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Fungal synthesis of chiral phosphonic synthetic platform – Scope and limitations of the method

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ABSTRACT

Chiral hydroxyphosphonates due to their wide range of biological properties are industrially important chemicals. Chemical synthesis of their optical isomers is expensive, time consuming and not friendly to the environment, so biotransformations are under consideration. Among others, these compounds act as enzymes inhibitors. This makes the bioconversions of phosphonates, especially scaling experiments, hard to perform. Biocatalysis is one of the methods that can be applied in synthesis of optically pure compounds. To increase the efficiency of the process with whole cell biocatalysts, it is essential to ensure optimal reaction conditions that minimize cellular stress and can enhance the metabolic activity of cells. The present investigation focuses on the scaling up of the kinetic resolution of racemic mixture of 2-butyryloxy-2-(ethoxy-P-phenylphosphinyl)acetic acid, applying free and immobilized form of the fungal biocatalysts and two operation systems: shake flask and recirculated fixed-bed batch reactor. Protocols of effective mycelium immobilization on polyurethane foams were set for *T. purpurogenus* IAFB 2512, *F. oxysporum*, *P. commune*. The best results of biotransformation were obtained with the immobilized *P. commune* in the column recirculated fixed-bed batch reactor. The conversion reaches 56% (maximal for the kinetic process) and the enantiomeric enrichment of the isomers mixture ranges between 82 and 93% (93% for ester of R_P,R conformation). All biocatalysts exhibit S_P-preference toward tested compound, what is essential because of importance of the phosphorus atom chirality for its biological activity.
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1. Introduction

The biological activity of many classes of compounds usually arise from their chirality, so obtaining enantiopure form of defined absolute configuration is crucial for the effectiveness and safety of pharmaceuticals [1]. Single isomers can be synthesized in chemical or chemoenzymatic processes [2]. Biocatalysis has become a common method, especially in the pharmaceutical industry, for the preparation of optically pure compounds [3]. It is caused by high regioselectivity and stereoselectivity of the reactions carried out mostly in water under mild conditions [4]. Such biotransformations are applied to resolve the racemic mixtures of chiral compounds or to create one or more chiral centers [5]. One of the limitations, that needs to be overcome to attain high productivity of bioconversions, is inhibition of the process by xenobiotic substrates, intermediates, products, and/or by-products. These compounds often interfere with important metabolic processes including cellular transport, energy generation, DNA synthesis and repair, protein synthesis and damage cellular infrastructures such as cell membrane, leading to decrease of the cell viability. These are the common reasons of the reduction of the biocatalysts capacity to obtain the desired product [6–9]. Immobilization is one of the methods, which allowed to increase biocatalyst stability, activity and to protect the fragile cells against the non-physiological environment. Also, high thermal stability, tolerance to pH variations and organic solvents are achieved due to the immobilization, these imply in the extension of the catalytic activity of cells. Moreover, such form of the biocatalyst can be easily removed from the reaction media, improving the products purity. What is more, possibility of biocatalyst re-use, reduces the cost of the process. Common method of whole cells immobilization is entrapment, which is based on the placement of the cells in fibers, membranes or encapsulation in semipermeable materials [10]. Polyurethane foams (PUF) are used as a carrier for the immobilization because of it good mechanical properties, high porosity, large adsorption surface and very low cost [11]. PUF have been used to immobilize enzymes and cells by adsorption and entrapment methods. Such biocatalyst find application in many processes e.g. fumaric acid production, vinegar production, biodegradation of

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chlorophenols, removal of heavy metals, manganese peroxidase production or decolourization of sugar refinery wastewater [12].

The aim of the study was to increase the scale of the biocatalyzed kinetic resolution of the racemic mixture of 2-butyryloxy-2-(ethoxyphenylphosphinyl)acetic acid (2, Fig. 1) performed by free and immobilized fungal biocatalysts, what is the continuation of the previous work [13]. *Penicillium commune, Talaromyces purpurogenus* IAFB 2511, *Fusarium oxysporum* and *Talaromyces purpurogenus* IAFB 2512 effectively hydrolyzed ester: 2-butyryloxy-2-(ethoxyphenylphosphinyl)acetic acid in the shake flask system. Current work focuses on the scaling up the process, what is difficult undertake, because of the nature of the substrate, which belongs to the family of the compounds known from their inhibitory activity against different enzymes. Also, desired products of biotransformation – chiral hydroxyphosphinates (1, Fig. 1) are predicted to be the molecules of possible biological activity. This assumption implies from the structural similarity to biologically important phosphates and from the structural analogy between α-hydroxyalkylphosphonic and α-hydroxycarboxylic acids, what is crucial for their antibacterial, antiviral, antibiotic, pesticidal, anticancer, and enzyme inhibitor properties [14]. Additionally, optically pure product: 2-hydroxy-2-(ethoxyphenylphosphinyl)acetic acid (1) can be used as chiral discriminator for analytical purposes (NMR spectroscopy) and building block of defined absolute configuration, applied for the synthesis of biologically active compounds such as pharmaceuticals or pesticides.

2. Materials and methods

All materials were purchased from commercial suppliers: Sigma Aldrich, Avantor, Biocorp and were used without further purification.

