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Biotransformations of organophosphorus compounds by biocatalysts with hydrolytic activity.

Summary

The current tendency to improve industrial processes, to reduce costs, and to increase the quality of products and also environmental and industrial requirements of chemical transformation has intensified the transfer of biocatalytic research results to industry [1]. Enzymatic processes have been implemented in a broad range of industries over the last decades due to their high specificity, rate of the reaction, and saving of raw materials, energy, reagents and/or water comparing to conventional processes. [2] Biocatalysis has become an important tool in meeting the growing demands of green and sustainable chemical production, especially in the pharmaceutical industry, where the biological activity of compounds is so important.

One of the most important groups of compounds used in chemical industry, due to their wide range of biological properties, are chiral hydroxy- and aminophosphonates. Phosphonates derivatives attracts considerable attention because of their antibacterial, antiviral, antibiotic and anticancer features, they have been also used as enzyme inhibitors and pesticides [3-5]. They constitute a synthetic platform for receiving a number of therapeutic agents. The chemical synthesis of their optical isomers is expensive, time consuming and not environmentally friendly, that is why biotransformations are becoming an increasingly attractive method of obtaining these compounds. Due to the strong dependence of the absolute configuration on activity of the phosphonic compounds, the synthesis of the optically pure enantiomers of these compounds is an important research topic.

As substrates, in conducted research, were used hydroxyphosphinate derivative - 2-butyryloxy-2-(ethoxyphenylphosphinyl)acetic acid, and aminophosphonic acids derivatives - *N*-acetyl-1-aminoethylphosphonic acid (Ala^{P}-Ac), *N*-acetyl-1-amino-2-methylpropyl phosphonic acid (Val^{P}-Ac), *N*-acetyl-1-amino-1-phenylmethylphosphonic acid (Phg^{P}-Ac).

2-butyryloxy-2-(ethoxyphenylphosphinyl)acetic acid and a product of its hydrolysis, have in their structures two stereogenic centers, so the racemic mixture of them is consisted of 4 stereoisomers. Studies were started from the attempts to separate the diastereoisomers with the use of chromatographic methods. The obtained one pair of enantiomers of 2-butyryloxy-2-(ethoxyphenylphosphinyl)acetic acid, was meant to be applied for biocatalyzed

resolution leading to enantiopure compounds. However, these efforts failed. An efficient method of 2-butyryloxy-2-(ethoxyphenylphosphinyl)acetic acid diastereoisomers resolution was not found. Only mixtures enriched with one pair of enantiomers were obtained: e.g. method with ethyl acetate/hexane/2-propanol (5:4:1 v/v) allowed to obtain small amount of butyryloxyphosphinate with molar ratio of diastereoisomers 1:0,042.

Next step of the research includes screening of microorganisms of hydrolytic activity towards tested compounds. Filamentous fungi, yeasts, bacteria, cyanobacteria and, in case of α-aminophosphonates, also enzymes were tested as biocatalysts. None of them was able to hydrolyze the amide bond in the investigated *N*-acetyl derivatives of α-aminophosphonic acid. Although lipases are not commonly used in hydrolysis of amide bonds, they active site is built of a catalytic triad: Ser-His-Asp/Glu, which resembles the construction of an active site in serine proteases, which are capable of hydrolyzing amide bonds. Compared to proteases, lipases exhibit greater activity and stability under difficult process conditions, what is the reason of searching for lipases of amidase activity.

Screening among tested biocatalysts, pointed that filamentous fungi and cyanobacteria were able to hydrolyze 2-butyryloxy-2-(ethoxyphenylphosphinyl) acetic acid. Nevertheless, cyanobacteria in spite of high activity, did not hydrolyzed tested compound enantioselectively. Butyryloxyphosphinate has been toxic to *Nostoc cf. muscorum* and *Nodularia sphaerocarpa* even at low concentrations (3.2 mM). The best results, both in terms of conversion degree and enantiomeric excesses, were obtained for *T. purpurogenus* KKP 2512, *P. commune, F. oxysporum, A. fumigatus* and *T. purpurogenus* KKP 2511. Fungi *M. circinelloides, P. purpurogenum, P. crustosum, P. lipolytic* and *P. funiculosum* also exhibited the ability to convert tested compound but with much lower enantioselectivity.

Biotransformation products were analyzed by ^{1}H and ^{31}P NMR. Chiral solvating agents were used to separate the signals derived from the enantiomers - quinine for the determination of the enantiomeric excess of hydroxyphosphinates and cyclodextrin in the case of aminophosphonates.

