives were hydroxylated at the first position forming corresponding *N*-oxides. Using these bacteria several new hydroxypyridines and hydroxypyrimidines, such as 6-amino-4-chloro-3-

hydroxypyridine, 6-amino-4-fluoro-3-hydroxypyridine, 2-amino-4,5-dihydroxypyridine, were synthesized for the first time.

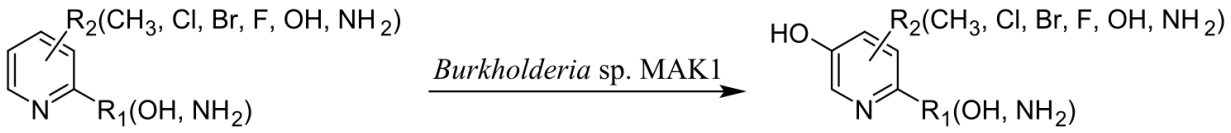


Fig. 11. Regioselective hydroxylation of pyridine derivatives by whole cells of *Burkholderia* sp. MAK1.

2,5-Dihydroxypyridines were further metabolized by *Burkholderia* sp. MAK1 cells. It was also shown that this whole cell biocatalyst could be easily entrapped in alginate gel.

RESUMÉ

Stereo- and regioselective synthesis of alcohols is a difficult task in organic syntheses. Therefore, the discovery of new and efficient biocatalysts as well as their investigation, application and improvement is very important for the development of target compounds.

In this study the process of DHAP synthesis was improved by creating a unique reactor which eliminates hydrogen peroxide. Moreover, the stereoselectivity and substrate specificity of PQQ-dependent ADH from *P. putida* HK5 and NADPH-dependent SDR TpdE from *R. jostii* TMP1 were investigated. The potential of these enzymes as efficient biocatalysts for the production of chiral alcohols was demonstrated. In addition, free and alginate gel-entrapped cells of *Burkholderia* sp. MAK1 were studied as a whole cell biocatalyst for the synthesis of hydroxylated *N*-heterocyclic compounds. 73 of 110 tested compounds were transformed by *Burkholderia* sp. MAK1. The analyses of the products revealed that the hydroxylation was regioselective. Thus, the cells of *Burkholderia* sp. MAK1 can be applicable for the regioselective synthesis of pyridinols.

CONCLUSIONS

1. A bioreactor consisting of Prussian Blue-modified carbon fibre with immobilized L-α-glycerophosphate oxidase as a working electrode eliminates hydrogen peroxide, thus it can be used for efficient synthesis of dihydroxyacetone phosphate.

2. Alcohol dehydrogenase IIG from *Pseudomonas putida* HK5 catalyzes enantioselective oxidation of *(S)*-(+) alcohols, therefore it can be applied for the kinetic resolution of racemic diols and amino alcohols.

3. NADPH-dependent TpdE protein from *Rhodococcus jostii* TMP1 is a classic short-chain dehydrogenase/reductase, which is able to reduce a variety of 2,3- and 3,4-diketones, acetoin and its derivatives.

4. TpdE catalyzes stereoselective reduction of diketones to optically active *(S,S)*-diols, therefore it can be used for the synthesis of chiral alcohols by asymmetric ketone reduction *in vivo* and *in vitro*.

5. *Burkholderia* sp. MAK1 bacteria catalyze the syntheses of *N*-alkyl-2,5-dihydroxypyridines, various 2-amino-5-hydroxypyridines and 2-amino-5-hydroxypyrimidines.

6. The 2-hydroxypyridine-induced *Burkholderia* sp. MAK1 bacteria catalyze the oxidation of pyridine, pyrazine, and their methylated derivatives yielding the corresponding *N*-oxides.

LIST OF PUBLICATIONS

Articles

1. Stankevičiūtė J., Kurtinaitienė B., Stankevičiūtė R., Meškienė R., Gasparavičiūtė R., Marcinkevičienė L., Laurinavičius V., Meškys R. (2014) Prussian Blue as an alternative to catalase: a bioelectrocatalytic system for production of dihydroxyacetone phosphate. *Chemija*, 2:115–118.

2. Kutanovas S., **Stankeviciute J.**, Urbelis G., Tauraite D., Rutkiene R., Meskys R. (2013) Identification and characterization of tetramethylpyrazine catabolic pathway in *Rhodococcus jostii* TMP1. *Appl Environ Microbiol*, 79:3649-3657.

3. Laurinavicius V., Razumiene J., Kurtinaitiene B., **Stankeviciute J.**, Meskys R. (2012) Reagentless and mediator-based electrochemical biosensors for food industry and medicine. *Proceedings of the IEEE Sensors*, 1549-1552.

4. Marcinkevičienė L., **Stankevičiūtė J.**, Bachmatova I., Vidžiūnaitė R., Chaleckaja A., Meškys R. (2012) Biocatalytic properties of quinohemoprotein alcohol dehydrogenase IIG from *Pseudomonas putida* HK5. *Chemija*, 23(3): 223-232.

5. Stankevičiūtė J., Kutanovas S., Rutkienė R., Ražanas R., Tauraitė D., Striela R., Meškys R. Purification and characterization of alcohol dehydrogenase TpdE from *Rhodococcus jostii* TMP1. Manuscript in preparation.

Conference posters

1. Stankevičiūtė J., Vaitekūnas J., Gasparavičiūtė R., Petkevičius V., Tauraitė D., Urbonavičius J., Meškys R. (2014) Whole cell regioselective hydroxylation of *N*-heteroaromatic compounds using *Burkholderia* sp. MAK1. FEBS-EMBO 2014, Paris, France.

2. Stankevičiūtė J., Vaitekūnas J., Gasparavičiūtė R., Petkevičius V., Tauraitė D., Meškys R. (2014) Oxyfunctionalization of pyridine derivatives using whole cells of *Burkholderia* sp. MAK1. Biocat2014, Hamburg, Germany.

