The Application of Fungi as Biocatalysts for the Synthesis of Optically Pure Phosphonates

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Abstract

Green chemistry is the field of science, which develops very dynamic during last years. It includes, besides other aims, the use of biocatalysts to obtain desired product of different interests. Biocatalysis is an effective and in many cases preferable alternative to the standard, chemical synthesis of optically active forms of fine chemicals, including phosphonates. Phosphonates exert biological effects as antibacterials, antivirals, herbicides, neuromodulators or chelating agents. As it is well known, usually enantiomers represent different biological activity and that is why they must be used as compounds of defined absolute configuration. The application of fungi imperfecti (Rhodotorula sp., Cladosporium sp., Verticillium sp. Geotrichum candidum etc.) with their own cofactor regeneration system offer wide range of enzymatic activities towards a number of non-physiological substrates and usually there are no side reactions except the expected ones.

Keywords: Fungi, Biocatalysis, Phosphonates

1. Introduction

Microorganisms have been used for many years for the biological transformation of organic compounds. Recently, the use of whole cells and their enzymatic systems to carry out stereospecific and stereoselective reactions has taken on greater significance, as these reactions have demonstrated their usefulness in the asymmetric synthesis of the molecules of biological importance [22, 27]. Bioconversions with living microbial cells, which have the ability to regenerate their own respective cofactors and exhibit a range spectrum of enzymatic activity, are very useful tool in bioorganic synthesis. They offer also a possibility to perform biocatalysis with cells in reaction media other than water. This is very
important when the substrates or products are unstable or insoluble in water and thus cannot be added to an aqueous medium. Several procedural solutions to this problem have been developed, including the use of biphasic media [8], biocatalysis in emulsions [21] or in anhydrous conditions (organic solvents, supercritical fluids or ionic liquids) [23,18]. It allowed increasing the substrates spectrum with the water insoluble compounds, however requires special biocatalyst preparation such as lyophilization and/or immobilization to stabilize the biocatalysts in such a reaction medium and protect the cells against toxic impact of organic solvent [20,12]. There is no doubt that the advantage of microbial biotransformations is the possibility to induce enzymes of defined, desired activity (which are not constitutively presented inside the microbial cells), simply by the suitable preincubation methods. This strategy includes temperature pretreatment, cultivation under anaerobic or starvation conditions, and the use of different sort of cultivation media with different source of supporting elements. Microbiological media in particular experiments, contain special chemical additives, which influence the particular enzymes activities or are an exogenous source of hydrogen for dehydrogenases cofactors regeneration systems [13,5].

2. Bioreduction of the structurally different oxoalkylphosphonates as the method to synthesis of the optically pure hydroxyphosphonates

Hydroxyphosphonates constitute a class of organophosphorus compounds of well recognized activity [24,14] and may also be considered as convenient substrates for the synthesis of amino phosphonic acids via the Mitsunobu reactions [11]. Among them phosphonic analogues of natural amino acids are of special interest as they are known to display diverse biological properties, applications of which range from medicine to agriculture [19].

A. Bioreduction of 1-oxoalkylphosponates.

1-Oxoalkylphosphonates (Scheme 1.), which are presumed to yield optically active 1-hydroxyphosphonates, are the class of substrates extremely unstable in water and easily decompose, yielding a wide variety of products, so their biotransformation had to be carried out in media other then water. Anhydrous hexane was the solvent of choice because it guaranteed stability of the substrates [13]. The biotransformation time was also limited by the stability of the substrates (1-6 days). The application of whole cells of microorganisms in completely anhydrous conditions required previous lyophilization and immobilization of the biocatalysts in order to protect them from the toxicity of the solvent. The Celite R 630 [20] was chosen as a support material for the immobilization, because this kind of support does not introduce any water into the bioconversion media.
As is known from literature [29, 30], baker’s yeast has a great potential as a catalyst for bioreduction of diethyl β-, γ-, δ-oxoalkylphosphonates but they were not effective for the biotransformation of the α-oxoalkylphosphonates because of low enantiomeric excess or chemical yield of the product [4].

