Synopsis of the PhD thesis

Application of cyanobacteria for the synthesis of chiral phosphonates

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Chiral phosponates synthesis *via* biocatalytic reduction of ketones with the use of non-photosynthetic and heterotrophic microorganisms or their purified enzymes is the method of choice. Phototrophic prokaryotes such as cyanobacteria have also been identified as a source of reductive activities, but in contradiction to other extensively examined bacteria and fungi, this field is still not fully explored. Despite the wide range of implemented industrial biotransformations, the practicability of technical applications of these methods is often limited by the lack of suitable biocatalysts for particular reactions and by difficulties with scaling up of the invented methods.

Cyanobacteria represent a remarkable, morphologically diverse and widely distributed group of photosynthetic prokaryotes with significant meaning in aquatic and terrestrial ecosystems. Microalgae have developed diverse mechanisms of adaptation to changing environment (desiccation, light, temperature, salinity, heavy metal ions presence). Cyanobacteria have gained a lot of attention in recent years because of their possible applications in bioremediation, cosmetic and food industry.

Presented dissertation reports effective employment of phototrophic microorganisms as biocatalysts for the production of chiral *β*-hydroxyphosphonates. The aim of this study was to explore the possibility of reduction of aliphatic and aromatic diethyl *β*-oxophosphonates by cyanobacterial photobiocatalytic system. Morphologically different strains of cyanobacteria: *Arthrospira maxima, Nostoc cf-muscorum, Leptolyngbya foveolarum, Geitlerinema* sp., *Nodularia sphaerocarpa* and *Synechococcus bigranulatus* were used for enantioselective bioreduction of selected, structurally different diethyl esters of oxophosphonic acids: diethyl 2-oxopropylphosphonate, diethyl 2-oxo-2-phenylethylphosphonate, diethyl 2-oxobutylphosphonate.

Among the tested cyanobacterial strains, the following ones were capable to chiral (*S*)-hydroxyphosphonates synthesis, within 7 days, under continuous illumination provided by different light source (Power Glo or Sun Glo fluorescent bulb): *Arthrospira maxima, Nodularia sphaerocarpa, Leptolyngbya foveolarum* and *Nostoc cf-muscorum*.

The mixtures of bioconversion products were analyzed by ^{31}P NMR technique. Optical purity of the products was estimated using quinine as a chiral solvating agent, what allowed achieving a shift difference of ^{31}P NMR signals, becoming from hydroxyphosphonates enantiomers – bioreduction products. The absolute configuration of *β*-hydroxyphosphonates was determined by Mosher's method and ^{1}H and ^{31}P NMR spectroscopic analysis of the arisen Mosher esters. α-Methoxy-α-trifluoromethylphenylacetic acid (MTPA) was used as chiral derivatizing agent.

Several efforts have been undertaken to optimize the bioconversion conditions. Results confirm that the relationship between nutrients (cultivation medium, presence of glucose) and physical factors, like illumination, and interactions between these factors affect the catalytic activity of photo-biocatalyst.

The efficiency of asymmetric hydrogen transfer was strongly dependent on the chemical structure of the substrates. *Nostoc cf-muscorum* was active toward the aromatic substrate, while filamentous strain of *Arthrospira maxima* toward aliphatic - diethyl 2-oxopropylphosphonate and these two strains, regardless of used illumination or environmental conditions (photoperiod, cultivation medium) were active, what allowed obtaining products with the optical purity up to 99% of *S*-isomer.