3. Stankevičiūtė J., Kutanovas S., Rutkienė R., Ražanas R., Tauraitė D., Meškys R. (2013) Purification and characterization of alcohol dehydrogenase TpdE from *Rhodococcus jostii* TMP1. The 5th Congress of European Microbiologists (FEMS), Leipzig, Germany.

4. Laurinavicius V., Razumiene J., Kurtinaitiene B., **Stankeviciute J.**, Meskys R. (2012) Reagentless and mediator based electrochemical biosensors for food and medicine. IEEE Sensors 2012, Taipei, Taiwan.

5. Stankevičiūtė J., Kutanovas S., Rutkienė R., Meškys R. Purification and characterization of alcohol dehydrogenase from *Rhodococcus jostii* TMP1. (2012) Biochemistry and Biophysics at Vilnius University, Vilnius, Lietuva. (1^{st} place in poster presentation section.)

6. Stankevičiūtė J., Kutanovas S., Rutkienė R., Meškys R. (2012) Purification and characterization of alcohol dehydrogenase from *Rhodococcus jostii* TMP1. 12^{th} International Lithuanian Biochemical Society Conference, Tolieja, Lithuania.

7. Astrauskaitė I., Užusienis T., **Stankevičiūtė J.**, Kurtinaitienė B. (2012) The use of glycerol-3-phosphate oxidase for the synthesis of dihydroxyacetone phosphate. 12^{th} International Lithuanian Biochemical Society Conference, Tolieja, Lithuania.

Conference oral presentation

Jonita Stankevičiūtė. (2012) The use of alcohol dehydrogenases for the syntheses of chiral compounds. 12^{th} International Lithuanian Biochemical Society Conference, Tolieja, Lithuania.

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I thank my biology teacher Rita Kareckienė and the head of the School of Young Biochemists Elvyra Kilikevičienė, who have encouraged my interest in biochemistry. I am very grateful to my family and friends.

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SANTRAUKA

Dihidroksiacetono fosfatas tapo svarbiu substratu stereoselektyvioje angliavandenių biosintezėje. Šio junginio gamybai naudojant L-α-glicerofosfato oksidazę reakcijos metu susidaro šalutinis produktas – vandenilio peroksidas, kuris negrįžtamai slopina fermentą. Siekiant padidinti dihidroksiacetono fosfato gamybos efektyvumą buvo sukonstruotas dihidroksiacetono fosfato bioreaktorius, kuriame darbinis elektrodas sudarytas iš Berlyno mėliu modifikuoto anglies pluošto ir imobilizuotos L-α-glicerofosfato oksidazės. Šiame unikaliame reaktoriuje vandenilio peroksidas buvo pašalinamas elektrochemiškai. Taigi, buvo patobulintas dihidroksiacetono fosfato sintezei skirtas bioreaktorius., kuriame papildomas fermentas vandenilio peroksido eliminavimui tapo nebereikalingas.

Šiame darbe pirmą kartą parodytas alkoholio dehidrogenazės IIG iš *Pseudomonas putida* HK5 gebėjimas enantioselektyviai oksiduoti įvairius pirminius ir antrinius alifatinius bei ciklinius alkoholius ir aminoalkoholius, pirmenybę teikiant *(S)*-enantiomerams. Dėl šios priežasties, alkoholio dehidrogenazė IIG iš *P. putida* HK5 gali būti pritaikoma enantiomeriškai grynų alkoholių gamybai kinetinio racematų atskyrimo būdu.

Rekombinantinės alkoholio dehidrogenazės TpdE iš *Rhodococcus jostii* TMP1 tyrimai parodė, kad šis fermentas pasižymi itin plačiu substratiniu specifiškumu ir gali stereoselektyviai redukuoti linijinius skirtingo ilgio 2,3-diketonus, įvairius aciloinus ir butan-3-ono-2-il- esterius. Remiantis GC-MS analizių rezultatais paaiškėjo, jog TpdE redukuoja abi 2,3- ir 3,4-diketonų ketogrupes, todėl produkto molekulėje susidaro du nauji chiraliniai atomai. Diketonų biokonversijos į atitinkamus diolius sėkmingai vyko katalizatoriumi naudojant tiek gryną fermentą, tiek visą *Escherichia coli* BL21 (DE3) ląstelę su rekombinantiniu TpdE. TpdE iš *Rhodococcus jostii* TMP1 dėl plataus substratinio specifiškumo, stereoselektyvumo ir darbo tiek *in vivo*, tiek *in vitro* yra patrauklus katalizatorius chiralinių alkoholių sintezėje asimetrinės ketonų redukcijos būdu.

Buvo ištirtas piridino darinių hidroksilinimas, kai katalizatorius – *Burkholderia* sp. MAK1 bakterija. Iš 110 išbandytų piridinų, pirazinų ir pirimidinų, 73 junginiai buvo oksiduojami. Esperimentiškai pagrįsta, kad *Burkholderia* sp. MAK1 katalizuoja

regioselektyvų hidroksilinimą: kai aromatinio heterociklo 2-oje padėtyje yra hidroksi- arba amino- grupė hidroksilinimas vyksta į *para* padėtį, tuo tarpu kai 2-oje padėtyje pakaitų nėra, arba yra metilo grupė susidaro atitinkami *N*-oksidai. Mūsų žiniomis šiame darbe pirmą kartą parodyta aminopiridinolių sintezė biokatalizės būdu. Taigi, ištirtas ir aprašytas naujas, ypač plataus substratinio specifiškumo biokatalizatorius – *Burkholderia* sp. MAK1, kurį galima pritaikyti regioselektyviai hidroksipiridinų sintezei.

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