There were two ways to improve such results: change the biocatalyst and/or add to the biotransformation medium some chemical additives that are known to influence the enzyme activity [9, 10]. Four strains of fungi, *Verticillum* sp., *Cladosporium* sp, *Rhodotorula rubra* and *Rhodotorula glutinis*, were the microorganisms of choice for a few reasons: they are known as biocatalysts [25, 2, 1], they are able to grow in the presence of phosphonates, and they preserve their reductive properties in anhydrous hexane. All of these microorganisms previously were used as biocatalysts to reduce 1-oxoalkylphosphonates in standard conditions of the biotransformation reaction (immobilized biocatalyst in anhydrous hexane without any additives) [4]. *Rhodotorula glutinis* turned out to be an efficient biocatalyst significantly increasing the yield of the reduction of compound 1a (1-oxoethylphosphonate, Scheme 1) to 81% with high enantiomeric purity (99%)- see Table 1- and, what was also important, after only 1 day of biotransformation. It is noteworthy that in this case, despite of the stability of the substrate, the prolongation of biotransformation to 6 days resulted in dramatic decrease of the yield and optical purity of reaction (correspondingly, 39% and 50%). The possible explanation for this is that, under extremely starving conditions of biotransformation, the product of the bioreduction could serve as a substrate for other enzymes, thus being slowly degraded. A similar effect was observed for the diethyl 1-oxopropylphosphonate (1b) which was reduced with *Verticillum* sp. with 52% yield and 99% e.e. within 1 day.
Table 1. The bioreduction of 1-oxoalkylphosphonates after one day of biotransformation – the best results.

<table>
<thead>
<tr>
<th>Compound</th>
<th>1a</th>
<th>1b</th>
<th>1d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microorganism</td>
<td>R. glutinis</td>
<td>Cladosporium sp.</td>
<td>Cladosporium sp.</td>
</tr>
<tr>
<td>Additive</td>
<td>no additive</td>
<td>ethyl alcohol or allyl bromide</td>
<td>allyl bromide</td>
</tr>
<tr>
<td>Yield [%]</td>
<td>81</td>
<td>80</td>
<td>33</td>
</tr>
<tr>
<td>Enantiomeric excess [%]</td>
<td>99</td>
<td>99</td>
<td>99</td>
</tr>
</tbody>
</table>

*Verticillum* sp. also reduced diethyl 1-oxo-3-phenylpropylphosphonate (1d), with the yield about 20% and 99% e.e. The compound 1c (1-oxo-*iso*-butylphosphonate) was not a good substrate for any microorganism used as a biocatalyst in this type of bioreduction [4].

Another way to increase the yield of the reduction of 1-oxoalkylphosphonates is the application of chemical additives, e.g., compounds influencing the activity of the enzymes involved in the bioconversion. It is known that two alcohols, ethyl or *iso*-propyl are sources of hydrogen for cofactor regeneration system [5] while additives such as allyl alcohol, allyl bromide and *iso*-propylmethyl ketone are in turn potent enzymes inhibitors [5, 9, 10]. The addition of chemicals allowed improving the results for three substrates- 1a, 1b and 1d - but this method failed for the compound 1c (Scheme 1). It is necessary to stress that for 1-oxoethylphosphonate the application of suitable additives gave the same or better results then in the case without any additives [4].

The use of additives changed the results in the case of the reduction of compound 1b. For this substrate a satisfactory yield of bioreduction (80%, 99%ee- Table 1) was reached using immobilized cells of Cladosporium sp. as a biocatalyst in the presence of ethyl alcohol or allyl bromide as additives. This strain was completely unable to reduce any substrate in the presence of any other chemical modulator, probably because of the inhibition of the enzymes involved in the process. The best result for the reduction of 1-oxo-3-phenylpropylphosphonate (1d) was observed for Cladosporium sp. in the presence of the allyl bromide (yield 33%, 99%- see Table 1). The chemical additives may influence in many different ways. Some of them inhibit enzymes, which displayed major reductive activity toward specific compounds (1c), and they influence the stability of the substrates (1b and 1c). Probably, there are several different oxidoreductases involved in the bioreduction of diethyl 1-oxoalkylphosphonates and this kind of compounds are accepted as substrates under unique conditions that have to be optimized individually. Additionally, the yield of the reduction is strongly dependent on the nature of the substrate. Steric
hindrances introduced by substituents that are in close proximity to carbonyl moiety either almost totally abort or significantly decrease their reduction by the biological system (compounds 1c and 1d).

B. Bioreduction of 2-oxoalkylphosphonates.

2-Oxoalkylphosphonates (Scheme 2) are chemically stable in water solutions therefore their stereoselective bioconversion is possible in such media.

\[
\begin{align*}
\text{1.} & \quad \text{biocatalyst} \\
\text{2.} & \\
\end{align*}
\]

- \text{a. R:H, R':H} \quad \text{Diethyl 2-oxopropylphosphonate}
- \text{b. R:CH}_3, \text{ R':H} \quad \text{Diethyl 2-oxobutylphosphonate}
- \text{c. R:C}_6\text{H}_5, \text{ R':H} \quad \text{Diethyl 2-oxo-2-phenylethylphosphonate}

Scheme 2. Bioreduction of 2-oxoalkylphosphonates.