Further studies on 2-butyryloxy-2-(ethoxyphenylphosphinyl) acetic acid have been focused on optimization of cultivation and biotransformation conditions to improve reaction effectiveness and on scaling the process. It is known, that lipase production can be increased, among others, at the fermentation stage. The secretion of these enzymes is conditioned by chemical factors of growth such as: composition of the culture medium, the type and concentration of carbon and nitrogen sources, and physical parameters such as: pH,

temperature or dissolved oxygen. In addition, microbial lipases are mostly inducible extracellular enzymes and their production increase at the presence of a lipid carbon source.

Therefore, the influence of culture parameters (carbon and nitrogen source, addition of lipase inducers and supplementation of SDS, DMSO and Tween) on the activity of *P. commune* was studied. The addition of DMSO and vegetable oils allow obtaining the most active biocatalysts. DMSO supplementation (1 or 2%) resulted in the increase in both activity and enantioselectivity of the process. For biotransformation with biomass cultivated on medium containing 1% DMSO, the conversion degree was 48%, and *e.e.* was in the range of 43-75%. Positive effects on the enantiomeric purity of obtained products were also noticed after addition to the cultivation medium oils such as olive oil, sunflower oil, linseed and grape oil.

The impact of various growth media on the activity of *P. commune* in the biotransformation of 2-butyryloxy-2-(ethoxyphenylphosphinyl) acetic acid was also studied. Among tested media, H1 and YEP medium turned out to be the most effective ones - the conversion degree of reactions was respectively 45 and 54%. Biomass grown on the other culture media exhibit low activity - the conversion degree of a substrate did not exceed 21%. The biomass grown on all tested media exhibited low enantioselectivity - *e.e.* did not exceed 39%. It was also observed that there was no relationship between the lipolytic activity of the tested strains, which was on high level (measured by hydrolysis rate of *p*-nitrophenol palmitate) and the efficiency of the conversion of 2-butyryloxy-2- (ethoxyphenylphosphinyl) acetic acid.

Another parameter, potentially influencing the biocatalysts activity, is the pre-incubation of biocatalyst under nutrient deficient conditions, before the biotransformation step. Application of this procedure caused a significant increase in enantioselectivity of the reaction catalyzed by *P. commune* (*e.e.* up to 90% and enantioselectivity E 52.8) comparing to the reaction without starvation pretreatment (*e.e.* and E up to 54% and 3.5 respectively). These approach allowed to develop a process of high efficiency and enantioselectivity. The use of *P. commune* biomass cultured on H1 medium supplemented with sunflower oil and preincubated under nutrient deficiency prior to the biotransformation stage, resulted in 49% conversion degree and enantiomeric excesses in a range of 82-90%.

So far, phosphinate bioconversion has been conducted only on a laboratory scale. The scale-up experiments are difficult, mainly due to the nature of the substrate. These compounds act as enzyme inhibitors, which makes the phosphinate bioconversion complex. Scale-up experiments were performed with the use of free and immobilized biocatalysts as well as two

operating systems - continuous shake culture and column with continuous recirculation of biotransformation medium. Protocols for effective immobilization on polyurethane foams have been developed for *T. purpurogenus* KKP 2512, *F. oxysporum* and *P. commune*. In contrast, the attempts of immobilization in calcium alginate were ineffective, what was proved for *P. commune* strain - the closure of the biocatalyst in the gel capsule hindered its contact with the substrate and resulted in a decrease in the efficiency of the process.

Based upon the results of the experiments performed on laboratory scale with 3.2 mM (50 mg) of substrate, the most active biocatalysts were selected: *P. commune*, *T. purpurogenus* KKP 2511, *F. oxysporum* and *T. purpurogenus* KKP 2512. The free cells of tested fungi were used in a process with double substrate concentration (6.4 mM - 100 mg). Under these process conditions, all biocatalysts exhibited high activity and enantioselectivity, but further scale up, to 32 mM substrate concentrations (500 mg), was ineffective. The decline in cell activity could be caused by toxicity of substrate.

To sustain the biocatalyst activity and to protect the viable cells, fungal mycelia were immobilized onto polyurethane foams and then applied for biotransformations. Immobilized *F. oxysporum* and *T. purpurogenus* KKP 2511 exhibit lower activity than free cells. In contrast *P. commune* and *T. purpurogenus* KKP 2512 remain active and stable at the presence of 3.2 mM (conversion degree and enantiomeric excesses were on similar level as in processes with free cells) and also 6.4 mM of substrate. However, further scale- up failed.

In order to increase the conversion degree for the process with high substrate concentration, immobilized biocatalyst (*P. commune* and *T. purpurogenus* KKP 2512) were loaded in to the column with continuous recirculation of reaction medium. Such system forces the flow of the reaction medium through the polyurethane foams, what improve the contact between biocatalyst and substrate. Application of the column recirculated fixed-bed batch reactor, fulfilled with immobilized *P. commune* mycelium, allow to hydrolyze tested compound at the concentration of 10.6 mM (500 mg) with 56 % of conversion degree and enantiomeric excesses in the range of 82-91%.

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