NMR (Nucleic Magnetic Resonance) spectra were measured on a Bruker Avance™ 600 at 600.58 MHz for ^{1}H; 243.12 MHz for ^{31}P and 151.02 MHz for ^{13}C in $CDCl_3$ (99.8% of atom D, contains 0.03% v/v TMS). Chemical shifts (δ) are reported in ppm. The biotransformation products were analyzed by ^{31}P NMR. In the case where signals of the enantiomers have not been separated to sample was added quinine as a chiral solvating agent (CSA).

2.1. Synthesis of 2-butyryloxy-2-(ethoxyphenylphosphinyl)acetic acid

Compound was synthesized according to the method described by Majewska [15]. The diastereomeric ratio of starting material was 1:0.7[(S_P,*S*) and (R_P,*R*) to (R_P,*S*) and (S_P,*R*)].

2.2. Bioprocess catalyzed by the free fungal cells – general procedures

Fungal strains *Talaromyces purpurogenus* IAFB 2512, *Penicillium commune* IAFB 2513 and *Talaromyces purpurogenus* IAFB 2511 were identified and deposited in Collection of Industrial Microorganisms (WDCM212) in Institute of Agricultural and Food Biotechnology (Poland). *Fusarium oxysporum* (DSM 12646) was purchased from German Collection of Microorganisms and Cell Cultures (Germany).

2.2.1. Microorganisms culturing

Microorganisms stored on potato dextrose agar (BIOCORP) were used for inoculum preparation on PDB medium (for *T. purpurogenus* IAFB 2511, *F. oxysporum, T. purpurogenus* IAFB 2512) and H1 medium (for *P. commune* IAFB 2513). H1 medium composition: 3% of bactopeptone, 0.5% of yeast extract, 0.1% of KH_2PO_4, 0.1% of $NaNO_3$, 0.05% of $MgSO_4$, 1% of lipase synthesis inductor (olive oil or sunflower oil) and 1 L of distilled water. 1 mL of inoculum (cells in logarithmic phase of growth) was added to the Erlenmeyer flask containing 100 mL of suitable medium. Particular amounts of applied biomass are presented in tables (Tables A.1–A.4 – Appendix). Biocatalysts were incubated at room temperature with shaking (135 rpm) until the log phase of growth was reached. Then the particular fungi were separated: *T. purpurogenus* IAFB 2512 (after filtration), *F. oxysporum* and *T. purpurogenus* IAFB 2511 (after centrifugation 20 min, 5000 rpm) and applied directly for the further steps of the process. Whereas *P. commune* IAFB 2513 (after filtration) was stored for 24 h under deficiency of nutrients source in 50 mL of distilled water, then filtered and applied for the bioconversion.

2.2.2. Biotransformation conditions

Biomass received after cultivation (Section 2.2.1), was suspended in 50 mL of distilled water containing different concentrations of the substrate, ranging from 3.2 to 32 mM (50–500 mg) and applied as ethyl acetate solutions (in 0.5 mL). Bioconversions were carried out at room temperature on a shaker pallet (135 rpm). After suitable time (3–7 days), when conversion degree reached up the 50%, biomass was removed by filtration or centrifugation, and supernatants were evaporated under reduced pressure using rotary evaporator. To check the stability of biocatalysts and the possibility of their reuse, the biomass received after biotransformation was placed in Erlenmeyer flask containing fresh biotransformation medium – 50 mL of distilled water with proper amount of substrate. The procedure of biotransformation was repeated sequentially three times (3 cycles).

2.3. Bioprocess catalyzed by the immobilized on polyurethane foams fungal mycelium

2.3.1. Immobilization on foams – general procedure

1 mL of fungal inoculum (Tables A.1–A.4 – Appendix) was added to the Erlenmeyer flask containing 100 mL of suitable medium and 20 cm^3 of foams (20 pieces of cubic foams with side 1 cm). Three types of open-pore foams with different pore size were tested: (a) S28089 foams: 740–1040 μm; (b) TM25133 foams: 1060–1600 μm and (c) S28280 foams: 2300–3300 μm (commer-

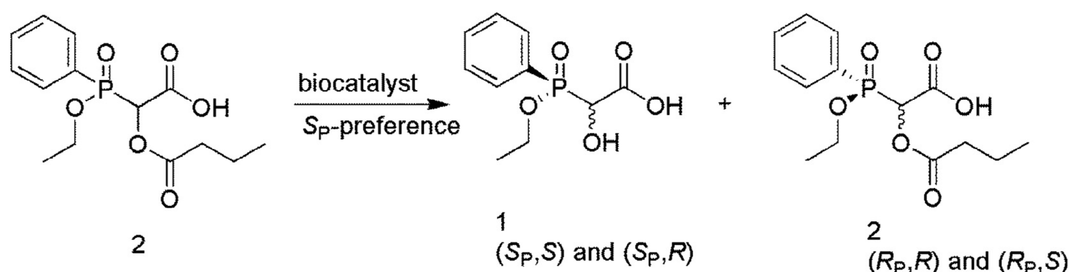

Fig. 1. Hydrolysis of ester: 2-butyryloxy-2-(ethoxyphenylphosphinyl)acetic acid.

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