Employing of *Nodularia sphaerocarpa* allowed obtaining the most spectacular result - diethyl (*S*)-2-hydroxy-2-phenylethylphosphonate with degree of conversion of 99% and the optical purity of 93%. This result was interesting, because in the case of efforts described in the literature, reduction of carbonyl functionality, which is situated directly next to the aromatic ring in phosphonic moiety failed. In the case of *Nodularia sphaerocarpa,* the effectiveness of diethyl (*S*)-2-hydroxy-2-phenylethylphosphonate biosynthesis was affected by substrates concentrations – conversion degree decreases with the substrate concentration increasing but enantioselectivity remain almost at the same level. Additionally, flow cytometry technique studies showed the excellent resistance of the cells of *Nodularia sphaerocarpa* against examined xenobiotic (diethyl 2-oxo-2-phenylethylphosphonate), microalgal cells remain viable even at the concentration of 10 mM. It seems that enzymes responsible for conversion of oxophosphonates are involved in secondary cells metabolism. This explains that such high xenobiotic concentrations do not affect the viability of the cells. Performed experiments allowed to observe other useful features of *N. sphaerocarpa* – independence on the bioconversion conditions (cultivation medium, light source, light cycle, shaking), in the case of diethyl 2-oxo-2-phenylethylphosphonate.

Determination of the effect of immobilization on the catalytic activity of biocatalysts was a significant step toward future research connected with scaling up the processes. To overcome the impact of *β*-oxoalkylphosphonates on living biocatalyst, cyanobacterial cells were entrapped in alginate matrix.

Ca^{2+}-alginate entrapped cells of cyanobacteria exhibit lower catalytic activity comparing to free cells of cyanobacteria, cultured under shaking conditions. The immobilized biomass of *A. maxima* exhibited the lowest biocatalytic ability to reduce of diethyl 2-oxopropylphosphonate, within the experimental period - the degree of conversion was up to 5%. Among tested strains of cyanobacteria, also the application of entrapped cells of *N. sphaerocarpa* resulted in decreasing of the conversion of *β*-oxophosphonates.
The important observation is also that entrapped cells of *Nostoc cf-muscorum* were more efficient biocatalysts than free cells, cultivated under stationary conditions.

The presented results confirm unique biocatalytic property of *N. sphaerocarpa* and its independence on tested externals, in the case of reduction of diethyl 2-oxo-2-phenylethylphosphonate. These results form the basis for efficient scaling-up of the laboratory-scale processes. The appropriate experiments were conducted to evaluate the possibility of the application of free-living and immobilized cells of *Nodularia sphaerocarpa* in the preparative scale batch culture (200 ml or 500 ml) and in the continuous-flow photobioreactor, respectively.

To develop an effective process of biotransformation, three models were used. They differ in the form of biocatalyst, type of bioreactor and conditions of the process. The volume of the culture of *N. sphaerocarpa* was gradually increased, from 200 ml (issue culture flasks) to 500 ml (Erlenmeyer flask).
The application of 200 ml culture of free cells of *N. sphaerocarpa* as biocatalyst was not effective for biotransformation of 1.3 mM of diethyl 2-oxo-2-phenylethylphosphonate. However, the use of 500 ml batch culture (Erlenmeyer flask) of free cells of *N. sphaerocarpa* towards the same substrate was the most effective one. The advantage of this entry was the use of high final amount of substrate - 5 mM, 640 mg. Moreover, this is the inexpensive solution, easy to operate and largely impervious to mechanical complications, comparing to continuous flow bioreactor (glass or plastic column) packed with immobilized cells of *N. sphaerocarpa* (1% w/v). The application of this model of bioreactor, in the case of bioconversion of 10 mM (384 mg) of diethyl 2-oxo-2-phenylethylphosphonate was ineffective (38%, 86% *e.e.*).

Thus, applying 500 ml batch culture allowed maintain metabolically active biomass of cells of *N. sphaerocarpa* and performed the effective process of reduction of diethyl 2-oxo-2-phenylethylphosphonate in the preparative scale process.

This dissertation presents the successful use of photoautotrophic microorganisms as biocatalysts for the production of chiral (*S*)-2-hydroxyphosphonates. Cyanobacteria represent diverse and not ordinary catalytic abilities to transform the *β*-oxoalkylphosphonates. Presented results confirm the unique features of *N. sphaerocarpa*, which can be consider as a new, effective photobiocatalyst for preparative scale application.