Different spices of \textit{Rhodotorula} (\textit{R. rubra}, \textit{R. gracilis}, \textit{R. glutinis}) were tested \cite{34} for the evaluation of the possibility to obtain optically pure enantiomers of diethyl 2-hydroxypropylphosphonate (Scheme 2, compounds 2a). Bioconversion protocol includes (as in the case of previously discussed 1-oxophosphonates) the addition of some chemicals as an enzymes activity affecting agents: ethyl, \textit{iso}-propyl and vinyl alcohols, allyl bromide, methylvinyl ketone and \textit{iso}-propylmethyl ketone. Experiments, which were done showed that only \textit{R. rubra} was active toward diethyl 2-oxopropylphosphonate and was able to reduce this substrate to corresponding alcohol of \textit{R} configuration no matter what biocatalytisis conditions were applied. However the chemical additives, in some cases, were factors which definitely increased the chemical yield of bioreductions e.g. ethyl chloroacetate - 40% chemical yield comparing to 10% for biotransformation without any additives \cite{34}. Correspondingly, for each set of experiments, were done biotransformations of acetophenone as a model substrate. It has to be stress, that these microorganisms- \textit{R. rubra} reduced acetophenone only to \textit{S}-phenylethyl alcohol. It seems that enzymes present in this species have a different stereospecificity and this is the real possibility to stereocontrol of the reaction, which increase range of possible applications.

For the enantioselective reduction of 2-oxobutylphosphonate (Scheme 2. \textbf{1b}) other kind of \textit{Rhodotorula} species- \textit{R. gracilis} were used. Chemical additives affecting the activity of particular enzymes \cite{4,9,10} were tested. The best result (yield 90.5%) was achieved for the bioconversions with \textit{iso}-propylmethyl ketone
as an additive. Other cases resulted in chemical yield ranged from 8% (iso-propyl alcohol) to 16.5% (allyl bromide) [3]. In the biotransformations with ethanol or ethyl acetate using as chemical additives or without any affecting factors the lack of the reactions was observed. Mosher’s method [7] allowed to define the absolute configurations of bioconversions product (diethyl 2-hydroxybutylphosphonate-1b) as an isomer of R- configurations. Summing up, the enantiospecificity of the both microorganisms- R. rubra and R. gracilis towards 2-oxoalkylphosphonates was the same R- isomer was obtained.

Biotransformations of the next substrate - diethyl 2-oxo-2-phenylethylphosphonate (compound 1, Scheme 3) lead to a new non-racemic derivative of hydroxyphosphonate [33]. Five fungal spices (Geotrichum candidum, Rhodotorula sp., Penicillium oxalicum, Cladosporium sp. and Beauveria bassiana), known from their various catalytic activities towards phosphonates [4, 34], were used as biocatalysts for the bioconversion of 2-oxo-2-phenylethylphosphonate into its asymmetric derivative. The resulting compound represents a structure, in which hydroxyl group of phosphonate (2) is phosphorylated (compound 3, Scheme 3).

Scheme 3. Microbial bioconversion of diethyl 2-oxo-2-phenylethylphosphonate.

This additional phosphonate group has to derive from the decomposition of some substrate molecule, what was possible for strains which are able to cleave the P-C bond especially in the case of Cladosporium sp. [26]. The biotransformation catalyzed by the cells of Cladosporium sp. was carried out under very mild conditions resulted in mixture of the products: compound 2 and 3 (Scheme 3). The yield of conversion was about 50% (product mixture 2 and 3) but the chiral O-phosphorylated hydroxyphosphonate constituted about 98% of the final mixture. Cladosporium sp. obligatory requires some amount of ethyl alcohol as an exogenous hydrogen source for cofactor regeneration system [32]. The absolute configuration of product 3 was tentatively assigned as “S” [33]. In the case of other microorganisms examined, compound 3 was also found as a major product.
of biocatalysis. The use of *Beauveria bassiana* allowed obtaining desired product (85% of the final products mixture), even without any additives. Supplementation of the biocatalytic medium with ethanol increased the amount of compound 3 (chemical yield still was 50%) for *Penicillium oxalicum*, *Geotrichum candidum* and *Rhodotorula rubra* whereas for *B. bassiana* or *R. glutinis* the addition of this factor resulted in decrease of final amount of *O*-phosphorylate derivative of the 2-hydroxy-2-phenylethylphosphonate. The structure of this compound was determined using NMR [33]. The authors of this paper speculate that there may be three enzymes of different activities involved in the described reactions: C-P liase, reductase and phosphotriesterase. One molecule of the substrate is cleaved by C-P liase [28], which resulted in two products: acetoephene and diethyl ester of phosphoric acid. The second molecule is reduced to corresponding hydroxyl derivative by reductase. Finally, hydroxyl group of the hydroxyphosphonate esterification is catalysed by phosphotriesterase.

3. Microbial kinetic resolution of racemic mixture of ethyl hydroxyl(phenyl)-methane(P-phenyl)phosphinate as an easy method to synthesize optically pure P-chiral phosphonate

Microbial transformations of hydroxyphosphonates (hydroxyphosphinates) containing one asymmetric centre have been investigated for several years [31], and the biocatalysis of compounds with two stereogenic centers was only a matter of time. For the enantioselective biotransformation of racemic ethyl hydroxyl(phenyl)methane(P-phenyl)phosphinate via oxidation process, were used fungal strains of different origin (*Penicillium oxalicum*, *Aspergillus flavus*, *Geotrichum candidum*, *Rhodotorula gracilis*) [15]. Biotransformation substrate-the racemic mixture was consisted of four stereoisomers: a left pair (*R*<sub>p</sub>*R*<sub>p</sub>), (*S*<sub>p</sub>*S*<sub>p</sub>) and a right one (*R*<sub>p</sub>*S*<sub>p</sub>), (*S*<sub>p</sub>*R*<sub>p</sub>) [17]. Isolated enantiomers of this compound are very attractive for further application as chiral buliding blocks in chemoenzymatic synthesis. Some effort has already been undertaken to separate all enantiomers of this compound but results, based on the lipase activity of the bacterial cells, were unsatisfactory. Although the enantiomeric excess of the resulting hydroxyphosphinate was 90% but both pairs of enantiomers were converted in 50%. Microbes preferentially hydrolyzed this compound with an (*S*)-configuration at the *α*-carbon, whereas no stereoselectivity was observed toward the phosphorus atom [17].

In discussed example, the way of biocatalyst preparation was crucial for microbial transformation of the substrate. Results were strongly dependent upon the sort of chemical additive (methyl-*iso*-propyl ketone, oxalacetic acid) used in the cultivation process. Chemicals allowed to “switching on” the oxidative activity of microorganisms. These chemical factors serve as an electrons acceptor and they allow obtaining the cofactors in oxidized form (Scheme 4). This process move the reaction balance to the oxidation of substrate and it is a good example of rationally projected biotransformation process.
The preincubation of biocatalyst cells in the presence of methyl-\textit{iso}-propyl ketone resulted in transformation of only one stereoisomer of the substrate. Remarkable results were obtained in the case of oxalacetic acid- because both pairs of diastereoisomers of ethyl hydroxyl(phenyl)methane(P-phenyl)phosphinate were resolved [15].

The absolute configuration of unreacted stereoisomers of ethyl hydroxyl(phenyl)methane(P-phenyl)phosphinate was determined by the Mosher’s method [7]. The result obtained, when methyl-\textit{iso}-propyl ketone was used as an additive show that only the (\textit{S}p\textit{S})- stereoisomer was transformed (yield was up 20%). Application of oxalacetic acid gave two diastereoisomers- (\textit{R}p\textit{R}) and (\textit{S}p\textit{R})- with excellent enantiomeric excess (>99%) and chemical yield up to 48% [15].

There are two possible mechanism of the presented biotransformation. First- the oxidized form of one isomer is unstable in water and splits (Scheme 5) and the second one is the selective O-dealkylation of the phosphinate [16]. The NMR analysis suggested the biooxidation process, because there are no other signals characteristic to organophosphorus compound.

**Scheme 4. Cofactors regeneration systems**
Scheme 5. Possible enantioselective biooxidation.

The biocatalytic process with whole cell of different fungus spices is the simple and chip alternative to synthesize optically pure organophosphorus compounds (hydroxyphosphonates, hydroxyphosphinates). There are a lot of possibilities to control the biocatalyst’s activity by medium or cells engineering.

References


[10] A.D.P. Forni, I. Moretti, F. Prati and G. Torre, (R)-(+) and (S)-(−)-ethyl 4,4,4-trifluoro-3-hydroxy butanoate by enantioselective baker’s yeast reduction, Enzyme and Microbial Technology, 25 (1999), 149-152.


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